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Multiphasic Growth Factor Release from Fibrin Microthreads

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MULTIPHASIC GROWTH FACTOR RELEASE FROM FIBRIN MICROTHREADS

A Major Qualifying Project Report:

Submitted to the Faculty of the

WORCESTER POLYTECHNIC INSTITUTE

In partial fulfillment of the requirements for the

Degree of Bachelor of Science

By:

______________________ ______________________

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Approved:

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Prof. George Pins, Major Advisor

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Authorship

All members of the project team contributed to the writing and editing of this report.

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Abstract

Biomaterial scaffolds have been designed to augment the body's wound healing process in cases of volumetric muscle loss (VML). Specifically, fibrin microthreads are advantageous due to their mechanical properties, uniaxial cell alignment, and low cytotoxicity. We designed a composite system consisting of a fibrin microthread bundle coated with a fibrin hydrogel to meet an unmet need of delivering multiple factors in different time domains to facilitate the regeneration of new skeletal muscle tissue. In natural wound healing, hepatocyte growth factor (HGF) is released 0 to 48 hours of and injury followed by insulin-like growth factor-1 (IGF-1) from day 2 to day 14. Each component of our system was loaded with different model proteins to simulate the release of HGF and IGF-1. Protein release from the microthreads was quantified with the bicinchoninic acid (BCA) assay and validated qualitatively with fluorescence microscopy. Hydrogels were fabricated with varying protein concentrations and protein release from the hydrogel component was validated with the BCA assay. The results demonstrated that we delayed protein release from bundled microthreads for 48 hours, followed by a continuous release until hour 150 and then a sustained release until hour 300. These results mimic the release profile of IGF-1 in natural skeletal muscle regeneration. Additionally, we loaded and released protein from hydrogels with a burst release in the first 48 hours, mimicking the release profile of HGF in natural regeneration. Further studies should use our results from the single components to fabricate and validate a composite system. This composite system has the potential to enhance skeletal muscle regeneration for patients in cases of VML.

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1. Introduction

1.1 Problem Definition

Musculoskeletal injuries are common in athletes and military personnel, with about 35-55% of sports injuries [Beiner, 2001] and 50% of all recent military injuries involving damage to the skeletal muscle [Fischer, 2009]. While skeletal muscle has the ability to repair itself after injury, this mechanism is insufficient in cases of volumetric muscle loss (VML), where over 20% of the muscle is removed or damaged. In these cases, the body's natural regeneration process lacks the capacity to repair the large amounts of lost muscle tissue. Instead, the body produces large amounts of collagen, or scar tissue, which has an impaired ability to contract, resulting in a loss of muscle function and permanent disablement [Turner, 2012].

Skeletal muscle regeneration begins with the degeneration of damaged tissue, followed by the regeneration of healthy muscle fibers formed by satellite cells recruited to the area. During this repair process, the body releases a host of growth factors in different time domains that are important in directing and facilitating the regeneration of the new tissue. Two growth factors that are particularly important in skeletal muscle regeneration are hepatocyte growth factor (HGF), which recruits satellite cells to the injury site, and insulin-like growth factor 1 (IGF-1), which stimulates satellite cell differentiation into new muscle fibers [Turner, 2012; Charge, 2004].

The innate regenerative process in skeletal muscle is partially dependent on the presence of HGF and IGF-1 in a specific, time dependent sequence: HGF must be present for the first 24-48 hours, while insulin-like growth factor 1 (IGF-1) must be present

after the first 24 hours and remain active for up to two weeks to fully heal minor skeletal muscle wounds [Grounds, 1991].

Current methods of treatment for VML injuries are autologous tissue transfer or, in cases of significant damage to the muscle or peripheral limb the wound is located on, amputation. While tissue transfer is a more favorable treatment than amputation, the process has several limitations. This method still may not establish full muscle functionality due to the incomplete restoration of skeletal muscle tissue and there is a possibility of failure due to necrosis [Turner, 2012]. Tissue transfers also create a new wound site, which increases the risk of infection and introduces the complication of donor site morbidity. This procedure is also difficult to execute properly due to its complexity and the requirement of multiple skilled surgeons. Therefore, there is a need for a treatment that enhances the body's natural regenerative capacity to be able to regenerate muscle tissue in cases of VML.

New treatment options are currently being developed in the field of biomedical engineering to regenerate muscle tissue. Many of these options incorporate implantable scaffolds made from different biomaterials such as fibrin or collagen [Ahmed, 2008; Liu, 2007]. These biomaterial scaffolds have the potential to aid in tissue regeneration through cell signaling and drug delivery via targeted release. These biomaterial scaffolds can be made in many forms including hydrogels and microthreads. Recent studies have focused on using microthread scaffolds over hydrogels because they have greater potential in skeletal muscle regeneration due to their natural structural homology to native tissues as well as their strength and controlled porosity [Cornwell, 2007].

Biopolymer microthreads have many benefits for *in vivo* wound healing such as localized growth factor delivery, increased wound healing, and directly aligned cell formation [Cornwell, 2007; Rovensky, 1994]. Microthreads are advantageous for skeletal muscle regeneration because they direct cell alignment along their length for contraction [Grasman, 2012]. In order to increase biocompatibility, many microthread scaffolds are fabricated using proteins naturally found in the body, such as fibrin or collagen. Microthreads can also be crosslinked to increase stiffness and strength, enhancing their mechanical properties and providing more support for the muscle tissue during the healing process [Cornwell, 2007]. These microthreads can also be braided or woven to form larger scale tissue constructs. Using microthreads as drug delivery vehicles could aid in proper delivery of necessary growth factors for rapid skeletal muscle regeneration and therefore potentially treat VML injuries.

This project optimizes fibrin microthread scaffolds to release two different growth factors with controlled, precise release rates for application as a targeted delivery system in wound sites. The application of fibrin microthreads loaded with growth factors has the potential to aid the body's natural skeletal muscle regeneration process, directing healthy skeletal muscle cell proliferation and regeneration to potentially heal VML injuries.

1.2 Project Goals

The aim of this project is to design and develop modifications to the current microthread fabrication process for the incorporation of multiple growth factors for use in VML injuries. These factors will be released in different time domains to mimic natural skeletal muscle regeneration. The first growth factor, HGF, will release between 0 and 48 hours after injury of the injury, while the second growth factor, IGF-1, will release from

day 2 to 14 injury. To do this, the project team loaded analogs of the growth factors onto fibrin microthreads in a process that ensures effective, uniform binding by splitting the system into two components: a fibrin microthread bundle loaded with IGF-1, coated with a HGF-loaded hydrogel. The release system will contain controllable parameters that will allow for adjustments of different factor concentrations and release rates. To validate the growth factor system, the project team will also develop an assay system protocol to measure release of each growth factor from the loaded microthreads to confirm the project's success.

2. Literature Review

2.1 Clinical Motivation

Skeletal muscle comprises over 40% of the human body mass and is responsible for all voluntary motion of the body [Charge, 2004]. It is composed of a highly complex structure that is susceptible to many types of injuries, caused by physical activity or due to traumatic events such as motor vehicle accidents or combat wounds in the military. Specifically, about 35-55% of all sports injuries cause injury to skeletal muscle in the body [Beiner, 2001]. In the military, musculoskeletal injuries now account for an estimated 50-70% of total combat injuries from 2001-2010 [Fischer, 2009]. As these injuries can vary in severity, it can be difficult to diagnose and characterize the full problem and therefore the treatment options vary between patients.

The ability of the muscle to regenerate after injury is also dependent on the severity or void produced in native tissue. Less severe injuries caused by small, repeated stresses on the body (such as with physical activity) are often repaired through skeletal muscle's innate ability for regeneration, as fully described in **Section 2.2**. However, this repair

process is somewhat limited as it can occasionally result in improper alignment of muscle fibers and therefore loss of optimal muscle function [Turner, 2012]. Endogenous skeletal muscle regeneration mechanisms are insufficient in cases of more extreme injuries, such as those caused by traumatic accidents, military battlefield injuries, and tumor removal [Turner, 2012]. Volumetric muscle loss (VML) is the clinical term that refers to these types of injuries in which the body's natural regenerative mechanism cannot compensate for the loss of muscle tissue, resulting in scar tissue formation and an irrecoverable loss of musculature. Research into methods of repair for VML has resulted in many clinical and experimental studies, but there are still few therapeutic strategies for adequate treatment of VML to restore healthy muscle.

The current standards of treatment for VML injuries are autologous tissue transfer or amputation [Sicari, 2012]. In autologous tissue transfer, healthy muscle tissue is grafted from a donor site to the injury [Tu, 2008]. However, this is a very complex procedure that requires a highly skilled surgical team and still has several limitations. Tu *et al.* describe several clinical challenges for surgeons in flap reconstruction, particularly in cases with large muscle injuries where improper assessment and removal of the damaged tissue may lead to further infection and necrosis [Tu, 2008]. The study also found that treatment of muscle trauma must be done no later than 72 hours after the injury to minimize infection and promote proper healing. After surgery, complications such as infection, donor site morbidity, and failure of the grafted muscle tissue caused by necrosis can result in a failed tissue transfer [Grogan, 2011].

In severe cases of VML where tissue is severely mangled, the only option may be amputation, resulting in the loss of limb and therefore permanent disability [Turner, 2012].

This procedure is not only debilitating, but it also requires further implementation of prosthetic devices and therefore additional surgeries and costs for the patient. While these therapeutic treatment options have improved in recent years, there is still a clear clinical need for more effective and less invasive strategies to treat VML injuries [Turner, 2012]. Current research into tissue engineering scaffolds have begun to address this need, but there are still several limitations, as these strategies have not yet found a way to mimic skeletal muscle's natural regenerative capacity in cases of VML. Therefore, there is a clear need for a treatment that enhances the body's natural regenerative capacity to be able to regenerate muscle tissue in cases of VML. The first step in creating an effective treatment for VML is to understand the mechanisms of skeletal muscle regeneration.

2.2 Skeletal Muscle Regeneration

Skeletal muscle is comprised of a large system of skeletal muscle fibers, blood vessels, nerve fibers, and layers of connective tissue that all help provide the overall integrity of the muscle. Each of these tissues is organized into a complex structure that is vital in giving the muscle its ability to voluntarily contract [Marieb, 2007]. This structure is shown in Figure 1 below. Briefly, skeletal muscle consists of many small individual muscle fibers, which are the contractile units of the system. These fibers are grouped together to form a bundle of muscle fibers, or fascicles, each of which is surrounded by a thin layer of connective tissue. Groups of fascicles are then bundled to form the overall structure of the muscle and covered with a thick outer layer of connective tissue, called the epimysium.

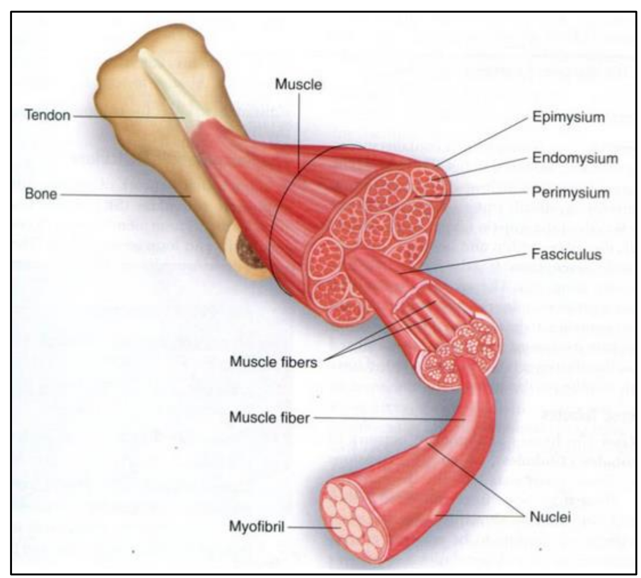

Figure 1: Components of skeletal muscle [Wilmore, 2004]

Immediately surrounding an individual muscle fiber, between the basement and plasma membranes, are muscle satellite cells. These cells are undifferentiated myogenic cells that remain on the fibers until activated during skeletal muscle regeneration [Charge, 2004]. The connective tissue layer surrounding the muscle fibers is primarily comprised of an extracellular matrix (ECM), which provides a stable environment for cell attachment and development, protecting the cells from stresses or loads that are directed at the tissue [Turner, 2012].

Skeletal muscle can be subjected to direct mechanical loads through day-to-day wear, extensive physical activity, or severe trauma. These mechanical loads disrupt the sarcolemma, or plasma membrane, of the myofiber, triggering the initial phase of skeletal muscle regeneration, muscle degeneration. Changes in the sarcolemma cause the membrane to become more permeable, which causes calcium to flow into the cell, activating the calcium-dependent proteases that rapidly disintegrate damaged muscle fibers [Turner, 2011]. These changes also trigger a release of growth factors (described further below), which activate an inflammatory response. Neutrophils invade the area

about 1-6 hours after injury, followed by the infiltration of macrophages about 48 hours after the injury. Macrophages begin to digest cell debris from necrotic myofibers through phagocytosis and may aid in activation of satellite cells within the damaged site [Charge, 2004].

This muscle degeneration is then followed by the stages of muscle repair, which involves proliferation and differentiation of satellite cells. Once activated by signals within the wound site, the satellite cells begin to proliferate. This is followed by terminal differentiation, in which the cells fuse to either existing damaged fibers (repair) or to one another to form new myofibers (regeneration) [Charge, 2004]. Once these myogenic cells fuse to one another, these myofibers increase in size and reorganize to form mature skeletal muscle tissue. Under normal conditions, these myofibers become indistinguishable from the previously undamaged muscle [Charge, 2004]. During this course of muscle regeneration, additional undifferentiated satellite cells remain on the periphery of the new muscle fiber for subsequent muscle repair [Charge, 2004].

After injury, muscle tissue releases a complex combination of growth factors in a sequential, time dependent order that is imperative for the control of muscle repair. Some of the key growth factors related to skeletal muscle regeneration include basic fibroblast growth factor (bFGF), leukemia inhibitory factor (LIF), vascular endothelial growth factor (VEGF), transforming growth factor-β (TGF-β), hepatocyte growth factor (HGF), and insulin-like growth factor (IGF-1) [Miller, 2000]. Of all of these factors, HGF and IGF-1 are perhaps the most important factors in regulating skeletal muscle regeneration as they are responsible for recruiting and stimulating satellite cells within the injury site to form new fibers [Turner, 2012; Charge, 2004].

HGF is one of the first growth factors to be released from the muscle extracellular matrix upon damage to the basal lamina of skeletal muscle [Charge, 2004]. It is the primary growth factor responsible for the recruitment and activation of satellite cells within the wound site [Miller, 2000]. The release of HGF within the muscle increase to concentrations on the order of nanograms per millimeter, compared to an order of picograms per millimeter within normal muscle tissue [Sheehan, 2000]. This increase in HGF triggers the migration of satellite cells from peripheral muscle fibers to the wound site [Philips, 1990]. After migration, HGF also induces these cells from a quiescent state into the first phase of the cell cycle [Miller, 2000]. However, previous studies have found that HGF may actually *inhibit* satellite cell proliferation and differentiation if present in the wound site longer than 2-3 days after injury [Miller, 2000]. Therefore, the timing of HGF treatment within the wound site after injury is vital in proper regeneration of skeletal muscle fibers.

IGF-1 appears at the injury site after the release of HGF, or approximately 2 days after injury, and remains active for about 12 days [Grounds, 1991]. IGF-1 is crucial for skeletal muscle regeneration as it is the primary growth factor responsible for myoblast proliferation and differentiation within the wound site [Charge, 2004]. Previous studies have shown that increasing the levels of IGF-1 within muscle cells results in an increased muscle mass, which is likely due to IGF-1's ability to promote myogenic cell survival and re-innervation of the muscle tissue [Caroni, 1990]. Similar to HGF, the timed release of IGF-1 is important, as satellite cell proliferation and differentiation occur only after about 2-3 days after injury [Grounds, 1991].

The systematic release of growth factors is essential to help cells regenerate functional skeletal muscle tissue. Without the properly timed release of growth factors, skeletal muscle may not regenerate properly. The dual release of HGF and IGF-1 are vital to the formation of mature, functional muscle. When creating devices or biomaterials to treat cases of VML, the addition of HGF and IGF-1 may accelerate regeneration of skeletal muscle. Various drug delivery techniques can be applied to deliver growth factors to affected muscle in a controlled, multiphasic manner.

2.3 Drug Delivery

The concept of drug delivery advanced the medical field by treating patients with pharmaceuticals in a controlled process. Most often, the drug is delivered via a loadable structure called a scaffold that allows for a targeted release of the drug in a specific area. Although some carriers, such as scaffolds, can be made into permanent structures, resorbable carriers are more desirable because the body metabolizes them after the drug has been delivered [Lavin, 2012]. The release of the drug from its carrier can be modified by altering its initial concentrations and release kinetics in order to meet the specific need of the patient. Drug delivery therefore is a vast field with many methods depending on the specific applications. A few of these methods and applications are described in the following sections.

2.3.1 Drug Delivery Methods

Areas under investigation for the controlled release of drugs to targeted areas are timed release rates and diffusion through scaffold degradation. These scaffolds allow the drug to release as the scaffold is broken down and metabolized by the body [Alsberg, 2003]. Diffusion scaffolds can be used for localized delivery to a specific, targeted location

in the body. This increases the efficiency of the biomaterial in cases where only one area of the body is in need of the drug.

Specifically, hydrogel scaffolds have been used in drug delivery because their degradation rate can be altered. They can be loaded with large molecules because the natural gaps within their structure [Jockenhoevel, 2001]. Microthread structures can deliver smaller molecules while maintaining scaffold support for cell alignment [Grasman, 2012]. Different scaffold geometries provide opportunities for different molecules to be delivered locally.

The rate of release and the concentration of factor released are influenced by the mode of scaffold degradation. Scaffolds that degrade through surface erosion consistently release the drug from the outermost surface while the scaffold maintains its integrity as its overall volume decreases in size. Often, this strategy is used to initiate the body's natural healing process by introducing growth factors needed in natural wound healing [Dhandayuthapani, 2011]. However, bulk erosion involves the breakdown of the structure as a whole, reducing the molecular weight of the scaffold gradually until complete degradation. This type of degradation is able to retain the geometry of the scaffold because of the pseudo-random degradation of the material [Dhandayuthapani, 2011]. Degradation by surface erosion can cause dosage dumping, in which large quantities of the drug are released at once. Bulk erosion allows for a more gradual release of the drug than with surface erosion due to the slower breakdown of the entire structure, as opposed to degradation at the surface. Depending on the desired application of a particular scaffold, different materials can be chosen so that it can degrade via either surface erosion, bulk erosion, or a combination of the two types. Typically, surface

degradation is used for short-term drug delivery, while bulk degradation is used more often for continual constant delivery of drugs for extended periods of time [Chen, 2010].

Effective drug delivery can be achieved by using a scaffold as a matrix that degrades over time, releasing the drug. Scaffold degradation is also critical for new tissue formation because it provides a framework for healthy tissue regeneration. The scaffold material, its geometry, and its rate of degradation can be tailored for optimum tissue regeneration, making these scaffolds useful in many areas of biomedical and tissue engineering [Alsberg, 2003].

2.3.2 Biomaterial Applications of Drug Delivery

The field of biomedical engineering has incorporated the use of drug delivery into biomaterials and various tissue engineering applications, which has led to many advances within the field [Friess, 1998]. Biomaterials and biomedical implants are designed with multiple functions, such as providing support for the injured tissue. Biomaterials can also be used to release growth factors to accelerate the healing process, ultimately yielding greater optimization of drug delivery techniques and faster healing processes. New medical devices with *in vivo* applications have the ability to perform in a multifunctional manner by delivering drugs as therapeutic agents to accelerate wound healing while providing mechanical structure and support to the damaged tissue, increasing their versatility [Malmsten, 2006]. The addition of a drug delivery component to an implant allows therapeutic agents to be released, to suppress infection at the site of the implant because they are found naturally in the body, limit the body’s reaction to the implant, accelerate wound healing, and/or facilitate tissue ingrowth. Scaffolds made from natural proteins, such as fibrin or collagen, do not elicit an increased immune response

when implanted into the body. Natural biomaterials specifically made to deliver multiple growth factors may further encourage cell proliferation for accelerated healing.

