ABSTRACT

Incisional hernias are a common clinical problem occurring in up to 10% of all patients undergoing abdominal incisions. Current repair techniques involve the placement of xenografts, allografts, or prosthetic biomaterials. Despite these techniques, the incidence of hernia recurrence ranges from 24% to 54%. In order to address these high recurrence rates, we propose using a skeletal muscle engineering strategy. To this end, the novel cyclic acetal biomaterial, 5-ethyl-5-(hydroxymethyl)-\(\beta,\beta\)-dimethyl-1,3-dioxane-2-ethanol diacrylate, was functionalized to promote skeletal muscle regeneration. It was found that this biomaterial promotes myoblastic cell attachment and proliferation as well as the delivery of functional insulin-like growth factor 1 proteins \textit{in vitro}; therefore demonstrating the scaffolds biocompatibility. Furthermore, mechanical properties of the scaffold were tested and the complex modulus was shown to decrease after a significant increase in initiator concentration. Overall, this work establishes the functionality of a degradable cyclic acetal as a scaffold for skeletal muscle engineering.
THE DEVELOPMENT OF EH NETWORKS FOR SKELETAL MUSCLE REGENERATION WITHIN ABDOMINAL WALL HERNIAS

By

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In 2004, the Society of American Gastrointestinal and Endoscopic Surgeons reported that approximately 600,000 hernia repair surgeries are performed every year in the United States.\textsuperscript{1-4} Epigastric, umbilical, inguinal and femoral hernias are all included in these figures, however the most prominent is the ventral or incisional hernia as they account for 36 percent of all hernia repairs with this number increasing every year.\textsuperscript{2} Incisional hernias develop when the skeletal muscle around an incision site is weakened such that it can no longer support the pressure exerted by the internal organs causing a rupture. This occurs in up to one out of every five patients that undergo abdominal incisions. With these overwhelming numbers, abdominal hernias are among the most common pathological conditions that afflict humans. Still, it is believed that these numbers are underestimated.\textsuperscript{5,6}

Once rupture occurs a portion of the abdominal viscera can become confined or entrapped in the defect leading to two common physical symptoms: pain and a visible protrusion. Viscera, such as the bowel, often get trapped in these abdominal defects, occasionally with grave consequences. When entrapment does occur, there is potential for strangulation, ischemia and possible necrosis of the viscera. If left untreated the end result could be the loss of the organ or possibly the death of the patient.

Current treatment methods for abdominal wall hernias involve pressure trusses, xenographs, allografts or the placement of a prosthetic biomaterial meshes.
Despite these available techniques, the incidence of recurrence ranges from 24 to 54%, still making hernia repair a matter of concern for today’s clinicians.\textsuperscript{7}

To address the clinical need for better hernia repair strategies tissue engineering approaches have been proposed. By using engineering methodologies to study the structure-function relationship inherent in normal and pathological mammalian tissues, one can create biological substitutes to restore, maintain or improve tissue function.\textsuperscript{8} Only through the combination of the system’s functional biology (cell to cell signaling) with the systems interaction characteristics (scaffolds) can methods or tissues be produced that can replicate or improve that found naturally.

To begin developing an alternative strategy for abdominal wall hernia repair, the interactions between a skeletal myoblastic cell population and a novel biodegradable scaffold made from 5-ethyl-5-(hydroxymethyl)-β,β-dimethyl-1,3-dioxane-2-ethanol diacrylate (EHD) were studied. Three aspects of these scaffolds were investigated: mechanical properties, myoblastic cell compatibility and protein interactions. First, to determine the mechanical properties of the crosslinked EH networks; the effect of initiator concentration on the complex modulus was examined. Second, by investigating the effect of initiator concentration on myoblastic cell attachment, the cellular compatibility of the network was determined. Third, once it was found that this network was a viable scaffold, the protein interactions were studied, specifically, the signaling pathways and delivery of insulin-like growth factor - 1 (IGF-1). The ability of a myoblastic cell population to maintain the IGF-1 signaling pathways while attached to the EH scaffolds was determined by studying the effects of adding increasing concentrations of IGF-1 to the culture media of
attached myoblasts. In further protein interaction studies, delivery of the IGF-1 was achieved through physical adsorption upon the scaffold surface. To quantify the IGF-1 delivery, the effects of increasing adsorbed IGF-1 concentrations on the release rate as well as myoblast proliferation were investigated.
Chapter 2: Tissue Engineering Approaches to Abdominal Wall Hernias

2.1 Abdominal Wall Physiology

2.1.1 Hernias and the Abdominal Wall Physiology

An incisional hernia has been defined as a breakdown or loss of continuity of a fascial closure. A discontinuity in the musculature can result from injuries or traumas, occurring by both direct or indirect means, as well as disease, aging, or a weakness from birth. With direct injuries, an object, such as a scalpel or a bullet, pierces the musculature layer creating the defect. Indirect injuries, on the other hand, occur when an object hits the body with enough force to create a defect without affecting the other layers of the abdomen; an example being a cyclist coming in contact with the bicycle handlebars during a crash. While direct injuries may involve more layers of tissue, it is the inability of the muscle to heal itself, in both cases, that leave the defect open and at risk.

The abdominal wall is made up of six layers: skin, subcutaneous fascia, musculature, transversalis fascia, preperitoneal tissue and peritoneum. Skin is composed of two layers: the dermis or corium and the epidermis. The dermis itself also contains two layers: the papillary and the reticular layers. Functioning as a connective layer, the papillary layer contains loose connective tissue that attaches to the epidermis. Beneath this, the reticular layer contains the capillaries, lymphatics, and nerve ending, as well as, the hair follicles, sebaceous glands, sweat glands and their ducts, and smooth muscle fibers. All of these elements are supported by a collagenous connective tissue. The epidermis is the uppermost layer of skin that is
made up of nonvascularized endothelial cells that form from the inside outward. Together these layers range in thickness from 1-3mm.

