Project ID: RLP 1201

Design of a Cell Delivery Vehicle for Volumetric Muscle Loss

A Major Qualifying Project Report submitted to the faculty of
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AUTHORSHIP

This report was written as a collaboration of project team members: Tuhina Bhattacharya, Megan Read, and Rebecca Sharpe. Each group member played a role in writing and revising each section for clarity, as well as ensuring that the views presented in the report were the views of the group as a whole.

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ABSTRACT

Volumetric muscle loss (VML) is a common result of traumatic injuries and myopathies. Large-scale muscle injuries deplete the native population of satellite cells in the tissue, which inhibits muscle regeneration, induces scar tissue formation, and causes loss of function. The purpose of this project was to design, produce, and experimentally test a cell delivery vehicle to aid in the treatment of VML. Ideally, this scaffold will mimic the native scaffold architecture and allow for human satellite cell attachment and transplantation without causing premature differentiation or cell death. The team began experimentation by evaluating the following materials on a two dimensional (2D) scale: fibrin, collagen I, laminin, chitosan, fibronectin, and vitronectin. Proliferating human satellite cells (hSC) were seeded on each substrate and the cell adherence was quantified at defined time points. Compared to fibrin, there was a statistically higher percent attachment for collagen I at 24 and 48 hours and for vitronectin and fibronectin at 48 hours ($p<0.01$). Additionally, collagen I at 24 hours had greater attachment than fibrin at any tested time point. Upon staining for myosin heaving chain, adhering cells cultured on collagen I were found to have a myogenic index of 32.1% compared to 25.1% for cells cultured on fibrin, indicating that they had a higher myogenic differentiation potential. The top candidate from 2D testing, collagen I, was then coated on bundled fibrin microthreads, and then stained to verify that the collagen I coating successfully adhered to fibrin. Next, bundled fibrin microthreads were coated with the top performing substrates: collagen I and vitronectin, prior to seeding cells. Collagen I coated fibrin microthread bundles were found to have a larger amount of cell attachment without premature differentiation compared to uncoated fibrin microthread bundles. Myogenic indexes were not calculated due to the low cell density on the control fibrin microthread thread bundle, however myosin was observed on both the fibrin microthread bundle and the collagen I coated fibrin microthread bundles. Furthermore, the collagen I coated bundles were found to have similar degradation properties as to the uncoated bundle, with both types of bundles being severed between 10 and 14 hours. The team recommends conducting further validation on the collagen I coated fibrin microthread design including conducting transplantation studies *in vivo* to determine its effect on engraftment.

EXECUTIVE SUMMARY

Introduction

Volumetric muscle loss is a common result of traumatic injuries and various myopathies. Specifically, over 50% of all combat-related injuries and 35-55% of all sports-related injuries affect muscle [1-2]. Myopathies with few effective treatments include muscular dystrophy and Rhabdomyosarcoma. These large-scale muscle injuries deplete the native population of satellite cells in the tissue, which inhibits muscle regeneration, induces scar tissue formation, and causes pain and loss of function.

In contrast to patients with volumetric muscle loss, humans are able to recover from minor muscle injuries due to the activation of the native pool of satellite cells found beneath the basal lamina of myofibers. These undifferentiated cells repair injuries by utilizing the scaffold remaining in the damaged muscle tissue to migrate to the injury, proliferate, differentiate, and fuse with each other and with the adjacent uninjured muscle fibers.

Autologous tissue transfer is the current standard of treatment, in which tissue from a donor site is used to treat the injury site. However, this creates donor site morbidity, increases the risk of infection, and does not completely restore muscle function due to altered muscle alignment and scar tissue formation [3]. Fibrin microthreads have been investigated as a potential scaffold system for satellite cell transplantation to facilitate cell axial alignment with the native tissue, but preliminary results *in vitro* have shown that activated human satellite cells (hSC) do not adhere as efficiently to fibrin as desired and undergo premature differentiation [4].

Consequently, the goal of this project is to design, produce, and experimentally test a cell delivery vehicle that promotes hSC attachment. Ideally, this scaffold will mimic the native scaffold architecture and allow for transplantation without causing premature differentiation or cell death.

Methods

Cell Culture

All experiments were performed using the viable non-adherent population of hSC cells between passages 8–15 (standard culture). Proliferation medium for these cells consisted of 54% DMEM

(Cellgro), 36% Ham's F12 medium (Cellgro), with 10% Fetal Clone III serum (Hyclone), and a proprietary growth factor cocktail. Differentiation medium consisted of 58.5% DMEM, 38.5% Ham's F12, 2% horse serum (Hyclone), and 1% Insulin-Transferrin-Selenium (ITS) (Cellgro).

Two Dimensional (2D) Testing

The team began experimentation by evaluating the ability of the following materials to produce an increase in hSC attachment on a 2D scale: collagen I (PureCol®, Advanced Biomatrix), laminin-111 (VWR), chitosan (Sigma), fibronectin (Gibco), and vitronectin (Gibco), with tissue culture plastic (TCP) as a coating control. The concentration of these materials was based on manufacturer recommendations. Fibrin was used as the control substrate and was made according to previously described methods [5]. Multi-well tissue culture plates were coated with each material prior to seeding proliferating human satellite cells. Phase contrast images were taken at 4, 8, 24, and 48 hour time points at 5X magnification. The number of adherent (morphologically flat and spread out) and non-adherent cells (morphologically spherical) was counted for each well (n). Approximately 1200-3000 cells were counted per well in order to arrive at the average percent of adherent cells.

Attached hSCs were then evaluated for their myogenic potential by calculating the myogenic index. After culturing for 6 days with differentiation medium, the cells were fixed and stained to fluorescently show cell nuclei and myosin heavy chain (MF20, Hybridoma Bank), to quantify differentiated myoctes. Images were taken at 20X magnification and the myogenic index was calculated by dividing the number of myosin positive cells by the total number of cells in the field of view.

Three Dimensional (3D) Testing

Collagen I was assessed as a coating on a 3D fibrin microthread scaffold. Fibrin microthreads were extruded according to previously described methods [5-6]. Threads were bundled in sets of ten by gluing the threads down onto a PDMS mold with the dimensions of the washer described by Grasman, and then dried overnight [5]. The fibrin bundles were coated by placing them in a 0.31 mg/mL collagen I solution in PBS (pH 7) or in a 0.02 mg/mL vitronectin solution in PBS (pH 7) for 30 minutes. The bundles were then removed from the solution and air dried for 1 hour. Collagen I coated and uncoated fibrin thread bundles were stained for pro-collagen I antibodies

(Procoll 1, Hybridoma Bank) to verify that the coating adhered to the thread. Threads were then sterilized and seeded with hSCs to ensure this procedure would not damage the coating or the threads [5]. Threads and attached cells were fixed and fluorescently stained to show cell nuclei and myosin heavy chain after 72 hours in growth medium and after 6 days in differentiation medium.

Results and Discussion

Two Dimensional Testing

The cell attachment properties for each substrate were assessed by comparing the percentage of attached cells in each well. Figure 1A and 1B show phase-contrast images of fibrin and collagen I coated wells used for cell counting. To assess the myogenic potential of the adherent cells, the cells were stained to show nuclei (blue) and myosin (red). A stained image of fibrin and collagen I, are shown in Figure 1C and 1D. There appears to be both a higher number of attached cells on collagen and a greater amount of cell nuclei in the myotubes.

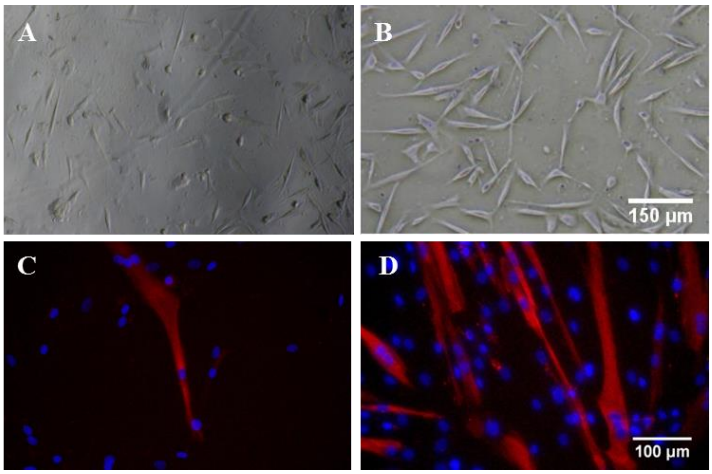

Figure 1: Attachment of satellite cells at 24 hours on fibrin (A) and collagen I (B) and myosin staining of fibrin (C) and collagen I (D).

Based on the results from an ANOVA and t-test ($p<0.01$), a statistical difference was found in the percent attachment of collagen at 24 and 48 hours and vitronectin and fibronectin at 48 hours compared to fibrin. Additionally, collagen I attachment at 24 hours and fibronectin at 48 hours were statistically greater than that of fibrin at any time point. Figure 2 below shows a graph of the percentage attachment of each material at 8, 24 and 48 hours.

The myogenic indexes for fibrin and collagen I were calculated by finding the percent of nuclei within the imaged myofibers. Cells cultured on collagen I had a myogenic index of 32.1% (n=2) compared to the 25.1% (n=2) for cells cultured on fibrin. All other myogenic indexes were less than 25%. A myogenic index of 30% or greater is ideal for cell therapy applications [7-8]. This shows that the attached cells on the collagen I coating had a higher ability to differentiate into myotubes. The cells also appeared to have the highest amount of proliferation on the collagen I coating.

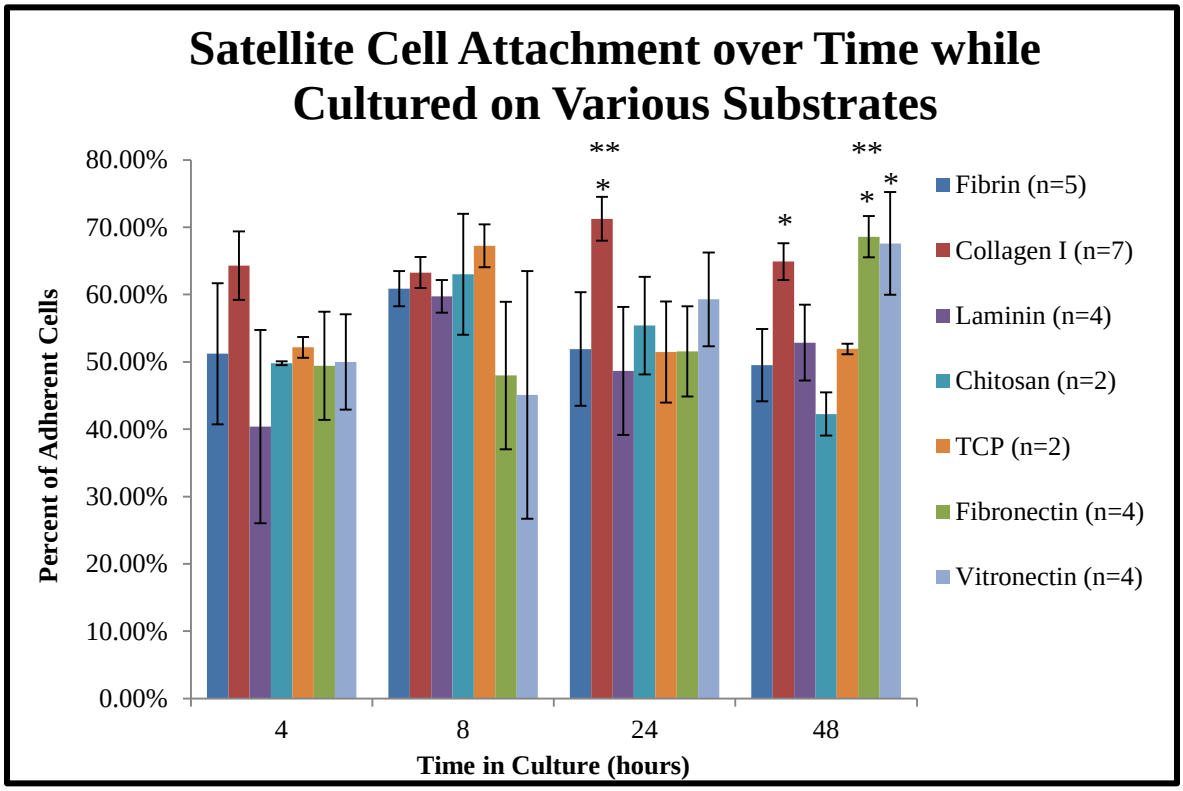

Figure 2: Satellite cell attachment over time while cultured on various substrates

** denotes p<0.01 for that time point, ** denotes p<0.01 in regards to fibrin at all time points*

Three Dimensional Testing

Based on its high performance in 2D testing, collagen I was chosen to be added to the fibrin microthread as a coating in an attempt to preserve the beneficial mechanical and degradation properties of fibrin, while increasing the cell attachment on the periphery of the threads. Collagen I was selected over vitronectin and fibronectin because of its improvement over fibrin

at all time points and because fibronectin and vitronectin may prove impractical for the laboratory due to their high cost. A collagen I coated microthread bundle was compared to an uncoated fibrin microthread bundle to assess coating efficacy by immunocytochemistry. The fluorescence images in Figure 3 below illustrate that the coating was successful. Addition of aprotinin, a protease inhibitor, (Sigma) was found to prevent hSC digestion of the coated microthread.

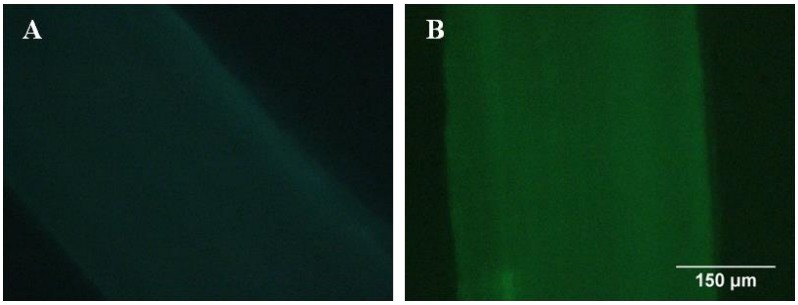

Figure 3: Pro-collagen I staining (green) for fibrin microthread bundle (A) and collagen I coated fibrin microthread bundle (B).

Satellite cells were stained with Hoechst to visualize the nuclei attached to the threads along the edges as well as in the middle. Very few cells were found on the fibrin microthreads as compared to vitronectin and collagen I coated fibrin microthread bundles. The greatest number of cells was found attached to the collagen I coated fibrin microthreads. The staining for myosin heavy chain showed that no cells were seen to have differentiated. This indicates that the attachment of the satellite cells to the fibrin, vitronectin, and collagen I does not catalyze differentiation. Satellite cells attached to the threads in an undifferentiated state, which indicates that these cells have a capability to proliferate along the thread.

Satellite cells attached to threads were cultured in differentiation medium for 6 days and then stained to fluorescently show nuclei and myosin. These images were difficult to process as the edges of the thread appeared to fluoresce as well. However, myotubes were seen indicating that the satellite cells maintained their ability to differentiate into myotubes while cultured on the microthreads. Myosin was seen on both fibrin microthread bundles and collagen I coated fibrin microthread bundles. The myogenic potential was not calculated due to the low cell density on fibrin threads, which may lead to unrepresentative results. Although the low cell density on uncoated fibrin microthread bundles did not allow the team to calculate the myogenic index, it

does support the previous conclusion that there was significantly higher attachment of satellite cells on the collagen I coated fibrin microthread bundle.

Future Work

Future experiments include assessing the effect of collagen I on hSC proliferation by conducting a BrdU assay, investigating the effect of collagen I on forcing the satellite cells down a myogenic pathway by staining for early muscle markers such as MyoD and Myogenin, and verifying that the mechanical properties of the coated microthreads are equal to the uncoated microthreads. Furthermore, alternative methods of cell seeding will be tested in order to assess cell attachment, premature cell differentiation, and myogenic potential. Ultimately, *in vivo* studies in small animal models will aid in determining whether using collagen I coated fibrin microthreads as a scaffold for delivering hSCs enables improved engraftment and muscle function.

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CHAPTER 1: INTRODUCTION

Humans are able to quickly recover from minor muscle injuries such as strains due to the activation of the native pool of satellite cells found beneath the basal lamina of myofibers. These undifferentiated cells repair minor injuries by utilizing the scaffold remaining in the damaged muscle tissue to migrate to the injury, proliferate, differentiate and fuse with each other and with the adjacent uninjured muscle fibers. A traumatic, large-scale muscle injury is defined by damage or loss of more than 20% of the native tissue. Large scale muscle injuries also deplete the native population of satellite cells in the tissue, which inhibits muscle regeneration, induces scar tissue formation, and causes long-term pain and loss of function.

Volumetric muscle loss is a common result of traumatic injuries and various myopathies. It specifically accounts for about half of all combat-related injuries, and also results from motor vehicle accidents and sports-related injuries. Myopathies with few effective treatments include muscular dystrophy and Rhabdomyosarcoma, both of which results in loss of muscle function due to loss of muscle mass and increased scar tissue formation.

Currently, the standard of treatment for volumetric muscle loss is autologous tissue transfer, in which tissue from another donor site is used to treat the injured site. There are many drawbacks associated with the current method as it creates a second morbidity from the donor site and increases the risk of infection. This method also does not fully restore muscle function, as there is still scar tissue formation and altered muscle alignment, resulting in long-term disabilities for patients. The only other option currently available is physical therapy, which cannot restore muscle function or trigger regeneration. It appears that patients would benefit from the design of an *in vitro* scaffold that could be used to transplant a human satellite cell population Fibrin microthreads have been investigated as a potential scaffold system for satellite cell transplantation to facilitate cell axial alignment with the native tissue. Implantations of this system with a differentiated cell population have shown a decrease in scar tissue formation *in vivo*, but fibrin lacks the capability to promote attachment with human satellite muscle cells and causes premature differentiation of attached cells. Thus a new scaffold system or a modification to the fibrin microthread system is necessary.

The goal of this project was to design, produce, and experimentally test a cell delivery vehicle for volumetric muscle loss. The vehicle was designed to ideally mimic the native scaffold architecture found in muscle tissue and also to allow for human satellite cell attachment and transplantation without causing premature differentiation or cell death. The following chapters of the project report contain a literature review describing candidate materials for the scaffold along with their associated advantages and disadvantages; a project strategy including objectives, functions, and means the team utilized in creating alternative designs; evaluation of the designs to find the best means of accomplishing the project goal; preliminary two dimensional evaluations of all materials to assess their attachment potential to human satellite cells; three dimensional testing of top two dimensional materials; discussion of all findings; and conclusions including future recommendations by the design team.

CHAPTER 2: LITERATURE REVIEW

This project focused on strategies to design a process for creating a viable *in vitro* scaffold and improving attachment of satellite cells to this scaffold. This chapter details background information and literature on skeletal muscle, muscle loss and regeneration, current engineering strategies for treatment of volumetric muscle loss, and potential methods for increasing satellite cell attachment.

2.1 Human Skeletal Muscle

Skeletal muscle is one the three types of muscle found in the human body, the other two being cardiac muscle and smooth muscle. Skeletal muscles are comprised of individual muscle fibers that contract and release upon neural stimulation. Increasing numbers of stimulated motor neurons leads to increasing contraction strength of the entire muscle. Muscle contraction allows for the movement of the bone and joints that they are attached to (via tendons). The following is an overview of the structure of skeletal muscle and how the human body naturally regenerates skeletal muscle in response to injury.

2.1.1 Structure of Skeletal Muscle

Fibrous connective tissue called fascia serve to separate skeletal muscle from surrounding muscles and hold them in position, using the strength and alignment of collagen fibers. Fascia are comprised of epimysium (outer layer) and perimysium (inner layer), the latter of which serves to compartmentalize the muscle tissue, as shown in Figure 4 below (Shier, et al., 2012). Each compartment is comprised of a bundle of fascicles, also known as myofibers, where each fiber is surrounded by a thin layer of collagen called endomysium. The diameters of myofibers range from less than fifty microns to a few hundred microns and their lengths range from a few millimeters to a few centimeters. Fasicles are surrounded by blood vessels (for nourishment) and nerves (for signaling). Myofibers can also be referred to as myocytes or muscle cells. Each of these fibers contain a bundle of myofibrils, which in turn contain actin (thin) and myosin (thick) protein filaments arranged in parallel. The striations seen in skeletal muscle are due to the arrangement of these filaments (Fox, 2011). The sarcoplasmic reticulum is a membrane that surrounds each bundle of myofibrils, which in turn is surrounded by a membrane called

sarcolemma. Another type of membrane is the transverse tubules, which extend into the muscle fiber perpendicular to the myofibrils.

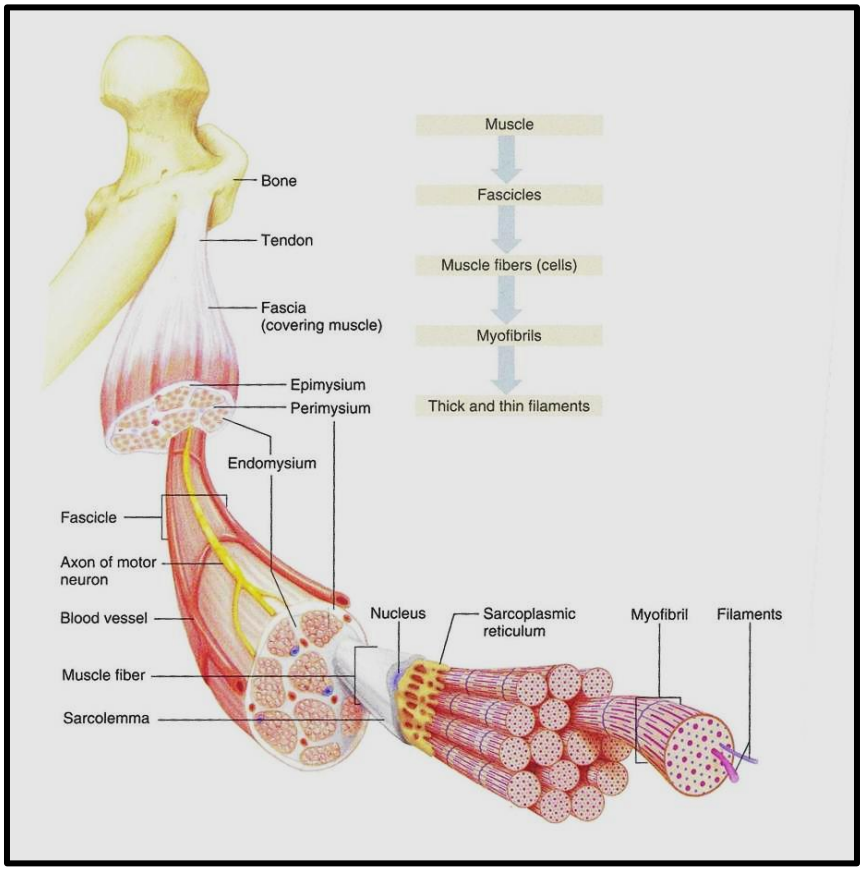


Figure 4: Structure of skeletal muscle (Shier, et al., 2012)

2.1.2 Skeletal Muscle Contraction

Myofibrils contain sarcomeres arranged in series along their lengths. The overlapping myofilaments myosin and actin enable muscles to shorten in response to signals from motor neuron axons located at the motor end plate (Shier, et al., 2012). Specifically, the sarcoplasmic reticulum transforms the electrical signal from a motor neuron axon into a chemical gradient of acetylcholine, which in turn binds to protein receptors on the muscle fiber membrane, increasing the membrane's permeability to sodium ions, which creates an electrical impulse that spreads in all directions. Once this impulse reaches the sarcoplasmic reticulum, the membranes become more permeable to calcium ions, which diffuse into the sarcoplasm of the muscle fiber. Muscle contraction itself can be described using the sliding filament model (Fox, 2011). The released

calcium ions bind to troponin, a protein on actin filaments, leading to tropomyosin, another protein on actin filaments, to be pulled aside, exposing binding sites on the thin filaments (Shier, et al., 2012). This enables cross-bridges to bind actin filaments (A band) to myosin filaments (I band), and then pull the actin filaments towards the center of the sarcomere, shortening the fiber and thus causing a contraction. This shortening of these bands can be seen in Figure 5 below. Adenosine triphosphate (ATP) binds to the cross-bridge, enabling the release of the cross-bridge from the actin filament. Following this, ATP breaks down into adenosine diphosphate (ADP) and a phosphate to provide the energy needed for the contraction. This cycle continues (as long as sufficient ATP is available) until the concentration of calcium ions is too low, at which point the muscle relaxes.

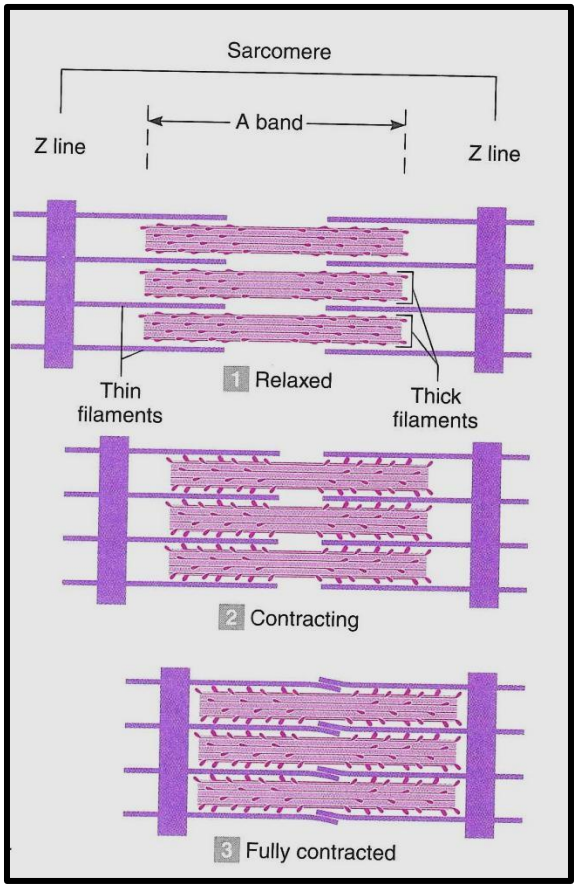


Figure 5: Skeletal muscle contraction (Shier, et al., 2012)

2.1.3 Skeletal Muscle Regeneration *in vivo*

In small scale muscle injuries (where less than 20% of muscle tissue is lost), the human body has the ability to regenerate healthy muscle in that area (Valentin, et al., 2010). Skeletal muscle cells arise from the fusion of myoblasts, which are muscle progenitor cells. As a result, myocytes are multinucleated cells. The basal lamina, which surrounds the sarcolemma, serves to provide mechanical support to the cells during cellular growth and development as well as during regeneration (Fox, 2011). Healthy muscle fibers are not able to divide to replace damaged fibers. Consequently, natural regeneration *in vivo* is a result of activated satellite cells – also known as quiescent progenitor cells, a type of stem cell – which are located between the sarcolemma and the basal lamina.

The regeneration of skeletal muscle *in vivo* consists of three phases: inflammation, tissue formation, and tissue remodeling. Inflammation occurs immediately after the injury and is characterized by necrosis of myofibers and an inflammatory response consisting of cellular signals, macrophage recruitment, and protease release (Turner and Badylak, 2012). Cellular signals from the injured and/or ruptured myofibers triggers macrophage recruitment, which promote inflammation through the secretion of pro-inflammatory factors such as tumor necrosis factor-alpha (TNF-α), remove the remains of the damaged myofibers, as well as support the proliferation of progenitor cells (Grefte, et al., 2007). The area of injury is also isolated with a contraction band of cells to prevent the proteases from destroying healthy muscle fibers in the area (Turner and Badylak, 2012).

In the tissue formation phase, macrophages conclude removing the damaged myofibers and decrease the inflammatory response. Following this, macrophages secrete growth factors such as fibroblast growth factor (FGF), hepatocyte growth factor (HGF), insulin-like growth factors (IFG-I and IFG-II), transforming growth factor-beta (TGF-β1), and interleukin-6 (IL-6) to recruit satellite cells to the area (Charge and Rudnicki, 2004). These progenitor cells go on to proliferate and differentiate in order to form new skeletal muscle tissue as depicted below in Figure 6 (Turner and Badylak, 2012). Satellite cell proliferation and differentiation is discussed further in Section 2.5.2.

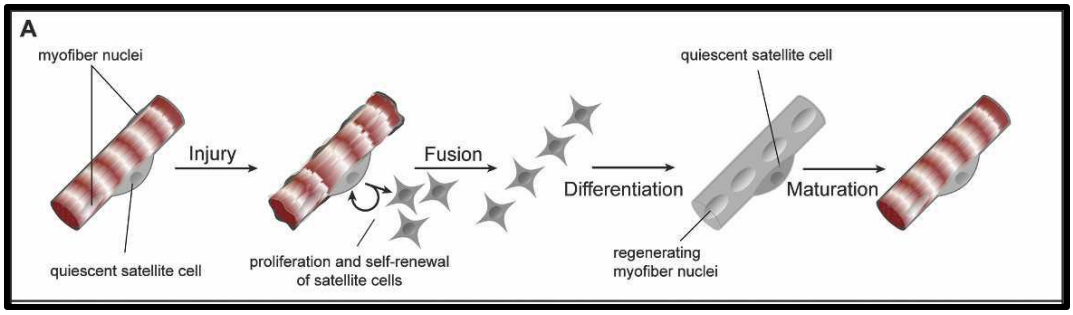


Figure 6: Satellite cells in muscle regeneration (A) (Shi and Garry, 2006)

Next, the newly regenerated tissue then undergoes tissue remodeling where the myofibers reorganize, skeletal muscle function is restored, and scar tissue is remodeled (Turner and Badylak, 2012). The fused myofibers align and fuse with each other, and mature to become a functional unit of contractile skeletal muscle. An increase in fiber diameter and the movement of the nuclei to the peripherals mark the myofiber maturation period (Grefte, et al., 2007). The muscle tissue then integrates with the surrounding muscle tissue through the revascularization (reestablishment of blood supply to allow oxygen and nutrients to reach the myofibers), reinnervation (reestablishment of neuronal connections), and further alignment (Turner and Badylak, 2012). Failure to revascularize the regenerated tissue leads to necrosis of the muscle tissue and failure to reinnervate the regenerated tissue leads to muscular atrophy. Finally, myofibroblasts further align the regenerated myofibers and replace the temporary extracellular matrix (ECM) (that had been formed during the tissue formation phase) with a permanent matrix (Grefte, et al., 2007). After the injured site has recovered the satellite cell pool is restored in the tissue so regeneration can occur for future injuries. In the case of large-scale traumatic injury and chronic injury, the cell pool is depleted and regeneration cannot occur (Shi and Garry, 2006).

2.2 Clinical Significance

Surgeons today have the difficult task of replacing functional muscle tissue following volumetric muscle loss, which is defined as the traumatic or surgical loss of skeletal muscle (Grogan and Hsu, 2011). Although skeletal muscle has the capacity for regeneration following minor injuries such as strains or sprains, there is a lack of effective therapeutic treatments for volumetric muscle loss. When a traumatic, large-scale muscle injury occurs, a minimum of twenty percent of the native tissue is damaged or lost. This amount of harm to muscle cells causes the tissue to lose its

ability to fully regenerate and instead there is scar tissue formation, denervation of neighboring muscle, and a loss of function. Injuries such as these occur most commonly as a result of combat injuries, motor vehicle accidents, sports injuries, and muscle-loss diseases (Valentin, Turner, et al., 2010). These muscle injuries all result in long term pain and physical disability for the patient (Longo, et al., 2012).

Traumatic injuries involve muscle cells that are damaged beyond repair, but do not necessitate limb amputation. This type of injury is a significant problem currently for the military. Injuries involving volumetric muscle loss such as lacerations are common on the battlefield, and about fifty percent of all injuries on the battlefield affect the musculoskeletal system (Fischer, 2009). Combat injuries, along with other traumatic injuries, are very difficult for reconstructive surgeons to treat as patients desire both cosmetic and functional restoration, but no current treatments exist that aptly satisfy these needs. The treatment option most commonly used for these patients is a tissue transplant, but there are many difficulties associated with this method including donor site morbidity and locating donor tissues (Mase, et al., 2010).

Rhabdomyosarcoma is one type of cancer found in the soft tissues of striated muscle and is one of the most common causes of skeletal muscle loss. Up to fifty percent of all soft tissue sarcomas in children are rhabdomyosarcomas (Andrassy, 2002). This cancer is treated with radiation, chemotherapy, and surgical removal of the sarcoma. The latter is a highly invasive procedure and involves the removal of not only the sarcoma, but also the surrounding tissue and muscle to guarantee the entire cancer is eradicated. In addition, a second surgery is often required to aid the recovery of muscle cells that were removed (Soft Tissue Sarcoma, 2011). These surgeries result in scar tissue formation and a loss of muscle function.

A major cause of skeletal muscle injury is myopathy, or muscle disease. The majority of these diseases cause chronic muscle injuries that cannot heal completely due to scarring and fat accumulation resulting from various reasons as show below in Figure 7. Muscular dystrophy is one common example of a myopathy and is identified by repeated cycles of muscle injury and regeneration in which the muscle is not fully restored and loses the ability to contract. As the chronic injury cycle continues the regenerative capacity of the native tissue diminishes as the satellite cell population is depleted. More scarring and fat accumulation occurs with each

additional cycle of the chronic injury (Brunelli and Rovere-Querini, 2008). Most patients with muscular dystrophy need wheelchairs for movement by their teens and die from loss of muscle mass impairing cardiac and pulmonary essential functions in their early twenties (Muscular Dystrophy Association, 2011).

