Electrospun Polycaprolactone Scaffolds for Small-Diameter Tissue Engineered Blood Vessels

DISSERTATION

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By

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Abstract

Cardiovascular disease is the leading cause of death in the United States with many patients requiring coronary artery bypass grafting. The current standard is using autografts such as the saphenous vein or intimal mammary artery, however creating a synthetic graft could eliminate this painful and inconvenient procedure. Large diameter grafts have long been established with materials such as Dacron® and Teflon®, however these materials have not proved successful in small-diameter (< 6 mm) grafts where thrombosis and intimal hyperplasia are common in graft failure.

With the use of a synthetic biodegradable polymer (polycaprolactone) we utilize our expertise in electrospinning and femtosecond laser ablation to create a novel tri-layered tissue engineered blood vessel containing microchannels. The benefits of creating a tri-layer is to mimic native arteries that contain an endothelium to prevent thrombosis in the inner layer, aligned smooth muscle cells in the middle to control vasodilation and constriction, and a mechanically robust outer layer.

The following work evaluates the mechanical properties of such a graft (tensile, fatigue, burst pressure, and suture retention strength), the ability to rapidly align cells in laser ablated microchannels in PCL scaffolds, and the biological integration (co-culture of endothelial and smooth muscle cells) with electrospun PCL scaffolds. The conclusions from this work establish that the electrospun tri-layers provide adequate
mechanical strength as a tissue engineered blood vessel, that laser ablated microchannels are able to contain the smooth muscle cells, and that cells are able to adhere to PCL fibers. However, future work includes adjusting microchannel dimensions to properly align smooth muscle cells along with perfect co-cultures of endothelial and smooth muscle cells on the electrospun tri-layer.
This document is dedicated to my family, both human and feline.
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Chapter 1: Literature Review

1.1 Introduction

Cardiovascular disease (CVD) is the leading cause of death in the United States and affects more than 1 out of every 3 adults [1]. As of 2013, it is estimated that 83.6 million adults in America are suffering from at least one type of CVD [1]. CVD is most commonly caused by atherosclerosis in which plaque deposits inhibit blood flow to the heart resulting in ischemia [2]. Plaque deposition frequently occurs in the coronary artery thus, coronary artery disease (CAD) is the most prevalent among the different forms of CVD. If left untreated, the plaque can grow large enough to block blood flow or pieces of the plaque can break away and block flow in smaller arteries downstream. Many patients with CAD receive coronary artery bypass grafts (CABG) or percutaneous coronary intervention (PCI). CABG is the preferred treatment because of its lower occurrence of cardiac or cerebrovascular events 1 year post CABG or PCI [3].

The saphenous vein is the most commonly used graft for a CABG procedure (Figure 1.1 [4]). However, this vein has a poor patency rate as occlusions occur in 10-15% of grafts after 1 year and almost 50% of grafts fail after 10 years [5]. Additionally, more than half of the individuals with CVD are over the age of 60 which decreases the
chance of having healthy veins for harvest and multiple surgeries are not ideal [1]. In contrast, allografts have long term patency but the risks of chronic inflammation limit this practice. A viable option is to use synthetic grafts instead of a native artery or vein. One complication with the use of synthetic grafts, especially small diameter (< 6 mm) grafts, is a low patency rate compared to native substitutes due to thrombosis or intimal hyperplasia [6-8].

Figure 1.1 Schematic of (a) the commonly used saphenous vein graft for CABG along with the second most commonly used internal mammary artery and (b) how CABG can restore blood flow [4].
1.2 Historical Background of Vascular Grafts

Over 100 years ago, Dr. Alexis Carrel implanted a human artery into a dog’s abdominal aorta. He also implanted one dog’s jugular vein into another dog’s thoracic aorta. Both dogs survived for 4 and 2 years, respectively [9]. Following his successful xenograft and allograft, Carrel attempted to intubate a rubber tube into the abdominal aorta of a dog. Knowing thrombosis would occur after the introduction of a foreign body, he coated his rubber tube with petroleum jelly and found that the dog’s circulation remained normal 15 months post-surgery [10]. Carrel continued his work by implanting glass or aluminum tubes into the thoracic aorta of dogs, trying to determine the ideal material for performing intubation. He was successful in that blood flow continued through an implanted glass tube for over 3 months [10]. More importantly, Carrel created techniques to improve the intubation process by preventing thrombosis. These included selecting the proper graft size, coating his tube with a more inert material (gold), and even the possibility of lining the tube with a vein to improve biocompatibility [10]. Carrel had the foresight to know that preventing thrombosis would be a key factor in creating successful vascular grafts.

In spite of Carrel’s efforts, it was not until the 1950’s that synthetic vascular grafts were seriously considered. Dr. Arthur Voorhees and colleagues created a graft from a plastic fine mesh cloth, termed Vinyon “N” cloth. They inserted their cloth tubes
into abdominal aortas of 15 dogs and found that 8 of the 11 dogs that were autopsied had functioning implants with the longest follow-up remaining healthy at 153 days [11]. Soon after in the late 1950s, the polyethylene terephthalate (PET) prosthesis (trademarked as Dacron® by DuPont) was introduced (Figure 1.2(a) [12]). However, due to reports of aneurisms and hemorrhage associated with PET, polytetrafluoroethylene (PTFE) (also trademarked by DuPont as Teflon®) became more prevalent in the early 1960s as a stiffer alternative to Dacron® [13] (Figure 1.2(b) [14]). Unfortunately, Teflon® was too rigid leading to the development of expanded PTFE (ePTFE), a porous version of the original PTFE that was trademarked as Gore-Tex® by Bob Gore (Figure 1.2(c) [15]). Dacron® and ePTFE have both been successful as large diameter grafts (> 6 mm). These large diameter grafts have succeeded where the small diameter versions fail because (1) the larger diameter deceases the chance of thrombosis leading to total occlusion and (2) larger grafts benefit from faster blood flows that make it more difficult for proteins and platelets to adhere (thoracic and abdominal aorta) [16,17]. However, using these materials in small diameter grafts such as coronary arteries or femoral-popliteal arteries where blood flow is much slower results in occlusion [16-18].
Small diameter grafts face the same challenges as larger diameter grafts such as maintaining mechanical strength for *in vivo* hemodynamic stresses, biocompatibility to minimize infection, and sutureability and handling ability for allowing ease of use during implantation. In addition, small diameter grafts must overcome challenges that result from their smaller diameter including thrombosis (clot formations) and intimal hyperplasia (luminal wall thickening) [16-19]. This has been associated with inadequate endothelial cell coverage on the luminal surface [19]. There remains a need for a more tissue engineered approach for small diameter grafts to overcome these problems with small diameter grafts.
1.3 Progress in Small Diameter Tissue Engineered Blood Vessels

Early research on vascular replacements set precedents for current tissue engineered blood vessels (TEBV) such as recognizing the need to minimize thrombosis in the graft, preventing hemorrhage, and ensuring appropriate values of compliance. While many different approaches can be used to create a TEBV, five methodologies are most commonly used: (1) endothelial cell (EC) lined scaffolds, (2) biopolymer-based scaffolds, (3) biodegradable synthetic polymer-based scaffolds, (4) self-assembled cell scaffolds, and (5) decellularized scaffolds [16, 20, 21].

1.3.1 EC Lined Scaffolds

All native blood vessels contain an endothelium- a monolayer of ECs lining the luminal wall in the vessel that plays a key role in angiogenesis and acts as a barrier between blood and the surrounding tissue [2] (Figure 1.3). More importantly, in the context of creating TEBV, the endothelium is responsible for the native non-thrombogenic properties in blood vessels [22]. Without an endothelium, platelets adhere to adsorbed proteins on the luminal wall resulting in thrombosis. In small diameter grafts, this leads to total occlusion and graft failure at a higher incidence and faster rate than larger diameter grafts. Herring et al. was the first group to examine the effects of an EC lining by comparing thromboresistance and histological appearance of EC seeded and
unseeded Dacron® grafts [23]. They found that the presence of an endothelium kept the seeded grafts 76.4% clot-free while the unseeded grafts were only 22.4% clot-free [23].

![Diagram of artery with ECs lining the luminal wall to provide non-thrombogenic properties to the vessel.](image1.png)

Figure 1.3 Schematic of the artery with ECs lining the luminal wall to provide non-thrombogenic properties to the vessel.

More recently, Zhang et al. investigated the adhesion and anti-thrombogenic properties of ECs on nanoporous electrospun scaffolds composed of both natural and synthetic polymers [24]. EC-seeded scaffolds were able to secrete prostacyclin (a molecule naturally secreted by ECs to prevent platelet adhesion and to act as a vasodilator) and were able to prevent platelet adhesion when inoculated with $1.4 \times 10^3$ cells/mm$^2$ and cultured for 7 days. However, any area of the electrospun scaffold that was exposed (i.e., not covered by ECs) allowed platelets to readily adhere due to the gelatin content of the scaffold [24] (Figure 1.4 [24]).
Figure 1.4 Zhang et al. demonstrates the non-thrombogenic properties of ECs when platelets (indicated with arrows) only adhere to exposed scaffold rather than areas covered by ECs [24].

For successful EC adhesion to scaffolding, biopolymers are considered as an ideal material because of their inherent biocompatibility and promotion of cell adhesion and retention.

1.3.2 Biopolymer-based Scaffolds

To increase the biocompatibility of vascular grafts, many groups have examined the use of biopolymers found in native vessels. The major proteins in the native vessel are collagen and elastin; they are the primary components of extracellular matrix (ECM) and provide mechanical strength and elasticity, respectively [2]. Weinberg and Bell have
been credited with first using collagen as substrates for cells in vascular grafts and were successful in demonstrating the biocompatibility of collagen by seeding their collagen gel with ECs, smooth muscle cells (SMCs) and fibroblasts [25]. While they were able to obtain proper integration of cells in their scaffold, the burst pressure obtained was less than 10 mm Hg in scaffolds fabricated without Dacron® [25]. This burst pressure value is significantly lower than the 2000 mm Hg clinically mandated burst pressure [26, 27]. Many groups thereafter have continued to use collagen as well as other biopolymers such as fibrin [28-30] or elastin [31, 32] due to their excellent biocompatibility to improve elasticity and strength. However, these reconstituted proteins have low tensile strengths and degrade quickly in vivo due to the abundance of biopolymer-specific enzymes [6]. Hackett et al. evaluated the degradation rate of an electrospun collagen scaffold versus an electrospun synthetic polymer (polycaprolactone) scaffold. The collagen scaffold completely degraded (via collagenase) in 36 hours while the synthetic polymer was able to resist degradation (via lipase) for 96 hours [33]. For this reason, many groups have explored using synthetic polymers as scaffolds for vascular substitutes because of their slower degradation rates and higher mechanical strengths.

1.3.3 Biodegradable Synthetic Polymer-Based Scaffolds

One option to increase the mechanical strength of vascular scaffolds is to use synthetic polymers rather than biopolymers. Non-degradable polymers such as Dacron® and ePTFE are used extensively in the clinic. However, biodegradable polymers can be
advantageous because they resorb in the body, ultimately leaving completely biological tissue behind [16, 20, 21]. Examples of commonly used biodegradable polymers are polyglycolic acid (PGA), poly-lactic acid (PLA), and polycaprolactone (PCL). PGA and PLA are both quickly resorbed (complete degradation ~ 6 months) by the body via hydrolysis and release of non-toxic degradable byproducts of glycolic acid and lactic acid, respectively [34, 35]. PCL is a slower degrading polymer (on the order of 2-3 years) but still elutes only non-toxic byproducts [36-38]. The advantages of synthetic polymers include: can withstand a wide variety of processing techniques (e.g., electrospinning, solvent casting, and three-dimensional printing), ability to control the microstructure (e.g., pore size and fiber diameter), the mechanical properties (e.g., tensile strength, stiffness, and compliance) can be tailored to meet necessary mechanical requirements, and the resorption rate can be adjusted to control degradation by synthesizing new polymers or creating copolymers. For example, polylactic-co-glycolic acid (PLGA) is a copolymer composed of PLA and PGA and the ratio of the two monomers drastically affects the degradation rate. When the monomers are used at a 1:1 ratio, degradation occurs the fastest (Figure 1.5 [35]) [35]. Additionally, many groups have been successful in obtaining adequate mechanical strengths with biodegradable polymers. Hassan et al. used poly(L-lactide-co-e-caprolactone) (PLCL) a copolymer of
Figure 1.5 Degradation properties of PLGA is dependent on the ratio of PLA and PGA [35].

PLA and PCL at 50:50 to make small diameter TEBV and compared mechanical strength (circumferential stress, longitudinal stress, modulus (E), suture retention strength (SRS), and burst pressure) to native rabbit aortas and ePTFE grafts prior to seeding cells. They found that their vascular graft had promising mechanical strengths in which the circumferential tensile strength was higher than either the rabbit aorta or the ePTFE graft. Maximum longitudinal stress was comparable to that of the rabbit and modulus was lower than the rabbit aorta but higher than the ePTFE. SRS and burst pressure values were all comparable to the rabbit aorta and ePTFE graft [39].

While biodegradable polymers have advantages over biopolymers such as the ability to provide adequate mechanical strength and tunability in manufacturing the scaffold, the biocompatibility of biopolymers is superior. Zhang et al. performed a study to evaluate the biocompatibility of electrospun PCL and collagen scaffolds and found that
fibroblasts adhered and proliferated better on collagen than PCL fibers as shown in Figure 1.6 [40]. To balance the mechanical and degradation property benefits found in synthetic polymer grafts with the less optimal cell-material interactions, surface modifications can be utilized to improve overall biocompatibility by minimizing inflammation post implantation [41] and optimizing protein adsorption to ultimately increase cell adhesion.
Figure 1.6 (a) Cell proliferation is greatest on collagen fibers and worst on PCL fibers. Additionally, electron images depict better cellular adhesion and growth on (b) collagen fibers rather than (c) PCL fibers. Graph and images reproduced from [40].
1.3.4 Surface Modifications to Improve Biocompatibility

Many groups have improved biodegradable polymer biocompatibility by plasma treatment [42-44], collagen coating [43], or the addition of proteins such as growth factors or arginine-glycine-aspartic acid (RGD) peptides to allow enhanced cell seeding [45-48]. Improving cellular attachment to the surface of the scaffold is important for biocompatibility and decreases the chance for thrombosis in the graft.

Plasma treating as an additive process can promote cell growth, as shown in the work by Ratner et al [42]. Deposition of an ultrathin plasma films containing oxygen or carbon monomers modifies the surface to enhance cell seeding of 3T3 fibroblasts, bovine aortic endothelium, human umbilical endothelium, and epithelial cells [42]. Plasma treating can also etch the surface of the scaffold to chemically change the composition. He et al. observed that air plasma significantly increased the hydrophilicity of their scaffold [43] and Perego et al. were able to increase cell adhesion after argon and oxygen plasma treatments [44]. Limitations of plasma treatment include stability of the surface modification with respect to time [49]; however, this method is fairly simple and is easily scaled-up [42-44].

Collagen coatings on synthetics have also been utilized to improve polymer scaffold biocompatibility [43]. He et al. electrospun poly (L-lactide-\textit{co}-\varepsilon\text{-}

\text{caprolactone}) \text{PLCL} and, after plasma treating with air, submerged the scaffold into a collagen solution. Human ECs attached better to these collagen-coated scaffolds than non-coated scaffolds. Additionally, they adhered just as well on coated scaffolds as on a tissue culture
polystyrene control [43]. Scaffolds in He et al.’s study were plasma treated to make the scaffold more wettable to collagen, demonstrating that combinations of surface modifications could be implemented, such as plasma treatment and collagen coating to improve cell seeding. A concern with collagen coating is loss of surface porosity although He et al. showed that surface porosity can be maintained [43].

A third method to enhance cell attachment to the polymer scaffold is the use of specific growth factors or peptide sequences. The RGD peptide sequence can be bound to the polymer scaffold to increase cellular adhesion [45]. To securely attach RGD peptides to the polymer, functional groups such as hydroxyl, amino, or carboxyl groups should be present. Since polymers typically do not contain these functional groups on their surface, functional groups must be added via blending, co-polymerization, or chemical/physical treatment. While many steps may be necessary to allow proper binding of the peptide to the scaffold, it creates a strong anchor for cell adhesion. Larsen et al. synthesized a polymer containing the RGD sequence and found that when they adsorbed their polymer surfactant solution onto ePTFE, EC growth rates were better than when seeded on native ECM proteins [46]. While PTFE is not necessarily a biodegradable scaffold, cell growth on this hydrophobic polymer shows that proteins adsorption can increase adhesion and growth [50]. Additionally, a variety of growth factors are available to improve cell seeding including vascular endothelial growth factors (VEGF), angiopoietins, platelet derived growth factor (PDGF) among others [47,48]. Crombez et al. demonstrated that VEGF promoted EC migration. Creating negatively charged surfaces on PTFE films to allow VEGF to bind was a lengthy and
complex procedure, involving ammonia plasma treatment, immersion in a solution of glutaric anhydride, activation with 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride, and reactions with human serum albumin and cis-aconitic anhydride. Though successful binding of VEGF to the polymer surface was observed and led to improved cell migration, the process was time consuming and labor intensive, making it difficult to utilize in a large-scale manufacturing setting.

An alternative strategy to promote cell attachment without the need for surface modification is to generate a completely cell-based vascular graft. This method eliminates the need for improving the biocompatibility of the “scaffold” as the cells will adhere to each other to make a vessel and generate their own, native ECM.

1.3.5 Self-Assembled Cell Scaffolds

L’Heureux and Auger et al. successfully created a novel TEBV that is entirely biologically based, composed of cultured human cells [51]. They harvested SMCs, fibroblasts, and ECs from umbilical cords of healthy newborn babies and cultured the SMCs and fibroblasts for 1 month to create cellular sheets that can be wrapped around an inert tubular support. After additional incubation to allow the media (SMC) and adventitia (fibroblast) layers to mature, ECs were added to an acellular fibroblast-derived sheet with SMCs and fibroblast sheets wrapped around to create a three-layered TEBV, a construct similar to native vessels. Native ECM proteins such as collagen and elastin were produced by the cells and present in the scaffold to provide mechanical strength for
the vessel. Platelet adhesion was also successfully inhibited by the engineered endothelium, a phenomena necessary for preventing thrombosis. Good handling and suturability were also achieved and burst pressures over 2000 mm Hg were obtained [51]. The only disadvantage of this method is the time necessary for cells to grow into cell sheets, typically on the order of at least 3-6 months [26, 51]. This not only creates clinical obstacles as a patient could need these vascular grafts immediately, but also greatly reduces their ability to be easily manufactured. Maintaining sterility for such a long period of time can be challenging, and the more time cells are in culture, the more quality assurance/bioburden checks are required. The expense in maintaining cultures for long periods of time is also high and the delicate process of transferring the cell sheets onto a mandrel to create a tube can significantly reduce the percentage of product that will proceed from initial fabrication to final product.