Adipose and connective tissue make up the subcutaneous fascia, transversalis fascia and preperitoneal tissue. All three layers contain fat cells and fat stores to varying degrees. The subcutaneous fascia layer varies in thickness from 0.5-6cm and changes gradually with position along the abdomen. The skin thickness, the quantity of fat cells and the amount of fat stored in these layers also varies dependently on the individual.

The musculature is made up of the transversus abdominis, internal oblique, external oblique and rectus abdominis. Each muscle has a contralateral mirror image, with each individual group having a unique arrangement of the muscle fibers. The fibers are arranged based on the group’s functionality and these bundles provide strength to the abdominal wall. The peritoneum lines the inside of the abdominal cavity, effectively containing the abdominal organs, which contains folds that vary in thickness throughout the abdominal cavity.

All of these layers are involved in hernia occurrence and repair to varying degrees, based on the injury sustained. Hernia repair research focuses mainly on the musculature and peritoneum, as they and the incorporated aponeuroses and muscular fasciae are the most affected.

2.1.2 Abdominal Wall Functionality

The critical function of the abdominal wall is to counteract the large pressure force exerted by the internal organs. Excess pressure in the abdominal cavity can
exploit the smallest weakness or disruption in the musculature. Many everyday activities are associated with a significant increase in this internal pressure force; laughing, coughing, vomiting, heavy physical exercise, or even just lifting your head and legs, standing or sitting upright can have an effect.\textsuperscript{4, 5, 9} When this weakness is exploited, the musculature can bulge or tear, consequentially relieving the built up pressure and creating a hernia. Depending on the size of the resulting hernia, symptoms may or may not be present. Some of the symptoms include: pain (especially in bending), intestinal obstruction or the presence of a visible bulge where the hernia sac is present.\textsuperscript{6, 9} Clinicians have various tools to diagnose hernias, including physical exams, ultrasound, CT (computer tomography) scans or any combination of the above.\textsuperscript{6, 7, 9, 14, 15}

2.2 Herniation

2.2.1 A Mechanism for Herniation

Several factors, including those that are patient oriented and others that are clinically based, increase the probability of an initial herniation as well as a hernia recurrence. A study of 850 hernia repair patients found that the average age of patients undergoing hernia repair surgeries was 54, with the ages ranging from as young as 13 to as old as 94. In addition, the study showed that patients with a body mass index greater than 40 were more susceptible to complications, longer surgery times, larger defects and a higher recurrence rate.\textsuperscript{16}

When isolating the data for incisional hernias specifically, it was found that patients that are of male gender, as well as, those who smoke or use steroids have
shown a greater propensity for occurrence and recurrence.\textsuperscript{5, 7, 9} Medical conditions such as constipation, prostatism, diabetes mellitus, pulmonary diseases, jaundice and several more have also been found to increase the probability of herniation.\textsuperscript{5, 7, 9} Each on its own can result in hernia development, but a combination of any of these risk factors can be detrimental. Furthermore, it has been found that incision and suture procedures, in addition to suture or prosthetic material choices and wound infection affect the probability of herniation.\textsuperscript{9}

Two types of incisions used in skeletal muscle surgeries are vertical and transverse incisions. Sutures used to closed these incisions exert an increased tension force on a small area in the muscle sometimes leading to pull through; especially in vertical incisions.\textsuperscript{9} To alleviate this tension and limit pull through different suture procedures have been utilized; two of the most common are the interrupted and continuous suture techniques.\textsuperscript{9} The interrupted suture technique is used occasionally, however, it is associated with higher tension in the defect, therefore increasing the likelihood of recurrence. This occurs if the sutures do not have uniform tautness or are not evenly spaced, leading to a risk of fascial necrosis. So to eliminate this effect, continuous suture techniques are used to evenly disperse the tension over the entire wound.\textsuperscript{9} Suture materials and wound healing effects are discussed in a later section.

2.3 Overview of Current Clinical Solutions

2.3.1 Clinical Approaches to Treating Abdominal Hernias

Due to the fact the abdominal wall hernias are so inconsistent in size, shape, position and severity, there is currently no accepted universal approach to treating
abdominal hernias. Techniques such as pressure trusses, sutures, the placement of a prosthetic biomaterial meshes, xenografts or allografts are all valid options.\textsuperscript{6, 17} With the exception of pressure trusses, all of these techniques require a surgical procedure. Pressure trusses apply mechanical force to the herniated area to close the defect. They are only used in patients with excessively large defects who are not suitable surgical candidates and are rarely used in developed countries.

The recurrence rate for suture only techniques is approximately 45%; recurrence is defined by the reopening of a previously treated abdominal defect.\textsuperscript{7} Using sutures alone in smaller hernias, which exhibit less tension, is a viable option, however when the defect size increases thus increasing suture tension, pull through becomes more common. Such high recurrence rates have prompted the development and advancement of a generation of tissue engineering grafts that help aid in the support of the surrounding tissue. Still, these materials must be secured to the abdominal wall to be effective and thus can harbor some of the same concerns as suture techniques. To help alleviate this complication current meshes are attached using “tension free” methods. In addition to lowering recurrence rates as much as 75%, tension free suturing also helps to decrease healing time and pain. This alone does not completely correct the problem as the overall recurrence rate is still approximately 24\%.\textsuperscript{7, 15, 18-20}

\textit{2.3.2 Criteria for Repair Techniques}

To be considered viable for clinical application, there are several criteria that surgical implants must meet. First, the prosthesis or tissue that is used for repair must
allow for the return of functionality to the tissue. In skeletal muscle, pliancy and mechanical strength are particularly significant. An implant must be able to move with the body to prevent additional stress and tension on the supporting tissue, as well as, the implant itself. Additionally, the implant must be able to withstand the changing pressure exerted by the internal organs. Anything weaker or less pliable than the native tissue could potentially increased risk of recurrence as well as affect the surrounding tissues.