2.3.3 Multiphasic Growth Factor Delivery

A current strategy for skeletal muscle regeneration includes the use of multiple growth factors in a scaffold to increase signaling cues for proliferation and differentiation of satellite cells. Using different factors in a single scaffold has yielded more promising regeneration results than single factor scaffolds, making this strategy more cost-efficient rather than using a single growth factor in a higher concentration [Basmanav, 2008]. By combining multiple growth factors on a single scaffold, smaller amounts of each growth factor are required to deliver the factors because the different growth factors each perform a specific job and one growth factor will not have to perform other growth factors' jobs. This means the scaffold is more efficient and there is less wasted factor [Basmanav, 2008]. In addition, by using smaller concentrations of each growth factor, the risk of cytotoxicity may be reduced by removing the need to administer repetitive doses. Multifactor scaffolds must release the growth factors sequentially to mimic the release of these proteins during native muscle regeneration.

One technique used to alter release kinetics of growth factors from a scaffold includes manipulating the binding mechanism between the scaffold material and the growth factor. Affinity-based systems use the natural binding sites that factors have on other molecules, such as the heparin binding sites on HGF [Cecchi, 2012]. Heparin-binding domains have been used in affinity-based systems to combine HGF with fibrin-based scaffolds [Mammadov, 2012]. Release kinetics may be varied using this method by changing the amount of heparin in the scaffold to increase the concentration gradient

and adjusting the heparin-to-growth factor ratio, or binding between the scaffold and heparin molecules [Mammadov, 2012; Bhang, 2006]. Each bond between molecules anchors the growth factor at the end of the heparin. Release may change according to the dissociation rates between these bonds, meaning the rate at which each link is broken determines the rate the growth factor releases from the scaffold. The bond strength is customizable by controlling the number of bonds that occur with this conjugation scheme and would dictate the rate of release. More bonds between molecules yields a stronger bond, producing a delayed release.

A second type of binding mechanism that can be used with scaffolds is a covalent linkage system, where the release of a growth factor is dependent on the rate at which the scaffold degrades [Ginty *et al.*, 2007]. With this method, altering the scaffold degradation or the enzyme sensitive linkages (covalent bonds) may control the type of degradation. Covalent linkage systems can also be used to create a continuous release profile [Chen, 2010]. The choice of scaffold material is essential in applying these binding mechanisms since the degradation of the chosen material also plays a role in the release rate of growth factor.

2.4 Fibrin

Fibrin is a primary component in the provisional matrix. This fibrous protein aids in blood clot formation at the site of an injury and has been the focus of many laboratories studying the wound healing process. Fibrin has been used in cell- and thread-based scaffolds for skeletal muscle regeneration and drug delivery because of its intrinsic signaling cues [Brown, 2014].

2.4.1 Fibrin in Biomaterial Applications

Fibrin is a plasma-derived protein that recruits cells to the site of an injury. During wound healing, thrombin cleaves two peptides from fibrinogen, a glycoprotein in blood plasma, to convert it into fibrin [Weisel, 2013; Brown 2014]. Platelets and fibrin aggregate into a fibrous mesh to create branched networks that form a blood clot and the provisional ECM [Brown, 2014]. This provisional matrix facilitates tissue regeneration by promoting migration, attachment, and proliferation of cells. It is believed that fibrin can be incorporated into scaffolds with therapeutic molecules such as growth factors to direct cell proliferation and outgrowth due to its intrinsic signaling cues [Proulx, 2011].

Biodegradable scaffolds are preferred over non-degradable scaffolds because they allow natural remodeling of the muscular ECM, which promotes proper alignment of the myofibers [Koning, 2009]. There are several advantages to using fibrin in tissue engineering. Fibrin is native to the body, resulting in low cytotoxicity and minimal inflammatory response when implanted [Vats, 2003]. To further limit the foreign body response, fibrin can be derived directly from a patient's blood [Jockenhoevel, 2001]. Fibrin is biodegradable and angiogenic, meaning it encourages neovascularization of new muscle tissue [Murphy, 2008].

Although there are many advantages to using fibrin in tissue engineered scaffolds and gels, there are also some limitations. There have been many studies on fibrin-seeded scaffolds mimicking wound healing *in vitro*, including hydrogels made from fibrinogen and cell-seeded fibrin gels on polymer meshes [Ahmed, 2008; Hokugo, 2006]. Hydrogels have potential for applications ranging from cardiovascular grafts to bioengineered cartilage, but their low mechanical strength makes them less desirable for functional load-bearing

tissue replacement such as ligament and muscle repair [Cornwell, 2007]. There have been several attempts, however, to improve these mechanical deficiencies by combining fibrin with other strengthening components such as polylactic acid [Ahmed, 2008]. Additionally, infectious agents may potentially be introduced when using proteins derived from human or other mammalian sources. Overall, hydrogels are advantageous in many applications, however, they may not be ideal for skeletal muscle regeneration due to their low strength.

Recent studies have focused on using thread-based scaffolds over gel-based scaffolds because they have greater potential in skeletal muscle regeneration due to their higher mechanical stability and controlled porosity [Cornwell, 2007]. Fibrin microthreads are advantageous over fibrin hydrogels because they are structurally similar to native tissues, allowing them to direct cell alignment along their length. Fibrin microthreads are can be used to enhance skeletal muscle regeneration because they are biocompatible, bioresorbable, and more mechanically stable than fibrin hydrogels.

2.4.2 Fibrin Microthreads for Skeletal Muscle Regeneration

Using the correct scaffold is important for treatment because it is responsible for delivering essential growth factors to the injured area. An ideal scaffold would accelerate healing, be biocompatible, and reduce scarring.

The morphology of fibrin microthread scaffolds directs the alignment of cells and cytoskeletal components along their length, leading to aligned matrix deposition and tissue regeneration [Cornwell, 2010]. This is ideal for skeletal muscle regeneration because the cells recruited to the scaffold align in a cable-like structure similar to healthy myofibers. Microthreads fabricated via coextrusion are more mechanically stable under

tension than hydrogels, making them more suitable for load-bearing applications, especially when the microthreads have been crosslinked. The increase in strength and stiffness from chemical or physical crosslinking may improve handling in the laboratory and allow direct implantation *in vivo* [Cornwell, 2007]. Ultraviolet (UV) light specifically may be used to increase the mechanical strength and structural stability of fibrin microthreads [Cornwell, 2007]. Recent studies have also used 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) to crosslink fibrin microthreads. This crosslinking technique has been found to enhance the ultimate tensile strength, increase the resistance to proteolytic degradation, and enhance cell attachment to the microthreads [Grasman, 2012]. Thread-based scaffolds can also be bundled, woven, or braided to increase their overall size and strength, making them more appealing for load-bearing applications such as skeletal muscle regeneration.

If used to enhance skeletal muscle regeneration, biodegradable scaffolds such as fibrin microthreads must fulfill specific requirements. Firstly, these scaffolds must facilitate alignment and arrangement of the newly formed muscle fibers along the length of the microthread. Many studies have shown that cylindrical thread-based scaffold structures smaller than 100μm facilitate the natural alignment and orientation of cells [Rovensky, 1994; Cornwell, 2010]. Fibrin microthreads have also shown an affinity for cells and growth factors, which could significantly advance *in vitro* wound healing research [Cornwell, 2007]. Due to their many advantages, the applications for fibrin microthreads range across many areas of study, including growth factor and drug delivery.

3. Project Strategy

3.1 Initial Client Statement

In order to fully understand the scope of the project, the design team analyzed the initial client statement presented to the team, which stated:

> *Design, develop, and characterize a fibrin microthread system that will facilitate the release of two different growth factors or therapeutic agents in a controlled, time dependent manner for skeletal muscle regeneration.*

To better understand the meaning of this client statement, the project team held informal meetings with their client and user to inquire about the intended use of the product, the general wants and needs of the client and user, and the overall scope of the project which was separated into two sections, the growth factor section and the assay system section. The team then brainstormed objectives and constraints to better define the project and begin the design process.

3.2 Clarification of Design Goals

Defining objectives and constraints allows all stakeholders to clarify the project scope and deliverables of a final product. Objectives are the aims of both sections of the project and include what the project team, clients, and users envision in an ideal final product. Not all of these objectives hold equal weight of importance, so it is important to rank them in order of priority. Constraints are the conditions of a design that must be met for the design to succeed.

3.2.1 Objectives

As stated previously, it was the design team's responsibility to produce an initial list of objectives. Since the team spilt the project into two categories, the growth factor system and the assay validation system, there are two separate lists of objectives. This

was to clarify which objectives corresponded to the growth factor microthread system versus the assay validation system. These objectives were reviewed several times by the user and client in order to satisfy the wants and needs of all stakeholders. After a series of revisions, the objectives were compiled into organized groups of primary and secondary objectives as seen in the indented objectives list below.

Growth Factor System

I. **Controlled** multiphasic release of growth factors from microthreads
   a. Repeatable release kinetics for each factor
   b. Adjustable release kinetics

II. **Modifiable** factor parameters
   a. Reproducibly vary the amount of growth factor loaded to microthreads
   b. Modulate the concentration of each factor released

III. **Efficient** loading of growth factors onto microthreads
   a. Repeatable
   b. Uniform attachment
   c. Storable
   d. Time efficient process
   e. Cost efficient process

IV. **Cost effective**
   a. Minimal loss of growth factor
   b. Use of readily available materials
   c. Labor efficient
   d. Time efficient

V. **User friendly** process

As shown in the indented objectives list, there are five top-level project objectives that related to the growth factor system: controlled multiphasic release of growth factors from microthreads, modifiable factor parameters, efficient loading of growth factors onto microthreads, cost effective, and user friendly. While these objectives are briefly defined below, a thorough explanation of these primary objectives and their sub-objectives can be seen in **Appendix A**.

The design team will first design for the multiphasic release of the growth factors from microthreads. The rate of release of the growth factors should be controllable so they are not all immediately released from the microthread at once, but instead follow a controlled, gradual release. To do this effectively, the methods developed must allow for repeatable release kinetics of each growth factor so the release of each growth factor from the microthread has the same rate within each "batch" of microthreads. Similarly, adjustable release kinetics will allow the design team to change the release as demanded by different tissue applications.

Another primary objective was to create a system with modifiable factor parameters. Doing so will accommodate for future research related to the optimal amount of growth factor present during wound healing. In order to complete this objective, the team must be able to vary the initial concentrations of each growth factor loaded onto the microthread in a repeatable fashion. It is also important that the team is able to regulate the concentrations of each growth factor released and determine standard units to measure said concentrations.

It is important to efficiently load the growth factors onto the microthread. To achieve maximum productivity with minimal wasted product, the team must uniformly bind growth

factors throughout the length of the microthreads. It is also important that the user can reproduce the same loading quality at any given time. The microthreads should also be storable such that the growth factors remain active from the time they are loaded until they are analyzed. Creating a cost effective and time efficient process will assure that the team can effectively use all purchased supplies and the growth factors can be efficiently loaded before the microthreads significantly degrade.

Cost effectiveness applies to many different aspects of this project. In order to create a cost effective microthread system, the materials and equipment used to bind growth factors must be readily available in the labs and the time taken to create and run the microthread and assay systems must be minimal. Minimizing the loss of growth factor avoids wasted product and costs, and labor efficiency means a minimal number of users will be required to run the system.

It is important that the process is user friendly and simple to understand. Ideally, there should be minimal adjustments made to the current microthread fabrication process with the addition of growth factors. This will limit the number of additional steps a user must take to recreate the design team's final product.

As mentioned in the project goals (**Section 1.2**), the design team must also design an assay system in addition to the growth factor system. This assay system will be used to test and validate the success of the growth factor binding and release from the microthreads. The final list of objectives for the assay system is shown below:

Assay System

I. **Repeatable** outcomes
    a. Accurate
    b. Precise
II. **Modifiable** factor parameters
    a. Various growth factor concentrations
    b. Different time domains
III. **Facile** validation process
    a. Detecting growth factor binding to microthread
    b. Growth factor release from microthread detection
    c. Bioactivity detection
IV. **Cost effective**
    a. Time efficient
    b. Labor efficient

As shown in the indented objectives list, the assay system had four primary objectives: repeatable outcomes, modifiable factor parameters, facile validation process, and cost effective. While these objectives are briefly defined below, a thorough explanation of these primary objectives and their sub-objectives can be seen in **Appendix A**.

When using an assay system to quantify growth factor binding and release, it is very important that the test is repeatable. A repeatable testing system should be accurate, meaning the measured values are as close to the true value as possible, and precise, meaning the results of all tests are consistent. The assay must also be sensitive enough to detect a range of protein concentrations (1-20 mg/mL).

An adjustable assay system with modifiable factor parameters will allow users to test various growth factors and varying concentrations released in different time domains. Creating a facile validation process will mean that the implementation of each assay would be simple and human error could be minimized. Each of the steps in the validation system must also be easy to perform. Specifically, the user must be able to easily use the assay to detect growth factor binding to the microthread, detect growth factor release kinetics from the microthread, and detect the bioactivity of the growth factors.

Cost effectiveness is also a primary objective of the assay system, which includes time and labor efficiency. Assuring that the costs of the chosen materials are minimal and the equipment is readily available will be beneficial to all stakeholders. Minimizing the number of users and the time needed to run the assay system will keep costs low and maximize labor efficiency.

Once these objectives were organized in the indented list, the team then put them into an objectives tree for easy visualization. These objectives trees for the growth factor system and assay system are seen below in Figure 2 and Figure 3, respectively.

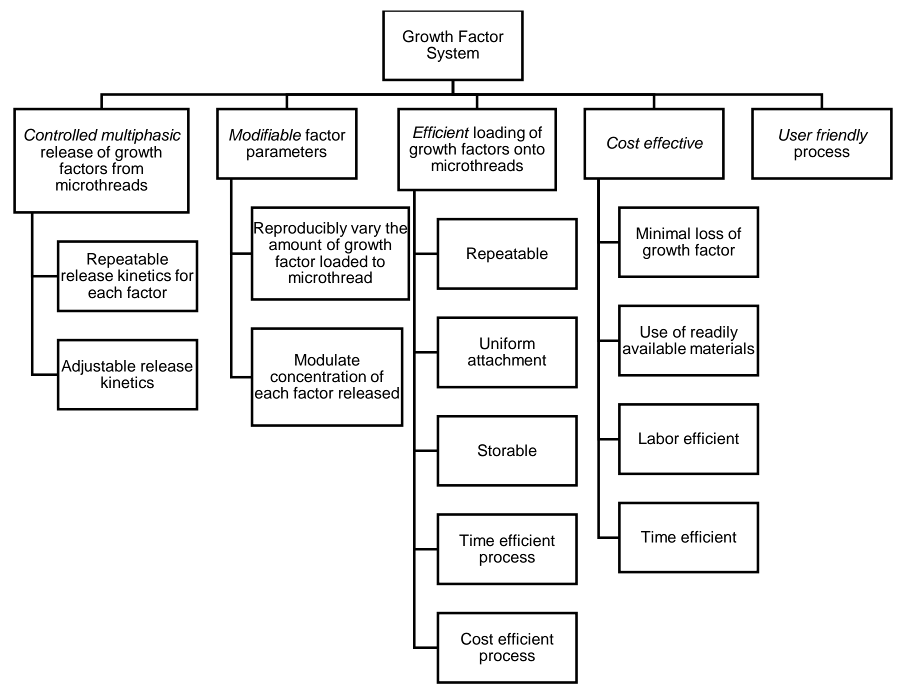

Figure 2: Growth factor system objective tree

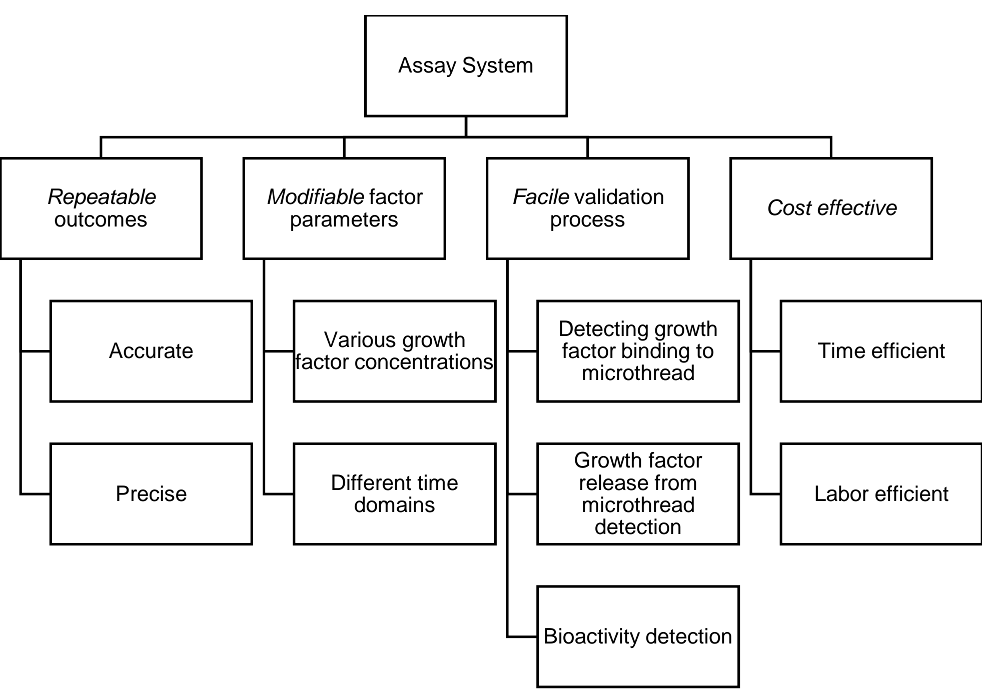

Figure 3: Assay system objective tree

3.2.2 Quantitative Analysis of Objectives

The design process relies heavily on strategic decision-making. Weighted objectives prioritize the goals of the project and give the design team direction when deciding on a project approach. Pairwise comparison charts are a common tool used to compare objectives to one another. When using a pairwise comparison chart to compare two items, the more important of the two receives a score of 1 while the less important receives a 0. If the compared items are equally important to the design, each is given a score of 0.5. Using this method, initial pairwise comparison charts were created and scored by the design team. Each pairwise comparison chart for the growth factor system and assay system can be seen in **Appendix B**.

The client and the user repeated this same process so that the design team could better understand their goals of the project. Once each party completed their evaluations, a condensed pairwise comparison chart was made to merge each of the three different scores. The team agreed that the client and user's scores should weigh more heavily than their own scores. Therefore, the weight of the client and user scores each equaled 37.5% of the total score while the weight of the design team's scores equaled 25%. To arrive at the final weighted scores, the totals in each of the rows were first normalized by adding one, followed by each of the normalized totals being multiplied by 0.375 or 0.25. A sample calculation can be seen in Table 1 below.

Table 1: Sample calculations of final weighted objectives scores

Project Objectives	I	II			III			IV			Total			Normalized Totals			Weighted totals			SUM	Weighted Score
I. Facile validation process		0	1	0	0	0	0	1	1	1	1	2	1	1+1=2	2+1=3	1+1=2	2*0.375=.75	3*.375=1.125	2*.25=.5	.75+1.125+.5=2.375	2.375/10=.24

In each row, the three weighted totals (each of which represented the design team, user, or client) were added up and divided by 10 (the total sum of all the weighted totals). This resulted in a weighted score for each primary objective on a scale of 0 to 1 with a value closer to 1 corresponding to a higher ranking. This process was repeated for each of the secondary growth factor objectives as well as all of the assay system objectives. The condensed charts for the growth factor system primary objectives and the assay system primary objectives can be seen below in Table 2 and Table 3, respectively.

