ABSTRACT

Title of Document: ELECTROSPUN SMALL-DIAMETER SILK FIBROIN VASCULAR GRAFTS WITH TUNED MECHANICAL AND BIOCOMPATIBILITY PROPERTIES AS TISSUE ENGINEERED SCAFFOLDS

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Almost 9 million people in the U.S. have peripheral arterial disease (PAD). In severe cases of PAD, arterial bypass surgery is performed to redirect flow around the problem area. However, for many elderly patients, this surgery is not feasible using the preferred autologous grafts because of the limited availability of tissue to use for grafting, so there is a clinical need for engineered vascular grafts. Engineered grafts are intended to replace native blood vessels by manipulating biomaterials to mimic the properties of the native vessel. Despite success in large diameter cases, small diameter grafts are still prone to a number of issues such as occlusion, hyperplasia, and thrombosis. Silk fibroin is a promising biomaterial for creating vascular grafts because of its demonstrated mechanical strength and biocompatibility. Our research established

a method for electrospinning the silk fibroin onto a rotating mandrel for seamless grafts. Mechanical testing, including burst pressure and tensile strength tests, compared the strength of our grafts to that of the autologous vessel. Finally, biochemical modifications, aimed at both recruiting and proliferating HUVECs on the grafts, increased cell proliferation on the grafts *in vitro*.

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Thesis submitted to the Honors College

of the University of Maryland, College Park, in partial fulfillment

of the requirements for the

Gemstone Program,

University of Maryland

2017

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Acknowledgements

We would like to thank everyone who supported us throughout this project. First, our mentor, Dr. Adam Hsieh, and other members of the OML lab — Dr. Hyunchul Kim, Poonam Sharma, and Lauren Resutek — for their guidance, resources, and assistance in our project. Our librarian, Eileen Harrington, and the Gemstone staff have continuously provided support. The Hu, Jay, Scarcelli, Kofinas, Fisher, White, and Kaplan (Tufts University) labs contributed resources, training, and advice. Finally, many thanks to our families and friends for believing in us throughout these past four years. Funding was provided by Gemstone, the UMD Libraries, and our Launch donors.

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*Note: * Indicates that a word can be found in the glossary.*

1. Introduction

Despite advances in healthcare within the past few decades, cardiovascular diseases remain the leading cause of death in Western countries.[1] Approximately one in three American adults has some form of cardiovascular disease, costing the U.S. nearly half a trillion dollars yearly.[1,2,3]

1.1. Peripheral Arterial Disease

Peripheral arterial disease (PAD) is a cardiovascular disease in which the arteries that supply blood to the peripheral microvasculature in the legs, stomach, arms, and head become obstructed.[4] Most cases of PAD are caused by atherosclerosis in the arteries leading to the microvasculature.[5] The disease, which involves symptoms such as cramping, pain, or tiredness, is frequently misdiagnosed.[5] If left untreated, PAD can result in gangrene, necessitating limb amputation.[5] In addition, PAD is of note because it is often an indicator for poor overall cardiovascular health and a warning for other cardiovascular diseases.

Particularly alarming is the high prevalence of the disease. Over 200 million people worldwide and 8.5 million Americans have PAD, costing U.S. hospitals over $21 billion per year by 2004 estimates[1] of direct costs.[5–8] The first response to PAD is risk factor modification, as PAD indicates that the patient is at a higher risk of lethal conditions such as cerebrovascular disease and coronary artery disease and more likely to suffer a stroke or myocardial infarction. These risk factor modifications include increasing exercise to prevent peripheral blood clotting, quitting smoking, managing diabetes, taking statins to control low density lipoprotein (LDL) levels, and using ACE inhibitors to reduce hypertension.[9,10] Severe PAD must be treated with

[1] This figure is based solely upon direct medical costs associated with PAD and does not include any of the indirect costs that are associated with the AHA estimate of heart disease's overall financial burden.

surgery, one of which involves the use of endovascular stents and balloon angioplasty to keep a conduit open. The other is arterial bypass, a critical procedure to address PAD high-risk patients who have multiple obstructed sites. Arterial bypass surgery provides an alternative route for blood flow in order to bypass the problematic area. Arterial bypass can help prevent complications such as cerebrovascular disease, myocardial infarction, and coronary artery disease.[11]

1.2. The Current Standard

Herein, we focus on PAD, which can be treated with peripheral arterial bypass surgery. This surgery is preferentially performed with autologous grafts harvested from the patient's great saphenous vein, small saphenous vein, or superficial arm veins.[12,13] However, there are many situations in which use of autologous grafts is not feasible due to inadequate sources of autologous tissue. Often, this is because the lumen desirable blood vessels have decreased diameters below 3 mm, which is insufficient to create an autologous graft.[11,14,15] Xenografts and allografts can also be used, but are problematic for multiple reasons. Namely, they have an increased risk of infection and the latter can, for unknown reasons, become aneurysmal in some patients. They also require complicated preparation steps and specialized storage.[16,17] As such, there is a need to develop viable engineered grafts that can be used off-the-shelf, without any preparation. In general, current engineered grafts have yet to perform adequately for treatment of PAD in the small diameter vasculature.[18]

The ideal vascular graft should be biocompatible, leak-proof, elastic, antithrombotic, conducive to cell growth, and resistant to infection or inflammatory responses.[11,18] Engineered grafts should aim to mimic both the structure and function of the native vessel.

Companies such as Terumo Vaskutek, Gore, and Jotec already offer commercially available synthetic grafts made from non-degradable materials such as Dacron (polyethylene terephthalate, PET) and Teflon (expanded polytetrafluoroethylene, ePTFE). These grafts have been successful for large vessels, in which there is high flow and low resistance.[1] However, these grafts are prone to problems in small-diameter vessels less than 6 mm in diameter.[1,11,18] Due to hypotensive flow in small-diameter vessels, grafts in this part of the vasculature often fail due to issues such as thrombosis, occlusion*, poor compliance, and intimal hyperplasia*. Furthermore, infection can require the removal of the graft. On the other hand, grafts using natural biological materials are susceptible to mechanical failure.[15]

Despite the wide range of approaches to producing small-diameter vascular grafts, there currently remains no good option. Therefore, the exploration of new biomaterials as potential solutions is needed. As such, engineered grafts made of synthetic biomaterials have emerged; however, there is currently not an ideal option for clinical use in small-diameter blood vessels.

Silk fibroin (SF) is one promising material for vascular grafts. SF is a protein produced by the *Bombyx mori* silkworm that has demonstrated biocompatibility* and mechanical viability both *in vitro* and *in vivo*, thus distinguishing itself as a potential biomaterial.[1,19–24] Furthermore, SF is amenable to electrospinning, a method of fabrication that is advantageous because it enables a high degree of control over fiber morphology.[1,25,26] Despite its potential, SF has yet to be a fully-researched and developed biomaterial capable of addressing current limitations of small-diameter vascular graft applications. We hope to expand upon the research using SF as a biomaterial for vascular tissue engineering.

Our research project addresses the clinical need for small-diameter vascular grafts, mitigating the limitations of current solutions that are commercially available. Furthermore, our

work presents an off-the-shelf alternative for bypass surgery in the small diameter vasculature. Specifically, we aimed to answer the following questions: **How can electrospinning parameters affect the silk fiber morphology in a small-diameter, electrospun SF vascular graft? Additionally, can Matrigel, an extracellular matrix protein extract, be used to improve the biocompatibility of the graft? Furthermore, can co-culture of endothelial cells with smooth muscle cells improve the biocompatibility of the graft? Finally, can the grafts withstand the pulsatile blood flow that would be applied to a vascular graft when inserted?** *It is hypothesized that fiber morphology of electrospinning with SF is a function of distance, voltage, and velocity. It is also hypothesized that Matrigel will improve the adhesion of vascular endothelial cells on the graft, and co-culture of endothelial cells with smooth muscle cells will improve proliferation of endothelial cells on the graft. Finally, it is thought that the grafts will be strong enough to withstand blood flow due to the strong nature of SF.* This thesis summarizes the work towards answering these questions and furthering the research of electrospun silk fibroin vascular grafts for small diameter tissue scaffolding applications.

1.3. Specific Aims

In order to answer our research question, we formulated the following specific aims, which we attempted to address through our experiments.

1. **Determine how electrospinning can be used to control fiber morphology in grafts.**
2. **Assess the *in vitro* biocompatibility of the electrospun silk scaffolds with vascular endothelial cells.**
3. **Test how Matrigel coating affects the biocompatibility of the graft *in vitro*.**
4. **Assess mechanical properties of the grafts.**

2. Literature Review

2.1. Overview

The following compilation of the scientific literature has helped us hone our research question and determine what issues in the field remain unaddressed. In order to understand how to improve vascular grafts, it is critical to first understand the natural anatomy and physiology of the native vessel that grafts seek to replace. This literature review will then elaborate on some basic tissue grafting principles, followed by the properties that an ideal vascular graft should have. The next section will discuss the use of silk as a biomaterial, specifically in grafts. The final portion of the literature review will cover ways to construct and modify the grafts to increase their patency*.

2.2. The Native Vessel

The goal of vascular grafts is to mimic the structure and functionality of a native artery. Therefore, an understanding of the native vessel is crucial to constructing a suitable graft.

2.2.1. Composition of Blood Vessels

The cardiovascular system, composed of arteries, veins, and capillaries, is responsible for transporting oxygen, nutrients, waste, and other biological factors throughout the body.[27,28] The arterial wall consists of three tissue layers, as seen in Figure 2.2.1.1. The outer layer consists of connective tissue that attaches the artery to surrounding tissue while allowing it to stretch and recoil.[28] The middle layer is made up of smooth muscle cells and elastic fibers. This layer is the predominant mechanical support for the vessel, and is the thickest layer in arteries because of the high blood flow.[28] The inner layer consists of a thin sheet of endothelial cells lining the innermost surface of the vessel. Endothelial cells are responsible for angiogenesis during tissue growth and repair.[29]

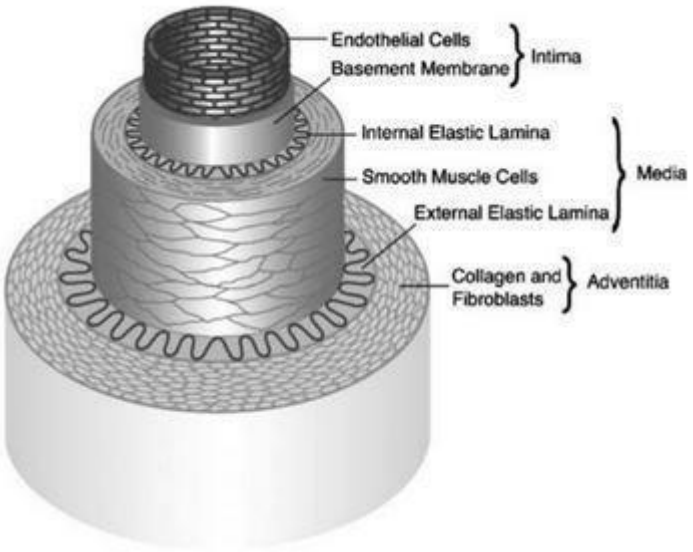


Figure 2.2.1.1. Breakdown of the layers that make up the vessel wall. Taken from Sarkar, Schmitz-Rixen, Hamilton, & Seifalian, 2007.[30] Reprinted with permission from Springer.

