Design and Analysis of a Collagenous Anterior Cruciate Ligament Replacement

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ABSTRACT

The anterior cruciate ligament (ACL) contributes to normal knee function, but it is commonly injured and has poor healing capabilities. Of the current treatments available for ACL reconstruction, none replicate the long-term mechanical properties of the ACL. It was hypothesized that tissue-engineered scaffolds comprised of reconstituted type I collagen fibers would have the potential to yield a more suitable treatment for ACL reconstruction. Ultra-violet (UV) radiation and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) were investigated as possible crosslinking methods for the scaffolds, and EDC crosslinking was deemed more appropriate given the gains in strength and stiffness afforded to individual collagen fibers. Scaffolds were composed of 54 collagen fibers, which were made using an extrusion process, organized in accordance with a braid-twist design; the addition of a hydrogel (gelatin) to this scaffold was also investigated. The scaffolds were tested mechanically to determine ultimate tensile strength (UTS), Young's modulus, and viscoelastic properties. Scaffolds were also evaluated for the cellular activity of primary rat lateral collateral ligament (LCL) and medial collateral ligament (MCL) fibroblast cells after 7, 14, and 21 days. The crosslinked scaffolds without gelatin exhibited mechanical and viscoelastic properties that were more similar to the human ACL. Cellular activity on the crosslinked scaffolds without gelatin was observed after 7 and 21 days, but no significant increase was observed with time. Although more studies are needed, these results indicate that a braid-twist scaffold (composed of collagen fibers) has the potential to serve as a scaffold for ACL replacement.

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1.0 Chapter 1
Introduction

1.1 Clinical Motivation

Every year in the United States alone, between 100,000 and 250,000 anterior cruciate ligament (ACL) injuries are diagnosed [1-4]. Seventy percent of ACL injuries occur in sports-related activities [5], and most of these ACL injuries are non-contact injuries (i.e., high-impact activities as well as cutting, pivoting, and other complex knee movements that cause excessive knee rotation or hyperextension) [6].

Annually, 50,000 ACL injuries result in reconstructive surgery of the ACL [1-4]. The "golden standard" for ACL reconstruction is the use of autologous tissue grafts (autografts), which are typically taken from the patellar tendon, hamstring tendons (semitendinosus-gracilis), or quadriceps tendon. Long-term, there is an 85-90% success rate for current ACL reconstruction approaches; however, for autografts, there is a risk of donor site morbidity, which can extend the rehabilitation time. Additionally, autografts may not be a viable option for patients who have already had an ACL autograft or patients without an appropriate tissue source [2, 7, 8]. Therefore, new techniques for ACL reconstruction need to be investigated.

1.2 ACL Background

Anatomy and Function

The human knee joint contains four major ligaments: ACL, posterior cruciate ligament (PCL), medial collateral ligament (MCL), and the lateral collateral ligament (LCL). The ACL is the most commonly injured ligament in the knee. The ACL's primary function is to stabilize the knee by resisting anterior and posterior dislocations of the tibia and femur, respectively. [9]

The ACL is approximately 38 mm in length and 10 mm in width [6], and it connects the femur to the tibia. More specifically, the ACL crosses the knee joint from the femur to the tibia in the lateral to medial and posterior to anterior directions. The ACL is connected to the femur

slightly posterior to the medial surface of the lateral condyle (LC), and to the tibia, anterior of the intercondylar region (ICR) [9] (Figure 1-1).

The ACL itself is composed of two ligamentous bundles, the posterolateral (PL) bundle and the anteromedial (AM) bundle. Both bundles help to stabilize the knee, but each bundle helps achieve stability differently because the bundles have different bone insertion sites. During knee extension, these differences cause the PL bundle to extend and tighten and the AM bundle to shorten, which in-turn leads to a natural twisting and untwisting of the ACL during knee extension and flexion [10].

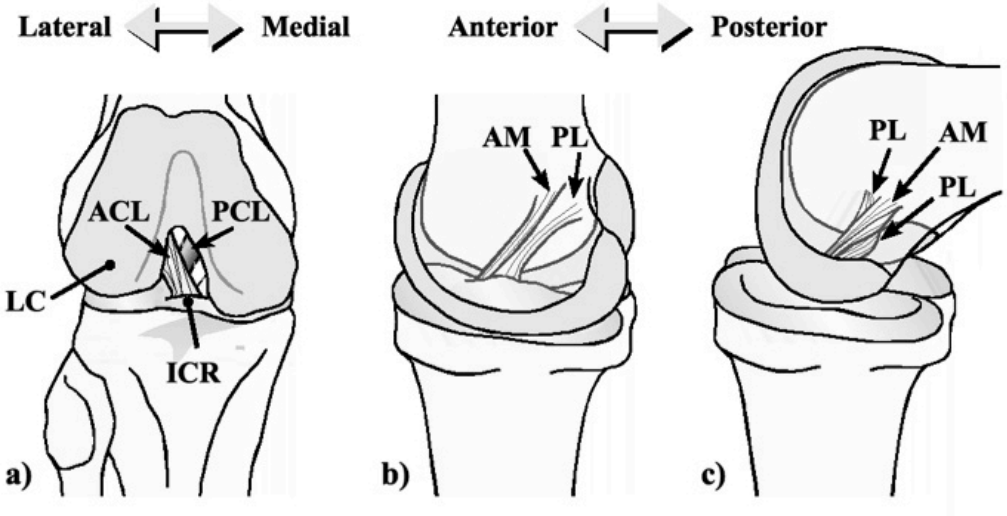

Figure 1-1: Anatomical drawing of the right knee from an anterior prospective (a), and medial perspective in extension (b) and flexion (c).

Composition and Structure

The ACL is a dense, highly organized connective tissue. The three primary components of the ACL are water, an organic matrix, and fibroblast cells. Water is the most abundant component of the ACL; it constitutes 65-70% of the ligament. Type I and type III collagens form 70-80% of the organic matrix dry weight with a ratio of 9:1 type I to type III collagen [11]. Type III collagen content increases during healing and development, but it is replaced by type I collagen during the tissue remodeling phase [12]. Elastin and proteoglycans are also found in the organic matrix and represent <5% and <1% of the dry weight, respectively [11].

All ligaments display a hierarchical organization (Figure 1-2) based on the collagenous matrix [12]. From a top-down approach, the entire ligament is surrounded by the epiligament.

The ligament is then broken down into bundled fascicles, which are separated by another connective tissue layer, the endoligament. The fascicles are composed of collagen fibrils and ligament cells are oriented along the long-axis of the ligament [13]; this is where the collagen fibrils interact with non-collagenous components and ligament cells. The collagen fascicles are organized in a crimped pattern reoccurring every 45-50 μm, and the collagen fibrils have a reoccurring crimp pattern every 67 nm [14]. Another level lower, the collagen fibrils are formed from microfibrils formed of collagen molecules, which are organized in a quarter-staggered arrangement. The formation of collagen fibrils is dependent on the interactions of fibril-forming collagens, fibril-associated collagens, non-collagenous molecules (such as proteoglycans like decorin), and enzymes that catalyze the formation of collagen crosslinks [15].

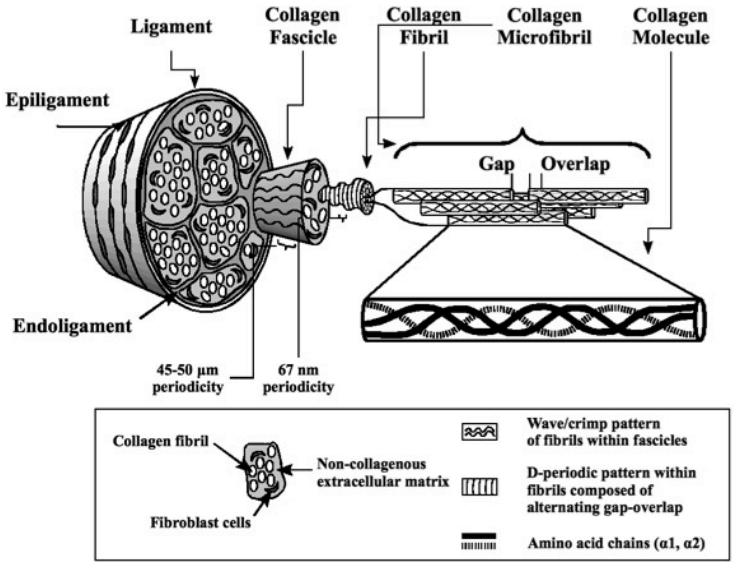

Figure 1-2: Hierarchical organization of collagen within ligaments such as the ACL.

Mechanical Behavior

Ligaments display a non-linear stress-strain relationship that consists of three regions: a toe region, linear region, and yield region (Figure 1-3). Initially, the crimped pattern of the collagen fibrils, previously described, straightens out resulting in deformation with low stresses; this process is associated with the toe region. The linear region is stiffer than the toe region and allows for recoverable deformation. The yield region is the start of permanent deformation, which tends to lead to failure. Damage is observed by a loss in stress, which indicates that collagen fibrils are breaking and can eventually lead to complete ligament rupture. Ligaments also display creep, stress-relaxation, hysteresis, and their mechanical properties are strain-rate dependent indicating that ligaments are viscoelastic. [16-19]

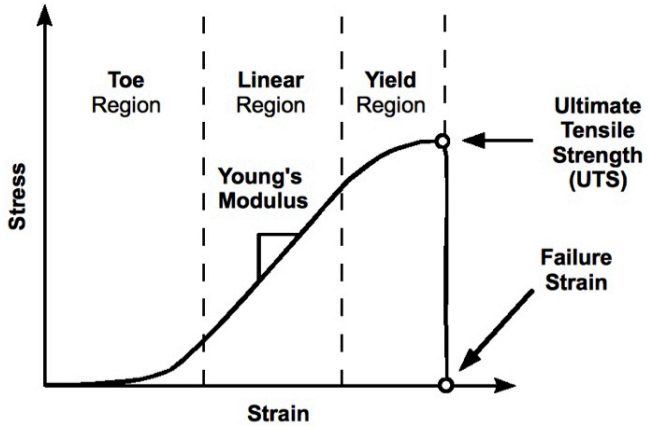

Figure 1-3: A representative stress strain curve for a ligament displaying the toe, linear, and yield regions.

1.3 ACL Replacements: Current Treatments

The ACL has poor healing capabilities due to a lack of vascularization and its being encased in synovial fluid [20]; therefore, following an ACL tear, surgery is typically needed in order to repair or replace the damaged tissue. There are two primary categories of ACL replacements: biological grafts and synthetic implants. Biological grafts include autografts, which are tissue grafts taken from the patient [2, 21-27], allografts, which are tissue grafts taken from a donor (including cadaveric tissue) [2, 21-27], and xenografts, which are tissue grafts taken from another species (i.e., porcine tissues) [21, 27, 28]. Synthetic implants include permanent replacements, augmentation devices, and scaffolds. Augmentation devices are designed to be implanted with biological grafts thereby protecting the graft in the early postoperative phase; whereas, scaffolds are designed to allow for new tissue growth [2, 21, 24-27, 29, 30]. Of all the options available for ACL replacement, none of them behave like the native human ACL long term [2, 21, 27].

Biological Grafts

Autografts

Autografts are typically considered the golden standard for current ACL replacements [2, 21-27]. Typical ACL autograft sites are the patellar tendon, which is the most common choice, semitendinosus-gracilis (hamstring) tendons, or quadriceps tendons. When harvesting tissue from patellar tendon, bony attachments from the patella and tibia are harvested as well in order to form a bone-patellar tendon-bone graft. This specific graft has yielded the best long-term results when compared to any other current replacement technique. Since autografts are tissue grafts taken from the patient, two surgeries are needed, one to harvest the tissue and another to replace the ACL. The tissue harvesting surgery may lead to donor site morbidity, which can further lead to pain, muscle atrophy, tendonitis and an increase in rehabilitation time. For example, patients with a hamstring tendon graft have been reported to suffer from hamstring weakness and saphenous nerve injury [31]. In addition to the second surgery, autografts are limited in supply, and they require revascularization, which prolongs the graft protection period [20]. On the other hand, autografts provide sufficient initial mechanical strength, and they promote cell proliferation and tissue growth. [2, 21, 24-27, 32-34]

Allografts

Allografts are tissue grafts from a donor, typically cadavers [2, 21-27], which eliminates the need for a second surgery. The popular harvesting sites for allografts are the patellar tendon, hamstring tendons, or the Achilles tendon. Allografts possess the same advantages as autografts; they provide sufficient initial mechanical strength and promote cell proliferation and tissue growth. In addition, allografts do not suffer from a lack of availability. The disadvantages associated with allografts are mostly centered on the patient's immune response and the necessity for sterilization, which typically alters the mechanical properties of the tissue. Allografts have the potential to transfer diseases, cause bacterial infections, and alter the healing process depending on the immunogenic response elicited by the replacement. Although allografts have some advantages over autografts, the disadvantages associated with allografts cause autografts to be the more popular replacement. [2, 21, 24-27, 33, 34]

Xenografts

Tissue grafts from other species (porcine and bovine being the classical examples) are called xenografts [21, 27, 28]. The advantages and disadvantages of this biological graft are the same as those associated with allografts, but the risk of disease transfer is elevated with transferring tissue between species Another disadvantage of xenografts is the need to pretreat them in order to remove the α-galactose (α-Gal) epitope, which is generally associated with hyperacute rejection, but pretreating xenografts makes them a viable option for ACL replacement [28]. [2, 24-26, 28, 35-37]

Stone *et al.* [28] showed that by removing the α-Gal epitope enzymatically, caused a necessary immunochemical modification of the tissue. In addition to removing the α-gal epitope, the cellular components were removed, the tissue was crosslinked in 0.1% glutaraldehyde (GA) for 12 hours and endcapping of glycine was done after chemical crosslinking in order to prevent any excess GA from causing additional reactions. After all these processes were done, the grafts were sterilized with electron beam radiation at 17.8 kGy in order to make the tissue an acceptable ACL implant. [2, 24, 25, 28, 38]

Synthetic Implants

As mentioned, there are three categories of synthetic implants: permanent prosthetics, augmentation devices, and scaffolds. Permanent replacement devices are intended to replace the ACL without having any soft tissue ingrowth. Augmentation devices are designed to provide initial protection while autografts, which are also implanted, mature and revascularize. Scaffolds are designed to help regrow native tissue without being accompanied by an autograft. [2, 19, 21, 24-27, 29, 30, 39].

Permanent ligament prosthesis allows patients to begin an aggressive rehabilitation routine since tissue maturation and revascularization is not desired [31]. One example of a permanent ligament prosthetic is Gore-Tex, which is a continuous low-density, high-strength polytetrafluoroethylene (PTFE) fiber looped and braided together [2, 21, 24-27, 29, 30]. Roolker *et al.* [20] performed a study that focused on the long-term results of the Gore-Tex prosthetic ligament as a permanent ligament replacement for the ACL and the PCL. There were 52 replacements total, 31 of which were ACL replacements and 21 were PCL replacements. It was reported that 19% of the replacements resulted in failure and the Gore-Tex replacement was removed, and only 17% of patients were satisfied with the long-term results. Failure of the Gore-Tex replacement resulted from rupture, sterile infection, or persistent instability. Subjectively, it appeared that patients with an ACL replacement suffered more pain based on the patients' complaints. The increase in pain was qualitatively supported by x-ray images that showed more degenerative changes in patients with the ACL replaced when compared to patients with a PCL replacement. In short, the overall properties for Gore-Tex prostheses tend to decrease over time, and they do not provide satisfactory long-term results. Permanent ligament prostheses, such as the Gore-Tex prosthetic ligament, tend to have superb short-term results, but poor long-term results [31], and are therefore recommended to only be used on knees that have had multiple operations in order to prolong total knee replacement or knee joint fusion since autografts tend to yield more satisfactory results [20].