1.3.6 Decellularized Scaffold

Decellularized scaffolds are native tissues that have been denuded of cellular components leaving behind an intact ECM with essential structural proteins such as collagen and elastin. These scaffolds possess several benefits including excellent mechanical strength because it maintains the native structure of the ECM as well as the native chemistry [52, 53]. Schaner et al. decellularized human saphenous veins (one of the most common vascular substitutes for bypass surgery) using sodium dodecyl sulfate (SDS) and evaluated burst pressure strength and SRS. When compared to fresh
saphenous veins, the burst pressure and suture retention values were nearly identical [52]. However, drawbacks included aneurysms, infections, and thrombosis that can occur if the decellularized graft does not re-endothelialize post-implantation, a problem common to glutaraldehyde-treated tissues [53]. Another disadvantage to this method is maintaining a source of tissue as well as providing adequate quality assurance as the source of the raw material (e.g., porcine) is inherently variable.

1.4 Limitations to TEBV Manufacturing

Many TEBV techniques have been utilized in attempts to create the perfect vascular graft possessing both excellent mechanical properties and biocompatibility. However, one of the limiting factors retarding clinical use of TEBVs is the time needed to create the substitute vessel. Two different TEBVs have shown encouraging results in early clinical trials [54-56], however only one has been implanted under arterial conditions and this particular TEBV requires 7-9 months to generate [56]. To develop a strategy for rapid manufacturing of TEBVs, we propose to engineer a tri-layered, synthetic vascular graft which both mimics the structure of the native vessel and possess cell-seeding channels for rapid cell organization within the layers (Figure 1.7).
Figure 1.7 Schematic of the proposed tri layer scaffold with cell seeding channels for rapid cell organization.
1.4.1 Materials Selection for TEBV

A few biodegradable polymers that have been most studied for TEBV are PLA, PGA, PLGA, and PCL. All these polymers possess non-toxic byproducts that degrade *in vivo* and are in devices that have already obtained Food and Drug Administration (FDA) approval. However, while their byproducts are substances already present in the body and non-toxic, high local concentrations near the degrading scaffold are a concern [57]. Additionally, if the scaffold degradation rate is faster than the rate of natural tissue regeneration *in vivo*, the possibility of an aneurysm exists [57]. Collagen is a natural polymer used for creating TEBVs, however reconstituted collagen does not possess the same mechanical strength as native collagen in the ECM [25-27] and often degrades far too quickly [6, 33] as mentioned earlier.

PCL was chosen because it is a slowly degrading polymer (~2-3 years), elutes non-toxic byproducts, and is biocompatible as evident through its wide array of FDA approved products (e.g., sutures and drug delivery) [36-38]. PCL degrades via hydrolysis of the ester linkages into water soluble hydroxycaproic acid monomers [36]. It is a semicrystalline polymer that degrades more slowly than many other biodegradable synthetics [37, 58]. This slow degradation rate allows adequate time for the cells to regenerate native tissue, ideally at the same rate as scaffold degradation. PCL also has a relatively low melting point of 60 °C allowing for easy manufacturing and scaffold fabrication [58].
PCL can be tailored to achieve appropriate mechanical strengths for a variety of tissue engineered applications. In the case of blood vessels, we have found that the three distinct layers found in native vessels can be created by initially dissolving PCL in different solvents. Electrospinning dissolved PCL in acetone creates dense fibers ~700 nm in diameter, in dichloromethane (DCM) creates very large porous fibers ~8 μm in diameter, and in hexafluoro-2-propanol (HFP) creates dense, stiff fibers ~700 nm in diameter with a higher moduli [57-62]. PCL can also be combined with other polymers to create unique properties to “fine tune” mechanical properties, degradation rate, porosity, and viscoelasticity [39, 58].

PCL not only elutes non-toxic byproducts but also allows successful cellular attachment and proliferation of a variety of cell types such as fibroblasts [63], smooth muscle cells [60, 62], chondrocytes [64], and mesenchymal stem cells [65-67]. In addition, cellular adhesion and proliferation can be improved by combining PCL with natural polymers such as collagen [68-70].

1.4.2 Scaffold Fabrication

Tissue engineering requires scaffolds that allow cells to infiltrate, adhere, and proliferate. Thus, it is ideal for engineered scaffolds to mimic native ECM. Some requirements for scaffolds include: (1) adequate porosity for cell migration while maintaining enough surface area for cell adhesion, (2) chemically encouraging cell adhesion, migration, and proliferation, (3) degrading at a similar rate to tissue.
regeneration, and (4) easy fabrication. Many different methods have been used to create tissue engineered scaffolds including filament extrusion [71], melt-molding and particulate-leaching [68], hydrogels [72], and electrospinning [31, 57-62, 63, 66, 67, 69, 70, 73]. While different methods have been successful in creating scaffolds for tissue engineering, one of the most unique properties of electrospun scaffolds is the similarity between physical morphology of electrospun fibers and native ECM fibers (Figure 1.8).

![Figure 1.8](image)

Figure 1.8 (a) Electrospun PCL fibers contain similar morphology to (b) native ECM decellularized from a porcine coronary artery.

Electrospinning creates fibers with diameter widths ranging from a few nanometers to several micrometers [74, 75], producing scaffolds that mimic the fiber arrangement and morphology of the native ECM. While fibers in the ECM have diameters generally on the order of ~100 nm, fiber diameters less than 100 nm and greater than 1 μm have been used to control cell morphology and development [74, 76, 77]. Additionally, fiber alignment has been found to affect cell migration and proliferation [78-80].
This method of fabricating scaffolds is fast, easily controllable, and compatible with a variety of synthetic and natural polymers. It can be used to control fiber alignment as well as diameter and porosity. To make electrospun scaffolds, a polymer solution is expelled through a syringe pump that has a positive (or negative) source. Opposite the needle tip is a collecting plate that can be stationary or rotating, and grounded or oppositely charged from the needle tip. The applied electric field causes the solution to exit the syringe in a cone-shape, called the Taylor cone, and forms a jet of polymer solution that is attracted toward the collecting plate. The majority of the solvent evaporates out of the solution before the jet contacts the collecting plate, although electrospun scaffolds are often exposed to a vacuum overnight to ensure that no residual solvent remains [81].

1.4.3 Cell Incorporation and Organization

In native arteries, SMCs are aligned circumferentially along the vessel wall and control dilation and constriction of vessels to regulate blood pressure and pump blood. The elongated morphology and aligned orientation of SMCs produce a contractile phenotype with presence of smooth muscle α-actin (α-SMA) to properly contract blood vessels [82-85]. Additionally, it is suggested that proper infiltration and alignment of cells in the scaffold can provide mechanical strength to the graft as cells are able to produce native ECM proteins such as collagen [86, 87]. Many groups have investigated
different options to align SMCs such as applying cyclic stretch [85, 88] and pulsatile perfusion [27, 84, 86, 87] bioreactors.

When a stretch bioreactor was applied to excised porcine aortic valves at 1.167 Hz (70 bpm), after 48 hours there was a 161.5% increase in collagen content (compared to a static control) as well as a significant up-regulation of α-SMA [85]. The increase in collagen content was either a result of increased collagen synthesis from the cells or decreased collagen degradation [85]. Another group seeded human coronary SMCs onto polyurethane scaffolds and cyclically stretched scaffolds 10% at 1 Hz for 4 weeks [88]. They found that cells were able to infiltrate the entire depth of the stretched scaffold while static scaffolds mainly had cells only present at the exterior regions [88].

Other groups prefer using pulsatile perfusion bioreactors to mimic the blood flow in native blood vessels in order to improve the properties of their graft. Syedain et al. seeded fibroblasts in fibrin gels around mandrels and after 2 weeks of static culture samples were transferred to a pulsed bioreactor where they were cultured for an additional 9 weeks [84]. They found that pulsed grafts produced scaffolds with 150% more collagen content and 73% higher burst pressure than non-pulsed grafts [84]. Additionally, another group used pulsatile perfusion on their EC and SMC seeded collagen-PLGA tubular scaffolds and observed increased nitric oxide (from ECs) and α-SMA (from SMCs) from pulsed scaffolds versus non-pulsed scaffolds after 3 weeks of culture [87]. Pulsatile perfusion was able to align the cells in the circumferential direction parallel to the direction of flow and histological examination using a
hematoxylin and eosin (H&E) stain revealed that cells on pulsed scaffolds were able to proliferate more than cells on their static counterparts [87].

While both cyclic stretch and pulsatile perfusion bioreactors have shown success by aligning SMCs, increasing cell proliferation, α-SMA production, collagen content, and mechanical strength, these methods are clinically impractical due to the time requirement of weeks to achieve these properties. Stickler et al. increased α-SMA and collagen in just 48 hours however, their study used excised tissue, rather than cell seeded scaffolds [86].

Thus another option to encourage alignment of SMCs is by creating microchannels that physically align the cells quickly. This micropatterning method may involve casting a polydimethylsiloxane (PDMS) mold followed by curing a polymer solution in the mold to create a polymer film having microchannels [82, 83]. An advantage of this technique is the ability to quickly align SMCs to induce a contractile phenotype with upregulated α-SMA in just 7 days [82]. However, a major limiting factor with micropatterning is the inability to use conventional lithography and micromachining with porous scaffolds such as electrospun fibers. Dong et al. were able to create microchannels on electrospun PLGA and PLCL scaffolds using UV lithography, but this method is extremely labor intensive and requires photomasks for patterning and several hours to degrade the scaffold. For this reason, we employed femtosecond laser ablation to rapidly and cleanly ablate microchannels in porous electrospun scaffolds [89].

Femtosecond laser ablation uses quick laser pulses that are able to cleanly remove electrospun scaffold without damaging surrounding fibers (Figure 1.9).
Figure 1.9 Femtosecond laser ablation is able to cleanly remove material in the scaffolds without melting or damaging surrounding fibers.

Electrospun PCL fiber morphology following both nanosecond laser ablation and femtosecond laser ablation were compared and melting was observed following exposure to the nanosecond laser, but not the femtosecond laser [90]. Femtosecond laser ablation is a simple single step process that can cleanly create microchannels in porous electrospun scaffolds while maintaining fiber morphology and porosity in surrounding fibers.
1.5 Proposed Tissue Engineered Blood Vessel

The envisioned engineered blood vessel builds on our expertise in tubular electrospinning [57, 60] to create a tri-layered scaffold composed entirely of synthetic biodegradable PCL. Electrospinning has the ability to create three distinct layers (Figure 1.10) by using different solvents, polymer concentrations, and electrospinning conditions.

Figure 1.10 Three distinct layers of electrospun PCL with different fiber size and porosity can be observed in the SEM image of the tri-layer structure.
The inner luminal wall is composed of 14 wt% PCL dissolved in acetone to create a dense layer of fibers that prevent ECs from penetrating through the scaffold after inoculation of the luminal surface. The goal is to create a uniform monolayer of ECs along this surface to prevent thrombosis in the blood vessel post-implantation. The middle layer (tunica media) is composed of 12 wt% PCL dissolved in DCM to create large diameter fibers and a porous layer to assist with SMC infiltration [91]. To encourage rapid SMC alignment in the circumferential direction, femtosecond laser ablation is utilized to create 100 μm wide and 100 μm deep channels down the length of the tube [60-62]. The final outer layer is composed of 5 wt% PCL dissolved in HFP. This layer will provide the bulk of the mechanical strength (i.e., burst pressure, tensile strength, and suture retention strength) thus these fibers are electrospun in the circumferential direction to create mechanically strong aligned fibers. A schematic of the tri-layer structure with distinct layers and ablated microchannels for rapid cell seeding is shown in Figure 1.11.
Figure 1.11 Schematic of creating the tri-layer scaffold with three distinct layers, ablated microchannels, and positions of where ECs and SMCs will be seeded.
1.6 References


2.1 Introduction

Coronary vascular disease is the leading cause of death in the United States. In spite of many medical advances, a critical need for synthetic vasculature remains. Over 80 million American adults have at least one type of coronary vascular disease and a coronary event occurs approximately every 25 seconds [1]. Additionally, more than 2 million Americans undergo cardiovascular surgery each year, many requiring coronary bypass grafting [2]. A major challenge associated with standard bypass procedures is that many patients, especially the elderly, do not possess vessels healthy enough for autologous transplantation [3]. In this context synthetic, polymer-based vasculature thus provides a promising alternative.

The challenge in creating synthetic grafts is a result of the multiple, often conflicting requirements they must meet: biocompatibility, mechanical properties, and ease of manufacture [4]. Current synthetic, small diameter (< 6 mm) coronary grafts frequently become occluded due to early thrombosis (blood clots) and late intimal hyperplasia (thickening of the inner wall of the vessel) [5]. To prevent thrombosis and
hyperplasia, a human endothelial layer can be formed on the luminal surface of the synthetic graft via inoculation of endothelial cells [6]. To possess the proper strength of adhesion and function, these cells must be grown on a substrate that inherently favors adhesion, organization and specific cell-cell communication. Studies have shown that the scaffolds upon which cells are seeded are the most important factor for subsequent in vivo success [7].

In addition to possessing biocompatibility and anti-thrombogenicity, well-designed scaffolds must also meet specific mechanical requirements to be effective. The requirement of 2000 mm Hg burst pressure is mandated by clinical concerns [6] as the arteries targeted for replacement typically have burst pressures above 2000 mm Hg [8]. Layering cell sheets has proven successful in creating burst pressures up to 3000 mm Hg along with minimal occlusions of the vessel [9]. However, this procedure requires weeks of cell culture to form the initial thin tissue sheets, followed by 8 more weeks of culturing to allow sheets to adhere to each other. Thus scaffolds which promote swift cellular organization and tissue maturation would be ideal.

In recognition of the need for multiple extracellular environments and cell types to comprise such burst pressure resistant, biocompatible synthetic vessels, we utilize our previously developed expertise in both tubular electrospinning [7] and laser ablation of electrospun fiber [10]. A trilayer tissue engineered vessel mimicking the structure and the properties of the extracellular matrix found within innermost tunica intima, the middle tunica media, and the outermost tunica adventitia of a normal blood vessel is envisioned. The tunica intima would consist of a low porosity layer to promote the
formation of a continuous, endothelial cell sheet lining the lumen. A high porosity layer with laser machined helical channels that allows smooth muscle cells to infiltrate the scaffold and promotes proper orientation, would be utilized to form the tunica media. Lastly, the outermost layer would provide the appropriate in vivo mechanical properties including burst pressure and integration into the host vessel.

To construct scaffolds that form the synthetic component of such tissue engineered vessels, electrospinning was employed as a well-established method for creating tissue engineering scaffolds [11-14] and direct-write femtosecond laser ablation were used. Polycaprolactone (PCL) was chosen because its biocompatibility and slow degradation rate in vivo allows sustained load bearing capabilities [15]. Additionally, the mechanical and microstructural characteristics of PCL scaffolds can be controlled by altering the solvents in which PCL is dissolved in prior to electrospinning [16, 17] and these processing variables can be exploited to create the desired trilayer construct. To produce microchannels on electrospun PCL fiber scaffolds, direct-write femtosecond laser ablation techniques were employed. Direct-write femtosecond laser ablation technique has been used only recently for microscale fabrication for biomedical applications [18] but has been shown to effectively generate linear grooves and microwells in PCL and PCL/gelatin nanofiber scaffold [10, 19]. While laser machining offers many benefits including programmable one-step processing and maintenance of sterility, the addition of laser machined microchannels in the middle layer (to assist cell infiltration) raises concerns that the strength and integrity of the resulting multi-layer scaffolds could be compromised.
The final realization of this idea will allow three-dimensional integration of cells within the wall of a tubular scaffold potentially \textit{during} a surgical intervention, thus eliminating the need for long-term cell culture. This process must also be highly predictable and reproducible. From a manufacturing viewpoint, this is challenging as the physical and biological properties of the ideal product will vary across the few hundred microns separating the inner and the outer walls of such tubes. These new capabilities in scaffold manufacturing require careful evaluation of the mechanical properties to ensure that these are preserved prior to the controlled integration of relevant cells. The present study investigates the mechanical properties of these tri-layer scaffolds. Tensile testing of these composites was performed to provide insight as to how the different electrospun layers interact with these deliberately introduced defects – and each other – by examining ultimate tensile stress, elongation, and Young’s modulus \((E)\).

2.2 Materials and Methods

2.2.1 \textit{Electrospun Polycaprolactone}

Three separate PCL solutions were prepared for electrospinning: (1) 14 wt\% PCL (Aldrich, Mw = 80,000) in acetone (Sigma-Aldrich) solution was prepared by heating the solvent and polymer to 60 °C with continuous stirring to dissolve the PCL; (2) 12 wt\% PCL in distilled (over calcium hydride (Aldrich)) dichloromethane (DCM) (Sigma-Aldrich) heated to 30 °C with continuous stirring to dissolve the PCL; (3) 5 wt\% PCL in
1,1,1,3,3,3-hexafluoro-2-propanol (HFP) (Aldrich) prepared by continuously stirring the PCL in solvent until dissolution. Hereafter, the 14 wt% PCL electrospun from acetone is referred to as acetone-derived, the 12 wt% PCL electrospun from dichloromethane is referred to as DCM-derived, and the 5 wt% PCL electrospun from HFP is referred to as HFP-derived.

Once these solutions were cooled to room temperature, they were loaded in 60-cc plastic syringes (BD, Luer-Lok) with 20-gauge blunt tip needles (EFD) and electrospun using a syringe pump (KD Scientific) and high voltage power supplies (Glassman). Both random and aligned fibers were created by electrospinning solutions onto a rotating mandrel with a 1.98 m circumference and 7.62 cm length attached to a drill and rotated at either 75 rpm or 450 rpm – linear speeds of 2.5 or 15 m/s – to obtain random or aligned fibers, respectively. Random acetone-derived and DCM-derived fibers were also electrospun onto stationary 3” x 3” (7.6 x 7.6 cm²) aluminum plates using the voltage potentials, flow rates, and source to ground (S-G) distances listed in Table 2.1. The length of time used for electrospinning was decreased to 5 minutes for acetone-derived and 10 minutes for DCM-derived when electrospun onto the plate. Random fibers electrospun on the mandrel at slow speeds were then compared to the random fibers electrospun on the stationary plate to ensure similar microstructures and a lack of alignment. After electrospinning, all samples were placed in a vacuum at room temperature overnight to ensure that residual solvents were removed [20-22].

To fully understand the behavior of the resulting trilayer structures, both single and bilayer electrospun sheets were fabricated. Bilayers were composed of a second
layer electrospun directly onto the first layer (acetone onto DCM or DCM onto HFP). To ensure that these layers did not delaminate from each other in the multilayer sheets post electrospinning, the S-G distance [23] could be shortened to 10 cm as needed (Table 2.1).