In addition to various cells, the blood vessel is held together by the extracellular matrix* (ECM), which consists of macromolecules that support the cells.[29] The ECM performs several important functions, such as providing structural support, bearing mechanical stresses, regulating cell proliferation and differentiation, modulating growth factors, and contributing to the plasticity of blood vessels.[31]

2.2.2. Angiogenesis and Arteriogenesis

Angiogenesis* and arteriogenesis* are both processes that are of interest in vascular tissue engineering. Angiogenesis refers to the process by which new capillaries sprout from existing vasculature.[29,32] While the process is complex, the basic stages involve the stimulation of endothelial cells by various growth factors to migrate, proliferate, and form capillary tubes.[29] Sensing and signaling by endothelial cells is essential to this process. These newly formed vessels are fragile as they consist only of endothelial cells and lack a smooth muscle layer.[32]

Arteriogenesis refers to the remodeling process by which existing blood vessels are enlarged.[32] Following endothelial cell proliferation, smooth muscle cells proliferate and migrate to expand the artery.[32] These cells secrete ECM proteins that form a new lamina intima layer.[32] Arteriogenesis is a response to excessive mechanical stress: endothelial cells sense shear forces and release molecules that are active in recruiting pericytes and smooth muscle cells.[29]

Both of these processes occur in the body as part of the body's natural repair mechanism. They are also of interest in tissue engineering, as properties that facilitate this regenerative response are desirable in any vascular graft.

2.3. The Basics of Grafts

2.3.1. Types of Grafts

Grafts are generally grouped into four main categories according to source material: autologous grafts, allografts, xenografts, and engineered grafts, as shown in Fig. 2.3.1.1. Autologous grafts use the patient's own tissues or tissues from someone of the same genome, namely monozygotic twins. These grafts are typically from the internal mammary artery or saphenous vein and are considered ideal due to minimal issues of immunological responses.[33] In fact, arterial autografts are often used due to their many benefits, such as resistance to infection, resistance to inflammation, and attachment to the native vessel wall.[18] Unfortunately, autologous grafts are not viable solutions for sick or elderly patients, as their tissue is not of high enough quality. The tissue quality determines the long-term outcomes and success of the graft.[18] Furthermore, autologous grafts can take weeks, if not months, of preparation and processing *ex vivo* before the surgery.[34]

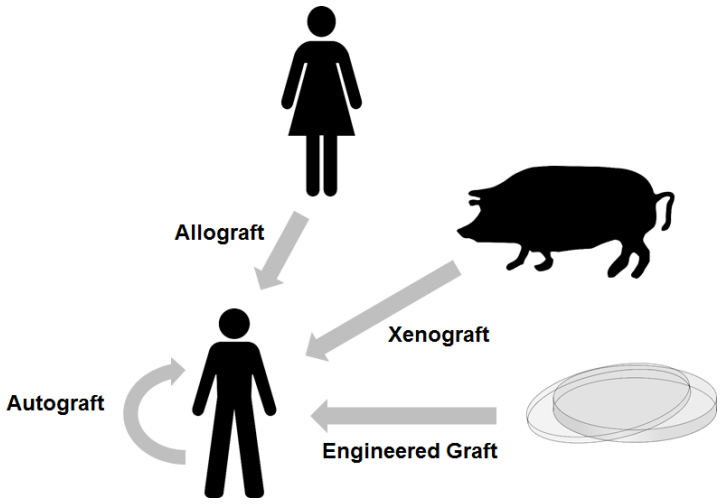


Figure 2.3.1.1. A graphical representation of autografts, allografts, xenografts, and engineered grafts.

Therefore, alternative grafts are necessary. Both allografts and xenografts are biological grafts that use foreign tissue. Allografts are fabricated from tissue harvested from a human donor and placed in another person's body.[35] However, issues include cost, availability, immune response, infection, and graft failure.[35] Furthermore, there is evidence that the processing required to sterilize allografts may compromise their mechanical and biological integrity.[36–38] Xenografts use tissues from other species as a source for graft material.[39] The use of xenografts has faced many ethical objections, regarding patient perspective and the humane treatment of the animals.[40] Additionally, unresolved biological questions related to transplant rejection and zoonotic infection generate uncertainty about the viability of xenografts.[41]

Engineered grafts are another alternative to autologous grafts. These grafts are advantageous because there are no limitations in terms of availability and because the broad range of possible materials and processes enables greater fine-tuning of biological and mechanical properties. Currently there are two main types of synthetic materials used for engineered grafts: PET* (Dacron) and PTFE* (Teflon). Both materials have many issues when used for small diameter applications, including both short and long-term problems; these issues

include stenosis*, thromboembolization*, calcium deposition, infection, and inability for the grafts to grow as pediatric patients grow.[33] A recent study found that PTFE bypass procedures had a 39% five-year primary patency rate.[42] Thus, many patients need to undergo multiple operations, which increases mortality rates.[33] For this reason, researchers are investigating the properties of other biomaterials that can be implemented to yield greater biocompatibility.

2.3.2. Tissue Scaffolding

One tissue engineering focus is the production of a tissue scaffold*, under which engineered grafts fall. Scaffolds provide a structure upon which cells can grow and secrete their own extracellular matrix (ECM), facilitating regeneration of the native tissue and neovascularization*.[43,44] The tissue scaffold provides support to the damaged tissue or organ until the full functionality is reestablished.[45] Vascular scaffolds should thus be created with a systems perspective and a focus on practical application.

Engineered grafts provide a scaffold to allow endothelialization of the surrounding tissue to help rebuild the natural vessel.[12] For a scaffold to function adequately, it must have appropriate architecture, biocompatibility, tissue compatibility, bioactivity*, and favorable mechanical properties.[43] In terms of architecture, the scaffold should not only have appropriate porosity* for cell growth, integration, and communication, but also retain mechanical stability during growth.[43] The scaffold should provide an environment compatible with the endogenous cells*.[46] The biomaterials of the scaffold may degrade at a rate that matches ECM production of the tissue and mimic the role of the ECM in normal tissues.[47] Different biomaterials interact with cells in a specific way that lead to various cell responses.[45] The scaffold may exhibit bioactivity by either interacting with the cellular components to facilitate growth or by acting as a delivery

system for cell-stimulating signals.[43] These qualities of the graft play an important part of regulating cellular functions and behavior, and consequently impact the growth of the tissue.[48]

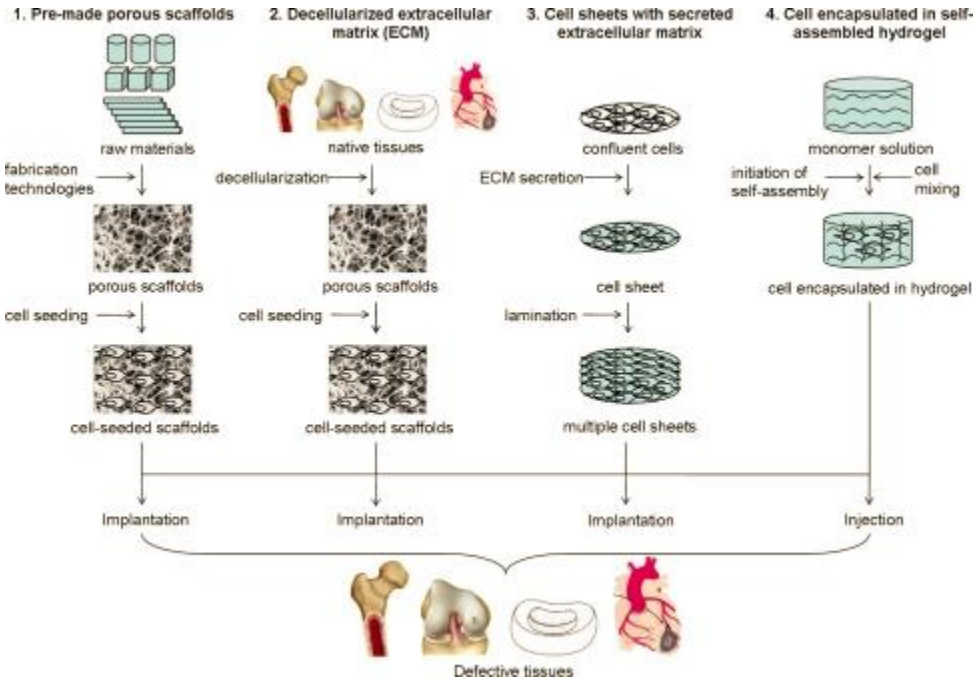


Figure 2.3.2.1. Diagram of various methodologies for constructing scaffolds from reference Chan & Leong, 2008 with permission of Springer. [43]

The four main types of tissue scaffolds are decellularized ECM, cell sheets with secreted cellular ECM, cellular encapsulated grafts, and pre-made porous scaffolds as shown in Figure 2.3.2.1.[39] Decellularized ECM is an approach that removes the allogenic or xenogenic cellular antigens from tissues and uses the remaining structural proteins to serve as the scaffold for the tissue that needs to be replaced.[49] While this is a common methodology for use in vascular grafts, a disadvantage to this method is the cost of obtaining the appropriate type of ECM and the possibility of an immune response due to incomplete decellularization.[39] The approach of producing cell sheets with secreted cellular ECM involves the use of enzymes to harvest ECM secreted by cells which have been seeded on a thermo-responsive polymer coated dish.[39] This approach is limited in its load bearing ability and is more advantageous in producing epithelium

and endothelium.[39] Encapsulating cells in self-assembled hydrogels is an approach that involves embedding living cells within a semipermeable membrane or a homogenous solid mass.[50] This method is advantageous for adapting to irregularly shaped defects, but tends to have poor mechanical qualities.[39]

A common and well-established method for producing scaffolds are pre-made porous scaffolds* in which either natural biological materials or synthetic biomaterials are processed using a variety of fabrication techniques including porogen* based, solid free-form, and fiber based.[51] Porogen based fabrication involves casted or extruded mixtures of materials and porogens in which the porogens are later sublimated, evaporated, or melted to make pores in the final scaffold.[39] Solid free-form techniques can involve laser sintering, stereolithography, and 3D printing to produce a solid structure.[39]

Lastly, fiber based production of scaffolds with electrospinning utilizes a voltage applied to a solution to produce a fibrous material. Due to the advantageous nature of the fiber based electrospinning technique in controlling pore size and producing large sheets of material, the focus of this project is on the pre-made porous scaffolds with fiber based production and particularly the different alterations of the scaffold in the development of vascular grafts.

2.4. Properties of Ideal Grafts

The ideal vascular graft is characterized by biocompatibility and mechanical properties post-implantation.[52] Mechanically, grafts placed in arterial circulation must be capable of withstanding long-term hemodynamic* stress without material failure, meaning that the construct used should perform its function in both high and low stress environments.[52] Thus, the graft must be able to sustain the same mechanical stresses as autologous blood vessels such as saphenous veins, which are considered to be the “gold standard” of small diameter vessel

replacement.[52] Although there are different measures for the mechanical strength of a saphenous vein, one record determines that the circumferential tensile strength is 1.8MPa with 242% strain, the longitudinal tensile strength is 6.3MPa with 83% strain, and the burst pressure ranges from 1680-3900 mmHg.[53,54] In addition, the graft must be able to pass a suture rip test so that it does not tear under the normal force exerted on it during surgical implantation.[12] The strain measurements suggest elasticity of the native vessel, which is critical to handling the pulsatile flow.[53]

In terms of biocompatibility, a tissue-engineered vascular graft should resemble a native saphenous vein in structure and function, specifically working to promote complete regeneration of the endothelium.[52] In addition to non-thrombogenicity* and degradability*, availability and simplicity of handling are desirable traits to minimize operating time, risk, and expense.[52] Although biological grafts possess mechanical properties that are already well suited for the stresses of the circulatory system, their flaws, such as post implantation healing complications and limited availability, have spurred efforts to improve upon engineered grafts through bioengineering approaches.