There are many more augmentation devices on the market than there are permanent prostheses. Some augmentation devices that have been tried are carbon fiber and Dacron prosthetics, the Leeds-Keio ligament, and the Kennedy ligament augmentation device (LAD). Carbon fiber augmentation devices are typically associated with the migration of carbon wear particles into the joint and regional lymph nodes. Dacron, which is successful in vascular surgery

implants, is composed of loosely woven velour surrounding four tightly woven polyester strips. This particular design has been reported to reduce the extent of graft abrasion while allowing for tissue ingrowth; however, long-term failure was reported for 37.1% and 47.5% of patients in two different studies performed by Richmond *et al.* and Barrett *et al.* [40, 41], respectively. The Leeds-Keio ligament is made from polyester that is woven into porous tubes and is anchored to the femur and tibia with bone plugs. Studies have shown neoligamentous growth, meaning aligned fibrous tissue growth, and non-neoligamentous growth. As a result, the Leeds-Keio ligament is generally described as being a more permanent load-bearing device. The Kennedy LAD is made of diamond-braided polypropylene and was originally designed to reinforce the weak area of the patellar tendon graft, which is believed to be the pre-patellar tissue area. [2, 19, 21, 24-27, 29, 30, 39]

Synthetic implants tend to have positive short-term results, but they tend to fail overtime because they are unable to mimic the mechanical behavior of the ligament long-term. Some reasons for this are that the implants tend to experience creep or fatigue, which alters their mechanical properties over time, and implant abrasion on the sharp edges of the bone tunnels can result in tissue damage or debris collection in the joint, which can cause inflammation and pain [2, 19, 21, 24-27, 29, 30, 39]. Of all the synthetic implants available, none are a viable alternative to the autograft, especially the patellar tendon graft, which is used in 90% of all ACL reconstruction surgeries [31].

1.4 Ligament Tissue Engineering

Even autografts, the “golden standard” for ACL replacement, do not do a sufficient job at mimicking the native ligament function, structure, and mechanical properties long term. The biggest problem for current replacement options is the inability for the grafts (synthetic or biological) to replicate the stress-strain behavior of the native ACL, which makes the current replacements highly susceptible to creep and fatigue [2, 21, 24-27]. As a result, the concept of ligament tissue engineering was developed. The overall goal of ligament tissue engineering is to develop a viable scaffold that begins the regenerative process and eventually produces a fully living regenerated ligament that is appropriate for long-term use [42]. Ideally, a tissue engineered scaffold should be biocompatible, be able to support the loads in the knee, reproduce the stress-

strain curve of the target tissue, promote cell infiltration and new tissue growth, and degrade at a rate that prevents stress-shielding [2, 21, 24-27, 43-45]. Many scaffolds have already been developed, both natural and synthetic, in hopes of producing a practical alternative to the current treatment options. Most scaffolds that have been developed and those that are currently being researched are polymer-based scaffolds because the mechanical properties of polymers can usually be altered systematically.

Cell Sources

In order to accelerate the tissue regeneration process and encourage cell infiltration, scaffolds are typically seeded with cells before implantation. Ideally, an autologous cell source would be used in order to eliminate risk of rejection; however, the cell source would need to be attainable without a second surgery in order to prevent donor site morbidity. Two primary cell types that have been investigated for the purpose of ligament tissue engineering are fibroblasts and bone marrow stromal cells (BMSCs) [21, 27, 42, 46-48].

There are a variety of fibroblasts that may accelerate healing in the ACL including ACL, MCL, and skin fibroblasts [42]. Mature ACL fibroblasts have received a lot of attention for ACL regeneration because they respond to static and dynamic stimulation and to specific growth factors. However, a reliable ACL fibroblast source has not been found [21, 27]. In addition, Bellincampi *et al.* [47] showed that skin fibroblasts provide better results than ACL fibroblasts in intra-articular environments.

Alternatively to fibroblasts, BMSCs, which are multipotent stem cells, are also heavily investigated as a cell source for ligament tissue engineering. By definition, stem cells can self-renew through mitotic divisions and differentiate into more specialized cell lineages. BMSCs can be found in the bone marrow and easily acquired through a needle biopsy. The number of BMSCs in the body decreases with age, but BMSCs in adults have the same ability to proliferate and differentiate as a newborn's BMSCs. It has been shown that BMSCs, given proper culture conditions, can differentiate into a variety of phenotypes including osteoblasts, chondrocytes, adipocytes, and fibroblasts. Since a suitable source of BMSCs can be found in adults, it is not necessary to take cells from embryos making BMSCs free of ethical issues. [21, 27, 42, 46, 48]

Based on the ability of BMSCs to organize cells and collagen in vitro, it has been hypothesized that implants seeded with BMSCs would increase the biomechanical properties

when compared to implants without BMSCs [46, 48]. In order to test this hypothesis, studies have been performed that inflict a tendon defect in New Zealand rabbits, and an implant either with or without BMSCs was implanted into each rabbit. Biomechanical comparisons of the two implants were done over time, and the results showed that implants seeded with BMSCs had better biomechanical properties when compared to implants without BMSCs [46, 48].

Synthetic Resorbable Polymer-Based Scaffolds

Scaffolds composed of synthetic resorbable polymers can degrade over time (i.e. they are biodegradable), which differs from the techniques discussed in the ACL Replacements: Current Treatments section. To be consistent with ligament tissue engineering's main goal, a scaffold should eventually degrade and leave only the newly formed fully living tissue, which makes scaffold biodegradation necessary. Similar to the synthetic implants previously discussed, synthetic resorbable polymers can be modified to change their mechanical properties (strength, extensibility, degradation rate, biocompatibility, etc.) in order to tailor the material to specific needs. Also, synthetic resorbable scaffolds do not have risks associated with disease transfer, and their risks for long-term foreign body response and mechanical failure are significantly less as a result of their biocompatibility [25].

Common synthetic polymers that have been studied for tissue-engineered ligament scaffolds include polyglycolic acid (PGA) [49], poly-L-lactic acid (PLLA) [49], polylactide-co-glycolide (PLGA) [49, 50], polycaprolactone (PCL) [26], polyurethane urea (PUU) [51], polydioxanone (PDS) [52], polyethylene glycol (PEG)-based polymers [53], and poly desaminotyrosyl-tyrosine ethyl carbonate (poly DTE carbonate) [51]. In this section, structural design and the addition of other components such as proteins, peptides, and growth factors will be discussed. Several studies discuss twisting or braiding as a way to increase the mechanical strength of a scaffold, and some also mention that the incorporation of extracellular matrix (ECM) proteins, peptides, and growth factors can increase scaffold biocompatibility and bioactivity [4].

The L-C Ligament™ (Soft Tissue Regeneration, Inc.) is a three-dimensional (3D) braided, degradable, tissue-engineered scaffold composed of PLLA fibers, which was developed by Laurencin and colleagues [26]. Cells can be seeded onto the PLLA fibers and/or they can be coated with fibronectin in order to improve biocompatibility and bioactivity. The L-C Ligament

is composed of braided PLLA fibers; however, the braiding is not uniform throughout. The design of the L-C Ligament is broken into three zones: two outer bone-attachment zones and an intra-articular ligament zone. The two outer bone-attachment zones have a higher braiding angle and smaller pores than the intra-articular ligament zone. This design was chosen because it replicates the same bone-tendon-bone scheme that the patellar tendon autograft produces [26]. The mechanical properties of the L-C Ligament have been reported to be very similar to native ACL tissue. In addition to the mechanical results, a favorable degradation rate and levels of cell attachment and ECM production has been observed [49, 50, 54]. The pore sizes and pore interconnectivity throughout the scaffold are important characteristics in order to ensure a favorable distribution of nutrients, cell migration, new tissue development, and metabolic waste removal throughout the scaffold [25].

The L-C Ligament was tested in vivo in adult New Zealand white rabbits in order to study tissue regeneration. There were two groups of scaffolds tested: a control group with no cells prior to implantation, and a group with cells seeded on the scaffold prior to implantation. The scaffolds were mechanically and histologically tested at three different time points: before implantation, 4 weeks after implantation, and 12 weeks after implantation. Mechanical testing showed that before implantation the scaffolds had properties very similar to the native rabbit ACL; refer to Table 1-1 for the study's results [54]. As time increased, the mechanical properties decreased for both groups of scaffolds; this trend was expected due to scaffold degradation over time. Even with scaffold degradation, the strength retention of the cell-seeded scaffolds after 12 weeks of implantation was better than an autograft, based on a study by Ballock *et al.* [55]. Ballock *et al.* [55] showed that after 6 weeks and 30 weeks of implantation, autografts exhibited only 6% and 15% strength retention, respectively. The cell-seeded scaffolds in the L-C Ligament study showed that after 4 weeks there was a 76% strength retention and a 30% strength retention at 12 weeks [54]. From the histological analysis after 12 weeks, both scaffold groups showed signs of tissue ingrowth and vascularization. The unseeded scaffolds showed a slightly larger fibrous capsule than the seeded scaffolds. The quality of neoligament production qualitatively varied between scaffold type; for the unseeded scaffolds the collagenous matrix was unaligned and had a higher proportion of phagocytic cells when compared to the seeded scaffolds. The cell-seeded scaffolds produced a more organized and denser collagenous matrix with signs of neovascularization [25, 54].

Table 1-1: Mechanical properties of scaffolds before and after implantation [54].

		Maximum Load, N	Extension at Maximum Load, mm	Elastic Modulus, MPa
Native Rabbit ACL		314.3±68	3.9±1.8	181.2±132.2
Before Implantation		332.2±19.6	4.7±0.24	354.4±68.5
4 weeks after Implantation	Unseeded scaffolds	209±3.5	3.5±0.59	103.0±53.9
	Seeded scaffolds	239.0±43	4.1±1.2	108.4±27.7
12 weeks after Implantation	Unseeded Scaffolds	35	11.4	3.0
	Seeded Scaffolds	92.8±18.4	3.9±2.0	53.8±15.1

Kimura *et al.* [56] reported on an ACL scaffold design with growth factor incorporation. This particular ACL scaffold was composed of two plain-woven PLLA braids coated with fibronectin, gelatin hydrogel with basic fibroblast growth factor (bFGF) incorporated in it, and a collagen membrane reinforced with PLLA microspheres. The gelatin supplemented with bFGF was placed between the PLLA braids, and the resulting structure was then wrapped with the collagen membrane that was reinforced with PLLA microspheres. This scaffold was tested *in vivo* in rabbits and it was found that exogenous bFGF enhanced overall tissue regeneration by stimulating cellular integration and ECM production [56].

Another recent ACL scaffold design is the braid-twist scaffold [57, 58]. As shown in the previously mentioned studies, braiding tends to increase the mechanical properties of the scaffold; more specifically, braids have the ability to bear and transfer axial loads, provide shear resistance, mechanical reinforcement, and extension under stress [59]. Twisting, on the other hand, alters the abrasion resistance, strength, and flexibility by adjusting the direction and the degree of twisting. Strength and abrasion resistance of yarns, formed by twisting fibers, increase as the amount of twisting increases until the yarn is wound too tightly. The yarn may be wound too tightly if the fibers become nearly perpendicular to the yarn's long axis, which decreases the strength and abrasion resistance [60]. Freeman *et al.* [58] developed a mechanically stable scaffold, which produced a larger load capacity and a greater degree of extensibility, by optimizing the combination of the braiding and twisting techniques. This scaffold's mechanical properties could be altered by changing either the braiding or twisting angles [58].

The braid-twist scaffold developed by Freeman *et al.* [58] mimics ligament organization. PLLA fibers were used to form the 3D scaffold. First, 9 groups of 6 PLLA fibers each were twisted to form 9 bundles. Three bundles were then twisted together to form 1 yarn, making a

total of 3 yarns. The twisting of the bundles and the yarns were done in a counter-clockwise direction. After 3 yarns were produced, they were braided together in order to make the braid-twist scaffold, refer to Figure 1-4 for a schematic. The twisting not only improved the mechanical properties of the scaffold, it also introduced a crimped pattern. The crimped pattern helps mimic the native ACL structure by creating toe and linear region in the stress-strain curve. Various twisting and braiding angles were tested mechanically in order to determine the optimal scaffold for ACL regeneration. The optimal design for the scaffold in order to produce an ultimate tensile strength (UTS) and Young's modulus higher than the human ACL and a toe region length comparable to the human ACL occurred when the 9 groups of fibers were twisted to 60°, then those 9 fiber bundles were twisted to 72° to form the yarns, and the braiding angle was ~69° (4 stitches per inch). Higher UTS and modulus values were chosen for the optimal properties to allow the scaffold to bear the necessary load during degradation [58].

The braid-twist scaffolds developed by Freeman *et al*. [58] mimic the human ACL's mechanical behavior during stress-relaxation. More specifically, there is a rapid decrease in stress until a plateau region is reached [57, 61, 62]. The final stress during the stress-relaxation tests was higher for the braid-twist scaffolds than for the human ACL. Therefore, a polyethylene glycol diacrylate (PEGDA) hydrogel was added to the scaffold in order to help reduce the final stress of the scaffold after stress-relaxation. It was found that as the amount of hydrogel added to the scaffold increased, the final stress decreased. Scaffolds with some hydrogel added, but not enough to encase the entire scaffold, yielded final stresses similar to human ACL [53].

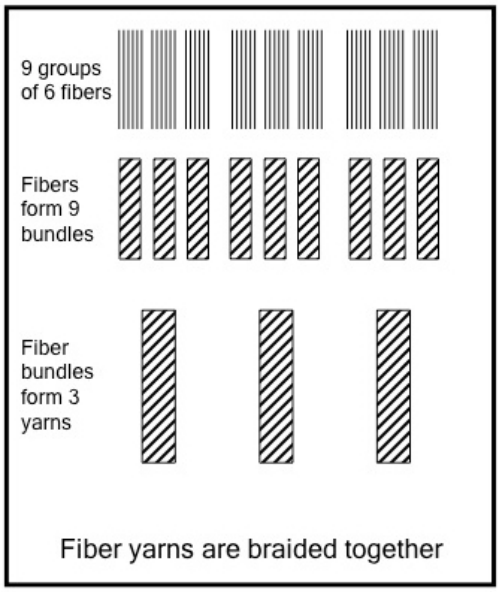

Figure 1-4: Schematic of the braid-twist scaffolds. Modified and included with permission from Freeman *et al.* [58] © Elsevier Science.

Natural Polymer-Based Scaffolds

Natural polymers are heavily studied in ligament tissue engineering because it is fairly easy to alter their degradation rate and mechanical properties by crosslinking the polymers. However, natural polymers are typically more expensive, have to be sterilized, which can alter their mechanical properties, and they carry a risk of disease transmission [2, 24, 25, 63-65]. There are a variety of natural polymers studied some of which include: collagen, silk, chitosan-based scaffolds, porcine small intestine submucosa, semitendinosus tendon, fibronectin/fibrinogen fibers, and enamel matrix [2, 24-27, 44, 45, 66-69].

Collagen type I and silk are the two primary natural polymers studied. Silk is currently used in a variety of medical applications, such as sutures [70]. Silk is composed of fibroin and sericin, but only the fibroin is used in medical applications because sericin is a potential allergen that may cause a foreign body response [27, 43, 70]. It has been shown that silk has excellent mechanical properties that can be easily tailored to match the properties of the native ACL, therefore it has been heavily studied as a potential material for ACL scaffolds [2, 24, 25, 27, 43]. However, for the purposes of this report, only studies pertaining to collagen will be discussed in this section.