<table>
<thead>
<tr>
<th>Solution (wt % PCL/Solvent)</th>
<th>Voltage (kV)</th>
<th>Flow Rate (mL.h⁻¹)</th>
<th>S-G Distance (cm)</th>
<th>Mandrel Speed (m.s⁻¹)</th>
<th>Time (h)</th>
</tr>
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<tbody>
<tr>
<td>14/acetone</td>
<td>24</td>
<td>16</td>
<td>10</td>
<td>2.5</td>
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<tr>
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<tr>
<td>5/HFP</td>
<td>25</td>
<td>15</td>
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Table 2.1 Electrospinning Parameters

The effect of laser machined microchannels on mechanical properties was observed by adding either 1 or 5 microchannels (described in the next section) into the gauge length of the DCM layer of the tensile specimens. Care was taken to avoid intersecting the edges of the gauge length to avoid edge effects. For the acetone-DCM bilayer, a DCM single layer was electrospun, tensile specimen were then cut, the microchannels were laser machined and finally, the tensile specimen was remounted on the mandrel to electrospin the acetone layer on top of the microchannels. For the DCM-HFP bilayer and acetone-DCM-HFP trilayer, the HFP layer was first electrospun followed by DCM deposition on top of the HFP layer prior to cutting the tensile specimen. A schematic for the envisaged trilayer tensile specimen is shown in Figure 2.1.
Figure 2.1 (a) A schematic for the desired trilayer tensile specimen with microchannels and (b) microchannels laser machined in the DCM layer as seen with SEM. The insert in (b) shows the eventual position of the laser-machined microchannels within a final tubular structure.
To fabricate microchannels on electrospun PCL nanofiber scaffolds, frequency doubled pulses from a mode-locked erbium-doped fiber laser intensified in a Ti:Sapphire regenerative amplifier laser (CPA2161, Clark-MXR) were used. The laser wavelength was $\lambda = 775$ nm. The maximum average output power of the laser was $P_{av} = 2.5$ W, pulse duration was $T_p = 150$ fs, pulse repetition frequency was $f_P = 3$ kHz and collimated beam diameter was 5 mm. The beam quality factors were $M_2 = 1.2$ in the vertical Y direction and $M_2 = 1.3$ in the horizontal X direction. Laser beam power was adjusted by a series of thin-film polarizing beam splitters and $\frac{1}{2}$ waveplates. The attenuated laser beam was focused on the material with a 50x infinity corrected microscope objective lens having numerical aperture $NA = 0.42$ (M plan Apo NIR 50x, Mitutoyo). Attenuated average laser power was measured by a power meter (PM100, Thorlab) placed under the laser focusing lens. Sample motion and focus lens positioning was provided by a computer controlled motion system (MX80L, Parker) with 0.5 μm resolution in the X, Y, and Z axes. The system was programmed to scan the electrospun nanofiber scaffolds under the focused laser beam in order to ablate 1 or 5 microchannels on the prepared tensile specimen (Figure 2.1).

The chosen dimensions of the microchannels were 100 μm wide, 100 μm deep and 1600 mm long. The X-Y motion system scanned the sample with linear paths at a scanning speed of 0.33 mm/s with a laser pulse energy of 3.33 μJ. To ablate to the desired width and depth of 100 μm, two layers consisting of 40 linear paths were used. After ablating the first layer, the focus position was adjusted downward by 50 μm to ablate the second layer with the corresponding linear path spacing equal to 2.5 μm. For
the 5 microchannel specimen, this process was repeated with a 100 μm spacing between microchannels (Figure 2.1). This process can then be translated into a tubular format as shown in Figure 2.1(b) (inset).

2.2.2 Tensile Test

Tensile specimens were produced from as-spun polymer sheets using a 2 mm thick aluminum tensile specimen template with a 20 mm gauge length and 2.4 mm width. PCL sheets were placed in the template with fibers positioned longitudinally in the template and surgical blades (Bard-Parker No. 15) were used to cut out the straight edges of the tensile specimen, while 3 mm dermal punches (Acuderm Inc.) were used to cut the radii, ensuring that the PCL was not torn or stretched during cutting. All tensile specimen thicknesses were measured and recorded prior to tensile testing with a digital micrometer by placing the specimen between two glass microscope slides and subtracting thicknesses of the slides by the total measured thickness. The acetone layer had a thickness of 54 ± 23 μm, DCM was 224 ± 16 μm, and HFP was 113 ± 25 μm (thin HFP) or 236 ± 15 μm (thick HFP), all with a sample size (n) ≥ 6. Tensile data was obtained using a 222.4 N loadcell (model 31, Sensotec) and a strain rate of 2.5 mm/min on an Instron load frame (model 1322) with lightweight carbon fiber grips (A2-166 Fiber Clamp Assembly, Instron). An n ≥ 5 was utilized for all conditions.

Some tensile curves did not display a defined failure point and instead exhibited extreme extensions of 500-700% representing the behavior of only a few fibers [23]. In
these cases the elongation was estimated based on extrapolating the slope of the first significant decrease to the point of intersection with the x-axis (elongation).

2.2.3 Scanning Electron Microscopy (SEM)

The microstructure of all three component layers – including the laser machined microchannels – was observed with a SEM (XL30 ESEM) at accelerating voltages of 10 - 12 kV. All samples were placed on carbon tape that was applied to aluminum SEM sample mounts (SPI Supplies) and then sputter coated (Pelco Model 3 Sputter Coater 9100) with ~100 angstroms of gold over a period of 60 s using an emission current of 15 mA.

2.2.4 Statistical Analysis

To determine the presence or absence of statistical difference between data sets, ANOVA and Tukey t-tests were performed using GraphPad InStat (version 3.06). P-values < 0.05 were considered statistically significant. All values are reported in mean ± standard deviation.
2.3 Results

2.3.1 PCL Single Layers

Random acetone fibers electrospun on a mandrel rotating at 2.5 m/s were compared to those electrospun on a stationary plate, both electrospun with S-G distances of 10 and 20 cm. Ultimate tensile strength (UTS), elongation, and Young’s modulus (E) of the acetone-derived fiber either remained constant or increased when spun on a rotating mandrel (Figure 2). The changes in UTS, E, and elongation for samples electrospun at a S-G distance of 20 versus 10 cm were not significant although this may have partly been governed by slight variations in layer thickness. However, elongation increased significantly when acetone-PCL was spun onto a rotating mandrel at a S-G distance of 10 cm (Figure 2.2; p < 0.001).
Figure 2.2 Mechanical properties of PCL single layers. Data points are labeled by solvent used (A = acetone, D = DCM, H = HFP), collecting ground (P = plate, M = mandrel), S-G distance (10 = 10 cm, 20 = 20 cm), number of channels (no channels = 0, 1 channel = 1, 5 channels = 5), and thickness of HFP for HFP layers (H = thin HFP, HH = thick HFP). For example, ‘AP20’ = acetone fibers electrospun onto a plate with a S-G distance of 20 cm.

Tensile data of single layer DCM scaffolds electrospun on the stationary plate were compared with those produced on the mandrel and no significant differences were
observed (Figure 2.2). However, there was a significant difference in the character of the tensile curves when comparing deposition on a plate to deposition on a mandrel. Samples from the plate failed gradually and did not exhibit a clear point of failure; in contrast, samples from the mandrel had an obvious point of failure (Figure 2.3(a) and (b)).

Figure 2.3 Failure of DCM fibers dramatically changes from (a) electrospun on a plate to electrospun on a mandrel with (b) no microchannels to (c) 1 microchannel to (d) 5 microchannels. Note that in (a) the x-axis extends out to 1400% elongation.
The presence of microchannels in the DCM layer was examined and no statistical difference was observed in UTS and E for as-spun DCM without microchannels when compared to the DCM containing 1 and 5 laser machined microchannels. However, a significant (p < 0.001) decrease in elongation was evident with 1 and 5 microchannels (Figure 2). The DCM tensile specimen containing 1 laser machined microchannel exhibited variable tensile behavior: 2 samples behaved similar to the original matrix without any laser machined microchannels, however, 5 other curves were dramatically affected by the presence of the microchannel and showed a gradual decrease in failure (Figure 2.3). The graphical data showed that all samples in the 5 microchannel group exhibited failure similar to each other without the appearance of the second linear portion that occurred in all of the DCM samples without microchannels (Figure 2.3(a)).

The tensile data illustrated that the thickness of the HFP layers electrospun on the mandrel at 15 m/s greatly influenced mechanical behavior. As thicknesses increase from 113 μm to 236 μm, the UTS increased by a factor of 7.5%, elongation by a factor of 2.6, and E by a factor of 2.92 (Figure 2.2). Tensile curves showed that failure occurred in a manner different than that of DCM samples (but similar to acetone fibers) in that the fibers failed abruptly instead of gradually (Figure 2.4).
Figure 2.4 Thickness of the HFP layers dramatically affects mechanical properties. ‘Thin’ (~113 µm) HFP layers fail before 30% elongation while ‘thick’ (~236 µm) HFP fibers fail after 60% elongation.

2.3.2 PCL Bilayers

Mechanical properties of acetone-DCM bilayers are shown in Figure 2.5. For the acetone and DCM bilayers, the initial use of a S-G distance of 20 cm [23] caused the acetone layer to delaminate from the DCM layer and resulted in consistent failure (of the acetone layer) at strains of only 137 ± 44% (Figure 2.5). As this was clearly not consistent with the goal of composite behavior, the S-G distance was decreased to 10 cm to deliberately cause fiber-fiber bonding at the interface. This bonding was intended to avoid delamination of the acetone layer from the DCM layer. Figure 2.5 shows that this decrease in S-G distance successfully delayed failure of the
acetone layer out to 336 ± 95% with little change in the final UTS, elongation, and E of the remaining DCM layer.

![Graph showing engineering stress vs. elongation](image)

Figure 2.5 The acetone layer fails at larger strains when the S-G distance is decreased from (a) 20 cm to (b) 10 cm and the presence of (c) 1 microchannel and (b) 5 microchannels impacts fiber failure.

The addition of laser machined microchannels into the DCM component of the bilayers did not significantly change UTS or E (Figure 2.6), however, elongation was significantly different for bilayers without microchannels versus those with either 1 or 5 microchannels. Graphically, the 1 microchannel bilayer tensile curves exhibited the same
slow gradual decrease to failure seen in tensile curves for DCM single layers containing 5 microchannels. However, the samples without microchannels and those with 5 microchannels behaved differently, exhibiting an abrupt failure (Figure 2.5).

Mechanical behavior was observed for HFP and DCM bilayers at two thicknesses of HFP: 113 ± 25 μm (‘thin’ HFP) and 236 ± 15 μm (‘thick’ HFP). The mechanical behavior of the thin HFP bilayers were predominantly dominated by the DCM layer as evidenced by the observation that the HFP layer failed in multiple locations along the gauge length while the DCM layer continued to experience strain. No delamination was observed between the HFP and DCM layers. Several of the bilayer samples displayed failure similar to that of DCM single layers alone, while others failed at relatively lower total elongations. The results for UTS, elongation, and E between samples without microchannels versus with 1 or 5 microchannels were similar with no apparent statistical difference (Figure 2.6).
Failure of the thick HFP-DCM bilayers was drastically different than that of the thin HFP bilayers (Figure 2.6). In the absence of microchannels, the bilayer showed true composite failure originating first in the HFP layer followed by gradual extensional...
failure characteristic of the DCM single layer. The HFP layer first exhibited mechanical properties very similar to those of the HFP layer alone while the remaining DCM layer displays elongation statistically identical to that of DCM alone. The HFP-DCM bilayer has an UTS of $5.22 \pm 0.84$ MPa and $E$ of $21.98 \pm 2.68$ MPa with HFP failure occurring at $48 \pm 3\%$. Failure of the DCM layer occurs at $2.05 \pm 0.31$ MPa which is slightly higher than the DCM alone and extends to $638 \pm 86\%$ elongation. With the addition of 1 and 5 microchannels, the bilayer mechanical behavior was surprisingly similar to that of the HFP and DCM bilayer without microchannels. Graphically, the DCM layer remaining after failure of the HFP layer was weaker than it was in the absence of microchannels. While there was no statistical difference in UTS and elongation of the samples with 1 microchannel or 5 microchannels, a decreasing trend of values (Figure 2.6) was observed. No statistical difference was present in the elongation for HFP, however, the elongations for DCM were statistically different between samples without microchannels and 1 microchannel ($p < 0.05$) or 5 microchannels ($p < 0.001$) samples.

2.3.3 PCL Trilayers

Acetone-DCM-HFP trilayers displayed a broad range of mechanical behaviors showing that the initial point of failure was statistically indistinguishable from each other in either the presence or absence of microchannels (Table 2.2 Mechanical properties of trilayers and Figure 2.7). The overall behavior of the trilayers was very similar to that of the HFP-containing bilayers regardless of the presence or absence of the acetone layer.
Graphically, we can observe that the presence of microchannels weakens the remaining DCM layer but this is visible only after the failure of the acetone and HFP layers (Figure 2.8). As before, the HFP layer dominates the overall behavior and the effectively useful properties of these composites.

<table>
<thead>
<tr>
<th>HFP Thickness (μm)</th>
<th>Channels (n)</th>
<th>UTS (MPa)</th>
<th>Elongation (%)</th>
<th>E (MPa)</th>
</tr>
</thead>
<tbody>
<tr>
<td>113</td>
<td>0</td>
<td>1.02 ± 0.20</td>
<td>404.77 ± 83.93</td>
<td>5.88 ± 0.53</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>1.18 ± 0.10</td>
<td>373.86 ± 153.66</td>
<td>7.19 ± 1.08</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>0.94 ± 0.14</td>
<td>293.52 ± 172.03</td>
<td>5.88 ± 0.74</td>
</tr>
<tr>
<td>236</td>
<td>0</td>
<td>4.47 ± 0.64</td>
<td>45.40 ± 6.80 (HFP)</td>
<td>18.85 ± 1.61</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>4.94 ± 0.40</td>
<td>48.33 ± 4.97 (HFP)</td>
<td>22.24 ± 3.11</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>4.85 ± 0.56</td>
<td>47.91 ± 3.28 (HFP)</td>
<td>21.93 ± 2.47</td>
</tr>
</tbody>
</table>

Table 2.2 Mechanical properties of trilayers.
Figure 2.7 Mechanical properties of PCL trilayers. Data points are labeled by solvents used for layers (ADH = Acetone-DCM-HFP), fiber layer failure for % elongation plots (D = DCM failure, H = HFP failure), thickness of HFP for HFP layers (H = thin, HH = thick), and number of channels (no channels = 0, 1 channel = 1, 5 channels = 5). For example, ‘ADH0’ = acetone-DCM-‘thin’ HFP trilayers with 0 microchannels.
Figure 2.8 HFP thickness has a significant impact on the failure of these trilayers as demonstrated in (a) trilayers with ‘thin’ HFP and (b) trilayers with ‘thick’ HFP.
2.4 Discussion

Femtosecond laser ablation is a unique tool allowing precision machining of polymer-based scaffolds, producing complex flow channel networks in electrospun scaffolds while preserving the underlying nanofiber structure [10]. By creating two- and three-dimensional patterns, favorable cell-cell interactions can be achieved. To best enable this manufacturing focus, we must develop the scientific knowledge needed to design and fabricate scaffolds with fully reproducible surface features at resolutions approaching 10 μm. The diameters and depths of desired helical passages and cavities are large compared to the diameter and the depth of material removed by a single pulse. Thus ablation must be incremental – as it is in this work – with each individual layer being ‘machined’ by pulses of laser energy that intersect (‘overlap’) in the axial and circumferential motion directions as individual laser pulses (typically 20 – 50 μm in diameter and depth) are superimposed to produce the overall cavity. In the eventual design, helical flow channels will circle around the tube and have cross sectional sizes of approximately 100 μm by 100 μm. Knowledge of the variation of strength in the presence of these microchannels is especially important because it can determine the size of the channels within the “tunica intima layer.” The process relationships derived during this initial work provide a starting point for this process study.
2.4.1 PCL Single Layers

The changes in UTS, elongation, and E observed in DCM electrospun fibers spun onto the mandrel rotating at slow speeds (2.5 m/s) versus the stationary plate are likely the result of decreased fiber-fiber bonding and slight differences in thickness (Figure 2.2). No alignment of these fibers [7, 23-27] was observed at these slow speeds. This comparison of deposition methods is critical to our long-term goal of fabricating tubular structures containing microchannels within the walls. To our knowledge, such tubes can only be fabricated by deposition onto a rotating mandrel. In a stationary plate, solvent vapors likely become trapped by successively arriving fibers during the electrospinning process and these trapped vapors can increase fiber-fiber bonding of relatively soft, as-deposited fibers. However, on a mandrel rotating at 2.5 m/s, 0.79 seconds separate each rotation allowing opportunities for more efficient solvent evaporation before additional fiber deposition occurs. Figure 2.9 shows SEM images of acetone-DCM fibers pulled to ~80% strain demonstrating fiber-fiber bonding in fiber arrays electrospun onto the stationary plate. Electrospun fiber arrays typically achieve larger tensile values when less fiber-fiber bonding exists because the fibers can move independently of each other [25]. The characteristic surface porosity of the DCM fibers [16, 17] is also visible.
Figure 2.9 SEM of fiber-fiber bonding present when acetone fibers (non-porous) are electrospun onto DCM fibers (porous) on after the composite has been extended to ~80% strain and ‘frozen’ into position [7].

The observation of no significant differences between the tensile data for random acetone fibers with a decreased S-G distance of 10 cm coincides with the work of Gaumer et al. in which fibers collected on a stationary plate exhibited less differences in tensile data at S-G distances of 10, 15, and 20 cm [23]. Random fibers deposited on the mandrel also exhibited no significant differences in tensile data because the mandrel was rotating at a speed of only 2.5 m/s. By decreasing the S-G distance, more fiber-fiber bonding occurs as less time is available for the solvent to evaporate before additional
fiber deposition. Fibers collected on a stationary plate follow this trend however, those collected on the mandrel do not.

Similar to the acetone results, the UTS and E of DCM fibers increased when the fibers were deposited on the rotating mandrel as a result of decreased fiber-fiber bonding. The elongation was slightly lower, but not significantly, when DCM fiber was collected on the mandrel.

With little statistical difference between the UTS and E of samples with and without 1 microchannel, electrospun DCM fibers clearly have some degree of damage tolerance. This is likely due to the fact that existing as-spun flaw sizes within these fiber arrays approach those of the microchannels; this explains the lack of sensitivity. Overall, the 1 microchannel samples behaved similarly to those fabricated without any microchannels. When 5 microchannels are ablated into the DCM fibers, decreasing UTS, elongation, and modulus were observed, implying that at some critical density these microchannels can compromise the mechanical properties of individual layers.

HFP thickness has a significant effect on mechanical properties, following the work of Drilling et al. [7]. Figure 2.2 Figure 2.4 show UTS, elongation, and E as a function of HFP thickness and these mechanical properties increase as thickness increases.
2.4.2 PCL Bilayers

Decreasing the S-G distance in acetone from 20 to 10 cm in the acetone-DCM bilayers showed that engineering the interface between two different fiber layers by controlling S-G distance offers clear benefits. The marked increase in elongation when the S-G distance was decreased proved that deliberate fiber-fiber bonding at the interface allowed the acetone layer to better elongate in tandem with the DCM layer before failing.