2.5. Silk

2.5.1. Intro to Biomaterials

A graft's mechanical strength and biocompatibility depend on many properties of materials, such as micro and nanostructure, crystallinity*, elasticity*, hydrophilicity*, porosity, surface properties, and degradation.[55] Many different materials offer a range of these properties, allowing optimization for the target purpose. Natural and synthetic polymers are the most commonly used biomaterials in engineered vascular grafts due to their availability and wide

range of properties. Natural polymers include collagen, fibrin, and gelatin. Common synthetic polymers include poly(lactic-co-glycolic) acid (PLGA), polycaprolactone (PCL), and PTFE.[56–58]

As they originate from the ECM, natural polymers are generally degradable and less likely to provoke a foreign body response. They possess properties that synthetic polymers may lack, such as hydrophilicity, cellular affinity, and reactive groups.[57] Despite these benefits, natural polymers are less easily processed and fail to exhibit the same level of mechanical strength as other polymers.[55]

On the other hand, synthetic polymers are easy to both manufacture and process with control; they are commonly used due to the advantageous mechanical properties they provide. Although they may induce more of a foreign body response than natural materials, they are largely biocompatible and degradable.[56] However, there have been documented incidents where synthetic polymers did not match well with native tissue, resulting in hyperplasia.[59] For the most part, synthetic polymers are less biocompatible than natural polymers.[57]

Choice of material is a major component of tissue engineering, as all materials have advantages and disadvantages. The nature and specific functions of the tissue being engineered are crucial in dictating which material properties should be prioritized. Aside from the most commonly chosen biomaterials, there are many novel biomaterials that warrant further study. In this thesis, we focus on silk fibroin, a biomaterial used for many tissue engineering applications.[60]

2.5.2. Silk Fibroin

Silk is obtained from the silkworm, *Bombyx mori*. During the late stages of larval development, *B. mori* synthesizes a large amount of silk in the silk glands.[61] The silk derived from the silkworm can be used for a variety of applications ranging from textiles to medicine.

Silk proteins are high in molecular weight and are made of two or more different subunits.[62,63] The silk that is produced from *B. mori* consists of two main proteins, sericin* and SF. Approximately 96% of the silk is derived from these two proteins, and about 60-70% of that is fibroin.[61] SF is best suited for tissue engineering applications due to its ready abundance, advantageous structural properties, and biocompatibility, which likely stem from the chemical structure.

SF is composed of heavy and light strands linked by disulfide bonds.[64] The heavy strand contains glycine, alanine, and serine residues, which form beta sheets in the secondary structure, creating a highly crystalline structure, as shown in Figure 2.5.2.1.[64] The homogeneity in the secondary structure of the silk is believed to give rise to some of silk's incredible mechanical properties.[21] Single stranded silk has been shown to have a modulus of elasticity of 16±1 GPa, a yield strength of 230±10 MPa, and an ultimate tensile strength of 650±40 MPa.[19]

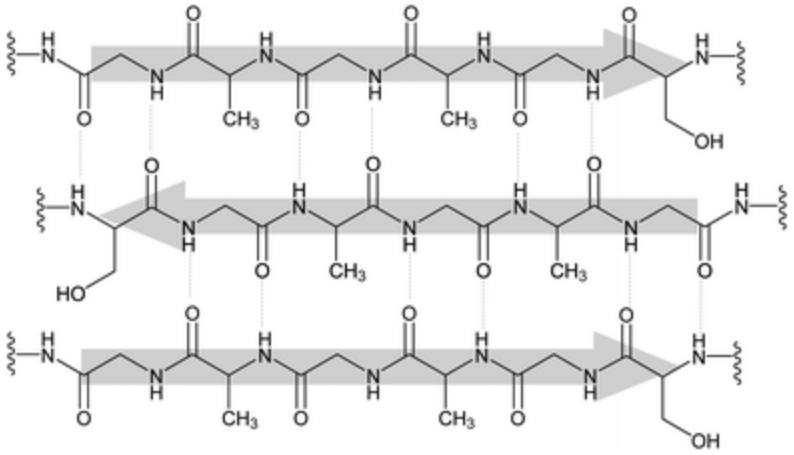


Figure 2.5.2.1. Shows the chemical structure of the antiparallel $(Gly\text{-}Ala\text{-}Gly\text{-}Ala\text{-}Gly\text{-}Ser)_n$ amino acid sequences of SF in the anti-parallel β-pleated sheet structure. Taken from Murphy & Kaplan, 2009 with permission from the Royal Society of Chemistry.[63]

The silk can be extracted from the cocoon of *B. mori* using a combination of degumming processes*.[12,65,66] It is essential that sericin be removed, as it is known to cause an immune

response *in vivo.*[21] The remaining SF elicits little to no immune response *in vivo.*[67] It is important to note that almost all non-autologous biomaterials will elicit at least some immune response, although for SF, this is normally sensitization* due to pre-exposure, not the more serious T-cell mediated hypersensitivity*.[17]

2.5.3. Silk-based Vascular Grafts

SF has been known to be an effective biomaterial for centuries and is commonly used in the medical world as sutures.[21,64,65] SF-based vascular grafts have been a relatively recent development. One of the first published papers attempting to use SF specifically for small-diameter vascular grafts was introduced in 2007.[68] The goal of this research by Lovett *et al.* was to develop small-diameter vascular grafts from SF microtubes. The study proved both the mechanical properties and biocompatibility properties of the grafts. The authors concluded that SF from *B. mori* could indeed be used to make vascular grafts, paving the way for further works that improve the SF vascular grafts through manufacturing changes and biochemical tailoring.[68]

One problem with current grafts is that the SF is brittle after fixation with methanol.[12] The search for elastic grafts that can better mimic the native vessel has turned researchers to crosslink the SF with poly(ethylene glycol) diglycidyl ether (PEG-DE), a flexible chain polymer. Varying the ratio of SF to PEG-DE can change the porosity of the graft. Overall, the researchers proved that PEG-DE is effective in increasing the flexibility of SF vascular grafts and for promoting cell proliferation.[69]

The possibility of improving the biocompatibility of previously fabricated SF tubes to develop a durable type of silk-based vascular graft for use *in vivo* is still being explored.[12] An issue that must be confronted when it comes to incorporating SF in vascular grafts is developing a small-diameter graft that simultaneously stimulates angiogenesis and mitigates the risk of

mechanical failure and thrombosis. As a result, researchers Liu *et al*. published a paper examining the success of a bilayered vascular graft based on SF that they created in order to improve mechanical strength, suture retention strength, and mechanical compliance. Based on this bilayered structure, Liu *et al*. focused on improving biocompatibility in order to reduce blood leakage and promote tissue regeneration* surrounding the vascular graft. Using a variety of polymers, the team ultimately found that the silk-based bilayer vascular graft achieved the highest suture retention rate in comparison to previous silk-based grafts.[12] A separate lab investigated this problem as well, finding a similar conclusion that silk fibroin produced outcomes better than PTFE non-degradable grafts.[70] Catto *et al*. recently published a paper that investigated 4.5 mm and 1.5 mm inner diameter electrospun SF vascular grafts mechanically, morphologically, *in vitro*, and *in vivo*.[52] This extensive study proved to be another testament to the utility of electrospun SF for small diameter vasculature.

2.5.4. Silk Fibroin Current Research

Within the human body, the environment of a vascular graft is much more complex. As a result, there have been many different studies that involve layering or blending various materials in conjunction with silk fibroin. A summary of a representative portion of the literature on the mechanical properties of SF grafts fabricated with a variety of fabrication methods and using different polymer combinations is shown in Table 2.5.4.1. These properties for the gold standard autologous grafts are shown in Figure 2.5.4.2. Particularly important to note is the wide variability in mechanical strengths that exist across the literature for both engineered grafts with SF and autologous grafts. Moreover, there have been many studies that targeted biocompatibility with various growth factors and antibodies, which aim to promote the growth of specific cell lines to the grafts.

Material	Fabrication Method	Burst Pressure (mmHg)	Suture Rip (N)	Longitudinal Ultimate Tensile Strength (MPa)	% Compliance per 100 mmHg	Reference
SF alone	Braided	804 ± 208	4.0 ± 0.5	7.5 ± 0.6†	2.4 ± 0.5	12
SF with 1% Heparin	Braided	779 ±195	4.7 ± 0.6	6.4 ± 1.5†	2.5 ± 0.4	12
SF with 3% Heparin	Braided	806 ± 185	5.6 ± 0.9	6.1 ± 1.5†	2.3 ± 0.5	12
SF alone	ES	344 ± 32 *	~0.75 ± 0.05	0.95 ± 0.09	~0.55 ± 0.30*	52
SF alone	Dipped	2780 ± 876	NR	NR	NR	68
SF with type 1 collagen	ES	894.00 ± 24.91	NR	NR	3.24 ± 0.58	71
SF alone	ES	575.67 ± 17.47	NR	NR	3.51 ± 0.42	71

Table 2.5.4.1. NR: parameter was not reported. *Estimated using formulas. †This graft was tested wet and dry, but the wet results are depicted here.

Material	Burst Pressure (mmHg)	Suture Rip (N)	Circumferential Ultimate Tensile Strength (MPa)	% Compliance per 100 mmHg	Reference
Human Saphenous Vein	~1300 ± 500	~2.6 ± 0.7	3.7 ± 2.0	3.4 ± 2.0	12,72
Porcine Internal Mammary Arteries	~2150 ± 250	1.0 ± 0.3	10.4 ± 7.1	11.2 ± 6.0	72
Human Saphenous Vein	1599 ± 877	1.92 ± 0.28	NR	0.7 ± 1.5	73,74
Human Internal Mammary Artery	3196 ± 1264	1.35 ± 0.49	NR	11.5 ± 3.9	73,74
Human Saphenous Vein	1680 ± 307	NR	NR	NR	75

Table 2.5.4.2. NR: parameter was not reported.

A native blood vessel is naturally composed of three major layers. Ding et. al. aimed to mimic saphenous veins for vascular grafts by combining three layers of silk fibroin. The inner layer was prepared with silk yarn using a braiding machine, which provided a unique structure for the graft. Afterwards, the inner layer was used as a mold as it was dipped into silk fibroin solution in order to add on the other two layers. Morphologically, this allowed the scaffold to be

biomimetic; the porous layer mimics the tunica media and the external layer simulates the adventitial layer.[76]

The previous study layered separate sections of silk fibroin coatings and mesh. Another study investigated a blend of silk fibroin and chitosan. Despite the strength silk fibroin provides, it is also highly brittle. However, by blending silk fibroin with other polymers the authors believed that the overall biomaterial would become more pliable. Chitosan from crustacean exoskeleton is structurally similar to glycosaminoglycans, which are naturally a part of the extracellular matrix. Additionally, chitosan has a free amine functionality that provides degradability in various biomedical applications.[77]

The end goal of bypass surgery is for the graft to integrate into the human body. This requires the graft to recruit cells and degrade appropriately as cells grow. There is a need to construct a smooth muscle layer; however, if the graft material is too dense and not sufficiently porous, smooth muscle cells have difficulty penetrating and establishing themselves. Yin et. al. used platelet-rich growth factor as an inducing factor in order to promote the growth of SMCs into the grafts. The addition of the growth factor produced grafts with larger pore size, which facilitated cell migration. Moreover, the packing of the silk fibers was looser, which likely resulted in high cellular penetration into the graft.[78]

2.6. Electrospinning

Electrospinning is a simple spinning method that is widely used in the fabrication of various nanoscale polymeric fibers and is defined as an electrostatic fiber fabrication technique.[79] Electrospinning utilizes electrostatic forces to produce fibers that have a larger surface area-to-volume ratio than those obtained from conventional spinning methods. Researchers have been able to develop electrospinning methods that allow for the creation of vascular grafts with silk.