Collagens are heavily studied for ligament tissue engineering because next to water, collagens are the most abundant constituents of native ligaments [11]. There are over 20 different

types of collagens, but types I, II, and III are the most abundant types, with type I being the most prevalent [15]. Type I collagen is composed of three amino acid chains that are each twisted in a left-handed helix. These three chains are then twisted around each other in order to form a right-handed triple helix. Two of the three chains, the $\alpha 1$ chains, are identical in sequence and length, while the other chain, the $\alpha 2$ chain, is slightly shorter in length with a slightly different amino acid sequence. The amino acids in these chains have a unique repeating glycine-X-Y sequence, where X and Y are occasionally proline and hydroxyproline, respectively [15]. Upon secretion into the extracellular space, bulky extensions called propeptides are enzymatically cleaved, resulting in rod-shaped triple helical molecules (295 nm in length and 1.5 nm in diameter); these collagen type I molecules are capable of self-assembling into highly ordered structures called fibrils [15, 71].

Fibril-forming collagens, like type I collagen, are commonly used in tissue engineering because their ability to self-assemble *in vivo* can be replicated to some extent under certain *in vitro* conditions [71, 72]. Therefore, type I collagen can be reconstituted and then crosslinked, chemically or physically, in order to alter its mechanical properties and degradation rate. Collagen scaffolds have been made in the form of gels, foams, films, and fibers. Collagen gels are not feasible for ligament regeneration because cells tend to sit on top of the gel, and they do not penetrate into the scaffold [25, 68]. From a mechanical standpoint, bundles of fibers yield the best results; however, bundles of collagen fibers are susceptible to creep, fatigue, and abrasive wear in long-term replacements [24, 25]. Studies have shown that neither the collagen gel or fiber bundles alone produce sufficient mechanical properties for ACL replacement, but with the aid of crosslinking and scaffold design considerations, the mechanical properties could be made sufficient [68].

There are two categories of crosslinking methods: chemical and physical. Chemical crosslinking forms bonds between different amino acids as a result of the chemical environment, whereas physical crosslinks cause bonds to form as a result of the physical environment (e.g. radiation exposure). For chemical crosslinking, two popular choices are glutaraldehyde (GA) [67, 73, 74] and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) [44, 66, 73]. Ultraviolet (UV) radiation [44, 66] and dehydrothermal (DHT) [44, 67, 74] are two popular physical methods. It is not uncommon to follow a DHT crosslinking method by a cyanamide crosslinking method in order to further increase mechanical properties [67, 74]. Physical crosslinking

methods typically do not introduce toxins or chemical residues, but the crosslinking generally is not sufficient for tissue engineering applications. On the other hand, chemical crosslinks have the ability to increase the mechanical properties more than physical methods, but they may have cytotoxic byproducts or residues [73].

When comparing GA to EDC, GA is believed to induce the higher crosslinking density. However, GA does introduce cytotoxic chemical residues, and its reaction mechanisms are not fully understood. It is known that GA is a "bridge-forming" crosslinker, which means that collagen molecules are linked by a bridging molecule derived from GA [73]. On the other hand, EDC is a "zero-length" crosslinker, meaning that the collagen molecules are directly linked to one another [66, 73]. After crosslinking with EDC, the remainder of the EDC can be rinsed free, and the only byproduct produced is a water-soluble urea; therefore, no toxins are introduced. It has also been shown that EDC promotes tissue in-growth, and therefore, it is a viable option for tissue-engineered scaffolds [66].

Studies have been done using type I collagen fiber bundles as ACL scaffolds. Results showed that these scaffolds had excellent cell attachment, proliferation, production of ECM, and biocompatibility, but the scaffolds did not posses the necessary mechanical strength. The scaffolds were arranged in parallel bundles, and it was determined that this design was a poor choice for ACL scaffolds because the scaffolds lacked structural reinforcement and were very susceptible to fatigue. Synthetic crosslinking or a different scaffold design, such as braiding and/or twisting, would have the potential to improve the mechanical properties of the scaffolds [24, 25, 43, 75].

1.5 Conclusions

ACL injuries that require surgery account for approximately 50,000 people per year in the United States. Among all the available replacement methods, the autograft is considered the "golden standard," but due to donor site morbidity, limited supply, and long-term problems, new techniques such as tissue engineering are being researched. From a ligament tissue engineering standpoint, a scaffold can help start the regenerative process, and eventually lead to a new, fully developed, living ligament. Both natural and resorbable synthetic polymer-based scaffolds are being explored as an alternative to current ACL replacement options; these scaffolds must be

biodegradable, biocompatible, support the loads of the knee, replicate the stress-strain curve of the native ACL, and promote cell infiltration and new tissue growth. Researchers are using various ways to try and achieve a viable long-term option including crosslinking the polymers and seeding them with cells such as fibroblasts or BMSCs. Scaffold design is also another key concept in developing a scaffold that will mimic the structure of the ACL. It has been shown that twisting and braiding tend to increase the mechanical properties of materials, making this a favorable design choice. Research in the ligament tissue engineering field continues with the hopes that it will eventually lead to a scaffold that can help regenerate the ACL, and that the new tissue will have all the same characteristics of the native tissue both mechanically and biologically.

1.6 Project Goals

The overall project goal was to develop a mechanically sufficient three-dimensional collagen scaffold that promotes cell growth for ACL replacement. In order to develop collagen scaffolds, a study was done on individual collagen fibers where UV and EDC were investigated as two crosslinking methods (Chapter 2). It was hypothesized that collagen fibers crosslinked with EDC would have mechanical properties that matched the human ACL better than UV crosslinked fibers. After choosing the optimal crosslinking method, the collagen scaffolds could be developed (Chapter 3). The addition of a hydrogel (gelatin) was investigated and it was hypothesized that the gelatin would increase the viscoelastic behavior of the collagen scaffolds, but that the collagen scaffolds without gelatin would have mechanical properties similar to the human ACL. It was also hypothesized that cells would proliferate on the collagen scaffolds over a 21-day period. Overall, it was hypothesized that crosslinked collagen scaffolds developed in this study would have potential to serve as a primary scaffolding material for ACL replacement.

1.7 References

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2.0 Chapter 2
Crosslinking of Reconstituted Collagen Type I Fibers

2.1 Abstract

Collagen type I has been investigated as the primary material in anterior cruciate ligament (ACL) scaffolds. The purpose of this study was to evaluate the effects of the crosslinking method, crosslinking time, fiber diameter, and fiber formation time on the hydrated mechanical properties of reconstituted collagen type I fibers. Ultra-violet (UV) radiation and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) (6, 10, or 25 mM) were investigated as the crosslinking methods. The ultimate tensile strength (UTS) and the Young's modulus were significantly higher for EDC-crosslinked fibers. The fiber diameter was varied by changing the diameter of the extrusion tubing (0.28 mm or 1 mm inner diameter). Smaller diameter fibers had significantly higher UTS and Young's modulus values compared to the larger diameter fibers, but the smaller diameter fibers were more fragile and weaker than the larger diameter fibers when considering structural properties such as the maximum load held by the fibers. A decrease in fiber formation time from 24 hours to 4 hours did not significantly affect the UTS or Young's modulus of the collagen fibers. The mechanical properties of collagen type I fibers crosslinked with 25 mM EDC for 4 hours with a decreased fiber formation time yielded a UTS within the range of the native human ACL and a Young's modulus that exceeded the range of the human ACL. These results suggest that collagen fibers made using the optimal protocol developed in this study have the potential to serve as the primary component of scaffolds for ACL replacement.

2.2 Introduction

ACL injuries are a growing clinical problem. There are between 100,000 and 250,000 annual anterior cruciate ligament (ACL) injuries per year in the United States alone, and roughly 50,000 of them result in ACL reconstructive surgery largely due to the ACL's poor healing capabilities [1-4]. Untreated ruptures result in pain, swelling, instability, and osteoarthritis [5]. However, current treatments are still in need of improvement. Autografts and allografts are popular choices for ACL reconstruction because they are both mechanically sufficient options. However, autografts can result in donor site morbidity, and allografts present risks of disease transfer and graft rejection [2, 5-13]. Synthetic options are also currently used for ACL reconstruction, but most of the synthetic options are only conditionally approved by the Food and Drug Administration (FDA). Permanent synthetic replacements tend to rupture or elongate over time, while degradable scaffolds, which are designed to slowly transfer the mechanical load to newly formed tissue, tend to cause stress shielding [2, 6, 9-11, 13-18]. As a result of the issues associated with the current techniques, ACL regeneration has been heavily studied in the ligament tissue engineering field.

Collagen type I is a natural polymer that has been heavily researched as the primary material for tissue-engineered ACL scaffolds because, aside from water, it is the primary component of ligaments. Throughout the rest of this chapter, use of the singular form "collagen" identifies collagen type I. The mechanical properties and degradation rate of collagen can be altered via a variety of crosslinking methods, which makes collagen an even more favorable option [2, 9, 10, 19-21]. Collagen scaffolds for ACL replacement have been used in the form of gels, foams, films, and fibers. However, collagen fibers yield the best results mechanically under tension [9, 10]. One example of a collagen-fiber based scaffold was described by Caruso *et al.* [5]. In their study, collagen scaffolds, consisting of 50 reconstituted bovine dermal collagen fibers aligned in parallel, were crosslinked using either ultra-violet (UV) radiation or 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC).

Crosslinking is a primary means to alter the mechanical properties of collagen; UV radiation, EDC, and glutaraldehyde (GA) are three commonly used crosslinking methods. Physical crosslinking methods, such as UV radiation, do not introduce toxins or chemical

residues, while chemical crosslinking methods typically do introduce toxins, such as GA [22]. UV radiation causes free radicals to form in the aromatic residues of the collagen molecules (i.e. phenylalanine and tyrosine); these free radicals are in turn thought to form covalent crosslinks [23]. GA can react with free amino groups (i.e. lysine, hydroxylysine, arginine, asparagine, glutamine) forming covalent "bridges" within and between collagen molecules [23, 24]. EDC is a "zero-length" chemical crosslinker that results in the formation of an amide bond between a carboxylic acid and a primary amine. Collagen contains some amino acids with carboxylic acid side chains and others with amine side chains; therefore, EDC can mediate the formation of direct or "zero-length" crosslinks between these amino acid pairs. As a result, EDC does not become incorporated into the collagen protein. Furthermore, the EDC does not introduce toxic byproducts; after the formation of the "zero-length" crosslinks, the only byproduct is a water-soluble urea derivative [5, 22]. Typically, chemical crosslinking methods increase mechanical properties more effectively than physical crosslinking methods [22]. There have been various studies done on comparisons between different crosslinking methods. Cornwell *et al.* [25] studied differences between dehydrothermal (DHT), UV, and EDC crosslinking methods on extruded collagen threads derived from rat tail tendon. Another study by Dunn *et al.* [18] investigated differences in the mechanical properties of extruded collagen fibers, derived from bovine corium (dermis), that were crosslinked with GA vapor or DHT followed with cyanamide vapor.

In the present study, UV and EDC were chosen as the two crosslinking methods to investigate in order to compare a physical and a chemical method. UV was chosen as the physical crosslinking method because it is a commonly used choice seen in other studies [5, 25-27]. EDC was chosen because it does not introduce toxins, which is greatly desirable for a scaffolding material for ACL reconstruction [5, 22]. The goal of the present study was to determine whether the use of UV radiation or EDC would increase the mechanical properties of collagen fibers to values similar to the native human ACL.

2.3 Materials and Methods

Reconstituted Collagen Fibers

Acid-soluble type I collagen was isolated from the rat tail tendons of Sprague-Dawley rats using procedures similar to those described by Pins *et al.* [25]. Collagen was dissolved in 10 mM hydrochloric acid (HCl, pH 2.0) for at least 4 hours at room temperature. The solution was then centrifuged at 30,000 x g at 4°C for 30 minutes. The supernatant was then sequentially filtered through 0.8μm, 0.65μm, and 0.45μm filters (Millipore). Sodium chloride (NaCl) was then added to the filtered solution for a 0.7 M solution, and then the precipitate was collected by a 1 hour, 30,000 x g centrifugation at 4°C. The precipitate was redissolved in 10 mM HCl (pH 2.0). The acid-soluble collagen was then purified with dialysis against 20 mM sodium phosphate dibasic (pH 7.4) for at most 16 hours at room temperature, after which dialysis was continued in a fresh 20 mM sodium phosphate dibasic (pH 7.4) bath at 4°C overnight. The collagen solution was then centrifuged at 30,000 x g for 1 hour at 4°C. The collagen pellets were dissolved in 10 mM HCl (pH 2.0) for 24 hours followed by a final dialysis against HCl (pH 2.0) for at most 16 hours at room temperature. The collagen concentration was then checked by placing known volumes of collagen in a 110°C oven until no further changes in mass occurred. The collagen solution was diluted as needed by adding more HCl (pH 2.0). Once the desired collagen concentration (10 mg/mL) was obtained, the solution was sterilized with chloroform (10% volume) at 4°C overnight. After sterilization, the collagen solution was placed into sterile 5 mL syringes and stored at 4°C until needed.

Self-assembled collagen fibers were made by extruding collagen and a fiber formation buffer (FFB) (135 mM sodium chloride, 30 mM Tris Base (Tris (hydroxymethyl) aminomethane), and 30 mM sodium phosphate dibasic, pH 7.4), simultaneously, through polytetrafluoroethylene (PTFE) tubing at a rate of 0.7 mL/min into a bath of FFB at 30°C. The fibers remained in the FFB bath for either 24 or 4 hours and were then placed in deionized water for 1 hour. The fibers were then taken out of the water and dried under their own weight. Kato *et al.* [29] performed various studies where the fibers were only allowed to soak in the FFB bath for 45 minutes; other researchers such as Dunn *et al.* [18] have adopted this fiber formation protocol. Therefore, by reducing the FFB bath from 24 hours to 4 hours, it is believed that proper fiber formation still occurs.

The effect of fiber diameter on strength was investigated by changing the PTFE extrusion tubing. PTFE tubing with either a 1 mm (18 gauge) or 0.28 mm (30 gage) inner diameter was used. The 1 mm inner diameter tubing was used during the optimization of the crosslinking method.

Crosslinking

Collagen fibers were prepared as previously described. Fifteen groups were established with sample sizes ranging from 9 to 11 fibers per group. One group of fibers was left uncrosslinked to act as a negative control (i.e., without synthetic crosslinking). All remaining groups of fibers were synthetically crosslinked with either UV or EDC, while varying different crosslinking parameters. UV crosslinking involved placing the fibers underneath a 254 nm UV lamp at a separation distance of 11.75 cm for different lengths of time (see Table 2-1 for the different UV exposure times). Aluminum foil was used to provide a reflective surface underneath the fibers. The EDC crosslinking protocol used was slightly modified from the EDC crosslinking protocol used by Caruso *et al.* [5]. EDC crosslinking involved immersing the fibers in various concentrations of EDC dissolved in a 90% acetone:10% water solution at room temperature for different lengths of time (see Table 2-1 for the different EDC concentrations and crosslinking times). After EDC crosslinking was complete, the fibers were then rinsed in 90% acetone:10% water for 8 hours followed by a 4-hour rinse in 50% acetone:50% water. The fibers were then rinsed in 0.1 M sodium phosphate (pH 7.4) for 2 hours and then rinsed in deionized water for 24 hours. Following this series of rinses, the fibers were dried at room temperature. Two additional factors were evaluated, namely, the inner diameter of the PTFE extrusion tubing (mentioned previously) and the time allowed for fiber formation (24 hours versus 4 hours).