Table 2: Combined pairwise comparison chart of growth factor system primary objectives

Primary Objectives	I			II			III			IV			V			Total			Weighted Score
	Client	User	Design	Client	User	Design	Client	User	Design	Client	User	Design	Client	User	Design				
I. Efficient loading of GFs				0	0	0	0.5	0.5	1	1	1	1	0	0.5	0	1.5	2	2	0.19
II. Controlled multiphasic release of GFs	1	1	1				1	1	1	1	1	1	1	0.5	1	4	3.5	4	0.32
III. Cost effective	0.5	0.5	0	0	0	0				0	0.5	1	0	0	0	0.5	1	1	0.12
IV. User friendly process	0	0	0	0	0	0	1	0.5	0				0	0	0	1	0.5	0	0.10
V. Modifiable factor parameters	1	0.5	1	0	0.5	0	1	1	1	1	1	1				3	3	3	0.27

Table 3: Combined pairwise comparison chart of assay system primary objectives

Project Objectives	I			II			III			IV			Total			Weighted Score
	Client	User	Design	Client	User	Design	Client	User	Design	Client	User	Design				
I. Facile validation process				0	1	0	0	0	0	1	1	1	1	2	1	0.24
II. Modifiable factor parameters	1	0	1				0	0	1	1	1	1	2	1	3	0.29
III. Repeatable outcomes	1	1	1	1	1	0				1	1	1	3	3	2	0.38
IV. Cost effective	0	0	0	0	0	0	0	0	0				0	0	0	0.10

Comparing the five primary objectives demonstrated that although all of the project goals were essential, some held more importance over others. Following the comparison of the growth factor system's primary objectives, the team determined that the controlled release of the growth factors was most important, followed by modifiable factor parameters and efficient loading. Cost effectiveness and ease of use were evaluated as the least important objectives. This process was repeated for the secondary level of each primary objective of the growth factor system (**Appendix B**).

The team also ranked the primary and secondary objectives of the assay system. The results of the pairwise comparison of the primary objectives ranked repeatable outcomes as the most important objective followed by modifiable parameters, a facile process, and cost effective, respectively. All of the sub-objectives under each primary objective were also compared (**Appendix B**). The objectives for the growth factor system and assay system are presented in order of importance (Fig. 2, Fig. 3) along with the sub-objectives.

Weighted objectives are used because the design process relies on a strategic approach to decision making. Including scores from the user, client, and design team, ensures every stakeholder contributes to the ranking. They also allow for the ranking to reflect who has more input in which areas of the design process.

3.2.3 Constraints

Constraints are the conditions that must be met for a design to succeed. Compiling a list of constraints was necessary to further define the scope of the project and give the team direction when making design decisions. In addition to the specific MQP project constraints (must be completed within the academic year and must be under the budget

of $624), there were constraints for the design process and the final product. After brainstorming, the design team created the list below (Table 4). Each of the constraints is related to either the growth factor system or the validating assay system.

Table 4: Constraints for growth factor and assay systems

Constraints
1. Materials must not be cytotoxic
2. Final product must be biocompatible
3. Addition of growth factor must not alter microthread geometry by more than 10%
4. Growth factor incorporation must be compatible with microthread production
5. Materials must be sterilizable with known processes and with on-site resources
6. Materials must be commercially available
7. Fibrin microthreads completely degrade after two weeks
8. Assay system must detect at least 1 mg/mL of protein

The final product must be biocompatible and non-cytotoxic so the loaded microthread is safe for the patient. The chosen process must use minimal amounts of growth factor due to the small surface area and volume of the microthread, and must not inhibit microthread processing or structure. The loaded microthreads must be sterilizable to ensure no contamination will occur since they are not currently fabricated in a sterile field. Any materials used must also be commercially available to accommodate for future research and studies. The *in vivo* degradation rate of the microthreads is a constraint because the scaffold must last long enough to fully released HGF and IGF-1 within 14 days to mimic their release in natural skeletal muscle regeneration [Grounds, 1991]. It is vital that the degradation kinetics of the microthread is not affected by the addition of growth factors. When the microthread scaffold degrades, its structure must also be

maintained in order to continue supporting cell infiltration. The assay system must be sensitive enough to detect concentrations of protein of 1 mg/mL. If the assay cannot detect the binding or release of such small concentrations, it will not be useful for this application.

3.3 Development of Revised Client Statement

Based on the results of the objective and constraint analyses, the design team revised the original client statement to better encapsulate the project goals. The revised client statement is as follows:

> *Design and develop fibrin microthreads that can release two growth factors in a time dependent, multiphasic manner. One growth factor should release within 48 hours, while the other should last up to 14 days. Design a protocol for a validation system to quantify loading and release kinetics of growth factors.*

The most important objective of this project was to develop a multiphasic growth factor release system using fibrin microthreads as a scaffold. The addition of growth factors demonstrates significant potential in skeletal muscle regeneration. The growth factors cannot compromise structural integrity or increase degradation rate of the microthreads. The system should be easy to use, time efficient, cost effective, and repeatable. Comprehensive characterization procedures should be developed to monitor the designed system.

3.4 Project Approach

Using the finalized client statement, the design team formed an initial project approach, including an analysis of the potential problems faced in the design process and solutions to those problems. Both the growth factor system and the assay system present challenges that the team addressed.

Two of the most important growth factors for skeletal muscle regeneration are HGF and IGF-1. To reduce costs, the team researched similar control molecules that have similar molecular weights as HGF and IGF-1 but are less expensive. Bovine serum albumin (BSA) can be used as a control protein for HGF because it has a similar molecular weight (70 kDa) to HGF (65-85 kDa) [Pietronave, 2010; Nakamura, 2010]. IGF-1 and ubiquitin from bovine are comparable in size with both molecular weights ranging between 6-7 kDa [Goldstein, 1975]. These control proteins were used to design a loading scheme for the microthread system at a reduced cost.

The release of the factors from the microthreads was a challenge for the team because it must be multiphasic. One phase involves an immediate, quick release of BSA while the other phase requires the long-term delivery of ubiquitin with a delayed response where the ubiquitin would not be released in the first 24-48 hours. Because fibrin microthreads only remain in the body for a maximum of two weeks, both factors must be completely released before the microthreads degrade. The desired outcome was a burst release of the BSA for the first 24-48 hours followed by a constant release of ubiquitin from the microthread. The team achieved a controlled, multiphasic release by creating a two-component composite system in which each component released one protein. To validate that the entire system was viable, each component was tested separately before combining both components in a composite system.

An appropriate assay system was required to measure and analyze the protein release kinetics. Assay kits with the desired sensitivity can be expensive; therefore the team used a bicinchoninic acid assay (BCA) kit because it was within the budget and modified the sample sizes accordingly to yield an accurate, measurable result.

In addition to using control molecules to reduce experimental costs, mathematical modeling was used to better understand the factors that can change the release kinematics of a typical hydrogel. These models were used to determine the diffusion mechanisms of proteins directly added to fibrin microthreads during fabrication.

3.5 Mathematical Models

Using release strategies from previous studies, the team modeled the release kinetics of growth factors releasing from a microthread structure. The release kinetics obtained by the team's multiphasic growth factor system should consist of two release profiles representing the two phases of release. The first phase of release correlates with HGF, consisting of a burst release for 24-48 hours and then a subsequent drop in release. None of the IGF-1 loaded onto the microthread should release during this time period. The initiation of the second phase would be marked with the release of IGF-1 and termination of HGF release. Next, a peak in the release of IGF-1 should remain consistent by releasing constant amounts of factor for 12 days, and then a drop in release once the scaffold has degraded. The top graph shown in Figure 4 below is representative of the desired release profile for HGF and the bottom graph is representative of the desired release profile for IGF-1 [Chen, 2010].

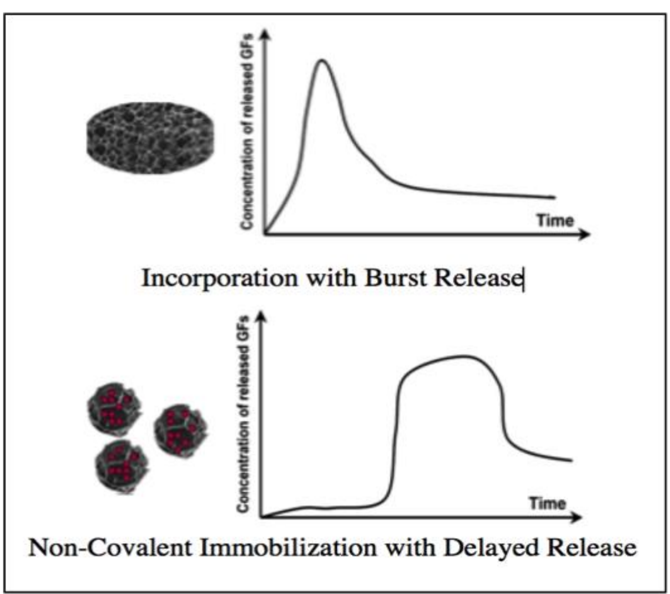

Figure 4: Release profiles of interest for HGF (top) and IGF-1 (bottom) [Chen, 2010]

When considering drug release from a fibrin microthread, the team first determined which method of mass transport was responsible for the movement of proteins from the scaffold to the surrounding medium. Initially, the empirical equation was the following [Datta, 2002]:

Equation 1: General mass transfer formula

$$\frac{\partial C_A}{\partial t} = D_{AB}\frac{\partial^2 C_A}{\partial x^2} - u\frac{\partial C_A}{\partial x} + r_A$$

Where C_A is the concentration of species A, D_{AB} is the diffusion coefficient that corresponds to the diffusivity of species A through material B, u is the mass average velocity, and r_A is the rate of mass generated within the system. For this application, we may consider species A as either growth factor being used (HGF or IGF-1). The material these growth factors are incorporated into is fibrin. Assuming there are no chemical reactions producing more growth factors within the system, we may remove the generation term. Assuming there is no movement of the media surrounding the matrix

and the matrix itself, the convection term may be negated. Finally, this leaves the diffusion term, which describes the mechanism for drug delivery of this fibrin matrix.

After establishing that the method of release is diffusion-based, the following equation was established as the basic equation for release [Saltzman, 2009]:

Equation 2: Rate of release formula

$$R = S_A * J$$

Here, R is the rate of release of a solute, S_A is the surface area exposed to the media, or the interface through which the solute will move, and J is the flux of release. Further, the flux may be described by the following equation [Datta, 2002]:

Equation 3: General flux equation

$$J = -D_{AB}\frac{dC_A}{dx}$$

When considering which mathematical model would be most appropriate for this project, certain characteristics were considered. We observed that a fibrin matrix degrades through bulk degradation, meaning that there are time dependent changes in the mass of the matrix itself. The process of bulk degradation begins with polymer swelling. Swelling occurs as the medium evenly disperses into the bulk of the polymer and alters the secondary and tertiary structures established by Van der Waal's forces, as well as the hydrogen bonds within the matrix. Due to this swelling, we may consider the specific transition and corresponding mass transfer that occur between the matrix's transitions from glassy to gel. This includes the relaxation of the polymer as well as diffusion before and after swelling. After this swelling, fibrolysis occurs and the cleavage of the polymer backbone begins. This occurs at a rate that is dependent on the material

itself. A formula that represents this transfer of mass in terms of the matrix is the following [Charlier*l*, 2000]:

Equation 4: Rate of mass transfer for degradable matrix

$$\frac{dM_W}{dt} = -kM_W$$

After integrating [Charlier, 2000]:

Equation 5: Integrated mass transfer equation

$$M_{W,t} = M_{t,0}e^{-kt}$$

In this equation, t is time, k is the degradation rate constant, $M_{W,t}$ is the polymer molecular weight at a given time, and $M_{t,0}$ is the initial molecular weight at t=0. In order to observe how molecular weight changes affect diffusion, the following relationship was used assuming the diffusion coefficient is inversely proportional to molecular weight [Lao, 2011]:

Equation 6: Relationship between diffusion coefficient and mass

$$\frac{D_t}{D_0} = \frac{M_{W,0}}{M_{W,t}}$$

Here, D_t is the diffusion coefficient at a given time t, D_0 is the initial diffusion coefficient, $M_{W,0}$ is the initial molecular weight of the polymer, and $M_{W,t}$ is the molecular weight at a given time. This correlation is then applied to the diffusion coefficient to produce a time dependent formula [Charlier, 2000]:

Equation 7: Change of diffusion over time for degradable matrix

$$D_t = D_0e^{kt}$$

Diffusion serves as the basic mechanism for passive delivery of protein from a fibrin microthread. There are various factors affecting the diffusion coefficient including

the interactions between the polymer and growth factors. This interaction includes any affinity that the growth factor may have for the matrix material. This affinity limits the amount of growth factor that may be diffused due to differences in the concentration gradient between the bulk and surrounding medium. In addition to factor-matrix interactions, structural changes in the matrix can affect the rate and kinematics of release. The size and shape of the protein loaded onto the polymer matrix also alters its path out of the matrix.

For this project, the team proceeded with a simple equation to assess the release of a given particle through a monolithic drug delivery device. This model assumes that the protein has no affinity to the matrix. Additionally, the geometry is assumed to be the simplest geometry of thin films with negligible edge effects. The following Higuchi model is therefore introduced [Zarzycki, 2010]:

Equation 8: Release from monolithic device

$$M_t = A\sqrt{D(2C_0 - C_s)C_s t}$$

Where M_t is the cumulative amount of drug released at time t, A is the surface area of the matrix which is exposed to release medium, D is the diffusivity of a drug in the polymer, C_0 is the initial concentration, and C_s is the drug solubility in the polymer. To visualize this release, a diffusion coefficient (D) was obtained from literature [Shkilnyy, 2012], while the following values were used to model and compute release of BSA from a fibrin gel:

$D = 1.86*10^{-7}$ cm^2/s

$C_0 = 5$ mg/mL

$C_s = 1$ mg/mL

$A = 1\ cm^2$

The value of C_0 was based on the concentration of BSA that may be loaded into hydrogels during preliminary studies. The value of C_s is 1 mg/mL based on the solubility of BSA in hydrogels. The cumulative amount of drug released was calculated with these values in Matlab using Equation 8. The release profile of this system is shown in Figure 6, below.

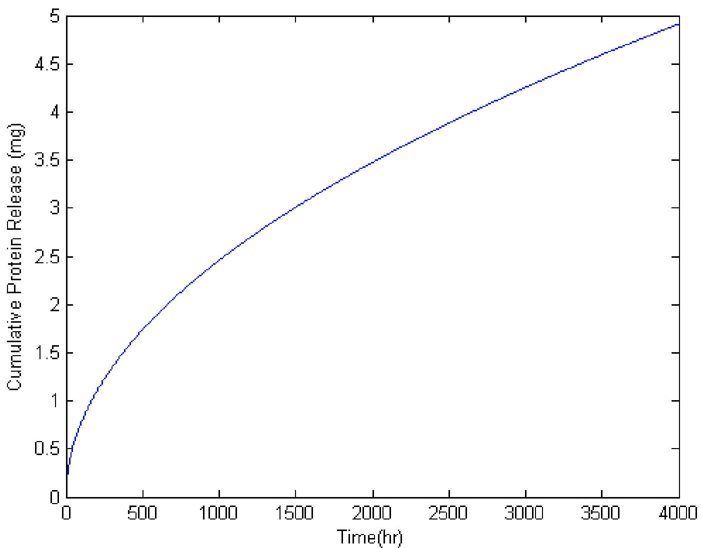

Figure 5: Approximation for release from monolithic device with initial constant values

According to this approximation, when simply considering the diffusion coefficient of a protein within a matrix above the solubility value, release would last up to approximately 6 months. However, in general different parameters may be adjusted to observe changes in release, such as changing the surface area to facilitate the release of protein. Although this parameter may not be adjusted for the application of this project, it is an important parameter that can change release by orders of magnitude. Additionally for the application of this project, the surface area of a microthread is large due to its cylindrical shape, versus a cube that only releases from a single face. In the case that the

surface area is increased by a factor of four, while keeping all other values consistent, the release profile changes to that seen below in Figure 7.

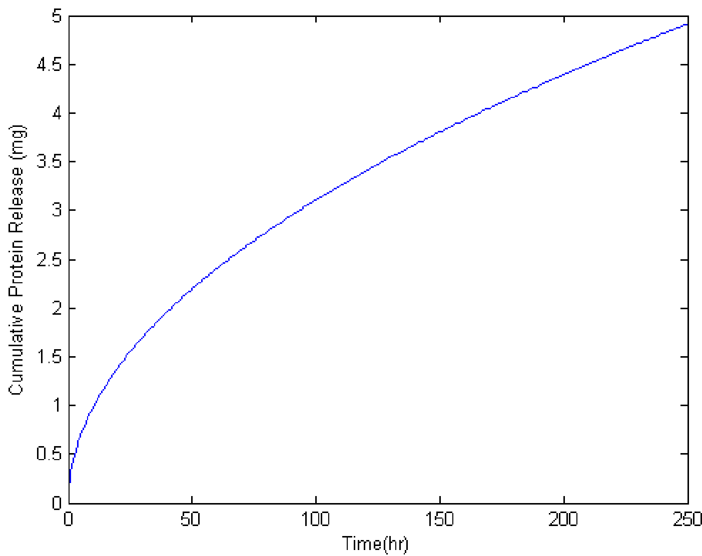

Figure 6: Approximation output following increase in surface area by factor of four

This approximation shows a pronounced decrease in time needed for the protein to release. The surface area is affected by the shape and size of the polymer delivery vehicle, which can be manipulated to obtain a desired release profile. Finally, if the diffusion coefficient is doubled, as may be the case for a smaller particle, the following graph is obtained (Figure 8):

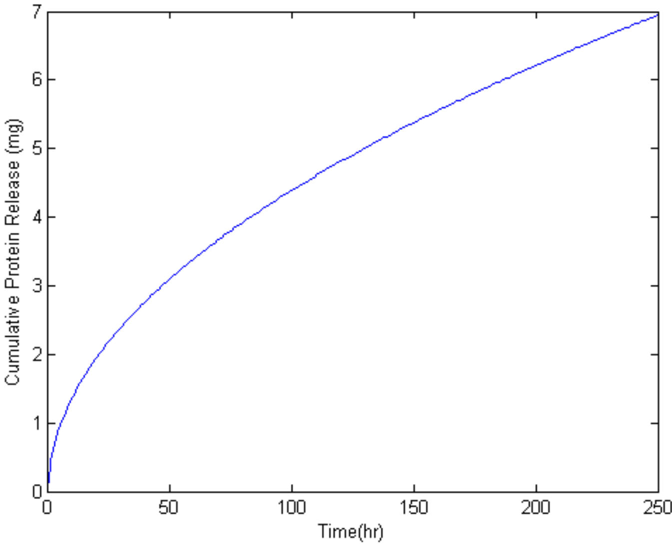

Figure 7: Approximation following increase in surface area and diffusion coefficient

Results indicate a shorter time of release when diffusion is increased. A higher diffusion value allows particles to more quickly travel from the matrix to the surrounding media, which explains the quicker release.

Through mathematical modeling, the team was able to better understand the changes that various geometries and particle-to-polymer relationships may have upon a monolithic delivery system. Of the parameters examined the tunable variable is determined to be the diffusion coefficient and concentration gradient. However, since dosing would be determined in a clinical setting of these, the primary variable of interest is diffusion. As was mentioned degradation is capable of changing diffusion in addition to other fabrication methods. Further, identification of other factors capable of changing the diffusion parameter should be explored. This includes scaffold alterations such as crosslinking and protein loading methods such as tethering which can both limit diffusion.

Alterations to the matrix include crosslinking, loading methods, and protein conjugations. Crosslinking the matrix without the growth factor will limit the amount of

swelling experienced by the fibrin polymer, delaying the release of factor. Release is also altered by the method used to load growth factors onto the matrix. When a growth factor is loaded into the bulk of the polymer, complete release occurs after the growth factor diffuses through the body of the matrix. Alternatively, when the growth factor is loaded onto the surface of the matrix, release is dictated by affinity present between the growth factor and matrix surface.

With all of these considerations, computational software can be used to produce a simplistic model to predict the release profile after alterations are made to the microthread scaffold or growth factors. Through mathematical modeling, the team was able to gain a better understanding of monolithic delivery systems and the variables that can be changed to obtain desired release profiles. A tunable matrix can be used to alter characteristics of protein loading and release.

4. Design Alternatives

4.1 Needs Analysis

After clarifying the design goals and developing a general project approach, the team analyzed the wants and needs of the client and user before creating potential design alternatives for the final product. The design team first met with the clients to determine overall project goals and attributes that the design should include. The team performed a needs analysis with the clients, organizing these aspects based on either "needs," what the product must have in order for it to be successful, and "wants," properties the clients would like the design to have, but may not be possible with respect to the constraints. Results of the needs analysis for the growth factor system are shown below in Table 5.