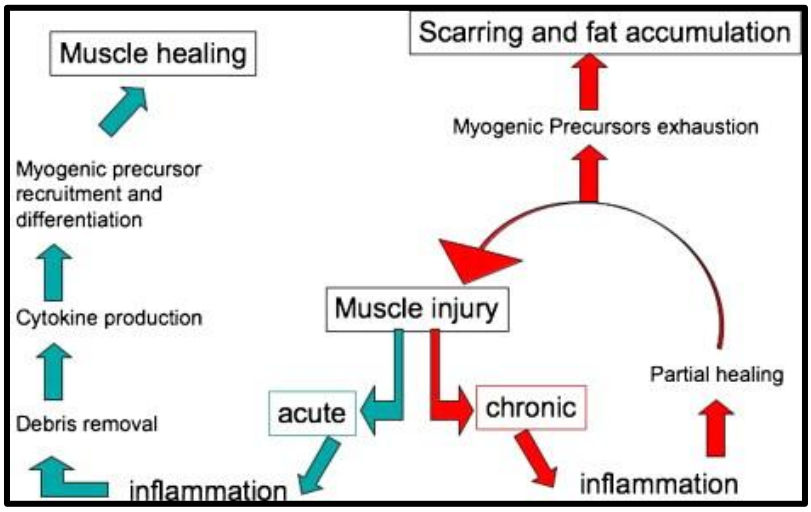


Figure 7: Acute vs. chronic muscle regeneration (Brunelli, 2008)

The above mentioned ailments all result in the destruction of the scaffold structure in native muscle tissue and the depletion of the body's natural pool of satellite cells. Volumetric muscle loss necessitates the design of a cell delivery vehicle that imitates the mechanical and structural properties of native muscle tissue and improves satellite cell attachment and implantation. This approach would be applicable for large-scale traumatic injuries and myopathies, both of which have little to no current effective treatments.

The current standard of treatment for volumetric muscle loss is an autologous tissue transplant. This procedure involves donor tissue being implanted at the injury site, but this practice only restores partial function and often alters anatomy and biomechanics in the recipient and donor sites. Most individuals should expect to always have a physical handicap for the rest of their lives. An additional drawback of this type of treatment is the creation of a second morbidity site with increased risks of infection and greater scar tissue formation (Valentin, Turner, et al., 2010). Because of these many negative aspects of current treatment, there exists a need for an improved therapeutic treatment for volumetric muscle loss.

2.3 Tissue Engineering Strategies

The field of tissue engineering provides alternative approaches to autologous tissue transfer in order to repair wounded musculature (Stern-Straeter, et al., 2007). To address the problem of volumetric muscle loss, researchers have taken various approaches to regenerate muscle growth and better the patient's daily life. These approaches fall into two main strategies: building skeletal muscle *in vitro* to be implanted into the wound site, or building a scaffold on which to seed myogenic stem cells and implanting it into the wound to promote regeneration as shown in Figure 8. These broad methods are also used in numerous applications of other tissue types to repair damaged areas of the body.

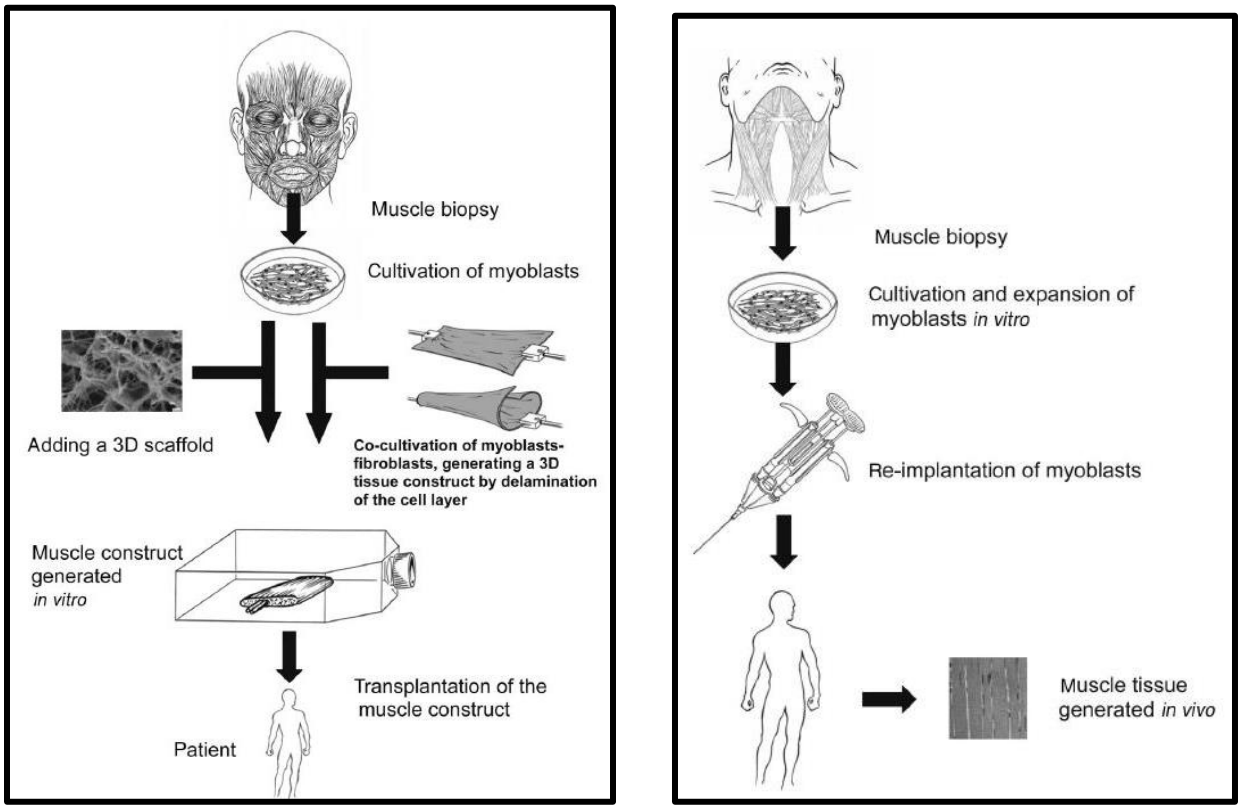


Figure 8: Methods of skeletal muscle tissue engineering (Stern-Staeter, et al., 2007)

Skeletal muscle tissue engineering by constructing 3D muscular tissue in vitro (left) and culturing myoblasts to be implanted in vivo to promote muscle regeneration (right)

2.3.1 Implantation of Preconditioned Engineered Tissue

Research conducted at Wake Forest Institute for Regenerative Medicine has shown that muscle tissue grown *in vitro* under cyclic mechanical stress generated a contractile response. The tissue produced a response that was 1% and 10% of the force observed in the native tissue. To their knowledge, this is the largest force generated from tissue grown *in vitro* (Moon, et al., 2008).

Most research in constructing three dimensional skeletal muscle tissue has been completed by culturing myoblasts on synthetic polymers or gel-based matrices with mechanical strain but not usually cyclical mechanical strain. The cyclic strain provides support to the formation of the skeletal muscle, preconditions it to generate a greater amount of force, and promotes alignment of forming myofibers (Moon, et al., 2008). Samples from both cyclically strained and unstrained tissues were stained and the morphology of each was compared. As seen in Figure 9, each sample differs in alignment and overall shape and form. The cyclical strain promoted alignment of the muscle fibers so that the fibers will contract in the same direction and generate a greater amount of force (Moon, et al., 2008).

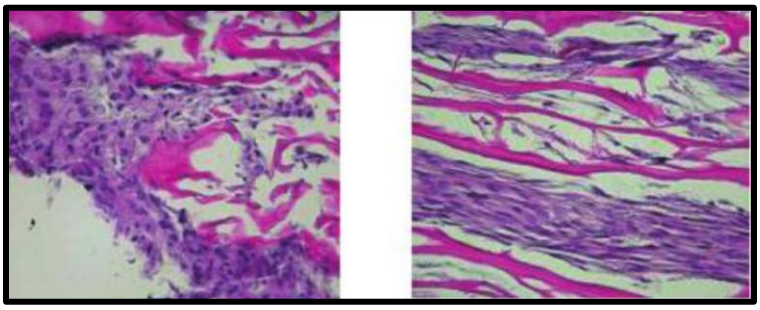


Figure 9: Tissue sample morphology (Moon, et al., 2008)

Morphology of tissue samples after growth under static conditions (left) and growth under cyclically strained conditions (right)

In addition to preconditioning skeletal muscle as it cultures, research has also been completed on preconditioning smooth muscle cells to develop tissue-engineered blood vessels that are able to withstand the mechanical stresses of circulating blood. It was shown that preconditioning in a bioreactor accelerates the formation of a muscular layer on the scaffolds. Additionally, the preconditioned vessels were able to activate calcium in response to depolarization (Yazdani, et al., 2009).

2.3.2. Using mammalian extracellular matrix as a scaffold

Another strategy in tissue engineering is to use the mammalian ECM as a scaffold to support and enhance tissue growth and repair. The matrix can be harvested from such sources such as the small intestinal submucosa (SIS) or the urinary bladder submucosa (Record, et al., 2001). One study completed in 1999 compared the performance of a SIS scaffold to a tendon autograft when they replaced the anterior cruciate ligament of sixty healthy goats. The failure force of each of the implants after 12 months was similar. This shows that SIS may hold promise as an absorbable bioscaffold for the musculature (Badylak, et al., 1999). .

In addition to using well-established sources for the ECM such as SIS and urinary bladder submucosa, the ECM of skeletal muscle may also be used. The ECM of each tissue type has particular structural and chemical qualities that benefit the native tissue and direct the tissue in growth and maturation (Wolf, et al., 2012). It would follow that a muscle ECM scaffold may be more suitable for reconstructing skeletal muscle than the ECM from the SIS. Myogenic cells were cultured on ECMs from skeletal muscle and SIS and then implanted in an injury site. The cells survived and proliferated *in vitro* on both scaffolds and the results were similar for both scaffolds 35 days post-surgery (Wolf, et al., 2012). This particular study showed that the harvesting site of the extra cellular matrix may not have a significant bearing on the outcome of the scaffold and tissue regeneration.

2.4 Scaffolds in Previous Research

Previous research involving satellite cells has focused on their abilities to stimulate muscle regeneration with the native muscle scaffold still intact. The majority of testing has been conducted on sites that were injured with an injection of myotoxin, or similar agent. There is a lack of research involving injury sites that mimic volumetric muscle loss with a significant portion of muscle tissue being surgically removed. It appears that if the native scaffold is destroyed, an *in vitro* scaffold would be needed to replace it in addition to the delivery of a population of satellite cells (Page, et al., 2011).

Adult and embryonic stems cells depend on specific conditions both physically and chemically, in order to differentiate into certain cell types. From studying this mechanism *in vitro*, researchers have shown that inducing differentiation of embryonic stem cells into a target cell

type can be a successful endeavor at times and a failure at other times (Evans, et al., 2006). Because of this and the destroyed muscle structure, researchers and tissue engineers have turned to 3D scaffolds to mimic the conditions *in vivo* to coax the cells into behaving as they would in the body. Scaffolds create an initial biochemical surface for the tissue until cells produce their own ECM (Bartolo, et al., 2008). In addition to providing a means to study cell behavior, scaffolds have been used to deliver differentiated cells to a specific site in the body These implanted cells then work to cure a disease or repair a defect (Evans, et al., 2006).

In order to deliver cells to a target area and facilitate tissue regeneration, a scaffold must primarily allow for cell attachment, proliferation, and differentiation. The structure also should retain cells, attach growth factors, and enable diffusion of cell nutrients and oxygen for subsequent growth. Furthermore, the scaffold should have the appropriate mechanical and biological environment to promote tissue regeneration in an organized way (Bartolo, et al., 2008). More specifically, a scaffold designed to promote muscle regeneration needs to be biocompatible, capable of three dimensional organization, promote native tissue growth and alignment, and be biodegradable yet mechanically stable (Page, et al., 2011). .

Researchers design scaffolds in various forms to be used in numerous applications for tissue engineering and regeneration. Each form serves a purpose as defined by the chemical and mechanical properties of the material and inherent properties of the shape. Broad categories of scaffolds include matrices, gels, and threads.

2.4.1 Matrix

A matrix, or network of materials, attempts to replicate the key conditions seen in the native ECM of the cell's environment (Borselli, et al., 2011). Also called semi-interpenetrating polymer networks, a matrix often combines materials to optimize their various properties as seen in a study completed by Battista and her colleagues at the Institute for Biomedical and Composite Materials in Italy (2005). Researchers combined the properties of collagen type I with laminin or fibronectin by interspersing either material at different concentrations within the matrix. These materials are discussed further in Section 2.5.1.

The study showed that structure and stiffness of the scaffold affected differentiation and organization of embryonic stem cells. The growth and differentiation of the embroid bodies were

inhibited when the elastic modulus of the scaffold increased from 16 to 34 Pa (Battista, et al., 2005). The composition of the matrix played an important role the development of the cells to differentiate into target cells. The scaffold material should provide mechanical and structural support as well as an assortment of macromolecular signals to direct tissue development.

The importance of the structure of the scaffold to support growth of the tissue is seen again in a study conducted at Harvard Medical School by Teng and his colleagues (2002). The group designed a scaffold with two layers to mimic the structure of the spinal cord as seen in Figure 10. The inner potion was similar to gray matter with a porous scaffold for cell seeding. The outer layer mimicked white matter with axially oriented pores for guidance and channels to allow fluid transport. This illustrates the variability of the scaffold form and the necessity that it must be similar to the structure in the native body so that the cells grow into and form similar tissue.

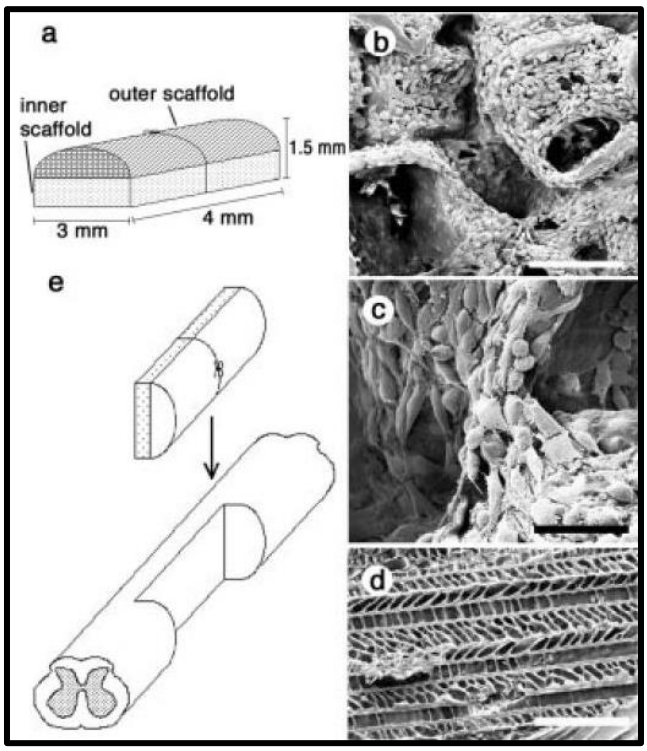


Figure 10: A scaffold built to resemble the structure of a spinal cord to regenerate nerve tissue (Teng, et al., 2002)

(a) Schematic of the conceptual design of the scaffold showing the inner and outer sections. (b and c) The inner scaffold seeded with neural stem cells – scale 200 µm and 50 µm. (d) Outer

section of the scaffold – scale 100 µm. (e) Schematic of surgical implantation of the scaffold into the spinal cord

2.4.2 Gel

Gels have been used specifically to transplant a target cell population. These scaffolds are often made porous so that cells are able to migrate within the structure which can aid in vascularization (Bartolo, et al., 2008). The ideal pore size for cellular adhesion and proliferation differs based on the cell type. For example, the ideal pore size for osteoblasts is between 200-400 microns, whereas the pore size of gels used for satellite cells is 500-600 microns (Cairns, et al., 2012). Hydrogel encapsulation provides the cell with a three dimensional environment similar to *in vivo*. This enables the cells to exhibit properties that are more similar to those shown in native tissue (Hunt and Grover, 2010). In one study, a macroporous scaffold of RGD-containing peptide alginate was designed to enhance cell viability during transplantation. The structure mobilized myogenic cells to disperse and engraft through a large wound (Borselli, et al., 2011).

In this particular study completed by Christina Borelli, alginate gels were cut into 5 mm squares, cooled to -80 °C and then lyophilized to make a porous anhydrous scaffold. It was shown that delivery of myogenic cells and growth factors from the alginate scaffolds reduced muscle inflammation and fibrosis as well as increased contractile function (Borselli, et al., 2011).

In another study, a porous hydrogel scaffold was used to deliver chondrocytes for the formation of neocartilage. The scaffold was made from hydrophilic polyethylene glycol (PEG) and hydrophobic polye-caprolacton (PCL). This scaffold was made porous by using a salt leaching method and studies showed that a higher PEG content increased cell growth. A PEG-PCL ratio of 14 to 6 in the scaffold was optimal for cartilage tissue formation in terms of collagen type II, aggrecan, SOX9, and COMP gene expression (Park, et al., 2007).

Despite the advantageous properties of gels, it has been shown that fibrin gels have poor mechanical stiffness. Researchers implanting a fibrin gel as a scaffold for cardiovascular tissue engineering for application as small blood vessels or heart valve prosthesis found that the gel is moldable and its degradation properties are controllable. However, the group found that the gel stiffness was too low for direct implantation (Jockenhoevel et al., 2001).

In many tissue engineering applications, efficient seeding methods are important and essential to the success of the scaffold. It has been shown that to save time and become more efficient, cells can be encapsulated into gels. In particular, a fibrin gel acts as a cell carrier and provides structural integrity to the growing tissue. When compared to a conventional seeding method, the cells (venous myofibroblasts) encapsulated in the gel resulted in less loss of collagen in the medium and a more mature ECM formed more quickly (Mol, et al., 2005).

Additionally, a hydrogel and cells encapsulated in microbeads were combined to create one scaffold. These two components complemented each other as the hydrogel matrix maintained the shape and volume of the desired implant while the beads degraded and released the cells into a porous scaffold. This method induced healthy spreading and elongated morphology for human umbilical cord mesenchymal stem cells. The hydrogel and mircrobead combination improved cell viability and myogenic differentiation of the mesenchymal stem cells (Liu, et al., 2012).

2.4.3 Thread

Scaffolds have been produced in the shape of a relatively small threads made up of various biopolymers. These threads can be applied to tissue regeneration because of their similarities to native tissue structure and biochemical state (Cornwell and Pins, 2010). Fibrin microthreads, as seen in Figure 11, have been shown to be a viable scaffold for fibroblast attachment, proliferation, and alignment (Cornwell and Pins, 2007; Grasman, et al., 2012). In addition to this, the fibrin microthreads have been used as a scaffold for delivering primary human skeletal muscle derived cells to regenerate muscle growth for *in vivo* studies. The threads allow for longitudinal growth and alignment of the cells along the axis of the threads (Page, et al., 2011). This leads to an aligned array of cells that could be used specifically for muscle regeneration.

The fibrin threads are made by coextruding fibrinogen and thrombin through small diameter polyethylene tubing and typically have a diameter of 50 to 100 μm. The alignment of fibers in the threads allows them to exhibit tensile strengths that are orders of magnitude greater than hydrogels (Cornwell and Pins, 2010). The ultimate tensile strength of the threads was 4.48 ± 1.79 MPa and failure strain was 0.31 ± 0.15.Finally, the modulus for the fibrin threads was 60.70 ± 25.71 MPa. In terms of cell viability and attachment, the fibrin threads supported more fibroblast attachment than polypropylene threads (Cornwell and Pins, 2007). .

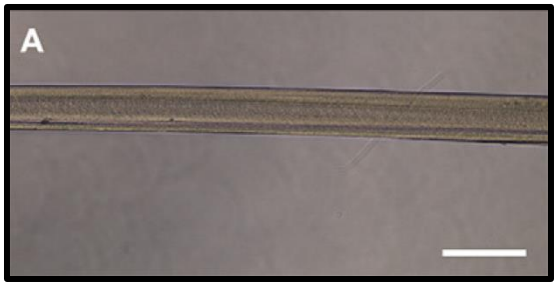


Figure 11: An uncrosslinked fibrin thread - scale 200 µm (Grasman, et al., 2012)

The mechanical properties of the fibrin threads can be adjusted by crosslinking, which will affect the overall behavior of the attaching cells and performance of the scaffold when implanted into the body. In one study the threads were crosslinked using carbodiimides in either an acidic or neutral buffer (Grasman, et al., 2012). These various treatments affected the tensile strength, modulus, and failure strain of the thread. When crosslinked in a neutral buffer, the threads were significantly stronger and stiffer than uncrosslinked threads. These threads were also comparable to native contracting muscle in terms of stiffness. The threads treated in a neutral buffer degraded by plasmin six times slower than uncrosslinked threads; however, those treated in an acidic buffer showed no significant signs of degradation over the course of seven days.

Interestingly, threads that showed a higher stiffness supported increased attachment of C2C12 cells, immortalized mouse myoblastic cells. It has been noted that softer substrates often trigger a proliferative response in cells while stiffer substrates induce differentiation (Grasman, et al., 2012). Myoblasts plated on gels with higher mechanical stiffness (13 to 45 kPa) increased in attachment, proliferation, and differentiation. Also, myoblasts showed increased differentiation when cultured on degradable scaffolds as opposed to non-degradable gels (Boontheekul, et al., 2007). This shows the importance of optimizing the mechanical properties of the scaffold to induce the desired characteristics in the target cell population. Additionally, the thread retains the biochemical ability to signal using integrins as seen in matrices. The threads have the potential to be woven, braided, and assembled into larger bundles to further promote cell alignment and orientation (Cornwell and Pins, 2010).

One major issue with current fibrin microthread techniques is that human satellite cells do not readily attach to the materials used and therefore a new cell delivery vehicle is required for the transplantation of these cells to the wound site. Currently most transplantation techniques of

satellite cells result in the differentiation before implantation or the death of the cell population (Boldrin, et al., 2007) In addition, the lack of rigidity in the threads makes them difficult to implant. The proposed novel scaffold should preserve the axial alignment properties of the current fibrin microthread system while increasing satellite cell attachment properties and minimizing differentiation.

2.5 Potential Means of Improving Cell Attachment

There are several means by which scaffold attachment properties could be improved specifically for human satellite cells. The material of the scaffold could be changed to induce attachment of satellite cells while hindering early differentiation. A variety of coatings could also be applied to the scaffold which would have the potential to increase cell attachment while not affecting the mechanical properties of the underlying scaffold. This coating could be added during the process of producing the scaffold or as an added step after the scaffold's creation. Growth factors, integrins, and proteins, known to inhibit differentiation, could also be used to affect the expression of attachment proteins and enable attachment of human satellite cells. Overall the scaffold should ideally mimic the native skeletal muscle ECM surface topography, mechanical stiffness, and chemical composition so it will promote enhanced cell interactions. The sections below provide a background on the specific materials, proteins, and growth factors that the team is considering to improve the attachment properties of the proposed scaffold.

2.5.1 Materials

Scaffolds for cell delivery and tissue regeneration are designed using a plethora of materials and coatings, taking advantage of the various mechanical and chemical properties of each material. Researches attempt to replicate the conditions in the ECM by using materials that are naturally found in the ECM to build a scaffold. Natural biomaterials found in the ECM often play important roles *in vivo* and usually contain sites for cellular adhesion. However, these materials show lot to lot variability and often have a limited range of mechanical properties (Willerth, and Sakiyama-Elbert, 2008). Advantages to using synthetic over natural materials include the ability to control degradation rate, mechanical properties, and shape directly and independently of each other. However, synthetic materials often lack sites for cell attachment and may produce unwanted by products following degradation *in vivo* (Willerth and Sakiyama-Elbert, 2008).

2.5.1.1 Fibrin

Immediately following an injury, a provisionally matrix forms that guides the repair and regrowth of the tissue. The matrix is made of mainly fibrin along with other extracellular proteins such as fibronectin and vitronectin. Fibrin acts as a scaffold for regeneration by promoting attachment, migration, and proliferation of cells around the wound site (Cornwell and Pins, 2007). It follows that fibrin would be an ideal material for a man made scaffold to deliver cells into the larger wound areas to facilitate large scale regeneration.

The nature of fibrin allows for cell adhesion and the material has a strong affinity for binding growth factors and cytokines found in the provisional matrix during wound healing (Cornwell and Pins, 2010). However, in the presence of cells fibrin often degrades rapidly. Fibrin degradation inhibitors such as aprotinin and factor XIII have been added to gels to maintain their structure over time (Hunt and Grover, 2010).

It has been shown that fibrin is a viable scaffold for cell delivery as discussed in previous sections. Fibroblasts attached to the thread and proliferated as the number of cells multiplied by three or four after two days of cell culture (Cornwell Pins, 2010).. Fibrin can be also modified via crosslinking and stretching to affect the mechanical properties of the material (Grasman, et al., 2012).

2.5.1.2 Collagen

Of the proteins in the human body, collagens are the most prevalent. They form the major portion of the ECM and they thus serve as the main structural component of all connective tissues (Shier, et al., 2012). There are many different applications with various cell populations and differentiation processes (bone, cartilage, heart, ligament, nerve, and vasculature) (Willerth and Sakiyama-Elbert, 2008). This biodegradable and biocompatible (due to being naturally occurring) polymer is approximately 300 nm long and a molecular weight of 300,000 g/mol (Gelse, et al., 2003). The collagen family can be broken down into the following types: fibril-forming collagens (I, II, III, V, XI), basement membrane collagen (IV), microfibrillar collagen (VI), anchoring fibrils (VII), hexagonal network-forming collagens (VIII, X), fibrin associated collagens with interrupted triplehelices (FACIT) (IX, XII, XIV, XIX, XX, XXI), transmembrane collagens (XIII, XVII), and multiplexins (XV, XVI, XVIII). All types of collagen consist of a

right-handed triple helix with three α chains. Non-collagenous domains attached to the central helix also play a role in providing structural support. C-propeptide helps to initiate the formation of the triple helix, N-propeptide plays a role in regulating primary fibril diameters, and telopeptides aid in covalent collagen crosslinking and linking other molecules to the ECM.

The fibril forming collagens are known for their ability to assemble into highly oriented aggregates (Gelse, et al., 2003). Among the 26 types of collagen identified thus far, Type I collagen is the most prevalent in the human body as well as the most researched. This form of collagen contains three polypeptide subunits, where the primary structure of repeating triplets (of glycine, proline, and hydroxyproline) creates a helical structure with high mechanical strength (Nair and Laurencin, 2007). The hydroxyproline aids in the formation of intramolecular hydrogen bonds to stabilize the helical structure (Gelse, et al., 2003). The glycine in collagen provides this polymer with increased flexibility (Nair and Laurencin, 2007). Type I collagen provides tensile strength, tensile stiffness, and torsional stiffness in body tissues such as bone and ligaments. Collagen fibrils are oriented differently in different tissues in relation to the mechanical strength needed in that area of the body (Gelse, et al., 2003). For example, they are oriented in parallel in tendon, but express random, interlaced orientation in skin.

Of the FACIT collagens, type IX has a region in the NC3 globular domain that enables the polymer to be very flexible and interact with proteoglycans and other parts of the ECM (Gelse, et al., 2003). This domain may also play a role in linking different types of collagen fibers together and enabling their interaction with other ECM molecules. Furthermore, microfibrillar collagen (type VI) is known for the long posttranslational processing they undergo to enable their high parallel alignment and aggregation into filaments to form networks in most types of connective tissue.

The primary type of collagen found in the basement membranes is type IV, which has the capability to integrate laminins into a two dimensional aggregate (Gelse, et al., 2003). Three domains form the structure of type IV collagen: the N-terminal 7S, the C-terminal globular domain, and the central triple helix with Gly-X-Y repeats. The heterotrimers aid in creating a network in the basement membranes of most embryonic and adult cells, while dimers interact with domains on type IV collagen to aid in cross-linking.

Collagen can be produced, processed, and modified in different forms, such as being made into sheets, foams, matrices, powders, and injectable solutions. Fibril-forming collagens spontaneously aggregate into ordered fibrillar structures *in vitro* and this ability stems from their structure, hydrophobic interactions, and electrostatic interactions. The fibril formation is largely affected by procollagen molecules and the formation of covalent crosslinks as well. Each type of collagen has its own advantages and disadvantages in regards to being used as a biomaterial. Collagen and ECM interaction can be mediated with biochemical factors such as glycoprotein VI, some proteoglycan receptors, and some integrins (Gelse, et al., 2003). It is through this signaling that different types of collagen promote different amounts of cell adhesion, proliferation, and differentiation. Collagen is also being used in making bioengineered skin for burn victims, diabetes and wound care (dressings and ulcer wounds) (Nair and Laurencin, 2007).

The high reactivity of collagen also aids in the substrate to be crosslinked in many different ways such as when the agents aldehydes, carbodiimides, posuccinimidyl ester polyethylene, and polproxy compounds in addition to cross-linking using thermal and energy irradiation, and chemically modifying the collagen to form gels (Nair and Laurencin, 2007). Collagen plays a large role in initiating the cascade for blood coagulation and thus is used in applications such as cardiovascular and spinal procedures (Gelse, et al., 2003). Succinylated collagen gels are used as drug delivery vehicles, protein delivery vehicles, as well as scaffolds in tissue engineering, due to allowing one to control the porosity, density, and degradation rate of these gels. Collagen also has the potential to enable storage and release of cellular signals and mediators such as growth factors and cytokines. For example, type IIA collagen has been shown to bind transforming growth factor-beta (TFGβ) and bone morphogenetic protein-2 (BMP-2). Studies have shown that the patient's immune response to a collagen vehicle is based on the species the collagen was isolated and/or derived from, the collagen processing method, and the site where the scaffold is implanted (Nair and Laurencin, 2007). Unfortunately, pure forms of certain types of collagen can be expensive and the mechanical properties and degradation times are not always consistent. As a result, current processes for producing human collagen using recombinant DNA are being developed.

2.5.1.3 Vitronectin and Fibronectin

Vitronectin and fibronectin are adhesive glycoproteins that play a role in directing cellular function in muscles (Sinanan, et al., 2008). Their adhesive properties are resultant of the Arg-Gly-Asp (RGD) peptide sequence present on both substrates that mediates cell attachment and cell spreading on an ECM with the aid of integrins (Schvartz, et al., 1999; Macri, et al., 2007). These proteins are expressed at myotendinous junctions within the extracellular matrix (ECM) of skeletal muscle (Seiffert, 1997; Kannus, et al., 1998). Both adhesive glycoproteins have been found to produce a similar level of expression for αv integrins (Sinanan, et al., 2008). Furthermore, both glycoproteins have been found to induce cell migration to a wound site (Macri, et al., 2007).

Vitronectin is one of the primary factors in serum that allows for cell attachment to tissue culture plastic (TCP), and tissue culture glass (Schvartz, et al., 1999). In addition to promoting and regulating cell adhesion, such as in osteoblasts, vitronectin controls enzyme cascades such as the ones that comprise the complement system and coagulation process (Armentano, et al., 2010; Seiffert, 1997). The glycoprotein also contains binding sites for thrombin-antithrombin III complex (TAT), plasminogen activator inhibitor-1 (PAI-1), collagen, and αv integrins (Schvartz, et al., 1999; Sinanan, et al., 2008). It also contains binding domains for plasminogen, heparin, and PAI-1. The collagen and glycosaminoglycan (GAG) binding domains on vitronectin enable anchoring to the ECM.

Based on *in vitro* studies, vitronectin is degraded by thrombin, elastase, and plasmin at various cleavage sites (Schvartz, et al., 1999). Since these factors are present in wound healing sites, vitronectin has a decreased ability to enable cell attachment and binding to plasminogen activator inhibitor-1. Vitronectin is expressed at high levels in skeletal muscle compared to other tissues, but its expression in skeletal muscle is less than that of fibronectin (Kannus, et al., 1998; Sinanan, et al., 2008).

Fibronectin has been shown to strongly stimulate endothelial cell differentiation and vascularization, using a network of materials in a study completed with embryonic stem cell-derived embryoid bodies (Battista, et al., 2005). This glycoprotein plays a large role in the wound healing process as there is often as absence of fibronectin in the wound bed of chronic

burns and wounds, as fibronectin aids in immobilizing growth factors at the wound site (Macri, et al., 2007). This substrate also contains binding sites for fibrin, and thus can be used in conjunction with fibrin in wound healing applications.

Fibronectin has also been reported to be present throughout muscle tissue, including the endomysium and perimysium (Kannus, et al., 1998). This protein is specifically found in the ECM between bundles of myofibers, and between collagen fibers at myotendinous junctions. Unlike vitronectin, fibronectin is a very minor receptor for αvβ3 and αvβ5, as blocking the interaction between the integrins and the substrate (fibronectin) resulted in only a 10-20% decrease in cell adhesion (Sinanan, et al., 2008). However, fibronectin plays a role in maintaining skeletal muscle integrity and can facilitate the removal of damaged myofibers by macrophages, as well as direct myogenic and non-myogenic cells within muscle compartments.