System reactions are the second criteria. Wound healing plays an important role in the regaining of functional tissue. It has been shown that devices that can decrease the inflammatory respond and body rejection to an implant or suture are better candidates for repair. Concurrently, any material that supports the growth of bacteria or the harboring of other harmful microorganisms would be detrimental to the wound healing process. Therefore, the tissue or prosthesis used would ideally support the ingrowth of the native tissue to regenerate the muscle without supporting the growth of harmful microorganisms. It should also limit adhesions of the abdominal viscera, as these adhesions are the leading cause of intestinal obstructions, as well as, they are responsible for chronic abdominal pain and 15-20% of cases of female infertility.

One major setback to the biomaterials used today is that they are left in the body permanently, making it possible for the implant to migrate or shrink thus rendering it ineffective. Therefore implants and suture materials that have the additional property of being absorbable are being studied. Being biodegradable allows the material to degrade and slowly leave the system as the host’s native tissues
grow into the defect. Optimization of all of these properties would give the ideal device for hernia repair.

2.3.3 Xenografts

In the beginning xenografts were used primarily as suture material. Tissues such as kangaroo, ox, deer and whale tendons have all been utilized. Catgut was also a common tissue used primarily to form suture materials. As the high recurrence rates of suture techniques have become more evident, patches have become more popular. Porcine small intestine submucosa (SIS) is being studied as a viable tissue patch. Taken from the small intestine of a pig, the cells are removed from the layers of tissue, leaving behind the extracellular matrix’s (ECM) supporting structure. Layers can then be combined as needed to increase the strength and size of the scaffold. Once implanted, this scaffold attracts host cells and begins to reorganize them in such a way that connective tissue fills the defect. An additional benefit to this graft is that over time it is reabsorbed into the body leaving behind only host tissue.

While all these materials help restore some functionality of the muscle there are two major limitations: disease transmission and host rejection. Several tests that are used to screen donor tissues for disease only test for known or prevalent diseases. This could leave the tissue unusable. Host rejection is an equally troublesome limitation. If the host’s body attacks the implant, the graft could become compromised and the defect will remain. Methods such as removing the cells of the grafts, as is done with the porcine SIS, may help to reduce the host rejection.
2.3.4 Allografts

Allografts are similar to xenografts and are especially useful because the donor tissues are more comparable to host tissues. They can be utilized in two ways. The first is as a whole tissue graft. This provides both a cellular component (for tissue regeneration) and the ECM component (a scaffold for host and donor cells). Another option is to separate the cellular component from the ECM and use them separately. The cells can be utilized in various therapeutic techniques and the ECM can be implanted as an acellular scaffold.\textsuperscript{25}

However, the core limitation of this technique is availability. There is a limit to how much tissue can be extracted from a donor without causing a defect that is of a critical size in which it does not heal itself. As with the xenografts, host rejection is still an issue; however the disease transmission risk is considerably lower.

2.3.5 Prosthetic Meshes

The placement of prosthetic meshes into defects is becoming a more frequent alternative to tissue implants as the number of biocompatible materials is increasing. These meshes give a greater control over the implant properties. Two common synthetic polymers that have been used extensively are polytetrafluoroethylene (PTFE) and polypropylene (PP). Natural polymers such as sodium hyaluronate (HA) and carboxymethylcellulose (CMC) are also used. By varying percentages of different monomers along with the crosslink density of the polymer, one can change the biodegradability and pliability of the resulting material. In addition, when examining processing techniques, different pore sizes, ranging from 3-500\textmu m can be
achieved. Not only can pore sizes be controlled but pore shapes can be altered and their distribution can be in either random or in ordered patterns.\textsuperscript{21,26} These processing techniques provide an added control over the mechanical and biological properties of the resulting material. Furthermore, these materials do not present the same risk for disease transmission due to the lack of a previous cellular component.

Biomaterials, unlike donor tissues or cells, are not limited in quantity or quality. With a larger supply of materials, defects of a larger size can be treated more effectively than with donor tissue. This technique is not without its drawbacks, however. To the body, the implant is seen as a foreign object; the risk of eliciting an inflammatory response is eminent. In abdominal hernias, fibrosis occurs and can provoke adhesions of the abdominal viscera to the defect site.\textsuperscript{22} This is just one of many concerns that are currently being addressed in research.

As mentioned previously, commonly used biomaterials are polytetrafluoroethylene (PTFE) and polypropylene (PP). PTFE is a hydrophobic, non-biodegradable polymer that is utilized clinically when formed into a fiber mesh.\textsuperscript{21} By adjusting the crosslink density of the polymer chains, one can ideally alter the adhesive and tissue ingrowth properties of the mesh; as is done with the Gore-Tex Dual mesh.\textsuperscript{21} This mesh’s bilayer design has a lower layer with a mesh size of 3µm and is meant to restrict tissue ingrowth, where as, the upper layer has a mesh size of greater than 100µm and is meant to provoke tissue ingrowth. In a study by Gonzalez et al, it was found that this change in mesh size does significantly affect tissue ingrowth, with the smaller pores being associated with inhibited tissue ingrowth.\textsuperscript{21} In
light of this, other studies have shown that this design still has a high rate of recurrence, infection and inflammatory response.\textsuperscript{27}

Polypropylene on the other hand is a slightly less hydrophobic, non-biodegradable polymer. It is formed into meshes that contain macropores as opposed to the micropores of the PTFE. These macropores help to trigger tissue ingrowth thereby improving the surgical integrity and strength of the implant.\textsuperscript{21, 22} Nevertheless, PP has no natural defense for adhesion formation. Therefore, the use of non-adhering agents such as hyaluronic acid and carboxymethylcellulose in addition to the PP is currently being investigated.\textsuperscript{22, 28} These glycosaminoglycans not only reduce the number of binding sites available on the PP, they also carry a charge, which can affect cell binding. An overall advantage of the PP grafts is that they have a decreased risk of infection and inflammation when compared to the PTFE grafts.\textsuperscript{27}