Table 5: Growth factor system needs analysis

Needs	Wants
Release GF-X for 24-48 hours	Uniform attachment
Release GF-Y with an initial delay of 24 hours and continued release for 7 days	Adjustable concentrations
Repeatable loading quality	Adjustable kinetics
Repeatable release kinetics	Apply to a variety of GFs
	Minimal loss of growth factor
	User friendly
	Cost effective
	Storable

Overall, the needs of the growth factor system were determined based on the main goals of the project. The release of growth factors in sequential order was essential to the design, as this is the purpose of the project. The design team determined that the final design must also show repeatable loading and release of growth factors onto the microthreads in order to fully characterize a system that would be useful to the client. The design team determined that although the objectives such as uniform attachment, adjustable concentration, and kinetics of the growth factor were important factors to consider, they were not critical to the overall integrity of the system. Similarly, objectives such as minimal loss of growth factor, user friendly, cost effective, and storable were considered non-essential objectives.

The assay system protocol is used to quantify the release kinetics of the growth factor system. The results of the needs analysis for the assay system protocol can be seen below in Table 6.

Table 6: Assay system needs analysis

Needs	Wants
Measure quantity of GF released at various time points	Facile validation process
Accurate and precise measurements	User friendly
	Use of readily available materials
	Labor efficient
	Time efficient
	Cost effective

The design team determined that the assay system must be able to measure the quantity of the growth factors released from the microthreads at varying time points. These measurements must be accurate and precise in order to clearly determine the success of the growth factor system. Objectives such as user friendly, use of readily available materials, cost, and labor efficiency were considered to be less vital to the success of the design. After performing the needs analysis, the design team began identifying necessary functions of the final product.

4.2 Functions

Based on the previously defined objectives and constraints, the team identified several functions that the system must be able to do in order to be considered successful. These attributes were organized into functions for the growth factor system and the assay

system protocol, and then were subsequently ranked in order of importance by the design team, based on the ranking of the objectives that these functions correlated to. These ranked functions for the growth factor system are listed in Table 7 below.

Table 7: Growth factor system functions

1. Control release of BSA for initial 48 hours from microthread
2. Control release of Ubiquitin from day 2 to day 14 from microthread
3. Load control molecules within 5 hours
4. Control rate of release
5. Adsorb varied amounts of control molecule
6. Minimize loss of control molecules
7. Control molecules attach uniformly to microthread
8. Minimize number of alterations made to the current fabrication process

In order to accurately mimic the body's natural release of HGF and IGF-1, the overall goal was to create a growth factor system that released the loaded factors in a sequential order. The design must release the first growth factor, or in this case, control protein, BSA, from the microthread from 0 to 48 hours, followed by the release of ubiquitin beginning after 48 hours until 2 weeks, or until the microthread fully degrades.

The design team determined that the system must quickly load the control molecules in order for the design to be time and cost efficient. Any loading method that required more than 5 hours in addition to the microthread fabrication process would not be actively pursued in order to increase efficiency.

Control rate of release refers to the adjustability of the system, as it is necessary for the design team to be able to adjust the rate of release according to the protein analogs

chosen so that they would release in the optimal time frame. This differs from the top two objectives (Controlled multiphasic release and Modifiable factor parameters) in that it relates to the design team being able to control the *rate* of release, rather than the specific time domains as in Functions 1 and 2 (Table 7).

The design team determined that the system must also allow for the adsorption of varied concentrations of growth factor onto the microthreads. This originates from the objective “modifiable factor parameters,” in which the design must be able to change the concentrations of loaded growth factors onto the microthread as the design team alters the control molecules and concentrations during preliminary testing.

The design must minimize the loss of growth factor during loading in order to stay within the financial constraints of the project. By minimizing the loss of growth factor, the design will maximize the amount of growth factor that can be used and reduce costs.

The system chosen must facilitate the uniform attachment of the growth factor to the microthread. Ensuring growth factors are uniformly loaded will increase loading efficiency, which will improve the other functions of the system.

The addition of growth factors to the current microthread fabrication process must minimize the number of alterations to this process. This function aligns with the “user-friendly” and “time-efficient” objectives.

The design team also used their objectives to define functions for the assay system protocol. These functions can be seen in Table 8.

Table 8: Assay system functions

1. Detect bound growth factor
2. Detect release of growth factor
3. Measure varied amounts of growth factor
4. Accurately and precisely measures concentrations of GFs over time
5. Measure over varied time domains
6. Minimize user labor
7. Minimize required purchases

Since the overall goal of the assay system is to validate our growth factor system, several functions in relation to the growth factors were necessary for the design. It was determined by the design team that the developed assay system protocol must be able to detect the presence of the growth factor, meaning that it can verify that our control proteins have attached to the microthread. Although detecting the release of the growth factor is the overall goal of the assay, detecting the bound growth factor was our top-ranked function as the design team determined that in order for the growth factor release to be analyzed, the growth factors must first bind to the microthread.

The assay system must also measure varied amounts of growth factor to ensure that if a different growth factor was chosen or if the loaded concentration of growth factor was modified, the users could still analyze data from the assay system. The system must measure this varying concentration accurately and precisely over varied time domains to ensure validation of the sequential release of growth factors. Finally, the assay system must minimize user labor and minimize required purchases in order to stay within the constraints of the project.

4.3 Development of Design Alternatives

After defining the functions of the final product, the design team held a brainstorming session with the client and user to develop conceptual design ideas for the growth factor system. The final list of conceptual designs was compared against the previously defined constraints and any conceptual designs that did not pass all of the constraints were eliminated. The remaining growth factor system designs were divided into two groups: single factor release and multiphasic release design alternatives. Five single factor release designs and three multiphasic release designs passed the constraint compatibility test.

4.3.1 Single Factor Release

Preliminary designs were separated into the two following categories of initial, rapid release and delayed, prolonged release. In order to simplify the biphasic release system the team addressed each phase as a single mechanism and considered what designs could produce the desired release. Once the team evaluated the designs for each category, quick release and slow release, the team proceeded to design a composite system that would combine the two mechanisms into one.

Coextrusion

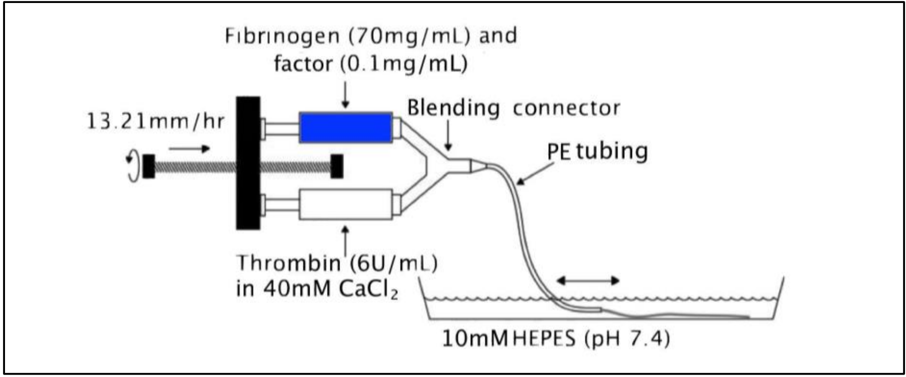

Figure 8: Coextrusion design alternative schematic

Pros	Cons
Repeatable release kinetics	May compromise microthread integrity
User friendly	
Uniform loading	
Reproducible	
Time efficient	

Coextrusion is a method where the factor would be directly mixed into the fibrinogen solution prior to the coextrusion with the thrombin and calcium chloride solution, as shown in Figure 5. The syringe containing the fibrinogen and factor solution (blue syringe, top) would be coextruded with the syringe containing thrombin and $CaCl_2$ (white syringe, bottom) to evenly distribute the factor throughout each single microthread. Once these microthreads are drawn there are no additional steps other than the necessary overnight drying. This method could be ideal for slow release (2-14 days).

Surface Adsorption

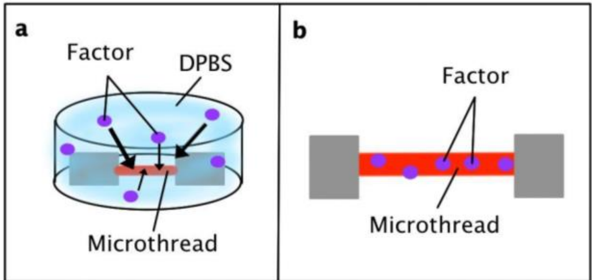

Figure 9: Surface adsorption design alternative schematic

Pros	Cons
Repeatable release kinetics	Release kinetics difficult to adjust
User friendly	Attachment may not be uniform
	Requires excess material

Surface adsorption is a method where the microthread would be submerged in an aqueous solution containing the desired factor in a buffer solution. Microthreads would be suspended in molds made of polydimethylsiloxane (PDMS) in a petri dish, which would then be filled with the fluid loaded with the factor (Fig. 6a). The factor would then adsorb onto the microthread so when the microthread is removed from the fluid it will be loaded with the desired factor (Fig. 6b). This method could be ideal for quick release (0-48hrs).

Adsorption Rosette

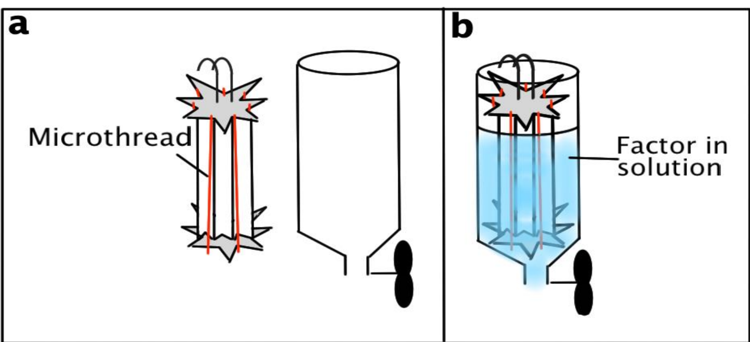

Figure 10: Adsorption rosette design alternative schematic

Pros	Cons
Adjustable release kinetics	Materials not readily available in most labs
Uniform attachment	
Reproducible	
Cost efficient	

An adsorption rosette is a PDMS mold used to perform surface adsorption or crosslink multiple microthreads at once using minimal crosslinking agents and buffer solution (Fig. 7a). Microthreads are placed in the rosette mold and placed in a crosslinker solution to evenly coat the surfaces to crosslink the growth factors to the microthreads. Alternatively, the growth factors could be adsorbed onto the microthreads in a buffer solution. The rosette mold can be placed in an apparatus with a drain at the bottom, adding the possibility of a gradient to each batch of microthreads (Fig. 7b). This method could be used to bind the growth factor for quick release (0-48hrs).

Fibrin Hydrogel Coating

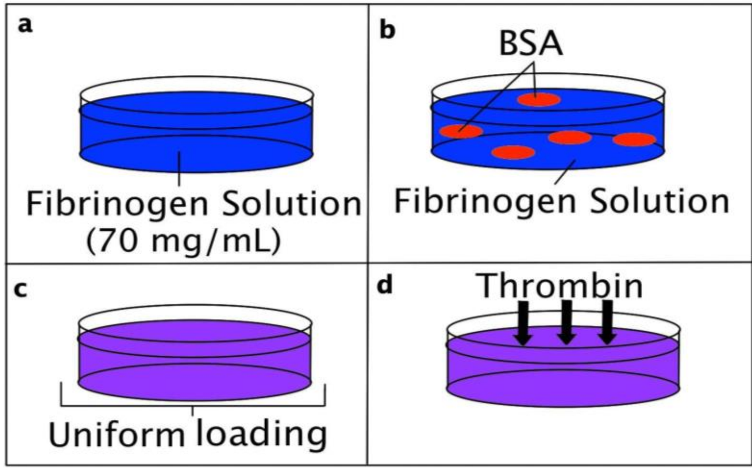

Figure 11: Fibrin hydrogel coating design alternative schematic

Pros	Cons
Reproducible	Not storable
Repeatable loading	Less user friendly
Uniform attachment	
Time efficient	

To create a fibrin hydrogel coating, the quick release growth factor was added to the fibrinogen solution (Fig. 8a-b). Using a fibrin hydrogel for the quick release growth factor would be beneficial because the microthreads can be evenly coated with a dipping technique performed after uniform loading had been achieved (Fig. 8c) and the thrombin had been evenly dispersed (Fig. 8d). The hydrogel also can be made to degrade faster than a microthread, enabling the desired burst release. Since the gelation time (Fig. 8d) could vary slightly depending on when the thrombin is added with each experiment, the fabrication process may be more variable than other methods. This technique is another method to crosslink growth factors to the microthread for quick release (0-48 hrs).

Crosslinking

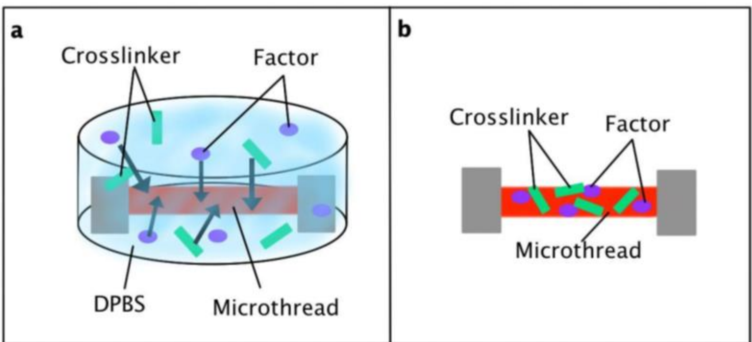

Figure 12: Crosslinking design alternative schematic

Pros	Cons
Adjustable release kinetics	Additional costs to include crosslinker
Reproducible	
Time efficient	
Minimizes loss of growth factor	
User friendly	

Crosslinking uses a crosslinker, such as 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) to reduce diffusivity of the growth factor through the microthread matrix, allowing the user to change the concentration of the crosslinker to change the rate of diffusion, delaying its release due to growth factor binding to the matrix. The crosslinker would covalently bind the factor to the fibrin in the microthread in an aqueous solution (Fig. 9a). Once removed from the aqueous solution the microthread would be loaded with the factor and crosslinked with the crosslinker (Fig. 9b). This method would be used for the slow release factor (2-14 days). If chosen, the team would have to determine the best crosslinker for this design by analyzing costs, product availability, and binding chemistries.

Protein Binding

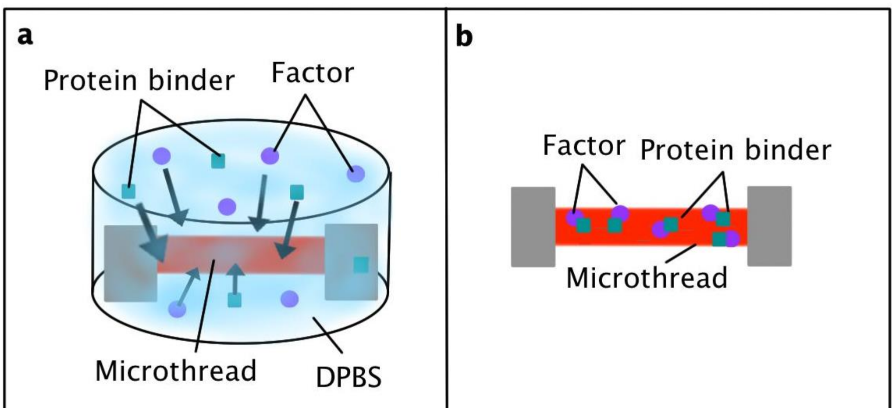

Figure 13: Protein binding design alternative schematic

Pros	Cons
Repeatable release kinetics and loading	Additional costs to include protein
Uniform attachment	Less reproducible

Protein binding involves adding the protein binder to the aqueous solution for surface adsorption (Fig. 10a). When the microthread was removed from the aqueous solution the factor would be bound to the surface with the protein binder (Fig. 10b). Protein binding would be ideal for slow release because the factor would be distributed throughout and bound to the microthread. The protein added would need to have the correct loading chemistries in order to sufficiently bind the factor to the fibrinogen. If this method was chosen, the binding affinities of the protein, factor, and fibrinogen must be compatible.

4.3.2 Multiphasic Release

Core/Shell Bundle

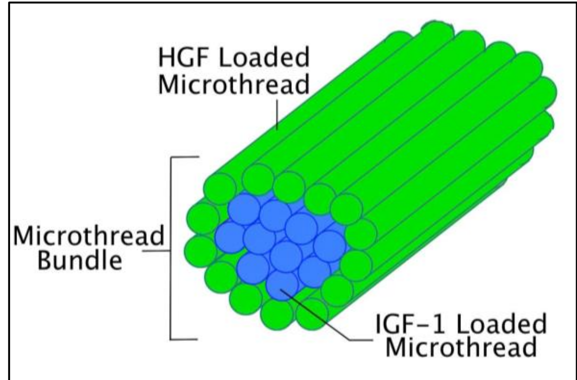

Figure 14: Core/shell bundle design alternative schematic

Pros	Cons
Crosslinked core bundle diffuses slower	Architecture is difficult to fabricate
Easier to uniformly attach growth factors	Bundling may affect cell migration
More repeatable release kinetics	

The core/shell bundle structure would have a slow release core of bundled microthreads with a single layer shell of microthreads bound with the quick release growth

factor (Fig. 11). The core would be fabricated by bundling single microthreads. The slow release growth factor would be bound to the core microthreads (blue) while the quick release growth factor would be bound to the shell (green). This method may be advantageous because the slow release core bundle would decrease diffusion due to the outer layer being exposed to the conditions in the body. It is also likely that users will be able to attach the growth factors uniformly to individual microthreads and produce repeatable release kinetics because of the organized architecture.

Dispersed Microthread Bundle

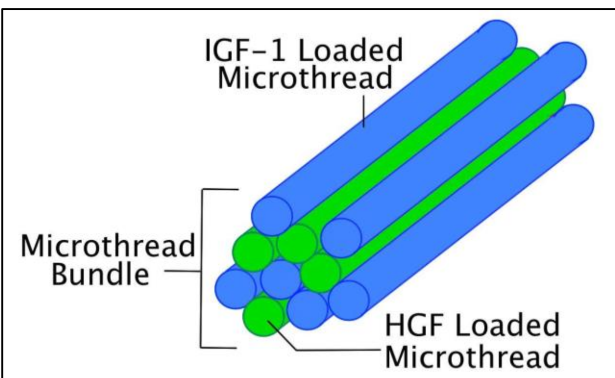

Figure 15: Dispersed microthread bundle design alternative schematic

Pros	Cons
Architecture easy to fabricate	Less uniform attachment
Time efficient	
Repeatable loading	

The dispersed microthread bundle includes randomly alternating slow release and quick release microthreads in a bundle (Fig. 12). The slow release microthreads would bound to the quick release growth factors. Since this method uses a randomly assorted bundle architecture, it would be easy to fabricate and be time efficient.

Hydrogel Coated Bundle

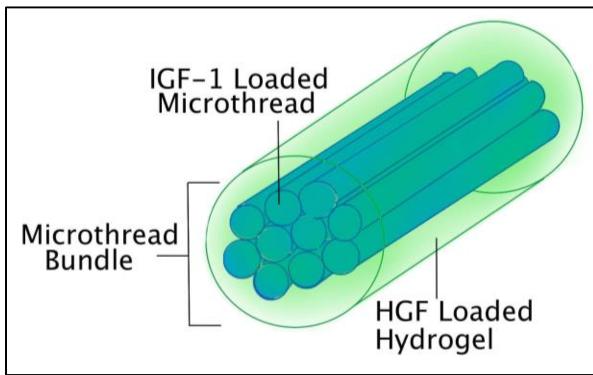

Figure 16: Hydrogel coated bundle design alternative schematic

Pros	Cons
More repeatable release kinetics	Bundling may affect cell migration
Can be applied to single microthread	Least time efficient

The hydrogel coated bundle is made out of a core of bundled microthreads and a fibrin hydrogel coating loaded with a quick release growth factor (Fig. 13). In order to create a multiphasic release, the core (blue) will only be loaded with slow the rate of growth factor while the hydrogel coating (green) is only loaded with the quick release factor. This design is likely to exhibit repeatable release kinetics and growth factor loading. The hydrogel coated bundle will also be the most time efficient process for the design team and user due to the dip coat method. This method would require the user to quickly dip the microthread bundles into the hydrogel solution before it fully solidifies. If needed, this method could also be applied to a single microthread rather than a bundle.