2.5.1.4 Laminin

Laminin can be found in the basement membranes of skeletal muscle as well as the capillaries of muscle-tendon units (Kannus, et al., 1998). Laminin plays an important role in cell migration, differentiation, and axonal growth (Kannus, et al., 1998; Gullberg, et al., 1995). In particular, it has been found to increase the ability of embryonic stem cells to differentiate into beating cardiomyocytes (Battista, et al., 2005). Other studies have shown that laminin can be incorporated onto substrates with microgrooves or microfilaments by covalent binding, physical adsorption, or electrospinning.

Electrospinning has been used to create tissue engineered scaffolds because it is a simple fabrication process to produce nano-sized and micro-sized synthetic polymeric fibers. These fibers are very similar in form and structure to natural protein fibrils in the extracellular matrix (ECM) (Koh, et al., 2008). Mimicking the properties of the ECM will help guide satellite cells in differentiation and muscle regeneration. Electrospinning uses an electric field generated by an applied voltage to create surface charges on a polymer solution. As these charges increase, a polymer jet stream forms through which fibers can be drawn. This technique has been used with PLLA, PGA, collagen, and gelatin for studies in bone, vascular, peripheral nerve, and other tissue engineered scaffolds. In addition, different materials can be use in conjunction during the electrospinning process to create coated materials. In one study, electrospun PLLA fibers were

coated with laminin and were found to increase neurite outgrowth. The directional guidance of the scaffolds created improved axonal outgrowth and improved rate of nerve regeneration (Koh, et al., 2008). These results could potentially apply to the directional regeneration of muscle tissue as well.

2.5.1.5 Chitosan

Chitosan is a natural material that has been investigated for several biomedical applications including cell implantation materials. It is a partially deacetylated derivative of chitin that has structural similarities to GAG (Tan, et al., 2008). Chitosan has been used as a wound treatment throughout history and recently has been shown to improve healing as a result of higher mitotic cells in the wound bed, greater macrophage infiltration, faster re-epithelialization, increased angiogenesis, and greater collagen deposition. These capabilities also resulted in less scarring of the wound and specifically increased wound healing rates and the regenerated area's strength. Additionally, when chitosan was researched as a biomaterial, it exhibited increased cell attachment and cyotokines and growth factor productions which could be relevant in the team's desired application. Chitosan also may provide antibacterial properties which may be beneficial for its use as an implantable biomaterial (Hamilton, 2006). Chitosan is positively charged and can be solubilized when the solution is below of pH of 6 to form tailorable gels. The degradation of chitosan materials in vivo releases non-toxic products (Hsieh, et al., 2005). In addition, chitosan allows rapid cell expansion and can maintain a differentiated cell type. However, a drawback of this material is that in its common form as a gel, it has poor mechanical stability and a faster rate of degradation (Tan, et al., 2008). Chitosan alone also has cytotoxicity issues and poor hydrophilicity (Hsieh, et al., 2005).

2.5.1.6 Hylaronan

Hylaronan (HA) is one of the major components of the ECM and contains sites for cell adhesion. It is upregulated during embryogenesis as seen in the culturing of embryonic stem cells and this material can also be used for the differentiation of adult stem cells (Willerth and Sakiyama-Elbert, 2008). HA consists of alternating units of N-acetyl-D-glucosamine and glucoronic acid, and it can have a molecular weight greater than a million grams per mole (Nair and Laurencin, 2007). HA is synthesized from the direction of hyaluronan synthase-1 (Has-1), Has-2, and Has-3,

which are transferases. Although other glycosaminoglycans in the body such as heparin sulfate covalently bind to proteins, HA does not. Furthermore, HA is water soluble, highly viscoelastic, and can form three dimensional structures with extensive hydrogen bonding while in solution. The high solubility of this polymer allows it to be made into different three dimensional constructs for various applications as well as being made porous through methods such as freeze-drying (Jiang, et al., 2011). For example, the product OSSIGEL® is a viscous HA compound with bFGF (basic fibroblast growth factor). This polymer can also be cross-linked by many physical and chemical means.

The half life of natural HA in the body (in terms of degradation) varies from a few minutes to a few weeks depending on the tissue location and properties. In wound healing applications, the degradation of HA can be reduced through chemical modifications such as crosslinking and esterification with ethyl/benzyl esters (such as HYAFF®, which has a degradation range from a few weeks to a few months) (Nair and Laurencin, 2007). This polymer degrades inside the body via free radicals in the ECM as well as through lysosomal degradation after endocytosis.

Natural HA also provides some of the structure in tissues such as in articular cartilage (Nair and Laurencin, 2007). Besides playing a structural role, HA has also been found to be involved in cell migration and differentiation, ECM regulation, metastasis, wound healing, and inflammation. HA is naturally produced by cells during wound healing, and aids in tissue repair by promoting mesenchymal and epithelial cell migration and differentiation. This behavior leads to increased collagen deposition as well. In addition, HA binds to a variety of cell surface proteins on various cell types. It regulates inflammatory cell recruitment, release of inflammatory cytokines, and cell migration and as a result is currently being used as a cell delivery vehicle for bone tissue regeneration and repair. The limitations associated with this material are related to its inadequate mechanical properties and rapid degradation rate by enzymes *in vivo* (Jiang, et al., 2011).

2.5.1.7 Silk Fibroin

Silk Fibroin (SF) is a structural protein that has been found to be non-toxic, non-immunogenic, and demonstrates supportive cell and tissue growth. It has been established as a biomaterial for tissue scaffolding mainly as a result of its slower degradation rate, desirable mechanical

properties, and its ability to form tissue conductive microstructures (Willerth and Sakiyama-Elbert, 2008; Garcia-Fuentes, 2009). SF is extracted from silkworm silk to be used as a scaffold for many *in vitro* applications such as tissue engineering of bone, cartilage, and ligaments. SF also has beneficial mechanical properties that resemble many structural tissues in the body and a longer degradation time than 3 weeks which makes it an ideal candidate for many of the above applications. The major drawback regarding this material is that no evidence has been presented to prove that SF alone can interact with cell receptors or actively trigger regenerative processes (Garcia-Fuentes, et al., 2009).

By combining various materials, the capabilities of a scaffold could be improved. One example is the mix of SF and HA. SF acts as the main structural and tissue conductive component by providing sound mechanical properties and slow biodegradation. HA, on the other hand, provides a biomimetic surface for the culture and ingrowth of cells such as mesenchymal stem cells. One example of this blend was processed so it formed porous microstructures to further enhance 3D tissue formation. Pores were created in the SF-HA blend using a mild water-based freeze-drying technique. Studies performed using SF and HA scaffolds in combination with human mesenchymal stem cells suggested that the scaffolds were able to trigger regenerative stimuli, which could be of future interest to research regarding the regeneration of a variety of tissues (Garcia-Fuentes, et al., 2009).

2.5.1.8 Bioglass

Bioglass has been investigated for use as a scaffold in the body for orthopedic applications. This non-crystalline material is nontoxic, biocompatible, and is composed of SiO_2, Na_2O, CaO, and P_2O_5, which release calcium, sodium, silicone, and phosphorous ions as degradation occurs (Rezwan, et al., 2006). Other ion releasing molecules found on particular types of Bioglass include magnesium oxide and potassium oxide. As a result, the metabolic activity of local cells increases and this behavior marks Bioglass as being a bioactive material. Specifically, osteoblasts have been found to proliferate and differentiate as a result of exposure to these ions (Jones, 2012). There is potential that these ions could have proliferative effects on skeletal muscle as well since they could up regulate the gene expression of growth factors affecting the

satellite cells, or potentially these ions could negatively affect their proliferation and differentiation (Rezwan, et al., 2006).

Bioglass is used on the market as a coating as well as porous solid (such as in bone fillers). It has also been considered for use in drug delivery. Due to the brittle nature of this ceramic, part of it can be converted to create a partially crystalline material (Jones, 2012). Bioglasses support enzymatic activities, vascularization, and cellular adhesion, and they can be easily processed to modify ion concentrations, shapes, and porosity. This material has a very slow degradation rate and also has high rigidity (Yao, et al., 2007). Both of these properties are adjustable based on which type of Bioglass is selected. Borate for example has about a 6 week degradation time tailored to mimic the length of time it takes a simple fracture to heal (Rezwan, et al., 2006).

2.5.1.9 Poly-γ-glutamic Acid (γ-PGA)

Poly-γ-glutamic acid (γ-PGA) is a water-soluble, biodegradable, and composed of only one type of amino acid. Modified forms of γ-PGA are used as drug delivery vehicles and bioactive tissue engineering scaffolds. γ-PGA has also been combined with FGF-2 and sulfonate to create γ-PGA-S, which has a lower anticoagulant activity and enhances FGF-2 activity. It can also be modified to exhibit thermosensitivity as well as a balance between hydrophobic and hydrophilic groups in the chain. Unfortunately; there is a limited availability of γ-PGA, which limits the number of research studies that can be done with pure γ-PGA (Nair and Laurencin, 2007).

As a result, γ-PGA has been used to modify chitosan matrices to create composite biomaterials of varying densities and porosity for tissue engineering applications. These studies concluded that composite matrices with γ-PGA created a more neural contact angle and thus encouraged cell attachment and proliferation (Hsieh, et al., 2005). It was also found that adding γ-PGA increased the mechanical properties of the matrix. In addition, increasing the amount of γ-PGA in the composite matrix, increased the adsorption of serum proteins and further smoothed the surface of the matrix, the latter of which is shown in the scanning electron microscopy (SEM) micrographs in Figure 12 below (Hsieh, et al., 2005).

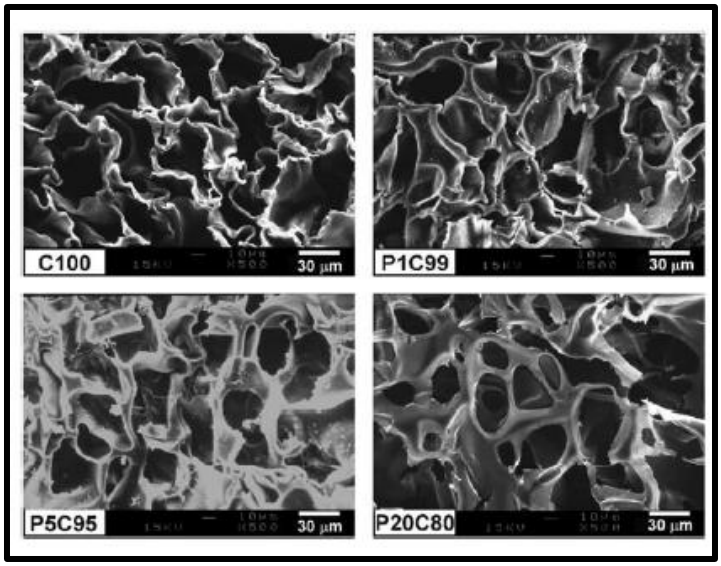


Figure 12: SEM micrographs of chitosan and γ-PGA porous composite matrices (Hsieh, et al., 2005)

Cross section of 100% chitosan matrix (C100), of 1:99 γ-PGA and chitosan matrix (P1C99), of 5:95 γ-PGA and chitosan matrix (P5C95), and 20:80 γ-PGA and chitosan matrix

2.5.1.10 Polylactic-co-glycolic acid (PLGA)

Polylactic-co-glycolic acid or PLGA is a synthetic material that is co-polymer based. Synthetic materials can be used as an alternative to natural materials and are often more reproducible due to their defined chemical composition. PLGA has been used in conjunction with stem cells and applied to adipose, bone, cartilage, muscle, and nerve tissue (Willerth and Sakiyama-Elbert, 2008). It is a highly biocompatible material and approved by the United States Food and Drug Administration (FDA) for certain applications. In one study, PLGA spheres were used with adipose tissue-derived adult stem cells (ADSCs), which have been reported to have myogenic capability under certain culture conditions. The spheres provided the appropriate rigidity and function similarly to porous scaffolds *in vivo* (Kim, et al., 2006). However, in the presence of cells, PLGA degrades into two monomers, which are natural metabolites. These monomers could have negative effects as they are acidic in nature (Willerth and Sakiyama-Elbert, 2008).

The spheres were loaded with ADSCs by culturing and stirring both together. Treated and untreated spheres were then implanted into the back of the neck of mice. Results were observed

after 30 and 60 days. The study showed that new muscle growth was present in the treated PLGA spheres but not in the untreated spheres as seen in Figure 13. Therefore, the spheres served as an adequate delivery system for the adipose tissue-derived adult stem cells and could act as a basis for muscle regeneration (Kim, et al., 2006).

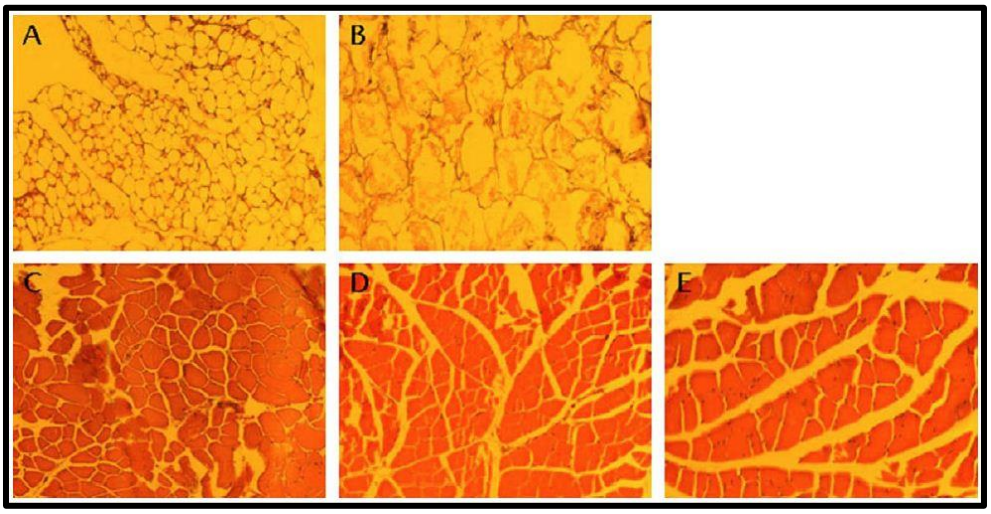


Figure 13: Hematoxylin and eosin staining showing newly formed muscle tissue of mice injected with PLGA spheres (Kim, et al., 2006)

(A) after 30 days (B) 60 days (C) PGLA spheres with ADSCx attached after 30 days (D), 60 days, (E) native muscle

In addition to being used as a scaffold for muscle engineering, PLGA has also been used in neural tissue studies (Willerth and Sakiyama-Elbert, 2008). In another study PLGA along with another copolymer was modified to by difference processing to resemble the spinal cord as discussed above (Teng, et al., 2002). The inner scaffold was made using a salt leaching process to make the material porous and mimic the gray matter of the spinal cord. The outer portion resembled white matter with radial porosity to encourage growth of long nerve endings. These studies showed the versatility of a material like PLGA, as it supports various cell types and can be made into micro spheres or molded to resemble the scaffold of the spinal cord. This also shows that the scaffold form and chemical properties greatly affect the behavior of each cell type.

2.5.2 Satellite Cell Expression

Satellite cells lack expression of myogenic regulatory factors, and therefore markers such as cell surface receptors, adhesion proteins, growth factors, and transcription factors can be used to identify satellite cells (Shi and Garry, 2006). A selection of these receptors and proteins are valuable for identification, regeneration, and controlled differentiation of satellite cells.

2.5.2.1 c-Met Receptors

Expression of the c-met receptor has been found to uniformly label the quiescent satellite cell pool found in muscle tissue. Met is a tyrosine kinase receptor for the cytokine ligand, hepatocyte growth factor. This receptor is expressed during early development and in adult skeletal muscle. Embryos with the absence of c-met are not viable and lack limb musculature. M-cadherin is a cell-adhesion molecule expressed in satellite cells that is up-regulated during muscle regeneration. Functions that have been proposed for this integrin include anchoring the satellite cell to its location and/or aiding in the migration of the cell to areas of injury to begin repair. Other integrins and adhesion proteins that serve as markers for satellite cells include vascular cell adhesion molecule 1 (VCAM-1), MyoD, myf5 from the family of myogenic regulatory factors (myf), myogenin, and neural cell adhesion molecule (NCAM) (Hill, et al., 2003). These integrins and proteins are shown below in Figure 14, illustrating which stage of muscle regeneration each is expressed.

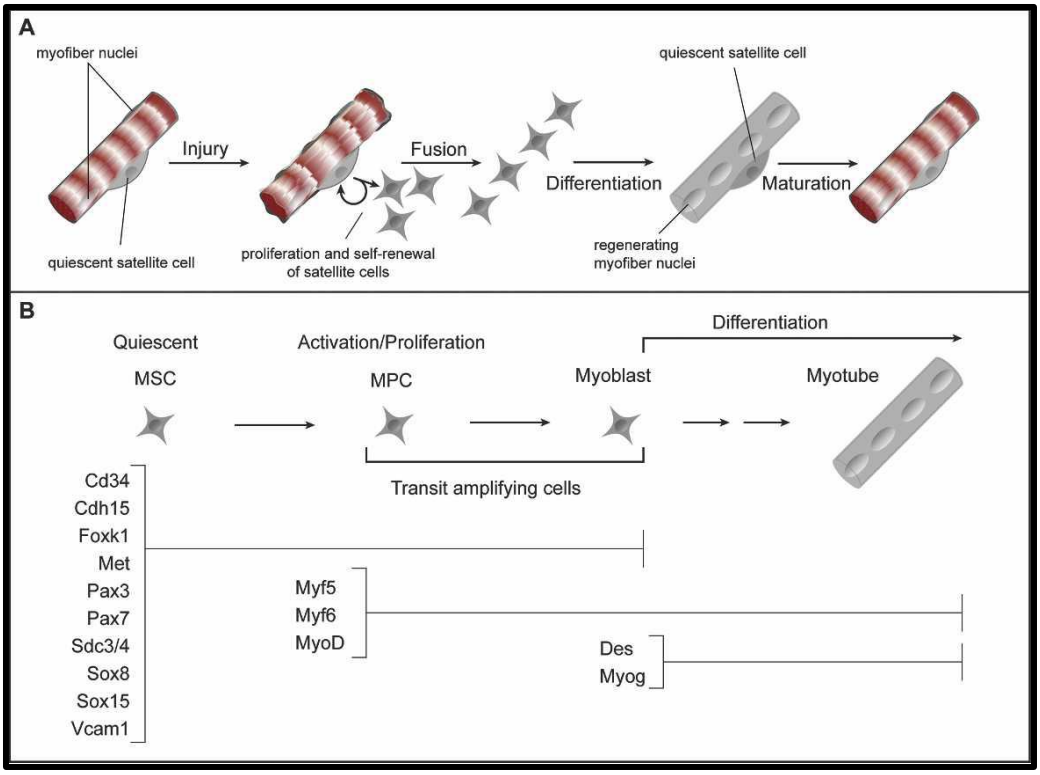


Figure 14: Schematic of satellite cell expression in muscle regeneration (B) (Shi and Garry, 2006)

2.5.2.2 Growth Factors

Insulin-like growth factors (IGF-1 and IGF-2) function as hormones and paracrine factors in skeletal muscle. Over expression of IGF-1 restores the regenerative capabilities of aging skeletal muscle by increasing satellite cell activation and proliferation and inducing recruitment of non-muscle stem cells. Myostatin is another growth factor that is expressed in skeletal muscle cells. Data from previous studies suggest that myostatin may serve a role in maintaining satellite cell quiescence and repressing self-renewal. In contrast, fibroblast growth factors (FGF) are released to promote the proliferation of activated satellite cells through a mechanism requiring the presence of heparin sulfate in the environment (Shi and Garry, 2006).

One study created a local muscle injury in rats to demonstrate and track the effects of satellite cells in muscle tissue regeneration. The hind leg of each rat was injured locally by either over stretching the muscle or injecting a myotoxin to induce satellite cell migration and regeneration. The study focused on how the satellite cells upregulated IGF-1 and mechano-growth factor

(MGF) in order to start muscle repair and adaptation (Hill, et. Al., 2003). Other studies have demonstrated that cultured murine myoblasts, derived from the satellite cell population, showed decreased engraftment efficiency when they were cultured and then transplanted. In contrast, when murine muscle stem cells were transplanted directly into the target area, they showed increased engraftment efficiency and muscle regeneration (Borselli, et al., 2011).

2.5.2.3 αvβ3 and αvβ5 Integrins

Sinanan studied the role of αv family integrins in cell adhesion (2008). Cell adhesion of human craniofacial muscle derived cells (hCMDC) to vitronectin was blocked from 100% adhesion (initial cell adhesion without anti-αvβ3 and anti-αvβ5) to 32% adhesion. However using anti-αvβ3 alone resulted in 83% adhesion, and using anti-αvβ5 alone resulted in 66% adhesion. This shows that the interaction between vitronectin and both αvβ3 and αvβ5 integrins play a large role in cellular adhesion. In humans, the expression of these two integrins decreases as myotubes begin to form (Sinanan, et al., 2008).

2.5.2.4 Tenascin C

Tenascins are a family of four extracellular matrix proteins. Each tenascin (-C, -R, -X, -W) has a specific expression pattern. Most ECM proteins promote cell adhesion, however some tenascins have been classified as anti-adhesive proteins (Chiquet-Ehrismann, 2004). Research from one study observed that by reloading the atrophied muscle of rats increased the tenascin-C expression around damaged muscle fibers (Falco, et al., 2011). Tenascin-C was classified as an adhesion-modulating protein. It supports neurite outgrowth and inhibits branching of newly formed axons (Chiquet-Ehrismann, 2004). The exact physiological role of tenascin-C remains unclear; however, one study demonstrated that tenascin-C deficient mice showed selective atrophy of fast-muscle fibers. Their research suggests that a pathway managed by tenascin-C improves muscle repair (Flück, et al., 2008).

CHAPTER 3: PROJECT STRATEGY

This chapter describes the steps taken to prioritize the various objectives and constraints that would result in a successful project. The project approach section outlines the steps necessary to create the proposed cell delivery vehicle.

3.1 Initial Client Statement

The following represents the initial client statement given to the team by the client, Dr. Raymond Page.

> *"Currently, the laboratory uses extruded fibrin microthreads with human skeletal muscle derived cells seeded onto the surface and transplanted into SCID mouse skeletal muscle injury models to study the effect of various cell derivation and culture methods on functional tissue regeneration. Results from initial histological studies indicated that the fibrin microthreads degraded rapidly and were essentially replaced with granulation tissue within 1-2 weeks of implantation. Some of the regenerated muscle fibers in the wound bed were the result of proliferation, fusion and maturation of human myoblasts transplanted with the fibrin microthreads. It is hypothesized that if the fibrin microthreads could be modified such that their degradation rate was slowed, enhanced cell engraftment and alignment could be achieved. Enhanced resistance to plasmin degradation in vitro has been accomplished by studies done in cooperation with the Pins lab (Worcester Polytechnic Institute, Worcester, MA) using NHS/EDC cross-linking where the degree of cross-linking is controlled by the pH of the reaction. However, cell adhesion studies have only been conducted with immortalized mouse C2C12 myoblasts. Recent advances in adult human primary satellite cell suggest that during the growth phase (prior to induction of differentiation) a significant population of cells exist in the non-adherent state. The adhesion properties of these cells to fibrin is unknown currently, however it is likely that they will exhibit reduced adhesion strength or delayed adhesion compared to traditionally cultured satellite cells.*

The goal of this project is to design a cell delivery system that preserves the axial alignment properties of the current fibrin microthread delivery system yet permits the transplantation of the non-adherent cell population."

Team design meetings and client interviews helped to clarify this initial client statement and create sets of objectives and constraints. These lists were used to generate a revised client statement that clearly and concisely described the problem and its desire solution.

3.2 Objectives

Through research, the team has compiled a list of objectives to describe the design problem. Objectives describe the desired attributes or characteristics of a designed device or system (Dym and Little, 2009). Designers often express objectives in terms of what the device will be and reflect what the client or potential users would like to see in the design. After examining the client statement, conducting a client interview, and researching cell delivery systems, the team compiled the following list of objectives.

Objectives:

- Reproducible
    - Cell attachment
    - Axial cell alignment
    - Properties of the scaffold
        - Strength
        - Elasticity
        - Surface topography
- Efficient
    - Cell attachment
    - Use of materials
- Useful
    - Promote regeneration of native muscle
    - Inhibit scar formation
- Implantable

    - Biocompatible
    - Biodegradable
    - Small in size
    - Simple to handle/implant
- Economical Solution

These objectives were then organized into an objectives tree as seen in Figure 15. The higher-level objectives of reproducible, efficient, useful, and implantable describe what attributes the design should have on a broad scale. Each higher-level objective was broken down into more descriptive, detailed objectives. The system and surrounding process must be reproducible in cell attachment, axial cell alignment, and properties of the scaffold. These attributes must be consistent from one test to the next so that each system is reliable and will produce similar results when implanted into the patient.

Similarly, the system needs to be efficient in both cell attachment as well as with the use of materials. The process will be optimized so that minimal waste will be generated during experimentation and from the final design. In order to optimize the cell attachment many methods will need to be tested and analyzed. This system must also be useful in that it will promote the regeneration of native muscle in traumatic injuries as well as inhibit scar formation. However, these last two objectives mandate *in vivo* testing and thus are outside of the scope of this project due to time constraints.

In addition, the system should be implantable into an animal model for further study. Therefore, the scaffold should be both biocompatible and biodegradable to minimize the adverse affects of implanting a foreign object into the body. The scaffold must remain in the body long enough to provide the needed support and alignment to the implanted cells but also degrade to minimize the negative response of the body. Moreover, the scaffold should be small in size so that it can be implanted easily within a muscular wound. Surgeons or researchers should also be able to implant the scaffold easily and it should be simple to handle. This includes having enough rigidity so that the scaffold stays aligned with the native muscle.

Finally, the design team's change to the current system should be economical. This system will be used in an academic laboratory, and the client would prefer to have a solution that is feasible in regards to the lab's budget.

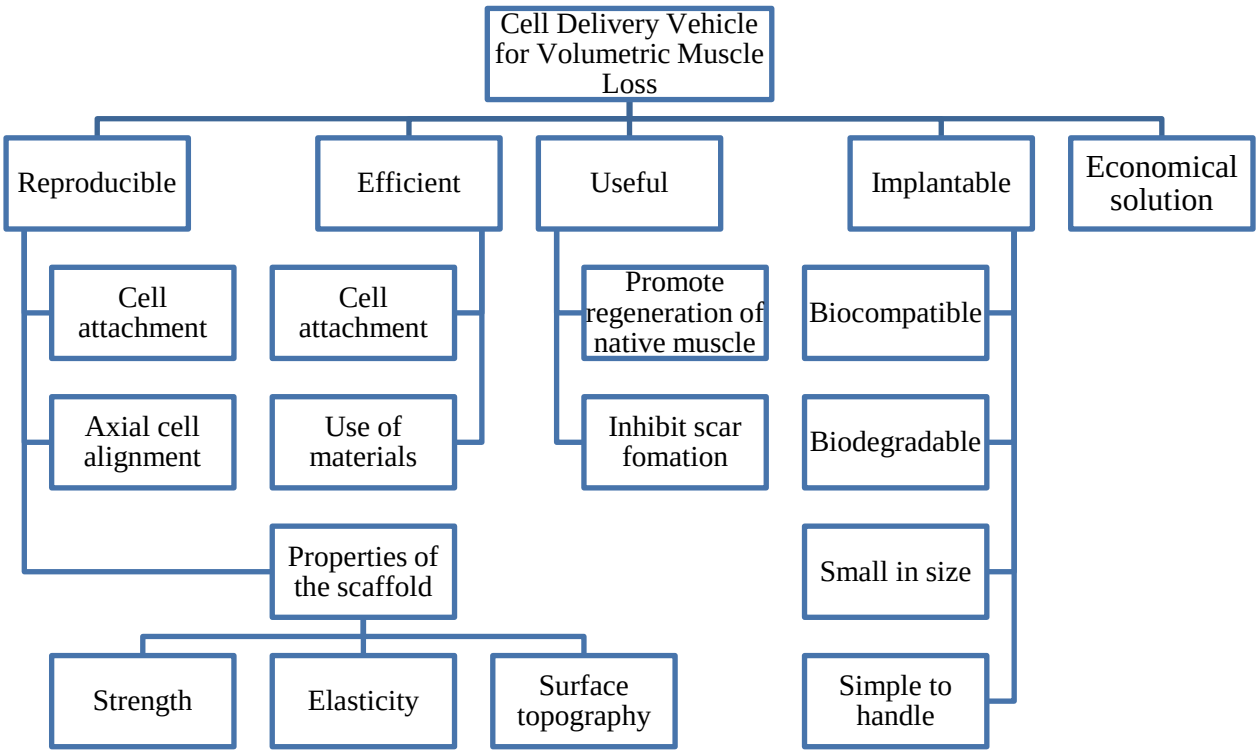


Figure 15: Objectives Tree

To rank our objectives in order of importance, they were organized into a Pair-wise Comparison Chart (PCC) by level. As seen in Table 1, the higher-level objectives were compared against each other and the final score was tallied to show its rank. Next, the detailed objectives were compared against each other, and each subset of objectives was also ranked as seen in Appendix A and Appendix B.

As seen by the Pairwise Comparison Chart for the broad objectives, usefulness of the design was ranked first. The system should be able to complete its primary goal of promoting the regeneration of native muscle and inhibiting scar tissue formation. If it is not able to meet these

goals then the system will not serve any purpose. Furthermore, the system should be implantable into an animal model or human patient. Without this attribute, there is no way to further research and experiment the design.

Table 1: Pairwise Comparison Chart - Broad Objectives

	Reproducible	Efficient	Useful	Implantable	Economical	Score
Reproducible	X	0.5	0	0	1	1.5
Efficient	0.5	X	0	0	1	1.5
Useful	1	1	X	1	1	4
Implantable	1	1	0	X	1	3
Economical	0	0	0	0	X	0

Ranked next, the design should be both reproducible and efficient. These are important objectives to the success of the system; however, being implantable and useful directly affects the functionality of the system, while reproducibility and efficiency describe the performance quality of the system. Reproducible and efficient were ranked as equally important attributes of the system. Having an economical solution to the design problem was ranked last as the client is more concerned with having a functional cell delivery system than a low cost design.

3.3 Design Constraints

Constraints serve as the boundaries for the design space and allow for the initial evaluation of design ideas. If these factors are not satisfied, the design is considered a failure (Dym and Little, 2009). The list of constraints below was derived from the initial client statement, client meetings, and team design meetings.

- The design must follow regulatory guidelines.
    - Safe for the user and patient
    - Sterile
- The design team is limited to the technology available at WPI
    - Manufacturing
    - Testing

- All testing must be performed *in vitro.*
- A maximum of 27 weeks is available.
- The team has a budget of $456 for purchasing additional substrates and biochemical factors for testing (those not already available in the laboratory).

Ultimately, if the final design is not sterile and safe for the user and the patient, the design cannot be implemented in the laboratory. The U.S. Food and Drug Administration is an example of a regulatory body that establishes measures for sterilization and safety to evaluate products prior to their commercial use. Although the design team is not designing a system for immediate release into the market, the regulatory measures will serve to assure the design is safe to use in an academic laboratory, including that of the client. Furthermore, the design team is also limited to the technology available at WPI for both manufacturing and testing the design alternatives. This confines the number of generated designs that can be pursued further into prototyping and testing, and also limits the types of experiments that can be performed. In addition, all testing must be performed *in vitro*, on 2D and 3D constructs. Furthermore, the entire project, including research, design, experimentation, and validation must be completed within 27 weeks. Finally, the team has a budget of $456 for the purchasing of substrates and biochemical factors that are not already available in the laboratory. All other expenses are provided by the client.

3.4 Functions

Based off the client statement there are four main functions of the combined process and device as seen in Figure 16 below. The process should transplant the non-adherent cell population (satellite cells), increase satellite cell attachment, not cause premature differentiation of the cells, and preserve the mechanical integrity of the current fibrin microthread system.

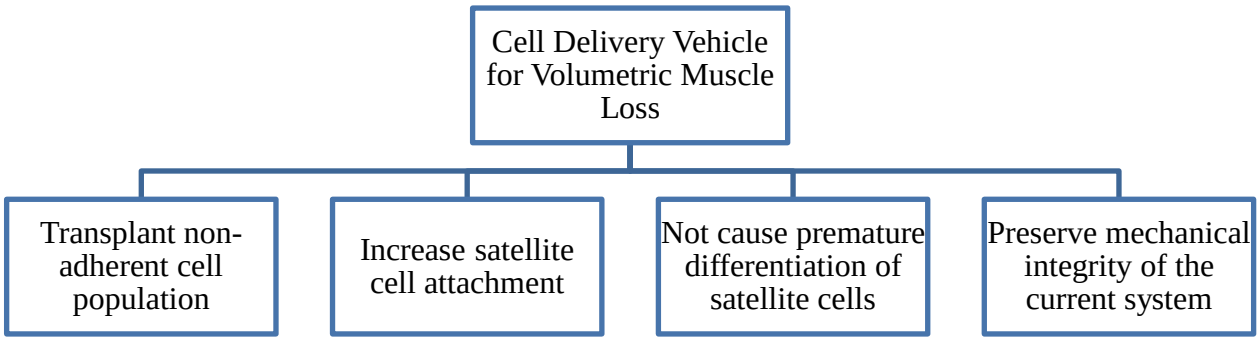


Figure 16: Functions Tree

The system should transplant the adherent cell population from one point to another. This function relates back to the primary goal of this project. If the system can not enable transplantation of satellite cells, then the system has no purpose and would not be useful to continuing research to promote muscle regeneration and inhibit scar tissue formation. One method of transplanting the satellite cells is to design a scaffold with compatible mechanical properties by modifying the scaffold form as seen in Appendix C. The general shapes a scaffold can take are a thread, matrix, or gel.