Table 2-1: Experimental design for the evaluation of UV and EDC crosslinking parameters, fiber extrusion diameter, and fiber formation time. Asterisks (*) denote conditions that were deemed optimal before moving forward to evaluate additional parameters/variables (i.e., UV versus EDC, EDC concentration, EDC crosslinking time, fiber extrusion diameter, and formation time). The selection of 8 hours for the optimization of the EDC concentration was based upon unpublished results by Cornwell *et al.* indicating a plateau of the UTS of their extruded collagen fibers after 4 hours of reaction with EDC [25].

Group #	Independent Variable	Crosslinking Method	PTFE Tubing, Inner Diameter	Fiber Formation Time
1	Negative Control	Uncrosslinked	1 mm*	24 hrs
2	UV Exposure Time	UV 15 min		
3		UV 30 min		
4		UV 1 hr		
5		UV 2 hrs		
6		UV 4 hrs		
7		UV 8 hrs		
8	EDC Concentration	EDC 6 mM, 8 hrs		
9		EDC 10 mM, 8 hrs		
10		EDC 25 mM*, 8 hrs		
11	EDC Crosslinking Time	EDC 25 mM, 1 hr		
12		EDC 25 mM, 2 hr		
13		EDC 25 mM, 4 hr*		
14	Fiber Extrusion Diameter	EDC 25 mM, 4 hr	0.28 mm	
15	Formation Time	EDC 25 mM, 4 hr	1 mm	4 hrs*

Mechanical Testing

Fibers were mounted on vellum paper frames with a 20 mm gage length prior to mechanical testing. Before testing, dry and hydrated fiber diameter measurements were taken using a Leica DM IL microscope (Leica Microsystems). Fiber cross-sectional areas were calculated using the fiber diameters by assuming a circular cross-section for the fibers. Hydration was accomplished by soaking the fibers in phosphate-buffered saline (PBS, pH 7.4) for 30 minutes. Uniaxial tensile tests were conducted on the fibers using a Tytron 250 Microforce Testing System (MTS Systems Corporation). Each fiber was mounted in custom-designed testing grips and immersed in a room temperature PBS bath. The sides of the vellum paper frames were cut before any tensile load was added. A 5 mN preload was obtained at a strain rate of 10%/minute. Once the preload was reached, the strain rate was increased to 10%/second and pulled until failure occured. Force-displacement data was converted to stress-strain data for determinations of ultimate tensile strength (UTS), Young's modulus, and strain at failure.

Statistical Analysis

Grubbs' test for outliers was used to detect any potential statistical outliers within each individual group. One-way analysis of variance (ANOVA) was done followed by a Tukey test to determine any significant differences between test groups. P-values < 0.05 were considered to be significant.

2.4 Results

All fibers that were crosslinked in this study had significantly higher UTS and Young's modulus values than the negative control group (group 1). However, the negative control group had a significantly higher failure strain than any of the crosslinked groups (groups 2-15).

UV Crosslinked Fibers

Fibers crosslinked with UV for either 4 hours or 8 hours had higher UTS values compared to any of the other UV groups (13.4 ± 4.9 MPa and 13.4 ± 4.5 MPa, respectively), but they were only significantly higher than the 15-minute group. The UV 8 hour group also had the highest Young's modulus (160 ± 48 MPa), which was significantly higher compared to any other UV group. Although the UV 8 hour group had a significantly higher modulus than the UV 4 hour group, the UTS appears to be decreasing after 4 hours, which could indicate collagen denaturation.

EDC Concentration Test

Three different EDC concentrations were tested: 6 mM, 10 mM, and 25 mM. All fibers were crosslinked in 90% acetone:10% water for 8 hours. There were no significant differences between the three groups for any of the three properties that were analyzed in this study (UTS, Young's modulus, failure strain). Although not statistically significant, there was a trend that showed that increasing the EDC concentration increased the UTS and Young's modulus. Figure 2-1 and Figure 2-2 show the UTS and Young's modulus results for the various EDC concentrations, respectively. Therefore, 25 mM EDC was chosen as the optimal EDC concentration.

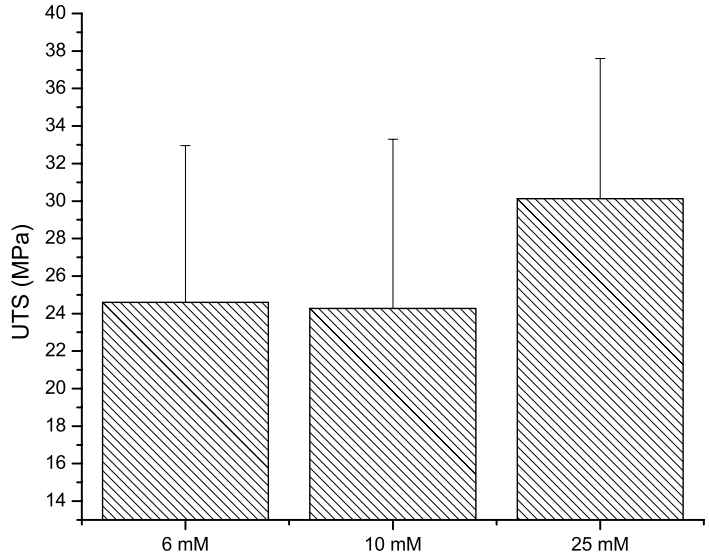

Figure 2-1: UTS results for fibers crosslinked with various EDC concentrations for 8 hours. A 24 hour formation time and 1 mm inner diameter extrusion tubing was used. No significant differences between the concentrations were observed.

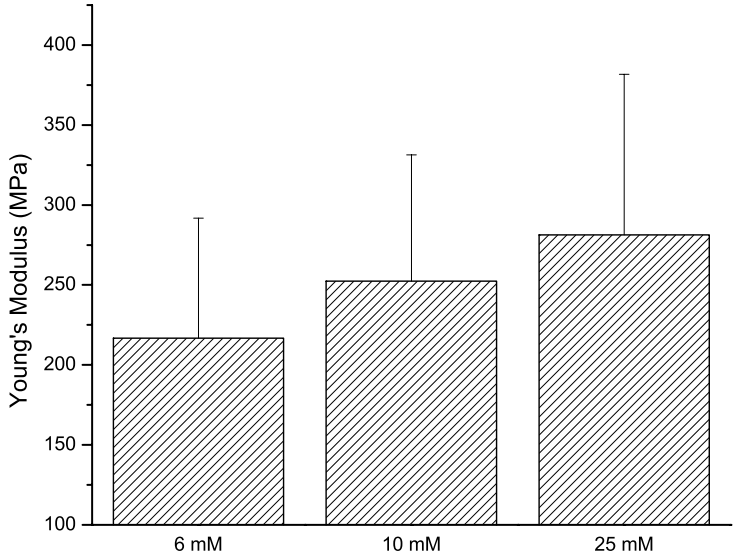

Figure 2-2: Young's modulus results for fibers crosslinked with various EDC concentrations for 8 hours. A 24 hour formation time and 1 mm inner diameter extrusion tubing was used. No significant differences between the concentrations were observed.

EDC Crosslinked Fibers

Groups 11-13 were crosslinked with 25 mM EDC, which was chosen based on the EDC concentration tests, but the crosslinking time was varied (1 hour, 2 hours, and 4 hours). Group 10

was also crosslinked with 25 mM EDC for 8 hours. Figure 2-3 and Figure 2-4 show the UTS and Young's modulus results for fibers crosslinked with 25 mM EDC for various times, respectively. Only the 4-hour crosslinking time yielded significantly higher UTS and Young's modulus values (41.4 ± 14 MPa and 361 ± 60 MPa, respectively) than the 1 hour and 2 hour groups. Although the 4-hour group did not have significantly higher results than the 8-hour group, the 8-hour group was not significantly different than the 1 or 2 hour group. Therefore, 4 hours was chosen as the optimal crosslinking time when crosslinking with 25 mM EDC in 90% acetone:10% water.

When comparing the optimal EDC group, 25 mM EDC crosslinked in 90% acetone:10% water for 4 hours, to the optimal UV groups, 4 hours and 8 hours, the EDC group displayed a significantly higher UTS (UV 4 hours: 13.4 ± 4.9 MPa, UV 8 hours: 13.4 ± 4.5 MPa, EDC: 41.4 ± 14 MPa) and Young's modulus (UV 4 hours: 118 ± 33 MPa, UV 8 hours: 160 ± 48 MPa, EDC: 361 ± 60 MPa). Therefore, 25 mM EDC for 4 hours in 90% acetone:10% water was selected as the optimal crosslinking method.

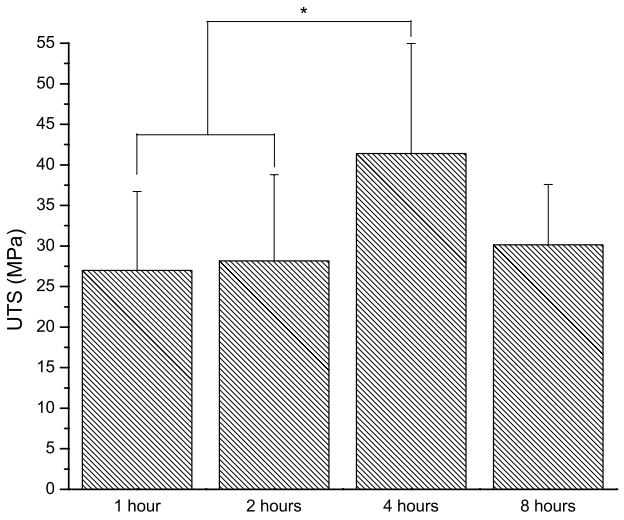

Figure 2-3: UTS results for fibers crosslinked with 25 mM EDC. A 24 hour formation time and 1 mm inner diameter extrusion tubing was used. A * denotes a significant difference between different crosslinking times ($p<0.05$).

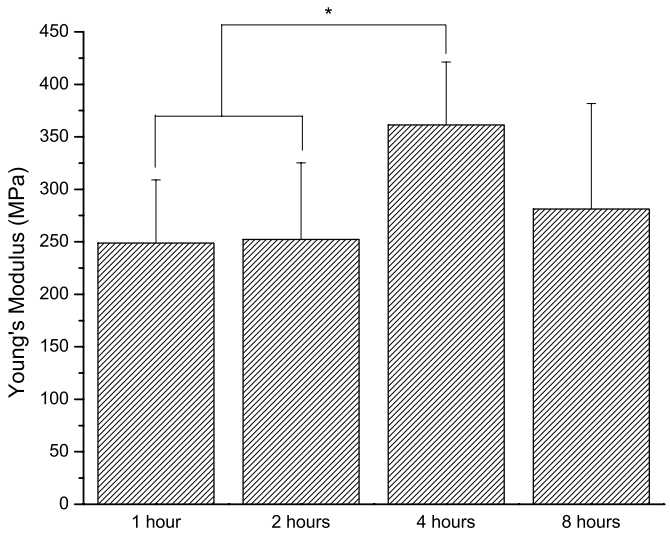

Figure 2-4: Young's modulus results for fibers crosslinked with 25 mM EDC. A 24 hour formation time and 1 mm inner diameter extrusion tubing was used. A * denotes a significant difference between different crosslinking times ($p<0.05$).

Fiber Diameter Tests

Once the optimal crosslinking method was chosen, the fiber diameter was varied. Fibers formed with 1 mm and 0.28 mm inner diameter extrusion tubing were compared. The smaller diameter group (23-46 μm wet diameters) had significantly higher UTS values (62.3 ± 21 MPa) compared to the larger diameter group (69-135 μm; 41.4 ± 14 MPa). The smaller diameter group also had a significantly higher Young's modulus than the larger diameter group (587 ± 123 MPa versus 361 ± 60 MPa, respectively).

The increased UTS and modulus of the smaller diameter group were likely caused by two mechanisms. First, it is plausible that the smaller diameter fibers had greater fibril alignment due to the greater confinement of the collagen solution during the fiber extrusion process. A greater alignment of collagen fibrils would be expected to increase mechanical properties, as described by Pins *et al.* [30] in the context of pre-stretching fibers. Secondly, it is also possible that the crosslinking solution penetrated the entire thickness of the smaller fibers easier than the larger fibers. If this is true, the smaller fibers would be more completely crosslinked and have more crosslinks per unit area than the larger fibers.

Although the smaller diameter group yielded higher mechanical properties than the larger diameter group, the larger diameter extrusion tubing (1 mm inner diameter) was chosen because

the smaller diameter fibers had UTS values significantly higher than that of the native human ACL (~38 MPa) [18, 31, 32]. Furthermore, the smaller diameter fibers were more fragile in terms of breaking loads, and thus, their fragility would have presented handling difficulties for our intended applications.

FFB Tests

Once the crosslinking method and extrusion tubing diameter were selected, the fiber formation process was altered. Groups 1-14 were formed by allowing the fibers to soak in the 30°C FFB bath for 24 hours before being rinsed with deionized water, but fibers in group 15 were only allowed to soak in the 30°C FFB bath for 4 hours before being rinsed with deionized water. The fibers were then crosslinked with 25 mM EDC for 4 hours in 90% acetone:10 % water. Mechanical tests showed that although the decrease in fiber formation time decreased the mechanical properties, this decrease was not statistically significant. The UTS decreased from 41.4 ± 14 MPa to 33.3 ± 14 MPa and the Young's modulus decreased from 361 ± 60 MPa to 312 ± 106 MPa when the fiber formation time was decreased from 24 hours to 4 hours. The decrease in mechanical properties, as a result of a reduced fiber formation time, was not significant. The UTS of the fibers is still within the range of the human ACL after the formation time was decreased from 24 to 4 hours.

2.5 Discussion

The primary goal of this study was to determine whether EDC or UV was a more appropriate crosslinking method for reconstituted collagen fibers for ACL reconstructive scaffolds. About 75% of the dry weight of a native human ACL is composed of collagens [33, 34], with 90% of these collagens being type I collagen [33]; thus type I collagen is an adequate polymer of choice for ACL scaffolds. The collagen type I fibers tested in this study had wet diameters ranging between 69 and 135 μm. The human ACL is composed of fascicles with diameters ranging from 20 to 400 μm; these fascicles are in turn composed of collagen fibrils [33]. Therefore, the fibers tested in this study are analogous in size to fascicles found in the human ACL.

It is necessary for extruded collagen fibers (also called reconstituted or self-assembled collagen fibers) to be crosslinked *in vitro* since their initial mechanical properties are insufficient for ACL replacement applications. It was hypothesized that crosslinking with EDC would yield more improved mechanical properties than crosslinking with UV, due to the following reasons: (1) UV-mediated crosslinking reactions are often limited to aromatic amino acids such as phenylalanine and tyrosine [26], which comprise only 1.3% and 0.3% of the amino acids in rat tail tendon collagen type I, respectively [35], and (2) although UV exposure can initiate crosslinking in collagen, this same exposure to UV radiation can also degrade collagen [26].