4.3.3 Assay System

During the brainstorming session previously mentioned, the design team, user, and client also developed conceptual design ideas for the assay system protocol to detect

the presence of protein on the microthreads. The design team then eliminated options that did not pass all of the constraints. The most feasible options were to use fluorescent proteins with microscopy and a bicinchoninic acid (BCA) assay with a spectrophotometer.

Fluorescent proteins or markers can be used with a fluorescent microscope to visualize changes in brightness over time that corresponds to protein release. Imaged microthreads will begin at a certain intensity level and will gradually dim over time as the fluorescent protein is released. These images can be used with the program ImageJ to calculate average pixel intensity values and quantify the change in brightness over time. For this application, Green fluorescent protein (GFP) is commonly used, but can be expensive. For this project, fluorescein isothiocyanate labeled bovine serum albumin (FITC-BSA) was used as the fluorescently labeled model protein, which emits in the green spectrum.

While fluorescence can be used to visualize protein release over time by comparing intensity values, it is not a method to quantify the concentration of factor being released. The BCA assay is a common tool used with a spectrophotometer or plate reader to quantify the concentration of protein released from media samples. When using the BCA assay, samples of the supernatant are taken at certain times determined by the user. These samples are then tested using a spectrophotometer to quantify the color changes across the samples.

The BCA assay is based on two mechanisms: the biuret reaction first causes a faint blue color in the sample, followed by the chelation of BCA that results in a strong purple color [Bainor, 2011]. The BCA assay quantifies the amount of protein within a sample without differentiating a single type of protein from another than may be present,

such as by products from a degrading microthread. By sampling both the releasant of an unloaded control microthread and a loaded microthread, a comparison can be made between the two samples so that the effects of normal degradation of the fibrin protein scaffold can be detected. Absorbance values read by the spectrophotometer are used to calculate the change in BSA concentration in the unknown samples over time by comparing the unknown samples to a standard curve created from a serial dilution of known concentrations of BSA.

The design team decided to use fluorescent microscopy and the BCA assay for single microthreads and microthread bundles respectively, since both were readily available, affordable, and sensitive enough to detect changes in protein concentration when used with microthreads.

4.4 Ranking Design Alternatives

The next step in determining a final design was to rank each design alternative for single factor release to determine credibility for use in the composite design. The following sections illustrate how the design team strategically analyzed the design alternatives.

4.4.1 Single Factor Release

The five design alternatives for single factor release were analyzed using Best of Class charts. These charts compare the ability of each of the conceptual designs to satisfy the previously defined objectives. Best of Class charts were made separately for quick and slow single factor release, as seen below in Table 9 and Table 10, respectively.

The design alternatives for quick release were ranked on a scale of 1-3 depending on how well they met each objective. A score of 3 meant the design alternative most closely met the objective while a score of 1 meant it did not meet the objective as well as

the other design alternatives. In the case of a tie, the team split the difference in score between the objectives. For example, if one alternative was better than the other two for a certain metric, the better design was given a “3”, while the remaining two were given a split score of 1.5. This technique is commonly used in Best of Class charts and guarantees proportional comparison. The team scored each design based on knowledge from literature and advice from the client and user. Weighted scores were calculated by multiplying the Best of Class chart scores by the objectives’ weights that the design team had previously assigned. Objectives with three stars (***) next to the title were multiplied by 3, while the objectives with two stars (**) next to the title were multiplied by 2. Objectives with 3 stars were considered necessary by the shareholders, 2 stars signified high importance, and objectives with no stars were determined to be less important. These weights were determined by the needs analysis seen in **Section 4.1**. The design alternatives with the highest total weighted scores were deemed to be the best choices for single factor release.

Table 9: Best of Class chart for single factor quick release

	Adsorption rosette	Fibrin hydrogel coating	Surface adsorption
Objectives/Metrics			
Repeatable release kinetics ***	1(3) = 3	2(3) = 6	3(3) = 9
Adjustable release kinetics ***	3(3) = 9	2(3) = 6	1(3) = 3
Reproducibly vary the amount of growth factor loaded to microthread ***	2(3) = 6	3(3) = 9	1(3) = 3
Repeatable loading **	2(2) = 4	3(2) = 6	1(2) = 2
Uniform attachment **	2(2) = 4	3(2) = 6	1(2) = 2
Modulate concentration of each factor released	1.5	3	1.5
Storable	3	1	2
Time efficient process	2	3	1
Cost efficient process	3	2	1
Minimal loss of growth factor	3	2	1
Use of readily available materials	2	3	1
Labor efficient	3	2	1
Time efficient (with respect to cost)	2	3	1
User friendly	3	1	2
TOTALS	47.5	55	30.5
Ranking	2	1	3

This Best of Class chart indicated that the best single factor quick release design alternative, given our constraints, objectives, and desired functions, would be the fibrin hydrogel coating. Although the adsorption rosette and surface adsorption were good options, the fibrin hydrogel coating would allow the design team to best conduct repeatable experiments and adjust growth factor release kinetics as necessary.

Since there were three slow release design alternatives they were ranked using the same system as mentioned above with the quick release alternatives. When the designs all met an objective or metric equally, each design alternative was given a score of 1. Weighted scores were also calculated as described above.

Table 10: Best of Class chart for single factor slow release

	Crosslinking	Protein binding	Coextrusion
Objectives/Metrics			
Repeatable release kinetics ***	1.5(3) = 4.5	1.5(3) = 4.5	3(3) = 9
Adjustable release kinetics ***	2(3) = 6	1(3) = 3	3(3) = 9
Reproducibly vary the amount of growth factor loaded to microthread ***	2(3) = 6	1(3) = 3	3(3) = 9
Repeatable loading **	1.5(2) = 3	1.5(2) = 3	3(3) = 9
Uniform attachment **	1.5(2) = 3	1.5(2) = 3	3(2) = 6
Modulate concentration of each factor released	2	1	3
Storable	1	1	1
Time efficient process	2	1	3
Cost efficient process	1	1	1
Minimal loss of growth factor	2	1	3
Use of readily available materials	1.5	1.5	3
Labor efficient	1.5	1.5	3
Time efficient (with respect to cost)	1.5	1.5	3
User friendly	2	1	3
TOTALS	37	27	116
Ranking	2	3	1

The results of the slow release Best of Class chart indicate that coextrusion is a preferred design. These methods received the same score for some objectives, however, the design team believed that coextrusion would allow them to more easily reproduce growth factor loading and adjust release kinetics. Coextrusion will also be more cost effective, time efficient, and easy to use.

4.4.2 Multiphasic Release

The three multiphasic design alternatives were evaluated in a Best of Class chart similar to **Section 4.4.1**. Each design was ranked on a scale of 1-3, similarly to the quick release single factor designs. Objectives were also assigned multipliers to create weighted objectives. The alternative with the highest total weighted score was deemed to be the best design alternative for multiphasic factor release. This Best of Class chart can be seen below in Table 11.

Table 11: Best of Class chart for multiphasic release

	Core/Shell Bundle	Dispersed Microthread Bundle	Hydrogel Coated Bundle
Objectives/Metrics			
Repeatable release kinetics ***	2(3) = 6	1(3) = 3	3(3) = 9
Adjustable release kinetics ***	3(3) = 9	2(3) = 6	1(3) = 3
Reproducibly vary the amount of growth factor loaded to microthread ***	2(3) = 6	1(3) = 3	3(3) = 9
Repeatable loading **	2(2) = 4	1(2) = 2	3(2) = 6
Uniform attachment **	3(2) = 6	1(2) = 2	2(2) = 4
Modulate concentration of each factor released	2	1	3
Storable	2	2	2
Time efficient process	2	1	3
Cost efficient process	2	2	2
Minimal loss of growth factor	1.5	3	1.5
Use of readily available materials	2	2	2
Labor efficient	2	3	1
Time efficient (with respect to cost)	1.5	1.5	3
User friendly	2	3	1
TOTALS	48	34.5	49.5
Ranking	2	3	1

According to the results of this Best of Class chart, the hydrogel coated bundle was the best design alternative, followed by the core/shell bundle and the dispersed microthread bundle. Because the team predicted the hydrogel coated bundle would

provide the most repeatable release kinetics and factor loading as well as the highest reproducibility, it was selected as the final design for further modeling and testing.

4.5 Preliminary Composite System Design

After the team decided to create a hydrogel coated bundle, the next step was to determine the specific design aspects of both the hydrogel and the microthread bundle. Within the hydrogel coating, quick release of HGF was modeled using BSA. The bundled microthreads contained ubiquitin to model the slow release of IGF-1. Release studies were then performed in parallel on the hydrogel and microthread components separately for prototype validation. The hydrogel experiments analyzed the release of BSA when loaded into hydrogels at various concentrations. Single microthreads were loaded with FITC-BSA to visualize the release of protein over time. Similarly, the microthread bundles were loaded with BSA and ubiquitin to analyze their release over time. The results of these studies would be used to combine the single components into a composite system.

To create the composite system, the ubiquitin-loaded fibrin microthread bundles would be coated with the fibrin hydrogel loaded with BSA. Possible methods for coating include dipping, rolling the bundle in a dish containing the hydrogel solution, painting or spraying the hydrogel solution onto the bundle. Crosslinkers such as EDC may also be used to ensure each protein is released within its designated time domain (quick or slow release). After creating a preliminary design for each component of the hydrogel coated bundle, the design team set up a series of experiments to validate the components.

5. Design Verification

5.1 Single Microthread Imaging

In order to validate the single components of the composite system, the design team first fabricated and imaged single fibrin microthreads. The team loaded single microthreads with FITC-BSA to analyze its release and any changes in microthread degradation over time using fluorescence microscopy. Although the slow-release factor would be incorporated into a microthread bundle in the final design, testing single microthreads was beneficial as it allowed the team to visualize protein loading and release over time without interference from other microthreads in a bundle.

Methods

Two groups of microthreads were fabricated and imaged: control microthreads and FITC-BSA-loaded microthreads. Control fibrin microthreads were fabricated using methods described previously by Grasman *et al.* [Grasman, 2012]. A full procedure on fibrin microthread fabrication can be seen in **Appendix C**. Briefly, 150 µL of thrombin (6 U/mL) was added to 850 µL of $CaCl_2$ solution (40 mM) and placed in a 1 mL syringe. Another 1 mL syringe was filled with 0.9 mL of fibrinogen (73 mg/mL) and 0.1 mL of DPBS, then both syringes were connected to a blender applicator tip with a polyethylene tube (inner diameter, 0.86 mm), as seen in Figure 14, below. The contents of the syringes were coextruded at a rate of 0.225 mL/min through this apparatus into a room temperature bath of HEPES buffer solution (pH 7.4). After incubating for 10 minutes in the buffer solution, the microthreads were removed, stretched, and dried overnight.

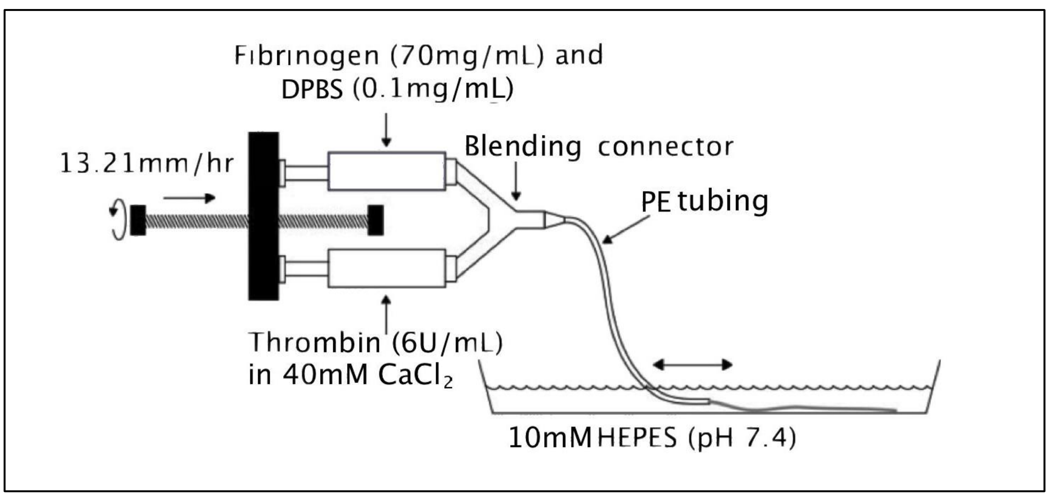

Figure 17: Schematic of adapted fibrin microthread extrusion system [Cornwell, 2007]

FITC-BSA microthreads were fabricated by adding 0.9 mL of fibrinogen (73 mg/mL stock solution) to 0.1 mL of FITC-BSA (10 mg/mL stock solution) for final concentrations of 70 mg/mL and 0.5 mg/mL, respectively. This solution was then coextruded with a thrombin-$CaCl_2$ solution. A complete protocol of this method can be seen in **Appendix D**.

Once fabricated, one control and one BSA microthread were each cut into three 3 cm pieces and secured to the bottom of separate wells in two 6-well plates using silicone glue and a syringe. Each microthread was soaked in isopropanol for 1 hour on a shaker to sterilize, then rinsed with deionized water (diH_2O) three times for five minutes. Each well was then filled with 2 mL of DPBS and the entire well plate was sealed with parafilm. The well plate containing FITC-BSA microthreads were wrapped in aluminum foil to limit exposure to light.

Control and FITC-BSA-loaded microthreads were imaged using a fluorescence microscope. Each microthread was imaged every 12 hours over a 72-hour period in two settings: FITC and bright field. In the FITC setting, the exposure was set to 1300 ms while in the bright field setting, the exposure was set to 18 ms. In the FITC setting, three images

were taken along the length of each microthread at a magnification of 10x. Each microthread was imaged once in the bright field setting at 10x.

The team then used ImageJ to measure the average number of pixels in each image in the FITC setting to quantify changes in microthread brightness over time. A detailed protocol for this process can be found in **Appendix E.** Briefly, the images from the FITC setting were opened in ImageJ the average pixel intensity of FITC-BSA and control microthreads at every sample time was analyzed. The team then ran this data with a one-way ANOVA and a Holm-Sidak post hoc analysis at a significance of $p<0.05$ ($n=3$ per group).

Results

The images taken during the 72-hour sampling period are seen below in Tables 12 and 13. Specifically, Table 12 illustrates one control and one FITC-BSA microthread at hour 0 and 72 in both the bright field and FITC settings. Table 13 illustrates a single FITC-BSA-loaded microthread at each sampling time.

Table 12: Single microthread images at hour 0 and 72 (scale bar = 100 μm)

Sample Hour		Control	FITC-BSA
0	Bright field setting		
	FITC setting		
72	Bright field setting		
	FITC setting		

Table 13: Single BSA-FITC microthread images over 72 hours (scale bar = 100 μm)

Sample Hour	FITC Setting	Sample Hour	FITC Setting
0		12	
24		36	
48		60	
72			

The average number of pixels for both control and FITC-BSA microthreads at each sampling time from the ImageJ analysis were also plotted against time (Figure 19).

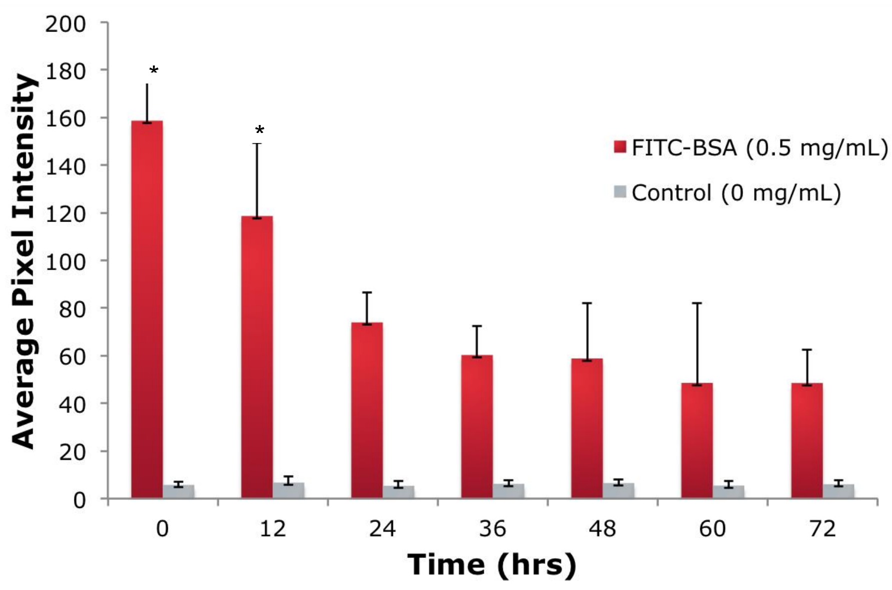

Figure 18: Average pixel intensity of FITC-BSA microthreads (n=3)

* denotes significance with respect to all other time points as determined by one-way ANOVA with Holm Sidak post hoc analysis (n=3 p<0.05)

The statistical analysis showed that all FITC-BSA groups were significantly different from the control group of the same sampling hour and there was no significant difference between controls. Within the FITC microthreads, the pixel intensity at hour 0 and hour 12 were significantly different than all other times, suggesting that the protein was loaded but had not released from the microthreads within 12 hours.

5.2 Microthread Bundle Release Studies

In conjunction with the single microthread release studies, the design team also fabricated and tested bundled microthreads to analyze the loading and release of protein over time. Bundling increased the concentrations of protein released to meet the minimum sensitivity of the BCA assay (5 μg/mL). Control bundles, bundles loaded with BSA and ubiquitin via coextrusion, and bundles crosslinked with ubiquitin and EDC were tested.

Methods

Control microthreads were fabricated as outlined in **Section 5.1** (full procedure in **Appendix C**). Once dry, the fibrin microthreads were bundled (full procedure for bundling can be found in **Appendix F**). Briefly, the fibrin microthreads were cut into 3 cm long sections. Ten of these 3 cm long segments were gathered, twisted, and secured together. They were then hydrated in DPBS for 15 minutes and dried for another 15 minutes.

BSA microthreads were fabricated by coextrusion following the procedure in **Appendix G**. Briefly, 0.1 mL of BSA solution (10 mg/mL) was added to 0.9 mL of fibrinogen solution (73 mg/mL) prior to coextrusion for final concentrations of 0.5 mg/mL BSA and 70 mg/mL fibrinogen. The 1 mL BSA-fibrinogen solution was then coextruded with a solution of 850 µL of $CaCl_2$ (40 mM stock solution) and 150 µL of thrombin (40 U/mL stock solution) into a room temperature HEPES buffer (pH 7.4). After 15 minutes the microthreads were removed from the bath to dry under their own weight overnight. The microthreads were sterilized using isopropanol prior to bundling.

Coextruded ubiquitin microthreads were fabricated using a similar procedure (**Appendix H**). Briefly, 0.1 mL of ubiquitin solution (10 mg/mL) was added to 0.9 mL of fibrinogen solution (73 mg/mL) prior to coextrusion for a final microthread concentration of 0.5 mg/mL ubiquitin and 70 mg/mL fibrinogen. The 1 mL ubiquitin-fibrinogen solution was then coextruded with a solution of 850 µL of $CaCl_2$ (40 mM stock solution) and 150 µL of thrombin (40 U/mL stock solution) into a room temperature HEPES buffer (pH 7.4). After 15 minutes the microthreads were removed from the bath to dry under their own weight overnight. The microthreads were sterilized using isopropanol prior to bundling.

Ubiquitin bundles were also fabricated using EDC as a crosslinking agent. A full procedure is in **Appendix I**. Briefly, dry control microthreads were sterilized with isopropanol and allowed to dry. The microthreads were then incubated at room temperature in a NaH_2PO_4 buffer for 30 minutes. Immediately after this buffer was aspirated, the microthreads were submerged in a solution of EDC (28 mM), *N*-hydroxysuccinimide (NHS, 16 mM), and ubiquitin (1 mg/mL) for two hours. Microthreads were then rinsed with diH_2O for five minutes three times and dried under their own weight overnight.

Each dry bundle was then glued to the bottom of separate wells in 6-well plates with silicone glue and submerged in 1.6 mL of DPBS. Samples were taken at hour 0, 6, 9, 27, 39, 51, 63, 75, 87, 111, 135, 154, 178, 202, 226, 255, 279, and 299 for a total of 18 sampling times. At each sample time, 80 µL of DPBS was pipetted from each dish and the supernatant samples were transferred to individual microcentrifuge tubes. To replace the sample volume, 80 µL of new, sterile DPBS (pH 7.4) was pipetted into each dish. These 80 µL samples were stored at -20°C.