Secondly, the system should increase satellite cell attachment so that cells can be transplanted from one point to another. Again, this function connects directly to the main goal of the project and is key to the success of the system. As seen in the functions means tree in Appendix C, there are many potential ways to increase the cell attachment. The broad means to accomplish this are using a material that allows for increased cell attachment, modifying the surface, and modifying the expression in the satellite cells. Each of these means contains sub-methods of achieving the function that will be explored in Chapter 4.

The system should also not cause premature differentiation of the satellite cells. The goal is to deliver the satellite cells before they have begun to differentiate so that the cells are at their full potential to proliferate to produce additional cells for muscle regeneration. Some experiments have shown that attachment and confluence of the satellite cells can induce differentiation. To avoid differentiation of the satellite cells, the expression of the cells could be modified by increasing or decreasing certain growth factors or modifying gene expression. Additionally, the

team could inhibit confluence of the cells by decreasing the culture time, decreasing the number of cells plated, or modifying the growth factors exposed to the cells.

Finally, the system should preserve mechanical integrity of the current fibrin microthread system. This includes maintaining axial alignment of the fibers to promote alignment of the cells and improving or maintaining the mechanical properties not well addressed in the current system. Most importantly, the axial alignment properties of the scaffold should be maintained. This enables the cells to align along an axial direction so that when the scaffold is implanted, the cells will proliferate and differentiate along the axis and align with native tissue fibers. The regenerated muscle would then contract in the same direction as the native tissue.

Mechanical properties to be improved or maintained in the new design are the stiffness, tensile strength, and diameter of the thread. This would be completed by changing the material or modifying it by crosslinking, stretching, freeze-drying etc. A stiffer scaffold would be easier to implant and would degrade slower, giving the cells the correct amount of time to align, proliferate, and differentiate.

3.5 Specifications

The team determined specifications that related to the functions such as preserving the mechanical integrity of the system or increasing the satellite cell attachment. Our specifications are listed below:

- Attachment
    - Greater than the fibrin microthread system
- Differentiation
    - Little to no differentiation is seen before implantation of the scaffold or without inducing differentiation
- Mechanical and Material Properties
    - Ultimate Tensile Strength > 4.5 MPa
    - Elastic Modulus > 60 MPa
    - Failure Strain ~ 0.31
    - If the scaffold is a gel, then ideal porosity to attract satellite cells is 500-600 microns

- Biodegradable by plasmin
    - Degradation time equal to or greater than fibrin microthreads
- If the scaffold is a thread, the hydrated diameter should be 55-65 μm

A specification for the target amount of attachment could not be set prior to testing since quantification of the current fibrin microthread system was among the first tasks of the design team. Currently, there is little attachment of the satellite cells to the fibrin microthreads so any improvement to this would be beneficial.

The system should not cause premature differentiation of the satellite cells. Therefore, little to no differentiation should be seen before implantation of the scaffold or before inducing differentiation in our experiments. This can be tested by staining the cells for certain proteins found in muscle fibers such as myosin heaving chain, MyoD or Myogenin.

The mechanical and material properties of the current system were quantified and can be modified by crosslinking and stretching. The ultimate tensile strength and elastic modulus of the current system could be increased in the new design. The increase in stiffness of the scaffold would lead to an increased ease of implantation of the scaffold. In addition, if the scaffold is a gel, the porosity of the surface is required to be between 500 to 600 microns in order to attract satellite cells and promote cell adhesion.

The scaffold must also be biodegradable, but must not degrade too quickly within the body. Previous studies with the fibrin microthreads showed that the threads might have degraded too quickly, which led to unaligned formation of myotubes within the regenerated tissue. The scaffold was not present long enough within the body to allow the cells to proliferate along the thread and align with the native tissue. Based on this study, the scaffold should remain in the body for more than one to two weeks to allow for sufficient alignment of the newly formed myotubes.

3.6 Revised Client Statement

As a result of client meetings and the above-mentioned objectives, functions, and constraints, a revised client statement was created. This states the necessary attributes of a successful final design and articulates how the team will evaluate design alternatives.

"*The goal of this project is to create a cell delivery vehicle that will ultimately inhibit scar tissue formation and facilitate muscle tissue regeneration. This will be accomplished by designing a system that permits the transplantation of human satellite cells and increases their attachment to a substrate that maintains the axial alignment of native tissue structure. Ideally this system will be efficient, reproducible, and simple to implant; however, it must be sterilizable and safe, without causing premature differentiation of the human satellite cells.*"

3.7 Project Approach

The objectives and constraints, as outlined in the previous sections will guide the direction of this project. However, in order to fulfill these objectives while remaining within the confines of the constraints, a series of design steps were taken. First, the attachment of satellite cells to the current system of fibrin microthreads was measured. This was accomplished by designing a 2D assay to save both time and materials, which enabled the team to quantify the amount of attachment on multiple substrates. The maximum amount of cell attachment was based on the area available for human satellite cells and also was determined through assay-based experiments. These experiments were feasible with the technology and resources available at Worcester Polytechnic Institute.

From this baseline, the design team brainstormed physical and biochemical methods for increasing the attachment of human satellite cells to the substrate. Possible ways of accomplishing this included changing the material of the 3D substrate, coating the substrate to enhance cellular affinity, coating the substrate to change the surface topography, and modifying the cellular expression of the satellite cells. Based on a numerical evaluation matrix, the designs that best met the team's objectives while complying with the design constraints were selected for testing. A number of assays were performed with each selected design to quantify the attachment of the satellite cells, as well as their myogenic potential. From this point, designs were combined to include the characteristics that produce optimum results. Next, the team moved from conducting testing in two dimensions to working in three dimensions. This was in the form of a microthread that has been modified based on the team's conclusions from the two dimensional testing.

The design of the cell delivery vehicle was driven primarily through testing and experimentation. This is due to the fact that there were many design alternatives that could increase cell attachment working by themselves or in conjunction with other designs. It was difficult to assess the efficiency, the possible negative effects on cellular growth, and integrity of each of these alternatives, without experimentation due to the current state of the literature regarding this particular design problem. In addition, the current amount of attachment was not yet quantified for human satellite cells and therefore testing was complete first to determine the baseline that the team worked from.

CHAPTER 4: DESIGN ALTERNATIVES AND EVALUATION

Generating design alternatives is an important and creative step in the design process. Having multiple options plays an important role in preventing design failure and also allows for the optimal design to be selected and further verified based on the revised client statement. Through tools such as a functions means tree and evaluation matrix, design ideas were generated and assessed. As seen in Section 2.4 and Section 2.5, research shows that there are numerous forms and materials that scaffolds utilize, depending on the behavior of the cells. As a result, the group arranged alternatives into the following categories: a change in the scaffold form, a modification to the scaffold material, an application of a coating, or a treatment to affect satellite cell expression. Each design alternative was evaluated based on its ability to satisfy the team's revised client statement. Specifically, the design needed to accomplish the system's main functions of allowing transplantation of the non-adherent cell population, increasing satellite cell attachment, preventing satellite cell differentiation, and maintaining the mechanical properties necessary for handling and implantation. Each of the broad categories of design alternatives were broken down into specific candidate materials that could be selected for the final design. The positive and negative aspects of each possible choice in regards to design objectives, constraints, and functions are detailed in the sections that follow.

4.1 Design 1: Modify the Scaffold Form

One of the functions of our design is that it should enable transplantation of the non-adherent cell population. In order to do this a physical construct or vehicle is need to move the cells from one point to another as seen in the functions means tree in Figure 16. Based on the discussion in Section 2.4, there are three main forms that scaffolds generally take, including threads, matrices, or gels. These scaffolds have been used in previous research; however, very little has been done working with satellite cells.

A thread as seen in Figure 17, will maintain the axial alignment of the cells to promote correct muscle regeneration. This scaffold allows for cell proliferation and differentiation along an axis so that myofibers will form in the same direction as the native tissue. However, satellite cells do not attach to the current system of fibrin microthreads very efficiently. The microthreads are

often more difficult to implant due to their mechanical properties and small diameter. A new form of the scaffold may be needed.

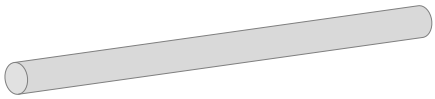


Figure 17: Conceptual design of a thread as the scaffold form

A matrix as seen in Figure 18 often combines multiple materials to take advantage of their various mechanical properties. Matrices take many overall forms and increase the surface area for cell movement, proliferation, and differentiation. However, unlike the thread, material fibers often spread in multiple directions and do not provide uniform axial alignment. This could be modified by attempting to align the fibers of the matrix by bundling threads in one direction.

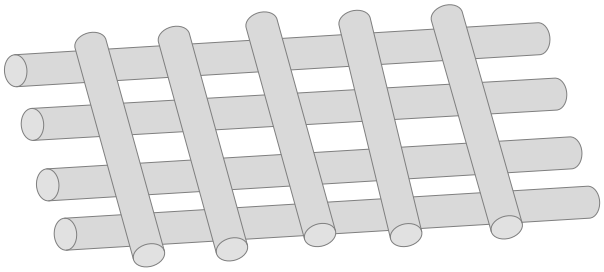


Figure 18: Conceptual design of a matrix as the scaffold form

The final broad form of the scaffold is a gel as seen in Figure 19. Gels occupy a more concrete space than the thread would and can be transported more easily. If the satellite cells were seeded within the gel, they may be able to be transported without needing to attach directly to a certain substrate. Additionally, gels can be made porous which often increases the vascularization and spacing of the resulting tissue as discussed in Section 2.4.2. However, the gel lacks the axial alignment as seen in the thread design. The cells may be transported more efficiently but myofibers would form in vary directions and not comply with the direction of the native tissue. This would result in inefficient muscle formation that would not be able to contract to the same level as the native tissue.

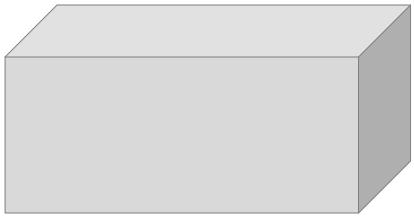


Figure 19: Conceptual design of a gel as the scaffold form

These forms represent the three major shapes that the scaffold can take. As discussed above, each form has advantages and disadvantages to maintain axial alignment of the cells as well as ease of transplantation of the non-adherent satellite cell population. These designs may be combined in the final design to utilize the benefits of each form.

4.2 Design 2: Modify the Scaffold Material

Other functions the team developed were the need of the design to increase human satellite cell attachment and to preserve the mechanical properties of a fibrin microthread system. This could be accomplished through altering the base material of any of the above scaffold forms. Depending on the selected material, different properties can be tailored to suit our needs. Ideally, the material would increase attachment while retaining the strength and increasing the stiffness and degradation time of the current fibrin microthread system. This would allow for improved implantation and increased effectiveness over other researched tissue engineering strategies for volumetric muscle loss. These materials would be evaluated using two dimensional preliminary testing, where each type of material will be sectioned to be an identical size and laid down at the bottom of the well plate with satellite cells would be seeded uniformly onto it to quantify attachment potential.

4.2.1 Fibrin

Fibrin is a logical choice for a scaffold material because of its inherent properties and function in the body. Fibrin promotes attachment, one of the team's main functions, and encourages migration and proliferation of cells around the wound site, which would promote muscle regeneration and inhibit scar formation, two main objectives. Fibrin also has a strong affinity for binding growth factors, which would make modifying the cell expression and chemistry easier to promote attachment of the satellite cells.

Additionally, it has been shown that fibrin microthreads have similar properties such as alignment and strength to that of a muscle fiber. It meets the team objectives of being biocompatible and biodegradable; however, the scaffold may degrade too quickly within the body. Other options may be explored to increase the degradation time such as crosslinking or stretching the scaffold. Fibrin is a versatile material in that it has been used in numerous scaffold shapes in combination with other materials such as collagen. Gels are another common form for fibrin but this scaffold often lacks the axial alignment as discussed in the previous design section.

Fibrin will be tested in the preliminary two dimensional assays as the control, as it shows potential for increasing satellite cell attachment or as base scaffold to which other materials can be attached. Multi-well plates will be coated with each potential material and cells will be seeded on top of the materials.

4.2.2 Collagen

A design alternative for changing the scaffold material is using various types of collagen. As discussed in Section 2.5.1.2, collagen is a critical structural component to the natural ECM. This material meets the team's objectives of having a biodegradable and biocompatible solution that is able to maintain axial alignment while providing structure for the scaffold. As discussed in Section 2.5.1.4, laminin is a natural component in the basement membranes of skeletal muscle and thus the interaction of collagen with this component can potentially promote cell attachment, differentiation, and axial alignment. Further options to explore in using collagen as a design alternative include crosslinking in order to improve mechanical properties and possibly increase satellite cell attachment, as well as changing the form of the scaffold from a microthread to a matrix or a sheet.

4.2.3 Hylaronan (HA)

Similar to collagen, HA is a major component of the ECM, as discussed in the literature review in Section 2.5.1.6. This material was proposed as a design alternative for this category because the material contains sites that encourage cell adhesion and it can be constructed and crosslinked in various three dimensional forms based on the application and cell type. The degradation rate can also be controlled by chemically modifying the material, which makes this candidate likely

to meet the team's objective of achieving a slower degradation rate than the current system. However this material was not tested due to availability and budget constraints.

4.2.4 Silk Fibroin

Silk has many mechanical properties that make it desirable as a scaffold material. Silk can be used as threads or a matrix that have high strength and stiffness coupled with a long degradation time. This biomaterial also has many favorable biocompatible properties such as being non-immunogenic, non-toxic, and inert. Silk has been found in previous studies to support cell and tissue growth and for tissue conductive microstructures, which would make the material perform well in the team's two dimensional testing. Silk scaffolds have been used for many in-vitro applications such as tissue engineering of bone, cartilage, and ligaments. The major drawback regarding this material is that no evidence has been presented to prove that a silk scaffold alone can interact with cell receptors or actively trigger regenerative processes. The design team decided not to test this material due to time and budget constraints.

4.2.5 Bioglass

The material properties of bioglass make it a strong competitor for a scaffold choice. This candidate allows the team to consider multiple scaffold forms since bioglass can be manufactured into a porous solid or an electrospun thread. Bioglass is biocompatible, non-toxic, and also bioactive. As the scaffold degrades bioglass releases a variety of ions, which may trigger cell migration and aid in muscle regeneration. The release of these ions upregulates gene expression of growth factors, which could be either a positive or negative effect on satellite cell attachment and differentiation. In addition, bioglasses support enzymatic activities, vascularization, and cellular adhesion, and they can be easily processed to modify ion concentrations, shapes, and porosity. This material also has a very slow degradation rate and also has high rigidity, which would be beneficial to the aforementioned design objectives, but is brittle which could be a major drawback. A majority of these mechanical and material properties are adjustable based on which type of bioglass is selected. Experimentation with different forms of bioglass could identify which ion released are favorable to satellite cell attachment without catalyzing differentiation. However, bioglass was not tested due to availability and budget constraints.

4.2.6 Poly-γ-glutamic Acid (γ-PGA)

Another option for changing the scaffold material is using γ-PGA, which was previously discussed in Section 2.5.1.9. This biodegradable material was chosen as a design alternative based on its history of being used as a vehicle for drug delivery as well as its use in creating various scaffolds. The team believes this material will perform among the best in encouraging cell attachment due to its porosity and its ability to create neutral contact angles when used in conjunction with other materials. Porous materials encourage cells to fill into the pores of the substrate and enhance cellular proliferation and differentiation. Furthermore, the neutral contact angles (neither completely hydrophilic nor hydrophobic) also encourage cell attachment. Section 2.5.1.9 describes other advantages of using this material, making it a candidate for the final design. However, γ-PGA was not tested due to availability and budget constraints.

4.3 Design 3: Coating the Microthread

Another potential method of increasing satellite cell attachment while preserving the scaffold's mechanical properties is to apply a coating on top of the base material. This would allow a scaffold to maintain its strength, stiffness, and degradation properties from the base material, while improving the attachment properties with a separate coating material. Furthermore, it allows for the combination of materials with complementary properties. In addition, the team could use the published protocols for fabricating and sterilizing fibrin microthreads as a basis for developing new fabrication, sterilization, and testing procedures. For two dimensional testing, the material would be dissolved in a buffer solution and then used to coat the bottom of the well. Cells would then be uniformly seeded in the coated well plate. If chosen for three dimensional testing, the dissolved solution would be used to coat the fibrin microthread scaffold in order to enhance cell attachment while maintaining the alignment of the microthread fibers. Selected coating materials are described below to illustrate their potential positive and negative qualities when used as a coating on the current system.

4.3.1 Collagen

In addition to using different types of collagen as a material in the system, collagen can also be used as a coating for the current fibrin microthread system. As mentioned previously in section

4.2.2, collagen is expected to meet the team's objectives of creating a biodegradable solution that encourages cellular adhesion, proliferation, alignment, and differentiation.

4.3.2 Vitronectin

Human vitronectin can be applied to the current fibrin microthread system as a biocompatible coating to increase the adhesion properties of the cell delivery vehicle. Similar to the collagen coating described above, this substrate can be used to coat the bottom of the plate well prior to cell seeding in two dimensional testing. This glycoprotein's RGD peptide sequence may promote cell attachment on the fibrin microthread while also promoting cell spreading on the ECM with the aid of integrins. Vitronectin has been shown to be more adhesive than fibronectin in regards to skeletal muscle. In addition, this coating could be used in conjunction with collagen for improved mechanical and chemical properties due to the collagen binding site on vitronectin, as discussed in Section 2.5.1.3. As a result, this coating has the potential to meet the objectives of reproducibly increasing satellite cell attachment, and promoting regeneration of native tissue. However, it may have a faster degradation rate than the uncoated fibrin microthread due to vitronectin being degraded by thrombin, which would decrease cell attachment over time.

4.3.3 Fibronectin

Fibronectin is also a biocompatible glycoprotein that can be used as a coating to the current fibrin microthread system to increase the attachment properties of the cell delivery vehicle, due to its RGD peptide sequence. Similar to the collagen and vitronectin coatings described above, human fibronectin can be used to coat the bottom of the plate well prior to cell seeding in two dimensional testing. In three dimensional testing, it may produce a more reproducible and efficient coating of the fibrin microthread due to fibronectin having binding sites for fibrin. Furthermore, fibronectin plays a role in directing cellular differentiation and function in muscle tissue as discussed in Section 2.5.1.3. Thus, using fibronectin as a coating may meet the objectives of reproducibly increasing satellite cell attachment, and promoting regeneration of native tissue. It may also prevent premature differentiation due to its ability to keep growth factors at the wound site.

4.3.4 Laminin

Laminin could also be a strong candidate material for satellite cell attachment since it is found naturally in the basement membranes of skeletal muscle as well as the capillaries of muscle-tendon units. Laminin also plays an important role in cell migration, differentiation, and axonal growth, which could aid in satellite cell attachment and perform well in the team's 2D and 3D testing. Laminin on a scaffold also directionally guides cells and was found to improve axial outgrowth and improved rate of nerve regeneration. This could possibly be applied to muscle fibers as well since they require directional guidance. The team could incorporate laminin onto substrates with microgrooves or microfilaments by covalent binding, physical adsorption, or electrospinning.

4.3.5 Chitosan

Chitosan could be used as a coating material for the fibrin microthread system as well. It can be dissolved by lowering the pH and turned into a gel. Its positive charge and structural similarities to GAG may result in favorable cell attachment and proliferation in the team's two dimensional assays. By coating a fibrin thread with chitosan, the team may be able to take advantage of chitosan's beneficial properties, while avoiding its poor mechanical properties as a gel. In addition, the fast degradation rate of the coating may be favorable since many properties of fibrin have been found to be beneficial post-transplantation and after cell attachment has already occurred. One drawback is that this material may cause the cells to prematurely differentiate or may have cytotoxic effects on the attached cell population. There are also a variety of forms of chitosan which would make replication and manufacture of a coated scaffold very difficult.

4.3.6 Bioglass

Bioglass also has the potential to be applied as a coating to a differing scaffold material. This could be a favorable decision since bioglass may have beneficial satellite cell adhesion properties and degradation properties, but it lacks the mechanical strength necessary for handling and implantation as described above in Section 4.2.5. In addition, the lengthy degradation time of many forms of bioglass may be altered depending on the thickness of the coating applied. In general, this coating would allow for many material properties to be tailored to find the ideal

candidate. By combining a strong material that does not allow for attachment of satellite cells with bioglass, there is the potential to create a scaffold with favorable implantation properties and improved satellite cell attachment properties. The design team chose not to test this material due to availability and budget constraints.

4.4 Design 4: Modifying Satellite Cell Expression

In order to regulate satellite cell differentiation as well as improve cell attachment properties, biochemical means can be applied to trigger various events. For example, several growth factors have been found to be up regulated in response to an injury. Some of these have been researched and are specifically used to modify satellite cell expression of attachment proteins. Growth factors can induce differentiation, migration, and proliferation and could be used to increase cell attachment without triggering differentiation before implantation is possible. In addition, some proteins have also been found to interact with these cells and affect differentiation and attachment. In the body, satellite cells interact with the ECM and many of these interactions are mediated by various proteins. By applying these proteins to the scaffold, it may be possible to increase cell attachment. Below are descriptions of various growth factors and proteins along with their potential outcomes.

4.4.1 Growth Factors

The application of a growth factor to the satellite cell population may be able to increase its attachment to the scaffold or delay its differentiation before implantation is possible. Growth factors can be applied through media changes during cell culture or prior to seeding the cells on a scaffold. Insulin-like growth factors (IGF-1 and IGF-2), for example, function as hormones and paracrine factors in skeletal muscle. Over expression of IGF-1 restores the regenerative capabilities of aging skeletal muscle by increasing satellite cell activation and proliferation and inducing recruitment of non-muscle stem cells. This could help with recruiting satellite cells for attachment to the scaffold as a precursor to the muscle regeneration process, which would be completed after cell transplantation. Myostatin is another growth factor that is expressed in skeletal muscle cells, which may serve a role in maintaining satellite cell quiescence and repressing self-renewal. This could be potentially helpful in maintaining the satellite cells in a quiescent state before transplantation and achieving the function of not inducing differentiation.

In contrast, fibroblast growth factors (FGF) are released to promote the proliferation of activated satellite cells through a mechanism requiring the presence of heparin sulfate in the environment. This could be beneficial in multiplying the number of cells so that an adequate number of cells is transplanted. Then the differentiation of the satellite cells would begin muscle regeneration and the wound healing process upon implantation.

4.4.2 Integrins

M-cadherin is one type of integrin that may be applicable for increasing satellite cell attachment to a scaffold with unfavorable cell attachment properties alone. Specifically, m-cadherin is a cell adhesion molecule expressed in satellite cells that is up-regulated during muscle regeneration. Functions that have been proposed for this integrin include anchoring the satellite cell to its location and aiding in the migration of the cell to areas of injury to begin repair, which is why this biochemical means may be very important to a scaffold trying to increase migration and attachment. Other integrins and adhesion proteins that serve as markers for satellite cells include vascular cell adhesion molecule 1 (VCAM-1) from the family of myogenic regulatory factors (myf), myogenin, and neural cell adhesion molecule (NCAM) (Hill et al., 2003). These integrins and proteins could be administered when the satellite cells are being seeded onto the scaffold to improve their attachment potential.

αvβ3 and αvβ5 integrins could also be used to modify cellular expression. Although further research is required to determine whether αv family integrins are expressed by human satellite cells specifically, it is still being considered as a design alternative for the modification of cell expression in order to increase cell attachment while taking advantage of the axial alignment of the current fibrin microthread system. This alternative can be applied to two dimensional testing by either selecting for cells that express a larger concentration of these integrins, which can be detected through immunocytochemistry. Increased expression of these integrins, which is expected to decrease as differentiation occurs and myotubes begin to form, could also be combined with a vitronectin coating for optimum cell adhesion, as previously discussed in Section 2.5.2.3.

4.4.3 Tenascin-C

Tenascins are a family of extracellular proteins that have been classified as anti-adhesive proteins. However, research has shown that tenascin-C expression increases around damaged muscle fibers. The studies suggest that a pathway managed by tenascin-C directs muscle repair. By increasing the tenascin-C in the scaffold, the muscle repair may be more directed and the satellite cells may be more efficient. However, the exact physiological role of tenascin-C remains unclear and this protein may inhibit adhesion of the satellite cells and negatively affect the outcome of one of the team's main objectives. The only benefit tenascin-C may have is increasing the efficiency of the wound healing processes once the cells have already been transplanted.

Tenascin-C may be used in the two dimensional testing to determine whether or not the protein promotes adhesion of the satellite cells. It if is determined that this EMC protein favorably affects the adhesion of satellite cells, the team will move to three dimensional testing to determine if it has the same effect on the full scaffold. Tenascin-C may be incorporated in the preliminary culturing of the satellite cells as well as in the plating on to varying materials for testing.

4.5 Design 5: Combining Candidates from Designs 1-4

If the above four design alternatives do not successfully satisfy the design objectives created by the team, then a combination alternative may be necessary. By merging the above techniques and materials, the team could create a system that outperforms any of the individual alternatives alone. Multiple scaffold forms can be combined to increase cell attachment while maintaining mechanical strength and alignment. A thread system implanted in a porous gel scaffold is one example of accomplishing this. Another option is to choose a new scaffold material and then apply a coating to it. Each material can be selected to target specific functions so the combination design meets all objectives. Biochemical means can also be paired with a specific scaffold or coating material. The material would be selected to interact with an applied growth factor or integrin to modulate satellite cell migration and attachment to the scaffold.

4.6 Preliminary Final Design

After generating design alternatives, the team ranked each category of design alternatives by comparing their ability to meet the design objectives while still abiding by the constraints. Due to the nature of this design process, not all of the objectives can be used to compare the design alternative categories without initial laboratory testing. Likewise, a specific material cannot be chosen as the final design without extensive laboratory testing.

Since the team chose only the objectives that could be tentatively compared the initial numerical evaluation matrix did not use weighted objectives as shown below in Table 2. In the objectives portion, as seen in the top half of the matrix, the number 1 indicates that the design alternative category is most likely to meet that objective when compared to the other categories. Coating the fibrin microthread had the best score, of 2.3. Modifying the scaffold material and modifying the satellite cell expression received the next best scores, of 2.5 and 2.6 respectively. The design alternative to modify the scaffold form received a 3.2, and the design alternative to combine two or more of the first four design alternatives received the worst score, of 4.4.

Table 2: Preliminary Numerical Evaluation Matrix without Weighting

	Modify the Scaffold Form	Modify the Scaffold Material	Coat the Fibrin Microthread	Modify Cell Expression	Combine Designs 1-4
Reproducible cell attachment	1.5	1.5	3	4	5
Maintain Axial Alignment	5	2	3	1	4
Reproducible material properties	5	1.5	1.5	3	4
Similar material properties to the current system	1	4	3	2	5
Efficient cell attachment	5	1.5	1.5	3	4
Biodegradable	2	5	3	1	4
Economical Solution	3	2	1	4	5
Average	**3.2**	**2.5**	**2.3**	**2.6**	**4.4**
Safe	Yes	Yes	Yes	Yes	Tentative
Sterile	Yes	Yes	Yes	Yes	Yes
Manufacturing Feasibility	Yes	Yes	Yes	Yes	Yes
Testing Feasibility	Yes	Yes	Yes	Yes	Yes
Feasible based on time	Yes	Yes	Yes	Yes	Tentative
Only *in vitro* testing	Yes	Yes	Yes	Yes	Yes
Within budget	Yes	Yes	Yes	Yes	Tentative

The design team believed that changing the scaffold form and changing the scaffold material would produce the most reproducible cell attachment due to the use of a single material in both cases. Modifying the satellite cell expression was thought to allow for maintaining the axial alignment of the current system since the scaffold would remain as a fibrin microthread. Changing the scaffold material and coating the microthread was thought to lead to more reproducible material properties since the beneficial mechanical properties of the microthread structure would remain in both cases. Furthermore, it was thought that obtaining similar material properties to the current system would arise from modifying the scaffold form since the scaffold would still be made of fibrin, which is the current material used. In addition, both changing the scaffold material and coating the microthread were thought to produce efficient cell attachment since ideally this would lead to better adhesive properties than the current fibrin microthread. However, fibrin is biodegradable, and thus modifying the satellite cell expression was the design

thought to be the most biodegradable design since the original fibrin microthread system would remain. Finally, coating the fibrin microthread was thought to be the most economical design alternative. Even if the substrate chosen for coating is expensive, coating the fibrin microthread with this substrate will be of a lower cost than making the scaffold entirely from this material. Furthermore, growth factors and integrins can be expensive, and thus changing the satellite cell expression would also be more expensive than coating the fibrin microthread. The low ranking of the combination design alternative is primarily due to the fact that combining a change to the scaffold form, material, coating, and/or satellite cell expression would not be as cost effective or reproducible due to the many variables involved.

In addition to having the best score on the numerical evaluation matrix, the team chose coating the fibrin microthread as the tentative final design over the other design alternatives because the system would take advantage of the beneficial and quantified mechanical properties of fibrin microthreads. Coating the fibrin microthread would also produce a scaffold with a hydrated diameter similar to the current system. Furthermore, there are published fabrication and sterilization procedures for producing and using fibrin microthreads for the application of skeletal muscle regeneration that could then be modified by the design team for coating the fibrin microthread. These implanted fibrin microthreads have been shown to reduce the formation of scar tissue and not incite a negative foreign body response upon implantation. However, human satellite cells have been found to have poor attachment to this substrate and pre-maturely differentiate and cease proliferation upon adhering to the scaffold. A coating would allow for a different substrate to interact with the cell population without changing the beneficial properties of the fibrin microthread system.

Furthermore, all five design categories abide by the design constraints, as shown in Table 2. The safety of design category five is listed as tentative because it requires laboratory testing prior to determining this. Depending on the selection of materials, the combination design may be unsafe for implantation inside the body. For example, using material A and coating B may lead to an acidic byproduct inside of the body. The feasibility in terms of time and budget is also listed as tentative for the Combination category because laboratory testing with alternatives from design categories one through four are required prior to determining whether the options in design category five are feasible within the time and budget constraints. For example, during two

dimensional testing, it may be found that material choice A requires a higher concentration, and therefore increases the price of the proposed combination with material B and coating A. Likewise, developing a particular type of scaffold may require more time than the current system. Combining it with another material that also requires extensive testing prior to use, may not be within the team's time constraints.

Within the proposed final design category of coating the fibrin microthread, the team compiled a list of materials that are more likely to meet the team's objectives, functions, and specifications, based on the literature. The following is an outline of the top material candidates for coating the fibrin microthread and the primary reasons for their consideration.

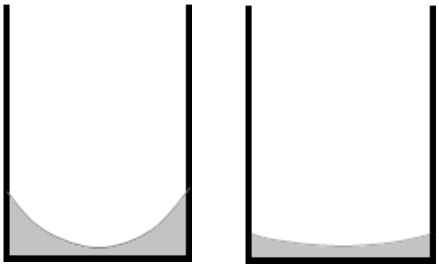
- Collagen Type I
    - Shown to promote cell attachment, proliferation, alignment, and differentiation
    - Most prevalent type of collagen in the human body
    - Versatile fabrication and processing (i.e. sheets, foams, matrices, powders, and injectable solutions)
- Fibronectin
    - Promotes cell attachment
    - Shown to direct myogenic and non-myogenic cells within muscle compartments
    - Aids in maintaining skeletal muscle integrity
- Vitronectin
    - Promotes and regulates cell attachment
    - Expressed at high levels in skeletal muscle compared to other tissues
- Laminin
    - Promotes cell migration, alignment, and differentiation
    - Naturally found in skeletal muscle and muscle-tendon units
- Chitosan
    - Positive charge, similar structure to GAGs, and allows for cell proliferation
    - Fast degradation time, non-hazardous by products
- Tissue Culture Plastic (TCP) was chosen to serve as an uncoated control.

Other potential candidates for coating the fibrin microthread that were not tested due to time and budget constraints include collagen Type IV, hylaronan (HA), and bioglass. Refer to Section 2.5.1 for a discussion on the properties of these materials and their impact on cell attachment, alignment, and differentiation.

4.7 Proof of Concept Testing

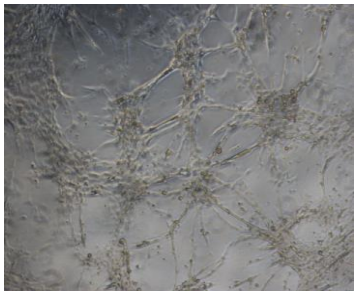
Proof of concept testing was done with a two dimensional assay with fibrin only. This allowed for the team to assess the potential of this assay in our design development. To show that the team will be able to evaluate certain variables of our design independently, tests were set up using thin fibrin gels. Thrombin and fibrinogen were combined in ratios identical to that of the fibrin threads to maintain similar mechanical properties. The mixture was added to a 24-well plate in varying volumes. It was found that the greater the volume the less uniform the surface was as the gel spread up ward on the sides of the well as seen in Figure 20.