Tensile data suggested that the addition of a single microchannel into the DCM component of the acetone-DCM bilayer did not greatly affect mechanical properties. This is expected as similar results were evident in DCM alone. The presence of 1 or 5 microchannels did not significantly affect the values of mechanical properties, however, graphically the failure was very different between samples with 1 microchannel and samples with 5 (Figure 2.5). In both cases, samples failed at a steady decline for 1 channel samples; failure occurred more abruptly for 5 channel samples. The greater resistance of the 5 channel specimens is likely due to the slightly thicker DCM layer (Figure 2.5). Additionally, Figure 2.10 shows acetone fibers aligning when electrospun over a channel however, remaining unaligned when fiber-fiber bonded to DCM fibers. This accounts for the abrupt failure observed with 5 microchannel acetone-DCM bilayers.

For the HFP-DCM bilayers, the DCM layer dominated during strain when the HFP layer was ‘thin’ (~113 µm). However, continued adherence of the HFP layer to the DCM layer even after its failure apparently served to concentrate stress in the DCM layer and led to its ‘early’ failure. When the HFP layer was ~230 µm thick, the HFP
dominated, providing much higher values of UTS, elongation and modulus. This again demonstrates that thickness – a typically unappreciated variable – has dramatic effects on mechanical properties.

![Figure 2.10](image)

Figure 2.10 Acetone fibers align over microchannels in the DCM layer, but remain unaligned when bonded to the DCM fibers when the acetone layer of the acetone-DCM bilayer is strained to ~ 80%. Channels are observed as the darker horizontal bands (ch).

2.4.3 PCL Trilayers

The addition of an acetone layer to the DCM-HFP bilayer (Figure 2.7) did not compromise any of the previously observed mechanical properties of the composite electrospun bilayers (Figure 2.6). In addition, the presence of microchannels also did not compromise mechanical properties of the scaffold. The DCM component of the trilayer failed earlier in the presence of microchannels however, this was not a concern since the mechanical properties provided by the HFP were sufficient to compensate for the early failure of the DCM layer.
2.5 Conclusions

Electrospun trilayer scaffolds were successfully created using acetone, DCM and HFP as the solvent for each of the three layers. Scaffolds were strained to failure to compare the relative mechanical properties of single layers, bilayers, and trilayers with and without laser ablated microchannels. Furthermore, the effect of having a ‘thin’ versus a ‘thick’ layer of HFP was investigated. A general theme suggests that tight control over the thickness of the individual layers will be necessary to successfully engineer load-bearing biomedical composites. Although the presence of laser machined microchannels sometimes had substantial effects on single layers and some of the bilayers investigated, in the ‘final’ trilayer little effect on UTS, elongation, and E was observed as the thickness of the HFP dominated the overall properties regardless of the nature of the other two layers. This clearly suggests that the concept of “vascular wall engineering” could potentially be successful in producing trilayer composite structures that can maintain the targeted mechanical properties while allowing for subsequent cell seeding. The next step in the process is to conduct burst pressure experiments of the final trilayer tubes to ensure that they can withstand burst pressures > 2000 mmHg which should translate into the necessary in vivo abilities.
2.6 References


Chapter 3: Fatigue

3.1 Introduction

Arterial tissue exhibits an impressive combination of high burst strength, elasticity, and fatigue resistance [1]. On average, human coronary arteries expand 8.2% and are exposed up to 120 mmHg of pressure per pulse [2]. With an average resting heart rate of 72 bpm, arteries will have experienced 3 billion cycles during an 80 year life. Tissue engineered vascular grafts suitable for clinical transplantation must avoid vessel dilation and creep which can cause catastrophic failure prior to replacement by native tissue.

Prior studies have examined burst pressure, compliance, and elasticity of tissue engineered blood vessels. However, scaffold fatigue is typically overlooked. Vascular grafts are often implanted into small animal models without prior observation of the effects of cyclic mechanical loading [3-5]. Mechanical assessment of grafts in vivo is important and beneficial but can be costly and time consuming if the graft fails immediately post implantation. One method improving the chances of successful in vivo testing is to evaluate fatigue of samples in vitro.
Groups that have examined graft fatigue properties typically seed cells prior to testing and understanding properties of the scaffold alone [6, 7]. Konig et al. fabricated vessels made from fibroblast cell sheets and evaluated effects of dynamic and static fatigue on burst pressure [6]. For dynamic fatigue studies, the pressure in a closed flow loop was cycled at 1 Hz for either 14 days at 120/80 mm Hg or 3 days for 600/300 mm Hg while static conditions maintained pressure at 250 mm Hg for 5 days. After fatigue, all samples were burst to failure and compared to unloaded controls. No statistical difference was observed between fatigued and unloaded samples. Wang et al. evaluated fatigue of tissue engineered vessels comprised of induced smooth muscle cells seeded onto unwoven polyglycolic acid fiber sheets [7]. These sheets were wrapped around silicone tubes in bioreactors in which PBS was pumped through the tubes at a pulsatile flow of 75 bpm. Flow rates started at 70-80 mL/min and gradually increased to 100-120 mL/min creating a radial distention of as much as 5%. The pulsatile strain improved mechanical properties (ultimate strength, elastic modulus, suture retention strength, and burst pressure) significantly compared to static strains however, the mechanical properties were only ~60% of those of human saphenous veins.

Synthetic polymer-based grafts constitute a promising alternative to autografts and cell sheet based therapies as their strong mechanical properties can withstand the high blood pressures experienced in vivo. A synthetic biodegradable polymer, such as polycaprolactone (PCL), is ideal because PCL is biocompatible, highly elastic, and degrades slowly in vivo [8-11]. Klouda et al. performed in vitro studies to better understand the mechanical and structural effect of fatigue on electrospun PCL and found
that testing sheet samples under in vivo conditions (gradually increasing strains from 2 to 10% over a period of 15 days at a 1 Hz frequency) showed no visual degradation within the scanning electron microscope (SEM) but the mechanical properties decreased following 15 days of exposure [12].

To reduce manufacturing time, we used electrospinning in conjunction with direct-write femtosecond laser ablation to engineer tubular scaffolds for vascular grafts to provide sufficient burst pressure and helical “inoculation channels” for directed smooth muscle cell (SMC) seeding. Use of different solvents can create electrospun fibers having vastly different microstructures and mechanical properties [9]. We previously examined the effect of femtosecond laser-ablated microchannels and discovered they did not necessarily disrupt the mechanical strength of tri-layered electrospun PCL scaffolds and ensured that cells are able to be seeded within them [9,12]. By demonstrating both desirable mechanical strengths and cell seeding, we are now interested in investigating the effects of fatigue on these scaffolds.

The current study examines the fatigue strength of single, bi-, and tri-layered scaffolds (Figure 3.1). All scaffolds were mechanically stimulated by applying cyclic tension at 10 and 25% strain at 1 Hz for 1 and 7 days followed by tensile testing to failure. Additionally, load with respect to position was collected in real time to quantify decay in load accumulation and any delay in force recovery. Macroscopic and microscopic structural analysis was performed on the samples before and after cyclic strain via SEM and image analysis to quantify changes in scaffold dimension, fiber diameter, and fiber orientation.
Figure 3.1 Electrospun PCL (a) tri-layer and single layers derived from (b) acetone, (c) DCM, and (d) HFP.
3.2 Materials and Methods

3.2.1 Electrospun Polycaprolactone

Three different solvents were used to create the three single layer scaffolds. 14 wt% PCL (Aldrich, $M_w = 80,000$) was dissolved in acetone (Sigma-Aldrich) (Figure 3.1(b)) by heating the solution to 60 °C. After cooling to room temperature, the solution was loaded into a 60-cc plastic syringe (BD, Luer-Lok) with a 20-gauge blunt tip needle (EFD) and electrospun using a high voltage power supply (Glassman) and a syringe pump (KD Scientific). Electrospun PCL fibers were collected onto a grounded 7.6 x 7.6 cm$^2$ aluminum plate at a source to ground (S-G) distance of 20 cm [14] for 30 s, a syringe pump flow rate of 16 mL/h, and a voltage potential of +24 kV. 12 wt% PCL was dissolved in distilled (over calcium hydride (Aldrich)) dichloromethane (DCM) (Sigma-Aldrich) heated to 30 °C with continuous stirring. Once dissolved, the solution was loaded into a 60-cc plastic syringe and electrospun with the following conditions: a S-G distance of 30 cm, a deposition time of 6 min, a flow rate of 15 mL/h, and a voltage potential of +23 kV. Lastly, 5 wt% PCL was dissolved in 1,1,1,3,3,3-hexafluoro-2-propanol (HFP) (Oakwood Products, Inc.) continuously stirred at room temperature. The fibers in this scaffold were aligned by electrospinning for 2 hours onto the circumference of a 57 cm diameter mandrel rotating at ~500 rpm (linear velocity ~15 m/s) with a S-G of 20 cm and a voltage potential of +25 kV. Two syringes were filled with solution and placed in a double syringe pump at a flow rate of 15 mL/h to expedite electrospinning.
For the bi-layered scaffold, 14 wt% PCL was dissolved in acetone (Figure 3.1(b)) and electrospun on top of the layer formed using 12 wt% PCL in DCM (Figure 3.1(c)). The S-G distance was increased from an initial distance of 10 to 20 cm during electrospinning via the use of a laboratory jack (VWR) to prevent delamination of the two layers. These were electrospun with the same conditions mentioned previously.

For the tri-layered scaffold (Figure 3.1(a)), first 5 wt% PCL was dissolved in HFP (Figure 3.1(d)) and electrospun onto bi-layered scaffolds adhered to the rotating mandrel to again create aligned fibers, this time in the third layer. The S-G distance was again increased from 10 to 20 cm during electrospinning to prevent layer delamination with all PCL solutions electrospun as previously described. After electrospinning, all samples were placed in a vacuum at room temperature for 12 hours to ensure that all residual solvents were removed [9, 14, 16].

3.2.2 Cyclic Mechanical Stimulation

Electrospun single layered, bi-layered, and tri-layered scaffolds were cut into 10 mm by 45 mm strips and placed into a custom-made, 12-sample bioreactor allowing for fatigue prior to tensile testing to failure. The samples were cut into strips and placed individually into a tensile tester mounted within a custom-made bath for real time mechanical analysis (Figure 3.2). All samples were mechanically stimulated at 10 or 25% strain at a rate of 1 Hz for 1 and 7 days in 37 °C PBS (Sigma) containing 0.2% sodium azide (Aldrich) to prevent bacteria growth. As a control, samples were placed in
a PBS solution containing 0.2% sodium azide at 37°C for 1 and 7 days without applied strain (0%). The PBS solution was continuously flowed through the bioreactor at 2 ml/min.

Figure 3.2 Setup of samples inside a custom made biobath attached to the tensile tester. Heated PBS continuously flowed through the bath.
3.2.3 Mechanical Properties

After cyclic mechanical stimulation in the 12-sample bioreactor, all samples were rinsed in distilled water for 1 h and cut into tensile specimens using a 2 mm-thick 310 stainless steel tensile specimen template having a 20 mm gauge length and a 2.4 mm gauge width. Wet scaffolds were loaded into the templates and cut out using surgical blades (Bard-Parker No. 15). Each of the conditions (0%, 10%, 25% for 1 and 7 days) for each of the scaffolds types (single, bi-, and tri-layered) had an \( n \) of at least 5. All tensile specimen thicknesses were measured and recorded prior to tensile testing with a digital micrometer. Tensile data was obtained using a 222.4 N load cell (model SM-50-294, TestResources) and a strain rate of 5 mm/min on a TestResources load frame (model 1000R12, TestResources) to determine ultimate tensile strength (UTS), elongation to failure, and modulus (E).

Data was collected from the real time mechanical analysis 30 times per second for 10 seconds every 24 hours for 7 days to create hysteresis curves describing each sample. This data was plotted to observe changes in the stress-strain curves for all sample conditions over 7 days. Additionally, energy dissipation data was evaluated by calculating the area of hysteresis curves at each time point using MATLAB (version R2012b) software.
3.2.4 Scanning Electron Microscopy

The microstructure of the electrospun single, bi-, and tri-layer specimens was assessed before and after cyclic mechanical stimulation using the SEM (Sirion, FEI) in secondary imaging mode at 5 kV. All samples were dried in air prior to mounting on aluminum SEM sample stubs using double sided carbon tape (Ted Pella). Samples were then sputter coated (Pelco Model 3 Sputter Coater 9100) with gold for 60 s using an emission current of 15 mA.

To image the cross-section of the single, bi-, and tri-layer scaffolds the samples were placed in liquid nitrogen for at least 5 minutes before cutting using a new knife blade. Samples were angled on the mounts with carbon tape, sputter coated with gold, and imaged in the SEM as previously described.

3.2.5 Statistical Analysis

SigmaPlot (version 12.0) was used to evaluate statistical difference between data sets by performing a One Way ANOVA with a Tukey post-hoc test. P-values less than 0.05 were considered statistically significant and values are reported as mean ± standard deviation.
3.3 Results

3.3.1 Single Layer Cyclic Mechanical Stimulation

Electrospun PCL fibers derived from acetone showed no significant changes in UTS and E following mechanical stimulation between 10 and 25% cyclic strain for 1 and 7 days versus controls (Figure 3.3 (a)). However, 25% strain resulted in a significantly lower elongation to failure compared to the controls. SEM image analysis revealed no obvious change in microstructure due to mechanical stimulation (Figure 3.4). Tensile data of cyclically strained single layer fiber scaffolds derived from both DCM and HFP showed no statistically significant changes in UTS, elongation, and E compared to the controls (Figure 3.3 (b) and (c)). This is confirmed by SEM in which no changes in microstructure observed for both scaffold types (Figure 3.4).
Figure 3.3 Tensile data for electrospun PCL single layer scaffolds pulled to failure for fibers derived from (a) acetone, (b) DCM, and (c) HFP.
3.3.2 Bi-layer Cyclic Mechanical Stimulation

Electrospun PCL fibers derived from both acetone and DCM were used to create the bi-layered samples. No statistical difference of UTS and elongation was observed as a result of mechanical stimulation to 10 and 25% strain for 1 or 7 days compared to controls. Modulus, however, significantly decreased after 7 days of PBS exposure in the controls compared to just 1 day of exposure. However, modulus significantly increased after 7 days of mechanical stimulation following 10% strain versus the 1 day of 10% strain, and was not statistically different in the 25% strain condition of 7 days versus 1 day (Figure 3.5). SEM imaging revealed that the microstructure of the fiber bi-layers are not vastly different following fatigue (images not shown).
Figure 3.5 Tensile data for electrospun PCL bi-layer scaffolds pulled to failure for fibers derived from both acetone and DCM.

### 3.3.3 Tri-layer Cyclic Mechanical Stimulation

Tri-layered samples were created by combining electrospun PCL fiber layers derived from acetone, DCM, and HFP. Statistical differences were observed in UTS for both the 1 and 7 day fatigued 25% strain values versus the 7 day control sample. Initial elongation to failure values were significantly decreased after mechanical stimulation compared to controls. Final elongation values were statistically identical following 7 days of 25% strain. Two elongation to failure values are reported because the aligned HFP layer fails first, creating a significant drop in the stress-strain curve. The other layers continue to elongate until complete failure of the tri-layer, constituting the second elongation value (Figure 3.6). The initial break in HFP is evident in SEM imaging where
holes are present in the HFP layer following exposure to 25% strain for 7 days (Figure 3.7). Additionally, modulus values all increased following fatigue compared to controls. SEM was only able to image the fibers derived from acetone and HFP as the DCM layer was ‘sandwiched’ between them and not visible.

Figure 3.6 Tensile data for electrospun PCL tri-layer scaffolds pulled to failure after cyclic strain.
3.3.4 Real-Time Mechanical Analysis

All scaffold types underwent energy dissipation analysis to observe how the samples changed over the 7 day period. Energy dissipation was calculated from the hysteresis curves. Generally, there was a significant drop (~4-5 times lower) in energy dissipation from the initial day 0 starting point to the following day 1 point. However, little significant change was observed between days 1 and 7 (Figure 3.8).
3.4 Discussion

3.4.1 Scaffold Mechanical Stimulation

Single layer scaffolds did not show any change in mechanical properties post strain with the exception of 25% strained acetone derived scaffolds. This implies that PCL single layer fibers did not degrade between 1 and 7 days of testing and could withstand continuous mechanical stimulation at 1 Hz with little change in properties.

For bi-layer scaffolds of acetone and DCM-derived fibers, no significant changes in UTS or elongation post fatigue were observed compared to controls. However, the observed decrease in E was unexpected. As fibers are cyclically strained, the polymeric
view of electrospun scaffolds [10] suggests that they would be expected to rearrange in the
direction of strain, ultimately increasing $E$ as more fibers carry load without
translation before failure. However, the observed decrease in $E$ could also result from
slight delamination of the two layers during mechanical stimulation. While the acetone-
and DCM-derived scaffolds appeared adhered together macroscopically, each layer has
its own specific mechanical properties. These differences may originate from broken
fiber-fiber bonds present in the control samples.

Tri-layer scaffolds experience an increase in UTS when cyclically strained to 25%
most likely as a result of the fibers rearranging in the direction of strain. This refers
specifically to the randomly aligned acetone and DCM-derived layers but could also
cause the aligned HFP-derived layer to become even more aligned. During fatigue, this
alignment could allow the tri-layer scaffold to withstand even higher forces before
failure.

Failure occurred at two points: (1) the initial elongation to failure in which the
HFP-derived layer fails followed by (2) the final elongation point and complete scaffold
failure. The initial elongation of mechanically stimulated scaffolds results in earlier
failure than the controls. This supports the idea that these fibers might rearrange in the
direction of strain. As fibers become aligned during fatigue, there is less potential for
fiber motion during testing to failure, resulting in scaffolds that fail earlier than non-
fatigued samples. The same must hold true for the final elongation of samples strained to
25% for 7 days as their elongation values were significantly lower than all other
conditions. Lastly, modulus was significantly increased after mechanical stimulation
compared to the non-fatigued control samples. This is appropriate because if the fibers rearrange in the direction of strain, the overall scaffold can carry higher loads in the direction of alignment since more fibers are now oriented in that direction. Consequently, the modulus increases since stronger scaffolds are also stiffer.

3.4.2 Real Time Mechanical Analysis

The observed decrease in energy dissipation between the initial mechanical stimulation and fatigue days 1 through 7 was not anticipated. It was discovered that this phenomena occurred because scaffolds were loaded into the tensile tester with room temperature PBS rather than being pre-heated to 37 °C. As the PBS solution increased to body temperature between day 0 (initial) and day 1, it was assumed that fiber resistance decreased. This resulted in the observed substantial changes in hysteresis curves. This theory was tested by loading a sample in PBS heated to 37 °C prior to collecting hysteresis data; it was found that the initial drop observed from previous hysteresis curves was not present.