The team then used the collected samples to perform a BCA assay, observe changes in absorbance using a spectrophotometer, and measure the concentration of each unknown sample based on a standard curve. First, a series of dilutions of known concentrations were prepared from a BSA ampule. The team prepared diluted BSA standards according to the Pierce™ BCA Protein Assay Kit instructions for the enhanced procedure. These dilutions and the supernatant samples would later be mixed with a working reagent (WR) solution to initiate BCA chelation. When preparing the working reagent, the team used a 50:1 ratio of reagent A to reagent B as suggested by the assay

protocol. An example of the volumes of reagent A (WR-A) and reagent B (WR-B) calculation is as follows:

(6 standards + 84 unknowns)*(3 replicates)*(200 μL of WR/sample) = 54,000 μL WR

1/51 = WR-B/54,000 → WR-B = 1,059 μL = 1 mL

50/51 = WR-A/54,000 → WR-A = 52,941 μL = 53 mL

After 1 mL of WR-B and 53 mL of WR-A were mixed in a beaker, 25 μL of each BSA dilution was pipetted in triplicate into each well of a labeled 96-well plate. Then, 200 μL of the WR solution was pipetted into each well containing the dilutions.

Each hydrogel supernatant sample (25 μL) was then pipetted in triplicate into each well of a separate, labeled 96-well plate. 200 μL of the WR solution was then pipetted into each well. Both well plates were then covered and incubated at 60°C for 30 minutes.

Once the plates incubated at room temperature for 2 minutes and cooled, they were placed in the SpectraMax Absorbance Microplate Reader. A program in SoftMax Pro was run to analyze the absorbance of each well plate at 562 nm and produce a standard curve with a linear fit and equation.

Results

The raw data from the spectrophotometer was analyzed in Microsoft Excel to create a standard curve with a linear fit line as seen in Figure 20 below.

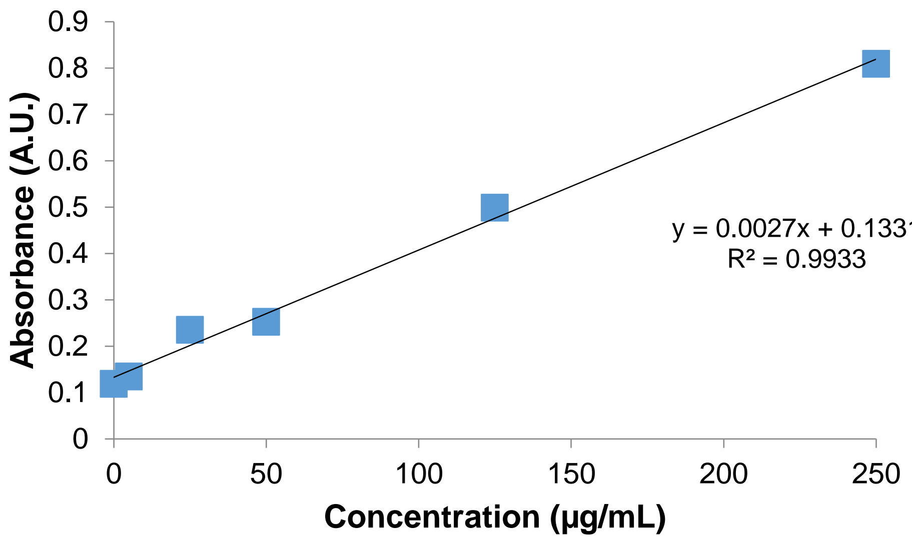

Figure 19: Standard curve from BCA assay

After plotting the absorbance values against the known concentration values, the formula obtained from the line of best fit was used to approximate the mass released by each bundle sample. Dilutions during sampling were accounted for and the volume each sample was submerged in was used to calculate the mass released (Figure 21). Next, the mass released values were normalized to the controls at each time point. The values of mass released obtained for the control group was subtracted from those of each experimental group. Each experimental group was normalized in order to observe only the release of loaded protein. Following this, the total percentage of release was calculated by considering the total mass of factor loaded onto each of the ten 3 cm long single microthreads in a bundle (Figure 22).

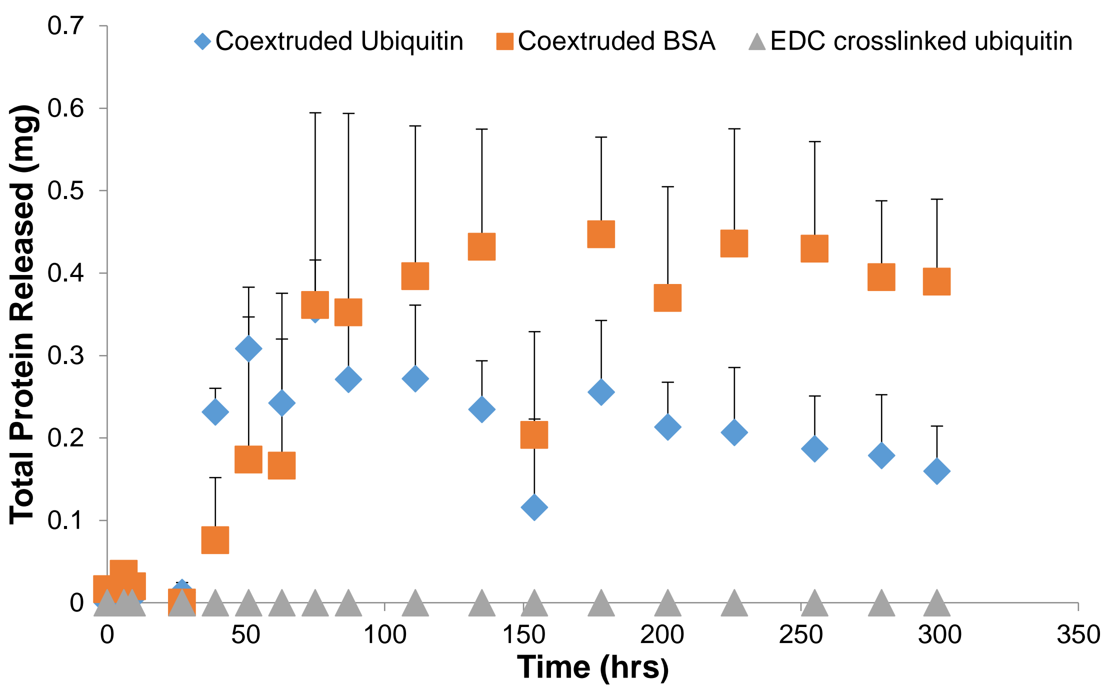

Figure 20: Cumulative protein release from bundled microthreads

The protein released was plotted against time to produce release profiles (Figure 21). The first observation was that no detectable protein released from the crosslinked bundle which could be explained by various factors including the concept that crosslinking reduced the swelling capability necessary for diffusion of the loaded protein, the disassociation of the ubiquitin protein from the fibrin matrix could produce a longer delay than that tested, ubiquitin binding to the matrix itself and not being able to diffuse out, and finally the loading efficiently of ubiquitin could have been low producing a lower concentration gradient. Any one or combination of these may produce the observed release profile, however for this application this profile is not desired therefore method of release is not compatible with natural skeletal muscle regeneration.

The coextruded ubiquitin release profile showed prolonged release consisting of a delayed release within 48 hours, following by a burst release from hour 48 to 168, and finally a sustained release until hour 300. In comparison to this, the coextruded BSA

showed similar profile but a higher release rate. This is most likely due to its larger size, which would cause larger pores and therefore an increased surface area over time for diffusion. The total percent of protein released was plotted (Figure 22).

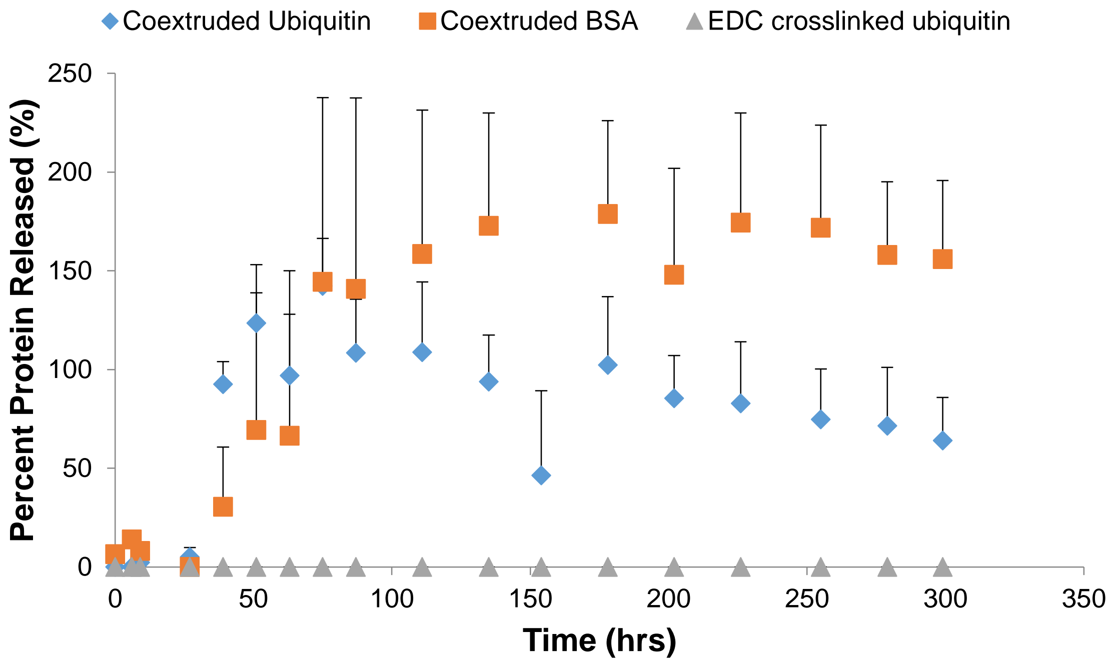

Figure 21: Percentage protein released from bundled microthreads

As was expected from the crosslinked ubiquitin results shown in Figure 21, the percentage release seen in Figure 22 is zero values because the results have been normalized. The BSA loaded bundles were shown to release more than 100% of loaded protein. This may be attributed to the increase in pore size produced in the microthread by the larger BSA molecule allowing more residual BSA in the microthread to be released rather than from the ubiquitin bundle. This would be true even in the case that the release is normalized to the control since this would have a smaller surface area. The coextruded ubiquitin bundle demonstrated the desired release profile and percent released for IGF-1 during natural skeletal muscle regeneration.

5.3 Hydrogel Release Studies

In the final design, the microthread bundles loaded with IGF-1 would be dipped in an HGF-loaded hydrogel. Protein release studies were conducted on hydrogel discs to determine the release kinetics of BSA and to validate the hydrogel component.

Methods

Hydrogels were fabricated with varying concentrations of BSA to analyze protein release over time. Hydrogels were fabricated with BSA concentrations of 1, 5, or 20 mg/mL (n=3). A control hydrogel was also made at 0 mg/mL BSA (n=3). The full procedure for control hydrogel fabrication can be seen in **Appendix J** and BSA-loaded hydrogels can be seen in **Appendix K**. Briefly, each BSA-loaded hydrogel was made by combining 0.45 mL of fibrinogen (73 mg/mL) with 0.05 mL of either 10, 50, or 200 mg/mL BSA in a 12-well plate to make final concentrations of 1, 5, or 20 mg/mL, respectively. This fibrinogen-BSA solution was then added to a solution of 0.075 mL of thrombin (40 U/mL stock solution) and 0.425 mL $CaCl_2$ (40 mM stock solution). The control hydrogels were made by combining 0.45 mL fibrinogen (73 mg/mL) with 0.05 mL of DPBS, which was then added to the thrombin and $CaCl_2$ solution and mixed on a shaker plate.

Once solidified, each hydrogel was sterilized with isopropanol and submerged in 2 mL of DPBS (pH 7.4) in separate wells of a 6-well plate and incubated at room temperature (Figure 23).

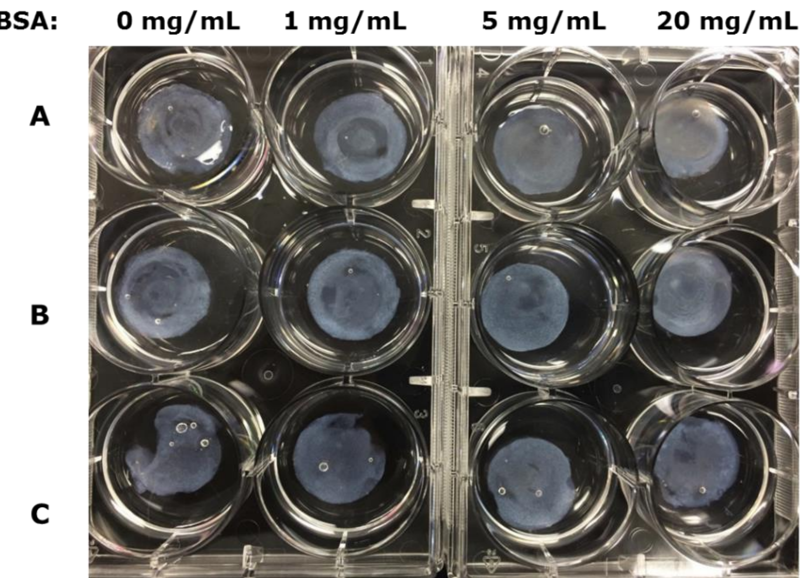

Figure 22: Fibrin hydrogels with varying BSA concentrations

To obtain supernatant samples, 240 μL of DPBS was pipetted from each well and transferred to individual microcentrifuge tubes. To replace the sample volume, 240 μL of new, sterile DPBS was pipetted into each well. The supernatant samples were stored at -20°C until analysis. Samples were taken at hour 0, 3, 9, 18, 30, 45, 60, 72, 84, 96, and 108 for a total of 11 sampling times.

The samples were run with a BCA assay as previously mentioned in the microthread bundle studies and incubated at 37°C as described in the Pierce BCA assay protocol. Concentration values were then calculated through the use of the collected absorbance values and the standard curve (Figure 24).

Results

The raw data from the spectrophotometer was analyzed in Microsoft Excel to create a standard curve with a linear fit line (Figure 24).

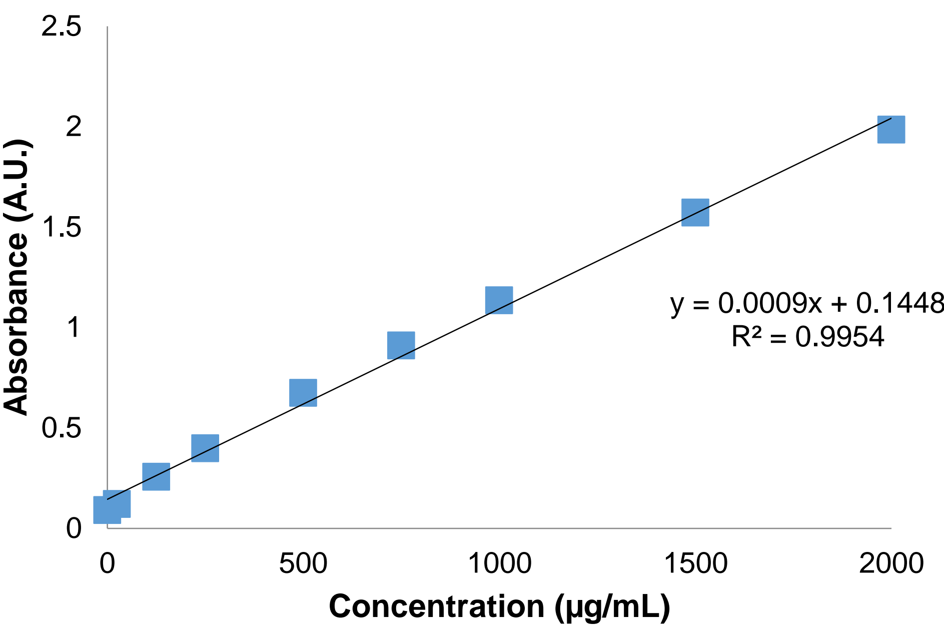

Figure 23: Standard curve from BCA assay

Similar to the microthread bundles, after plotting the absorbance values against the known concentration values, the formula obtained from the line of best fit was used to find the mass released by each hydrogel sample. Dilutions during sampling were accounted for and the volume in which each sample was submerged in was used to calculate the mass released of all experimental groups (Figure 25). Again, since protein release was observed from the control hydrogel all experimental groups were normalized to the control at each time point.

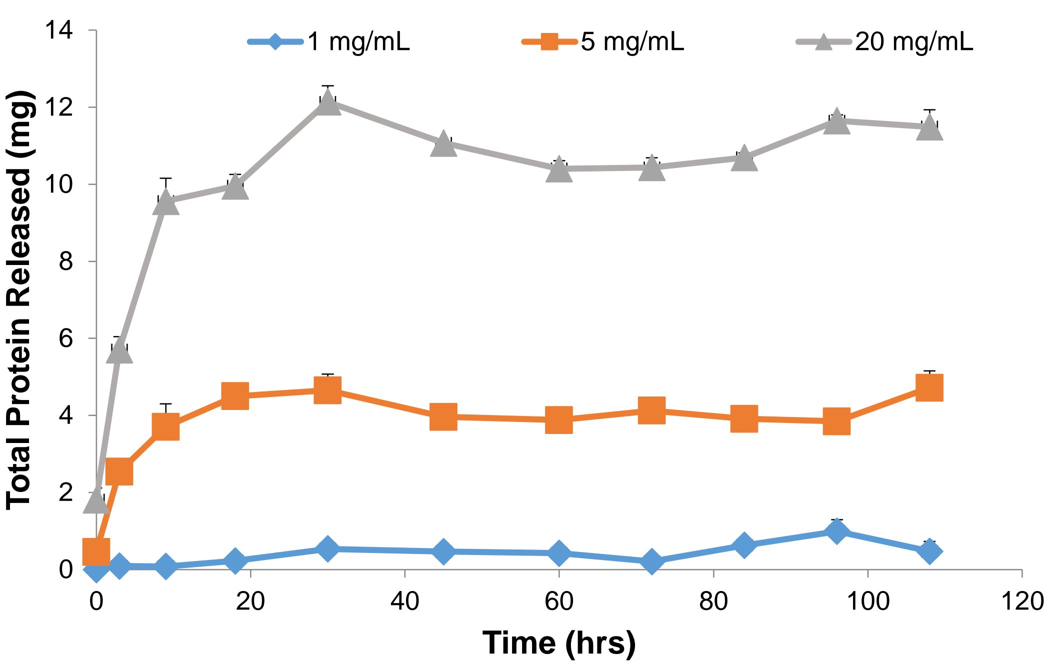

Figure 24: Cumulative BSA released from hydrogels

Results showed protein release from all experimental with a higher rate of release during the initial 30 hours of sampling, with the 20 mg/mL hydrogel having the highest initial rate of release and 1 mg/mL having the lowest (Figure 25). These differences in rate of release may be explained by the higher concentration gradient that exists in the 20 mg/mL rather than that which exists in the 1 mg/mL. Following this, the percentage of release was calculated by considering the total mass of BSA loaded onto a 1 mL hydrogel for each experimental group including 1 mg, 5 mg, and 20 mg for each concentration. The percentage of release was plotted against time to produce the cumulative percent release of BSA (Figure 26).