1. Set up a simulation using fibrin



2. Cultured C2C12 mouse myoblastic cells on fibrin gels

3. Differentiated cells and fixed with 2% parformaldehyde



4. Stained for: Nuclei (Hoechst 33342, blue); Myosin (MF20, red)

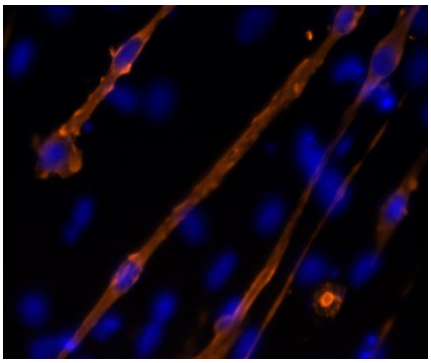


Figure 20: Methods used to determine the proof of concept

For this experiment, C2C12 cells were used since they are easier to handle, reproduce quickly, differentiate easily, and were largely available in the lab. The cells were cultured on the gels and then differentiation was induced by lowering the serum concentration and adding insulin. The cells were fixed with 2% paraformaldehyde and then stained with Hoechst 33342 to mark the nucleus and MF20 to stain for myosin, a protein found in muscle fibers.

From this proof of concept, the team determined that 24-well plates can be coated with various materials and C2C12 cells are able to grow and differentiate on the substrate. The fixing and staining method also worked well however, during the staining the substrate often detached from the well plate and was washed off during washings. In subsequent testing, it is important to gently wash the cells, as satellite cells are likely to be more sensitive. A similar method of analysis will be used in two dimensional testing to narrow down the material selection for the final scaffold.

CHAPTER 5: DESIGN VERIFICATION

Various material substrate options were evaluated to confirm which choices would be best for the final design. The current system uses a fibrin substrate, and the team began by quantifying the cell attachment and myogenic potential of this material. Collagen I, fibronectin, vitronectin, laminin, chitosan, and TCP (coating control) were also assessed through two dimensional assays and compared to fibrin. The two dimensional assay involved applying a coating of each material to wells in a 4-well plate. Cells were then seeded in each well and imaged at various time points to assess attachment. Differentiation medium was then introduced to the wells and after several days the cells were fixed and stained for myosin to evaluate the myogenic potential of the cells that adhered to each substrate. The cell culture protocols, assay preparation, and evaluation steps are described in detail in this chapter.

5.1 Culturing Human Skeletal Muscle Primary Cells (hSC)

In order to test our alternative designs, it was necessary to grow a sufficient number of satellite cells. This involved making several types of media, thawing vials of frozen cells, feeding the cells as they grew, subculturing them periodically, and cryopreserving them in between periods of testing. For both our two dimensional and three dimensional testing, only the non-adherent population of these cultures were used because they have been previously found to have a higher myogenic potential. However the non-adherent population is much smaller in number compared to the adherent population in each flask. As a result, several flasks were plated at a time in order to have enough non-adherent cells to use for testing.

5.1.1 hSC Culture Media

Media composition is a key factor to maintaining viable cells in the desired stage of the cellular life cycle. Variable factors in media such as pH, growth factors, concentration of serum, metabolite and ion concentration, and in some cases antibiotics must all be controlled (Freshney, 2005). Three types of media were used for this project: hSC proliferation medium, hSC differentiation medium, and freezing medium. In all cases, antibiotics were not used in order to prevent the chances of concealed contamination as well as to reduce the chances of developing

antibiotic resistant microbes in the laboratory. All stock solutions were pre-warmed to 37°C prior to media preparation.

The base of the proliferation medium was comprised of 54% Dulbecco's Modified Eagles Medium (DMEM) 1X with 4.5 g/L glucose and sodium pyruvate (without L-glutamine and phenol red) (Cellgro), 36% Ham's F-12 1X with L-glutamine (Cellgro) (60:40 ratio between DMEM and Ham's F-12), and 10% Fetal Clone III (FCIII) (Hyclone). 4mM L-glutamine (Cellgro) was then sterilely filtered through a syringe and added to the base medium. This base medium, termed DF12, can be stored at 4°C for up to two months. A proprietary growth factor cocktail (growth factors from PeproTech) was added to a 100 mL aliquot of DF12 at a time because the longevity of the medium after adding growth factors reduces to about 14 days. The selection of these growth factors is based on previous unpublished research. This medium was also stored at 4°C and warmed to 37°C prior to use in cell culture.

Differentiation medium was comprised of 58.2% DMEM 1X with 4.5 g/L glucose and sodium pyruvate (without L-glutamine and phenol red) (Cellgro), 38.8% Ham's F-12 1X with L-glutamine (Cellgro) (60:40 ratio between DMEM and Ham's F-12), 2% horse serum (Hyclone), and 1% insulin-transferrin-sodium selenite (ITS) (Cellgro). Similar to the proliferation medium, the differentiation medium was stored at 4°C and warmed to 37°C prior to use in cell culture.

Freezing medium was made for the cryopreservation of hSC cells. This medium comprised of the proliferation medium described above and 10% dimethyl sulfoxide (DMSO) (Cellgro) by volume. Appendix F.1 contains detailed procedures for the making of all three types of media.

5.1.2 Thawing Cells

It is essential to thaw quickly because the DMSO used in the freezing medium is toxic to the cells in addition to the cells being in a fragile state from being frozen for a long period of time (Freshney, 2005). The initial vial of cells thawed were removed from the liquid nitrogen tank and quickly thawed to 37°C. The cryovial was then taken inside of the biosafety cabinet and the DMSO inside of it was neutralized with 1 mL of DF12 medium with growth factors, added drop by drop. This was then added to a centrifugue tube containing DF12 medium with growth factors, prior to centrifuging. After centrifuging, the supernatant was aspirated and the cell pellet was resuspended in the desired amount of DF12 medium with growth factors. To plate cells,

proliferation medium was added to T75 tissue culture flasks and the appropriate volume of cell suspension was added (with a total volume of 8 mL per T75 flask). Appendix F.2 contains a detailed procedure for thawing cells. In addition to the thawing of the initial vial of cells, cells were also thawed after being cryopreserved in between periods of laboratory work according to the same procedure.

5.1.3 Cell Culture Maintenance and Subculturing

After plating the human satellite cells the flasks were kept in a 37°C incubator with 5% CO_2 in air (standard culture). The cells were fed every 48-72 hours, according to the protocol in Appendix F.3. When cell culture flasks became 70% confluent (as observed under light microscopy), the cells were subcultured to prevent premature differentiation into myotubes.

To subculture the hSC population of each T75 flask, the non-adherent cells and culture medium was transferred into a 15 mL centrifuge tube. The flask was then washed with deionized phosphate buffered saline (DPBS), which does not inhibit trypsin. Next, 3 mL 0.05% trypsin EDTA was added and the flask was observed under light microscopy as the adherent cells started to detach. After two minutes the flask was checked to see if most of the adherent cells had detached and the trypsin was neutralized by adding the same volume of culture medium. Trypsin is a protease that functions to break protein bonds between cells and the flask surface as well as the protein bonds between adhering cells (Freshney, 2005). Thus, if the trypsin is left in the flask for too long (without neutralizing with culture medium), it will start breaking down the proteins inside of the cells, leading to cellular death. The entire contents of the flask were then transferred to another 15 mL centrifuge tube, and a 50 µL aliquot was taken out for cell counting. The cells sample from each tube was then counted according to the protocol in Appendix F.4.

After centrifuging both tubes and aspirating the supernatant, the cells were resuspended in 1 mL of proliferation medium. For the non-adherent cells, the pellet was very small and thus about 0.5 mL of the supernatant was left when aspirating, whereas the adherent cells created a larger pellet and thus nearly all of the supernatant was aspirated out. Based on the number of cells in each of the tubes, they were plated on T75, T150, or T175 tissue culture flasks. A detailed protocol on subculturing hSC cells can be found in Appendix F.5

5.1.4 Cryopreservation

Cells were cryopreserved in between periods of laboratory work in order to preserve their capacity to proliferate. This can be accomplished by keeping the primary cells at a low passage number and thus preventing them from reaching cellular senescence (Freshney, 2005). Although antibiotics were not used in the proliferation and differentiation media in order to prevent the concealment of contamination and to reduce the chances of developing antibiotic resistant microbes, in this case antibiotics such as gentamicin were not used because they are known to interfere with animal cells in frozen state. It is critical to freeze slowly in order to minimize cell stress during the freezing process.

The hSC cells were first centrifuged, resuspended (after aspirating the supernatant), and counted for the number of viable cells according to the protocol in Appendix F.4. Freezing medium was prepared as described in Section 5.1.1. In December of 2012, approximately 350,000 cells were frozen in each cryovial, and in April of 2013, approximately 500,000 cells were frozen in each cryovial, with a 1 mL volume in each cryovial. Extra caution was taken to not have the freezing medium come into contact with skin due to the toxicity of DMSO. The cryovials were immediately transferred to a Nunc cooler, which was then placed in a -80°C freezer where the temperature decreased 1°C per minute. After approximately 2 days, the cryovials were transferred to liquid nitrogen for long term storage. Health of the cells was verified by thawing one of these vials and culturing them for 4 days. A detailed procedure for cryopreservation can be found in Appendix F.6.

5.2 Design of Two Dimensional Assay Testing Procedure

To determine which materials promote cell adhesion most successfully, the materials were initially tested in two dimensions. This saved both materials and time in preparation and enabled the team to test various materials before designing the final three dimensional scaffold. The top material choices were narrowed down at the conclusion of the two dimensional assays. This allowed for designing the scaffold variable by variable to combine the top characteristics of a scaffold for delivering satellite cells from one point to another.

5.2.1 Coating Tissue Culture Plates for Testing

To test the materials in two dimensions, solutions were made of each material and then coated on the bottom of a 4-well or 24-well plate. The layer of material was made thin enough so that cells could still be imaged through it using a light microscope, but thick enough so that the entire surface of the well was covered. The experiments occurred in nine sets with varying materials. Multiple iterations were assessed using fibrin, collagen I, fibronectin, vitronectin, laminin, and chitosan. Refer to Appendix F.8 for detailed coating procedures.

Fibrin Coating:

To coat the wells with fibrin, all materials were kept on ice prior to and during the coating to slow the polymerization process and ensure an even layer of fibrin on the well. Components were mixed in the same concentrations and ratios as is used in making the fibrin microthreads. Thrombin (EMD Millipore) at a concentration of 40 U/mL was mixed with 40 mM cold calcium chloride dehydrate (JT Baker) in a sterile environment and kept on ice. Next, fibrinogen (EMD Millipore) at a concentration of 70 mg/mL was added to the solution and 100 µL of the solution was immediately aliquoted to one well of a multi-well plate. The solution was swirled to cover the bottom of the well and then 70 µL of the solution was removed to produce a gel with a thickness of 150 µm. The gel was covered and incubated at room temperature for 12 hours. The gel was rinsed once with PBS before the cells were seeded onto the substrate.

Collagen Type I Coating:

Collagen I (PureCol®, Advanced Biomatrix) was diluted to 0.31 mg/mL with 30% ethanol according to the Grace Bio-Lab procedure for short term cell culture experiments. The solution was spread over the surface of the well as described in the fibrin coating protocol and 70 µL was removed. The plate was then covered and incubated at room temperature for 12 hours. The gel was rinsed once with PBS before cells were seeded onto the substrate.

Fibronectin Coating

Human fibronectin (Gibco) was diluted to 0.08 mg/mL in PBS, based on the manufacturer's recommendation of using 2.0 µg/cm^2 for coating applications. The solution was spread over the surface of each well as described in the fibrin coating protocol and 70 µL was removed. The

plate was then covered and incubated at room temperature for 12 hours. Each well in this case is 2 cm^2. The wells were rinsed once with PBS prior to cell seeding.

Vitronectin Coating

Human vitronectin (Gibco) was diluted to 0.02 mg/mL in PBS, based on the manufacturer's recommendation of using 0.5 $\mu g/cm^2$ for coating applications. The solution was spread over the surface of each well as described in the fibrin coating protocol and 70 µL was removed. The plate was then covered and incubated at room temperature for 12 hours. Each well in this case is 2 cm^2. The wells were rinsed once with PBS prior to cell seeding.

Laminin Coating:

Stock solution of laminin (laminin-111 (VWR)) was diluted to 50 µg/mL in PBS. Enough solution was added to cover the bottom of each well and was allowed to incubate at room temperature for 12 hours. The laminin solution was then aspirated and the well was washed once with PBS. The cell suspension was then added to the well without allowing the laminin coating to dry.

Chitosan Coating:

5 mg/mL of deacetlylated chitosan (Sigma) diluted in 1% acetic acid was diluted further in PBS to result in 0.5 mg/mL chitosan. 100 µL of the chitosan solution was then pooled over the surface of each well and then 70 µL of the solution was removed, similar to the fibrin protocol. The plate was allowed to incubate at room temperature for 12 hours. The gel was rinsed once with PBS before cells were seeded onto the substrate.

5.2.2 Cell Culture for Two Dimensional Assays

After each well was rinsed once with PBS, the cell suspension was added. Non-adherent human skeletal muscle cells were seeded at a density of 50,000 cells per well in growth medium, which allowed the team to quantify both the rate of attachment of the cells and the myogenic potential of the cells in the same assay. This high number of cells produced a greater confluence as the cells grew and proliferated over three days. Cell-cell interaction aids the fusion and differentiation of the cells, which was why this level of cell density was chosen.

The cells were cultured in growth medium for three days. At this point the viability of the non-adherent cells was determined by removing 10 µL of medium and cells, staining these with trypan blue, and then counting the number of live and dead cells as described in Appendix F.4. This was converted into a percentage to determine the viability of the non-adherent cell population at 72 hours. After this phase of growth, the medium was switched to differentiation medium without growth factors and a lower serum concentration. This caused the cells to begin differentiating into muscle fibers. After six days of differentiating, (nine total days in culture), the cells were fixed and stained for myosin and Hoechst 33342. Throughout the cell culture time period cells were imaged using phase contrast at predetermined time points. This process is illustrated in Figure 21 below.

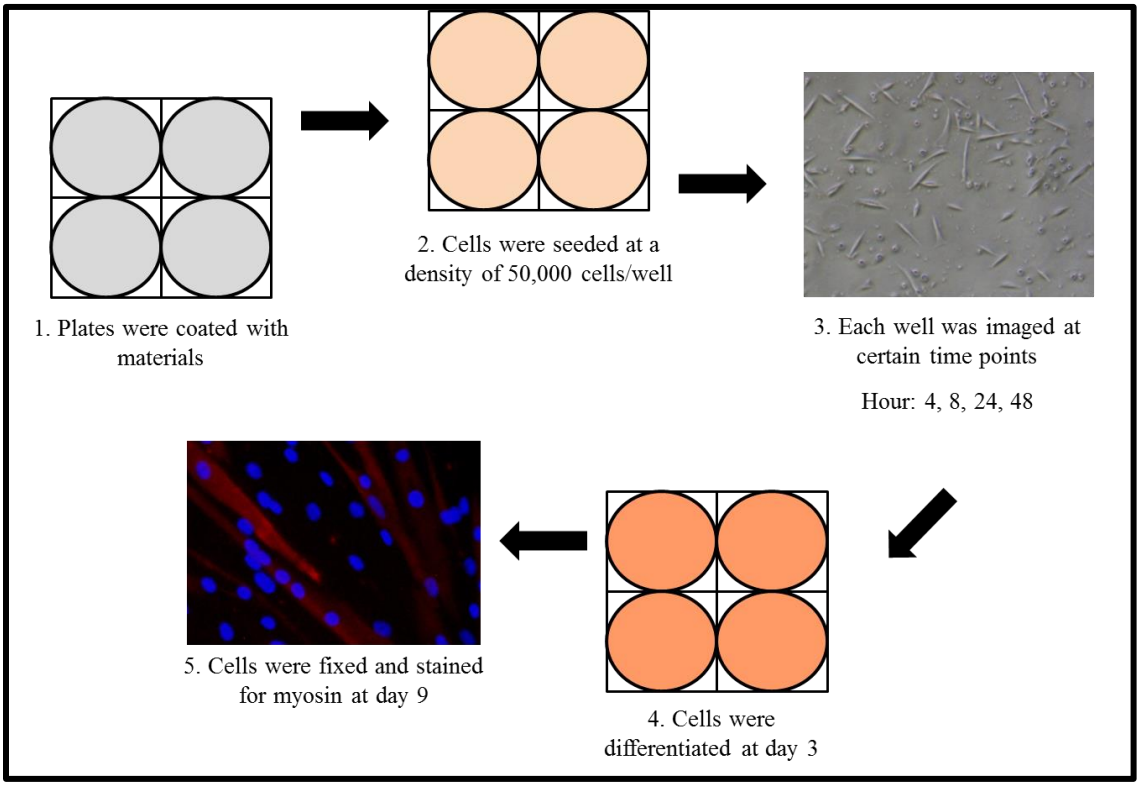


Figure 21: Cell culture protocol and timeline for two dimensional assays

5.2.3 Imaging and Counting Procedure for Cell Attachment

To quantify the attachment rate of the satellite cells to the various substrates, images were taken at predetermined time points throughout the growth phase of cell culture as seen in Figure 21. Cells were imaged using phase contrast at hours 24, 48, and 72 for the first iteration of the

experiment to determine the broad rate of attachment over three days. Since a high attachment percentage was seen after 24 hours, the team chose to focus on the early time points, and thus the plates were imaged at hours 4, 8, 24, and 48 hours for the remaining assays. This allowed the team to analyze how quickly and efficiently the satellite cells attach.

Each well was divided into six regions, as seen in Figure 22 below, before cell seeding. Six images, one per region, were taken at predetermined time points using phase contrast at 5X magnification. In addition to the one image per region, abnormal cell morphologies or surface structures were also imaged and noted. The non-adherent and adherent cells, which are distinguishable by cell appearance and morphology, were counted for each image using ImageJ software (NIH). Then the average number of each cell type was determined per region and compared to other materials by the percentage of adherent cells. The percent of adherent cells was calculated by dividing the number of adherent cells by the total number of cells. This value was compared across time points and substrates.

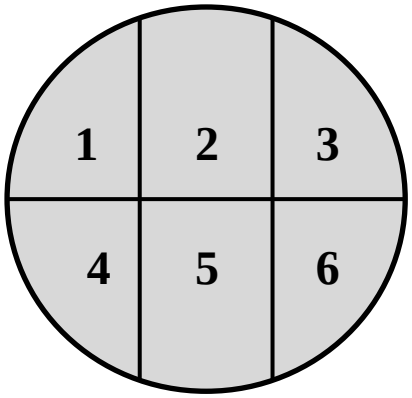


Figure 22: Schematic of a divided well to aid in cell counting

While assessing the ability of each substrate to promote hSC attachment during assays 1 and 2, the team found that it was often difficult to count the number of cells in the middle of each well (bottom of region 2 and top of region 5) as the cells would aggregate to create a giant mass. In some cases the cells from these aggregates would move to other parts of the well that were not as crowded. One way the team could minimize the chances of these aggregates appearing is to resuspend the cells more prior to seeding them on the coated wells and to also distribute the cell suspension across the well rather than pipetting them into the center of the well. Another way to address this issue would be to place the multi-well plate on a plate shaker after seeding the cells.

5.2.4 Immunocytochemistry for Quantifying Myogenic Potential

To determine the myogenic potential of the satellite cells on various substrates, the cells were fixed with 2% formaldehyde for 15 minutes at room temperature. The cells were then stained for myosin using mouse anti-myosin (MF20; 1:500) and labeled with goat anti-mouse IgG Alexa 568 (1:500) and counterstained with Hoechst 33342 at 1 µg/mL for 10 minutes at room temperature. Six images were taken from each treatment and analyzed using florescence microscopy to determine the average myogenic index for each material. The myogenic index is defined by the number of nuclei in myosin positive cells divided by the total number of nuclei. Through these calculations, the effect of various materials on the differentiation of satellite cells was determined.

5.3 Quantification of Two Dimensional Assay Testing

The team analyzed the effects of various substrates as well as various culture conditions on the attachment of hSC. Cells were cultured on various substrates including fibrin, collagen I, fibronectin, vitronectin, laminin, chitosan, and tissue culture plastic (TCP), in hSC proliferation medium with growth factors.

5.3.1 Quantification of Attachment of Satellite Cells to Various Substrates

The team began the two dimensional assays by quantifying the satellite cell attachment to fibrin, the current substrate for delivering satellite cells to animal models for preclinical testing, and then compared these results to those of the other tested substrates. The evaluation of satellite cell attachment occurred in two stages. Cells were seeded on the substrate at a concentration of 50,000 cells per well and were imaged using light microscopy at various time intervals. First, the team quantified the attachment of satellite cells to the substrate in 24-hour increments and then focused on the earlier time points to determine the rate and efficiency of cell attachment to the fibrin. Non-adherent hSCs at between passages 8 and 15 were seeded on the substrate for each assay.

As expected, the percentage of adherent cells increased over time which can be seen qualitatively in the sample images in Figure 23 and Figure 24 below. The team distinguished between adherent and non-adherent cells by morphology as the non-adherent cells are spherical in nature

and the adherent cells are flat and spread out on the surface. One problem in this approach was that it was difficult to count the number of cells when they became close to 90% confluent in the center of the well. It was also difficult to distinguish between cells and debris as the confluence increased. Although the cells in each well appear in layers under phase contrast (and thus not all of the cells can be focused on at the same time), approximately 200-500 cells are counted per region per well, and thus approximately 1200-3000 cells are counted per well in order to arrive at the average percent of attached cells. Based upon these methods, the number of adherent cells increased over time from 4 hours to 24 hours. These images were analyzed and the percentages of adherent cells were evaluated quantitatively.

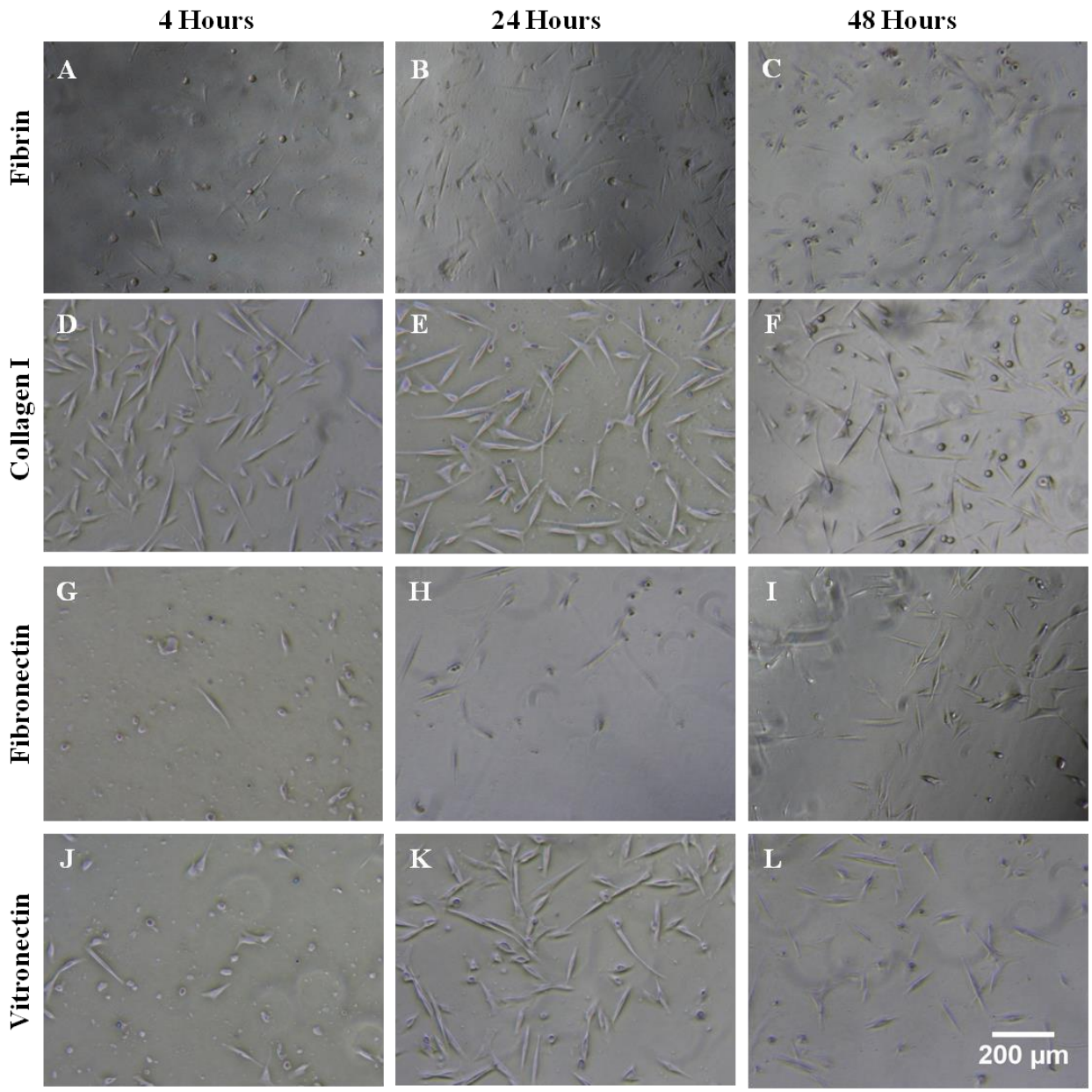


Figure 23: Attachment of hSCs to fibrin, collagen I, fibronectin, and vitronectin over time

Cells were imaged using light microscopy at 5x magnification at 4 hours (A, D, G, J), 24 hours (B, E, H, K), and 48 hours (C, F, I, L) on fibrin (A-C), collagen I (D-F), fibronectin (G-I), and vitronectin (J-L).

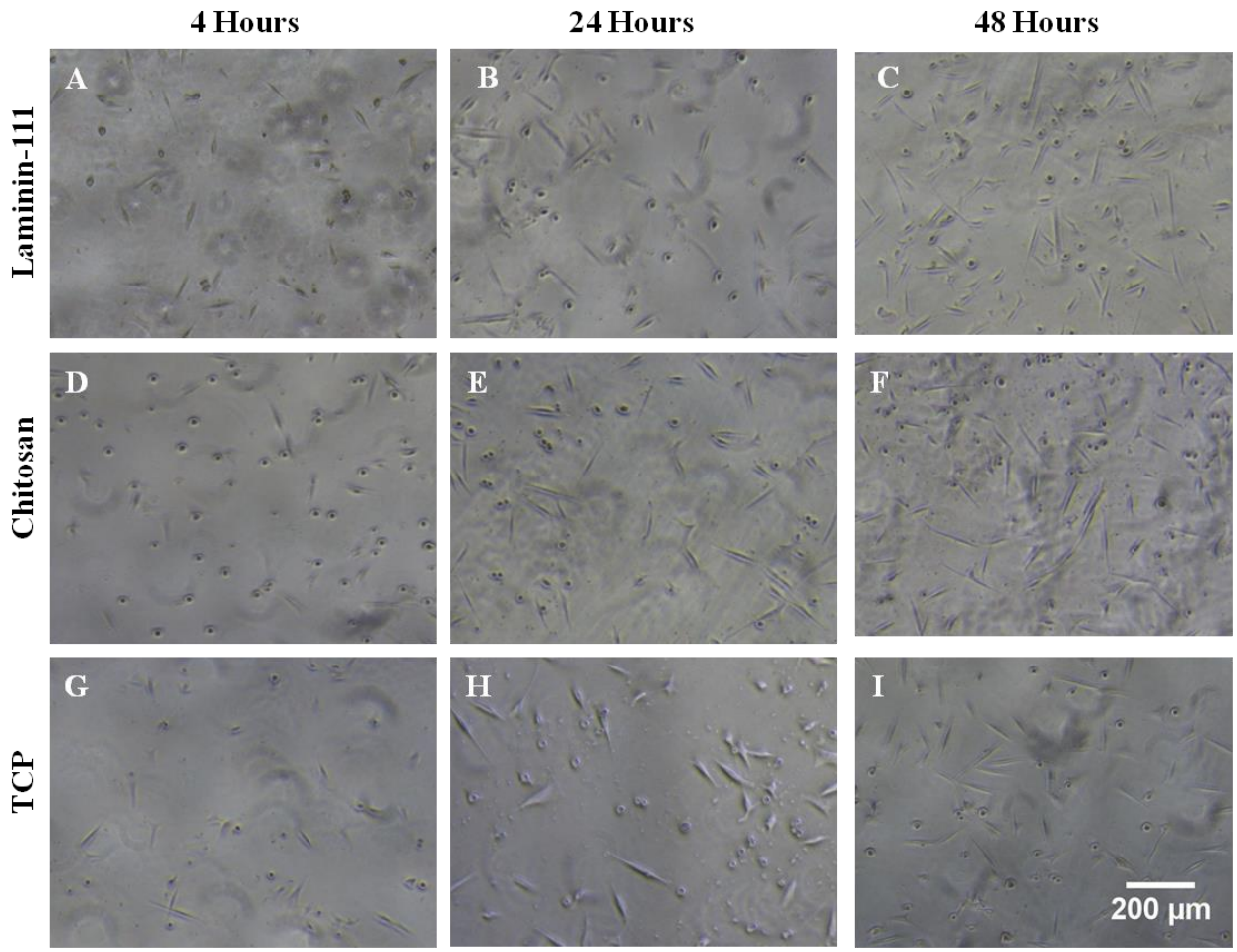


Figure 24: Attachment of hSCs to laminin, chitosan, and TCP over time

Cells were imaged using light microscopy at 5x magnification at 4 hours (A, D, G), 24 hours (B, E, H), and 48 hours (C, F, I) on laminin (A-C), chitosan (D-F), and tissue culture plastic (G-I).

As expected, the attachment percentage increased from 4 hours to 24 hours for each substrate in each assay. However, in terms of the individual assays, there was not always an increase in attachment between 24 hours and 48 hours. For the incidences where there is a decrease in attachment between hour 24 and hour 48, one explanation could be that the cells are lifting off of the substrate and/or reaching maximum attachment due to the cell cycle stage the cells are in during that time point. Similarly, the observation of having approximately equal attachment between hour 24 and hour 48 could be explained as being related to the stage of the cell cycle the cells are in during that time point, or due to the increased cell density (due to proliferation since the initial seeding of 50,000 cells per well) creating cell competition. Quantitative results of the

average percent attachment of satellite cells on each of the tested substrates across all attachment assays are shown in Figure 25 and Table 3 below. For cells cultured on fibrin, laminin, chitosan, and TCP, the peak average percent attachment occurred at the 8 hour time point. This finding is in agreement with the finding during Assays 1 and 2 where it was found that imaging every 24 hours did not allow for seeing smaller changes in percent cell attachment, and that shorter time increments between imaging were required in order to better characterize the attachment behavior. For cells cultured on collagen I, the imaging time point with the highest percent attachment was 24 hours. Furthermore, cells cultured on fibronectin and vitronectin had the highest percent attachment occur at the 48 hour imaging time point.