2.4 Tissue Engineering Approaches to Treat Defects

To construct more ideal devices, tissue engineering approaches are being applied. By using engineering methodologies to study the structure-function relationship inherent in normal and pathological mammalian tissues, one can create biological substitutes to restore, maintain or improve the tissue function.\textsuperscript{8} Only though the combination of the functional biology of the system with the systems interaction characteristics can methods or tissues be produced that can replicate or improve that found naturally. This approach began in the late 1880s with xenografts and it has evolved to the development of autographs and biodegradable synthetics.\textsuperscript{18}
2.4.1 First Generation Drawbacks

While each current hernia solution has different limiting factors, one that holds true for all current treatments is that there is a presence of an inflammatory response after implantation. In the inflammatory response blood fills the defect, allowing proteins to form a provisional matrix, providing a scaffold to facilitate wound healing by helping resolve and reorganize incoming cells, proteins and signals. Once this has occurred, acute inflammation begins with the infiltration of fluid, plasma proteins and leukocytes. Following acute inflammation is the formation of granulation tissue, which is characterized by the proliferation of capillaries and fibroblasts. However, when the inflammatory stimuli persists chronic inflammation results; which is characterized by the presence of macrophages, monocytes, and lymphocytes with vascularization beginning to occur. Finally, fibrous tissue is formed when foreign body giant cells incorporate into the granulation tissue.

The degree to which this inflammatory response occurs is responsible for how well the defect heals. Wound infection can profoundly affect this response, thus making it essential to treat and prevent. It has been reported that chronically draining sinuses will sometimes develop after a surgical procedure, become infected and cause complications; one complication being recurrence.

Therefore current technologies attempt to control not only this response but also wound infection and have been successful to a degree. An implant’s topography has a significant influence on the extent to which the foreign body reaction occurs and consequently the amount of fibrous scar tissue formed. Advances in processing techniques have given clinicians the advantage in creating specifically patterned...
topographies to control cell infiltration. One example would be the choice of a porous implant (natural or synthetic) that would incorporate more cells during the inflammatory response than a non-porous material.

2.4.2 The Tissue Engineering Solution: Muscle Regeneration

For hernia repair an ideal cell population would be the skeletal muscle progenitor cell: the satellite cell. This would facilitate skeletal muscle regeneration as opposed to fibrous scar formation. However, since satellite cells are not present in the prosthesis previous to implantation, they must migrate and compete with the granulation tissue and foreign body giant cells to enter the matrix. With such an inadequate number of satellite cells available compared to the competing cells, the musculature is replaced almost solely by fibrous scar tissue as opposed to skeletal muscle. Regenerating the skeletal muscle in the defect could ultimately restore function and decrease the hernia recurrence. To create the ideal implant, the development and regeneration processes of skeletal muscle must first be understood.

During muscle development mononuclear cells, called myoblasts, align parallel to one another and fuse to produce multinucleated myotubes. The myotubes then undergo a maturation process, during which they become innervated and vascularized, resulting in the myofiber structure. Myofibers are bundled parallel to one another by aponeuroses to form functional skeletal muscle. When electrically stimulated these bundles contract simultaneously, leading to the most distinctive characteristic exhibited by skeletal muscle: voluntary movement.
Muscle regeneration is a slightly more complicated process as it involves satellite cells. These mononuclear cells are present in skeletal, smooth and cardiac muscles. In skeletal muscle specifically, they reside between the basal lamina and the existing myofiber it encapsulates.\textsuperscript{32-34} While the cells appear to be associated with the specific myofiber, this is not the case, as is shown by the presence of a gap between the plasma membrane of the myofiber and the membrane of the satellite cell. This gap can range in size from 15-60nm with little protrusion of the basal lamina into the space. It is possible that this disassociation is the key to the mobility of these cells.\textsuperscript{31}

The ability to migrate is vital, as the cells are found disbursed randomly throughout the myofiber with which they reside and are not always found in the same density in all skeletal muscles.\textsuperscript{31} Regardless of the reason for the defect, whether it be trauma, injury or disease, the process for regenerating muscle does not change.\textsuperscript{31}

When trauma to the musculature occurs, the available satellite cells become activated and migrate to the site of the defect and begin proliferating. The newly produced cells have two options: to replenish the satellite cells reserve and return to quiescence or remain activated and migrate to the site of the defect. If the second is the cells fate, it will differentiate into a myoblast and align parallel with each other and the remaining myofibers, as was done in muscle development. Fusion begins and the resulting myotubes undergo the maturation process, becoming functioning myofibers. To utilize this process current studies are focused on defining cell signaling pathways, migration, attachment, proliferation and differentiation of several types of cells, in the regeneration of tissues.\textsuperscript{35-37}
2.4.3 A Novel Construct

Polymeric scaffolds allow researchers and clinicians to control the properties of the repair meshes that are used. The newest front in tissue engineering is the use of biodegradable polymers. Most clinically used biodegradable polymers degrade into acidic products. This creates an environment that elicits an inflammatory response and limits tissue healing. By using EHD, a novel construct can be created which will degrade into neutral products, decreasing the inflammatory response and creating a better environment for tissue healing.

A two-layer design will be used to develop this construct. A layer of porous EH scaffold will be created and implanted facing the abdominal wall. This scaffold will provide the structural stability for the construct, serve as a delivery vehicle for different proteins and provide an area for muscle regeneration to occur. By altering the porosity of this layer, the tissue ingrowth, proteins delivery rate and possibly the degradation rate of the implant can be controlled. However, to address the concern of adhesions from the peritoneal tissue and abdominal viscera the EH scaffold will be coated with a film of poly(ethylene glycol) (PEG). PEG is currently used in clinical implants and has been shown to prevent the attachment of different cell types; this coating would be in contact with the underlying fascia on the peritoneal surface. Overall this construct would create an environment were skeletal muscle can integrate freely into the EH scaffold while the PEG coating would significantly decreasing the number of adhesions from the peritoneum and abdominal viscera.