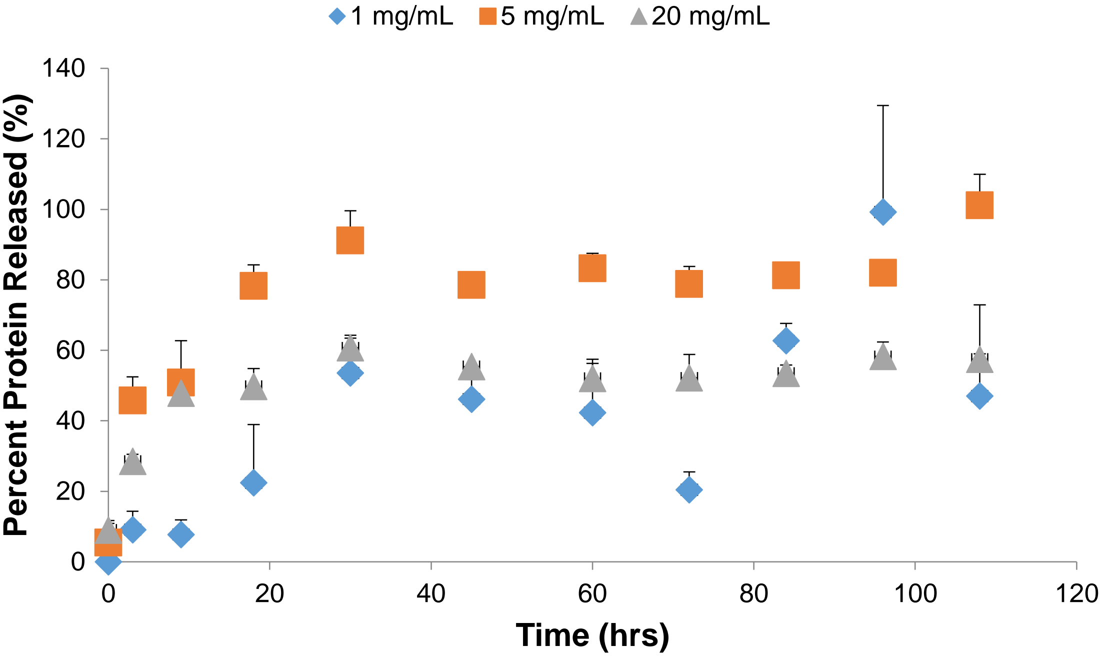

Figure 25: Percentage BSA release from hydrogels

The results of percentage release (Figure 26) were normalized to the control at each time point. The 20 mg/mL hydrogel was loaded above the polymer solubility concentration causing uneven dispersion and therefore cause the protein to not fully diffuse. The BCA assay may not have been able to accurately detect the protein released from the 1 mg/mL hydrogel because of its low concentration. The 5mg/mL hydrogel was loaded with a lowest concentration of protein that produced the desired release profile so it was chosen as the optimal release profile to model HGF. Complete release of the loaded BSA from the 5 mg/mL hydrogel occurred by hour 30, which falls within the targeted time frame. This profile best represents the release of HGF during natural skeletal muscle regeneration and potentially would work best for the composite system.

6. Discussion

6.1 Single Microthread Imaging

The combination of fluorescent images and ImageJ pixel intensity analysis are effective tools for analyzing BSA release from single microthreads. Although the averages calculated from the ImageJ analysis are arbitrary values, they supplement the release data from samples used for the BCA assay with a visual verification that the images at each sample time decreased in intensity. The decreasing average intensity values correspond to dimming microthreads imaged in the FITC setting, which can be inferred as a release in BSA. Compared to the controls, the FITC-BSA-loaded microthreads showed a greater decrease in intensity over time.

The average intensity of the FITC-BSA microthreads decreased by nearly four times in the first 11 hours but showed little change from hour 11 to 72. These results could correlate to a burst release of FITC-BSA from the microthread in the first 11 hours. Because these microthreads will be loaded with the slow release control protein in the final composite system, this burst release was not desired. The release from microthreads should be delayed by at least 24 hours and sustained for a longer period of time. Bundling several microthreads could prevent this immediate release. With a small surface area and high concentration of BSA, it is expected that a large amount of the BSA would be released quickly. By completing this experiment with more samples, a clearer trend in release could be determined. When compared to microthread bundles, the burst release should only be evident in the single microthreads. Multiple tests would also eliminate possible outliers caused by user error during imaging. Inconsistent values for the FITC-BSA microthreads could have also been due to bleaching from over-exposure to light, causing a higher standard deviation between images.

The control average intensities were slightly variable over time, but were not significantly different from one another and averages were well below the lowest value for the FITC-BSA microthreads. Variability in the control averages could have been due to inconsistencies in imaging. Increasing the sample size in future experiments should eliminate this variability.

Overall, this release study proved that FITC-BSA could be bound to single microthreads when added to fibrinogen before coextruding. FITC-BSA released from the microthreads with an initial burst release followed by a gradual release for 72 hours. Future experiments should be done to increase sample sizes and generate consistent release profiles for both control and FITC-BSA-loaded microthreads.

6.2 Microthread Bundles

To validate the microthread bundle core for the composite system, three experimental groups and a control group were tested. The crosslinked ubiquitin group did not release any protein in amounts detectable by the BCA assay. This was unexpected but useful information for further experimentation with crosslinking small molecules, like IGF-1. The team had a crosslinked ubiquitin group in case the coextruded ubiquitin group released too quickly. All other bundles were analyzed by the changes in absorbance using a spectrophotometer, and the concentration of each unknown sample was calculated based on a standard curve.

These results showed that all bundles followed a similar release profile with a delay in release for the first 48 hours, followed by a burst release for the remainder of the first week and then sustained release for the duration of the sampling period. The control bundles showed this release profile because the microthreads are made of fibrinogen,

which is detected as releasate by the BCA assay. To account for this, we normalized our results to the releasate from these control bundles and found the coextruded BSA and ubiquitin experimental groups were comparable in release between hours 0 and 48. As expected, coextruded ubiquitin had a higher release profile than coextruded BSA between hour 48 and 168 because it is a smaller molecule and therefore diffuses faster as the microthread degrades. This BSA release profile differs from the profiles seen in the hydrogel experiments because here the BSA is releasing from microthreads, which have a denser matrix than a hydrogel. BSA has a larger molecular weight than IGF-1 and is not comparable in size, however, this experimental group showed that microthread integrity can withstand loading of larger molecules. Coextruded ubiquitin had a higher amount of protein released from hour 168 to the end of the study for the same reason.

All experimental groups showed data points after 168 hours are that were lower than the previous data points. This can be explained by loss of material potentially due to the proteins sticking to the lab equipment such as micropipette tips, and well plates since these materials were not coated with anything to prevent protein binding. This could cause the absence of protein that is reflected in the lower data points on the graph. However, since both coextrusion methods showed the desired release profiles, this method can be used to mimic IGF-1 release in natural skeletal muscle regeneration.

Ubiquitin (8.5 kDa) has a similar molecular weight as IGF-1 (7 kDa), suggesting that this method would work for coextruding IGF-1 in the final composite design. It also showed the best sustained release during week two, which is ideal for this design because it mimics the natural release in the body during muscle regeneration.

Overall, this experiment showed coextruded ubiquitin is the best choice for future testing with the composite system because it shows a molecule of similar size as IGF-1 releasing in the proper time domain. Future experiments should be done to increase sample sizes and generate consistent release profiles for all experimental groups in this study. Future studies should also include the coextrusion of IGF-1 in the composite system.

6.3 Hydrogel

In order to simulate quick release of an HGF-like factor from a hydrogel, three hydrogels were fabricated with varying BSA concentrations and sampled for 48 hours. The concentrations of BSA were chosen based on previous research and guidance from the client and user. The higher BSA concentration of 20 mg/mL used in one type of hydrogels may explain why it was much more opaque than other hydrogels. Although there was no noticeable visual change in opacity for this hydrogel over time, there was still some release of BSA as was quantified through the use of the BCA assay. The control, 1 mg/mL, and 5 mg/mL hydrogels formed clear, rigid gels while the 20 mg/mL hydrogel formed an opaque, rigid gel. Results obtained through testing this component will be used in future experiments when creating the composite system.

Following the sampling and quantification of protein release through the BCA assay, results showed variations among the concentrations used. After 48 hours, all of the hydrogels demonstrated a burst release comparable to that of HGF during natural skeletal muscle regeneration. To account for the releasate from the control, all hydrogels were normalized at each time point to the value of protein released from the control hydrogel. The percentage of protein release for the 5 mg/mL hydrogel best demonstrated

release kinetics of the quick release phase while using the minimal amount of BSA. In addition the percentage release graphs showed varied rates of release observed in the slopes of each release profile. This showed that an increased concentration of BSA loaded to the hydrogel yielded a higher percentage of protein released during the first 48 hours of the initial burst release phase. Finally since rates of release were different for each concentration, these differences were most likely due to the limiting release factors from the hydrogel matrix. Limiting factors may be the dissolution rate of the protein through the polymer matrix, the diffusion of the protein through the surrounding medium, or lowering concentration gradient due to release. To obtain more accurate results with a higher R^2 value, the sampling volumes should be smaller to reduce the impact of each dilution on the released amount. These results will be used in future experiments with the hydrogel component tested for release kinetics as a coating since the surface area will increase allowing more release.

7. Final Design and Validation

7.1 Project Accomplishments

The main focus of this project was to design a multiphasic system to release two different growth factors in a time dependent manner that could be applied to fibrin microthreads. Methods of loading each independent growth factor were designed and validated. Analysis techniques were used to determine the optimal loading strategies for each growth factor.

All fluorescence imaging of single microthreads showed statistical significance between microthreads loaded with FITC-BSA and unloaded control microthreads at each time point. This confirmed the presence of FITC-BSA on microthreads, demonstrating that protein could be loaded onto microthreads without the addition of any binding factors. Release was observed through the decrease in average pixel intensity over time. These results were interpreted to indicate the microthread structure integrity can withstand the addition of loaded factor via coextrusion. This confirmation allowed the team to test different loading strategies with bundling experimentation.

Microthread bundle experiments showed that coextruded ubiquitin microthreads (1 mg/mL) had the optimal release profile. The coextrusion method ensures uniform loading because the factor is mixed directly into the fibrinogen solution prior to coextrusion (Fig. 14). This method also yielded a delay in release until hour 40, followed by distinct, burst release from hour 48 to hour 168 and then sustained release for the duration of the study. This confirms that a molecule with a molecular weight of 8.5 kDa can be loaded via coextrusion onto microthreads, bundled, and released in the desired time domain. Future experiments using IGF-1 coextruded in microthreads should be performed to validate this.

Ideally, different concentrations of IGF-1 would be compared to determine the optimal amount if factor necessary for the microthread bundle component.

The hydrogel coating was fabricated with uniform loading achieved through the stirring technique. The hydrogel with 5 mg/mL BSA showed the desired release rate. There was an initial burst release from hour 0 to hour 48, followed by a sustained release for the duration of the study. This confirms that a molecule with a molecular weight of 66 kDa can be loaded and released in the desired time domain from a hydrogel. Future experiments using HGF instead of BSA in hydrogels should be performed to validate this. Ideally, different volumes of hydrogels should be compared to determine the optimal coating size for the hydrogel component.

Using the individual results from the single components, a hypothesis can be drawn for the composite system. The coextruded ubiquitin microthread bundle and the 5 mg/mL BSA concentration hydrogel showed the proper time dependent release profiles, therefore suggesting that the combination of these two single components combined would potentially make a functional composite system. Each single component would have to be fabricated with IGF-1 and HGF, tested and validated separately before experiments with the composite system can be performed. Alterations to the current single components may be necessary. For example, the number of single microthreads within the bundle may need to be increased or decreased depending on the results of future experimentation. The thickness of the hydrogel component may also need to be adjusted, pending what the composite results yield. The coating method may need to be adjusted from the current dip coat idea. Painting, spraying, or rolling the hydrogel onto the microthread bundle could be possible design alternatives.

7.2 Impact Analysis

An impact analysis was conducted on our composite design, addressing the following topics: economics, environmental impact, societal influence, political ramifications, ethical concerns, health and safety issues, manufacturability, and sustainability.

Economics

Economics relates to the manufacturing, distribution, and consumption of products. Because fibrin microthread testing is currently limited to *in vitro* studies in small laboratories, there will be minimal economic impact from our composite design in the foreseeable future. The results from this project will be used to conduct additional studies before potentially moving to clinical trials. There are currently no animal or human clinical trials being conducted due to the high costs and limited research data available. After more studies are done with fibrin microthreads incorporating growth factors, a reliable source of materials will be required. Should the hydrogel coated bundle be made in mass production, sterile and reliable fibrinogen, thrombin, HGF, and IGF-1 would need to be in constant supply. A patient's willingness to pay for the product would also factor into the costs of producing and distributing it. If a patient's need for the product outweighs the potentially high costs, it will make a positive impact on society and the economy.

Environmental Impact

As the production of fibrin microthreads advances, the demand for fibrinogen and thrombin from animal sources will increase. With this increase in demand, there will need to be an increase in required sources such as land, energy, food, and labor to maintain the higher animal source population. The increase in required energy and the resulting

waste may have a significant effect on the environment. Food sources and housing for the sources may require land allocation from otherwise untouched environments. However, if the disposal of waste is properly regulated and minimal resources are used for production, or if renewable energy sources are used, the production of fibrin microthreads will have minimal effects on the environment while still meeting growing demands.

Societal Influence

This product has potential to greatly affect society in a positive way. When fibrin microthreads are used with growth factors to aid in skeletal muscle regeneration, the quality of a patient's life who is suffering from VML will greatly improve. A dependable cure for VML will increase patient compliance and overall happiness during treatment. In addition, the research done prior to this project as well as the results from the experiments conducted will aid in other laboratories using fibrin microthreads for therapeutic applications. The success in other laboratories using tissue engineering to improve native muscle, tendon, and ligament regeneration will positively affect society.

Political Ramifications

This design currently has minimal political ramifications. Microthread research is still in the preliminary research stage so the commercial market is marginally affected. If this design reaches a clinical setting, there may be research laboratories outside of the United States who may find this technology useful, however the impact on the global market as a whole is relatively small. After fibrin microthread technologies become commercialized, they may become relevant in the political sphere.

Ethical Concern

Since this product will be used in the medical field, there are ethical concerns, but they are minimal. The sourcing of blood products from animals for implantation in the human body may be one ethical concern, however, fibrinogen and thrombin could ultimately be patient specific. This would eliminate concerns about sourcing from human cadavers, stem cells, and animals. Overall, this product was designed to improve the quality of life for patients with VML, so the minimal ethical concerns are outweighed by the positive societal impact.

Health and Safety Issues

This product has the potential to greatly improve the health of patients with damaged skeletal muscle through the use of microthreads as growth factor delivery vehicles. The use of biological materials that can be sterilized with isopropyl alcohol or ethylene oxide should reduce health and safety concerns. Following continued research and development of fibrin microthreads, extensive tests and clinical trials will need to be conducted to ensure the product is safe and reliable. Once these tests are completed and the FDA approves the product, it will be considered safe for the majority of the population. However, individuals may have adverse side effects such as allergic reactions, so each patient should be tested for compatibility with this product before it is implanted. In addition, the fibrinogen, thrombin, and growth factors would need to be tested to ensure the animal sources had no prior conditions that may affect the microthreads. It would be ideal to find a reproducible, consistent source that could be used in a commercial setting.

Manufacturability

Standard fibrin microthreads are currently manufactured by a process using a coextrusion system developed by the Pins lab at WPI. Microthreads are manufactured in small batches on location and by hand; the threads are not prefabricated at this point due to concerns with maintaining the threads in a sterile environment. The novel process described in this report to create a composite system loaded with proteins is repeatable, does not involve any complex machinery, and utilizes materials that are readily available from commercial sources such as Sigma Aldrich and Life Technologies. As this product is further developed, the fabrication process may be made shorter to decrease labor costs and accommodate for increased demands. Streamlined methods for fabricating microthreads and hydrogel coatings loaded with growth factors would improve this product's manufacturability in the future.

Sustainability

Ensuring this product and its materials do not deplete resources is necessary when analyzing its sustainability. Since the main materials of this product (fibrinogen, thrombin, and growth factors) are naturally occurring and can be derived from human or animal sources, they are renewable and not easily depleted. Much of the equipment used to fabricate microthreads (coextrusion machine, blunt end tip, baking pan) is reusable, increasing sustainability. The non-reusable equipment such as the syringes or polyethylene tubing can be sent to a reprocessing plant to be recycled. This increases the sustainability of the extrusion system and is an added benefit to the product.

8. Conclusions and Recommendations

The main goal of this project was to design and develop modifications to the current microthread fabrication process for the incorporation of two growth factors to release in different time domains to mimic natural skeletal muscle regeneration. Through extensive research and client meetings, the project team designed a composite system with two single components, a hydrogel coating and microthread bundle, each to be loaded with different growth factors to release in different time domains. The project team was able to quantify loading and release kinetics of control proteins from both the hydrogel and microthread components using a BCA assay and fluorescence microscopy. These results suggest that each component could be implemented into a composite system.

Future work on this system should include fabricating the composite system and producing release studies for the system to determine proper release kinetics from the composite system. After a complete analysis of the composite system, future work could also include adding HGF and IGF-1 to the system to measure release kinetics of the growth factors versus our control proteins. The project team also recommends increasing sample sizes to produce more consistent results. A more sensitive assay system, such as an ELISA, could also be used for better results from the composite system.

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Appendices

Appendix A: Descriptions of Growth Factor System and Assay System Objectives

During the design process, the team made a list of primary objectives for the Growth Factor (GF) system and Assay system. This appendix gives a more detailed description (in italics) for each primary and secondary objective for both systems (in normal font). This section is to help clarify what each objective means in detail.

Growth Factor System

Efficient loading of growth factors (GFs) onto microthreads

What percentage of the GFs bond to the microthreads? How much is lost?

- Uniform attachment
  - *Generate uniform attachment of GFs onto the microthreads*
  - *Constant/even throughout, consistent coating and concentration*
- Storable
  - *The microthreads must be storable so the GF remains active from the time we load threads until they are analyzed*
- Repeatable
  - *Obtain the same loading quality each time GFs are added to microthreads*
- Cost efficient process
  - System that *minimizes waste of purchased GFs*
- Time efficient process
- Storable
  - *Add GF before significant degradation of microthreads*

Controlled multiphasic release of GF from microthreads

Should control slope

Control the rate of release of the GF from the microthread so that the GF is not immediately dumped, and instead follows a gradual release through bulk degradation (or similar process)

- Adjustable release kinetics
  - *Be able to change as experimentation demands*
- Repeatable release kinetics for each factor

        - *The release of GF from microthreads should have the same slope within each "batch". Each loaded microthread should produce an initial dosage of GF followed by a slower linear release of GF.*

Cost effective

Highest % of GF possible ends up in the body (minimum waste i.e.: unbound, stuck in pipet tip, left behind in well, lost during fabrication, etc.)

- Minimal loss of growth factor
    - *The addition of GF onto the microthreads should be time efficient and stable to minimize the loss or deactivation of GF*
- Use of readily available materials
    - *The equipment used for the addition of GF onto threads should be currently available in most labs*
- Time efficient
    - *Quick process will save money*
- Labor efficient
    - *Process requires minimal effort*

User friendly process

Simple to understand how to complete GF loading onto threads

Process of GF addition must easily build on current microthread procedure of fabrication

Minimal adjustment to current process (extra steps, added time, etc.)

Modifiable factor parameters

The concentration of each GF in the microthreads must be adjustable to accommodate for future research related to the optimal amount of GF present at a wound

- Reproducibly vary the amount of GF loaded to microthread
    - *Type, concentration, rate, etc. of GF can all be altered*
- Modulate concentration of each factor released
    - *Regulate and determine standard units for ease of measuring concentrations*

Assay System

Facile validation process

The implementation of each assay should be a simple process to minimize human error

Designed with standardized units or dimensions for easy assembly and repair

Use predetermined amount/concentration of GF

- Detecting growth factor binding to microthread
    - *Easy validation process for growth factors that were added to microthreads via addition of stains to growth factors*
- Growth factor release from microthread detection
    - *Easy validation process for detecting the release kinetics from the threads*
- Bioactivity detection
    - *Easy process for detecting the functionality of the system*

Modifiable factor parameters

The assay system should be adjustable to allow staining of different GFs

- Various growth factor concentrations
    - *Assay system should be able to detect varying concentrations of growth factors in samples*
- Different time domains
    - *Assay should be able to measure multiphasic release at different times. (Over span of hours, days, etc.)*

Repeatable outcomes

- Accurate
    - *The assay system should be accurate. Measured levels must be as close to the true value as possible*
    - *The assay should be sensitive enough to detect small concentrations and sample sizes*
    - *Results show low standard of deviation*
- Precise
    - *The assay should be precise to ensure that the results of all tests are consistent*

Cost effective

The cost of chosen stain(s) must be minimal

The equipment used to conduct assay, analyze data, and collect data must be accessible in most labs

- Time efficient
    - *The process must be time efficient to reduce the effects of GF loss*
- Labor efficient
    - *Process should be time efficient in order to minimize labor cost*

Appendix B: Calculations of Weighted Objectives for Growth Factor System and Assay System

This section evaluates the importance of each objective for the design team, client, and user. It uses a pairwise comparison chart (PCC) to rank each objective against each other to give each objective a numerical score against another, which could then be summed for a total value corresponding to their overall ranking. This helped the design team prioritize the objectives when making decisions.