During the present study, several different parameters were investigated, including the method of crosslinking (UV versus EDC), UV exposure time (15, 30, 1, 2, 4, and 8 hrs), EDC concentration (6, 10, and 25 mM at 8 hrs exposure), EDC exposure time (1, 2, and 4 hrs), PTFE extrusion tubing inner diameter (1mm versus 0.28 mm), and fiber formation time (24 hrs versus 4 hrs). After analyzing the data, it was determined that EDC is a more appropriate choice than UV for crosslinking our extruded collagen fibers. More specifically, crosslinking with 25 mM EDC for 4 hours yielded promising results, and we therefore believe that this crosslinking protocol will aid in our pursuit to fabricate a resorbable collagen scaffold with application to ACL tissue engineering. Furthermore, 1 mm inner diameter extrusion tubing was found to be favorable for handling of our resulting fibers, and a 4-hour fiber formation time was found to be sufficient to expedite the fiber formation process, while retaining the desired mechanical properties.

Mechanical properties of the human ACL have been shown to vary with age; Noyes and Grood [32] reported an average UTS of 37.8 ± 9.3 MPa, a Young's modulus of 111 ± 26 MPa, and a failure strain of 60.25 ± 6.78% for the ACLs of human donors between the ages of 16 and 26 years. In comparison, the ACLs of human donors between the ages of 48 and 86 years exhibited an average UTS of 13.3 ± 5.0 MPa, a Young's modulus of 65.3 ± 24.0 MPa, and a failure strain of 48.5 ± 11.9%. The mechanical properties of these bone-ACL-bone specimens were determined at a strain rate of 100%/second, which is a strain rate that is akin to that experienced in certain accidents [32]. Note that when comparing to the native human ACL, data obtained from younger donors (16 to 26 years of age) in Noyes and Grood's study were used for the following reason. Noyes and Grood discussed that the mode of failure of the ACLs from the two age groups were different with younger ACLs failing within the ligament substance and

older ACLs failing at bone-ligament attachment sites. These differences were attributed to a weakening of bone and ligament tissues with age and other age-related factors [32]. Thus, it was implied that data from younger human ACLs is more representative of the properties of human ACL tissue, and in particular, that data from younger human ACLs is a more appropriate target for the design of mechanically robust scaffolds for ACL reconstruction.

As mentioned, the data from younger ACLs reported by Noyes and Grood were acquired with a strain rate of 100%/second. In the present study, individual collagen fibers were tested at a strain rate of 10%/second. In order to assess the effect of strain rate on our collagen fibers, two additional groups of fibers were formed and crosslinked according the optimal protocol chosen in this study. These two groups of fibers were then tested at 10%/second (n=13) and 100%/second (n=11); average increases of 32.4% and 31.6% were observed in the UTS and Young's modulus, respectively. However, these increases in the UTS and Young's modulus for the higher strain rate were not statistically significant.

2.6 Conclusions

In summary, the optimal protocol for forming and crosslinking type I collagen fibers chosen was a 4 hour fiber formation time using a PTFE tubing with an inner diameter of 1 mm followed by 4 hours of crosslinking with 25 mM EDC in 90% acetone:10% water. This protocol resulted in an average UTS of 33.3 ± 14 MPa, a Young's modulus of 312 ± 106 MPa, and a failure strain of 13.3 ± 1.8%. The individual collagen fibers produced in this study have an UTS comparable to the native human ACL and a Young's modulus that exceeds the native ACL, but the failure strain is much lower than the native ACL. However, it was still hypothesized that collagen fibers formed by the optimal protocol in this study would have the potential to serve as a primary component in a scaffold intended for ACL reconstruction.

2.7 Acknowledgements

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3.0 Chapter 3
Design and Analysis of Braid-Twist Collagen Scaffolds

3.1 Abstract

Collagen type I fiber-based scaffolds for anterior cruciate ligament (ACL) replacement were evaluated for their mechanical properties and their ability to promote cell survival and growth. These collagen fiber-based scaffolds were assembled by twisting and braiding reconstituted collagen type I fibers according to a braid-twist scaffold design. Scaffolds were then either uncrosslinked, crosslinked after the addition of a hydrogel (gelatin), or crosslinked without gelatin. Scaffold crosslinking was performed with 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) (25 mM) for 4 hours. Ultimate tensile strength (UTS), Young's modulus, and viscoelastic properties of the scaffolds were evaluated with strain to failure and stress relaxation tests. In order to assess cell response on the scaffolds, primary rat fibroblast cells, isolated from rat lateral collateral ligament (LCL) and medial collateral ligament (MCL), were seeded upon the scaffolds. Cell activity was evaluated after 7, 14, and 21 days of seeding using a Cell Titer 96® AQueous One Solution Cell Proliferation Assay (MTS Assay). The mechanical testing results showed that among the three scaffold groups, the crosslinked scaffolds without gelatin displayed strength (UTS), stiffness (Young's modulus), and viscoelastic properties that were closest to the human ACL. Improvements are still desired to improve the mechanical compliance and ductility of these scaffolds. Cell activity was observed on all cell-seeded scaffolds by day 7, but by day 21 only the crosslinked scaffolds without gelatin had significantly higher cellular activity than the negative controls. Although we would still like to further improve mechanical properties and cell-scaffold interactions, we believe that these scaffolds have the potential to serve and contribute towards an ACL replacement strategy.

3.2 Introduction

The anterior cruciate ligament (ACL) has poor healing capabilities, making ACL ruptures a clinical problem. There are an estimated 50,000 ACL ruptures per year in the United States alone [1-4]; ACL ruptures typically cause pain, swelling, and joint instability and require surgery in order to reconstruct the tissue. Currently, the autograft is the gold standard in ACL replacement, but an autograft procedure requires two surgeries and typically results in donor site morbidity [2, 3, 5-8]. Other options available include allografts, xenografts, and synthetic implants, but each has associated problems [8]. As a result of these problems, alternative approaches are being investigated.

Resorbable scaffolds for tissue regeneration are being investigated in the tissue engineering field. For ACL replacement, a tissue-engineered scaffold should be biocompatible, biodegradable, be able to support physiological loading of the knee, replicate the mechanical properties of native ACL tissue, and promote cell infiltration and new tissue growth [2, 3, 5-8]. Collagen type I has been investigated as a primary material in ligament scaffolds because of its biocompatibility, strength, and degradation rate, which can all be controlled via crossinking [9-11]. In addition to collagen type I, silk is another popular natural polymer studied in the ligament tissue engineering field [8, 12-15]. Silk is mainly composed of fibroin and sericin, but due to a potential antibody response, fibroin is the only component of silk that is used [8, 12, 13]. Altman *et al.* [12] developed a 6-cord wire-rope silk fiber matrix displaying a hierarchical design. This 6-cord wire-rope silk fiber matrix consisted of 30 parallel fibers per bundle, 6 twisted bundles per strand, 3 twisted strands per cord, and 6 cords per ACL scaffold. The 6-cord wire-rope silk fiber matrix demonstrated structural properties comparable to the human ACL, but a smaller cross-sectional area [12, 16]. This particular design resulted in a significant decrease in stiffness without affecting the ultimate load, when compared to scaffolds with a parallel arrangement of fibers. Additionally, human bone marrow stromal cells (BMSCs) have been observed to grow and proliferate on the silk fiber matrices [12]. Some concerns raised about silk include a slow degradation rate due to its β-sheet structure [3, 13, 17] and the potential to cause an antibody response [6, 8, 12, 13].

In previous studies, ligament scaffold designs have involved bundles of fibers in a parallel arrangement [9-11], but other studies [18-20] have shown that a braid-twist scaffold design may be more favorable in replicating the properties of native ligament tissue. Freeman *et al.* [19] showed that braiding provides a strong structure, making the scaffold easier to handle, and twisting helps to replicate a crimped pattern, similar to the crimp pattern found in native ligaments. The crimp pattern is critical in replicating the toe region observed in the stress-strain curves of ligaments. The combination of twisting and braiding has also provided significant increases in ultimate tensile strength (UTS) and ultimate strain, as opposed to braiding alone, which can help mimic the mechanical properties of the native ACL [20].

Collagen type I fibers were studied in Chapter 2 as the primary material for the braid-twist scaffolds that will be discussed in this chapter. As in the previous chapter, the use of the singular form "collagen" represents collagen type I. Scaffolds comprised of collagen fibers have been studied by previous investigators [9-11], but in order for collagen to be mechanically sufficient as a ligament scaffolding material, it needs to be synthetically crosslinked. The crosslinking method chosen in the present study was 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC). Studies have shown that collagen fibers crosslinked with EDC can still be degraded in the presence of proteolytic enzymes, making EDC an acceptable method of crosslinking for resorbable scaffolds [9, 21]. Furthermore, based on our work presented in Chapter 2, collagen fibers crosslinked with EDC display similar mechanical properties to the human ACL.

The goal of this study was to investigate a braid-twist scaffold made with natural polymers, specifically collagen type I. This braid-twist scaffold design was previously developed by Freeman *et al.* [20] but was made with synthetic polymers. In addition to a collagen fiber braid-twist scaffold, a composite scaffold with the inclusion of a hydrogel, gelatin, was evaluated. Each scaffold type was assessed for its mechanical properties and for its ability to promote cell growth. Hydrogels absorb and release water during loading and unloading; therefore, the addition of a hydrogel was hypothesized to help better mimic the viscoelastic behavior seen in ligaments. The addition of a hydrogel to fiber-based scaffolds has been shown to exhibit mechanical properties similar to the human ACL [2, 10, 18, 22]. Gelatin (denatured collagen) was selected as the hydrogel component as it is biocompatible, biodegradable,

commercially available, non-immunogenic, and has been shown to promote cell proliferation [23, 24].

3.3 Materials and Methods

Acid-soluble type I collagen was isolated from the rat tail tendons of Sprague-Dawley rats using the following procedures, which are similar to those described by Pins *et al.* [25]. Collagen was dissolved in 10 mM hydrochloric acid (HCl, pH 2.0) for at least 4 hours at room temperature. The solution was then centrifuged at 30,000 x g at 4°C for 30 minutes. The supernatant was then sequentially filtered through 0.8μm, 0.65μm, and 0.45μm filters (Millipore). Sodium chloride (NaCl) was then added to the filtered solution for a 0.7 M solution, and then the precipitate was collected by a 1 hour, 30,000 x g centrifugation at 4°C. The precipitate was redissolved in 10 mM HCl (pH 2.0). The acid-soluble collagen was then purified with dialysis against 20 mM sodium phosphate dibasic (pH 7.4) for at most 16 hours at room temperature, after which dialysis was continued in a fresh 20 mM sodium phosphate dibasic (pH 7.4) bath at 4°C overnight. The collagen solution was then centrifuged at 30,000 x g for 1 hour at 4°C. The collagen pellets were dissolved in 10 mM HCl (pH 2.0) for 24 hours followed by a final dialysis against HCl (pH 2.0) for at most 16 hours at room temperature. The collagen concentration was then checked by placing known volumes of collagen in a 110°C oven until no further changes in mass occurred. The collagen solution was diluted as needed by adding more HCl (pH 2.0). Once the desired collagen concentration (10 mg/mL) was obtained, the solution was sterilized with chloroform (10% volume) at 4°C overnight. After sterilization, the collagen solution was placed into sterile 5 mL syringes and stored at 4°C until needed.

Self-assembled collagen fibers were made by extruding collagen and a fiber formation buffer (FFB) (135 mM sodium chloride, 30 mM Tris Base (Tris (hydroxymethyl) aminomethane), and 30 mM sodium phosphate dibasic, pH 7.4), simultaneously, through polytetrafluoroethylene (PTFE) tubing at a rate of 0.7 mL/min into a bath of FFB at 30°C. The fibers remained in the FFB bath for 4 hours and were then placed in deionized water for 1 hour. The fibers were then taken out of the water and dried under their own weight. A 4 hour formation time was selected based on the results discussed in Chapter 2.

Scaffold Design

A braid-twist design was selected for this study using the protocol developed by Freeman *et al.* as discussed below [20]. Each scaffold consisted of 54 reconstituted collagen fibers. The fibers were cut to 160 mm lengths, and then the following protocol was used. Nine groups of six 160 mm length reconstituted collagen fibers were made. Each group was twisted in a counter-clockwise direction in order to achieve a twisting angle of 60 ± 4.5°, which formed a fiber bundle. Three bundles were then twisted in a counter-clockwise direction around one another to a twisting angle of 72 ± 2.3°, forming a yarn (3 yarns per scaffold). The 3 yarns were then braided together (manually) at a braiding angle of 69 ± 2.7° in order to form one scaffold.

The twisting and braiding angles chosen for this study were determined from an optimization study performed by Freeman *et al.* [20], which showed that this particular combination of twisting and braiding angles yielded the best results for a fibrous poly (L-lactic acid) (PLLA)-based ACL replacement. Twisting and braiding angles were determined using images taken with a stereoscope (Vision Engineering, New Milford, CT). To see a schematic of the braid-twist scaffold protocol, refer to Figure 3-1. Braiding and twisting angles were defined as the angle between the fiber and a line perpendicular to the scaffold's long axis (Figure 3-2). Fiber twisting to form bundles and fiber bundle twisting to form yarns were done using a Conair device (Model QB3ECS, Conair Corporation, East Windsor, NJ). Fibers were twisted for 5 seconds in order to form bundles at the desired twisting angle, and then bundles were twisted for 3 seconds in order to form yarns at the desired twisting angle. In order to achieve a braiding angle of 69°, the yarns were braided together at 4 stitches per inch. A stitch is defined as the incorporation of each yarn once. These twisting and braiding angles were optimized by Freeman *et al*. in a previous study using the same scaffold design [20].

The average scaffold length was 94 mm, but only a 20 mm gage length was needed for mechanical testing and a 12 mm scaffold length for the cell study. Therefore, the original ~94 mm long scaffolds were cut into multiple smaller scaffolds. Prior to cutting these longer scaffolds, high strength 2-Ton epoxy (Devcon, Riviera Beach, FL) was used to delineate the smaller scaffolds to be obtained from each ~94 mm long scaffold, to ensure that the braiding and twisting angles would be unaffected.

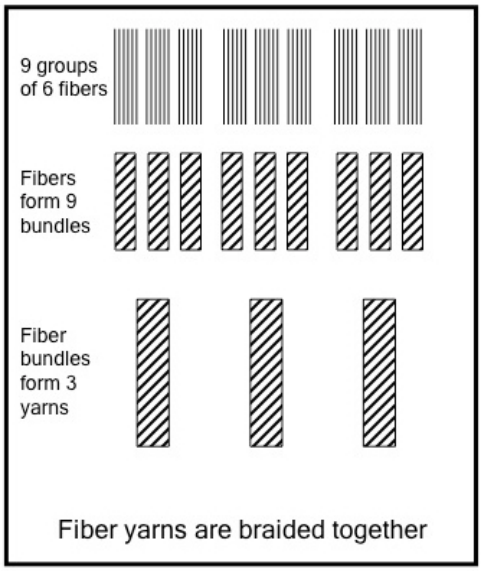

Figure 3-1: Schematic of the braid-twist scaffolds. Modified and included with permission from Freeman *et al.* [20] © Elsevier Science.

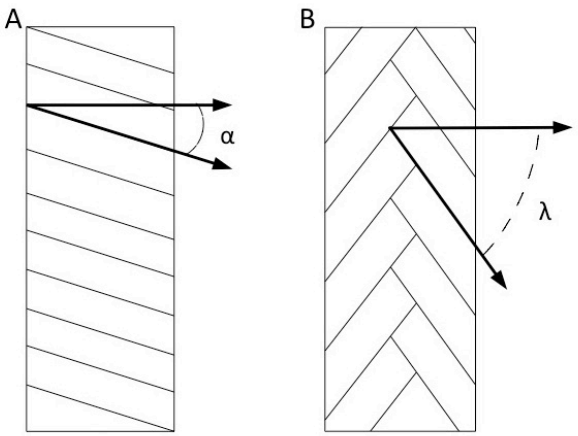

Figure 3-2: Definition of the twisting angle, α, (A) and the braiding angle, λ, (B). Modified and included with permission from Freeman *et al.* [20] © Elsevier Science.