The work presented in this study addresses the initial stages of developing the EH scaffold. To begin, both the mechanical stability and myoblastic cell interactions
with the scaffold were studied. Once these had been established, further tests were performed to determine the interactions between the IGF-1 protein, myoblastic cell population and the EH scaffold.
Chapter 3: Novel Biodegradable Scaffolds for Skeletal Muscle Regeneration

3.1 Introduction

The objective of this project was to determine the feasibility of 5-ethyl-5-(hydroxymethyl)-β,β-dimethyl-1,3-dioxane-2-ethanol diacrylate (EHD) networks as a biomaterial for abdominal wall hernia repair. Incisional hernias are a common clinical problem occurring in up to ten percent of all patients undergoing abdominal incisions. Current repair techniques involve the placement of a prosthetic biomaterial, xenografts, or allografts. Despite these techniques, the incidence of hernia recurrence is in excess of ten percent. This high recurrence rate is due to many things including the use of non-biodegradable scaffolds and fibrous scar tissue formation. Therefore we hypothesize that developing a biodegradable biomaterial that can exhibit mechanical properties similar to skeletal muscle while incorporating myoblastic cells for skeletal muscle regeneration would be advantageous for hernia repair strategies.

The novel property of the EH biomaterial is that it lacks the carboxyl groups in other biodegradable polymers, producing byproducts that are neutral as it degrades. This prevents additional premature degradation of the scaffold due to high acidity and decreases the inflammatory response of skeletal muscle tissue surrounding the implant. With such desirable degradation qualities, adding a cellular component to this scaffold would create a functional biomaterial specific to abdominal wall hernia repair.
This study investigated the initial steps of creating an EH scaffold in an effort to develop a novel EHD based hernia repair construct. To begin, the mechanical properties of the radically crosslinked EH networks were determined by studying the effects of increasing initiator concentration on the complex. Next, the cellular interactions between the network and a myoblastic cell population were established by investigating the effects of increasing initiator concentration on myoblastic cell attachment. Then, to ensure that the IGF-1 protein signaling was unaffected by the scaffold, the effect of increasing IGF-1 concentrations in the growth media on cell proliferation was tested on attached myoblastic cells. Finally, to determine the ability of the EH scaffold to serve as a delivery system for IGF-1, the effects of increasing the concentration of absorbed IGF-1 on release and myoblastic cell proliferation were characterized.

3.2 Materials and Methods

3.2.1 Materials

Benzoyl peroxide (BP), \(N,N\)-dimethyl-\(p\)-toluidine (DMT), 5-ethyl-5-(hydroxymethyl)-\(\beta,\beta\)-dimethyl-1,3-dioxane-2-ethanol diacrylate (EHD) and bovine serum albumin (BSA) were obtained from Sigma-Aldrich (Milwaukee, WI, USA). Reagent grade acetone was purchased from Fisher Scientific (Pittsburgh, PA, USA). F-10 ham media (F-10), fetal bovine serum (FBS), penicillin/streptomycin (Pen/Strep) and trypsin/EDTA were received from Invitrogen (Carlsbad, California, USA). Collagenase P was ordered from Roche Applied Sciences (Indianapolis, IN,
USA). Recombinant human insulin-like growth factor-1 was acquired from R&D Systems (Minneapolis, MN, USA).

3.2.2 EH Network Synthesis

EH sheets were fabricated in a glass plate mold. To assemble this mold, capillary tubes were secured onto one of two glass plates using vacuum grease. Together, the plate and capillaries made up the walls and bottom of a rectangular well. Once this was done, a 0.81 M stock solution of BP in acetone was prepared. Working solutions were subsequently made from this stock based on the desired fabrication parameters. EHD monomer was then mixed with the working initiator solution until one phase was present. The accelerant DMT was added to the solution at a concentration of 0.5µL / g of EHD monomer. The solution was mixed again and poured into the well of the previously prepared plate. A second plate was used to cover the solution and the system was placed in an oven at 30°C for 20min. A uniform thickness of approximately 0.8mm was achieved with the system.
Figure 1. (a) Chemical structure of the monomer 5-ethyl-5-(hydroxymethyl)-β,β-dimethyl-1,3-dioxane-2-ethanol diacrylate (EHD). (b) Chemical structure of the resulting crosslinked EH networks. (c) An EH network sheet made with 0.58M BP solution.

3.2.3 Skeletal Myoblast Isolation and Harvest

Skeletal muscle was harvested and isolated from the hind limbs of Wistar Hannover GALAS rats. Once the muscle was removed, it was rinsed three times with F-10 Ham media containing 10% Pen/Strep, diced, and then digested in 10mL of collagenase P solution for 2 hours at 37°C and 5% CO₂. After this time, 100µL of trypsin/EDTA solution was added and the cell solution was mixed and placed back
into the incubator for 30min at 37°C and 5% CO₂. The trypsin and collagenase P solution was neutralized with 22mL of growth media, composed of F-10 Ham media containing 10% FBS and 1% Pen/Strep. The cell suspension was passed through three filters with 100µm, 70µm and 40µm mesh sizes respectively. The remaining cells were spun down and the pellet was resuspended in growth media. This was done twice more: once with growth media and once with F-10 Ham media only. The resulting suspension was plated on a T-25 culture flask and was placed in the incubator at 37°C and 5% CO₂. The growth media was changed the day after harvest. At day 3 the cells were passaged and reseeded on a new T-25 culture flask. Growth media was changed again at day 6 and finally on day 8 the cells were twice washed with PBS, trypsinized, and seeded according to the study parameters.

3.2.4 Dynamic Mechanical Analysis

The mechanical properties of EH networks were analyzed by dynamic mechanical analysis, reflecting the mechanical forces experienced in the abdominal wall. Five different networks were formed using initiator solution concentrations of 0.08, 0.15, 0.23, 0.48 and 0.81M. During fabrication 2.25mL of the desired initiator solution was used for every 2.0g of EHD monomer. The resulting sheets were cut into 13mm x 30mm rectangles. Using the Q-800 dynamic mechanical analyzer (TA Instruments, New Castle, Delaware) the samples were placed in the single cantilever clamp and held isothermally at 37°C. One end of the network was then oscillated over a frequency range of 1-100Hz at an amplitude of 15µm. This experiment was
run with a sample size of 6 and the values for the complex modulus were reported as a mean and standard deviation of these samples at a frequency of 10Hz.