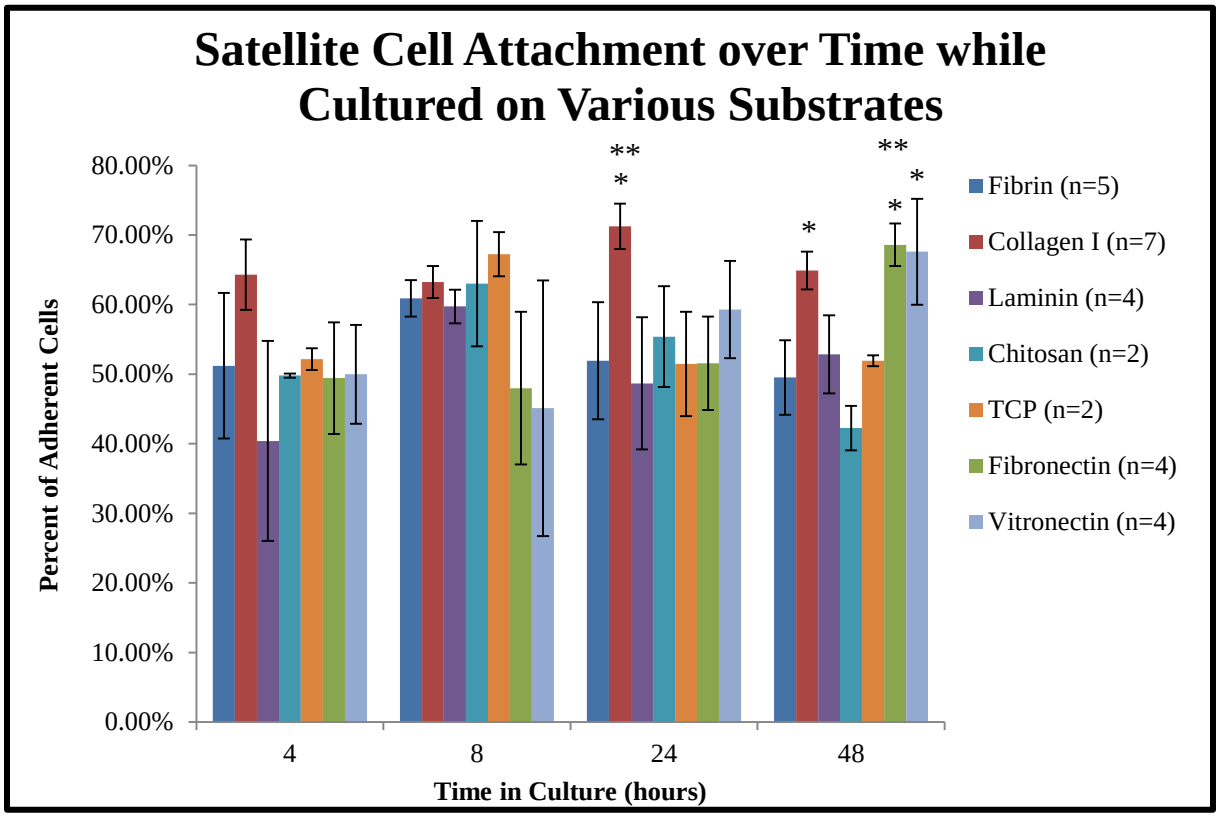


Figure 25: Satellite cell attachment over time while cultured on various substrates

** denotes p<0.01 for that time point, ** denotes p<0.01 in regards to fibrin at all time points*

Table 3: Average percent attachment of human satellite cells over time while cultured on various substrates

	Average Percent Attachment ± STDEV			
	4 Hours	**8 Hours**	**24 Hours**	**48 Hours**
Fibrin (n = 5)	51.2 ± 10.5	60.88 ± 2.6	51.9 ± 8.4	49.5 ± 5.4
Collagen I (n = 7)	64.3 ± 5.1	63.3 ± 2.3	71.3 ± 3.3	64.9 ± 2.7
Fibronectin (n = 4)	49.4 ± 8.1	48.0 ± 11.0	52.0 ± 6.7	68.6 ± 3.1
Vitronectin (n = 4)	50.0 ± 7.1	45.1 ± 18.4	59.3 ± 7.0	67.6 ± 7.6
Laminin (n = 5)	40.4 ± 14.4	59.7 ± 2.4	48.7 ± 9.5	52.9 ± 5.6
Chitosan (n = 2)	49.8 ± 0.2	63.0 ± 9.0	55.4 ± 7.3	42.3 ± 3.2
TCP (n = 2)	52.1 ± 1.5	67.2 ± 3.2	51.5 ± 7.5	51.9 ± 0.8

It should also be noted that in most cases, duplicate wells in each assay produced very similar attachment percentages. This can indicate a number of things such as the wells were coated to the same level, the quantification method chosen was reasonable, and/or that the cellular behavior was representative of the material it is on. However, these ideas cannot be confirmed without further testing.

5.3.2 Quantification of Satellite Cell Viability

In order to determine whether the non-adherent satellite cells after 72 hours of culture on the given substrate were viable, the viability of this population was quantified during attachment assays 1 and 2. This was performed by taking out a small sample of cell suspension from each well, staining with trypan blue, and counting the number of live cells and dead cells in the sample (as described in the procedure for counting cells in Appendix F.4). The satellite cells cultured on fibrin had a high viability for both assays 1 and 2. In assay 1 the average viability of the non-adherent cells was 91%. For assay 2 the viability was even higher at 97%. This shows that cells grew healthily on the fibrin substrate without any apparent cytotoxicity.

The viability of the non-adherent cell population cultured on collagen I was also determined. The non-adherent cells in the collagen I wells of assay 1 had an average viability of 80%, while the non-adherent cells in the collagen I well of assay 2 had a viability of 93%. These viability values show that the percent of viable attached cells is even higher than the percent of attached cells reported in this section, since there are some dead cells among the non-adherent population.

Viability testing was not performed for subsequent assays due to the high viability obtained during assays 1 and 2.

5.3.3 Statistical Analysis of Attachment Assays

An analysis of variance (ANOVA) was completed using Microsoft Excel to test for significant differences between multiple means. An ANOVA test allows for the acceptance or rejection of the null hypothesis, which is that there is no statistically significant difference between the means of the samples. The ANOVA test reports a p-value, f-value, and f-critical value for each data set. We rejected or failed to reject the null hypothesis based on the outputted p-value from the T-test. This value represents the probability of error involved in rejecting the hypothesis of no differences between the two categories of observations in the population. A higher p-value corresponds with less reliability in a result. For example, a value of 0.05 indicates that there is a 5% probability that the relation between the variables found in our sample is due to random chance. This means that the results of our analysis could be replicated 95% of the time. Customarily, in many areas of research, the p-value of 0.05 is treated as border-line acceptable error level. A higher degree of certainty is associated with a lower p-value, and for this reason, we have selected 0.01 as our critical value (StatSoft, Inc., 2011). If the calculated number resulting from the T-test is below 0.01 then the null hypothesis is rejected, while if the value is greater than 0.01 we fail to reject the null hypothesis. An F-value over F-critical also leads to rejection of the null hypothesis. This statistical test was completed for each time point to compare the attachment of various materials. The reported p-values, f-critical, and f-values for each test are displayed in Table 4 below. Statistically significant differences were identified at all time points.

Table 4: Reported results from ANOVA

Time Point	P-Value	F-Value	F-Critical
4 hours	0.01	3.72	2.49
8 hours	0.01	3.64	2.55
24 hours	0.00	7.13	2.43
48 hours	0.00	16.97	2.53

Although a one-way ANOVA test can identify which data sets have significant differences in their means, it cannot show where the difference stems from. A t-test can be done to compare the means between two samples, and also reports a p-value to accept or reject the null hypothesis. Specifically a one-tailed t-test was completed comparing the values for fibrin to each competing material. Unequal variances were assumed between the means. A one-tailed t-test was selected because the team is only interested in seeing if the value of the competing material is significantly higher than that of the control fibrin. Table 5 below reports the p-values for the t-tests of each material for 4, 24, and 48 hours. For collagen I at 24 and 48 hours, and vitronectin and fibronectin at 48 hours, the results of this test rejected the null hypothesis, and as a result, these time points and materials perform better than fibrin.

Table 5: T-test values for each two dimensional material versus fibrin at 4, 24, and 48 hours

** Denotes a statistical significance p<0.05, ** p<0.01*

Material vs. Fibrin	Collagen I	Laminin	Chitosan	TCP	Fibronectin	Vitronectin
4 Hours	0.03*	0.15	0.75	0.84	0.78	0.83
8 Hours	0.14	0.49	0.79	0.16	0.04*	0.18
24 Hours	0.00**	0.61	0.56	0.97	0.94	0.13
48 Hours	0.00**	0.37	0.10	0.29	0.00**	0.01**

Additionally, collagen I at 24 hours was found to be statistically better than fibrin at any time point, with the p-values also reported in Table 5 as well. As a result, collagen I appeared to be the top performing material in the two dimensional analysis for attachment in regards to attachment and attachment time, with fibronectin and then vitronectin being strong competitors.

Table 6: Reported p-values from a t-test comparing each two dimensional material vs. fibrin at all time points

** denotes p<0.05, ** denotes p<0.01 for all time points*

	Collagen I		Fibronectin	Vitronectin
	24 hours**	**48 hours***	**48 hours****	**48 hours***
4 Hours	0.00	0.02	0.01	0.02
8 Hours	0.00	0.03	0.01	0.18
24 Hours	0.00	0.00	0.00	0.01
48 Hours	0.00	0.00	0.00	0.01

5.3.4 Quantification of Myogenic Potential of Satellite Cells

In addition to the percent of cell attachment, the team also analyzed the myogenic potential of the cells by evaluating the myogenic index. The myogenic potential of the satellite cells that were able to attach to the substrates were fixed and stained for myosin heavy chain, an early muscle marker, which shows the commitment of the cell to continue and carry out differentiation into myofibers. The number of myosin positive cells was divided by the total number of cells to determine the myogenic index. All of the substrates tested for percent cell attachment were also assessed for myogenic index except for chitosan, due to the low number of adhering cells found on that substrate.

Based on the team's client statement, not only does the selected final design need to increase the amount of hSC attachment to the substrate, but it also needs to promote the adherence of satellite cells that have the potential to continue differentiating into myofibers. As described in Section 5.3.2, this was tested by first changing the cell culture medium in the multi-well plate from proliferation medium (DF12 with growth factors) to differentiation medium (DF12 with 2% HS and 1% ITS). After six days the cells were fixed and stained to fluorescently show myosin (which indicates which cells have committed to carrying out differentiation into myofibers) and

nuclei (which indicates the presence of a cell at that location). Images were taken at 32X and 20X magnification, and the myogenic index was calculated by dividing the number of myosin positive cells by the total number of cells in the field of view. Figure 26 below shows sample images of the myosin heavy chain staining of the adherent cells on both fibrin and collagen I.

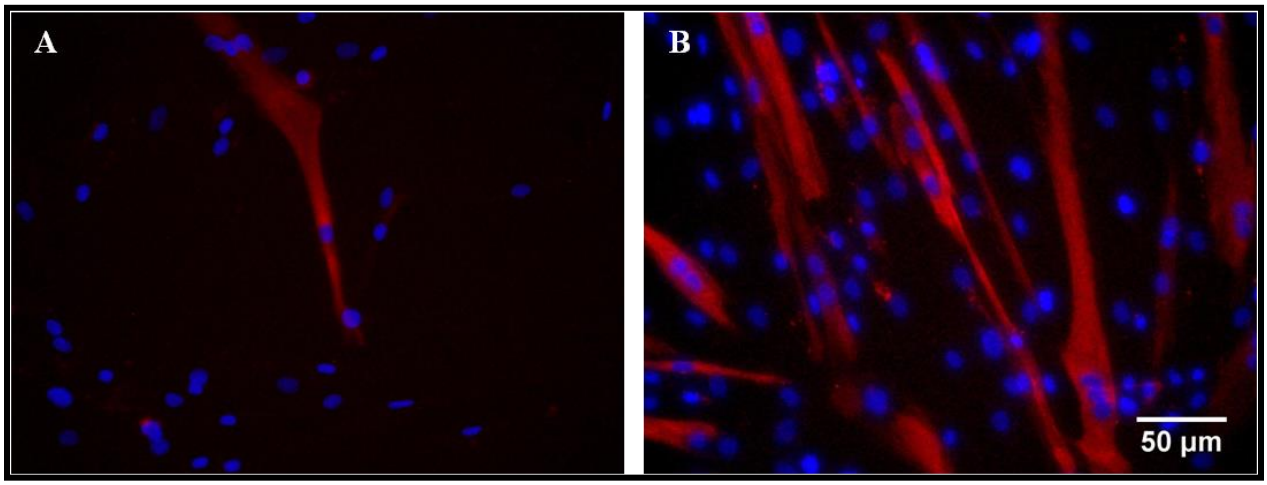


Figure 26: Immunocytochemistry of hSCs after 9 days of culture on a fibrin substrate (A) and collagen I substrate (B)

Red: Myosin (MF20), Blue: Nuclei (Hoescht), images taken at 32X magnification.

As shown in Table 7 below, the satellite cells cultured on a fibrin coating for 9 days was determined to have an average myogenic index of 25.1%, indicating that about a quarter of the cells had begun to differentiate. Cells cultured on fibronectin and vitronectin had similar values of 24.3% and 24.2% respectively. Laminin and TCP had the lowest values for myogenic index (15.5% and 15.9% respectively). Cells cultured on collagen I had the highest index of 32.1%.

Table 7: Myogenic potential of satellite cells cultured on various substrates

Substrate	Myogenic Index	Number of Wells
Fibrin	25.1%	n = 2 (averaged)
Collagen I	32.1%	n = 3 (averaged)
Fibronectin	24.3%	n = 1
Vitronectin	24.2%	n = 1
Laminin	15.5%	n = 1
TCP	15.9%	n = 1

One reason for the low myogenic index values for some of the substrates could be due to decreased initial attachment during the growth phases of cell culture. The lower number of cells on the surface led to a smaller cell density and increased distance between the cells. The distance could have an effect on cell-cell signaling which impaired the ability of the cells to differentiate. The timing of differentiation is important in the end goal of the delivery vehicle. One of the objectives of the vehicle is to deliver undifferentiated cells that are able to proliferate and migrate to the wound but yet maintain their ability to differentiate. The differentiation must occur after sufficient proliferation of the satellite cells throughout the wound and not before delivery.

However, cells cultured on collagen I had the highest average myogenic index of 32.1%. This indicates that not only did collagen I promote the greatest percent cell adhesion during two dimensional testing, but that the adhering cells were more likely to mature into myofibers compared to the adhering cells on the other substrates. First, the substrate itself could be promoting cell behavior and thus be causing the overall increase in myogenic potential for the seeded population. Second, the number of cells initially attached during the first three days of growth (during cell-substrate adherence testing) is much larger for collagen I compared to fibrin and thus there was a higher cell density in these wells after changing the medium from proliferation medium to differentiation medium. This higher cell density could possibly increase cell-cell communication, which in turn could improve the cells' ability to differentiate. Since the client has asked the team to develop a vehicle that can deliver undifferentiated cells that have the potential to differentiate, but that first can proliferate and migrate to the wound site, it is

important to find a material and/or coating that prevents premature differentiation of the satellite cells while still promoting cell attachment.

Overall, a myogenic index of 30% or more is considered effective for cell therapy applications (Lafuste, et al., 2005; Lapan, et al., 2012). However the myogenic index can also be related to the passage number of the cells used to determine the myogenic index, as cells at lower passage numbers tend to have greater potential to differentiate compared to cells at higher passage numbers approaching senescence.

CHAPTER 6: FINAL DESIGN AND VALIDATION

Taking into consideration time, budget, and laboratory capability constraints, the team selected the design that had the greatest potential to increase attachment. A successful final design will allow for optimum and reproducible satellite cell attachment, prevent premature differentiation, maintain axial alignment, and have desirable mechanical properties that simplifies the implantation process. The materials that had the best performance in two dimensional testing were selected for use as a coating of a fibrin microthread for three dimensional testing. The thread extrusion, coating, cell seeding, and evaluation processes are described in detail below.

6.1 Final Design

Potential substrate coatings were initially investigated through two dimensional assays and the final design alternative was a fibrin microthread with a coating. Collagen I, fibronectin and vitronectin showed the best performance in the two dimensional testing in comparison to laminin, chitosan, and fibrin. Additionally, collagen I cultured for 24 hours and 48 hours and fibronectin cultured for 48 hours outperformed fibrin at any time point. The myogenic index for collagen I also appeared higher than fibrin while vitronectin and fibronectin were very comparable to fibrin. The benefits of moving forward with the design alternative of coating fibrin microthreads include that the mechanical properties have already been assessed and these threads have been successfully implanted over a muscle defect in an in vivo study. There are already established methods of creating this scaffold and seeding cells on it as well. This will allow for the improvement of cell attachment properties of the scaffold without compromising the existing favorable properties. As a result of material and time limitations, the team decided to test a collagen I and a vitronectin coating on a fibrin microthread bundle. Although fibronectin appeared to perform better in two dimensional attachment testing, its ability to be coated on fibrin microthreads is currently being evaluated concurrently in another laboratory. As a result the team wanted to investigate the potential of another material to be coated on the fibrin microthread system.

6.2 Final Design Fabrication

The selected design alternative of coating a fibrin microthread was assessed in comparison to the control of a bare fibrin microthread. These scaffolds were coated, sterilized, hydrated, and seeded with cells as described below in this section. In addition, cells were stained before and after the addition of culture media to assess cell attachment, premature differentiation, and myogenic potential.

6.2.1 Extrusion and Bundling of Fibrin Microthreads

Fibrin microthreads were extruded in a HEPES bath by combining thrombin, calcium chloride, and fibrinogen at a neutral pH. The threads were allowed to sit in the bath between 10-15 minutes and were then removed and stretch to about two times their original length. These threads were allowed to dry and then were placed into a tin foil pouch. The threads were stored in a desiccator until further use, with all threads being used within 2 weeks. The detailed protocol for the extrusion of these threads is depicted in Appendix F.12 (Grasman, et al., 2012).

PDMS molds were made to imitate the dimensions of the stainless steel washers used in previous studies (Grasman, et al., 2012). Fibrin microthreads were placed into bundles of 10 threads and secured with tape. The PDMS molds were slid beneath the thread bundles and silicone glue was used to adhere the threads to the molds as shown in Figure 27. Care was taken to avoid glue on any part of the thread in the middle of the mold. The glue was allowed to cure for 24 hours, and any unused bundled threads were stored in a desiccator until needed.

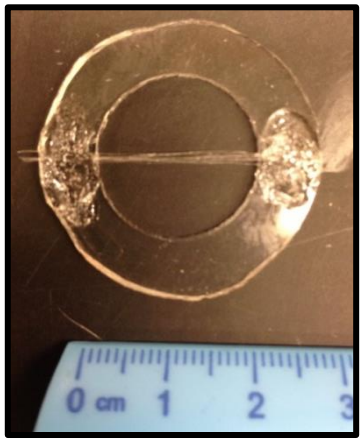


Figure 27: Ten fibrin microthreads bundled and glued to a PDMS mold

6.2.2 Method for Coating Fibrin Thread

PDMS molds with bundles of fibrin microthreads were placed in a 6-well coating plate. The threads were coated by placing 750 µL of solution in the center of each mold for 1 hour. 0.31 mg/mL collagen I in PBS (pH 7) and 0.02 mg/mL vitronectin in PBS (pH 7) were each used to coat fibrin microthreads, while the control threads were placed in PBS for 1 hour. It was necessary to ensure coatings had a pH greater than 6 to avoid the threads dissolving as a result of the acidic content. After 1 hour, the solutions were aspirated and the PDMS molds were transferred to a raised platform to dry for several hours. Care was taken to avoid the threads contacting any surface while drying to maintain an even coating and to prevent breakage.

6.2.3 Verification of Coating on Fibrin Thread

To determine whether or not the fibrin microthread had been successfully coated with collagen I, the thread was fluorescently stained. Threads were fixed with cold methanol for 15 minutes at room temperature. The threads were then stained for collagen I using mouse Pro-collagen I (1:100) and labeled with goat anti mouse IgG (H+L) Alexa 488 (1:500) at room temperature. Five images were taken at specific points along the thread: at the center, two at each end, and two in between the center and the end of the thread. Images were taken at an exposure time of 0.5 seconds. Figure 28 below shows representative images of the middle of the thread. The amount of collagen I bound to each thread as indicated by the amount of florescence was compared to the fibrin thread. As a result of this staining, the collagen I coating's capability to adhere to the fibrin microthread was verified. Images were taken at 20X magnification at an exposure time of 0.282 seconds.

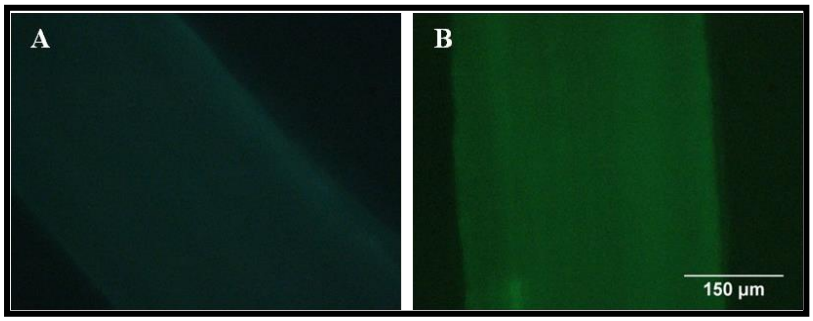


Figure 28: Fluorescence microscopy of collagen I coated fibrin microthreads

Images of the fibrin microthread bundle (A) and collagen I coated fibrin microthread bundle (B). Green: Pro-Collagen I. Images were taken at 20X magnification.

6.2.4 Fibrin Microthread Sterilization and Cell Culture

A 6-well plate was modified according to a previous study to optimize it for the seeding of hSCs onto fibrin microthreads, as shown in Figure 29 below (Grasman, et al., 2012). Each well has a raised Thermanox coverslip on a PDMS post. This will allow the threads to sit flush on the coverslip, which will aid in cell attachment.

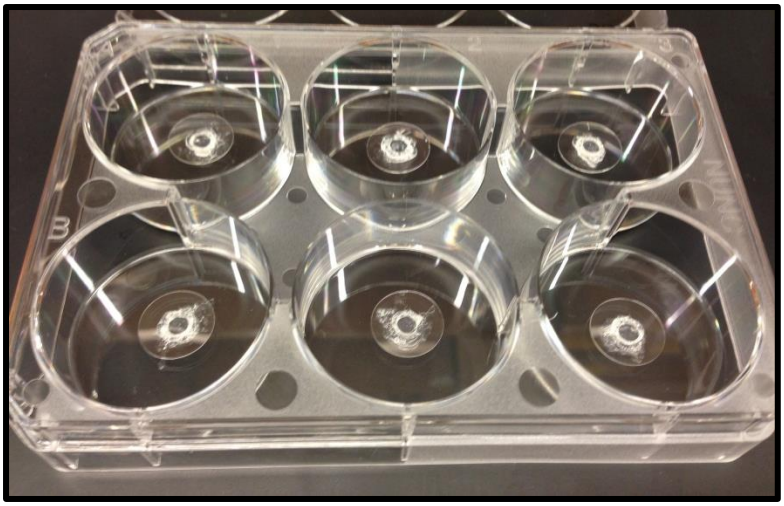


Figure 29: 6-well plate for microthread seeding

The wells were filled with 70% ethanol to sterilize them for 1 hour in a laminar flow hood. Then the ethanol was aspirated from each well, while being careful not to disturb the threads. The threads were then washed three times with sterile DI water. After this wash step, the threads were allowed to air dry for 3 hours. The threads were then rehydrated with a drop of PBS on each coverslip for 1 hour, which was later replaced with a 150 µL drop of hSC cell suspension (200,000 non-adherent hSC cells/mL) with the addition of aprotinin (50 µg/mL) to inhibit the cells' secretion of protease which breaks down fibrin microthreads. The 6-well plate was then placed in an incubator. After 24 hours, the PDMS washer was transferred to a new 6 well plate with sterile tweezers and covered with growth factor proliferation medium for an additional 24 hours (Grasman, et al., 2012). The threads were imaged at 0, 24 and 48 hours from initial seeding to qualitatively assess cell attachment and proliferation. After 72 hours it was observed that the

cells were beginning to detach from the threads and re-attach to the culture plate wells. This could be a result of proliferation or caused by changes in the cell cycle. At this time point one of each coating was removed and placed in a separate 6-well plate for staining in order to assess the possibility of premature differentiation. For all remaining wells, the growth medium was replaced by differentiation medium and fed every other day for a total of 6 days.

6.3 Qualitative Assessment of Three Dimensional Testing

Three dimensional testing was carried out to assess the capability of a coated microthread to improve satellite cell attachment and prevent premature differentiation, while maintaining cells with a myogenic potential. Fibrin microthreads bundles, as well as vitronectin and collagen I coated microthread bundles were evaluated using staining techniques described above. The number of cells attached to each thread was observed using a Hoechst 33342 stain, while premature differentiation was assessed by staining for myosin heaving chain. The adhering cells were also cultured in differentiation medium and stained to assess the myogenic potential of the attached cells. The results of this testing is described below.

6.3.1 Assessment of Satellite Cell Attachment to Microthread Bundles

Satellite cells were fixed and stained after 72 hours in growth medium as well as after 6 days in differentiation medium to assess cell attachment and confirm that the transport vehicle does not cause premature differentiation and does not inhibit induced differentiation. At these time points the threads and attached cells were fixed with formaldehyde and stained for nuclei using Hoechst 33342 and myosin heavy chain using MF20 according to the procedures in Appendix F.9 and F.10 used for the two dimensional analysis. Images were taken at 10x magnification at an exposure time of 0.014 seconds for Hoescht 33342.

As seen in Figure 30, satellite cells were stained with Hoechst 33342 to visualize the nuclei attached to the threads along the edges as well as in the middle. Cells were also found in various planes, showing that they attached in three dimensions and migrated within the bundled thread structure. Very few cells were found on the fibrin microthread bundle as depicted in Panel A of Figure 30. A greater number of cells were found on vitronectin coated fibrin microthread bundle as seen in Panel B; however, the greatest number of satellite cells was found on collagen I coated

fibrin microthread bundle as seen in Panel C. Further testing is needed to verify and validate this outcome.

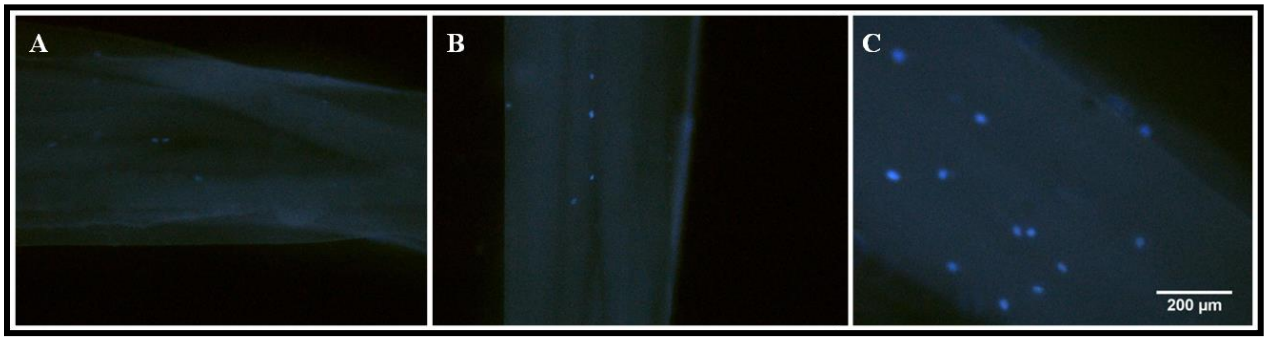


Figure 30: Florescence microscopy of satellite cells attached to microthreads

Satellite cells attached to fibrin threads (A) and vitronectin coated fibrin threads (B) and collagen I coated fibrin microthreads (C). Blue: Nuclei (Hoescht). Images were taken at 10x magnification.

6.3.2 Assessment of Satellite Cell Premature Differentiation and Myogenic Potential

The satellite cells attached to the threads were also stained using MF20, a marker for myosin heavy chain. Images were taken at 32X magnification at an exposure time of 0.062 seconds for Hoescht (blue) and 0.5 seconds for MF20 (red). As seen in Figure 31, no cells were seen to have differentiated, which would have been marked by distinct, red, tube like structures around the nuclei of the cells. This shows that attachment of these cells to the above substrates does not catalyze premature differentiation. Satellite cells attached to the threads in an undifferentiated state, which also means that these cells have the capability to proliferate along the thread. One consideration to note is that satellite cells often proliferate by detaching from the substrate, dividing, and then attaching once again to the substrate. By this mechanism, satellite cells may detach, divide, and then attach to the surface of the plate due to the force of gravity as seen in phase contrast imaging. As a result, different seeding methods should be investigated to optimize continual satellite cell attachment.

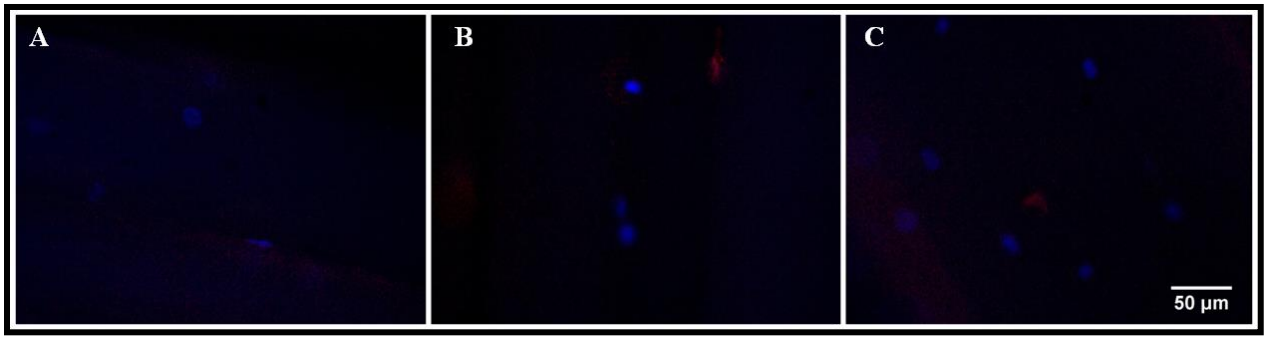


Figure 31: Florescence microscopy of undifferentiated satellite cells attached to microthreads

Satellite cells attached to fibrin threads (A) and vitronectin coated fibrin threads (B) and collagen I coated fibrin microthreads (C) showing the lack of myosin. Red: Myosin (MF20) Blue: Nuclei (Hoescht), Images taken at 32X magnification.

After 72 hours in growth media, threads with attached cells were transferred to differentiation medium for 6 days to ensure that the satellite cells maintained their myogenicity. Images were taken at 20X magnification at an exposure time of 0.062 seconds for Hoescht (blue) and 0.5 seconds for MF20 (red). These images were difficult to process as the edges of the thread appeared to fluoresce as well. However, myotubes were seen indicating that the satellite cells maintained their ability to differentiate into myotubes while cultured on the microthreads. Myosin staining was seen on both fibrin microthread bundles and collagen I coated fibrin microthread bundles. However, as seen in Figure 32, a greater number of cells attached to the collagen I coated fibrin microthread. Differentiated cells were seen in the middle of thread as indicated by the red staining for MF20. The myogenic potential was not calculated due to very low attachment for the fibrin sample and a low sample size. These two factors could lead to unrepresentative results. Although the low cell density on the uncoated fibrin thread did not allow the team to calculate the myogenic potential, it does support the previous conclusion that there is significantly lower attachment on the uncoated fibrin thread compared to the collagen I coated fibrin thread.

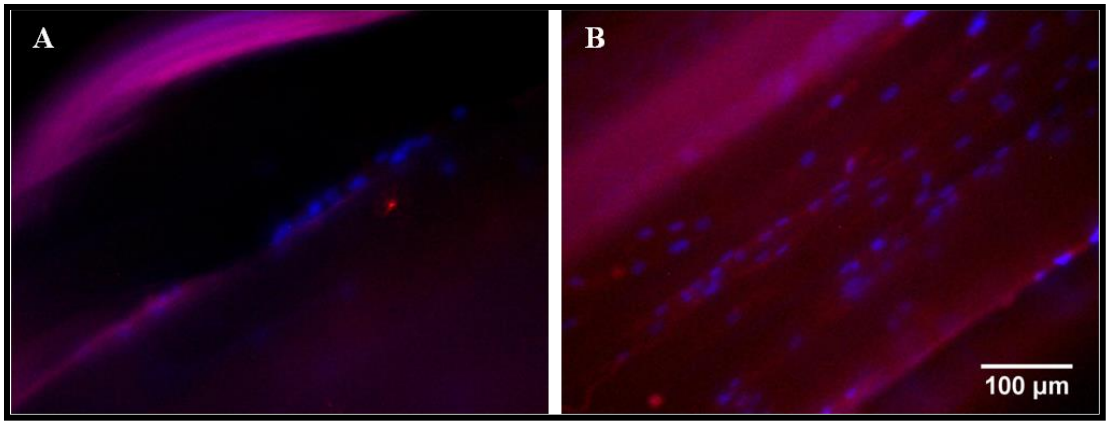


Figure 32: Florescence microscopy of differentiated cells on microthread bundles

Satellite cells attached to fibrin threads (A) and collagen I coated fibrin microthreads (B) showing differentiated cells. Red: Myosin (MF20) Blue: Nuclei (Hoescht). Images were taken at 20X magnification.

6.3.3 Degradation Time of Collagen Coated Fibrin Microthreads and Fibrin Microthreads

As a result of the higher cell attachment and lack of premature differentiation associated with collagen I coated microthreads, the team decided to conduct degradation testing with only collagen I and a control. This testing would help to ensure that the addition of a collagen I coating was not negatively impacting biodegradable property of fibrin microthreads. Two PDMS thread bundles were prepared as described above, and one was coated with collagen I as previously stated while the other served as an uncoated control. These bundles were placed in a sterile 6 well plate and were sterilized with 70% ethanol for 1 hour. The threads were then rinsed with sterile tris-buffered saline (TBS, 25 mM Tris–HCl) (Sigma) with 0.9% NaCl (pH 7.5) 3 times and then hydrated with TBS for 30 minutes. 3 Images were taken of each bundle at 10X magnification as the 0 hour time point. Plasmin (Calbiochem) (0.1 U/mL) in TBS was then prepared and 750 µL of the solution was placed in each well (Grasman, et al., 2012). Threads were imaged at 0 – 14 hours to assess the degradation of both bundles. Representative images of the bundles at 0 and 8 hours are shown below in Figure 33. Both showed a significant reduction in diameter size during the testing period. Additionally, the fibrin bundle was severed at 13 hours (n = 1), while the collagen I coated bundle was broken at 10 hours (n = 1). Repetition of this testing would be necessary to further quantify these results, but it was shown that the application

of a collagen I coating to a fibrin microthread bundle does not impede the threads ability to degrade over time.