Crosslinking

Scaffolds were prepared as previously described and divided into three groups: control (n=6), crosslinked without gelatin (n=7), and crosslinked with gelatin (n=6). The control group was left uncrosslinked to act as a negative control. The crosslinked without gelatin group was crosslinked using EDC. The crosslinked scaffolds with gelatin were immersed in a 10% gelatin solution at 37°C for 45 minutes and allowed to dry at room temperature prior to crosslinking in EDC.

The two crosslinked scaffold groups were crosslinked using the optimal method selected in Chapter 2: 4 hours of crosslinking with 25 mM EDC in 90% acetone:10% water. Briefly, the fibers were immersed in 25 mM EDC dissolved in 90% acetone:10% water for 4 hours to allow for crosslinking to occur. Following the crosslinking, the scaffolds were subjected to the following rinses: an 8 hour rinse in 90% acetone:10% water, a 4 hour rinse in 50% acetone:50% water, a 2 hour rinse in 0.1 M sodium phosphate, and then a 24 hour rinse in deionized water. After the rinses were complete, the scaffolds were dried at room temperature. Scaffolds were then placed under the 254 nm ultra-violet (UV) lamp of a biological safety cabinet for 5 minutes per side; the separation distance between the scaffolds and the UV source of the biological safety cabinet was approximately 58 cm. This UV exposure was performed because it is necessary for the scaffold sterilization process, and a direct comparison between the mechanical tests and cellular activity tests was desired.

Mechanical Testing

Scaffolds were mounted on vellum paper frames with a 20 mm gage length prior to mechanical testing. For the control group and the crosslinked without gelatin group, the average fiber diameter was determined using a Leica DM IL microscope (Leica Microsystems). A circular cross-section was assumed, allowing us to determine the cross-sectional area of the average fiber, which was multiplied by the number of fibers within one scaffold. For the crosslinked with gelatin group, both width and thickness images were taken using a stereoscope (Vision Engineering, New Milford, CT). Image analysis was performed with OpenOffice.org's Draw program to determine the dimensions of each scaffold. A rectangular cross-section was assumed for the crosslinked with gelatin scaffolds. For all three groups, both dry and hydrated images were taken. Hydration was achieved by soaking each scaffold in phosphate-buffer solution (PBS, pH 7.4) for 30 minutes.

Uniaxial tensile tests were performed on the scaffolds using a Tytron 250 Microforce Testing System (MTS Systems Corporation). Each scaffold was mounted into custom-designed testing grips and immersed in a room temperature PBS bath for testing. The sides of the vellum paper frame were cut after each scaffold was loaded into the bath and before any tensile load was introduced. Scaffolds were preloaded to 100 mN at a strain rate of 10%/minute. Once the preload was reached, the scaffolds were pulled to failure at a strain rate of 10%/second. Force-

displacement data was converted into stress-strain data in order to determine the ultimate tensile strength (UTS), Young's modulus, and strain at failure.

Stress-relaxation tests were performed on the crosslinked without gelatin (n=4) and crosslinked with gelatin groups (n=4). Prior to testing, cross-sectional area was determined as previously described. Scaffolds were tested using a Tytron 250 Microforce Testing System (MTS Systems Corporation) in a room temperature PBS bath. All scaffolds were subjected to a series of two stress-relaxation tests (5% strain and 12% strain). Scaffolds were preloaded to 100 mN at a strain rate of 10%/minute, then strained to 5% strain at a rate of 10% second, held for 30 minutes, then strained to 12% strain at a rate of 10%/second, and finally, held for another 30 minutes. 5% and 12% strain were selected based on the uniaxial tensile tests to failure, where 5% strain marked the beginning of the linear region and 12% strain was located well into the linear region. Although it has been shown that testing longer than 1000 seconds does not provide an increase in the accuracy of calculated rates of stress-relaxation [26], a 30 minute hold (1800 seconds) for stress relaxation was selected in order to compare to Pioletti, *et al*. [27]. Stress relaxation data for each scaffold was normalized by the maximum stress attained at each strain level.

Model

A simple model, similar to the one used by Freeman *et al.* [20], was developed in order to determine the affect of the braiding angle on the failure rate of the scaffolds. Figure 3-3 shows a free body diagram of the scaffold and an individual yarn, which was developed in order to determine the forces on an individual yarn. The braid was assumed to be symmetric and therefore, that each yarn would experience the same forces. For this model, the yarns were modeled as solid rods. As seen in Figure 3-3, T is the tensile force placed on an individual yarn, which is one-third of the tensile force placed on the entire scaffold (T_S), since a uniform force distribution due to symmetry of the three yarns was assumed. T_B is the tensile force on an individual yarn in the direction of the path of that yarn in the braid. B is the force placed on a yarn by the other two yarns in the braid, which is the force preventing each yarn from becoming straight when pulled in the axial direction, and λ is the braiding angle. By balancing the forces in the x and y directions the forces on an individual yarn were determined, refer to Equations (1) – (4).

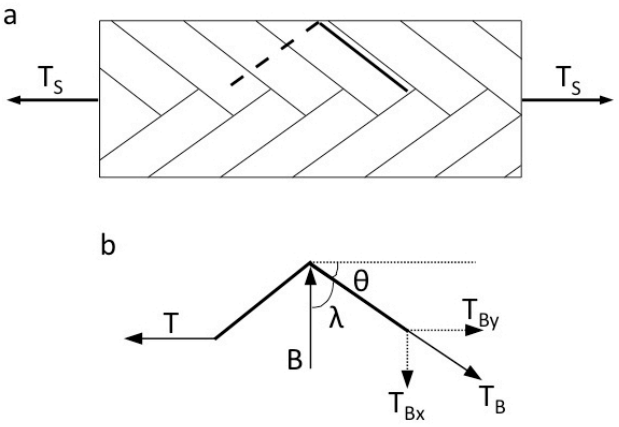

Figure 3-3: Free body diagram of the scaffold (a) and an individual yarn (b) used to determine the forces on each yarn. Modified and included with permission from Freeman *et al.* [20] © Elsevier Science.

$$T = T_{By} = T_B \cos\theta, \quad T_B = \frac{T}{\cos\theta} \tag{1}$$

$$B = T_{Bx} = T_B \sin\theta \tag{2}$$

$$B = T \tan\theta \tag{3}$$

$$\theta = 90 - \lambda \tag{4}$$

Cellular Activity

Primary fibroblast cells were isolated from the lateral collateral ligaments (LCLs) and medial collateral ligaments (MCLs) of Sprague-Dawley rats. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 4.5 g/L each of glucose, L-glutamine, and sodium pyruvate (Cellgro, Manassas, VA) supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin. This cell culture medium was replaced three times per week.

Scaffolds with and without gelatin (n=4 per group) were cut into lengths of 12 mm and glued using a Silastic® medical adhesive (Dow Corning, Hemlock, MI) into the wells of a 24 well Ultra-Low Attachment tissue culture plate (Corning, Corning, NY). Scaffolds were sterilized by immersion in 70% ethanol for 30 minutes and then removed. Scaffolds were UV irradiated for 5 minutes on each side [19]. Scaffolds were then soaked in DMEM overnight under

standard culture conditions (37°C, 5% CO_2). Cells were seeded at 50,000 cells per well (passage 2). The DMEM was changed three times per week over the three week study.

Cell activity was measured after 7, 14, and 21 days in culture using the Cell Titer 96® AQueous One Solution Cell Proliferation Assay (MTS Assay) (Promega Corporation, Madison, WI). The reagent of this assay contains MTS (a tetrazolium compound) that is reduced by metabolically active cells into a formazan dye, which is optimally measurable at an absorbance wavelength of 490 nm. At each time point, the old media was removed and fresh media plus the MTS reagent were added. Samples were then incubated for three hours and the absorbance measured at 490 nm using an Absorbance Microplate Reader (ELx800™, BioTek Instruments, Winooski, VT). Since Ultra-Low Attachment tissue culture plates were used and the media was replaced three times per week, it was assumed that all cells tested with the MTS assay were attached to the scaffold; cells not attached to the scaffold would have been removed with media during media replacements.

The dimethylmethylene blue (DMMB) assay was performed to determine the concentration of the proteoglycans that the cells excreted. The DMMB assay was performed on crosslinked scaffolds with and without gelatin (n=4 per group) after 7, 14, and 21 days in culture and on negative controls (digested scaffolds with no cells seeded). The DMMB assay protocol was adopted from Billington *et al.* and Alves da Silva *et al.* [28, 29]. Briefly, for every time point, the following solutions were made fresh: phosphate buffer (PB) (69 mM NaH_2PO_4 and 32 mM Na_2HPO_4, pH 6.5), 2.5% (w/v) solution of papain in PB, 0.78% (w/v) solution of cysteine-hydrochloric acid (cysteine-HCl) in PB, and 1.9% (w/v) solution of ethylenediaminetetraacetic acid (EDTA) in PB. Papain is a protease that degrades proteins; papain is activated and stabilized in the presence of cysteine-HCl and EDTA [30]. Furthermore, papain displays optimal enzymatic activity at 65^{o}C [31]. In order to digest the collagen scaffolds and release proteoglycans from the extracellular matrix produced by the cells, each scaffold was placed in a 1.5 mL microcentrifuge tube with 350 μL of PB, 100 μL of the papain solution, and 50 μL each of the cysteine-HCl and EDTA solutions. The tubes were then vortexed for 10 minutes and then incubated overnight at 65°C. Following the overnight incubation, solutions containing the enzymatically-digested scaffolds were centrifuged at 13,000 rpm for 10 minutes. Supernatants were collected and stored at -20°C until the DMMB assay was performed. Solutions for the DMMB assay were prepared as follows. 16 mg of DMMB was dissolved in 5 mL of ethanol and supplemented with 2 mL

formic acid and 2 g of sodium formate. The total volume of the DMMB solution was then increased to 1 L using deionized water and stored at room temperature in a bottle covered with aluminum foil to block exposure to ambient light. The glycosaminoglycan (GAG) standards were prepared by dissolving chondroitin sulfate (Sigma C-4384) in deionized water at a concentration of 1 mg/mL; this stock solution was then serially diluted from 40 to 0 μg/mL in 5 μg/mL increments to establish a standard curve. For each GAG standard and scaffold supernatant, 80 μL was transferred into two wells (40 μL per well) in a 96 well plate. To each well, 250 μL of the DMMB solution was added, and the absorbance read at 525 nm.

Statistical Analysis

For the strain to failure and stress relaxation data, Grubbs' test for outliers was used to detect any potential statistical outliers within each scaffold group. For mechanical tests, MTS, and DMMB data one-way analysis of variance (ANOVA) was done followed by a Tukey's test to determine any significant differences between test groups. P-values < 0.05 were considered to be significant.

3.4 Results

Mechanical Properties

Figure 3-4 shows an image of a dry and wet uncrosslinked scaffold. Figure 3-5 represents an example of a stress-strain curve for one of our EDC-crosslinked scaffolds without gelatin during a strain to failure test. From the stress-strain curve, the UTS, Young's modulus, and failure strain were determined. Table 3-1 shows the average UTS, Young's modulus, and failure strain from the strain to failure tests for all three groups tested; one of the gelatin-based scaffolds was excluded from further analysis because it was found to be a statistical outlier when performing Grubbs' test for outliers. The EDC-crosslinked scaffolds without gelatin had a significantly higher UTS and Young's modulus compared to those scaffolds with gelatin and to the control scaffolds, and the control group had a significantly higher failure strain than either of the two crosslinked scaffold groups.

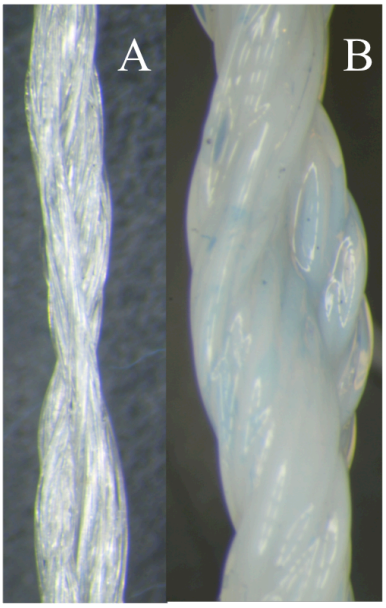

Figure 3-4: A picture of a dry braid from the uncrosslinked control group (A), and a picture of a wet braid from the uncrosslinked control group (B). The image in (B) is zoomed in compared to the image in (A).

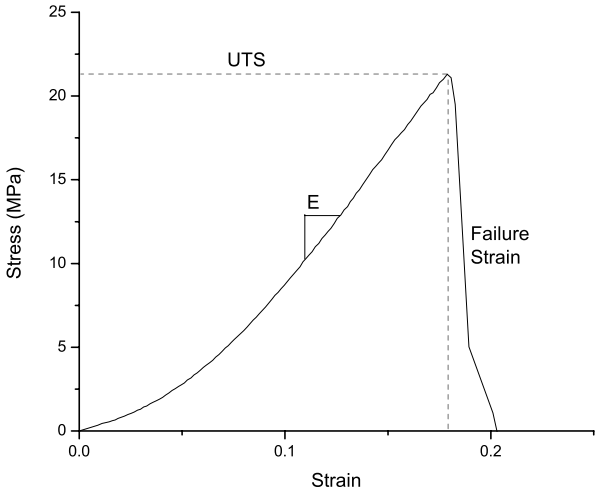

Figure 3-5: Representative stress-strain curve for the crosslinked scaffolds without gelatin. E represents the Young's modulus and is taken to be the slope of the linear region of the stress-strain curve.

Table 3-1: Mechanical properties obtained from strain to failure tests of our collagen fiber braid-twist scaffolds. A * denotes significant differences between other test groups ($p<0.05$).

Group	UTS (MPa)	Young's Modulus (MPa)	Failure Strain (%)
Control (uncrosslinked, without gelatin)	0.843 ± 0.052	1.95 ± 0.20	44.6 ± 2.9*
EDC-crosslinked, with gelatin	1.07 ± 0.061	6.32 ± 0.95	20.0 ± 2.6
EDC-crosslinked, without gelatin	19.3 ± 3.1*	148 ± 17*	18.0 ± 2.3

The stress-relaxation data was normalized for each strain level (5% and 12%). Figure 3-6 and Figure 3-7 show the average stress-relaxation response for both the scaffolds without and with gelatin, respectively, as well as data for the human ACL at 8% and 12% strain [16, 27]. During the first 100 seconds, the scaffolds relaxed very quickly, after which they slowly relaxed and eventually approached an equilibrium stress.

The absolute values of the stress-relaxation slopes (the slope of a line tangent to the stress-relaxation curve within a selected time interval) from different time periods were determined and can be seen in Table 3-2. Differences between the two groups during the same time interval were analyzed. The scaffolds with gelatin had a significantly higher stress-relaxation slope than the scaffolds without gelatin during the 600-800 second time interval during the 5% strain stress-relaxation; no other significant differences were observed.