An advantage of electrospinning is that it yields fibers of consistent thickness, which have many outstanding properties, such as large surface area, tunable surface morphologies, and superior mechanical performance.[20] The versatility of electrospinning is useful for human transplantation because the electrospinning solution conforms to the shape of the collector. The use of an appropriately shaped collector allows for it to be specific and fine-tuned for the physiological role that it is needed for.

Electrospinning is also useful because it enables the development of nanofiber-based biomaterial scaffolds.[80] The scaffolds are effective for tissue engineering and regenerative medicine because they mimic the nanoscale properties of fibrous components of the native ECM.[80] Vascular grafts have been made from electrospun fibers and research has supported the potential of electrospun silk scaffolds in vascular tissue engineering both biologically and mechanically.[80]

2.6.1. Parameter Control

Since electrospinning has many parameters to be considered, the variables can be customized in order to produce different kinds of grafts based on specific requirements. The three main categories for these parameters are solution, process, and ambient parameters.[81] These include, but are not limited to, the viscosity and electrical conductivity of the solution, applied voltage, flow rate*, distance between the syringe tip and collector, and humidity and temperature of the electrospinning environment.[53] Each parameter can affect fiber characteristics, and if manipulated correctly, can produce nanofibers of desired morphology and diameter.

Solution parameters can alter fiber morphology when electrospinning. By increasing either the concentration or the viscosity of the solution, the fiber diameter and porosity of the

scaffold increase. However, when increasing the electrical conductivity of the solution being used, there is an observed decrease in fiber diameter.[53]

Changes to the electrospinning set-up can also lead to changes in the fiber morphology. An important parameter is the applied voltage to the solution. By using a higher voltage, there is greater stretching of the solution, which leads to reduction in the fiber diameter and rapid evaporation of the solvent from the generated fibers.[81] Rotating rods can also help with the nanofiber alignment during electrospinning; the rotational speed of the rod can be changed to create scaffolds with aligned nanofibers and simple patterns.[82] Experiments with changes to the flow rate have shown that with an increase in flow rate, there is an increase in fiber diameter. Flow rate must be monitored, as a high flow rate would prevent proper solvent evaporation, and beads would form; a lower flow rate ensures solvent evaporation during the electrospinning process.[53] Another parameter that can be altered to control fiber morphology is the distance between the source and the collector.[83] By increasing the distance between the syringe that is dispelling the solution and the collector, the fiber diameter decreases. Gaumer *et al*. found that increasing this distance increased the tensile strength.[84]

Mechanical performance is largely determined by fiber diameter, porosity, and alignment of fibers. By altering the parameters, the structure of the graft changes, thus affecting the mechanical performance.[53] Since electrospinning can be utilized to a high degree of specificity, it is one of the most effective techniques for creating vascular grafts.

2.6.2. Environment for Cell Growth

The fiber thickness in electrospun grafts will affect the pore size on the surface of the grafts. This has the result of creating environments that have varying degrees of cell infiltration, and can ultimately determine the effectiveness of the graft within the human body, influencing

the regeneration and remodeling process.[69] It has been shown that thicker fibers are more conducive to larger pore sizes, which enables cells to infiltrate and attach to the graft.[69] A study by Yin and colleagues came to the same conclusion, finding that grafts with a large pore size significantly enhanced cellular migration and infiltration, as opposed to the cells remaining confined to the graft surface.[78]

In addition to cell infiltration, the permeability of the graft can greatly affect the ability of nutrients and metabolites to diffuse through the outer layer of the graft to the inside cells; high levels of porosity and larger fiber diameters lead to greater permeability.[85] This in turn can lead to an increase in the metabolic activity of the cells inside the graft; Sisson and colleagues found that with a larger fiber diameter, cells had dramatically more metabolic activity.[86] It was also found that larger fiber diameters have a positive impact on cell growth, significantly increasing the rate of growth due to the greater volume and surface area available on the larger fibers. Additionally, because larger fiber diameters lead to larger pores, the cells perceive the structure as three-dimensional, creating a more appropriate environment for cell growth and proliferation.[86]

With the evolution of scaffold design for tissue engineering, electrospinning has attracted interest due to its ability to produce nanofibrous scaffolds mimicking certain fibrous structures of the native ECM.[20] Electrospinning is simple and effective, allowing for quick and easy fabrication of nanofibers that can be used in a wide range of biomedical applications. As such, electrospinning is used as the primary method of graft creation for this project.

2.7. Addition of Biomacromolecules

2.7.1. Explored Biomolecule Additions

Biomacromolecules can provide biological cues to aid cell adherence and enhance certain biocompatibility properties of vascular grafts.[56] In situations in which scaffolding and cell growth need to be improved, various proteins and polysaccharides can be added. For example, fibrin, a structural biopolymer that is similar to collagen, is distilled from blood plasma.[58] Fibrin is a structural derivative of fibrinogen. Within the body, fibrin facilitates blood clotting by holding platelets together.[87] Fibrin has excellent biocompatibility and degradability, promoting cell adhesion and proliferation.[57] Fibrin gel has been shown to stimulate the production of elastin and collagen, two proteins essential to the structural integration of the graft with native tissue.[87] However, because fibrin aids clotting, thrombogenesis needs to be considered.

Attaching anticoagulant* agents to grafts has also been explored; the most commonly researched agent is heparin which has shown reduced thrombogenicity and increased patency in an eight week period.[88] Heparin* is a glycosaminoglycan* that has anticoagulant activity as well as growth factor and glycoprotein interaction.[89] Furthermore, heparin coatings provide a surface for heparin-binding growth factors, which together stimulate angiogenesis and are involved in early development and wound healing.[90]

Elastin*, a main structural component of blood vessels, provides vessels with elastic recoil, durability, and resilience, helps prevent cell migration and proliferation, and improves attachment of endothelial cells.[91] Thus, elastin provides the characteristic of elasticity, positive cell interactions and biocompatibility that are lacking in current grafts.[91]

Other biomacromolecules such as growth factors can be implemented in order to promote the growth of cells onto vascular grafts. For example, vascular endothelial growth factor*

(VEGF), a glycoprotein manufactured by T cells, macrophages, and endothelial cells, helps stimulate angiogenesis by binding to one of two receptor tyrosine kinases.[92] Another prominent growth factor is epidermal growth factor* (EGF), which works in a manner similar to VEGF. After graft implantation, EGF continually deposits and activates platelets, which was shown to occur for a year in a canine model.[93] Improved graft skeletal construction via synthetic polymers or biologically derived structural proteins are bonded to bioactive cytokines and growth factors to induce a favorable host response.[93]

2.7.2. Matrigel

Matrigel*, derived from the ECM, is used in cell culture as a microenvironment similar to native tissue in order to stimulate cell growth.[94] As an alternative biological enhancement, Matrigel has the advantage of being composed of many growth factors (epidermal growth factor, basic fibroblast growth factor, transforming growth factor, insulin-like growth factor, nerve growth factor, and platelet-derived growth factor) and proteins (collagen IV, enactin, laminin, and peptides) derived from the extracellular matrix, which provides an environment conducive to cell growth.[94] Matrigel has an average modulus of approximately 450 Pa and softens below 37° C.[95] As an applied enhancement for vascular grafts, Matrigel has previously been investigated for an *in vivo* study as an ECM mimic to retain endothelium through shear forces.[96] Because of its ability to stimulate differentiation of many cell types, Matrigel has been commonly used to study angiogenesis and tumor formation.[97] However, since Matrigel is not a well-defined matrix, the use of this basement membrane substitute can generate inconsistency in experimental trials.[94]

2.7.3. Co-culture

The co-culture of cells involves the culture of two different cell types in one region. Four types of co-culture include: culture of cells on either side of a membrane, culture of one cell line

on a gel containing the other cell line, culture of cell types in microspheres within the same environment, and direct co-culture.[98] In direct co culture, the two cell lines are side by side or one on top of the other, similar to the cellular structure of the vessel wall.[98] The culture of different cell types together allows for communication between the cell lines and may alter the behavior of either cell type. A previous study by Xiaohui Zhang investigated a dynamic co-culture of smooth muscle cells and endothelial cells seeded on electrospun silk vascular grafts in a bioreactor system.[96] However, the focus of the study was on the impact of dynamic flow conditions and smooth muscle cell response. More investigation would be necessary to determine the co-culture effect in a static environment and endothelial cell response.

2.8. Graft Mechanical Testing

Mechanical testing is used to determine the physical strength necessary for the vascular graft to mimic a healthy human blood vessel. It evaluates the impact of natural stressing or loading and the graft's ability to respond under hydrated conditions.[99] Testing includes burst strength* and tensile strength*. The structural support and physical capability of the graft may alter its biocompatibility since form defines function. Though the graft's structural character may change *in vitro,* its initial strength is critical to ensure reliable durability.[53]

2.8.1. Burst Strength

The burst pressure test is designed to evaluate how well a graft can withstand blood pressures similar to those within the body's vascular system, including the difference in pressure between systole* and diastole*. This test measures how much pressure the graft can take, leading to an expansion of diameter and surface area, before the pressure of the fluid running within the graft causes failure. The burst strength is directly related to the graft's ability to retain the initial dimensions after implantation.[100] Small diameter vasculature must withstand single pulse

pressures of between 80 and 120 mmHg.[101] Other engineered synthetic grafts have aimed for a burst strength of at least 1300 ± 500 mmHg, achieving the strength of an autologous saphenous vein, which is often the standard for bypass surgery.[68]

There are various ways to perform this test. Water can be pumped through the graft causing a gradual increase in pressure within until the graft reaches its limit.[102] The graft can also be submerged in water and the air pressure gradually increased in 1-mmHg increments until a CO_2 leak is observed in the form of a bubble, or until the graft bursts.[103] Air or water can be pumped directly through the graft by attaching one end of the graft to a syringe pump, a machine that will release the fluid at a constant rate. The pressure of the graft is then monitored by a pressure gauge which is attached to the closed system.

2.8.2. Tensile Strength

Tensile tests measure the resistance of a material to a static or applied force by pulling apart an object that is secured on both ends. The Longitudinal Tensile Strength (LTS) is a crucial test that measures the graft's resistance to the internal longitudinal stresses that it would be subjected to in the body after implantation.[102] To determine LTS, a segment of a graft with a known cross-sectional area is mounted in a tensile testing device. The machine then pulls the graft segment with a known amount of force on the long axis of the graft until it breaks, at which point the peak force value is recorded to calculate the LTS.[104]

2.9. Motivation

The literature review and background presented above shows what research has been done with small diameter grafts and silk fibroin in particular. Previous work with electrospun SF has highlighted the promise of these grafts. Research has also shown the general biocompatibility and strong mechanical strength of SF; electrospinning has been shown to demonstrate an

immense amount of control over the graft fabrication. However, electrospun SF grafts in the literature thus far still does not meet the strength or biocompatibility of the native vessel. As a result, our work sought to investigate biochemical modification of the grafts as one approach to mimic the native environment of a vessel in order to increase the ability of cell growth and electrospinning as a means to control fiber morphology and strength.