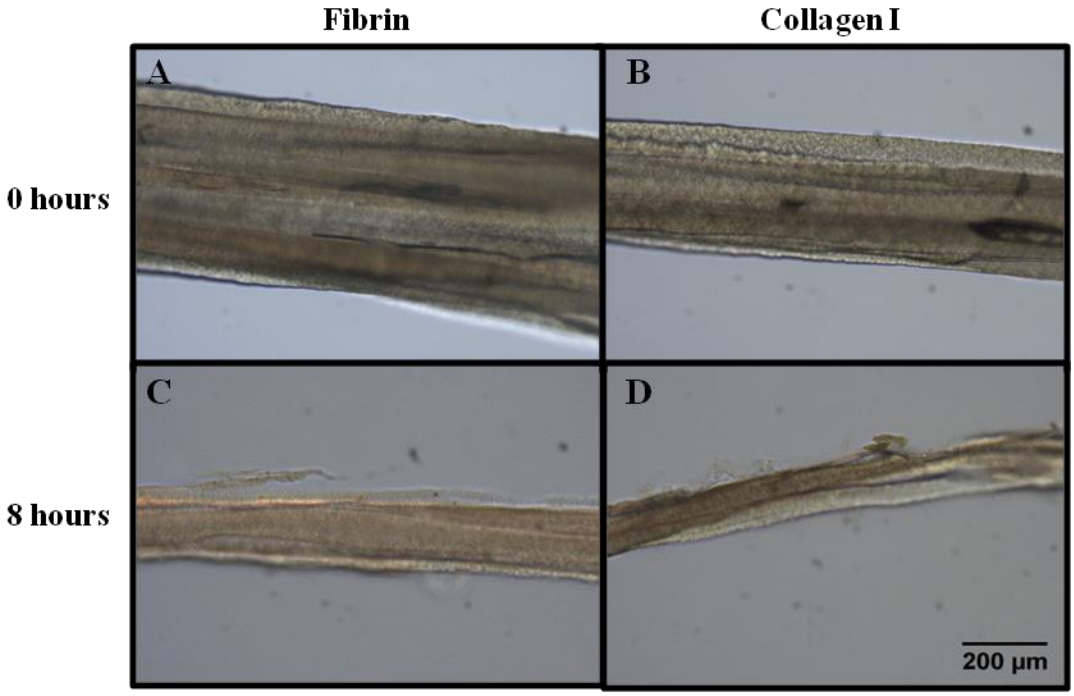


Figure 33 : Degradation of a fibrin microthread bundle and a collagen I coated fibrin microthread bundle after being placed in a plasmin (0.1U/mL) solution for 0 and 8 hours.

CHAPTER 7: DISCUSSION

Initially, the team set out to create a cell delivery vehicle that preserves the axial alignment properties of native muscle and permits the transplantation of a non-adherent satellite cell population. The success of the project was based on the initial objectives, functions, and constraints. The final design increases cell attachment in comparison to the currently used fibrin microthreads without causing premature differentiation. It also maintains the axial alignment of native muscle tissue. In addition the design is efficient, implantable with biodegradable and biocompatible properties, and is an economical solution. Furthermore, the design is sterile and safe. Based on the design process, two dimensional, and three dimensional testing completed by the team, these parameters were successfully completed and cumulate in a final design of a collagen I (0.31 mg/mL) coated fibrin microthread bundle. Further description of the final design is discussed in this chapter.

7.1 Device Design

The team's decision to coat the fibrin microthread as opposed to changing the scaffold form, scaffold material, or the satellite cell expression, allowed the final design to take advantage of the beneficial mechanical properties of the initial fibrin microthread system as well as the material's lack of foreign body response. A collagen microthread on the other hand may incite a negative response once implanted because of its similarities to scar tissue. Furthermore, the team was able to fabricate and sterilize the control fibrin microthreads based on published procedures, while developing new procedures for coating the fibrin microthread and testing its ability to promote cell attachment and myogenic potential.

Two dimensional testing showed that 0.31 mg/mL collagen I had improved cell attachment that was statistically significant in comparison to a fibrin coating. Collagen I promoted cells to attach and proliferate with a higher myogenic potential. Immunocytochemistry verified that it was possible to coat a fibrin microthread bundle with collagen I. Three dimensional analysis illustrated that the collagen I coated fibrin microthread bundle had improved cell attachment properties over fibrin microthreads alone, yet the coating did not cause premature differentiation of the attached satellite cell population which still had myogenic potential. Degradation testing showed that the collagen I coating did not negatively impact the fibrin microthreads' ability to

degrade over time. As a cumulative result, this testing showed that the final design has efficient, improved cell attachment, lacks premature differentiation, maintains axial alignment, is biodegradable, and is simple to handle. Furthermore, it meets the design constraints of being sterile as well as safe for the user and patient, as the team was able to successfully culture cells on the scaffold. The design is also be biocompatible as fibrin microthreads have previously been implanted in vivo and shown to not exhibit a negative foreign body response.

7.2 Device Limitations

The primary limitation of the final design is that it does not directly inhibit scar tissue formation and promote cell engraftment since *in vivo* testing was not within the scope of this project. However, prior to *in vivo* testing, additional *in vitro* tests are recommended, as outlined in Chapter 8. Additional limitations include consistent collagen I coating of the fibrin microthread, and the partial dependence of the percent cell attachment on the satellite cell cycle. Lastly, further testing would be needed to ensure that the addition of collagen I on the surface of the threads would not negatively impact its biocompatibility.

7.3 Manufacturability

The team's design can be manufactured as a kit for academic or commercial use. The fibrin microthreads can be drawn automatically, as shown by VitaThreads, LLC (Worcester, MA). Research on how to automate the stretching and drying of these threads is currently being conducted by other research teams. Once dried, the threads can be sterilized and shipped in double sterile packaging along with frozen collagen I solution for coating prior to use. Instructions for thawing and coating the collagen I can be provided with the packaging, in addition to recommended cell seeding densities. Furthermore, this design can be easily scaled-up and scaled-down since larger scale applications can use more or fewer collagen I coated fibrin microthread bundles. The primary market for this product is small academic laboratories and research institutions interested in skeletal muscle regeneration for the treatment of volumetric muscle loss. This product could also be used in other tissue regeneration applications as well if the cell type used for the application was found to perform as proficiently as human satellite cells.

7.3 Economical Impact

Due to the selected coating being collagen I, the design is cost efficient compared to other substrates that was found to promote human satellite cell attachment such as fibronectin and vitronectin. Collagen I is available at various suppliers nationwide and worldwide.

Developing an *in vitro* cell delivery vehicle greatly reduces costs and risks during the research phases, and thus it allows for increased spending on *in vivo* animal trials and clinical trials once the design is complete. Designing a cell delivery vehicle that can eventually help treat those suffering from volumetric muscle loss will reduce the amount of government spending on research regarding treating and curing muscular dystrophy, Rhabdomyosarcoma, and sarcopenia, and thus allow for the funding of other ailments.

7.4 Environmental Impact and Sustainability

This system used primarily polystyrene flasks, well plates, and glass to design a method for improving the attachment of satellite cells to fibrin microthreads. The substrates tested are naturally occurring components and would not have a harmful effect on the environment. The flasks and plates used to culture our system are made from biodegradable materials so that, when disposed of all components of the system, there is minimal amount of negative effects on the environment. The culturing system is made to be easily sterilized and reused when possible, which reduces waste and the associated costs for removal. .

7.5 Societal Influence

The research from this project could potentially aid in muscle regeneration and also provides new information for any studies using human satellite cells. If the design is eventually released commercially, volumetric muscle loss and scar tissue formation that was previously without treatment besides autografts may now show muscle tissue recovery. This could aid patients with traumatic injuries, Rhabdomyosarcoma, and myopathies such as muscular dystrophy, while promoting awareness in regards to these ailments. This research could also aid other studies involving human satellite cells or any of the tested materials. The team believes that this study showed some insight into the cell cycles, attachment potential, and myogenic potential of human satellite cells and the growth factors they are exposed to during proliferation.

7.6 Political Ramifications

Currently, the device has no foreseeable political ramifications as the human satellite cells that were cultured are widely accepted in the research and public communities. However, improved muscle regeneration therapies using the device could have potential effects in military medicine and muscular disease treatment. This could have a small impact on the distribution of national funds for research purposes to improve and scale up the system as well as to implement this system in clinical settings.

7.7 Ethical Concerns

If our device was found to be successful *in vitro*, it is probable that future testing would involve *in vivo* testing in a rat model as was previously done with fibrin microthreads (Page, *et al*., 2011). By testing a large volume of materials in two dimensional assays and then evaluating the top material candidates through three dimensional testing to select a final design the team avoided the unnecessary use of animal models. Furthermore, future *in vivo* testing will require a reduced number of animal models, due to the statistically large number of *in vitro* tests conducted. We are also using human skeletal muscle cells that were provided by a male, 19 year old donor, which may be of concern to some. However, the donation does not affect the person's quality of life in any way and also can be fully recovered from.

7.8 Health and Safety Considerations

This system was not used clinically or for *in vivo* studies, therefore there was no potential for health or safety concerns in the functions of our system. In addition, harmful or toxic substances were avoided while designing and using this system. While designing and using the system, all safety protocols were implemented and followed according to the protocols in the laboratories at Worcester Polytechnic Institute's Gateway Park. To ensure sterility of the components of our device all testing with satellite cells was completed in a Biosafety Level II laminar flow hood.

CHAPTER 8: CONCLUSIONS AND FUTURE WORK

The design team was able to address the primary objectives as set forth by the revised client statement in Section 3.2 and Section 3.6 respectively. The collagen I coated fibrin microthread bundle increases human satellite cell attachment while preventing premature differentiation as shown in three dimensional testing discussed in Section 6.3.1 and Section 6.3.2. This system is sterile and safe, as the coated bundle was sterilized in ethanol and the viability of the cells cultured on the final design remained high. Due to the team's decision to coat the fibrin microthread instead of changing the scaffold material or form, the final design maintains axial alignment. The system is also biodegradable, as shown in Section 6.3.3. Finally, the biomaterial chosen for coating the fibrin microthread is an economical solution since it has a much lower cost than some of the other materials tested during two dimensional and three dimensional testing, such as fibronectin and vitronectin.

As future work, the design team recommends further verification and validation of the collagen I coated fibrin microthread bundle selected as the final design. The following is a list of *in vitro* and *in vivo* tests that would further characterize the final design and aid in determining whether the designed cell delivery vehicle promotes cell proliferation, cell myogenicity, and cell engraftment, while inhibiting scar tissue formation.

- Assess the effect of collagen I on proliferation of the satellite cells by conducting a Bromodeoxyuridine (BrdU) assay.
- Investigate the effect of collagen I on forcing the satellite cells down a myogenic pathway using early muscle markers such as MyoD and Myogenin. This will help determine whether or not this substrate causes satellite cells to begin differentiation while still in growth medium and will aid in determining the cell culture protocol for seeding the delivery scaffold for *in vivo* applications.
- Test other methods of cell seeding on fibrin microthreads such as tube rotation to maximize cell adherence.
- Verify that the mechanical properties of the coated fibrin microthreads, such as their ultimate tensile strength (UTS), fatigue strength. Ideally, these mechanical properties will be equal or better than the uncoated fibrin microthreads.

- Conduct additional degradation testing of collagen I coated and uncoated fibrin microthread bundles.
- Conduct additional three dimensional testing with higher cell concentrations for fibrin microthread bundles, collagen I, vitronectin, and fibronectin coated microthread bundles to assess cell attachment, premature differentiation, and myogenic potential.
- Test various concentrations of collagen I coatings to find which best improves cell attachment.
- Conduct *in vivo* testing with small animals to assess the ability of collagen I coated fibrin microthreads to inhibit scar tissue formation and promote cell engraftment. This would also allow for evaluation of the presence of a foreign body response resulting from the collagen I coating. Due to the large amount of *in vitro* testing conducted in this project, as well as the additional *in vitro* testing being suggested as future work, the number of animals needed for *in vivo* testing can be minimized.

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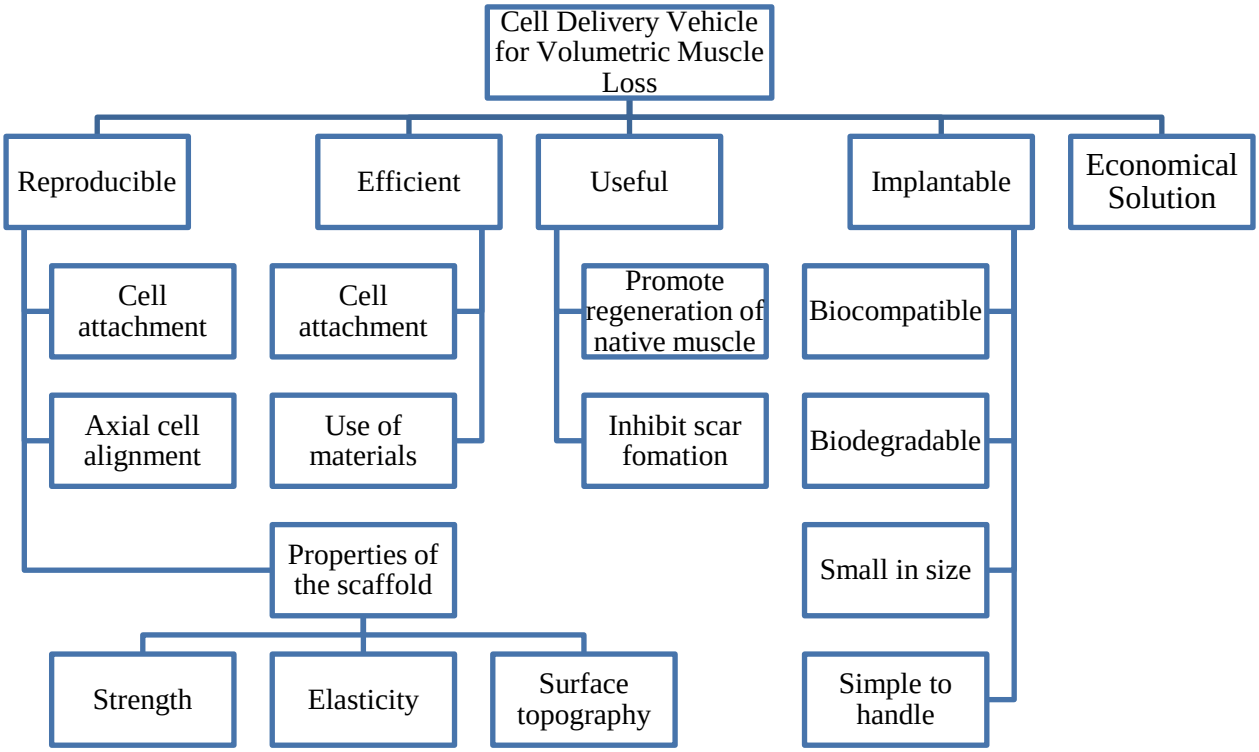
APPENDICES

Appendix A: Objectives Tree



Appendix B: Pairwise Comparison Charts

Table 8: Pairwise Comparison Chart - Reproducible

	Cell Attachment	Axial Alignment	Properties of the Scaffold	Score
Cell Attachment	X	1	1	2
Axial Alignment	0	X	1	1
Properties of the Scaffold	0	0	X	0

Table 9: Pairwise Comparison Chart - Efficient

	Cell Attachment	Use of Materials	Score
Cell Attachment	X	1	1
Use of Materials	0	X	0

Table 10: Pairwise Comparison Chart - Useful

	Promote regeneration of native muscle	Inhibit scar formation	Score
Promote regeneration of native muscle	X	1	1
Inhibit scar formation	0	X	0

Table 11: Pairwise Comparison Chart - Implantable

	Biocompatible	Biodegradable	Small	Simple to handle/implant	Score
Biocompatible	X	1	1	1	3
Biodegradable	0	X	1	0.5	1.5
Small	0	0	X	0	0
Simple to handle/implant	0	0.5	1	x	1.5

Appendix C: Functions Means Chart

Cell Delivery Vehicle for Volumetric Muscle Loss

- **Transplant non-adherent cell population**
    - Develop a scaffold with compatible mechanical properties
        - Gels
        - Threads
        - Matrices
- **Increase satellite cell attachment**
    - Use a material that allows for increased cell attachment
        - Fibrin, collagen, HA, silk, gelatin, PLA-gamma, PEG+PLLA, Bioglass
    - Modify expression in satellite cells
        - Integrins
            - αvβ3 and αvβ5 integrins
        - Growth factors
            - FGF-2, IFG-I, IFG-II, and TGF-β1
        - ECM proteins
            - Tenascin-C
    - Modify the surface
        - Crosslink the surface
            - Physical: UV
            - Chemically: EDC, genipin, succinalyate
        - Coat the surface
            - Vitronectin, fibronectin, laminin, collagen, fibrin
        - Modify the surface topography
            - Freeze drying, porosity, salt leaching
- **Not cause premature differentiation of satellite cells**
    - Modify expression in satellite cells
        - Growth factors
        - Gene expression
    - Inhibit confluence

        - Seed fewer cells
        - Change culture time
        - Growth factors
            - Reduce time of exposure
            - Reduce concentration
- **Preserve mechanical integrity of the current system**
    - Maintain axial alignment
        - Structural materials to make an aligned scaffold
    - Improve the mechanical properties not well addressed by the current system
        - Stiffness, tensile strength, diameter

Appendix D: Work Breakdown Structure (WBS)

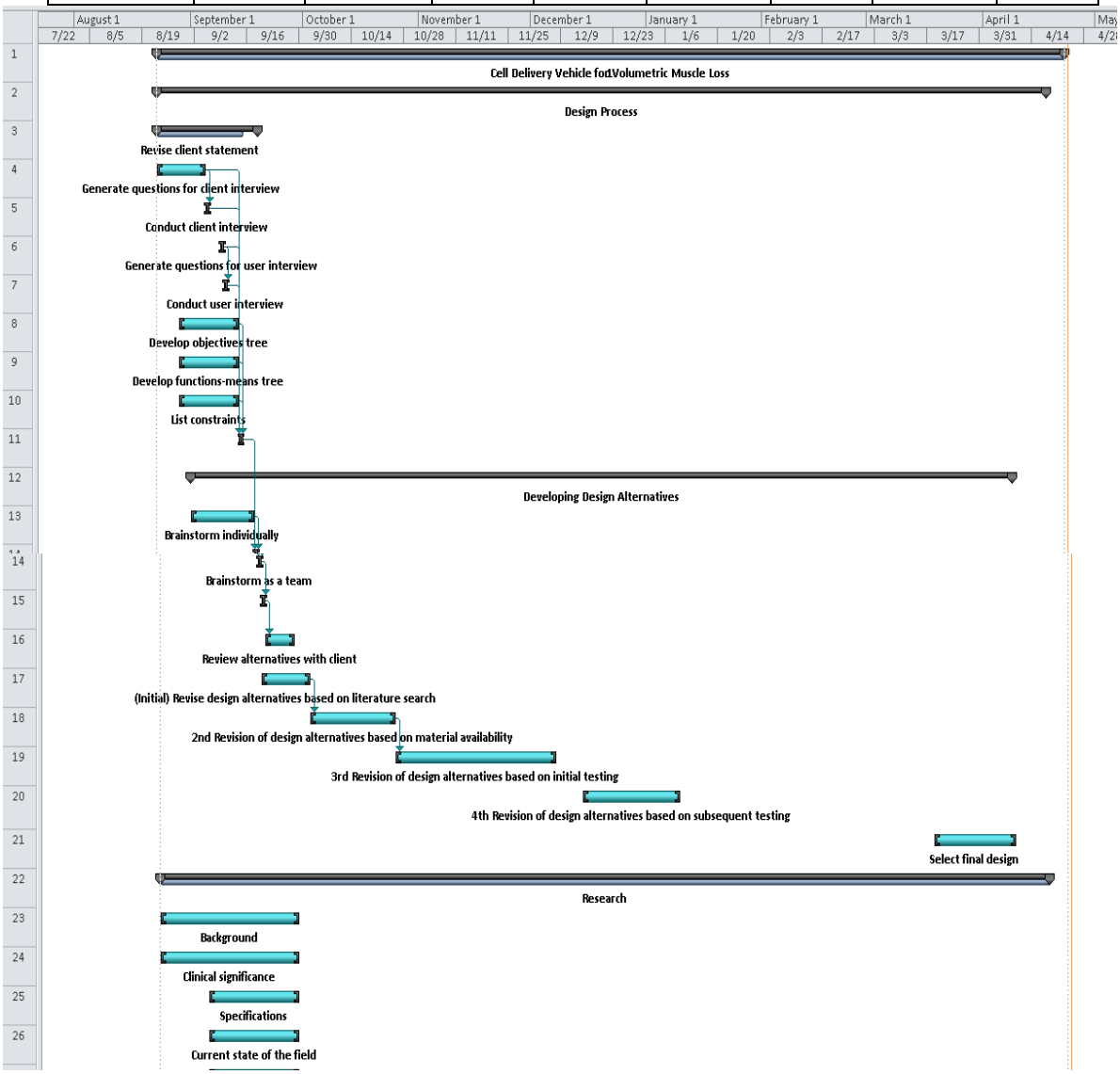
1. Cell Delivery Vehicle for Volumetric Muscle Loss
1.1. Design Process
1.1.1. Revise client statement
1.1.1.1 Generate questions for client interview
1.1.1.2 Conduct client interview
1.1.1.3 Generate questions for user interview
1.1.1.4. Conduct user interview
1.1.1.5. Develop objectives tree
1.1.1.6. Develop functions-means tree
1.1.1.7. List constraints
1.1.1.8. Revise client statement for clarity and completeness
1.1.2. Developing Design Alternatives
1.1.2.1 Brainstorm individually
1.1.2.2. Brainstorm as a team
1.1.2.3. Categorize alternatives
1.1.2.4. Review alternatives with client
1.1.2.5. (Initial) Revise design alternatives based on literature search
1.1.2.6. 2nd Revision of design alternatives based on material availability
1.1.2.7. 3rd Revision of design alternatives based on initial testing
1.1.2.8. 4th Revision of design alternatives based on subsequent testing
1.1.2.9. Select final design
1.1.3. Research
1.1.3.1 Background
1.1.3.2 Clinical significance
1.1.3.3 Specifications
1.1.3.4 Current state of the field
1.1.3.5. Previous research and tissue engineering strategies
1.1.3.6. Tissue engineering scaffolds
1.1.3.7. Material methods of improving attachment
1.1.3.8. Biochemical methods of improving attachment
1.1.3.9. Testing methods and protocols
1.2 Documentation
1.2.1. Weekly reports
1.2.2. MQP report chapters
1.2.3. Research notes
1.2.4. Laboratory notebook
1.2.5. Meeting minutes
1.2.6. Project Presentation Day - write IEEE abstract

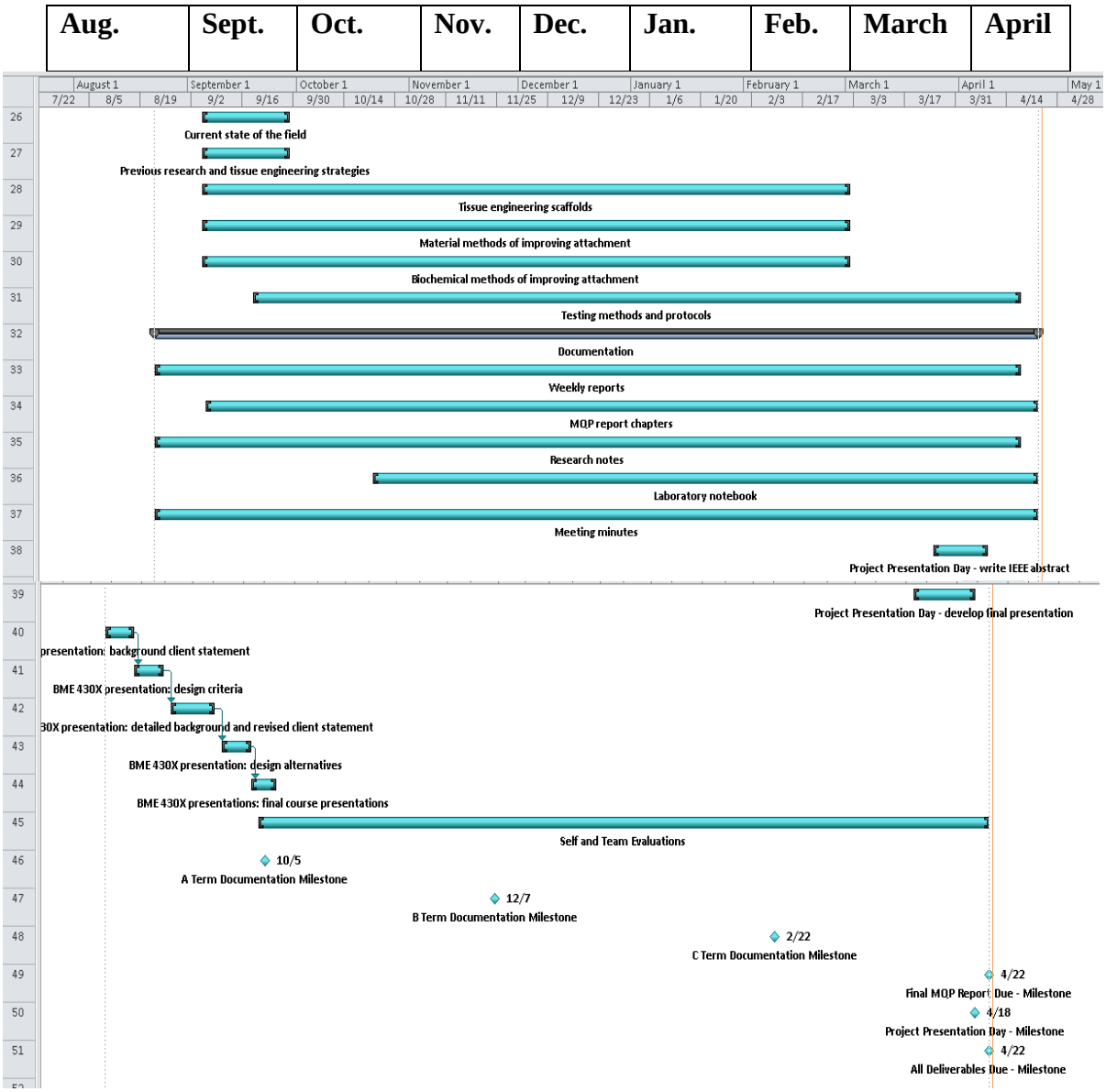
1.2.7. Project Presentation Day - develop final presentation
1.2.8. BME 430X presentation: background client statement
1.2.9. BME 430X presentation: design criteria
1.2.10. BME 430X presentation: detailed background and revised client statement
1.2.11. BME 430X presentation: design alternatives
1.2.12. BME 430X presentations: final course presentations
1.2.13. Self and Team Evaluations
M.1. A Term Documentation Milestone
M.2. B Term Documentation Milestone
M.3. C Term Documentation Milestone
M.4. Final MQP Report Due – Milestone
M.5. Project Presentation Day – Milestone
M.6. All Deliverables Due – Milestone
1.3. Project Management
1.3.1. Organize and maintain electronic files
1.3.2. Manage team communications
1.3.3. Create and periodically modify Gantt chart and WBS
1.3.4. Create and modify agendas
1.3.5. Create and modify to-do lists
1.3.6. Scheduling
1.4. Laboratory Testing
1.4.1. Order/Obtain Laboratory Materials
1.4.1.1. Obtain and maintain satellite cells
1.4.1.2. Order/Obtain cell culture materials
1.4.1.3. Order/obtain candidate materials for preliminary testing
1.4.1.4. Order/obtain candidate biochemical factors for preliminary testing
1.4.1.5. Order/obtain candidate materials and biochemical factors for 3D testing
1.4.2. 2D Assays
1.4.2.1. Quantify the current system (2D) in terms of satellite cell attachment
1.4.2.2. Quantify the current system (2D) in terms of myogenic potential
1.4.2.3. Quantify the satellite cell attachment on various coatings (2D)
1.4.2.4. Quantify the myogenic potential of satellite cells cultured on various coatings
1.4.3. 3D Assays
1.4.3.1. Make fibrin microthreads
1.4.3.2. Bundle, sterilize, coat, and hydrate threads
1.4.3.3. Verify the coating of the bundled fibrin microthreads
1.4.3.4. Determine the satellite cell attachment on the current system (3D)
1.4.3.5. Determine the myogenic potential of satellite cells cultured on the current system (3D)

1.4.3.6.Determine the satellite cell attachment on bundled fibrin microthreads coated with top alternative substrates
1.4.3.7. Determine whether satellite cells cultured on bundled fibrin microthreads coated with top alternative substrates undergo premature differentiation
1.4.3.8. Determine the myogenic potential of satellite cells cultured on bundled fibrin microthreads coated with top alternative substrates
1.4.4. Assay imaging
1.4.5. Immunocytochemistry
1.4.6. Degradation testing
1.4.7. Data analysis
1.4.8. Lab training
1.4.9. Satellite cell passaging and maintenance

Appendix E: Gantt Chart

Aug.	Sept.	Oct.	Nov.	Dec.	Jan.	Feb.	March	April

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10/14
10/28
11/11
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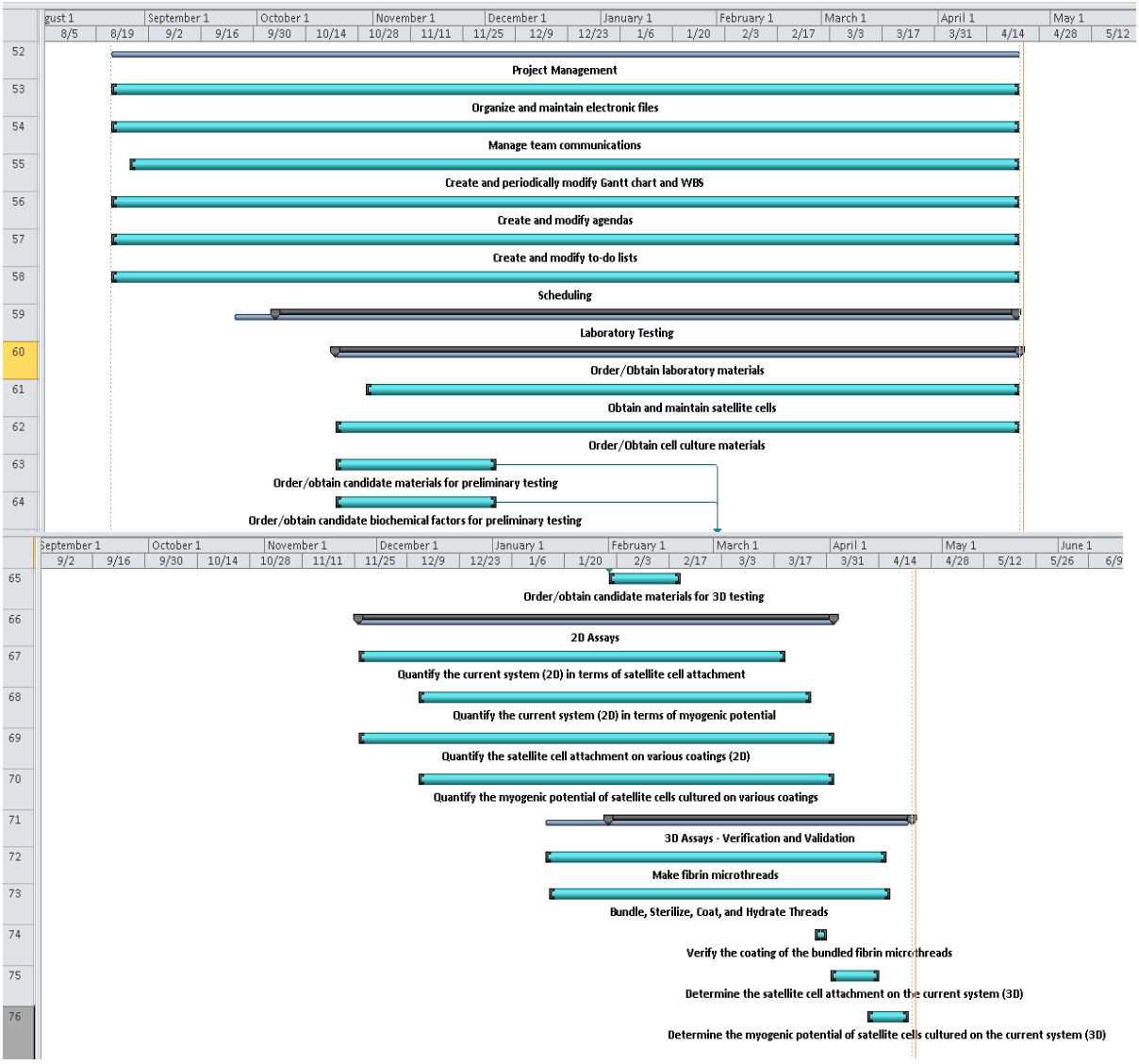
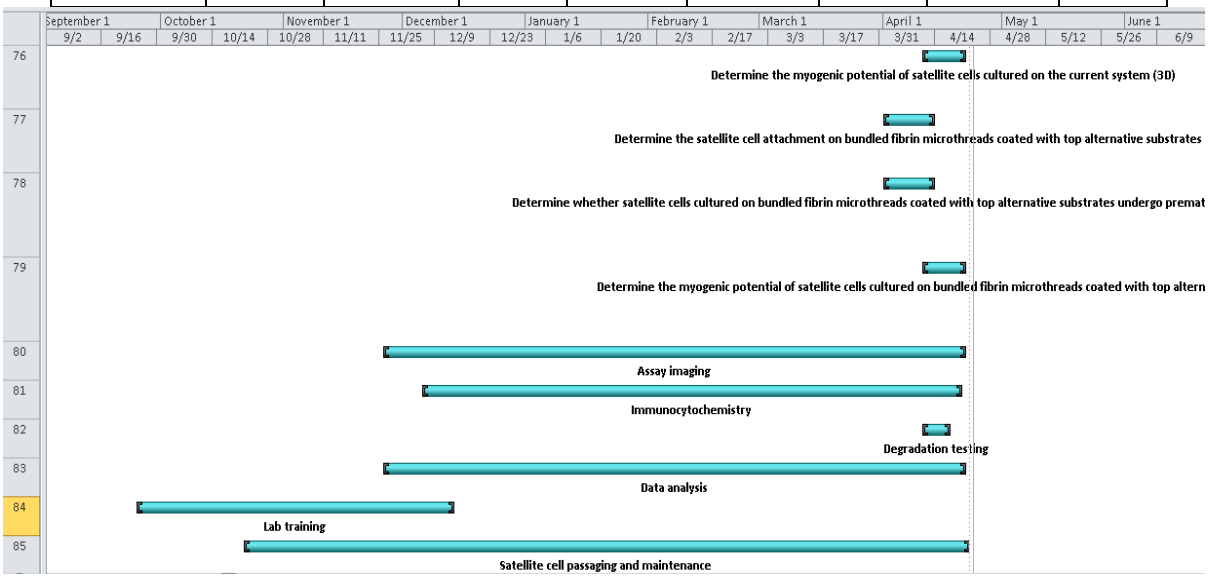
12/9
12/23
1/6
1/20
2/3
2/17
3/3
3/17
3/31
4/14
4/28
Current state of the field
Previous research and tissue engineering strategies
Tissue engineering scaffolds
Material methods of improving attachment
Biochemical methods of improving attachment
Testing methods and protocols
Documentation
Weekly reports
MQP report chapters
Research notes
Laboratory notebook
Meeting minutes
Project Presentation Day - write IEEE abstract
Project Presentation Day - develop final presentation
presentation: background client statement
BME 430X presentation: design criteria
30X presentation: detailed background and revised client statement
BME 430X presentation: design alternatives
BME 430X presentations: final course presentations
Self and Team Evaluations
10/5
A Term Documentation Milestone
12/7
B Term Documentation Milestone
2/22
C Term Documentation Milestone
4/22
Final MQP Report Due - Milestone
4/18
Project Presentation Day - Milestone
4/22
All Deliverables Due - Milestone

Aug.	Sept.	Oct.	Nov.	Dec.	Jan.	Feb.	March	April



Aug.	Sept.	Oct.	Nov.	Dec.	Jan.	Feb.	March	April



Appendix F: Experimental Protocols

F.1 Human Satellite Cells (hSC) Media Preparation

Warm each component of media to 37°C prior to making media. Always warm media to 37°C prior to use in mammalian cell culture.

hSC Proliferation Medium (DF12) with Growth Factors

Media Component	Final Concentration	Notes
DMEM 1X w/ 4.5 g/L glucose and sodium pyruvate (Cellgro)	54%	Without L-glutamine and phenol red
Ham's F-12 1X with L-glutamine (Cellgro)	36%	
Fetal Clone III (FCIII) (Hyclone)	10%	
4 mM L-glutamine (Cellgro)		
Proprietary growth factor cocktail (PeproTech)		

1. Mix 60:40 (v:v) of DMEM:F12 + 10% FCIII
2. Add 4mM L-Glutamine to DMEM in the following manner:
    a. Per 500mL medium (FV), add 0.2922g L-Glutamine to 12mL medium then sterile filter through a syringe into stock media bottle.
3. Add the growth factors one by one (names and concentrations proprietary) to the DF12:
4. Resuspend contents slowly with a serological pipet to mix.
5. Store medium at 4°C and warm to 37°C prior to use.

hSC Differentiation Medium

Media Component	Final Concentration	Notes
DMEM 1X w/ 4.5 g/L glucose and sodium pyruvate (Cellgro)	58.5%	Without L-glutamine and phenol red
Ham's F-12 1X with L-glutamine (Cellgro)	38.5%	
Horse serum (Hyclone)	2%	
Insulin-Transferrin-Selenium (ITS) (Cellgro)	1%	

1. Mix 60:40 (v:v) of DMEM:F12
2. Add horse serum and ITS
3. Resuspend contents slowly with a serological pipet.
4. Store medium at 4°C and warm to 37°C prior to use.