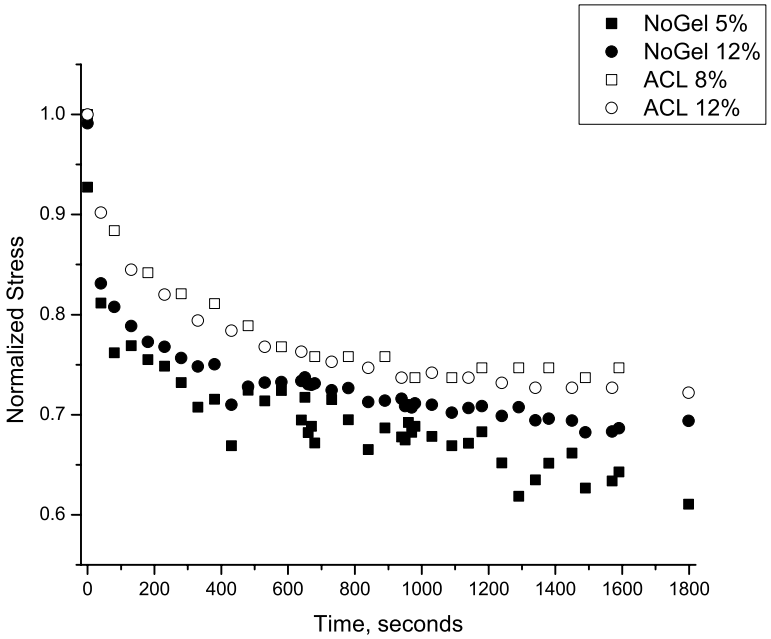

Figure 3-6: Results from stress relaxation tests for the crosslinked scaffolds without gelatin (NoGel) at 5% strain and 12% strain. Human ACL stress-relaxation data at 8% strain and 12% strain are also shown on the graph [27]. All stresses have been normalized for each relaxation.

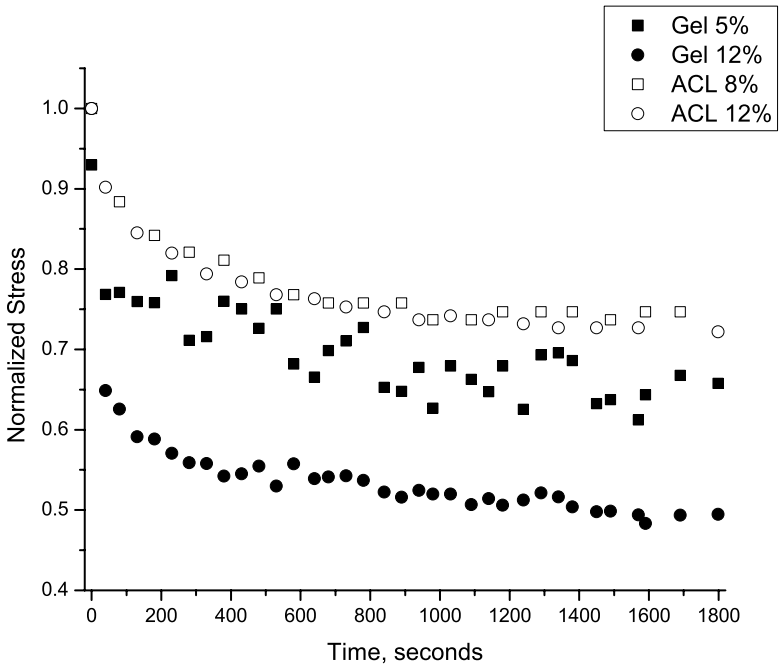

Figure 3-7: Results from stress relaxation tests for the crosslinked scaffolds without gelatin (Gel) at 5% strain and 12% strain. Human ACL stress-relaxation data at 8% strain and 12% strain are also shown on the graph [27]. All stresses have been normalized for each relaxation.

Table 3-2: Absolute value of stress-relaxation slopes (x 10^{-4} sec^{-1}). A * denotes a significant difference to the other test group during the same time interval and strain level.

	5% strain		12% strain	
Time Interval	NoGelatin	Gelatin	NoGelatin	Gelatin
0-50s	19.8 ± 11	29.0 ± 6.0	26.1 ± 9.0	34.2 ± 3.9
50-100s	3.92 ± 2.9	17.1 ± 17	5.11 ± 2.4	6.72 ± 0.87
100-200s	3.09 ± 0.81	6.11 ± 7.3	2.63 ± 0.74	2.78 ± 0.44
200-400s	2.03 ± 2.2	1.45 ± 0.99	1.08 ± 0.28	1.46 ± 0.20
400-600s	1.21 ± 1.2	1.26 ± 1.1	0.600 ± 0.28	0.565 ± 0.32
600-800s	0.700 ± 0.48	1.72 ± 0.52*	0.737 ± 0.37	0.505 ± 0.15
800-1000s	0.443 ± 0.35	1.10 ± 0.68	0.380 ± 0.089	0.658 ± 0.49
1000-1200s	0.456 ± 0.21	1.37 ± 1.2	0.298 ± 0.19	0.569 ± 0.24
1200-1400s	0.907 ± 0.56	0.543 ± 0.34	0.330 ± 0.11	0.221 ± 0.087
1400-1600s	0.621 ± 0.44	0.519 ± 0.27	0.347 ± 0.25	0.203 ± 0.18
1600-1800s	0.814 ± 0.69	0.722 ± 0.27	0.356 ± 0.17	0.371 ± 0.37

Cell Study

For the MTS cell activity assay, the following results were observed (Figure 3-8). Both groups of scaffolds, with and without gelatin, on day 7 and the scaffolds without gelatin on day 21 were significantly higher compared to the negative control of blank wells with no cells. Between days 7 and 14, a decrease in cell activity was observed for both types of scaffolds but

only crosslinked scaffolds without gelatin were significantly lower. Although day 21 values increased for both types of scaffolds, only crosslinked scaffolds without gelatin were significantly higher than the respective day 14 results. The DMMB assay was performed on a negative control for both groups, crosslinked scaffolds with and without gelatin with no cells. None of the cell-seeded scaffolds had a significantly higher proteoglycan content compared to the negative controls at any time point.

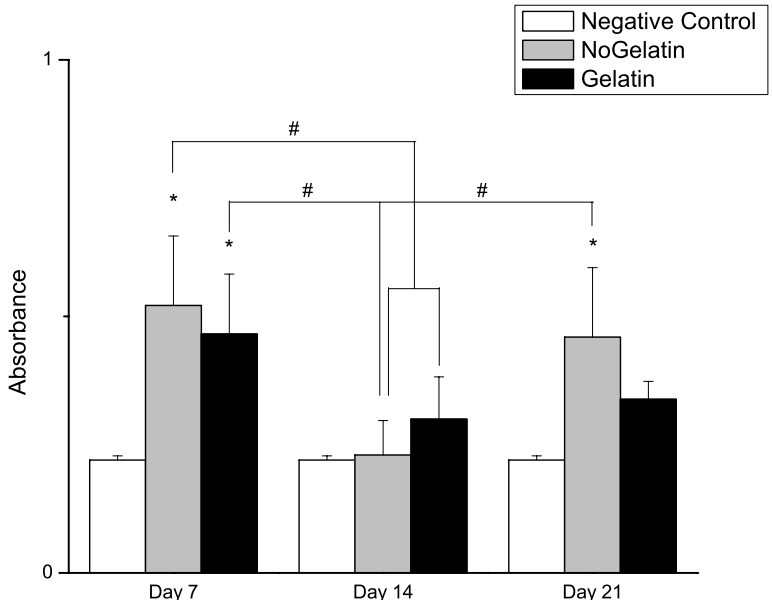

Figure 3-8: MTS results for the duration of the cell study. A * denotes significant differences from the negative control ($p<0.05$). A # denotes significant differences between test groups and time points ($p<0.05$).

Twisting and Braiding Angles

After mechanical testing was complete, twisting and braiding angles were investigated due to the discrepancy in dry fiber diameters between the PLLA fibers (0.295 ± 0.044 mm) used by Freeman *et al.* [20] and the uncrosslinked collagen fibers (0.061 ± 0.007 mm) produced in this study. The first twisting angle, individual fibers twisted to form fiber bundles, could not be determined with the images obtained. The second twisting angle, fiber bundles twisted together to form yarns, was 79.98 ± 1.03°. The braiding angle was 76.43 ± 2.10°. The angles of the collagen-fiber braid-twist scaffolds were slightly higher than that determined for the PLLA fiber braid-twist scaffolds developed by Freeman *et al.* [20].

Model

The forces preventing the braided yarns from becoming straight can be explained to some extent by the model described earlier (Figure 3-3 and Equations (1) – (4)). By experimenting with different braiding angles, the model suggests that by increasing the braiding angle (fewer stitches per inch), the forces preventing the braided yarns from becoming straight decrease in a linear fashion. However, based on the expected braiding angle in this study (69°), the forces preventing the braided yarns from becoming straight were 12.8% of the breaking load (Equation (3)). Therefore, at failure, an optimal scaffold in this study would experience a force of approximately 1.013 N acting to prevent each braided yarn from becoming straight in addition to the stress induced by the axial load. Based on the actual braiding angle (76.43°) each yarn experienced a force of 0.637 N that prevented it from becoming straight, 8.05% of the breaking load.

3.5 Discussion

When comparing the three different groups in the strain to failure tests, the crosslinked scaffolds without gelatin yielded significantly higher UTS and Young's modulus values when compared to the crosslinked scaffolds with gelatin and the uncrosslinked control group. When comparing the hydrated cross-sectional areas of the three groups, the crosslinked scaffolds without gelatin had the smallest area (0.411 mm^2), the scaffolds with gelatin had an intermediate area of 1.16 mm^2, and the control group had the largest area of 1.70 mm^2. Although the gelatin was able to fill in the area between the fibers, the control scaffold group still had a higher cross-sectional area most likely because crosslinking may prevent complete swelling of the hydrogel and the fibers. It is also believed that the gelatin either prevented the EDC from sufficiently crosslinking the collagen fibers or reacted with the EDC itself since it is denatured collagen. These hypotheses are supported by our mechanical testing results, since no significant difference was observed between the crosslinked scaffolds with gelatin and uncrosslinked control group, with the exception of failure strain. In order to improve the mechanical properties of the scaffolds with gelatin, the fibers should be crosslinked prior to the addition of the gelatin, and then further crosslinked as needed by the gelatin.

Younger humans (16-26 years of age) have been shown to have stronger and stiffer ACLs than older humans (48-86 years of age) [16]. Therefore, when designing a scaffold for human ACL replacement, the scaffold should meet the requirements for a younger adult in order to avoid rupture. Noyes and Grood [16] showed that ACLs from younger human donors exhibited a UTS of 37.8 ± 9.3 MPa and a Young's modulus of 111 ± 26 MPa. The optimal collagen fiber braid-twist scaffold in this study, which was determined by the UTS and Young's modulus values obtained from the strain to failure tests, was the EDC-crosslinked scaffold without gelatin; it yielded a UTS of 19.273 ± 3.074 MPa and a Young's modulus of 147.935 ± 17.186 MPa. The UTS of these braid-twist scaffolds is lower than the human ACL, but when considering the standard deviations of the Young's modulus, there is an overlap. However, the human ACL data obtained by Noyes and Grood was acquired using a strain rate of 100%/second, which was chosen to simulate fast strain-rates (i.e., in the range of strain rates experienced during accidents). Ideally, the scaffold would serve to act as the ACL during the rehabilitation time for the patient, and therefore, would probably not be subjected to strain rates as high as 100%/second. Over time, the body would be expected to remodel the ACL scaffold and generate new ligament tissue, which would ideally have the same mechanical properties as a native healthy ACL and thus be able to withstand higher strain rates.

Although the properties of the braid-twist scaffolds developed in this study were not tested at 100%/second and therefore cannot be directly compared to the properties of the human ACL determined by Noyes and Grood [16], a strain rate comparison study on individual reconstituted collagen fibers was conducted. These individual collagen fibers were fabricated using the same formation and crosslinking processes as the fibers in the braid-twist scaffolds and were then tested at either 10%/second (n=13) or 100%/second (n=11) until failure. The strain rate comparison study showed that as the strain rate increased, the UTS and Young's modulus appeared to increase; however, the differences were not statistically significant. A 32.4% increase in the UTS and 31.6% increase in the Young's modulus was observed for these individual collagen fibers.

When comparing the collagen fiber braid-twist scaffolds developed in the present study to the PLLA braid-twist scaffolds developed by Freeman *et al.* [20], the collagen fibers in the present study were much smaller than the PLLA fibers used by Freeman *et al.* An individual PLLA fiber consisted of 30 microfibers plied together; the collagen fibers in this study were

more similar to the size of these individual microfibers than to the PLLA fibers. The PLLA scaffold had a UTS of 81.6 ± 1.6 MPa and a Young's modulus of approximately 741 MPa, both of which are much higher than the human ACL. Although the collagen scaffolds had a lower UTS and Young's modulus compared to these PLLA scaffolds, the collagen scaffolds were more comparable to the human ACL. A disadvantage of having a scaffold with mechanical properties that greatly exceed the native ACL is the possibility of stress shielding upon implantation (i.e., when a scaffold imbalances the mechanical loading of surrounding tissues due to a mismatch of mechanical properties). Freeman *et al.* [18] also investigated adding a hydrogel to the scaffold design used in previous work [20]. In Freeman *et al.* [12], the scaffolds were covered with 10% poly (ethylene glycol) diacrylate (PEGDA). The addition of the hydrogel resulted in a significant decrease in mechanical properties; the UTS decreased to 36 ± 3 MPa and the Young's modulus decreased to 437 ± 38 MPa, which is more comparable to the human ACL. This decrease should be expected as a result of the increase in cross-sectional area brought about by the incorporation of a hydrogel. Furthermore, the hydrogel was shown to improve the viscoelastic properties of the scaffolds when subjected to stress-relaxation tests, making the addition of hydrogel a viable addition to the braid-twist scaffold design [18].

Caruso *et al.* [9] performed a study using bovine dermal type I collagen. Their scaffolds consisted of 50 fibers aligned in parallel. The fibers in the Caruso *et al.* study had dry diameters of approximately 60 μm and were made using a similar process to the protocol used in the present study. The fibers used in the crosslinked without gelatin scaffolds in the present study had dry diameters of 65 μm. However, Caruso *et al.* had a 24-hour crosslinking time in 25 mM EDC, and their scaffolds were tested at 1000%/minute after being hydrated for 1 hour in PBS. In the study done by Caruso *et al.* [9], the crosslinked scaffolds had an average breaking load of approximately 2.77 N and a stiffness of 1.43 N/mm, whereas the crosslinked without gelatin scaffolds in the present study had an average breaking load of 7.92 N and a stiffness of 2.80 N/mm. Although, the material properties (i.e., UTS, Young's modulus, etc.) were not determined in the study by Caruso *et al.*, their dry fiber cross-sectional areas were very similar, and the scaffolds in the present study yielded higher breaking loads and stiffness values even though they were tested at a slower strain rate.