3. Electrospinning of small-diameter grafts

3.1. Methods

3.1.1. Materials

Silk cocoons were provided by Mulberry Farms (Fallbrook, CA). Sodium carbonate, lithium bromide, and polyethylene oxide (900 kDa avg MW) were purchased from Sigma-Aldrich (St. Louis, MO). Slide-a-Lyzer dialysis cassettes (3.5 kDa MWCO, 12 mL) were obtained from Thermo Fisher Scientific (Waltham, MA).

A Gamma High Voltage 30 kV high voltage power supply was borrowed from the Bing Research Lab (Ormond Beach, FL). KD Scientific Legato 210 syringe pump was borrowed from the White Research Lab (Holliston, MA). Materials also include MSC Direct 3/16 inch stainless steel rods, a Gates ¼ inch width and 2/25 inch pitch timing belt, CML Supply 16-gauge needles, and an UXCELL 12V DC motor. BD Luer-Lok 10 mL syringes were purchased from Becton, Dickinson, and Company and a Sparkfun DC wall adapter power supply was purchased.

3.1.2. Silk Extraction

The silk is extracted from the cocoon of *B. mori* using a degumming process.[12,65,66] The first steps remove the sericin, while the latter steps dissolve and purify SF to obtain a 6-8% w/v solution. First, the cocoons were boiled for 30 minutes in 0.02 M Na_2CO_3 to separate the fibers. Then, the fibers were rinsed with dH_2O for 20 minutes in three separate cycles. The excess dH_2O was squeezed out and the extract was then dried overnight. After that, 9.3 M of LiBr was poured on top of the silk fibers and incubated at 60ºC for four hours. Then, dialysis was performed against ultrapure H_2O for 72 hours. Finally, the SF was centrifuged for isolation; the resulting SF can be stored for up to one month at 4ºC.[65] A 5% aqueous polyethylene oxide (PEO) solution was prepared to increase the viscosity of the silk fibroin solution. The PEO was prepared by

adding 1 g PEO to 20 mL DI water. Because the PEO solution was highly viscous, the solution was prepared at least 4 hours in advance to use and was left spinning on a magnetic plate. For electrospinning, a solution of 6.4% silk fibroin was combined with 5% PEO.

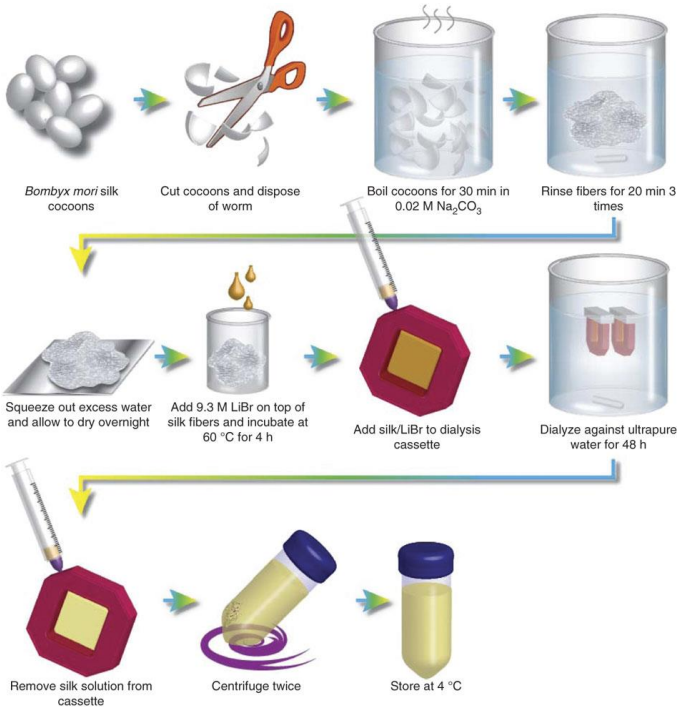


Figure 3.1.2.1. Workflow for processing of the silk fibers. Reprinted by permission from Macmillan Publishers Ltd: Nature Protocols (Rockwood *et al*., 2011), copyright 2011.[65]

3.1.3. Preliminary Work

3.1.3.1. Dipping Grafts

A regular dipping method was utilized to produce cylindrical grafts (Figure 3.1.3.1.1). Two solutions that were used in alternation were 16% (w/v) concentrated SF/poly(elthylene glycol) (PEG) solution and methanol. By alternating the solution in which the rod was dipped for various cycles, a layered graft was produced.

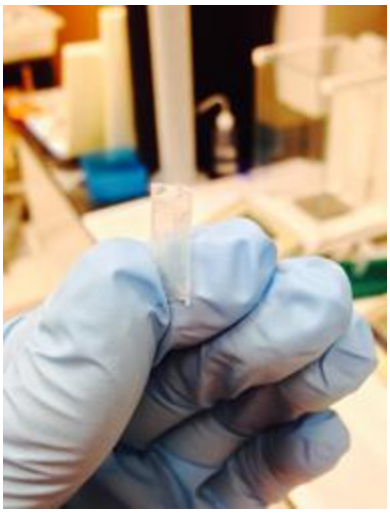


Figure 3.1.3.1.1. SF graft produced from the dipping method.

3.1.3.2. Electrospinning Sheets

Before electrospinning cylindrical grafts on a custom-made mandrel, the SF/PEO solution was electrospun onto a larger cylindrical rotating mandrel (Figure 3.1.3.2.1). The sample could easily be removed from the mandrel and rolled out to become a sheet. These samples were used for preliminary mechanical and biocompatibility testing.

Figure 3.1.3.2.1. Electrospinning set-up for sheet samples

3.1.4. Electrospinning Setup

The electrospinning apparatus consisted of three components: the voltage source, the syringe pump, and the rotating mandrel.

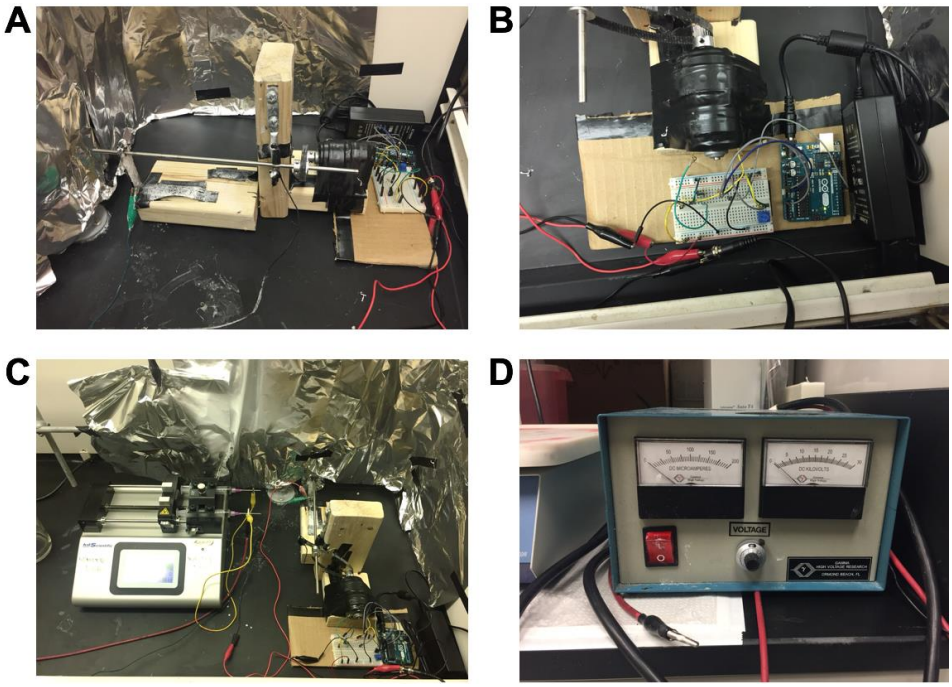


Figure 3.1.4.1. Electrospinning set-up utilized for production of cylindrical grafts. (A) Grounded collecting rod coupled to motor (B) Arduino board for motor (C) Solution source and collector (D) Voltage source used throughout electrospinning process.

The voltage source had two leads: a ground wire that was connected to the L-bracket and a positive output that was directly connected to the needle of the syringe (Figure 3.1.4.1A, C). The rotating mandrel was grounded on both the left and right side through the attachment of a wire in parallel to the ground wire from the voltage source (Figure 3.1.4.1C, D). 10mL syringes were used to hold the silk solution, and were fitted with 16-gauge needles. The syringes were placed into the syringe pump and positioned at a fixed distance from the stainless steel rod.

The rotating mandrel held a stainless steel rod between a stainless steel L-bracket and a stainless steel hook, both attached to the wooden base (Figure 3.1.4.1A). As mentioned before, a

DC motor was mounted on the other side and was connected to the stainless steel rod by a timing belt (Figure 3.1.4.1B). The motor was powered by a DC wall adapter, which was connected to the breadboard for power modulation (Figure 3.1.4.1B). The breadboard was connected to an Arduino circuit board that controlled the rotation speed of the motor (Figure 3.1.4.1B). The circuit diagram of the motor's control is shown in Figure 3.1.4.2. The motor rotated at 1074 rpm.

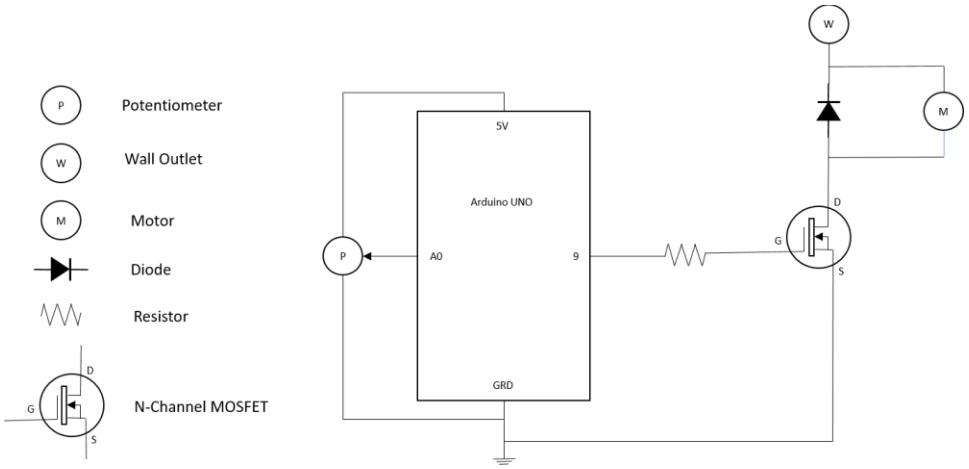


Figure 3.1.4.2. Circuit diagram of the electrospinning setup consisting of the motor that rotated the mandrel. The N-Channel MOSFET was used to modulate the speed of the motor that was controlled by a potentiometer.

3.1.5. Electrospinning Operation

The SF/PEO solution was mixed for approximately 15 minutes in order to make a homogenized solution of SF and PEO before electrospinning. The syringe was loaded with solution and the positive lead from the voltage source was connected to the 16G needle. The rotating mandrel was set to a speed of 1074 rpm, and the syringe pump was set to a rate of 0.02 mL/min. After turning the mandrel and syringe pump on, the voltage was turned on and slowly increased from 0 kV to 12.5 kV.

After the designated volume of solution was dispensed, the graft with the supporting rod was submerged in 90% (v/v) methanol for 15 minutes.[65] This graft was then placed in a water bath on a shaker for approximately 24 hours. After partially drying the graft, a KimWipe was

used to slide the graft off from one end of the rod. The graft was then stretched out to the original length and stored at room temperature in a sealed petri dish.