Freezing Medium

Freezing Solution: Media Component	Final Concentration
hSC Proliferation Media without growth factors	90%
DMSO (Cellgro)	10%

1. Transfer the appropriate amount of hSC proliferation medium without growth factors (see protocol above) to a 15 mL centrifuge tube (90% of desired final volume).
2. Add DMSO (10% of final volume) to the centrifuge tube using extra caution.

   Note: Wearing gloves for this procedure is highly recommended.
3. Resuspend the contents of the second tube to mix.

F.2 Thawing Cells

1. Remove desired number of cryovials from liquid nitrogen.
2. Place vials in water bath at 37°C to thaw (use immediately, or keep on dry ice until ready to thaw).
3. Add 5 mL of fresh medium (hSC proliferation media with growth factors) to 15 mL centrifuge tube.

4. Introduce 1 mL of hSC proliferation medium with growth factors to the contents of the cryovial drop by drop.
5. Add the contents of the cryovial to the 15 mL centrifuge tube and centrifuge at 1100 RPM for 6 minutes.
6. Aspirate the supernatant taking care not to disturb the cell pellet.
7. Resuspend the pellet in 1 mL of hSC proliferation medium with growth factors.
8. Add 8 mL of hSC proliferation medium with growth factors to each T75 flask (the number of flasks used depends on the desired cell seeding density).
9. Add the appropriate amount of cell suspension to each flask.

F.3 Feeding hSC Cells

1. Observe the cells using light microscopy for possible contamination, cellular morphology, and confluence.
2. Transfer half of the cell suspension (non-adherent cells and culture media) to a 15 mL centrifuge tube and spin at 1100 RPM for 6 minutes.
3. Aspirate the supernatant without disturbing the cell pellet, leaving about 0.5 mL at the bottom of the tube.
4. For a T75 flask, add between 3.5 mL to 4 mL of warm hSC proliferation medium with growth factors and resuspend the pellet. For a T150 flask, add 7.5 mL – 8 mL of medium.
5. Transfer the contents of the 15 mL centrifuge tube to the same cell culture flask.
6. Repeat steps 1 – 5 for additional flasks.

F.4 Counting Cells with a Hemocytometer

1. Add 40 µL of PBS to a clean microcentrifuge tube.
2. Pipet 10 µL of cell suspension into the microcentrifuge tube. Be sure to resuspend the sample prior to taking this aliquot out.
3. Add 50 µL of trypan blue to the contents of the microcentrifuge tube and resuspend the entire contents to mix.
4. Clean the hemocytometer and place the coverslip on top.
5. Load 10 µL of the microcentrifuge tube contents to each side of the hemocytometer, taking care not to overflow either side.

6. View the grid on the hemocytometer under 10X magnification using light microscopy. Focus in on the gridlines.
7. For each side of the hemocytometer, count the cells in the 4 large squares (Figure 34 below illustrates one of these squares) in the corners and the square in the middle. Count the cells on the top and right edges of the squares but do not ocunt the cells on the bottom and left edges of the squares.

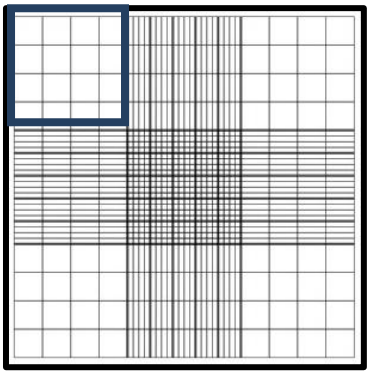


Figure 34: Schematic of one side of a hemocytometer

8. Count the number of dead cells in each of these 10 squares. Dead and dying cells will stain blue.
9. After determining the total number of cells in all 10 counted regions (both sides of the hemocytometer) multiply the number by the dilution factor (10 µL of cell suspension in 100 µL total volume indicates a dilution factor of 10), and then multiply this number by 10^4 to calculate the number of cells per mL.

    i.e. If 25 total cells are counted across the 10 regions, there are 2,500,000 total cells per mL.
10. Convert the number of dead cells into the number of dead cells per mL using the information in step 9.

    i.e. If 4 dead cells are counted across the 10 regions, there are 40,000 dead cells per mL.
11. To calculate the percent viability subtract the number of dead cells per mL from the total number of cells per mL and then dividing that number by the total number of cells per mL. Then multiply this number by 100.

    i.e. For a count resulting in 25 total cells and 4 dead cells:

((2,500,000 – 40,000) / 2,500,000) * 100 = 98.4% cell viability

F.5 Subculturing hSC Cells

When cells reach 70% confluency they need to be subcultured according to the following protocol for each T75 flask. Reduce and/or scale-up volumes as appropriate for T25 flasks and T150 flasks respectively.

1. Transfer the entire cell suspension (non-adherent cells and culture media) into a 15 mL centrifuge tube.
2. Wash the flask with 5 mL of DPBS, taking care to swirl the DPBS across the entire bottom surface of the flask.
3. Trypsinize the adherent cells by adding 3 mL of 0.05% trypsin EDTA.
4. Observe detachment under light microscopy to confirm release. Let the flask stand for up to 2 minutes at room temperature.
5. Deactivate trypsin with 3 mL of proliferation medium.
6. Transfer the entire contents of the flask to a 15 mL centrifuge tube.
7. Centrifuge the non-adherent and adherent cells at 1100 RPM for 6 minutes.
8. Aspirate the supernatant, taking care not to disturb either cell pellet. For the tube containing the non-adherent cell population, leave about 0.5 mL of supernatant.
9. Determine the number of cells per mL in each population by following the protocol in Appendix F.4.
10. Add 8 mL of hSC proliferation medium with growth factors to each new T75 flask and plate the desired concentration of cells. Depending on the application, non-adherent and adherent cells may be combined.

F.6 Freezing Cells

1. Trypsinize and resuspend the cells based on the protocol in Appendix F.5.
2. Count the cells and calculate the total number of cells using the protocol in Appendix F.4.
3. Centrifuge the cells at 1100 RPM for 6 minutes.
    1. Prepare freezing medium according to the protocol in Appendix F.1.

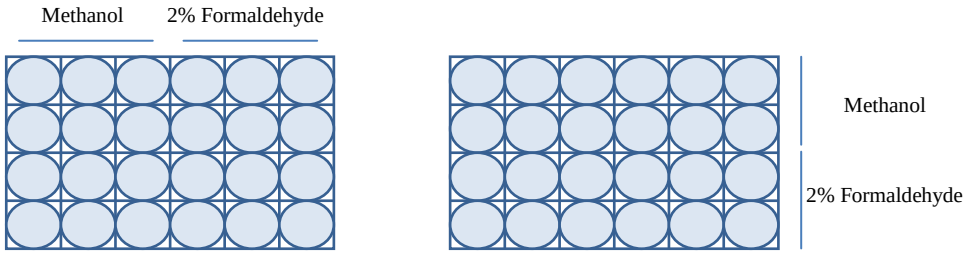
4. Aspirate the supernatant and add freezing medium slowly while mixing the contents gently. Cells are typically resuspended to 0.5 to 1 million cells/ml in freezing medium and pipetted into cryovials.
5. Aliquot 1 ml into each cryovial. Tighten the caps well. Do not overfill cryovials.
6. Place cryovials into the freezing container and transfer the container into the -80°C freezer overnight, then transfer vials to liquid nitrogen for long term storage.

F.7 Fixing cells with 2% Formaldehyde or Cold Methanol

Procedure for 24-well or 4-well plate

Note: Scale-up volumes when using a 6-well plate

1. When cells reach 70% confluency remove medium by aspiration.
2. Wash cells 1X with 0.5 ml/well PBS and let it sit for 5 min.
3. Remove PBS by aspiration and start fixing cells with 2% formaldehyde or methanol. If you have to fix with both reagents make sure you label the sides of 24-well plate before you start:



4. Add 0.5 ml/well 2% formaldehyde and incubate cells at RT for 15 min or add 0.5 ml/well cold methanol and incubate cells at RT for 15 min
5. Wash each well 3X with 0.5 ml/well PBS and each time let PBS sit in the wells for 5 min.
6. Remove PBS by aspiration and add 0.5 ml/well 0.1 % NaAzide in PBS.
7. Parafilm plates carefully and keep at 4^{o}C.

Note: Cold methanol and 2% formaldehyde fixations each have a different mechanism of fixing. Formaldehyde does not permeabilize the cell membrane but is gentler on the cells whereas methanol breaks through the membrane and cells often detach from the bottom of the plate. For this reason the team used 2% formaldehyde fixation for the hSCs.

F.8 Coating 24-well Plates

Fibrin (yields 500 µL *fibrin*)

1. Place all materials and the 4-well plate on ice for 10 minutes
2. Mix 37.5 µL of 40 U/mL thrombin (EMD Millipore) and 212.5 µL of 40 mM cold calcium chloride dehydrate (JT Baker) under the hood and keep on ice
3. Add 250 µL of 70 mg/mL fibrinogen (EMD Millipore) to the solution
4. Immediately aliquot 100 µL of solution to the well and remove 70 µL of solution leaving a thin layer on the well

   Note: The volume aliquoted can be adjusted when only coating one well to enough solution to cover the surface of the well

5. Incubate the gel at RT for 30 minutes to produce a gel with a thickness of 150 µm
6. Store the gel with 1 mL PBS per well at 4°C until further use

Collagen I for short term cultures

1. Dilute collagen I (PureCol®, Advanced Biomatrix) solution to 0.31 mg/mL with 30% ethanol and spread over surface of sterile glass coverslip.
2. Air dry in a tissue culture hood.
3. Cells can be seeded directly on the collagen surface.
4. Collagen coating prepared in this way tends to detach from the glass in long-term cultures.

Laminin

1. Stock solution can be prepared by dissolving 1 mg/ml laminin-111 (VWR) in PBS. Filter sterilize and freeze in aliquots.
2. Diluted stock solution to 10-100 ug/ml in basal medium or PBS.
3. Add enough solution to pool over surface of sterile glass coverslip.
4. Incubate several hours at room temperature.
5. Aspirate to remove laminin and rinse coverslips with medium or PBS.
6. Immediately add cell suspension or growth media. Do not allow coating to dry.

7. Coating the glass coverslip first with polylysine or polyornithine and then laminin may increase the concentration of laminin applied using this method.

Chitosan

1. Dilute deacetlylated chitosan (Sigma) in 1% acetic acid to 5 mg/mL.
2. Dilute solution in PBS to result in 0.5 mg/mL chitosan.
3. Pool 100 µL of the chitosan solution over the surface of each well and cover the surface fully.
4. Remove 70 µL of the solution to leave a coating of 30 µL.
5. Air dry the plate at room temperature for 12 hours.
6. Rinse the gel once with PBS before seeding the cells onto the substrate.

Vitronectin

1. Dilute stock human vitronectin (Gibco) to 0.02 mg/mL in PBS
2. Pool 100 µL of the vitronectin solution over the surface of each well and cover the surface fully
3. Remove 70 µL of the solution to leave a coating of 30 µL
4. Air dry the plate at room temperature for 12 hours
5. Rinse the gel once with PBS before seeding cells onto the substrate

Fibronectin

1. Dilute human fibronectin (Gibco) to 0.08 mg/mL in PBS
2. Pool 100 µL of the vitronectin solution over the surface of each well and cover the surface fully
3. Remove 70 µL of the solution to leave a coating of 30 µL
4. Air dry the plate at room temperature for 12 hours
5. Rinse the gel once with PBS before seeding cells onto the substrate

F.9 Immunocytochemistry for Myosin

Procedure for 24 or 4 well plate:

Note: Scale-up volumes when using a 6-well plate

1. Aspirate PBS (0.1% NaAzide) from the wells to be assayed, do not let wells to be dried.
2. If cells are fixed in formaldehyde and the antigen is intracellular, permeabilize cells by incubating in 0.5 ml of 0.1% Triton X-100 in PBS for 20 min.
3. Wash each well 1X 0.5 ml with PBS/Tween with 5 min wait.
4. Block by incubating cells with 0.5 ml of blocking solution at RT for 15 min.

   (Blocking solution: 5% Serum in PBS – which is 25 µl FBS + 475 µl PBS per well)

5. Wash 1 X with 0.5 ml PBS with 5 min wait.
6. Incubate with 1º Ab (Myosin: MF20, Hybridoma Bank) diluted according to the value needed for the experiment in PBS/Tween for 30 min at RT.

   (1º Ab ratio is 1:500 use 0.5 µl 1º Ab + 250 µl PBS:Tween per well)

7. Aspirate 1º Ab and wash each well 3 X 0.5 ml in PBS/Tween with 5 min wait.
8. Incubate with 2º Ab (Alexaflouro 568: S) (at 4µg/ml) for 30 min in PBS/Tween at RT.

   (2º Ab ratio is 1:500 use 0.5 µl 1º Ab + 250 µl PBS:Tween per well)

9. Aspirate 2º Ab and wash each well 4X with 0.5 ml PBS with 5 min wait.
10. For counterstaining add 0.5 µg/ml Hoechst 33342 (for nuclear antigens) to last wash (stock is 1 mg/ml) and incubate for 10 min at RT.

    (0.25 µl Hoechst stock + 250 µl PBS per well)

    Note: Do not counterstain with DAPI if nuclear antigens are to be detected. It is best to avoid counterstaining unless specific counts of positive cells are to be obtained. In this case, counterstain with Hoechst only.

11. Wash 2X with 0.5 ml PBS with 5 min waiting periods.
12. If cells will not be mounted on coverslips they can be kept in 0.1% NaAzide in PBS at 4^{o}C.

F.10 Hoechst 33342 Live-Cell Staining

1. Prepare stock solution of Hoechst 33342 to a final concentration of 2.0 mM in ddH2O. (Store solution at 4°C in the dark. MW is 624.0.)
2. Aspirate media from all wells
3. Add 1 ml PBS (with Ca++/Mg++) to the wells to rinse the cells twice.

4. Aspirate PBS.
5. Make a 1:500 dilution of the stock solution with complete media.
6. Add 1 mL per well of the above solution to each well.
7. Incubate the plates at 37 C for 15 minutes.
8. Rinse five times with PBS (with Ca++/Mg++)
9. Image the wells using a fluorescent microscope using an emission of 340-380 nm.*

Note: Make sure to protect dish from light as much as possible. Hoechst stain will gradually disappear from live cells.

F.11 Fibrin Thread Extrusion Protocol (Grasman, et al., 2012)

Fibrinogen Aliquot Preparation

Materials:

- Fibrinogen (F8630, Sigma)
- Thrombin (T4648, Sigma) – 1 KU
- Calcium Chloride ($CaCl_2$; MW: 110.99)
- Sodium Chloride (NaCl; MW: 58.44)
- HEPES (MW: 238.3)

Procedure:

HEPES buffered saline (HBS) preparation

1. Definition: HBS contains 20 mM HEPES and 0.9% (w/v) NaCl
2. Add the following reagents to 200 mL
    a. 2.25g of NaCl
    b. 1.1915g of HEPES
3. pH solution to 7.4 using NaOH/HCl.
4. Bring final volume to 250 mL.
5. Store at room temperature.

Fibrinogen aliquots (70 mg/mL)

1. Measure 14.3 mL of HBS into a 50 mL conical tube.

2. Weigh 1.00 gram of fibrinogen and pour into conical tube.
3. Put conical tube on rocker plate, adjusting the position every 30-40 minutes until fibrinogen goes into solution.

   Note: Never shake/vortex fibrinogen solution as it will fall out of solution and bind to itself.

4. Incubate conical tube at 37 C overnight to ensure fibrinogen is completely dissolved.
5. The next morning, measure 1 mL aliquots in eppendorfs and store at -20 °C.

Thrombin aliquots (40 U/mL)

1. Add 25 mL HBS to bottle of 1KU thrombin, mix well.
2. Aliquot 200 µL into eppendorfs and store at -20 °C (Final concentration: 8U / 200 µL).

Calcium chloride preparation (40 mM)

1. Add 0.1776 g of $CaCl_2$ to 40 mL of diH_2O.
2. Store at 4 °C.

HEPES buffer bath stock solution

1. Definition: Stock solution will be prepared at 10X of 10 mM HEPES buffer (100mM).
2. Add 23.83g of HEPES to 900 mL of diH_2O.
3. pH to 7.4 using NaOH/HCl*

   Note: This will require large amounts of base.

4. Bring final volume to 1000 mL.
5. Store at room temperature.

Prepared by: Jonathan Grasman

Extrusion Procedure

Materials:

- Fibrinogen aliquot (warmed to room temperature)
- Thrombin aliquot (warmed to room temperature)
- Calcium chloride solution (40mM, warmed to room temperature)

- HEPES buffer bath stock solution (10X)
- Metal non-stick pan
- 25 Gauge blunt end needle
- 0.86 mm I.D. polyethylene tubing (Intramedic PE90 427421)
- 2 1 mL syringes
- Blending connector (SA-3670; Micromedics, MN)

Setup:

1. Place blunt end needle (25 gauge, BD) into 0.86 mm I.D. polyethylene tubing. (can be reused)
2. Leur lock blunt end needle/tubing onto the front end of blending connector.
3. Turn syringe pump on.
    a. Press SELECT.
    b. Toggle to Table, press SELECT.
    c. Toggle to Bec. Dic. Plastic, press SELECT.
    d. Toggle to 1 cc 4.70 mm, press SELECT.
    e. Enter volume: 1.0 mL, press ENTER.
    f. Enter extrusion rate: 0.225 mL/min, press ENTER.
4. Place a metal non-stick pan next to the syringe pump.
5. Prepare 300 mL of 1X HEPES buffer solution (30 mL of stock solution and 270 mL diH_2O), pH to 7.4
6. Fill pan with 300 mL HEPES buffer solution
7. Add 150 µL of thrombin aliquot to 850 µL of calcium chloride solution, mix well.

Extrusion:

1. "Prime" 2 1 mL syringes by moving the plunger several times.
2. Collect all of the thrombin and fibrinogen solutions into 1 mL syringes.
3. Invert syringe, remove all bubbles, and ensure that both syringes have equal volumes.
4. Place each 1 mL syringe of fibrinogen and thrombin solutions into the back end of the blending applicator, with the thrombin on the side with an "o" and the thrombin on the side marked with a "T"

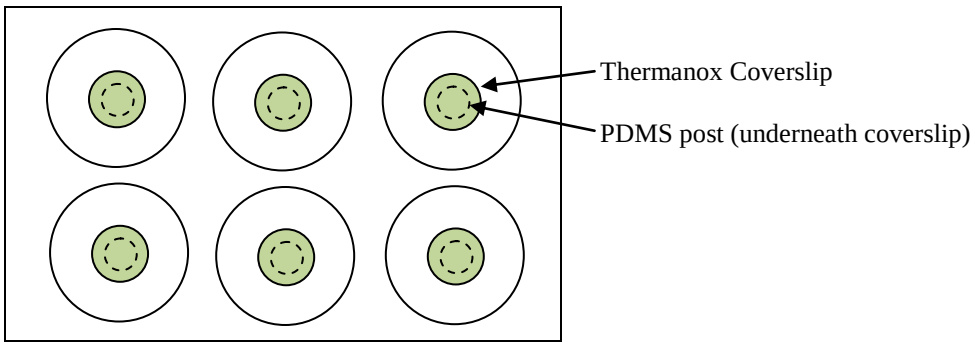
5. Secure syringe/blending applicator construct into syringe pump
6. Press RUN on the syringe pump and wait for fibrin solution to flow out of the tip of the tubing.
7. Using a metal bar if necessary, draw threads into the buffer solution, taking 6-10 seconds to draw each thread.
8. If the pump does not automatically stop when the syringes empty, press STOP.
9. Wash tubing/blending applicator with cold water and a 5 mL syringe, plugging the other opening with your thumb (at least 5 water rinses per blending applicator opening).
10. Flush water out of blending applicator/tubing repeating step 9 using an empty 5 mL syringe.
11. Fibers can be removed from the bath after 10-15 minutes and stretch threads to make 3 thread along the cardboard box (~7.5 inch threads).

F.12 Preparation of 6-well Plate for Microthread Cell Seeding (Grasman, et al., 2012)

1. Using a 0.75 inch diameter biopsy punch, remove pieces of PDMS from a mold that is about 2 cm thick.
2. Cut circular outer diameter of approximately 1.188 inches around each punch.
3. Adhere each PDMS 'post' to the middle of each well in the 6 well plate using silicone glue, allow 5 minutes for glue to set.
4. Place a Thermanox coated coverslip, coated side facing up, on top of the PDMS post in each well, using silicone glue to adhere*

   *After 5 minutes to set, test coverslips and PDMS posts to ensure that they are properly adhered.

5. Use a stainless steel washer that is 0.75" ID x 1.188" OD to trace a pattern on a PDMS mold that is about 0.005" thick.
6. Using a biopsy punch that is 0.75" ID, remove the centers of the PDMS washer molds.
7. Cut the remaining mold away from the 'washer' with scissors to create 6 PDMS molds for use in thread bundling and seeding.



F.13 Bundling Fibrin Threads

1. Remove 10 fibrin microthreads from cardboard drying box with tweezers and secure together in the center of a thin strip of tape.
2. Secure the opposite end of the threads in the center of another piece of tape while attempting to keep threads taunt and close together.
3. Slide the PDMS molds underneath the threads and apply silicone glue to adhere threads to the molds. Allow a cure time of 24 hours.
4. Cut threads so that individual molds can be removed. Store in a desiccator until use.

F.14 Coating Fibrin Microthread Bundles

Collagen I

1. Place PDMS mold with bundled threads in 6-well plate.
2. Prepare collagen I solution by diluting it in PBS (0.31 mg/mL).
3. Place approximately 0.75 mL of collagen I solution in the center of the PDMS mold, ensuring that the threads are completely submerged.
4. Incubate for 1 hour at room temperature
5. Remove PDMS mold, elevate so that the threads do not contact any surfaces, and allow to dry 1 hour at room temperature

Vitronectin

1. Place PDMS mold with bundled threads in 6-well plate
2. Prepare vitronectin solution by diluting it in PBS to a concentration of 0.02 mg/mL.

3. Place approximately 0.75 mL of vitronectin solution in the center of the PDMS mold, ensuring that the threads are completely submerged.
4. Incubate for 1 hour at room temperature
5. Remove PDMS mold, elevate so that the threads do not contact any surfaces, and allow to dry 1 hour at room temperature

F.15 Immunocytochemistry Collagen I Coated Fibrin Microthread Bundles

1. Place two PDMS molds of a collagen I coated fibrin microthread bundle and an uncoated fibrin microthread bundle in a 6-well plate.
2. Wash threads with 1x with 1 mL of PBS for 5 min at RT.
3. Fix threads with cold methanol for 15 minutes at room temperature by adding 0.50 mL to the center of the mold and submerging the thread
4. Wash each thread 3X with PBS with 5 min wait by submerging the thread (approximately 1 mL).
5. Block by incubating threads with blocking solution at RT for 15 min by submerging the threads (approximately 0.50 mL per well).

(Blocking solution: 5% Serum in PBS – which is 25 µl FBS + 475 µl PBS per well)

6. Wash 1 X with PBS with 5 min at RT
7. Incubate with 1º Ab (Procoll I, 1:100, Hybridoma Bank) diluted according to the value needed for the experiment in PBS/Tween for 30 min at RT.

(1º Ab ratio is 1:100 use 10 µl 1º Ab + 1000 µl PBS:Tween per thread)

8. Aspirate 1º Ab and wash each well 3X in PBS/Tween with 5 min wait by submerging the thread.
9. Incubate with 2º Ab (IgG(H+L)-Alexa 488, 1:500) for 30 min in PBS/Tween at RT.

(2º Ab ratio is 1:500 use 2 µl 1º Ab + 1000 µl PBS:Tween per thread)

10. Aspirate 2º Ab and wash each well 4X with PBS with 5 min wait (approximately 0.750 mL per well)
11. Store with sodium azide (approximately 2 mL per well), wrapped in parafilm at 4°C.

F.16 Cell Culture for Fibrin Microthread Bundles (Adapted from Grasman, 2012)

1. Place PDMS 'washers' with bundled threads into the prepared 6-well plate from Appendix F.12.
2. Fill each well with 70% ethanol to sterilize the wells and threads for 1 hour.
3. Move plate into a laminar flow hood.
4. Aspirate ethanol from the wells and wash with sterile DI water 3 times.
5. Let threads dry uncovered for 3 hours in hood.
6. Hydrate threads by placing a 150 µL drop of PBS on each coverslip. Let threads sit in PBS for 1 hour.
7. Aspirate PBS from threads and place a 150 µL drop of hSC cell suspension (200,000 cells/mL) on each coverslip.
8. Place plate in incubator for 24 hours.
9. Move washers into a new, normal 6-well plate and fill wells with 1 mL proliferation medium with growth factors.
10. Image each well at 24 and 48 hours, taking 5 pictures along the length of the thread at 10X magnification.
11. At 72 hours, replace growth medium with differentiation medium.
12. Replace differentiation medium every other day for a total of 6 days.

F.17 Immunocytochemistry for Myosin Staining on Fibrin Microthreads Bundles

1. The protocol for staining 24-well plates was followed as seen in Appendix F.9
2. Volumes were increased to 1.5 mL for washing and 0.5 mL for blocking, primary antibody, secondary antibody, and counterstaining. All volumes were added to the center of the PDMS mold with care taken to completely submerge the threads.

F.18 Degradation of Coated and Uncoated Fibrin Microthreads

1. Place one uncoated fibrin microthread and one coated microthread into a 6 well plate.
2. Sterilize each bundle by submerging threads and PDMS molds with 70% ethanol for 1 hour.

3. Rinse each bundle with TBS 3 x and then add 0.50 mL of TBS to each well to hydrate threads for 30 minutes.
4. Image each bundle 3 times along the length of the thread bundle at 10X.
5. Aspirate TBS and replace with 0.750 mL plasmin (0.1 U/mL in TBS).
6. Image threads 3 times at 10X at various time points to assess degradation until the thread is severed.

Appendix G: Lab Usage Report

Table 12: Lab Usage Report (October 2012 – April 2013)

Item	Amount Used and/or Allocated to the Design Team
DMEM (Cellgro)	1350 mL
Ham's F12 (Cellgro)	900 mL
FCIII (Hyclone) – check IEEE	225 mL
L-Glutamine (Cellgro)	1.344 g
Proprietary Growth Factor Cocktail (growth factors from PeproTech)	----
DMSO (Cellgro)	900 µL
T25 cell culture flasks (Nunc)	2
T75 cell culture flasks (Nunc)	23
T150 cell culture flasks (Nunc)	6
T175 cell culture flasks (Nunc)	12
100 mm plates (Nunc)	11
24 well plates (Celltreat)	5
4 well plates (Nunc)	13
6 well plates (Nunc)	10
35 mm plates (Nunc)	8
Intrigrid Petri Dish (100 x 15 mm with 13mm square grid)	9
Thermanox plastic coverslips 13mm diameter	30
Insulin-Transferrin-Selenium (ITS) (Cellgro)	3 mL
Horse serum (Hyclone) – check IEEE	10 mL
Gentamicin (Lonza)	68 µL
Fungizone/Amphotericin B	1.46 mL
Vial of hSC cells	2 (500K each)
Trypsin-EDTA (0.25%)	30 mL
Thrombin (40 units/mL) (EMD Millipore)	1.395 mL
$CaCl_2$ dehydrate (40mm) (JT Baker)	2.613 mL
Fibrinogen (70 mg/mL) (EMD Millipore)	8.00 mL
Bovine Collagen I (PureCol®, Advanced Biomatrix)	7.9 mL
Laminin (2.27 mg/mL) (VWR)	22 µL – Pins Lab
Chitosan (Deacetylated, Sigma)	50 mg aliquot
Human Fibronectin (Gibco)	32 µL
Human Vitronectin (Gibco)	68 µL
5% serum (blocking solution)	1.94 µL
2% Triton X-100	18.25
Pro-collagen I primary antibody	10.8 µL
Countimouse IgG secondary antibody – Alexa 488	2.2 µL
Methanol	1.1 mL

2% Formaldehyde	9.25 mL
MF20 and secondary antibody	22 µL and 22 µL
Hoechst 33342 (1 mg/mL)	11 µL
250 mL bottle for media (Nalgene)	3
100 mL bottle for media (Nalgene)	4
Cryovials (Nalgene)	7
PDMS	8 in^3 Pins Lab
HEPES (Calbiochem)	24 g
Silicone adhesive (Locktite)	1 tube
Plasmin (0.1 U/mL in TBS) (Calbiochem)	3 mL – Pins Lab
Aprotinin aliquots	270 µL – Pins Lab

Items not counted: Microcentrifuge tubes, conical tubes, serological pipettes, pipette tips, glass Pasteur pipets, trypan blue, DPBS, PBS, 0.22 µm filters, parafilm, needles, syringes, pH strips, and vellum paper.