3.2.5 Myoblastic Cell Compatibility

Myoblastic cell attachment was investigated on EH networks that were formed using 0.75mL of either 0.34M or 0.58M initiator solution for every 2.0g of EHD. These resulting networks were cut into disks with a diameter of 2.0cm. They were subsequently washed three times for 15min each with phosphate buffer saline solution (PBS), acetone and PBS again, and then they were sterilized by exposure to UV light overnight. Each disk was placed in a 12 well plate. Stainless steel inserts were fabricated by cutting 1.5cm sections from a ¾” stainless steel polished pipe purchased from Stainless Steel Stock (Friend, NE). These inserts were filed down until the edges were smooth, washed thoroughly and sterilized through autoclaving. They were then placed onto the network to prevent them from floating in the culture media. A myoblastic cell population was seeded onto the center of the EH disks at a seeding density of 100,000 cells per network. After 4 and 6h the disks were washed twice with PBS and the remaining cells were lifted with trypsin/EDTA and counted using a hemacytometer.

3.2.6 Dosed IGF-1 Induced Myoblastic Cell Proliferation

To examine the effect of EH networks on IGF-1 signaling, skeletal muscle cells were isolated and networks made from the 0.58M initiator solution were prepared. Myoblastic cells were cultured on each disk for 48h to promote cellular
attachment before testing for cellular proliferation. After 48h, the growth media was changed and IGF-1 was added at concentrations of 0ng/mL (control group), 10ng/mL and 15ng/mL of IGF-1. All media was supplemented with FBS to provide proteins vital to the cell attachment. At Day 0, when the experimental media was added, it was found that there were $0.1 \times 10^6 \pm 0.07 \times 10^6$ cells per disk. After 3 and 5d the cells were lifted and counted using a hemacytometer. This experiment was done in triplicate and the values are reported as the mean and standard deviation of the total number of cells for each group.

3.2.7 Absorbed IGF-1 Release Profile and its Effect on Myoblastic Cell Proliferation

In order to determine the activity of IGF-1 absorbed on the surface of EH networks, IGF-1 was physically adsorbed onto the network and the IGF-1 release and myoblastic cell proliferation was characterized. IGF-1 powder was reconstituted in PBS with 0.1% BSA to a concentration of 100µg/mL. Solutions of the IGF-1 stock were diluted to 10ng/mL, 50ng/mL and 150ng/mL. 1mL of the IGF-1 working solutions was added to the surface of the network and was allowed to evaporate overnight in a sterile laminar flow hood to physically adsorb the IGF-1. Once dry, 2mL of fresh PBS was added to each network and the networks were placed in the incubator at 37°C and 5% CO₂. At 0.25, 0.5, 0.75, 1, 2, 4, 6, 12, 24 and 48h a 300µL sample was removed and fresh PBS was added to maintain total volume. All sample aliquots were stored at 4°C until analysis. The samples were analyzed using a Human IGF-I Quantikine ELISA Kit (R&D Systems, Minneapolis, MN). To investigate the myoblastic cell proliferation, myoblastic cells were cultured on the IGF-1 loaded
disks with growth media being changed on day 3. All media was supplemented with FBS as was done in the previous proliferation study. After 3 and 5d the cells were lifted and counted using a hemacytometer. Both studies were done in triplicate and the values were reported as the mean and standard deviation of the absolute amount of IGF-1 and the percent of loaded IGF-1 released from the network, as well as the total number of cells on each network respectively.

3.2.8 Statistical Analysis

Statistical analysis was performed on all data using an ANOVA and Tukey’s pairwise comparison tests.

3.3 Results

3.3.1 Dynamic Mechanical Analysis

In this mechanical study, the frequency at which the sample was oscillated was varied between 1 and 100hz. Initial data analysis displayed that this material had a complex modulus that had less than 8% variance across the entire frequency range. Therefore reported values were chosen from the middle of the experimental range at 10Hz to eliminate any end effects. Figure 2 shows that the complex modulus was found to be statistically similar for all networks made from BP solutions between 0.07 and 0.48M. However, comparison of the experimental groups that contained samples made from the 0.07 and 0.81M BP solutions show that the complex modulus decreased significantly from \(4.7 \times 10^2 \pm 1.2 \times 10^2\) MPa to \(2.5 \times 10^2 \pm 99\) MPa respectively.
Figure 2. Complex modulus as a function of initiator concentration. A frequency sweep of 1-100Hz was run on a Q-800 dynamic mechanical analyzer (TA Instruments) with parameters of 37°C and 15µm amplitude. Results for 10Hz are reported.

3.3.2 Myoblastic Cell Attachment

To investigate the biocompatibility of the EH scaffolds, myoblastic cell attachment was tested. Figure 3 demonstrates that when comparing the 0.34 and 0.58M BP networks, there is a slight increase in myoblastic cell attachment as the initiator content is increased. However, upon closer study of the results this trend was found to be insignificant. It was also found that the percent of myoblastic cells attached to the experimental group and the tissue culture polystyrene (TCPS) were statically similar at all time points; showing that the material is biocompatible.
3.3.3 IGF-1 Induced Myoblastic Cell Proliferation

To increase cell proliferation and ultimately cell number, IGF-1 was added to the culture media at concentrations of 0ng/mL (control group), 10ng/mL and 15ng/mL. As mentioned above, cells were allowed to attach to the EH network for 48h prior to IGF-1 addition; therefore all results were normalized to data taken on the day of IGF-1 addition (Day 0). In Figure 4, at day 3, the cell numbers for the control, 5ng/mL and 10ng/mL groups were as follows: $0.65 \times 10^6 \pm 0.10 \times 10^6$, $0.61 \times 10^6 \pm 0.11 \times 10^6$ and $0.64 \times 10^6 \pm 0.13 \times 10^6$. It was found that there was no significant difference between all groups at day 3. However, by day 5 the groups containing growth media supplemented with IGF-1 had significantly more cells than the control group: the values for the control and experimental groups were $0.65 \times 10^6 \pm 0.17 \times 10^6$, $1.0 \times 10^6 \pm 0.34 \times 10^6$ and $1.1 \times 10^6 \pm 0.28 \times 10^6$ respectively. While there were no statistical differences between the experimental groups at these doses, this study
displays the result that the supplementation of IGF-1 to the growth media increases the proliferation of our attached cell population.