3.1.6. Electrospinning Optimization

In order to determine the correct specifications to properly electrospin a SF/PEO graft, we had to troubleshoot an array of selected parameters based on the needle gauge, rate of dispensing, and distance from the mandrel. Rotation speed of the mandrel (1074 rpm), the voltage applied to the syringe (12.5 kV) and the amount of SF/PEO solution used (5 mL) were kept constant to control for variance between grafts for parameters not being tested. After multiple trials, it was determined that the control graft would be electrospun 11.5 cm away from the mandrel, at 0.02 mL/min, using a 16G needle. Upon production of control grafts, the distance from the mandrel and the applied voltage were changed to obtain ideal pore size and fiber diameter.

3.1.7. Surface Analysis and Morphology

A small portion of the grafts were visualized under SEM (Hitachi S-3400 Variable Pressure SEM). To reduce charging of the nonconductive polymer surface, the specimens were coated using a Denton DV-502A carbon evaporator for 3-5 minutes. The specimens were held under vacuum at about $2*10^{-5}$ torr throughout the process and the current through the filaments did not exceed 35 A. A motor rotated the sample to get an even coating of carbon on the surface. SEMs employed a technique of 5-7 kV with a probe current between 55 and 65 µA. Although charging did still occur, it was minimized by the carbon coating and provided clearer images than variable pressure SEM without any coating. Images were taken at a range of magnifications. The captured image was then analyzed for fiber diameter in ImageJ.

3.2. Results & Discussion

3.2.1. Characterizing Extracted Silk Fibroin

A batch of 5 g of silk cocoons typically yielded 30 mL 7-8% (w/v) silk fibroin solution. A nanodrop UV-Vis was performed on the initial silk fibroin solution in order to confirm that the solution is pure (Figure 3.2.1.1). The ratio of the 260/280 nm peaks was calculated to determine purity; the ratio for the SF solution was about 0.5, indicating that the solution was pure. At first, PEG was used in order to increase the concentration of the silk fibroin. Silk fibroin was added to a dialysis cassette, which was left to spin in a solution of PEG. Every 2 hours for a total time frame of 10 and 20 hours, small samples of silk fibroin were removed from the dialysis cassette to determine the concentration. In the time frame analyzed, there was a linear relationship between the dialysis time in PEG to the concentration of the overall silk fibroin (Figure 3.2.1.2).

Although using PEG as a method of concentration worked for dipped grafts, dialysis against PEG did not produce a solution that was conducive for electrospinning. To alleviate this problem, 5% PEO was directly added to the silk fibroin solution because it increased the viscosity of the solution slightly and induced pore formation in the scaffolds.

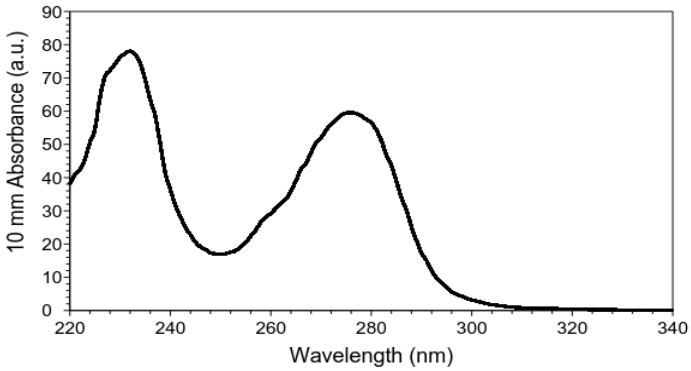


Figure 3.2.1.1. Representative Nanodrop UV-Vis spectrum of SF. Purity can be approximated by ratio of nucleic acids (abs. 260nm) to protein (abs. 280nm).

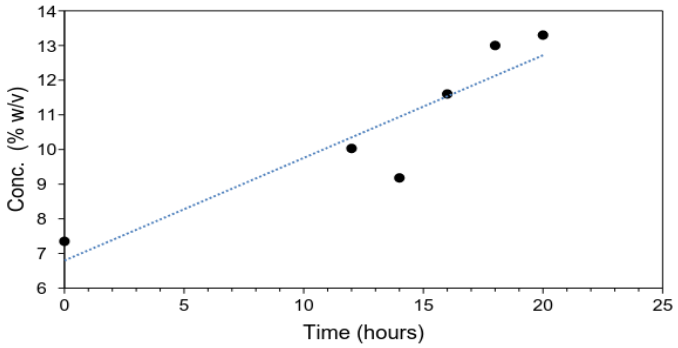


Figure 3.2.1.2. Time study of silk dialyzed with PEG. Concentration increases during dialysis, and after dialysis for 20 minutes enter suitable range for electrospinning.

3.2.2. Optimization of Electrospinning Parameters

Of the many parameters that could have been modified to fine tune each electrospinning set-up, the first that had to be addressed were the needle gauge to be used and the solution dispensing rate. It is common for electrospinning set-ups to utilize needle gauges between 16 and 20. It was determined that 16 and 18 gauge needles worked the best for this set-up; 20 gauge needles caused beading of the SF solution. 16 gauge needles were chosen because they were predominantly used for electrospinning in the past. Additionally, a blunt tip needle was used because a beveled tip would cause the solution to be dispensed at an angle.

The next parameter that had to be set for the controls was the dispensing rate. The syringe pump was set such that the dispensed solution would not form beads at the tip of the needle. The speed that was best suited for this set-up was 0.02 mL/min. The optimal dispensing rate varied with the gauge of the needle and voltage applied, but as it was determined that the gauge and voltage were to be kept constant, the control rate was solidified at 0.02 mL/min for the set-up.

One of the major parameters that had to be set was the solution composition. Pure SF was not conducive to electrospinning and dialyzing against PEG did not produce a solution acceptable for electrospinning. To circumvent this, PEO was added to the SF solution, as it increased the viscosity of the solution enough to allow formation of the Taylor cone and

prevented bead formation at the tip of the needle (Table 3.2.2.1).

Rate	**0.01mL/min**		**0.02mL/min**		**0.03mL/min**	
Gauge	PEO Present	PEO Absent	PEO Present	PEO Absent	PEO Present	PEO Absent
16	Success	Fail	Success	Fail	Fail	Fail
18	Success	Fail	Success	Fail	Fail	Fail

Table 3.2.2.1. Efficacy of various parameters on electrospun graft viability.

Since voltage level changes fiber morphology, the voltage source was used at a set 12.5 kV for all grafts.[45] Previous research have used voltages in the range of 12 kV to 30 kV; since the electrospinning setup was semi-exposed and open to the environment, 12.5 kV was a safer option as opposed to using a higher voltage.

The distance from the tip of the syringe needle to the stainless-steel rod also affected fiber morphology. This varied widely between previous experiments in literature; a distance of 11.5 cm was arbitrarily chosen as the control. Grafts were created using 1.5 times and 0.5 times the control distance to determine if varying distance positively affected the mechanical and biocompatibility properties of the graft.

3.2.3. Surface Analysis and Morphology

The morphology of the grafts was imaged with SEM. Comparing the SEM images obtained to those from literature, it is clear that the grafts do not exhibit much similarity. In previous literature, electrospun silk fibers are distinctly separated and straighter; from Figure 3.2.3.1, it can be seen that the fibers are closely entangled and have a winding pattern in all grafts. This could be the result of environmental factors and/or parameter variation. Because the electrospinning was performed in a chemical hood, there was a constant wind gradient created from the fans within the hood that could have altered the path of the fibers from the needle to the rotating mandrel. The wind could also have effected change on a physical level; the force of the wind could have affected the fiber diameter by shaping the SF. Additionally, there was no control of the temperature and humidity that the electrospinning was performed at; these ambient

factors have been shown to affect fiber morphology and could have altered the overall graft structure.[45]

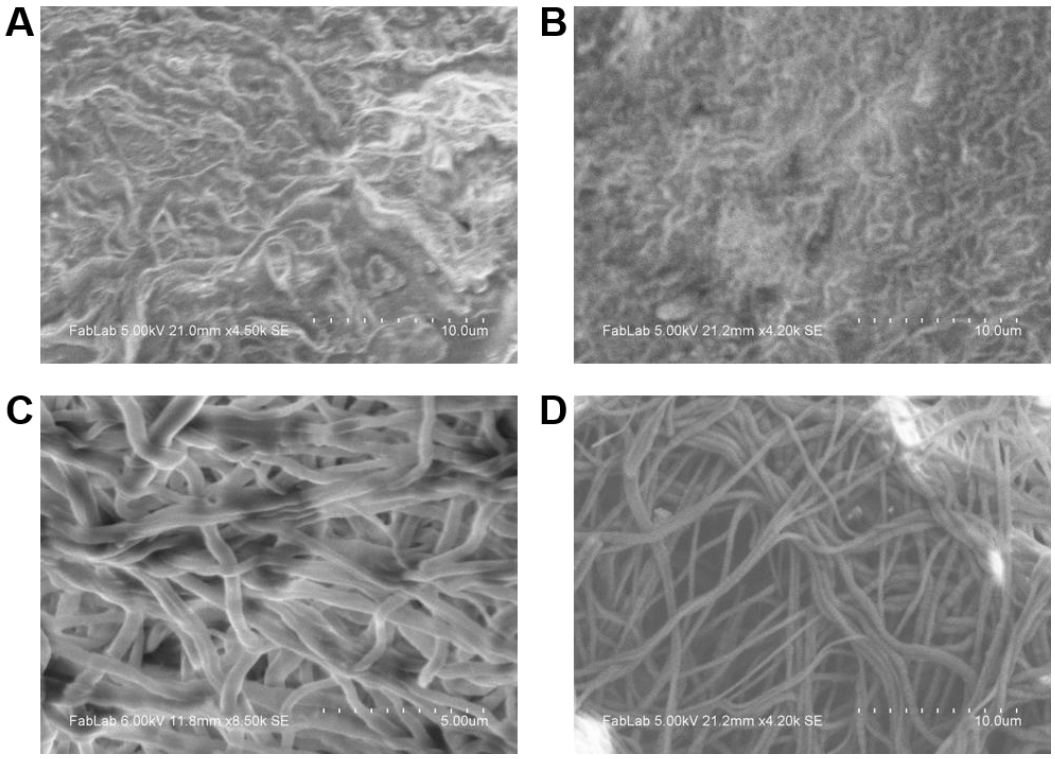


Figure 3.2.3.1. SEM images were taken of electrospun grafts to show fiber diameter at an electrospinning distance of (A) 5.75 cm (B) 7.50 cm (C) 11.5 cm (D) 17.25 cm.

Parameter variation could also be a contributing cause for the dissimilarity of the grafts. Due to the widely varied voltages and distances used in electrospinning procedures, it is expected that electrospun grafts will have different characteristics. The parameters chosen for this experiment were optimized for an electrospinning set-up in a chemical hood in order to ensure safety and efficient use of space; this differs from most other set-ups in literature and could have affected the results obtained. In order to ensure a safe working environment, the only parameter changed was electrospinning distance; the distance was varied to determine the effect of source-to-collector distance on fiber diameter. As seen in Figure 3.2.3.3, the fiber diameters fluctuated within set distances.