3.5 Conclusion

The fatigue properties of arterial tissue are extremely important to predict in vivo success. Mechanical stimulation to strains of 10% (similar to those found in the native environment) and 25% (an admittedly extreme situation) were compared to non-fatigued
controls after 1 and 7 days of fatigue. General trends suggest that UTS and modulus increase while elongation decreases as a result of fibers rearranging during strain in the direction of alignment. Future work should involve examining the effect of fatigue in the two-dimensional platform with the addition of ablated cell-guiding microchannels and ultimately three-dimensional platform in which physiologically relevant 3 mm tubes will be evaluated.
3.6 References


4.1 Introduction

Native blood vessels experience many difference forces *in vivo* and these forces must be considered and evaluated in order for tissue engineered blood vessels (TEBV) to function properly and successfully. Vascular grafts must meet specific mechanical requirements such as tensile strength, burst pressure strength, and suture retention strength (SRS). While the tensile and fatigue properties of our grafts have been evaluated in Chapters 2 and 3, respectively, in this chapter more clinically-specific factors known as burst pressure and SRS are examined.

Having the appropriate burst pressure in TEBV is critical because graft rupture can result in aneurysms, hemorrhage, and death. Conversely, if burst pressure is too high this is typically associated with compliance mismatch and can ultimately result in eventual intimal hyperplasia and graft occlusion. On average, the systolic blood pressure for healthy adults is 120 mm Hg of pressure per pulse; those diagnosed with hypertension may exhibit blood pressures above 180 mm Hg. However, to ensure a large safety factor, the imposed requirements of 2000 mm Hg for replacement vessels are mandated by both clinical concerns and the fact that autografts (most commonly the saphenous vein (SV))
and internal mammary artery (IMA)) possess burst pressures around 2000 mm Hg or higher [1].

These levels of burst pressure have been achieved via biological methods (i.e., mechanical stimulation, long term culture) (Figure 4.1) [1]. However, these methods can require months to mature in vitro and are both costly and impractical from both the manufacturing and the clinical viewpoints.

Additionally, the SRS of TEBVs are of considerable interest to vascular surgeons because, despite new methods for anastomosis [2] of TEBVs to native vessels, sutures remain the most commonly used method [3]. Clinically, the SRS needs to be at least 2.0 N or greater [4, 5]. However, groups have used SRS as low as 50 gmf (0.49 N) as a criteria allowing for further clinical trials [1]. This research focuses on an analysis of
different PCL fiber orientations, thicknesses and thermal properties and their impacts on SRS in electrospun vascular tissue equivalents. The objectives are to (1) examine the effects of fiber alignment (parallel versus orthogonal to the direction of strain) on SRS in PBS media; (2) compare these to randomly oriented fiber; (3) examine the comparative behavior of in vivo tissues under similar circumstances, and (4) compare microstructural changes both during and after failure.

4.2. Materials and Methods

4.2.1 Electrospinning PCL Scaffolds

Electrospun tri layer sheets were prepared as follow: the inner layer (tunica intima) 14 wt% PCL (Aldrich) was dissolved in acetone (Fisher) and was continuously stirred at 60 ºC until dissolution occurred, the middle layer (tunica media) 12 wt% PCL in distilled (with calcium hydride (Sigma-Aldrich)) dichloromethane (DCM) (Fisher) solution was prepared by heating the solution to 30 ºC with continuous stirring, and the third outer layer (tunica adventitia) 5 wt% PCL in 1,1,1,3,3,3-hexafluoro-2-propanol (HFP) (Oakwood Chemical) was prepared at room temperature (RT) with continuously stirring to dissolve the PCL. After all solutions were homogeneous and cooled to RT they were placed inside a 60 cc plastic syringe (BD Luer Lok) with a 20-gauge blunt tip needle (EFD). The electrospinning conditions were the following: randomly oriented PCL dissolved in acetone fibers were made with a voltage potential of 25 kV, a flow rate
of 15 mL/h, and a source to ground distance (S-G) of 20 cm [6]. Randomly oriented PCL dissolved in DCM fibers used the same flow rate but a voltage potential of 23 kV and a S-G distance of 30 cm. Randomly oriented PCL dissolved in HFP fibers were created with the same conditions as PCL in acetone fibers, while aligned fibers were made by collecting fibers on a rotating mandrel (2.01 m circumference and 15.2 cm length) attached to a drill revolving at 500 rpm (a net linear speed of 16.8 m/s) and a voltage potential of 18 kV, a flow rate of 4 mL/h and a S-G distance of 18 cm. All randomly oriented fiber scaffolds were collected on a stationary 7.5 cm x 8.0 cm aluminum plate. Additionally, small diameter electrospun tubes were created with a 304 stainless steel rod (outer diameter = 3 mm, inner diameter = 2 mm, and length = 5 cm) rotating at 9,000 rpm (a linear speed of 1.4 m/s). Samples were electrospun for the following target thicknesses: acetone derived = 100 μm, DCM derived = 200 μm, and HFP derived = 100 μm (for single layers and tri-layers) and 300 μm (for single layers only). To remove any residual solvent that remained after electrospinning, samples were placed in a vacuum at RT overnight [7].

4.2.2 Burst Pressure Strength Testing

To obtain burst pressure strengths, angioplasty dilation balloon catheters (expired catheters kindly donated by The Ohio State University Medical Center Radiology Department) were carefully inserted into these grafts and inflated via the use of a syringe pump and distilled water. Samples were expanded by pumping water into the balloon on
the catheter at 20 mL/min until failure (Figure 4.2). Pressure was recorded by a pressure transducer (Honeywell 4OPC150G) and diameter with a laser micrometer (Keyence LS-7001) and LabView [8]. Figure 4.3 shows a schematic of the burst pressure setup.

Figure 4.2 A 3 mm diameter electrospun PCL tubular scaffold shown after applying burst pressure to failure. The dilation balloon catheter is expanded with distilled water and can be seen inside the graft.
Figure 4.3 Schematic of the burst pressure setup. A syringe pump pushes distilled water into a balloon catheter that is inserted inside the electrospun PCL tube. LabView records pressure and diameter.

4.2.3 Suture Retention Strength Testing

SRS testing was performed on electrospun scaffolds as well as on native and decellularized porcine coronary arteries (generously donated by Professor Matt Allen of the Department of Veterinary Clinical Science at The Ohio State University) according to the American National Standard Institute - Association for the Advancement of Medical Instrumentation (ANSI/AAMI/ISO 7198:1998/2010) using the straight-across procedure. Briefly, rectangular strips 2 cm in length and 1 cm in width were first created. Three silk sutures (3-0 Ethicon with a FS-1 reverse cutting needle) were inserted 2 mm from one end and 2.5 mm apart from each other, looped, and secured with seven knots. The
sample was secured onto lightweight carbon fiber grips (A2-166 Fiber Clamp Assembly, Instron) in a tensile tester (Instron, model 1000R12) using a 50 lb load cell (Test Resources, MTestW R system) and pulled at a rate of 50 mm/min. The SRS is considered the maximum force required to pull the sutures through the sample. The same procedure was repeated with the porcine samples, native and decellularized, using two sutures. All samples were tested in wet conditions using phosphate buffer saline (PBS) at 37 °C.

4.2.4 Scanning Electron Microscopy (SEM)

Scanning electron microscopy (SEM) (FEI Quanta200) was used to observe the microstructure of all scaffolds before and after burst pressure and SRS testing. Samples were adhered with conductive carbon tape to aluminum SEM mounts (SPI supplies) and sputter coated with gold (Pelco Model 3 Sputter Coater 9100). Over a period of 60 s with an emission current of 15 mA ~100 Å of gold was applied to the surface of samples. Samples were imaged using secondary electron mode at 3-15 kV.

4.2.5 Statistical Analysis

Data is presented as mean ± standard deviation. Statistical analysis was performed by means of a non-parametric test, ANOVA, using Minitab® (version 16.2.3)
to test the p-values for the SRS and burst pressure analysis. Statistics were deemed significant when p < 0.05.

4.3 Results

4.3.1 Burst Pressure Strength

The tri-layer tubes exhibited burst pressures much higher than the 2000 mm Hg target. Tri-layers with a HFP layer ~500-600 μm thick possessed burst pressures of 14200 ± 3330 mm Hg (n = 4). Tri-layers with a HFP layer only ~200-300 μm thick exhibited an average burst pressure of 9690 ± 746 mm Hg. Bi-layer samples composed of PCL fibers electrospun from acetone and DCM solutions had significantly lower burst pressure values, 1610 ± 324 mm Hg. These values are plotted in Figure 4.4.
Figure 4.4 Burst pressures of electrospun PCL tri- and bi-layer tubes. All conditions were statistically different from each other.

Trilayer samples were examined in the SEM to investigate structure mechanisms that may be responsible for the observed high burst pressure values. The acetone-derived PCL fiber layer appeared to melt into a film during expansion of the grafts as observed in a sample inflated to failure (Figure 4.5).
Figure 4.5 SEM revealed that acetone-derived PCL fibers inside a tube expanded to failure transform from a fiber morphology into a film-like structure.

4.3.2 Suture Retention Strength

There was significant difference observed in the SRS based on the thickness and orientation of as-spun scaffolds. SRS values were more directionally insensitive to random and orthogonally aligned HFP derived PCL fibers (Figure 4.6). Additionally, SRSs of electrospun PCL fibers (HFP derived) were comparable (when scaffold thickness was > 100 μm) to values obtained from decellularized porcine coronary arteries (2.08 ± 0.64 N).
An interesting phenomenon was observed from SRS testing that, to our knowledge, has not been previously reported. When sutures were pulled through both native tissues and electrospun PCL scaffolds a V-shape tear occurred (Figure 4.7 and Figure 4.8) suggesting that the synthetic PCL scaffolds fail in a manner similar to native tissues. The sutures pulled in tension drastically effected the orientation of fibers (Figure 4.9) and histograms illustrated how fiber alignment changed during SRS testing (Figure 4.10).
Figure 4.7 A V-shape tear is observed from SRS testing in electrospun PCL scaffolds as seen in situ with a high speed camera and post-testing SEM.

Figure 4.8 A V-shape tear is observed following SRS failure in decellularized porcine coronary arteries observed with SEM.
Figure 4.9 Microstructure of HFP-derived PCL fibers following SRS failure at (a) an area near the grip, (b) the middle of the scaffold, and (c) the site of failure near the V-shape notch.

Figure 4.10 Histograms demonstrating how fiber alignment changes during SRS testing. Calculated fiber angle from the 0° horizontal reference at (a) an area near the grip, (b) the middle of the scaffold, and (c) the site of failure near the V-shape notch.
4.4 Discussion

The burst pressure values observed in the tri-layered grafts are far higher than the clinically mandated 2000 mm Hg. However, this could have been due to the existence of a highly bonded layer of PCL fiber would create a more network-like structure that could better resist expansion forces. From SEM, electrospun fibers appeared to fuse into a film at the high pressures (above 2000 mm Hg) applied internally. It would be more physiologically relevant to first soak the scaffolds in cell culture medium or PBS at 37 °C and then burst in this liquid environment to better mimic the eventual in vivo application; perhaps this could prevent fiber fusion. Additionally, these experiments have not accounted for the presence of ablated microchannels in the middle DCM layer. However, as demonstrated in Chapter 2, if the outer 5% PCL dissolved in HFP layer is thick enough it will dominate and provide the bulk of the needed mechanical properties of the graft.

The SRS values obtained from the electrospun PCL fibers are above the necessary 2.0 N requirement when the thickness of the scaffold was 300 μm. However, these values were only around 1.0 N with the thinner scaffolds. More work needs to be done in order to evaluate SRS of tri-layer scaffolds, rather than HFP derived single layers, but results so far are promising. The significance of the formation of the V-shape notch after SRS failure demonstrates that electrospun PCL scaffold failure is similar to native tissue. Additionally, the microstructure of electrospun fibers moves and re-aligns in the direction of strain, providing a better understanding of how our scaffolds fail during SRS testing.
4.5 Conclusion

While more work needs to be done to properly evaluate burst pressure and SRS, preliminary work suggests that electrospun PCL tri-layers are able to provide adequate mechanical strengths. Tri-layers provided more than enough burst pressure and single layer HFP scaffolds provided adequate SRSs. Electrospun fibers underwent significant structural reorganization at high pressures and strains thus it is important to understand these microstructural changes to understand how mechanical properties are affected by burst pressure and SRS testing. Although, it should be noted that the fiber bonding observed at high strains in burst pressure may not be clinically relevant as it is unlikely for the body to reach such high pressures *in vivo*. 


Chapter 5: Vascular Wall Engineering Via Femtosecond Laser Ablation Scaffolds with Self-Containing Smooth Muscle Cell Populations

5.1 Introduction

Over 80 million people in the United States suffer from coronary vascular disease; within this group more than 176,000 patients annually undergo coronary artery bypass grafting [1]. The lack of healthy autografts, especially in the elderly, is a major obstacle to successful treatment [2]. Synthetic vascular grafts made of non-degradable polymers, such as polyethylene terephthalate (Dacron®) or expanded polytetrafluoroethylene (ePTFE), are commonly used for bypass grafting. Unfortunately, in vivo small (< 6 mm) diameter synthetic grafts often exhibit compliance mismatch, rapid plaque formation and occlusion of the lumen [3, 4].

To provide a replacement vessel with biomimetic function and properties, tissue engineering strategies have been investigated such as cell sheets or cells cultured on biopolymers, such as collagen, to form engineered vessels [5-7]. A key component of these strategies is promoting proper cell organization and orientation within the vessel, especially in the tunica media (middle layer) of the vessel. Bioreactors with cyclic stretch [8], pulsatile perfusion [9], and microchannels [10, 11] have all been successfully
utilized to direct smooth muscle cell alignment. However, these methods can require up to 5 months from initial cell culture to final vessel formation [6] which is incompatible with many clinical applications.

Micropatterning on electrospun nanofiber scaffolds is quite difficult using conventional lithography or mechanical micromachining due to their highly porous nature. Recent work by Dong et al. involved micropatterning poly(lactic-co-glycolic acid) (PLGA) and poly(L-lactide-co-ε-caprolactone) (PLCL) nanofiber scaffolds using UV photolithography [12]. However, such techniques require photomasks for patterning and several hours to sufficiently degrade the nanofiber scaffold. To more rapidly engineer microchannels within three-dimensional scaffolds for directed cell alignment, femtosecond laser ablation has been utilized [13-15] due to its flexibility and capabilities for precise material removal [16, 17]. Femtosecond laser ablation is a non-clean room fabrication method that requires only a single step. Femtosecond laser ablation minimizes thermal effects on the surrounding materials due to laser pulses that are relatively shorter than thermal diffusion. For instance, Choi et al examined relative morphological change in polycaprolactone (PCL) following irradiation with nanosecond and femtosecond lasers [18]. Significant melting was observed after nanosecond laser ablation but not following femtosecond laser ablation. Femtosecond laser micromachining of porous nanofiber scaffolds provides unique advantages and can produce surface features in which resolution is limited only by the inherent porosity of the material. Femtosecond laser ablation is an effective method for three-dimensional structuring of nanofiber scaffolds made of biodegradable polymers while concurrently
retaining the fibrous microstructure [18-20]. Electrospun PCL tubes have demonstrated burst pressures above the clinically-mandated level of 2000 mm Hg [21]. In a prior study, direct-write femtosecond laser ablation was employed to create a series of microchannels (100 μm deep, 100 μm wide, and 1.6 mm long) running the length of tri-layered three dimensional (3D) electrospun scaffolds [22]. The tri-layer scaffold was comprised of a low porosity electrospun fiber layer creating a surface for endothelial cell attachment (tunica intima), a high porosity fiber layer containing femtosecond laser ablated microchannels that facilitate directed seeding, infiltration and organization of smooth muscle cells (tunica media) and a mechanically robust electrospun fiber layer to provide appropriate mechanical strengths (tunica adventitia). If designed correctly, this tri-layered structure can include microchannels that do not compromise the overall mechanical properties [22]. However, the ability of these tri-layered scaffolds to promote cell attachment, viability, and organization has not been investigated.

This study was undertaken to first examine the effect of materials selection on cell viability on a biodegradable polymer, PCL. The ability of the laser ablated microchannel described above to direct cell seeding and organization was then evaluated. Flow within this microchannel was initially studied by injecting fluorescent polystyrene beads through the microchannel. Cell seeding followed the successful demonstration of bead flow. In addition, two dimensional (2D) fluid modeling using computational fluidic dynamics (CFD) software was employed to estimate the fluid profile within the microchannel.
5.2 Materials and Methods

5.2.1 Scaffold Preparation

To fabricate the tunica intima equivalent that also serves to “seal in” cells seeded into the laser-ablated channel, a PCL solution was prepared by dissolving 14 wt% PCL (Sigma Aldrich, $M_w = 80,000$) in acetone (Sigma-Aldrich) heated to 60 °C with continuous stirring to dissolve the polymer. Once cooled to room temperature, the solution was placed in a 60-cc plastic syringe (BD Luer-Lok) equipped with a 20-gauge blunt tip needle (EFD). The solution was electrospun using a syringe pump (KD Scientific) at a flow rate of 16 mL/h and a high voltage power supply (Glassman) set to a voltage difference of 24 kV. Electrospun fibers were collected onto standard glass microscope slides (Gold Seal, Ted Pella) placed upon a 7.6 cm x 7.6 cm steel plate covered with aluminum foil at a distance of 20 cm from the needle tip for 10 min.

To fabricate the tunica media equivalent into which microchannels were ablated, a 12 wt% PCL solution in distilled (from calcium hydride (Sigma Aldrich) dichloromethane (DCM) (Sigma Aldrich) was created by heating the solvent and the PCL pellets to 30 °C with continuous stirring. After cooling to room temperature, the solution was placed in a 60-cc syringe and electrospun at a flow rate of 15 mL/h, a voltage potential of 23 kV and a source to ground distance of 30 cm. Fibers were collected on a 7.6 cm x 7.6 cm steel plate covered with aluminum foil for 10 min. After
electrospinning, all samples were placed in a room temperature vacuum overnight to ensure that any residual solvents in the scaffold were eliminated [23-25].

To fabricate a potentially more biologically acceptable substrate than PCL, a solution of acid-soluble bovine collagen (Kensey Nash) at 10 wt/vol% in 1,1,1,3,3,3-hexafluoro-2-propanol (HFP) (Sigma-Aldrich) was prepared. This solution was stirred for 48 h on a stir plate to fully dissolve the collagen in HFP. The solution was poured into a 20-cc plastic syringe (BD Luer Lok) with a 14-gauge blunt needle tip (EFD) and electrospun with a voltage potential of 30 kV onto an 8.5 cm x 8.5 cm aluminum foil covered brass plate placed 20 cm away from the needle tip using a flow rate of 4.0 mL/h. Approximately 5 mL of solution was electrospun to create the scaffold. After electrospinning, all collagen scaffolds were physically cross-linked by vacuum dehydration at 140 ºC for 24 h, followed by chemical crosslinking in 5 mM N-(3Dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (6) (EDC) (Sigma-Aldrich) in pure ethanol for 24 h [26].