Growth Factor System

B1: First level objectives combined PCC

Project Objectives	I			II			III			IV			V			Total			Normalized Totals			Weighted Totals			SUM	Weighted Score
	Client	User	Designers	Client	User	Designer	Client	User	Designer	Client	User	Designe	Client	User	Designers											
I. Efficient loading of growth factors onto microthreads				0	0	0	0.5	0.5	1	1	1	1	0	0.5	0	1.5	2	2	2.5	3	3	0.94	1.13	0.75	2.81	0.19
II. Controlled multiphasic release of growth factors from microthreads	1	1	1				1	1	1	1	1	1	1	0.5	1	4	3.5	4	5	4.5	5	1.88	1.69	1.25	4.81	0.32
III. Cost effective	0.5	0.5	0	0	0	0				0	0.5	1	0	0	0	0.5	1	1	1.5	2	2	0.56	0.75	0.50	1.81	0.12
IV. User friendly process	0	0	0	0	0	0	1	0.5	0				0	0	0	1	0.5	0	2	1.5	1	0.75	0.56	0.25	1.56	0.10
V. Modifiable factor parameters	1	0.5	1	0	0.5	0	1	1	1	1	1	1				3	3	3	4	4	4	1.5	1.5	1	4	0.27
																									15	

B2: Second level objectives combined PCC for objective I

Second Level Objectives																										
I. Efficient loading of growth factors onto microthreads	A			B			C			D			E			Total			Normalized Totals			Weighted Totals			SUM	Weighted Score
A. Uniform attachment				1	0.5	1	0	0	1	1	1	1	1	1	1	3	2.5	4	4	3.5	5	1.5	1.31	1.25	4.06	0.27
B. Storable	0	0.5	0				0	0.5	0	0	0.5	1	0	0.5	1	0	2	2	1	3	3	0.38	1.13	0.75	2.25	0.15
C. Repeatable	1	1	0	1	0.5	1				1	1	1	1	1	1	4	3.5	3	5	4.5	4	1.88	1.69	1	4.56	0.30
process	0	0	0	1	0.5	0	0	0	0				0.5	0.5	0	1.5	1	0	2.5	2	1	0.94	0.75	0.25	1.94	0.13
process	0	0	0	1	0.5	0	0	0	0	0.5	0.5	1				1.5	1	1	2.5	2	2	0.94	0.75	0.5	2.19	0.15
																									15	

B3: Second level objectives combined PCC for objective II

II. Controlled multiphasic release of growth factors from microthreads	A			B			Total			Normalized Totals			Weighted Totals			SUM	Weighted Score
A. Adjustable release kinetics				0	0	0	0	0	0	1	1	1	0.38	0.38	0.25	1	0.33
B. Repeatable release kinetics for each factor	1	1	1				1	1	1	2	2	2	0.75	0.75	0.5	2	0.67
																3	

B4: Second level objectives combined PCC for objective III

III. Cost effective	A			B			C			D			Total			Normalized Totals			Weighted totals			SUM	Weighted Score
A. Minimal loss of growth factor				1	0	1	1	1	1	0.5	1	1	2.5	2	3	3.5	3	4	1.31	1.13	1	3.44	0.34
B. Use of readily available materials	0	1	0				1	0.5	1	0.5	1	1	1.5	2.5	2	2.5	3.5	3	0.94	1.31	0.75	3	0.3
C. Time efficient	0	0	0	0	0.5	0				0	0.5	1	0	1	1	1	2	2	0.38	0.75	0.5	1.63	0.16
D. Labor efficient	0.5	0	0	0.5	0	0	1	0.5	0				2	0.5	0	3	1.5	1	1.13	0.56	0.25	1.94	0.19
																						10	

B5: Second level objectives combined PCC for objective IV

V. Modifiable factor parameters	A			B			Total			Normalized Totals			Weighted Totals			SUM	Weighted Score
A. Reproducibly vary the amount of growth factor loaded to microthread				0.5	0.5	1	0.5	0.5	1	1.5	1.5	2	0.56	0.56	0.5	1.63	0.54
B. Modulate concentration of each factor released	0.5	0.5	0				0.5	0.5	0	1.5	1.5	1	0.56	0.56	0.25	1.38	0.46
																3	

Assay System

B6: First level objectives combined PCC

Project Objectives	I			II			III			IV			Total			Normalized Totals			Weighted totals			SUM	Weighted Score
I. Facile validation				0	1	0	0	0	0	1	1	1	1	2	1	2	3	2	0.75	1.13	0.5	2.38	0.24
II. Modifiable factor parameters	1	0	1				0	0	1	1	1	1	2	1	3	3	2	4	1.13	0.75	1	2.88	0.29
III. Repeatable outcomes	1	1	1	1	1	0				1	1	1	3	3	2	4	4	3	1.5	1.5	0.75	3.75	0.38
IV. Cost effective	0	0	0	0	0	0	0	0	0				0	0	0	1	1	1	0.38	0.38	0.25	1	0.10
																						10	

B7: Second level objectives combined PCC for objective I

I. Facile validation process	A			B			C			Total			Normalized Totals			Weighted totals			SUM	Weighted Score
A. Detecting growth factor binding to microthread				1	1	0	1	0.5	0	2	1.5	0	3	2.5	1	1.13	0.94	0.25	2.31	0.39
B. Detecting growth factor release from microthread	0	0	1				0.5	0.5	1	0.5	0.5	2	1.5	1.5	3	0.56	0.56	0.75	1.88	0.31
C. Detecting bioactivity	0	0.5	1	0.5	0.5	0				0.5	1	1	1.5	2	2	0.56	0.75	0.5	1.81	0.30
																			6	

B8: Second level objectives combined PCC for objective II

II. Modifiable factor parameters	A			B			Total			Normalized Totals			Weighted totals			SUM	Weighted Score
A. Various growth factor concentrations				0.5	0.5	0	0.5	0.5	1	1.5	1.5	2	0.56	0.56	0.50	1.63	0.54
B. Different time domains	0.5	0.5	1				0.5	0.5	0	1.5	1.5	1	0.56	0.56	0.25	1.38	0.46
																3	

B9: Second level objectives combined PCC for objective III

III. Repeatable outcomes	A			B			Total			Normalized Totals			Weighted totals			SUM	Weighted Score
A. Accurate				1	0.5	1	1	0.5	1	2	1.5	2	0.75	0.56	0.50	1.81	0.60
B. Precise	0	0.5	0				0	0.5	0	1	1.5	1	0.38	0.56	0.25	1.19	0.40
																3	

B10: Second level objectives combined PCC for objective IV

IV. Cost effective	A			B			Total			Normalized Totals			Weighted totals			SUM	Weighted Score
A. Time efficient				0.5	0.5	1	0.5	0.5	1	1.5	1.5	2	0.56	0.56	0.50	1.63	0.54
efficient	0.5	0.5	0				0.5	0.5	0	1.5	1.5	1	0.56	0.56	0.25	1.38	0.46
																3	

Appendix C: Coextruded Control Fibrin Microthread Procedure

This procedure was adapted from previous publications [Cornwell, 2007], [Grasman, 2012] and was used for fabrication of fibrin microthreads. This details how the design team fabricated the microthreads used in the control groups in the experiments.

Materials

- 1 mL Fibrinogen (MP-151122) of 70 mg/mL stock solution
- Thrombin (SIGMA-T4648-1KU), 150μL (warm to room temperature)
- 850 μL of $CaCl_2$ 40 mM stock solution
- HEPES salt
- DPBS (without Calcium and Magnesium)
- Metal non-stick pan
- 25 Gauge blunt end needle (1)
- 0.86 mm I.D. polyethylene tubing (Intramedic PE90 427421)
- 1 mL syringes (2)
- Blending connector (SA-3670; Micromedics, MN)
- pH meter

Procedure

1. Prepare 300 mL of 1X (10 mM) HEPES buffer solution as follows:
    a. Make 100 mL of 10mM HEPES (10X)
        i. Add 2.6g HEPES salt to 100 mL of deionized water
        ii. Fully dissolve solid
    b. Add 250 mL deionized water to 30 mL of Hepes buffer stock solution
    c. Adjust the pH to 7.4
    d. Bring the total volume to 300mL using deionized water
2. Place blunt end needle (25 gauge, BD) into 0.86 mm I.D. polyethylene tubing
3. Luer lock the blunt end needle/tubing assembly onto the front end of blending connector
4. 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 4.70mm, press SELECT
    e. Enter volume: 1.0mL, press ENTER
    f. Enter extrusion rate: 13.21 mL/hr, press ENTER
5. Acquire a metal non-stick pan and place it next to the syringe pump

6. Fill pan with 300 mL HEPES buffer solution (prepared in step 1)
7. Add 850 µL of $CaCl_2$ stock solution to 150 µL of thrombin aliquot, mix well
8. Prime 1 mL syringes by repeatedly moving plungers up and down
9. Label one syringe with the word thrombin and one with fibrinogen
10. Mix 0.9 mL of fibrinogen with 0.1 DPBS together
    a. Collect the fibrinogen and DPBS solution in the 1 mL syringe labeled fibrinogen
11. Collect the thrombin solution into the 1 mL syringe labeled thrombin
12. Invert syringes to remove bubbles
13. Have equal volumes of solution in both syringes
14. Place the 1 mL syringe of fibrinogen and DPBS solution in the back of the blending applicator, secure syringe/ blending applicator construct into syringe pump
15. Place the 1 mL syringe of thrombin solutions into the back end of the blending applicator, secure syringe/blending applicator construct into syringe pump
16. Press RUN on the syringe pump
17. Once fibrin solution begins to flow out of the tube, draw threads into the buffer solution (taking about 10 seconds to draw each thread)
18. The pump will automatically stop (if it does not, press STOP)
19. Wash tubing/blending applicator with cold water and a 5mL syringe, plugging the other opening with your thumb (at least 5 water rinses per blending applicator opening). Remove all residual water out of blending applicator/tubing
20. Repeating step 9 using an empty 5 mL syringe
21. Remove fibers from the bath once solidified (10-15 minutes)
22. Acquire cardboard box, stretch single thread from pan over box to form 3, 7.5 inch threads, secure along the cardboard box
23. Leave stretched fibrin threads to dry overnight.

Appendix D: FITC-BSA Loaded Microthreads Procedure

This was the procedure used to fabricate FITC-BSA loaded microthreads for single microthread imaging, as described in **Section 5.1** of the report.

Materials

- Fibrinogen (MP-151122) stock solution (70 mg/mL)
- Thrombin (SIGMA-T4648-1KU) stock solution (40 U/mL)
- 40 mM $CaCl_2$ stock solution
- FITC-BSA stock solution (24 mg/mL)

Procedure

Note: Conduct all of the following procedure in a dark room to maintain integrity of the FITC-BSA.

1. Add 30 mg of fibrinogen to room temperature fibrinogen stock solution for a final concentration of 100 mg/mL.
2. Combine 0.958 mL of 100 mg/mL fibrinogen solution with 0.083 mL of FITC-BSA stock solution in a 1 mL aliquot.
3. In another aliquot combine 0.850 mL of CaCl2 solution with 0.150 mL of thrombin stock solution.
4. Proceed with extrusion and stretching process as dictated in the Coextruded Control Fibrin Microthreads procedure (Appendix C).

Appendix E: ImageJ Pixel Intensity Procedure

The team used the following procedure for quantifying the pixel intensity within each of the single Microthread images, as described in **Section 5.1** of the report.

1. Open image from the FITC setting (.png file) within ImageJ
2. Split channels into the red, green, and blue components by going to Image → color → split channels
3. Since FITC fluoresces in the green channel, only the gray scale image of the green channel from the original image was used
4. Draw a 50x50 pixel square onto the image
5. Move the square so that it is entirely over the microthread within the image
6. Go to Analyze → measure to get the mean pixel intensity with the box
7. Move the same box and measure along the length of the microthread for a total of 4 times
8. Move the same box and measure to select a total of four areas of the background of the image (no part of the microthread should be included in the box).
9. A mean pixel intensity of all 8 data points should now be within the small pop-up window on ImageJ. Save this file and open within Excel
10. Take an average of each the 4 microthread pixels and 4 background pixels
11. Subtract the average pixel intensity for the background from the average number for the microthread
12. This results in total average pixel intensity on each microthread within the image.
13. Repeat this process to find total average pixel intensity for all control and FITC-BSA microthread images at each time point.

Appendix F: Coextruded Microthread Bundling Procedure

The team used this procedure when microthreads were bundled for testing. Bundling allowed for a magnified concentration of protein in each sample for the BCA to detect during testing, as described in **Section 5.2** of the report.

Materials

- Fibrin microthreads (dried)
- 35 mm diameter petri dish
- 7 mL DPBS (without calcium and magnesium)

Procedure

1. Remove fibrin microthread from drying apparatus or storage
2. Cut microthread into sections 3 cm in length
3. Secure 10 microthreads to one area along the rim of the petri dish using tape
4. Twist each individual microthread together so that each is connected within a bundle
5. Secure loose end of twisted microthreads directly across from the other secured end using tape
6. Fill petri dish with DPBS, fully submerging twisted microthread bundle (7mL)
7. Hydrate microthreads for 15 minutes
8. Remove DPBS from petri dish
9. Allow microthreads to fully dry (15-20 minutes)
10. Remove bundle from petri dish by cutting at each secured edge

Appendix G: Direct Addition of BSA to Coextrusion Procedure

This procedure was used to create BSA loaded microthreads. It is an altered version of the general coextrusion microthread procedure described in **Appendix C**.

Materials

- 0.9 mL Fibrinogen (MP-151122) of 73 mg/mL stock solution
- 1 mL of BSA (SIGMA-A9418) of 10 mg/mL stock solution
- Thrombin (SIGMA-T4648), 150 μL (warm to room temperature)
- 850 μL of $CaCl_2$ 40 mM stock solution
- HEPES salt
- DI water
- Metal non-stick pan
- 25 Gauge blunt end needle (1)
- 0.86 mm I.D. polyethylene tubing (Intramedic PE90 427421)
- mL syringes (2)
- Blending connector (SA-3670; Micromedics, MN)
- pH meter

Procedure

Follow steps 1-9 from Coextruded Control Fibrin Microthreads Procedure (Appendix C)

1. Carefully mix fibrinogen and BSA solution
2. Collect 1 mL of mixed solution from step 1 into a 1 mL syringe

Continue to follow steps 11-25 from Coextruded Control Fibrin Microthreads Procedure (Appendix C)

Appendix H: Direct Addition of Ubiquitin to Coextrusion Procedure

This procedure was used to create BSA loaded microthreads. It is an altered version of the general coextrusion microthread procedure described in **Appendix C**.

Materials

- 0.9 mL Fibrinogen (MP-151122) of 73 mg/mL stock solution
- 0.1 mL of ubiquitin (SIGMA-U6253) of 10 mg/mL stock solution
- Thrombin (SIGMA-T4648), 150 µL (warm to room temperature)
- 850 µL of $CaCl_2$ 40 mM stock solution
- HEPES salt
- DI water
- Metal non-stick pan
- 25 Gauge blunt end needle (1)
- 0.86 mm I.D. polyethylene tubing (Intramedic PE90 427421)
- mL syringes (2)
- Blending connector (SA-3670; Micromedics, MN)
- pH meter

Procedure

Follow steps 1-9 from General Coextruded Fibrin Microthreads Procedure (Appendix C)

1. Carefully mix fibrinogen and ubiquitin solutions
2. Collect 1 mL of combination into a 1 mL syringe

Continue to follow steps 11-25 from General Coextruded Fibrin Microthreads Procedure (Appendix C)

Appendix I: Loading of Ubiquitin to Microthreads with EDC/NHS Crosslinking

This procedure was used to create EDC crosslinked ubiquitin loaded microthreads. It is an altered version of EDC crosslinking [Grasman, 2012].

Materials

- Fibrin microthreads
- Ubiquitin (SIGMA-U6253)
- DPBS (without calcium or magnesium)
- Sodium phosphate monobasic, monohydrate (NaH2PO4; MW: 137.99)
- N-Hydroxy-succinimide (NHS; MW: 115.09)
- N-(3-Dimethylaminopropyl)-N'-Ethylcarbodiimide hydrochloride (EDC; MW: 191.7)
- One-well plate coated in PDMS (1 per crosslinking batch)
- PDMS frames (2 per crosslinking batch)

Procedure:

Fibrin microthread preparation

1. For each crosslinking plate, align 2 sets of PDMS frames distanced to fit inside a one well plate.
2. Insert 10 microthreads into the frames using forceps so they are taut between the frames. **Be careful, microthreads break under too much pressure.**
3. Place frames into well plates by positioning the well plate over the frames and inverting everything. Pull the frames to the edge of the plate so threads are taut.

NaH_2PO_4 buffer preparation (100 mM)

1. For every 60 mL, add 0.8279 g NaH_2PO_4.
2. pH solution to 7.4 using NaOH/HCl.

Hydration of microthreads

1. Slowly add 30 mL of NaH_2PO_4 buffer to well plates.
2. Incubate at room temperature for 30 minutes.
3. Remove liquid carefully with aspirator, avoid touching microthreads.

EDC and ubiquitin buffer preparation

1. Definition: Contains 28mM of EDC, 16mM of NHS and 1 mg/mL ubiquitin.
2. Add 0.5 mL of DPBS to 5mg of ubiquitin
3. For every 30 mL of similar buffer, add 0.0552 g of NHS, 0.1610 g of EDC and 0.1 mL of ubiquitin to remaining NaH_2PO_4 buffer.

EDC Crosslinking

1. Immediately after removing buffer in *hydration of microthreads* add 30mL EDC/NHS/ubiquitin NaH_2PO_4 buffer to well plates
2. Incubate at room temperature for 2 hours.
3. Remove liquid with aspirator, avoid touching microthreads.
4. Rinse plates with 30 mL diH_2O for 5 minutes.
5. Remove liquid with aspirator, avoid touching microthreads.
6. Repeat steps 4 and 5 two more times.
7. Remove frames from plates, being careful to keep microthreads intact, and allow to dry under the tension of their own weight overnight.

Appendix J: Fibrin Hydrogel Procedure

This procedure was used to create a fibrin hydrogel that was used as our control for single factor release testing, as described in **Section 5.3** of the report.

Materials

- Fibrinogen lyophilized powder (MP-151122)
- Deionized water
- Thrombin (SIGMA-T4648) stock solution 40 U/mL

Procedure

1. Dissolve 0.04 mg of fibrinogen into 10 mL of deionized water in a 1.5 cm petri dish
2. Place petri dish with solution on mixing plate with the rotation speed on low and leave until powder is completely dissolved.
3. Add 0.075 mL of thrombin stock solution to the solution of fibrinogen
4. Leave dish on mixing plate for 30 seconds
5. Remove onto stationary lab table to allow complete gelation of hydrogel

Appendix K: BSA-Loaded Fibrin Hydrogel Procedure

This procedure was used to create fibrin hydrogels loaded with three different concentrations of BSA: 1 mg/mL, 5 mg/mL, or 20 mg/mL, as described in **Section 5.3** of the report. These hydrogels were used to validate the hydrogel component of our composite system.

Materials

- Fibrinogen lyophilized powder (MP-151122)
- Deionized water
- Thrombin (SIGMA-T4648) stock solution 40 U/mL
- BSA (SIGMA-A9418)

Procedure

1. Dissolve 0.04 mg of fibrinogen into 10 mL of deionized water in a 1.5 cm petri dish
2. Place petri dish with solution on mixing plate with the rotation speed on low and leave until powder is completely dissolved
3. Add 10 mg (for 1 mg/mL concentration), 50 mg (for 1 mg/mL), or 200 mg (for 20 mg/mL) of BSA powder to the fibrinogen solution while on the mixing plate
4. Add 0.075 mL of thrombin stock solution to the solution of fibrinogen
5. Leave dish on mixing plate for 30 seconds then remove to allow gelation.