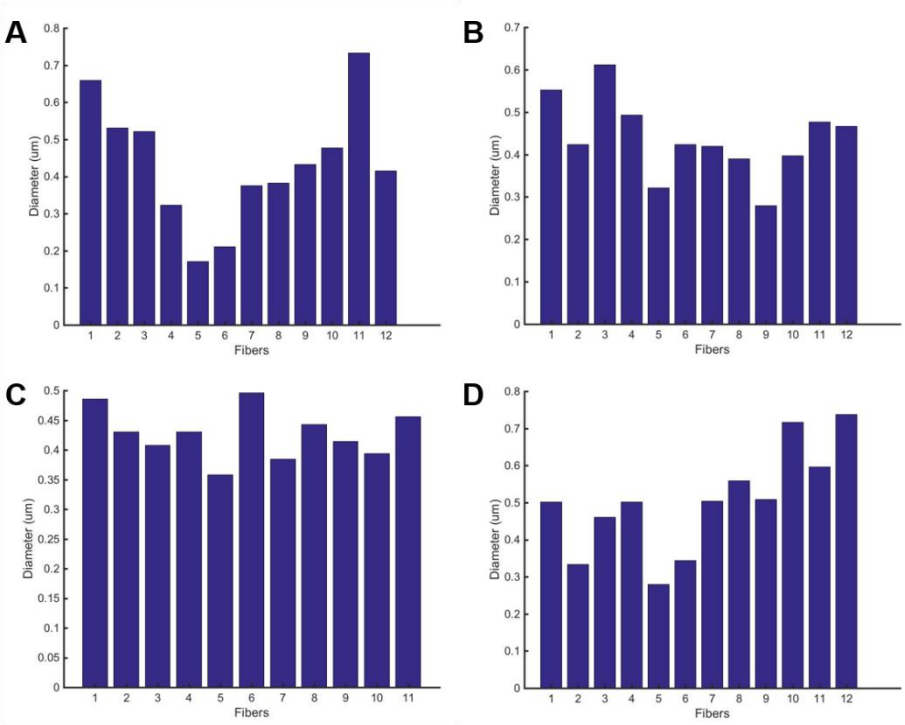


Figure 3.2.3.2. Diameters of random fibers throughout quadrants of the SEM images of SF grafts electrospun at a distance of (A) 5.75 cm (B) 7.50 cm (C) 11.5 cm (D) 17.25 cm.

However, the average fiber diameters across all grafts were within standard deviations of each other (Figure 3.2.3.3). This concludes that there is no significant difference between the grafts that were electrospun at different distances. This is most likely due to the conduction of the experiment in a chemical hood, in which a constant air stream disrupted the electrospinning process.

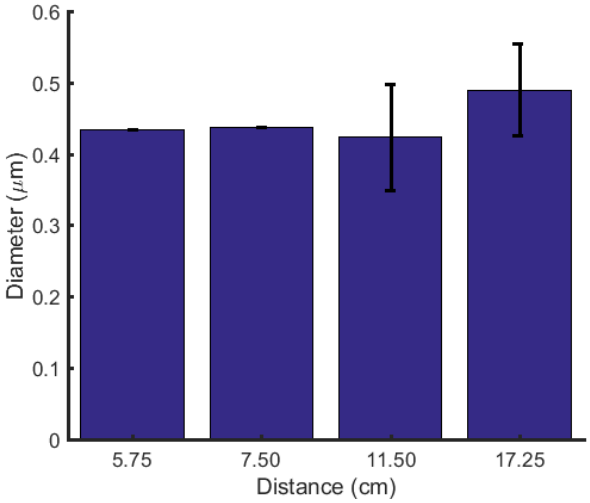


Figure 3.2.3.3. Averages of the fiber diameters for the SF grafts electrospun at all of the distances. Standard deviation bars are shown. *One sample only.

3.3. Conclusion

The procedure for extracting SF and concentrating with PEO to create the necessary viscosity was standardized. The diameter of individual fibers did not change much as a result of the varying electrospinning distances. Hypothetically, by changing the distance, the fiber morphology would change. However, due to the constant wind produced by the fans in the chemical hood, the fiber diameters were disrupted. Future work needs to be done with varying distances at larger significance in order to investigate whether the distances would impact the fiber diameters.

4. Improving Biocompatibility of SF Vascular Grafts

4.1. Methods

4.1.1. Materials

Human umbilical vein endothelial cells (HUVECs) were purchased from Lonza (CC-2517) along with the appropriate growth media, EGM-2MV Bulletkit growth media (CC-3202). Human coronary artery smooth muscle cells (HCASMCs) were also purchased from Lonza (CC-2583) as well as its SmGM-2 Bulletkit growth media (CC-3182). Dulbecco's phosphate buffer solution (DPBS) was purchased through Thermo Fisher Scientific. Hank's Balanced Salt Solution (HBSS), Ethidium homodimer (E1169), and Calcein AM (C3099) were purchased from Life Technologies. GFR Matrigel was purchased from Corning (#356320, Corning, NY). Triton X-100 and bovine serum albumin were purchased from Sigma Aldrich. Antibodies were purchased from Abcam: mouse monoclonal anti-CD31 (ab9498), rabbit polyclonal anti - α smooth muscle actin (ab5694), goat anti-mouse IgG Alexa Fluor 594 conjugate (ab150116), and goat anti-rabbit IgG Alexa Fluor 488 conjugate (ab150077).

Use of cell culture hood, cell culture materials, and Olympus IX81 inverted microscope was provided by the Orthopaedic Mechanobiology Laboratory (University of Maryland, College Park). Imaging for the immunofluorescence experiment was performed in the Optic Biotech Laboratory (University of Maryland, College Park).

4.1.2. Preparation for Cell Culture

To prepare grafts for cell culture, samples of electrospun silk were cut and placed in individual wells of a well plate. For sheets, 10mm x 10mm squares were cut out of the center of the sheet; for cylinders, segments of approximately 10mm long were cut. The SF samples were sterilized using UV light for 20 minutes, then soaked in DPBS for 30 minutes.

4.1.3. Treating the Grafts with Matrigel

A basic SF graft was treated with growth factor reduced Matrigel with the goal of improving the bioactive properties of the graft. Matrigel was aliquoted and frozen at -20°C until use to reduce freeze/thaw cycles. Matrigel requires all pipette tips and tubes to be frozen prior to use, as it gels at room temperature. Grafts were cut and sterilized as described in Section 4.1.2. Solutions of 1%, 10%, and 50% Matrigel were prepared by dissolving frozen aliquots of stock Matrigel in cold DPBS. Then, instead of soaking grafts in DPBS after sterilization, grafts were soaked in Matrigel solution for 30 minutes to be used for biocompatibility testing.

4.1.4. Cell Culture

Two cell lines of interest in vascular regeneration were used to assess our grafts. HUVECs were cultured in EGM-2MV Bulletkit growth media. HCASMCs were cultured separately in SmGM-2 Bulletkit growth media. Cells were expanded in T-75 tissue culture flasks, harvested via trypsinization at passage eight, and seeded at a density of approximately 2 million cells/mL on both pre-prepared tubular and sheet scaffolds. Initially, 500µL were applied to each scaffold; after 3-5 hours media was added to each well to increase the total volume to those recommended by the manufacturer for extended cell culture.

4.1.5. Co-culture Model

HUVECs were seeded on the outer side of tubular and sheet scaffolds, and HCASMCs were seeded on the inner side of tubular and sheet scaffolds. Cells were seeded in 50% EGM-2MV growth media and 50% SmGM-2 growth media and grown over the course of several days.

4.1.6. Cytotoxicity Assay

In order to assess cell viability, a live-dead assay was performed following standard protocols.[105] Briefly, electrospun scaffolds were UV sterilized and then soaked in DPBS.

Scaffolds were then seeded with HUVECs and cultured for one day. Following standard live-dead protocols,[105] scaffolds were soaked in HBSS to dilute excess media and other reagents. The scaffolds were then incubated in 0.2% ethidium homodimer and 0.04% calcein AM in HBSS for 30 minutes in the dark. The scaffolds were observed via fluorescence microscopy through an Olympus IX81 inverted microscope.

4.1.7. Immunofluorescence

In order to assess co-culture behavior between HUVECs and HCASMCs, an immunofluorescence experiment was conducted. Electrospun cylindrical scaffolds were cut, UV sterilized and then soaked in DPBS. Scaffolds were seeded with either HUVECs only, HCASMCs only, or both HUVECs and HCASMCs in a direct co-culture.[98] These cultures were maintained in EGM, SBM, and 50% EGM-50% SBM media for HUVEC, HCASMC, and co-culture scaffolds, respectively. Scaffolds were fixed at days 1, 4, and 7 by rinsing with DPBS, soaking in 10% neutral buffered formalin for 40 minutes, and rinsing three times with DPBS for 10 minutes per rinse. Samples were stored in DPBS at 4°C until scaffolds from all time points were fixed.

Scaffolds were permeabilized in 0.1% Triton-X 100 for 20 minutes, blocked with 2% BSA in DPBS for 35 minutes, and incubated with a prepared dilution of primary antibodies overnight at 4°C. Between each step, all scaffolds were rinsed with three 10 minute long DPBS washes. All steps were performed at room temperature, except for the incubation with primary antibodies. HUVEC cells were incubated with a mouse anti-CD31 antibody, HCASMCS were incubated with a rabbit anti-α smooth muscle actin, and co-cultures were incubated with a solution containing both antibodies. All primary antibodies were diluted 1:100 in 1% BSA. The following day, all samples were washed three times in PBS: one hour-long rinse and two 10

minute rinses. Scaffolds were incubated in the appropriate prepared secondary antibody solution for 1.5 hours in the dark. HUVECs were incubated with an anti-mouse IgG Alexa Fluor 594 antibody, HCASMCs with an anti-rabbit IgG Alexa Fluor 488 antibody, and co-cultures with both. All secondary antibodies were diluted 1:1000 in 1% BSA solution. After incubation with secondary antibodies, the scaffolds were rinsed again three times with PBS for 10 minutes per rinse, then stored in PBS at 4°C shielded from light. Immediately prior to imaging, all scaffolds were incubated in a solution of DAPI diluted at 1:1000 in DPBS, for 15 minutes at room temperature. Scaffolds were rinsed one time with DPBS and imaged. To obtain clearer images, individual scaffolds were transferred from the well-plate to a glass-bottomed dish for imaging. Images were taken at 40x magnification.

Four immunofluorescence controls were included along with the samples taken from each timepoint. All controls were expected to be negative on all channels. One scaffold seeded only with HUVECs was incubated with the HCASMC primary and secondary antibodies (anti-α smooth muscle actin and anti-rabbit IgG) to check the specificity of the HCASMC antibodies; conversely, one HCASMC scaffold was incubated with the HUVEC primary and secondary antibodies (anti-CD31 and anti-mouse IgG) to check the specificity of the HUVEC antibodies. To check for cross-reactivity between the primary and secondary antibodies, one co-culture scaffold was incubated with anti-CD31 and anti-rabbit IgG and one co-culture scaffold was incubated with anti-α smooth muscle actin and anti-mouse IgG.