5.2.2 Smooth Muscle Cell Culture and Viability Assay

To prepare the scaffolds for inoculation of cells, PCL-acetone, PCL-DCM and collagen scaffolds were cut into 2 cm x 2 cm squares, placed into 24-well plate cell crowns (Scaffdex Oy), disinfected in 70% ethanol for 24 h, rinsed twice in phosphate buffered saline (PBS, 24 h between rinses), rinsed 5 times in HEPES buffered saline (15-
20 minutes between rinses) and lastly, rinsed in proliferative smooth muscle cell culture medium (SMC-PM) [27] (Lonza) in preparation for cell inoculation.

Human coronary artery smooth muscle cells (HCASMCs) (Lonza) were cultured at 37 °C and 5% CO₂ in SMC-PM with the medium exchanged every other day. Once cells reached 70% confluence, HCASMCs were harvested and inoculated onto PCL-acetone, PCL-DCM and collagen scaffolds at 50,000 cells/cm². Scaffolds with cells were then incubated at 37°C and 5% CO₂ in SMC-PM for 30 min before 5 mL of medium was added into each well of the 6-well plates (Techno Plastic Products). Cell-seeded scaffolds were cultured for 5 days with medium exchanged daily. HCASMC viability was assessed at days 1, 3, and 5 (n = 7 per group, per time point) using a CellTiter 96 AQueous Non-reactive Cell Proliferation 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) Assay (Promega Corp.). At each time point, a 4 mm biopsy was removed from each sample and incubated with the MTS-phenazine methosulfate (PMS) solution for 3 hours at 37 °C and 5% CO₂. Following this incubation, the medium was removed and its absorbance was read at 490 nm using a plate reader (Gemini Spectramax, Molecular Devices). Average absorbance ± standard deviation was reported.
5.2.3 Laser Ablation

Frequency doubled pulses from a mode-locked erbium-doped fiber laser intensified in a Ti:Sapphire regenerative amplifier laser (CPA2161, Clark-MXR) were used to create microchannels on electrospun PCL scaffolds. The laser wavelength was $\lambda = 775$ nm. The maximum average output power of the laser was $P_{av} = 2.5$ W, pulse duration was $T_p = 150$ fs, pulse repetition frequency was $f_p = 3$ kHz and collimated beam diameter was 5 mm. The beam quality factors were $M^2 = 1.2$ in the vertical Y-direction and $M^2 = 1.3$ in the horizontal X-direction. Laser beam power was adjusted by a series of thin-film polarizing beam splitters and half waveplates. The attenuated laser beam was focused on the material with a 50x magnification infinity corrected microscope objective lens having numerical aperture $NA = 0.42$ (M plan Apo NIR 50x, Mitutoyo). Attenuated average laser power was measured by a power meter (PM100, Thorlab) placed under the laser focusing lens. Sample motion and focus lens positioning was provided by a computer controlled motion system (MX80L, Parker) with 0.5 $\mu$m resolution in the X, Y and Z axes. The system was programmed to scan the electrospun nanofiber scaffolds under the focused laser beam in order to ablate the microchannel. The chosen dimensions of the microchannels were 100 $\mu$m wide, 100 $\mu$m deep, and 10 mm long. The X-Y motion system scanned the sample with linear paths at a scanning speed of 0.33 mm/s with a laser pulse energy of 3.33 $\mu$J. To ablate the desired width and depth of 100 $\mu$m, two layers consisting of 40 linear paths with spacings of 2.5 $\mu$m were used. After
ablating the first layer, the focus position was adjusted downward by 50 μm to ablate the second layer.

5.2.4 Fluorescent Bead Flow Analysis of Microchannels

After laser ablation, a plastic Teflon 25-gauge blunt needle tip (EFD) was cut at the end to create a slight taper that was then secured to the scaffold containing laser ablated microchannels, taking care to position the plastic needle tip directly above one end of the microchannels. With the needle tip secured, the scaffolds received another layer of electrospun 14 wt% PCL in acetone with the same parameters used previously although only 1 min of electrospinning time was used to create a thin (80 ± 12 μm) fibrous layer that permitted imaging but was thick enough to retain either beads or cells flowing down the length of the microchannels (Figure 5.1).
Fluorescent, Nile red polystyrene beads with a diameter of 20 µm (Spherotech) were diluted in deionized water to 0.2 wt/vol% and placed in a 5-cc plastic syringe (BD Luer-Lok). A 33” extension with both female and male luer-lok (Medex) was used to attach the plastic needle tip to the syringe. Using a syringe pump, the fluorescent bead solution was flowed into the microchannels at the flow rate of 1 mL/h. An upright fluorescent microscope (Nikon Eclipse LV150) was used to record bead motion down the microchannel at a recording rate of 50 frames per second. The video file was then extracted into time-lapse image files using Image J Software.
5.2.5 Flow Field Modeling in the Microchannels

Numerical simulation was carried out to model flow behavior in the microchannel ablated within the nanofiber-based porous scaffold. Modeling can produce predictions of cellular deposition useful in future adaptations of this work in more extensive and complex microchannel geometries. Consider helically arranged microchannels running down the length of a hypothetical 2 cm long tubular construct 0.3 cm in diameter. If each microchannel occupies 0.1 cm tube length, the total microchannel length within the tube will be 188.5 cm. Thus, an understanding of how far cells can travel in the face of continuing media loss into the surrounding walls is of interest. However, to circumvent the complexity of modeling flow through the surrounding porous medium, a simplified 2D duct flow model with constant flow out velocity boundary conditions on the walls was used to elucidate the experimental flow pattern. With the assumption of constant physical properties, the steady-state laminar incompressible flow is governed by the continuity and Navier-Stokes (N-S) equations in the forms of where \( \mathbf{V} \) is the velocity field, \( \rho \) is the density and \( \mu \) is the dynamic viscosity.

\[
\nabla \cdot \mathbf{V} = 0
\]

\[
\rho \nabla \cdot (\nabla \mathbf{V}) = -\nabla p + \mu \nabla^2 \mathbf{V}
\]

The flow through a microchannel having a length of 10 mm and width of 100 μm (Figure 5.2) is simulated. The fluid was assumed to be distilled water and physical
properties at room temperature were used for the computation. A uniform velocity profile with a magnitude of 2.78 cm/s obtained from experimental data was assigned at the inlet; the outlet was set at atmospheric pressure. A constant lateral velocity was used at the walls defined by:

\[ fV_{inlet}W = 2V_{wall}L \]

where \( f \) is the total fraction of volume flow rate flowing out from the wall, \( W \) and \( L \) are the width and the length of the microchannel and \( V_{inlet} \) and \( V_{wall} \) are the velocities at the inlet and the wall, respectively (Figure 5.2). Therefore, \( V_{wall} \) can be determined for different values of \( f \). If \( f = 1.0 \), the entire flow originating from the inlet will flow through the wall and the velocity at the outlet will be zero. The computational domain was meshed with uniform grids 4 \( \mu m \) in size using GAMBIT preprocessor software. The
governing equations were solved by FLUENT (ver. 6.3, ANSYS Inc.) based on the finite volume algorithm.

5.2.6 Smooth Muscle Cell Microchannel Seeding

To assess the ability of the microchannels to guide cell seeding and to promote cellular organization, HCASMCs were inoculated into laser ablated microchannels utilizing the same apparatus used to introduce the fluorescent beads. Cell organization was qualitatively assessed after 24 hours of culture using immunocytochemistry and confocal microscopy. Once the HCASMCs reached 70% confluence (described in 5.2.2), they were harvested and 500,000 cells were resuspended in 1 mL of medium and placed in a 1 mL insulin syringe (BD slip tip) attached to the tapered plastic needle tip adhered to the scaffold by electrospinning as before. The scaffold and needle tip were previously sterilized in 70% ethanol for 24 h then conditioned in SMC-PM for 30 min in a 37 °C incubator prior to seeding. The resuspended cells were introduced into the microchannels (~ 500,000 cells per microchannel) at a rate of ~500 μL/min and the scaffold returned to medium in a 37 °C incubator. Following 24 h in culture, the HCASMC-scaffold constructs were fixed with 4% paraformaldehyde (Sigma Aldrich) and stained with DAPI (Invitrogen) to visualize the nuclei and Alexafluor 488 phalloidin (Invitrogen) to visualize F-actin. Samples were examined using confocal microscopy (Olympus FV1000-Filter).
5.2.7 Scanning Electron Microscopy (SEM)

Scanning electron microscopy (FEI Quanta200 and FEI Sirion) was utilized both to examine microstructure of the laser ablated microchannels as well as the post-seeded presence and distribution of beads and cells. All samples were adhered with conductive carbon tape to aluminum SEM sample mounts (SPI Supplies), sputter coated with ~100 angstroms gold (Pelco Model 3 Sputter Coater 9100) over a period of 60 s using an emission current of 15 mA and imaged using secondary electron mode at 5-10 kV.

5.2.8 Statistical Analysis

Average fiber diameter and pore size on the surface of as-spun PCL scaffolds were measured from SEM images using image analysis software (Image J). Averages and standard deviations of fiber diameter and pore size were determined using Minitab (version 16.1.1). Analysis of variance (ANOVA) was used to determine p-values for the MTS assay. P-values < 0.05 were considered statistically different.
5.3 Results

5.3.1 Electrospun Scaffolds

Utilizing the conditions given in the materials and methods section, the sheet thicknesses of electrospun fibers produced were: (1) 14 wt% PCL dissolved in acetone, 358 ± 33.5 µm; (2) 12 wt% PCL dissolved in DCM, 178 ± 23.9 µm and (3) 10 wt/vol% collagen dissolved in HFP, 348 ± 70.5 µm. While DCM-based electrospinning can create pore diameters large enough to allow full infiltration of seeded cells [28], the acetone-based electrospun PCL contains pore diameters that are too small – regardless of sample thickness – to allow cell infiltration [29]. Electrospun DCM-based scaffolds had a pore area of 1788.8 ± 757.3 µm² and acetone-based scaffolds had a smaller pore area of 92.4 ± 30.7 µm². These values are statistically different (p-value = 0.005).

5.3.2 Cell Viability

To assess the effect of scaffold chemistry on HCASMC viability, cells were cultured on PCL-acetone, PCL-DCM and collagen scaffolds for 5 days. The MTS assay revealed no statistical difference (p-value = 0.296) in viability (Figure 5.3) among HCASMCs grown on all three scaffold types at any time point. As a result, all subsequent studies were performed using PCL-based scaffolds.
The viability of smooth muscle cells grown on electrospun PCL scaffolds showing no statistical difference to those grown on collagen. Electrospun solutions are: 14 wt% PCL

5.3.3 Laser Ablated Microchannels

The femtosecond laser ablation conditions utilized produced microchannels 100 μm wide, 100 μm deep, and 10 mm long. Femtosecond laser ablation successfully produced a microchannel in the PCL scaffolds without substantially altering the microstructure of the fibers. No significant melting of fibers either in or surrounding the microchannels themselves was observed. Additionally, the margins appear to be uniform down the length of the microchannels.
5.3.4 Bead/Cell Seeding in the Microchannels

Flow tests using the 20 μm diameter fluorescent beads were conducted using the 100 μm by 100 μm by 10 mm length microchannels. The thin electrospun layer capping the microchannels did not appear to obstruct bead flow during the experiment. The fluorescent beads stayed within the microchannels (Figure 5.4) and successfully flowed down the length of the microchannels without any apparent buildup or obstruction. The velocity of the beads in the microchannel was 0.6 cm/s as determined from the time-lapse images (Figure 5.5). This velocity was measured near the edge of a microchannel.
Figure 5.5 Time lapsed images for fluorescent beads flowing down the microchannel. The velocity was calculated to be 0.6 cm/s between the 8 and 9 mm point along the microchannel.
Confocal microscopy (Figure 5.6) and SEM (Figure 5.7) confirmed that cells stayed within the microchannels and were denser toward the edges of the channels. Both

Figure 5.6 Cells stained with DAPI (nuclei) and Alexafluor 488 (actin) are visible down the entire length of the microchannel as shown via confocal microscopy.
the nuclei and F-actin were observed within the cells; however, fiber auto fluorescence often made it difficult to resolve the DAPI-stained nuclei. At the conclusion of the seeding process, the thin layer of fibers covering the microchannels was removed for imaging. SEM was then used to show that the cells line both the bottom and the walls of the microchannels. Cells were visible down the entire length of the microchannels. The presence of F-actin fibers (especially evident in Figure 5.6) suggests that the cells did adhere.

Figure 5.7 SEM of smooth muscle cells adhered inside the microchannel after 1 day in culture. (a) Cells are maintained within the channel and (b) exhibit visible extensions across the width of the channel.

5.3.5 Flow Field in Microchannels

Pressure driven flow into the microchannels was simulated using CFD software. To study the effect of fluid loss, the flow patterns of different values of \( f \) ranging from 0 to 1 with increments of 0.1 were studied. As shown in Figure 5.8, the velocity profiles along the microchannels were plotted for \( f = 0.5, 0.8, \) and 1. It is shown that the flow
velocity is reduced toward the end of the microchannel due to the fluid loss from the wall. Additionally in Figure 5.8, the maximum velocity ($u_{\text{max}}$) in the center of the microchannel is plotted under different degrees of fluid loss ($f$) into the walls. Since flow is in the micron scale, the viscous force is dominant. The Reynolds number ($\text{Re}$) calculated based on the width of a microchannel is $\sim 2.2 \times 10^{-7}$. Therefore, the entrance effect is minimal and the fluid is quickly accelerated to the peak value in a short distance as seen in Figure 5.8. After reaching a peak value, the maximum velocity decreases linearly for different $f$ values. As $f$ becomes larger, the velocity decrease becomes greater.
Figure 5.8 Comparison of the (a) fluid profile with different fractions of fluid volume loss ($f = 0.5, 0.8$ and $1$) along the microchannel and (b) maximum fluid velocity ($u_{\text{max}}$) at the center of microchannel width with different fractions of fluid volume loss ranging from $f = 0$ to $1$ into electrospun fiber walls.
5.4 Discussion

Previous methods of creating tissue engineered scaffolds included both biopolymers and non-degradable polymers. Others have successfully used cell-seeded biopolymers to construct small diameter vascular grafts, where smooth muscle cells were mixed with collagen type I to make a tube followed by endothelial cell seeding in the luminal surface to engineer vessels that were histologically similar to native vessels [7]. In spite of their similarity to native tissues, biopolymers often lack the appropriate mechanical strength. Synthetic, non-degradable polymers can exhibit the opposite trend: strong mechanical properties but poor biocompatibility [3, 4]. We first showed that smooth muscle cell viability on the electrospun scaffolds investigated was not strongly dependent on scaffold processing (Figure 5.3) [30,31]. Thus, the scaffold was fabricated using only one polymer – PCL – due to its well-known biocompatibility, slow degradation rate [32], ease of electrospinning [25,33], and mechanical capabilities in electrospun form [21, 22, 34].

In addition to promoting cell adhesion and maintaining viability, scaffolds for engineered blood vessels must promote proper cell organization and orientation, especially in the tunica media of the vessel. Healthy smooth muscle cells exhibit a spindle-like morphology and are oriented in a helical pattern around the vessels causing blood to be pumped through the vessel each time the smooth muscle cells contract [35]. To encourage proper alignment of smooth muscle cells, many strategies have been investigated including the use of microchannels [10,11]. Smooth muscle cells inoculated
into microchannels created using UV microembossing and subsequent lift off from a carrier substrate film exhibit a high level of cellular alignment, proper contractile morphology and α-actin production [10]. However, to form these channels, multiple time consuming steps are required including silicon mold manufacturing, polydimethylsiloxane (PDMS) daughter mold forming and solvent-polymer solution casting [10]. In the current study, femtosecond laser ablation created 100 µm x 100 µm x 10 mm channels (Figure 5.4, Figure 5.5, and Figure 5.7) in 3D PCL scaffolds in a single step process with precise material removal and minimal alterations to fiber morphology [16,17]. Femtosecond laser ablation is a unique tool allowing precision ablation of polymer-based tissue scaffolds, producing complex flow channel networks in electrospun scaffolds while preserving the underlying nanofiber structure [18]. By creating 2D and 3D microchannel patterns, favorable cell-cell interactions can be achieved. Femtosecond lasers produce short duration (FWHM~150 fs) pulses which, when focused to a spot size of several microns, provide large irradiance (>TW/cm²), fluence and photon flux. As a result, the beam-material interaction is non-linear and energy absorption occurs only when the fluence is above a distinct material dependent threshold. When used for ablation, the femtosecond laser can remove material within a region smaller than the spot size; microscale resolution is enhanced vis-à-vis nanosecond lasers. Using this ability to form channels in a 3D scaffold is a large step forward in the fabrication of more complex tissue engineering structures. The precision and reproducibility obtained by femtosecond laser ablation allows for a range of patterns to be ablated into polymer scaffolds for tissue engineering applications.
Lim et al. recently studied chemical compositions before and after femtosecond laser ablation on PCL/gelatin nanofiber scaffolds using attenuated total reflection Fourier transform (ATR-FTIR) infrared and x-ray photoelectron spectroscopy (XPS) techniques [20]. They found no significant changes in chemical state following femtosecond laser ablation. Rebollar et al [36] used FTIR and time of flight secondary ion mass spectroscopy (ToF-SIMS) to study the chemical modification of PCL nanofiber after femtosecond laser irradiation and also found no significant change in chemical composition.

Average fiber diameters ($n = 50$) were measured from SEM images for as-spun acetone PCL surface and bottom surface of microchannel produced by femtosecond laser ablation. Average fiber diameter was found to be $0.96 \pm 0.51 \, \mu \text{m}$ for as-spun PCL, while the average fiber diameter for the bottom surface of microchannel was $1.14 \pm 0.71 \, \mu \text{m}$. As before, no significant change in average fiber diameter can be observed following femtosecond laser ablation. In addition, no change in pore size was observed. Before femtosecond laser ablation, the equivalent circular pore diameter was $9.61 \pm 5.5 \, \mu \text{m}$, afterwards it was $11.1 \pm 8.3 \, \mu \text{m}$.