![Graph showing cell proliferation with IGF-1 concentrations](image)

**Figure 4.** Total number of cells attached to EH networks after 3 and 5d. It was shown in this study that only the experimental IGF-1 groups at day 5 were statistically different.

### 3.3.4 Absorbed IGF-1 Release Profile

To investigate the use of this material as a delivery device, IGF-1 was physically adsorbed onto the EH surface and allowed to release into fresh PBS over time. To obtain release profiles of the IGF-1 three concentrations of 10ng, 50ng, and 150ng were adsorbed onto the network and release was characterized over 48h. Figure 5a displays the absolute amount of IGF-1 released. The 50ng and 150ng loading groups released $16 \pm 0.32\text{ng}$ and $17 \pm 0.12\text{ng}$ of IGF-1 respectively over this time period. These two groups follow a similar trend and are significantly similar for all point except 2h and 48h. The 10ng loading group however, released $1.7 \pm 0.038\text{ng}$ over 48h. This group was statistically lower than the higher loading groups.
at all time points as was expected. However, as a comparison, Figure 5b shows that when you look at the percent of loaded IGF-1 released there is a clear trend. The 50ng loading group released a significantly higher percent of IGF-1 than both the 10ng and 150ng loading group after 48h. Moreover, the 10ng loading group released a significantly higher percent of IGF-1 than the 150ng loading group. The values for these percents after 48h were $32 \pm 0.64\%$, $17 \pm 0.29\%$ and $11 \pm 0.081\%$ IGF-1 loaded respectively. These networks were able to sustain the release of structurally intact IGF-1 over a period of 48h.
Figure 5. (a) Total IGF-1 released from the surface of the EH network. The 10ng loading group released a significantly lower amount of IGF-1 than the 50ng and 150ng loading groups. The 50ng and 150ng groups released statistically similar amounts of IGF-1 at all time points except for 2 and 48h. At these time points the 150ng loading group released significantly more IGF-1.
(b) Percent IGF-1 released from the surface of the EH network. The 10ng loading group released a significantly higher percent of the loaded IGF-1 than the 150ng loading group. The 50ng group released the highest percent of the loaded IGF-1 and was statistically different from both the 10ng and 150ng loading groups.
3.3.5 Adsorbed IGF-1 Induced Myoblastic Cell Proliferation

To investigate the functionality of the adsorbed IGF-1, cells were attached to the loaded networks and proliferation was measured. It was found, in Figure 6, that the 50ng and 150ng loaded networks had significantly more cells attached to the networks than either the 10ng loading group or the control at day 3. By day 5 all groups were shown to be statistically similar. Also, the control at day 5 was statistically higher than at day 3, showing a slower increase in cell numbers than the IGF-1 loaded network groups.

![Figure 6](image)

**Figure 6.** Total number of cells attached to IGF-1 loaded EH networks after 3 and 5d. It was shown in this study that only the networks that contained 50ng and 150ng of IGF-1 had significantly more cells attached at day 3 than the 10ng and control groups. It was also found that by day 5 all groups statistically similar cell numbers.

3.4 Discussion

In this study, we hypothesize that developing a biodegradable biomaterial that can exhibit mechanical properties similar to skeletal muscle while enhancing native
myoblastic cell signaling will increase skeletal muscle regeneration within a hernia defect and would be advantageous for hernia repair strategies. Specifically, fabrication parameters of the EH network as well as its biological function were investigated; mechanical strength, cellular attachment, IGF-1 induced proliferation, IGF-1 release and cellular proliferation upon a loaded scaffold were all examined. The results of these studies showed the ability of an EH scaffold to function as a portion of a viable clinical construct for abdominal hernia repair.

Firstly, the mechanical properties of the crosslinked network were studied by investigating the effects of increasing initiator concentration on the complex modulus. We hypothesized that by increasing the BP content the mechanical properties, specifically the complex modulus, would increase. However, it was found that over the range of networks made with 0.08 to 0.48M BP solutions there was no significant effect on the complex modulus. In this range of initiator concentrations, the additional radicals in the system did not significantly change the crosslink density; therefore the increase in initiator content had no effect on the mechanical properties. When looking at the effects from 0.08 to 0.81M BP, however, the complex modulus decreased significantly. The data shows that increasing the amount of initiator to this concentration not only increases the number of free radicals formed in the monomer solution, but it also increases the probability that the radicals will encounter each other or that cyclic polymer chains will be formed. These phenomena decrease the number of radicals and monomers that are participating in the crosslinking, thus lowering the complex modulus. Current clinical implants have higher mechanical strengths than skeletal muscle, which is a contributing factor to implant failure. The
initial mechanical testing has shown that the modulus of this material is still
significantly higher than abdominal wall skeletal muscle, which has reported young’s
modulus values of 42.5 ± 9.0kPa in the transverse plane and 22.5 ± 2.6kPa in the
sagittal plane. Therefore to achieve more desirable mechanical properties porous
scaffolds will ultimately be used.