The stress relaxation tests on our EDC-crosslinked collagen fiber braid-twist scaffolds displayed similar trends to the human ACL's tested by Pioletti and Rakotomanana [27]. Pioletti

and Rakotomanana performed stress-relaxation on human ACL's at four different strain values: 8% strain, 12% strain, 14% strain, and 16% strain. Figure 3-6 and Figure 3-7 show the results of our crosslinked scaffolds without and with gelatin, respectively, along with the human ACL data obtained by Pioletti and Rakatomanana at 8% and 12% strain. The human ACL has a cross-sectional area of 44.4 mm^2 [16] whereas our scaffolds have cross-sectional areas of 1.18 mm^2 and 0.411 mm^2 with and without the inclusion of gelatin hydrogel, respectively. At the end of the 30-minute hold, the human ACL had a normalized stress of 0.7474 and 0.7216 at 8% and 12% strain, respectively. Our scaffolds without gelatin had normalized stress values of 0.6197 ± 0.197 and 0.6822 ± 0.117 at the end of the 5% and 12% strain holds, respectively, and our scaffolds with gelatin had values of 0.6576 ± 0.016 and 0.4946 ±0.036 after the 5% and 12% strain holds, respectively. The human ACL relaxed more at a higher strain level (i.e., 8% versus 12% strain); this increased relaxation at higher strains was seen in our scaffolds with gelatin (i.e., 5% versus 12% strain). Both groups of braid-twist scaffolds relaxed more than the human ACL, but the stress-relaxation behaviors were still similar (Figure 3-6 and Figure 3-7), with the exception of the stress-relaxation at 12% strain for the scaffolds with gelatin, which displayed a notable increase in stress relaxation compared to the human ACL (Figure 3-7). Again, the stress-relaxation slopes between our two scaffold groups (with and without gelatin) were analyzed, and with the exception of one time interval, there were no statistical differences observed. The scaffolds in this study, both with and without gelatin, generally presented similar viscoelastic properties to the human ACL, which is consistent with earlier work done with braid-twist scaffolds containing a hydrogel component [18].

When comparing the mechanical properties of our collagen fiber braid-twist scaffolds to our individual collagen fibers discussed in Chapter 2, on average, the individual fibers had higher UTS and Young's modulus values but lower failure strains. Three possible reasons for this decrease in the UTS and Young's modulus when moving from an individual fiber to the scaffold are torsional strain effects, less crosslinking, and the forces preventing the yarns in the braid from becoming straight. The scaffolds were crosslinked as a whole; therefore, the EDC may not have crosslinked the collagen molecules in the scaffold as effectively as the molecules in an individual fiber. The forces preventing the braid from becoming straight was explained, to some extent, by the model. Although the model showed that the force preventing the braid from

becoming straight was 8.06% of the breaking load, it is hypothesized that this force contributes to the failure of the scaffold.

The MTS assay showed that cells were attached and active 7 days after seeding for both groups of scaffolds and 21 days after seeding for the scaffolds without gelatin. Both our work and previous work by Freeman *et al.* [19], using the braid-twist scaffold design with PLLA fibers, showed that this design did not inhibit cell proliferation. Also, from previous studies it is known that cells have attached and proliferated on collagen. For example, Cornwell *et al.* [21] performed studies on collagen threads crosslinked with EDC using normal human dermal fibroblasts and showed that cells did attach and migrate on EDC crosslinked collagen fibers. In another study using bovine corium collagen, human dermal fibroblasts were seeded at 50,000 cells per scaffold on collagen scaffolds consisting of 200 fibers aligned in parallel. Over an 8-day period, an increase in cell number was observed [32]. Based on the literature, the lack of increasing cellular activity over time on our scaffolds may be associated with surface area limitations, seeding technique, or cell type. It is known that LCL and MCL fibroblasts have higher proliferation rates than ACL fibroblasts, but over a 4 day period (seeding at passage 3) there are no significant differences between the proliferation rates of the knee ligament fibroblasts. Therefore, changing the cell type to patellar tendon fibroblasts [33], mesenchymal stem cells [34], or skin fibroblasts [35], which all have higher proliferation rates than ACL fibroblasts, may improve the cellular activity on our scaffolds. Although cellular activity was established, the cells did not produce significant levels of proteoglycans, as measured by the DMMB assay. These results may be attributed to the cell source, since proteoglycans make up less than 1% of ligaments [36]. Thus, the LCL and MCL fibroblasts may not have produced proteoglycans at detectable levels for the DMMB assay.

In the future, twisting time and stitches/inch would need to be correlated to twisting and braiding angles, respectively, in order to produce collagen fiber braid-twist scaffolds with the desired angles. The twisting and braiding angles for scaffolds produced in this study were higher than expected. Based on trends observed by Freeman *et al.* [20], the higher twisting angle of the yarns (79.98 ± 1.03° for the present study compared to 72 ± 2.3°) likely caused an increase in failure strain and UTS, although the magnitude of the increase is unknown. It is also likely that the Young's modulus was not affected by the slight increase in twisting angle, but the length of the toe region may have decreased. The increase in the braiding angle (76.43 ± 2.10° for the

present study compared to 69 ± 2.7°) may have resulted in a decrease in the length of the toe region, but the UTS, Young's modulus, and failure strain were probably not affected based on trends observed by Freeman *et al.* [20], who studied a variety of twisting and braiding angles.

3.6 Conclusions

In summary, EDC-crosslinked collagen fiber braid-twist scaffolds without gelatin have mechanical results that may be acceptable for ACL replacements. The UTS of these braid-twist scaffolds is less than that of the native human ACL; however, our scaffolds without gelatin had a cross-sectional area of 0.411 mm^2, which is much smaller than the native human ACL (44.4 mm^2), and would therefore need to be scaled-up for human ACL replacement. The addition of the gelatin hydrogel component to the scaffold significantly decreased the mechanical properties, but this is most likely a result of the gelatin blocking the fibers from being crosslinked. More specifically, the gelatin could have hindered diffusion of EDC through the collagen fibers and/or the gelatin, being denatured collagen, could have reacted with the EDC. Both of our EDC-crosslinked collagen fiber scaffolds, with and without gelatin, exhibited similar trends to the human ACL in terms of the extent of stress relaxation. However, more studies are needed to evaluate the cellular activity on these scaffolds. In the future, using a different cell source, increasing the surface area for cells to attach, and improving cell-seeding techniques are all ways that should improve cellular response. Future work should also focus on investigating mechanical properties after scaling up the overall scaffold size and incorporating the gelatin after crosslinking the collagen fiber scaffolds.

3.7 Acknowledgements

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3.8 References

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4.0 Chapter 4
Conclusions and Future Work

4.1 Project Conclusions

The overall project goal was to investigate the mechanical properties of crosslinked reconstituted collagen fiber braid-twist scaffolds with and without gelatin, and the response of primary rat lateral collateral ligament (LCL) and medial collateral ligament (MCL) fibroblasts seeded upon these scaffolds. However, before the fabrication of the braid-twist scaffolds, the method of crosslinking was optimized with individual reconstituted fibers. Two crosslinking methods were examined, ultra-violet (UV) radiation and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC). Fibers crosslinked with UV were crosslinked for 15 minutes, 30 minutes, 1, 2, 4, or 8 hours. Crosslinking times of 4 or 8 hours were deemed optimal for UV crosslinking. Three different concentrations of EDC, 6, 10, and 25 mM, were studied with a crosslinking time of 8 hours. After comparing the results of the different EDC concentrations, the data showed that 25 mM EDC yielded the best rests. Although there were no statistical differences between the concentrations, there was an increasing trend of increased strength with increasing concentration. Once the EDC concentration was determined the EDC crosslinking time was varied (1, 2, 4, or 8 hours), and a crosslinking time of 4 hours was determined to be optimal. The EDC crosslinked fibers yielded significantly better results than UV crosslinked fibers when using the ultimate tensile strength (UTS) and Young's modulus as the selection criteria.

After the crosslinking method was chosen, 25 mM EDC in 90% acetone:10% water for 4 hours, the braid-twist scaffolds were constructed. Each braid-twist scaffold was composed of 54 reconstituted collagen type I fibers. Scaffolds were made using the protocol developed by Freeman *et al.* [1]. Briefly, a group of six fibers were twisted into a fiber bundle. Three fiber bundles were then twisted around one another to form a yarn. Three yarns were then braided together in order to produce one scaffold. It was expected that the fiber groups would be twisted to 60° to from bundles, the bundles twisted to 72° to form yarns, and the yarns braided at an angle of 69°; however, due to differences in fiber diameters between our study and Freeman *et*

al. [1] (0.061 ± 0.007 mm and 0.295 ± 0.044 mm, respectively), the bundles were twisted to 79.98 ± 1.03° to form yarns and the yarns were braided at an angle of 76.43 ± 2.10° (the other twisting angle was not measured). All angles are measured from the fiber to a line perpendicular to the scaffold's long axis. The scaffolds were cut into the desired size of 20 mm gage length for mechanical tests and 12 mm for the cell study; epoxy was used to maintain the twisting and braiding angles of the scaffolds. Gelatin was added to some samples and then samples were crosslinked (except for the uncrosslinked negative control group) as previously described.

Scaffolds were hydrated and tested under uniaxial tension at 10%/second until failure occurred in order to determine the UTS, Young's modulus, and failure strain of the scaffolds. Based on the UTS and Young's modulus, crosslinked scaffolds without gelatin showed to be significantly stronger and stiffer than crosslinked scaffolds with gelatin and the uncrosslinked negative control group. However, it is hypothesized that the gelatin prevented the EDC from crosslinking the collagen molecules effectively or the gelatin, since it is denatured collagen, reacted with the EDC. For the scaffolds without gelatin the UTS was 19.273 ± 3.074 MPa, the Young's modulus was 147.935 ± 17.189 MPa, and the failure strain was 18 ± 2.3%. In comparison to the human anterior cruciate ligament (ACL) data reported by Noyes and Grood, the human ACL has a higher UTS (37.8 ± 9.3 MPa) and failure strain (60.25 ± 6.78%) but a lower Young's modulus (111 ± 26 MPa). Considering the standard deviations of the Young's modulus there is overlap between the braid-twist scaffolds and the human ACL. However, Noyes and Grood's human ACL data was obtained at a strain rate of 100%/second in order to simulate injury conditions, so no direct comparison can be made.

The crosslinked braid-twist scaffolds both with and without gelatin were also subjected to two stress-relaxation tests for 30 minutes each. The first 30 minute hold (relaxation) was at 5% strain, which represents the beginning of the linear region in a typical stress strain curve from our braid-twist scaffolds, and the second 30 minute hold (relaxation) was at 12% strain, which was well into the linear region of the stress strain curve. Both groups of scaffolds exhibited very similar strain-dependent trends (i.e. changes in the residual or equilibrium stress at different strain levels) when compared to the human ACL, but the crosslinked scaffolds without gelatin matched the viscoelastic properties of the human ACL better [2].

In addition to the mechanical characterizations, cellular activity on crosslinked scaffolds with and without gelatin was examined. Scaffolds were placed in 24 well Ultra Low Attachment

well plates and both primary rat LCL and MCL fibroblasts were seeded together at a density of 50,000 cells per well. Cell viability was assessed at three time points, days 7, 14, and 21, using a Promega Cell Titer 96® AQueous One Solution Cell Proliferation Assay (MTS assay). Proteoglycan concentration was also measured at the three time points using a dimethylmethylene blue (DMMB) assay. On day 7, the MTS assay showed significantly higher results compared to a negative control for both groups, and on day 21 the samples without gelatin had significantly higher MTS results compared to the negative control. No other time points yielded significant MTS results. The DMMB assay showed no significant results for either group at any time point, but this could be a result of proteoglycans making up less than 1% of the dry weight ligaments [3]; and therefore, the LCL and MCL fibroblasts would not produce a large enough amount of proteoglycans in order to be detected.

In summary the collagen braid-twist scaffolds have potential to serve as an ACL ligament replacement mechanically. Furthermore, these scaffolds are composed of a natural polymer collagen type I, which as been shown to be biocompatible and biodegradable. However, approaches to improve proliferation and attachment rates of cells on these scaffolds need to be investigated more thoroughly to improve the potential of these scaffolds for tissue-engineered ligament applications.

4.2 Future Work

One limitation in the present study was the cross sectional area of the scaffolds. In the future, we would like to increase the cross-sectional area of our scaffolds to better mimic the size of the native human ACL. Although the material properties such as the UTS and Young's modulus are on the same order of magnitude as those of the human ACL, structural properties such as maximum force and stiffness diverge by multiple orders of magnitude. An increase in the scaffold's cross-sectional area could be obtained by increasing the number of collagen fibers used to build the scaffold. This could be done in a variety of ways, but one would be to increase the number of fibers in each of the groups that are twisted to form the fiber bundles, or another would be to add another step of twisting fibers before creating the fiber bundles, also known as plying the fibers together. Plying the fibers together would be similar to the structure of the PLLA fibers used by Freeman *et al.* [1], who first presented this design for ACL scaffolds. In

order to improve the UTS and Young's modulus of the scaffolds with a hydrogel component, the braid-twist scaffolds could be crosslinked before adding the hydrogel. After the addition of the hydrogel, the crosslinked collagen fiber braid-twist/hydrogel composite could be crosslinked to introduce crosslinks between the collagen fibers and the hydrogel component.

The human ACL is viscoelastic, meaning it is strain rate dependent. Therefore, since no direct comparison of the mechanical properties between the braid-twist scaffolds and human ACL [4] can be made, additional tests are required. Either the braid-twist scaffolds could be tested at a strain rate of 100%/second or human ACL samples would need to be tested at 10%/second for comparison purposes. Since 100%/second simulates strain rates commonly seen in injuries [4], it would be more beneficial to study human ACLs at a lower strain rate that does not typically cause injury.

There are multiple ways to improve cell attachment and verify cellular activity on the scaffolds. An increase in surface area for cells to attach to should help increase the number of cells on each scaffold. This can be achieved by increasing the size the scaffold as previously described. In addition to increasing the surface area, the cell type could be changed since it is known that ACL fibroblasts have low proliferation rates [5-7], and studies have shown that the proliferation rates of LCL and MCL fibroblasts do not significantly differ from ACL fibroblasts with increasing culture time and passage number [8]. For example human dermal fibroblasts [5], mesenchymal stem cells [6], or patellar tendon fibroblasts [7] could be used since they have higher proliferation rates than ACL fibroblasts. The seeding density could also be increased, as well as improving the seeding technique. Seeding on fibrous, three-dimensional scaffolds can be difficult because the cells can either fall through the scaffold or not attach to the scaffold One way to improve the cell seeding technique would be to embed the cells in a hydrogel before adding the hydrogel to the scaffold. Gentleman *et al.* [9] performed a study where rat skin fibroblasts were embedded in a collagen type I solution. The cell/collagen solution was then added to a collagen fiber scaffold and incubated under standard culture conditions for 30 minutes, causing the cell/collagen solution to gel. A similar protocol could be used to improve the cell-seeding technique.

A tissue-engineered scaffold should allow for cellular infiltration in order to get tissue growth through the scaffold. Therefore, in addition to improving cellular attachment and proliferation on the scaffolds, the cellular distribution would also need to be investigated, which

could be done by using fluorescence staining and imaging the cell-seeded scaffolds. The addition of a hydrogel could potentially inhibit cellular infiltration as a result of a decrease in pore size, which has been observed by Freeman *et al.* [10]. However, the use of a biodegradable hydrogel, a requirement for a tissue-engineered scaffold, should exhibit gradually increasing pore sizes with time since the hydrogel would be degraded overtime, but the degradation rate and cellular infiltration over time would need to be investigated. Another option would be to investigate adding channels in the hydrogel for cellular infiltration without disrupting the braid-twist structure.

Eventually, the scaffold should degrade leaving the patient only new living tissue, so scaffold degradation would need to be examined. EDC crosslinked collagen fibers have been shown to degrade over time [11, 12], but scaffold design and crosslinking time would impact the degradation rate. Ideally, the degradation rate should be tailored to match the rate of new tissue growth.

Overall, cellular proliferation needs to be increased on the scaffolds since promoting cell proliferation and new tissue growth are two requirements for a tissue-engineered scaffold. The mechanical properties as well as the biodegradation rate would need to be investigated after increasing the size of the scaffolds via increasing the number of collagen fibers and changing the hydrogel protocol. The ideas discussed in this section describe a few ways that this project could evolve in the future.

4.3 References

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