We envision a tubular construct (Figure 5.9) that makes use of this overall strategy by introducing appropriately distributed cellular populations within a
mechanically capable, cylindrical scaffold. We have previously shown that a thick enough layer of fiber spun from HFP can successfully compensate for the presence of laser ablated porosity [22]. Seeding of representative cell populations – both endothelial (EC) and smooth muscle cells (SMCs) – should modulate the thrombogenicity of the endothelial layer by covering the synthetic luminal surface with adherent endothelial cells [34,37]. Signaling between these cell populations plays a significant role in blood vessel function. At the interface between blood and the vessel wall, ECs are directly exposed to blood flow-induced shear stress. When exposed to shear stress healthy ECs secrete nitric oxide (NO) that prevents clotting [38-42]. These shear stresses also influence the
signaling between ECs and the surrounding SMCs even though they are not in physical contact. Furthermore, (1) ECs cultured with SMCs align with blood flow more rapidly [42]; (2) EC response to shear stress is improved if neighboring SMCs are preconditioned with cyclic strain [43]; (3) EC/SMC co-culture improves both SMC proliferation [44] and EC lifetime [45]; and (4) shear stress decreased inflammation in ECs if they were located near SMCs. To successfully maintain ECs in an engineered environment that allows them to adhere in the presence of shear stress while producing appropriate amounts of NO, ECs must be co-located with SMCs. Key to achieving this goal in a tubular format is a helical microchannel running down the length of a cylindrical tissue engineered vessel. This microchannel would sit within the “tunica media” layer and seeded SMCs could infiltrate the microchannel to create the cell-cell signaling necessary for the appropriate EC adherence. We currently envision that the cells so introduced would be a population derived from the patient’s own cells expanded ex vivo. As such, it would exist within a cell media similar to that used here and the net viscosity would be nearly identical.

In contrast to DCM, which creates pore diameters that allow full infiltration of cells [28], acetone-based electrospun PCL creates thing, dense fibers containing pore diameters that are too small to allow cell infiltration [29]. In the final construct, this layer will be ~ 100 μm thick and lined with endothelial cells to imitate the tunica intima. The “tunica media” layer consists of the DCM-based electrospun PCL into which SMCs should proliferate [25] (Figure 5.9) following introduction via the laser ablated microchannel. Femtosecond laser ablation successfully created microchannels in this
layer of the scaffold without disrupting the microstructure of neighboring fiber; the absence of any melted fibers recapitulates the results of Choi et al. [18] and Lim et al. [20].

During the seeding experiments, the thin acetone-derived fiber layer electrospun over the microchannels successfully ensured that neither beads nor cells could escape. Because electrospinning is typically not a conformal technique, no fibers were deposited inside the relatively small microchannels. However, this bead- and cell-impermeable layer was thin enough to allow direct imaging of as-deposited beads and cells.

Fluorescent bead seeding allowed imaging of bead motion (Figure 5.5) inside the microchannels due to their relatively large size (20 μm diameter), fluorescent intensity and the slow flow rate (1 mL/h). Their size retained the beads inside the ablated microchannels and prevented infiltration out into the surrounding scaffold.

HCASMC seeding was similarly successful and showed that the cells were both deposited and remained in the microchannels. A slight gap in cell coverage running down the length of the microchannel (Figure 5.6) is likely due to cells either adhering preferentially to the walls of the microchannel (due to flow into the walls) or perhaps the loss of cells when the thin layer of fibers covering the microchannel was removed to allow SEM imaging. According to the flow model, flowing cells likely experience a slower velocity at the walls of the microchannel (Figure 5.8) as opposed to the maximum velocity observed in the middle of the microchannel. This could also explain why cells are observed along the walls of the microchannel in confocal microscopy. Under confocal microscopy, the cells in the “gap” can be more easily resolved (than those on the
walls) because they reside only on a single plane: the bottom of the microchannel. This phenomenon became clearer when viewed under the SEM; the cells on the bottom of the microchannel appear to have expanded to cover the width and length of the microchannel (Figure 5.7). Due to the greater depth of field available in the SEM, more cells are visible across the microchannel. With 500,000 cells inoculated into the microchannel, it is curious what happened to all the smooth muscle cells as only ~500 cells (assuming confluent smooth muscle cells are ~ 12,000 cells/cm²) could have adhered when calculating cells/cm². The total surface area of the 100 um x 100 um x 1 cm channel was .0402 cm² when the top ‘sealing’ layer of nanofiber was included. There was some evidence for fractured cells in the microchannel after SEM analysis – confirming presence of cells on the roof of the microchannel prior to removal of this layer. The question of where excess cells went still remains, since a factor of 1000x cells were not present. Perhaps some cells were still in the syringe and needle tip during inoculation or maybe non-adherent cells died and were flushed away during scaffold rinses.

In the final scaffold design, SMC will be inoculated in DCM-based nanofiber scaffolds and not the acetone-based fibers described here. The flow velocity would have been indeterminate if DCM-based scaffolds were used because the microbeads would have most likely dispersed throughout the scaffold [31] and would not have remained within the microchannel. The acetone-based fibers utilized in this study allow for estimates of flow rate within the microchannel.

The velocity of the beads in the microchannel was found to be 0.6 cm/s using time lapse images from Figure 5.5. Additionally, the mean input velocity was calculated by
dividing the volumetric flow rate by the cross sectional area to arrive at 2.78 cm/s. If there is no fluid loss, theoretically a fully developed parabolic velocity profile should be obtained in which the maximum velocity in the center of the microchannel will be 1.5 times the mean velocity [1], resulting in \( u_{\text{max}} = 4.125 \) cm/s. However, the fluid diffuses in the lateral directions along the microchannel because of the porous microstructure inherent to electrospun scaffolds (Figure 5.2). Fluid is transported into the nanofiber walls via a combination of pressure gradients and capillary action. This explains why the calculated experimental velocity was less than the calculated analytical velocity. In Figure 5.8, the fluid velocity linearly decreases as the front moves toward the end of the microchannel. When the fluid loss \( f \) is equal to 1, fluid velocity is zero near the end of the microchannel. To establish the proper \( f \) value, the calculated bead velocity was compared with the numerical simulation for different values of \( f \). From the experiment, bead flow velocity was 0.6 cm/s at between 8 and 9 mm of the microchannel length. As a result, the proper \( f \) range is from 0.9 to 1 (Figure 5.8(b)). The value of \( f \) could be related to the porosity of the PCL nanofiber. Since electrospun PCL nanofiber has a high porosity, > 90\% [46], more fluid losses are expected through the porous walls in the microchannel. This result is consistent with that of other studies [47].

While this technology has considerable potential to allow targeted 3D cell seeding throughout both tubular and non-tubular electrospun scaffolds, faithful vasculature reconstruction would require that we introduce the vasa vasorum interna, externa, and the venuous vasa vasorum to provide the appropriate blood supply. Utilization of femtosecond laser technology during the electrospinning process could be used to create
the necessary passages assuming that anatomically accurate details are available.

However, given that saphenous vein transplants arrive with neither the vasa vasorum externa nor the venuous vasa vasorum intact/useful, the lack of such passages may not prove a substantial barrier to the application of our current level of sophistication.

5.5 Conclusion

In this study, both microbeads and HCASMCs in media were flowed through femtosecond laser-ablated 100 µm x 100 µm x 10 mm microchannels to evaluate flow velocity and cell seeding. Bead seeding allowed estimation of fluid flow velocities on the order of 0.6 cm/s, slower than the analytical velocity. This slower rate results from fluid losses through the nanofiber scaffold. HCASMCs were seeded successfully down the 10 mm length of the microchannel. The cells not only stayed within the walls of the channel but also adhered to the walls of the scaffold.
5.6 References


Chapter 6: Biological Integration

6.1 Introduction

Chronic thrombosis is the leading cause of failure in synthetic small diameter vascular grafts. To prevent thrombosis, a fully confluent monolayer of endothelial cells (ECs) lining the luminal wall (i.e., an adherent endothelium) is needed [1]. The thromboresistant properties of the endothelium inhibit platelet adhesion, aggregation, and recruitment. As early as the 1970s, Herring et al. investigated the ability of ECs to inhibit thrombosis on Dacron® grafts. They observed that an endothelium was capable of keeping EC seeded grafts 76.4% clot-free, while unseeded grafts were a mere 22.4% clot free [2]. Forty years later, the creation of endothelial layers on grafts continues to be explored. Zhang et al. seeded ECs onto electrospun fibers composed of both natural and synthetic polymers and showed that this successfully prevented platelet adhesion [3]. Any area of the electrospun scaffold not covered by ECs readily adhered platelets, demonstrating how easily thrombosis can form on scaffolds without a complete endothelium [3]. Thus, one way to successfully prevent thrombosis of small diameter vascular grafts (< 6 mm in diameter) is by recreating the endothelium present in native vessels within tissue engineered blood vessels (TEBVs).
Smooth muscle cells (SMCs) play a complementary role in native blood vessels by providing structural support and controlling vascular tone in the medial (middle) layer [4]. In native arteries, SMCs are aligned and thus fully functional arteries require aligned SMCs for proper contraction and adequate mechanical strengths [4, 5]. Groups have successfully aligned SMCs by applying cyclic stretch and pulsatile perfusion; however these techniques are time consuming as alignment requires weeks to induce. A more rapid alternative is to initially align SMCs by seeding into microchannels. Using microchannels, Shen et al. were able to quickly align SMCs and induce a contractile phenotype with upregulated smooth muscle actin in only 7 days [5].

To make a completely functional TEBV, scaffolds must be populated with both ECs and SMCs. For example, grafts with a confluent EC monolayer on the luminal wall successfully delayed loss of patency compared to their unseeded counterparts; however, without the presence of SMCs the contractile response is significantly lower than that found in native vessels [4, 6]. Thus, a greater understanding of how these two cell types interact in vitro is needed to develop a stable co-culture system for TEBV. Several co-culture systems have been previously investigated: culturing on opposite sides of a membrane, culturing ECs onto collagen gels with SMCs, using microcarrier ECs or SMCs, conditioning media, and culturing ECs directly onto SMCs [7]. To our knowledge, co-culture has not been investigated on electrospun PCL scaffolds. Diban et al. evaluated EC and SMC culture on PCL films and hollow fibers created by phase inversion [8]; several groups have performed co-cultures on electrospun fibers but co-culture on electrospun PCL has not been studied.
The goal of this study was to assess the suitability of electrospun PCL as a platform for TEBV development by investigating methods for EC and SMC co-culture to facilitate endothelium development and medial layer function. This includes evaluating cell viability on electrospun PCL scaffolds, establishing appropriate co-culture medium first in 2D and then 3D platforms, and determining the optimal inoculation density.

6.2 Materials and Methods

We conducted many cell culture experiments in an attempt to understand cell (both EC and SMC) adhesion/proliferation on electrospun polycaprolactone (PCL) with the ultimate goal of examining the interaction of ECs and SMCs in co-culture. Cell culture experiments include examining scaffold type, cell type, medium used, inoculation density, and the type of data collected (viability assays or samples for histology, fluorescent confocal microscopy or SEM).

6.2.1 Scaffold Fabrication

To engineer a dense fibrous scaffold (Figure 6.1(a)) adequate for EC surface adhesion without cellular infiltration, 14 wt% PCL (Sigma-Aldrich, M_w = 80,000) was dissolved in acetone (Sigma-Aldrich) by heating the solution to 60 °C with continuous stirring. After cooling to room temperature, the solution was placed in a 60-cc plastic syringe (BD Luer-Lok) with a 20-gauge blunt tip needle (EFD). The solution was
electrospun with a syringe pump (KD Scientific) at a flow rate of 16 mL/h, a high voltage power supply (Glassman) with a voltage potential of 24 kV, and a 7.6 cm x 7.6 cm steel collecting plate wrapped with non-stick aluminum foil positioned 20 cm from the needle tip. As solution was electrospun onto the collector, the acetone solvent evaporated leaving pure PCL fibers. To ensure that no residual solvent was present, scaffolds were placed in a room temperature vacuum chamber overnight [9-11].

Electrospun collagen scaffolds (Figure 6.1(b)) were created as a potentially more biologically acceptable scaffold than PCL. A solution of 10 wt/vol% acid-soluble bovine collagen (Kensey Nash) in 1,1,1,3,3,3-hexafluoro-2-propanol (HFP) (Sigma-Aldrich) was prepared by continuously stirring the solution at room temperature for 48 h to dissolve the collagen. The solution was poured into a 20-cc plastic syringe (BD Luer Lok) with a 14-gauge blunt tip needle (EFD) and electrospun with a flow rate of 4.0 mL/h and a voltage potential of 30 kV onto an 8.5 cm x 8.5 cm brass plate covered with aluminum foil. The source to ground (S-G) distance was 20 cm and approximately 5 mL of solution was electrospun per scaffold. All electrospun collagen scaffolds were physically cross-linked via vacuum dehydration at 140 °C for 24 h and then chemically cross-linked in 5 mM N-(3Dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (6) (EDC) (Sigma-Aldrich) in pure ethanol for 24 h [12].

To fabricate a porous scaffold (Figure 6.1(c)) that SMCs could infiltrate and ideally fill the entire thickness of the scaffold, 12 wt% PCL was dissolved in distilled (over calcium hydride (Sigma-Aldrich)) dichloromethane (DCM) (Sigma-Aldrich) by heating the solution to 30 °C with continuous stirring. The solution was put in a 60-cc
syringe and electrospun with a flow rate of 15 mL/h, a voltage potential of 23 kV, a S-G distance of 30 cm, and fibers were collected on a 7.6 cm x 7.6 cm steel plate covered with aluminum foil [10, 11]. Again, electrospun scaffolds were placed in a vacuum overnight to remove any residual solvent.

Bi-layer scaffolds that contain one layer of dense fibers and one layer of porous fibers were also used for co-culture experiments. These scaffolds were created by first electrospinning a layer of 14 wt% PCL dissolved in acetone solution followed by direct deposition of electrospun 12 wt% PCL dissolved in DCM solution fibers on top. To ensure that the two layers did not delaminate, the S-G distance for the PCL in DCM solution was initially 15 cm but was increased 30 cm after 1 minute of deposition [11].
Figure 6.1 SEM images of electrospun scaffolds of (a) PCL derived from acetone, (b) collagen, and (c) PCL derived from DCM.
To prepare electrospun scaffolds for inoculation, they were first disinfected in 70% ethanol for 24 h, rinsed twice in sterile phosphate buffered saline (PBS, 24 h between rinses) (Sigma), rinsed 5 times in HEPES buffered saline (15-20 minutes between rinses) and lastly rinsed and incubated in cell culture medium (a step dependent on the type of cell used) for cell inoculation. Scaffolds were either cut into squares and placed into 6-, 12- or 24-well cell crowns (Scaffdex Oy) or remained as a 7.6 cm x 7.6 cm scaffold sheets for culture.

### 6.2.2 Smooth Muscle Cell Culture

Two types of SMCs were used: human coronary artery smooth muscle cells (CASMCs) (Lonza) and human aortic vascular smooth muscle cells (HA-VSMCs) (ATCC). Additionally, different SMC mediums were compared such as smooth muscle basal medium (SmBm) (Lonza), Dulbecco’s modified Eagle medium (DME) (Invitrogen), SMC proliferative medium (SMC-PM) [7], SMC quiescence medium (SMC-QM) [7], and nutrient mixture F-12 Ham Kaighn’s modification medium (F-12K) (Sigma). Supplements added into each SMC medium type are listed in Table 6.1.
Cell culture technique and procedures for SMCs remained the same regardless of cell line and medium type. SMCs were cultured at 37 °C and 5% CO₂ in SMC medium (DME, SMC-PM, or F-12K) with medium exchanged every other day. Once cells reached 70% confluence, SMCs were harvested and inoculated onto the scaffold (electrospun PCL, electrospun collagen, TCPS, or glass disks). SMCs were inoculated
onto sterile scaffolds and cultured anywhere from 7 to 18 days with medium exchanged daily when using plates and every other day when using dishes.

6.2.3 Endothelial Cells

Two types of ECs were used: primary human dermal microvascular endothelial cells isolated in our lab (CE759) and human umbilical vein vascular endothelial cells (HUVECs) (ATCC). Additionally, different mediums were compared such as Medium 131 (Invitrogen), DME medium, and EC-proliferative medium (EC-PM) [7] in order to find an alternative to the expensive complete medium provided from the manufacturer. Supplements added into each EC medium are listed in Table 6.2.

<table>
<thead>
<tr>
<th>Medium Name</th>
<th>Base Medium</th>
<th>Supplements</th>
</tr>
</thead>
<tbody>
<tr>
<td>131</td>
<td>MCDB 131</td>
<td>Microvascular Growth Supplement</td>
</tr>
<tr>
<td></td>
<td>Sodium Bicarbonate (1.18 g/L)</td>
<td></td>
</tr>
<tr>
<td>DME (for EC)</td>
<td>DME</td>
<td>FBS 20%</td>
</tr>
<tr>
<td></td>
<td>Sodium Bicarbonate (1.5 g/L)</td>
<td>PSF 10%</td>
</tr>
<tr>
<td>EC-PM</td>
<td>DME</td>
<td>Fetal Bovine Serum (5%)</td>
</tr>
<tr>
<td></td>
<td>CuSO₄ (3x10⁻⁶ g/L)</td>
<td>Porcine Serum (5%)</td>
</tr>
<tr>
<td></td>
<td>HEPES (0.05 M)</td>
<td>PSF (10%)</td>
</tr>
<tr>
<td></td>
<td>Proline (0.05 g/L)</td>
<td>bFGF (10x10⁻⁹ g/L)</td>
</tr>
<tr>
<td></td>
<td>Alanine (0.05 g/L)</td>
<td>PDGF (10x10⁻⁹ g/L)</td>
</tr>
<tr>
<td></td>
<td>Glycine (0.02 g/L)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Ascorbic Acid (0.05 g/L)</td>
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</tr>
</tbody>
</table>

Table 6.2 Base medium and supplements for EC mediums.
Cell culture technique and procedures for ECs remained the same regardless of cell line and medium type. ECs were cultured at 37 °C and 5% CO₂ in EC medium (131, DME, or EC-PM) with medium exchanged every other day. Once cells reached 70% confluence, ECs were harvested and inoculated onto the scaffold (electrospun PCL, electrospun collagen, TCPS, or glass disks). ECs were inoculated onto sterile scaffolds and cultured anywhere from 7 to 18 days with medium exchanged daily when using plates and every other day when using dishes.

6.2.4 Cell Viability

AlamarBlue (AB) (Invitrogen), 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) (Sigma-Aldrich), and 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) (Promega).

AB was used to evaluate cell viability in the scaffolds following manufacturer’s instructions. Briefly, a solution of AB was mixed with cell culture medium at 1:10 (AB:medium), then added directly to the wells containing scaffolds inoculated with cells at the desired time point, and finally placed in the incubator for 4 h at 37 °C and 5% CO₂ and shielded from light. After incubation, the solution was transferred to a new plate and read with a fluorescence absorbance reader (Tecan Genios Pro) at an absorbance of 570 nm. Average absorbance ± standard deviation was reported.