To investigate the biological functionality of the network, specifically the
cellular interactions, the effects of increasing initiator concentration on the myoblastic
cell attachment was tested. The myoblastic cells were expected to have a similar
percent attachment to the EH networks as the tissue culture polystyrene control. It
was found that at both time points the 0.58M BP system had a slightly higher percent
of myoblastic cell attachment than the 0.34M BP system, however closer
investigation showed that all groups performed statically similar at both 4 and 6h. It
has been shown in previous work that the 0.58M BP solution reacts more completely
during network formation, leaving behind less toxic unreacted materials. This
increase in unreacted materials from the 0.58M BP system to the 0.34M BP system
could have resulted in the decrease in attached cells that was observed in this study.
Therefore, the 0.58M BP formulation was used for all other studies.

Protein interactions, specifically IGF-1 signaling pathways, were investigated
by determining the effects of increasing the IGF-1 concentration in the culture media
on cell proliferation. It was expected that IGF-1 would retain its signaling pathways
and increase the proliferation of attached myoblastic cells. 10ng/mL and 15ng/mL of
IGF-1 were added to the culture media everyday for 5d. It was found that at day 3, all
groups had significantly similar cell growth. However, by day 5, both the 10ng/mL
and 15ng/mL group had significantly more cells that the control group but significantly similar cell numbers to each other. This indicates that repeat exposure to the IGF-1 had a long-term effect on the cell proliferation. It also shows that increasing the concentration from 10ng/mL to 15ng/mL did not have a significant effect on the cell proliferation perhaps due to the small difference in concentrations. This study demonstrates that the EH network does not affect the myoblastic cell’s ability to utilize the IGF-1 protein and increase proliferation.

To be effective, the EH scaffold should be able to deliver the IGF-1 protein and enhance the IGF-1 signaling *in vivo*. Therefore, the release rate of adsorbed IGF-1 from the network must be characterized. The effect of physically adsorbing increasing concentrations of IGF-1 on total IGF-1 released into PBS over time was measured. 10ng, 50ng and 150ng of IGF-1 were adsorbed onto the network and release was characterized over 48h. These loading values correlate with the total amount of IGF-1 added over the 5d period in the first proliferation study. It was expected that similar trends and percents released would be seen over the time period, however this was not the case. The 10ng group showed an increase in release then began to level off around 2h, where it settled at $1.7 \pm 0.029\text{ng}$ or $17.0 \pm 0.29\%$ of the loaded IGF-1 at 48h. While the absolute amount of IGF-1 released was significantly lower than both of the other groups, this group released the second highest percent release. The release from the 50ng and 150ng groups was sustained until after 12h. The final amounts for these groups were $16.0 \pm 0.32\text{ng}$ and $17.0 \pm 0.12\text{ng}$ respectively. This correlates to $32.0 \pm 0.64\%$ and $11.0 \pm 0.081\%$ of the loaded IGF-1 releasing from the network after 48h. While these two groups have released...
statistically similar amounts of IGF-1, for all points except for at 2h and 48h where the 150ng loading group had a higher release, they did not release similar percents. The 150ng loading group had the lowest percent release where the 50ng loading group released the highest percent of IGF-1. All percent data groups were statistically different. These results show that only a small fraction of the IGF-1 adsorbed was released over this time period. This may be a result of a structural change in the IGF-1 protein during the loading process. If this is the case, these mutated proteins cannot be accounted for using the Elisa assay and can only be deemed functional though cellular tests. However, this data does show that the network can be functionalized to release IGF-1 into the local environment, therefore showing its ability to deliver IGF-1 to native skeletal muscle in vivo.

To determine if the IGF-1 that was on the network was still active, proliferation tests were preformed on the loaded disks. In these tests, the effects of increasing adsorbed IGF-1 on the myoblastic cell population were studied. It was found that the networks loaded with 50ng and 150ng of IGF-1 had significantly more cells than the control and 10ng loaded networks after 3d. However, by day 5 all of the groups had become significantly similar. These results coincide with the release study results. The 50ng and 150ng loading groups showed significantly similar proliferation, which coincides with the release data. The 10ng loading group, however, released significantly less IGF-1 and this was reflected in the cell numbers after 3d. By day 5, there was no additional IGF-1 being released from any of the networks, therefore proliferation had slowed and all groups were statistically similar.
This data indicates a short-term increase in myoblastic cell proliferation with respect to absorbed IGF-1 concentration.

This study, when compared to the previous IGF-1 induced proliferation study showed two different trends. When myoblastic cells were exposed to lower concentrations of IGF-1 continuously there was a delayed increase on myoblastic cell proliferation. However, when the same total amount of IGF-1 was added in a burst at one time point, the results were a short-term increase in myoblastic cell proliferation. Overall, a similar increase in cell proliferation was seen in both studies, with the adsorbed IGF-1 exhibiting an increase in proliferation sooner than IGF-1 in culture media.

These studies suggest that the EH scaffold would be a favorable material that could be used to facilitate myoblastic cell attachment and proliferation. When combined with the fabrication and degradation properties, these materials are very promising in the field of tissue engineering, more specifically for skeletal muscle engineering. This work explores the scaffold parameters that are most desirable for skeletal myoblastic cell attachment and proliferation in an effort to advance abdominal wall hernia repair. An abdominal wall hernia repair scaffold would have mechanical properties similar to skeletal muscle as well as be able to induce myoblastic cell attachment and proliferation. From this study, we have found that the novel cyclic acetal biomaterial EHD could be used clinically to support the muscle, and along with the incorporation of IGF-1 onto the network surface, induce myoblastic cell attachment and proliferation.
3.5 Conclusions

This work examined the ability of the novel cyclic acetal biomaterial EHD to function as an abdominal wall hernia repair implant. To do this, tissue engineering principles were employed to test this material for mechanical and biological compatibility. Mechanical properties of the material were tested, as well as cellular attachment, IGF-1 induced proliferation and adsorbed IGF-1 release and induced proliferation. The results showed that these networks could be altered based on the concentration of initiator solution used during fabrication. Also, the myoblastic cell population attaches to the networks similarly to the tissue culture polystyrene control and proliferate upon these networks in the presence of IGF-1. Finally, it was determined that IGF-1 can be released from the network after adsorption and continue to induce proliferation upon a myoblastic cell population.
References