MTT is used to measure cell metabolism within the scaffolds. 4-mm punch (Miltex) biopsies were taken from the scaffolds and placed into wells of a 24-well plate.
Next, 0.5 mL of sterile 0.5 mg MTT/mL PBS was dispensed in each well with the biopsies and incubated for 3 h at 37 °C and 5% CO₂. After incubation, the MTT solution was removed and replaced with 0.5 mL methoxyethanol (Fisher Scientific) and mixed for 3 h on a rocking plate. Lastly, the medium was removed and its absorbance was read at 590 nm using a plate reader (Gemini Spetramax Molecular Devices). Average absorbance ± standard deviation was reported.

For the MTS assay 4 mm biopsies were removed from the scaffolds and incubated with the MTS-phenazine methosulfate (PMS) solution at 1:5 MTS-PMS solution to medium. Following a 3 h incubation at 37 °C and 5% CO₂, the solution was removed and its absorbance was read at 490 nm using a plate reader. Average absorbance ± standard deviation was reported.

6.2.5 Histology Sample Preparation

Samples of cells incubated on electrospun scaffolds were analyzed via histology sections to observe cell penetration depth within the scaffold rather than just being limited to the scaffold surface. Scaffold sections were embedded in cross section and cryosectioned. Samples were stained with hematoxylin and eosin and imaged with light microscopy.
6.2.6 Fluorescent Confocal Microscopy

To differentiate cells in co-culture, cells within scaffolds were first rinsed twice in PBS for 5 minutes/rinse, fixed in 4% paraformaldehyde (PFA) (Sigma) in PBS for 1 hour at room temperature, and rinsed twice again in PBS for 5 minutes/rinse. Afterwards, confocal microscopy was utilized to distinguish cells via nuclei (DAPI, Invitrogen), smooth muscle actin (α-SMA, Invitrogen), and platelet endothelial cell adhesion molecule (PECAM-1, Santa Cruz Biotechnology, Inc.). Secondary antibodies were used to distinguish the primary antibodies using Alexafluor 594 with PECAM-1 (to fluoresce red) (Invitrogen) and Alexafluor 488 with α-SMA (to fluoresce green) (Invitrogen). DAPI fluoresces blue. Samples were imaged using confocal microscopy (Olympus FV1000-Filter).

6.2.7 Scanning Electron Microscopy (SEM)

Scanning electron microscopy (FEI Quanta200 and FEI Sirion) was used to examine the microstructure of scaffolds as well as the post-inoculated presence and distribution of cells. All samples were adhered to aluminum SEM samples mounts (SPI Supplies) with conductive carbon tape and sputter coated with ~100 Å of gold (Pelco Model 3 Sputter Coater 9100) over a period of 60 s with an emission current of 15 mA and imaged using secondary electron mode at 5-10 kV. All cell cultured samples were fixed in 4% PFA and PBS prior to mounting and sputter coating.
To ensure viability of cells on the synthetic PCL scaffolds, many experiments were performed to obtain both numerical results (AB, MTT, or MTS) and images (confocal, histology, or SEM). Electrospun collagen and glass disks served as controls. It is historically known that our purchased cell lines perform well on 2D glass substrates, thus electrospun collagen served as our control for 3D substrates. For viability assays, \( n \geq 6 \) and for collecting samples for image analysis, \( n \geq 2 \). Most experiments collected data on days 1, 3, 5, and 7, where cell viability assays were performed and samples were removed from culture and prepared for image analysis. Infection of plates reduced the length of some experiments to only 5 days and resulted in the repetition of many experiments. Cells were inoculated initially at 5000 cells/cm\(^2\), however, inoculations increased to as high as 500,000 cells/cm\(^2\) because cells did not proliferate if the inoculation density was too low. While cell viability of both ECs and SMCs needed to be evaluated, complications in culturing ECs resulted in most experiments examining SMCs (specifically CASMC) in complete medium (SmBm) from the manufacturer. Table 6.3 shows experiments used for evaluating viability.
<table>
<thead>
<tr>
<th>Expt</th>
<th>Scaffold</th>
<th>Cell Type</th>
<th>Plate Size</th>
<th>Inoculation Density</th>
<th>Test Method</th>
</tr>
</thead>
<tbody>
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<td>CASMC</td>
<td>Dish</td>
<td>24-well plate</td>
<td>d1, d3, d5, d7, AlamarBlue, Confocal, SEM</td>
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<tr>
<td></td>
<td>12 mm glass disk</td>
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<td></td>
</tr>
<tr>
<td></td>
<td>14% PCL/ACE</td>
<td>CE759</td>
<td>Dish</td>
<td>24-well plate</td>
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<tr>
<td></td>
<td>12 mm glass disk</td>
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<td>CASMC</td>
<td>Dish</td>
<td>24-well plate</td>
<td>d1, d3, d5, d7, AlamarBlue and Confocal</td>
</tr>
<tr>
<td></td>
<td>12% PCL/DCM + O2 Plasma</td>
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<td>14% PCL/ACE</td>
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<td>24-well plate</td>
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<td>400,000 cells/cm2</td>
<td>d5, d7, d9, MTS and Confocal</td>
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Table 6.3 Experiments performed for evaluating cell viability.
6.2.9 Co-Culture of ECs and SMCs

As the co-culture of endothelial cells and smooth muscle cells is proposed to form the most functional TEBV, it was necessary to attempt to optimize culture medium, inoculation density and cell interaction on the scaffolds.

6.2.9.1 Medium Formulation

Many experiments were performed to analyze cell viability (through assays and microscopic imaging) when cultured in different mediums. In general, cells grow excellent in complete medium (131 or SmBm) provided from the supplier, however this medium is extremely costly and a more generic alternative was needed. After cells were expanded and frozen in large quantities, generic, cheaper mediums were tested such as DME, SMC-PM, SMC-QM, and F-12K. Once cheaper alternatives were established on 2D substrates (TCPS or glass), it was necessary to determine the ideal co-culture medium that could sustain both ECs and SMCs as these cell types are grown in different base mediums when cultured alone (used both 2D (TCPS or glass) and 3D (electrospun) substrates). This focus was based off of that of Lavender et al. [7] in which they observed co-cultured ECs were most successful if SMCs were in a quiescent rather than a proliferative state. Thus, it was necessary to ensure that cells could survive in the quiescent/proliferative mediums prior to co-culture. Additionally, co-culture medium was combined ratios (1:1 or 1:5) of EC and SMC medium [7].
In general, experiments were carried out for 7 days with data collected on days 1, 3, 5, and 7, or 1, 4, 7. For viability assays (AB, MTT, or MTS) $n \geq 6$ and for collecting samples for imaging (confocal, histology, or SEM) $n \geq 2$. A list of the experiments that evaluated cell culture medium is in Table 6.4.
Table 6.4 Experiments used for evaluating mediums used in single culture and co-culture of ECs and SMCs.
6.2.9.2 Inoculation Density

Determining the proper inoculation density is important because if there are too few cells, there is not enough cellular interaction occurs to maintain healthy cell cultures. HA-VSMC were inoculated at either 100,000 cells/cm² or 500,000 cell/cm² onto electrospun PCL fibers derived from DCM. The medium used was F-12K and scaffolds were placed in a 12-well plate. Samples were removed at d5, 7, and 9 for the MTS viability assay and for preparing samples for imaging (histology or confocal).

6.3 Results and Discussion

6.3.1 Cell Function on TEBV Scaffolds

SMC were cultured on electrospun PCL scaffolds (both acetone and DCM derived) and electrospun collagen (control) for 5 days. No statistical difference of viability was found between cells cultured on PCL-acetone, PCL-DCM, or collagen at any time point (Figure 6.2). This is perhaps because the SMC medium rich in proteins adsorbed onto the electrospun fibers as the scaffolds were incubated in medium for at least 30 minutes prior to cell inoculation.
Figure 6.2 MTS assay indicating that CASMC are able to proliferate on electrospun PCL scaffolds just as well as on the collagen controls.

EC viability on PCL was assessed both using PCL-acetone and PCL-DCM. Unfortunately, very low EC adhesion and viability were observed and by 3 days in culture no EC’s remained on the scaffolds (data not shown). SEM revealed that ECs were not adhered well to the fibers, and instead were visible as small balls on the fibers. During subsequent medium changes these poorly adhered ECs were easily rinsed off which explains why no ECs were present after day 3. Attachment Factor was then utilized before scaffold incubation in medium (prior to EC inoculation). However, efforts of cell attachment continued to be unsuccessful on these electrospun fibers.
6.3.2 Co-culture Medium Assessment

Several medium types were evaluated to identify the optimal culture environment for the co-culture of the SMCs and ECs. The medium studies were first completed using 2D platforms, TCPS and glass disks, due to simplicity and ease of assessment. Studies were then completed using the 3D fiber TEBV scaffolds.

SMCs were cultured in complete SmBm and supplemented DME medium. In DME, SMC viability, as measured by AlamarBlue, decreased significantly after 5 days in culture compared to the SmBm group (Figure 6.3). Thus our generic SMC medium was not adequate as a replacement for SmBm possibly due to lack of nutrients and proteins.

![Graph showing results from SmBm vs homemade DME medium.](image)

**Figure 6.3** Results from SmBm vs homemade DME medium. CASMC viability decreases after d3 when cultured in DME medium. * = p < 0.05 indicating statistical significance.
It is possible that the DME medium utilized pushed the SMCs toward a quiescent state leading to reduced cellular metabolism and proliferation at days 5 and 7. To promote proliferation during the SMCs mono-culture, alternate mediums were investigated. Lavender et al. [7] developed a two-stage medium system in which a proliferative medium, SMC-PM, was used to culture SMCs followed by inducing quiescence with SMC-QM prior to EC inoculation. The SMC-PM medium appeared promising for SMC culture after observing images obtained from confocal microscopy where cells were inoculated on glass slides (Figure 6.4). Unfortunately, the ECs had difficulty reaching the necessary density of a confluent monolayer.
Figure 6.4 Confocal microscopy revealing DAPI stained nuclei, (a) α-SMA in CASMC, and (b) PECAM-1 in CE759 on glass slides at d5.

6.3.3 Cell Inoculation Density

3D platforms such as electrospun PCL require a much higher seeding density than traditional 2D platforms due to the increased amount of surface area (especially for SMC
in the porous PCL scaffolds derived from DCM). Inoculation densities of 100,000 cells/cm² versus 500,000 cells/cm² were evaluated. The higher density produced better cell proliferation results via MTS assay, histology sections, and confocal (Figure 6.5) and SEM microscopy. However, histology and microscopy revealed that SMCs did not infiltrate the porous (PCL-DCM) electrospun scaffold but rather remained on top (Figure 6.6). The SMCs did not infiltrate the porous, large diameter, DCM derived fibers was unexpected given the porosity of the scaffold. This could be due to surface tension within the scaffold that creates a barrier to SMC penetration of the scaffold.

![Image](image_url)

Figure 6.5 SMCs inoculated at (a) 100,000 cells/cm² do not proliferate as well as (b) 500,000 cells/cm².
Figure 6.6 SMCs remain on top of the electrospun DCM derived PCL scaffolds rather than infiltrate illustrated by both (a) histology sections and (b) SEM.
6.4 Conclusions

Successful TEBV requires the co-culture of ECs and SMCs. However, determining the ideal cell culture medium, substrate, inoculation density, and cell lines to use proved to be a challenging task. Nevertheless, valuable information was obtained: SMC were able to proliferate on electrospun PCL scaffolds (although techniques to improve infiltration throughout the scaffold remain to be determined), cells produced better cell proliferation data when inoculated at higher densities, and determining the optimal co-culture medium was essential as ECs and SMCs were generally cultured in different mediums when cultured as single cells. The switch over to HAECs is promising as this cell line has expanded very well in culture so far and is assumed to be highly compatible with HA-VSMCs.
6.5 References


Chapter 7: Conclusions and Future Work

Tri-layer scaffolds were manufactured by electrospinning different PCL solutions, creating three distinct layers, each with their own unique properties to mimic the layers present in native vessels. The inner layer was electrospun from a solution of PCL dissolved in acetone to create dense fibers (pore size ~90 μm and thickness of scaffold ~100 μm) that support the attachment of endothelial cells (ECs) to form the endothelium. The middle layer was electrospun from a solution of PCL dissolved in dichloromethane (DCM) to create a highly porous layer (pore size ~1800 μm and thickness of scaffold ~200 μm) to allow infiltration of smooth muscle cells (SMCs). It is within this layer that microchannels were ablated via femtosecond laser ablation to assist in rapidly aligning SMCs during inoculation. The third and final layer was electrospun from a solution of PCL dissolved in 1,1,1,3,3,3-hexafluoro-3-propanol (HFP) onto a rotating mandrel to create dense aligned fibers that provide the mechanical strength for the scaffold (thickness of scaffold ~100 or 300 μm).
7.1 Mechanical Properties

The presence of cell seeding microchannels were intended to guide the SMCs, however, there was concern that the mechanical strength could be compromised by creating these defects in the scaffolds. The presence of microchannels decreased mechanical strength of single layer, bi-layer, and tri-layer samples (but only when the HFP derived layer was thin). A thicker HFP derived layer in tri-layer samples was able to dominate and prevent significant changes in mechanical strength when microchannels were present.

Although no significant reductions in mechanical properties were observed when 1 or 5 channels were ablated into tri-layer sheets, it is not yet known how these channels will affect the mechanics of a tubular construct or at the density of channels envisioned in the final construct. The next step would be to ablate microchannels into electrospun tubes as the final construct will be in tubular form with 100 μm wide x 100 μm deep channels spaced 100 μm apart from each other. It is hypothesized that if the outer HFP derived layer is thick enough, it will be able to dominate again as observed in the 2D electrospun sheet platform. There is concern, however, that these dominating HFP fibers may cause the overall graft to be too stiff and can result in compliance mismatch. Thus it may be necessary to find the thinnest thickness for the HFP derived layer that is still capable of maintaining adequate mechanical properties. Other alternatives include using a different polymer or solvent to electrospin in order to create a fibrous scaffold that may be able to provide more elasticity while maintaining strength.
Though we did not believe that the presence of microchannels weakens mechanical strength of electrospun PCL tri-layer scaffolds initially, it was important to understand how multiple expansion/contraction cycles affected the structural and mechanical properties of the constructs. After cyclic strain at 25% for 7 days, holes began forming in the robust, stiff, aligned HFP derived layer in the tri-layer scaffolds. However, this did not occur when scaffolds were strained to 10%, a strain magnitude much closer to what is observed in a typical in vivo setting. Additionally, mechanical properties (UTS, modulus, and elongation) were not drastically altered due to fatigue. While these results suggested the tri-layer scaffold platform would be able to withstand the strains found in the native vessel, it was unknown how the microchannels would alter this response or how moving from a sheet format to a tube format might alter the response. Additionally, the constructs were subject to only 7 days of cyclic strain which is the equivalent of 604,800 cycles, far short of the $9.43 \times 10^8 - 1.25 \times 10^9$ cycles the construct would experience in an estimated 30-40 year graft lifespan. As a result, long term cyclic testing should be performed to provide an estimate of the life of the materials. Higher frequency fatigue testing could also be performed with the caveat that the strain rate might strongly influence how the material responds to the applied tension.

To evaluate mechanical strength of scaffolds during expansion/contraction under normal blood flow, tubular scaffolds could be tested on a pulsatile perfusion flow loop that contains medium or even blood to simulate in vivo conditions and pulses flow at ~70 bpm.
While burst pressure was unnaturally high within the electrospun tri-layer tubes, this data does not compensate for (1) a 37 °C water-based environment that could potentially deteriorate and weaken/soften the PCL fibers or (2) presence of laser ablated microchannels. Thus to properly assess the burst pressure, these additions should be added to the experimental setup.

Additionally, mechanical properties have only been evaluated for electrospun PCL scaffolds without the presence of cells. The effects of both ECs and SMCs on PCL scaffolds could drastically alter the mechanical strengths- possibly weaken due to fiber decomposition or strengthen from the effects of an EC monolayer or aligned SMCs. The duty of SMCs in blood vessels is to allow contraction/dilation, thus, this phenomena needs to be evaluated as well once it is determined how to properly align SMCs on electrospun fibers.

7.2 Biological Integration

Since the presence of microchannels did not appear to affect overall mechanical properties of electrospun tri-layer scaffolds, the ability of channels to contain cells needed to be evaluated. To simplify testing methods, 20 μm beads were flowed into ablated microchannels in acetone derived PCL scaffolds. The velocity of beads within the channel was calculated to be 0.6 cm/s, far slower to the calculated computational velocity of 4.125 cm/s due to fluid loss through the channel walls.
SMCs were also flowed through the ablated microchannels to observe if cells were able to (1) adhere to the PCL fibers, (2) fill the entire length of the microchannel, and (3) align in the desired orientation. SMCs were able to successfully adhere to PCL fibers as well as fill the entire length of the microchannel, however, instead of aligning in the desired direction of the channel, the cells spanned across the channel in the perpendicular direction. It is expected that a thinner microchannel, perhaps 50 \( \mu \text{m} \) instead of 100 \( \mu \text{m} \) would solve this problem.

Obvious next steps include repeating these experiments in DCM derived fibers since this is the SMC containing layer. Thus, 20 \( \mu \text{m} \) beads will be flowed through ablated microchannels in electrospun DCM derived fibers to determine the ideal flow rate to inject beads, to observe how the beads spread out of the microchannel into the surrounding porous fibers, and to calculate velocity of beads through the porous DCM fibers. Since there was significant fluid loss within ablated microchannels in acetone derived fibers, it is expected that beads must be introduced into the channel at higher flow rates in DCM derived fibers to ensure beads adequately fill the entire length of the channel.

Once the appropriate flow rate is determined, SMCs can be used to determine if they can (1) fill the entire microchannel, (2) infiltrate into the surrounding DCM derived fibers, and (3) align in the direction of the channel.

A variety of types and combinations of SMCs, ECs, and cell culture medium were evaluated in hopes of determining the ideal methodology to successfully co-culture SMCs and ECs in electrospun PCL scaffolds. However, success of cellular integration
was not attained and it still being assessed. Hopefully by using SMCs and ECs derived from the same tissue (human aortic vascular SMCs and human aortic ECs), greater success can be obtained.

The best combination of cell culture medium is still being determined as well. It is still unknown if ECs should be inoculated onto quiescent SMCs or if ECs are able to help migration of SMCs into the scaffold rather than just remaining on the surface of porous electrospun PCL fibers.

Additionally, cells have only been cultured on acetone and DCM derived single layers and bi-layers. While HFP derived scaffolds are composed of the same PCL material post-electrospinning, it is necessary to ensure that stiffness of this layer is not detrimental to cells.

7.3 Concluding Remarks

While there is much work to be done in the future, great progress has been made in the envisioned electrospun PCL tri-layer graft. The search for the “holy grail” of small diameter vascular grafts still remains and the work presented confirms the complexity of creating such a graft. The ideal small diameter vascular graft is affordable with off-the-shelf availability and is comparable to native tissue with appropriate mechanical strengths (tensile, fatigue, burst pressure, SRS, compliance) as well as exhibit excellent biocompatibility to increase patency in vivo by decreasing risk of thrombosis and intimal hyperplasia.
References


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