4.2. Results

4.2.1. Cytotoxicity Assay

Representative Live-Dead images of HUVECs seeded on the electrospun silk scaffolds on day 1 show a majority of live cells on both sheet (2D) and cylindrical (3D) scaffolds (Figure

4.2.1.1). The majority of both pictures demonstrated live cells (green) and very few dead (red spots indicating stained nucleus). The scaffold itself was also found to be slightly stained red which was consistent with auto-fluorescence of silk under ethidium homodimer staining.[106]

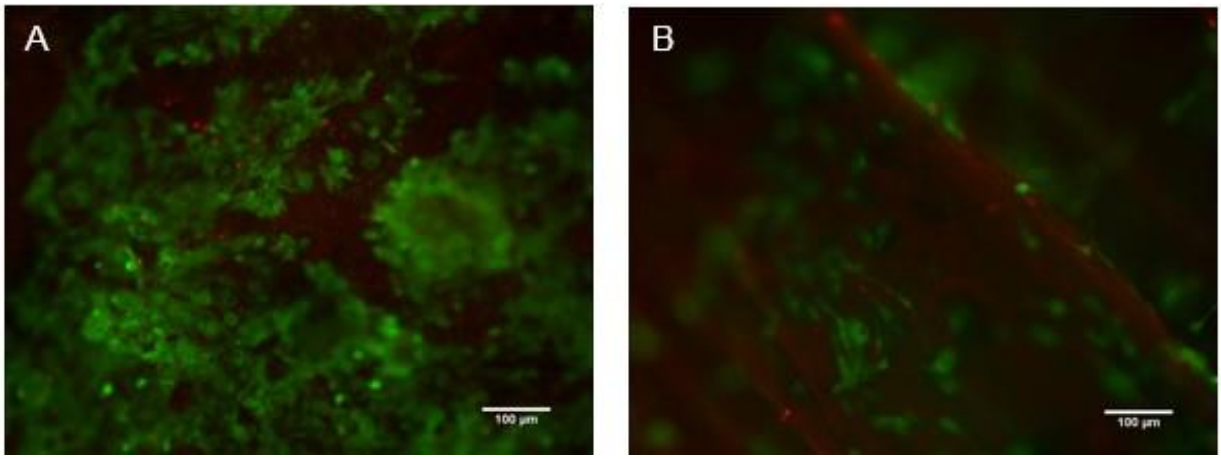


Figure 4.2.1.1 (A) Live-Dead assay image of HUVECs seeded onto electrospun sheet (B) Live-Dead assay image of HUVECs seeded onto control 3D cylindrical scaffold. Two images of two samples were taken at 10x and representative images selected; scale bars represent 50µm.

4.2.2. Matrigel

Live-Dead images of HUVECs seeded on the electrospun silk scaffold sheets with varied Matrigel concentrations were collected on day 1. In Figure 4.2.2.1, a majority of the cells on all of the electrospun scaffolds are live and viable as demonstrated by the majority of green stained cells to red stained nuclei. A single-blinded imaging procedure was used to avoid introducing bias by the person seeding the various Matrigel-coated grafts. Overall, there was an increase in the amount of cells attached to the scaffold with increasing concentration of Matrigel from qualitative assessment of Live-Dead assay photographs. This is consistent with previous research demonstrating the increase in cell adhesion with the addition of Matrigel.[96]

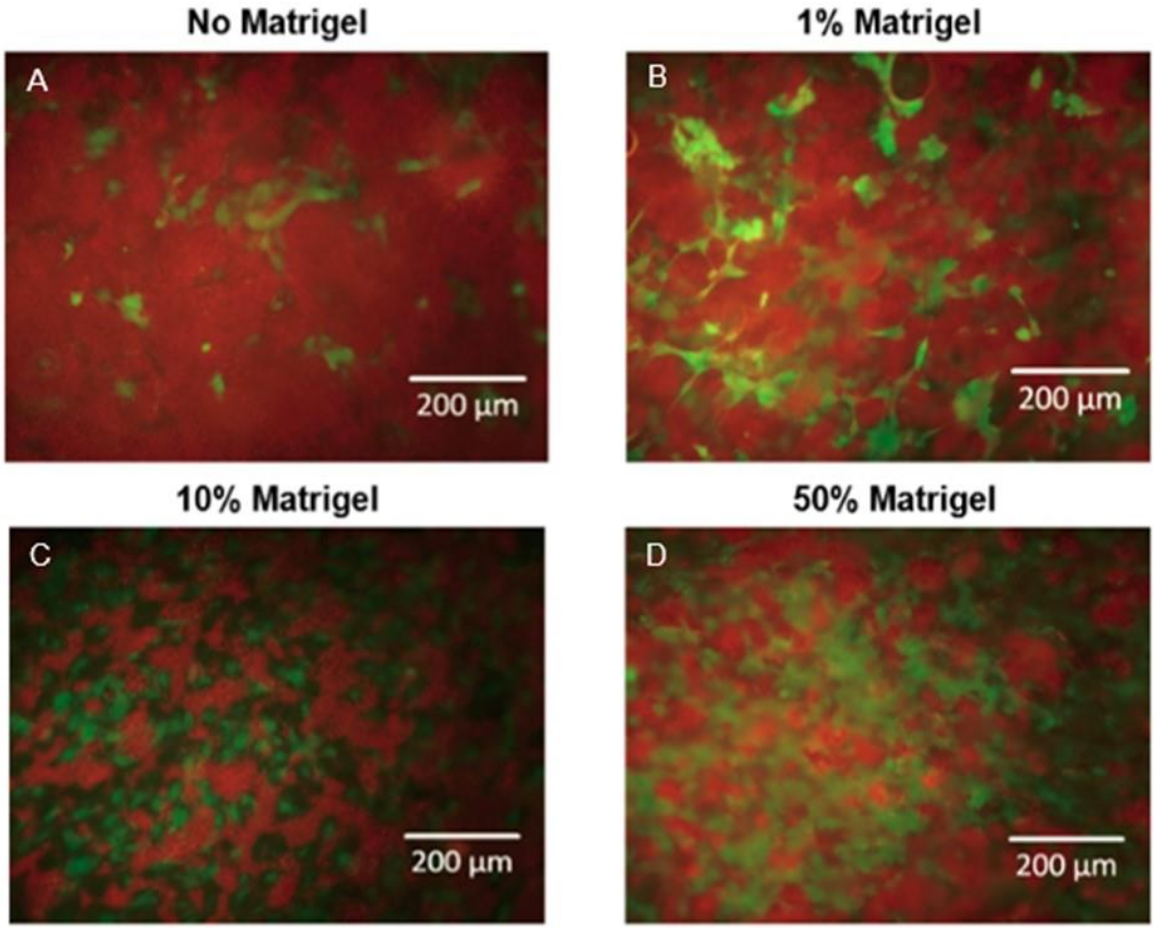


Figure 4.2.2.1 Live-Dead assay image of HUVECs seeded onto electrospun sheets. Sheets varied in concentration of Matrigel including (A) no Matrigel, (B) 1% Matrigel added (C) 10% Matrigel added. (B) 50% Matrigel added. Using a single-blind procedure, four images were taken at 10x and representative images selected; scale bars represent 200µm.

4.2.3. Immunostaining

The effects of a co-culture model on the morphology of HUVECs cultured on a cylindrical SF electrospun graft were investigated through immunofluorescence staining, as shown in Figure 4.2.3.1. Previous research demonstrated that direct contact co-culture systems may be used as a representative model of a healthy native vessel.[107] Therefore, in order to closely investigate the SF electrospun grafts in a native environment, a co-culture model with 50%

HUVEC and 50% HCASMC experimental group was chosen to be compared against a HUVEC-only experimental group over the course of seven days.

The controls included in the immunofluorescence experiment were all negative (not shown), as expected, indicating that the antibodies chosen were specific to their respective cell-types and not cross-reactive. However, the immunolabelling of α-smooth muscle actin and the DAPI staining were unsuccessful: it was not possible to distinguish between the cells and the scaffold. It is unclear if this is due to scaffold fluorescing or if the cells were not sufficiently labelled or stained. However, the labelling of CD31 (a HUVEC marker) yielded clear results, enabling us to study the morphology of HUVECs in the two culture models over time. The morphology of HUVECs in the co-culture model demonstrated a more web-like net morphology than the HUVECs in the independent culture. This may be due to the formation of junctional complexes which have been shown to be enhanced in endothelial cell and smooth muscle cell co-culture models.[108]

Over time, the co-culture HUVECs showed a trend of consistent concentration on the grafts while in the HUVEC-only model there were demonstrated regions of lower HUVEC concentration in later time points. It is hypothesized that HCASMC is involved in HUVEC proliferation, as prior research has demonstrated that direct contact with smooth muscle cells plays a role in regulation of endothelial cell proliferation.[108] Overall, the co-culture model played a role in HUVEC morphology and apoptotic behavior that is more consistent with a healthy native vascular environment.

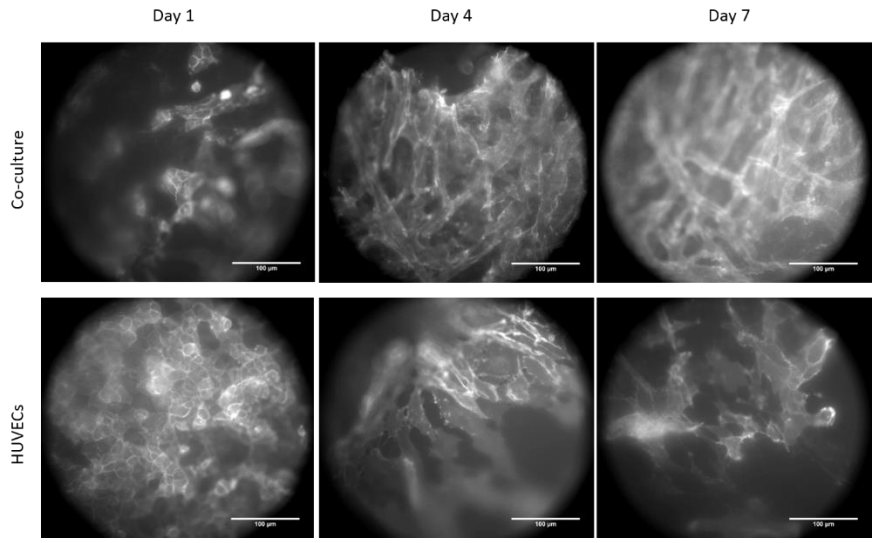


Figure 4.2.3.1 Single-channel fluorescent images of HUVECs in both co-culture and HUVEC-only seeded control 3D cylindrical scaffolds over a period of 7 days. CD31, an endothelial cell membrane protein, was immunolabeled with Alexa Fluor 494; immunolabelling of α-smooth muscle actin and DAPI staining yielded poor images and are not included.

4.3. Conclusion

The viability and attachment of HUVECs to the electrospun grafts was evaluated using a Live-Dead assay. Both electrospun sheets and cylindrical grafts were evaluated to be biocompatible and HUVECs were shown to adhere to both regardless of shape. This was consistent with previous studies, which have demonstrated the biocompatibility of silk.[109] Our method of electrospinning resulted in a biocompatible surface for native human endothelial and smooth muscle cells to grow.

The enhanced adhesion effects of Matrigel were evaluated using a Live-Dead assay. The results of initial testing suggested a dose-dependent increase in cells with an increase in Matrigel applied to the grafts at lower concentrations. Matrigel was not seen to affect the cytotoxicity of the grafts. Overall, it was found that increased adhesion of HUVECs to electrospun SF vascular grafts was consistent with prior research demonstrating that Matrigel enhances adhesion.

A co-culture system of 50% HUVECs and 50% HCASMCs was compared against an HUVEC-only culture model using immunofluorescence over the course of 7 days. An observed difference in morphology of HUVECs in the co-culture model may be the result of increased tight-junction formation in the presence of HCASMCs. A reduction of apoptosis observed in the co-culture model compared to the HUVEC-only model could be due to a reduction in apoptosis which has been observed in previous co-culture models. Inclusive of both of these observations, our experimentation suggests that the co-culture of HUVECs and HCASMCs results in better controlled proliferation of HUVECs and a closer model to a native healthy vessel.

As a whole, both Live/Dead and immunofluorescence testing demonstrated the biocompatibility of electrospun SF vascular grafts. Matrigel testing suggests that the cellular adhesion of the grafts can be improved by the use of culturing with Matrigel. Co-culture testing suggests the biocompatibility of the grafts can be improved through the use of a co-culture methodology to improve endothelial cell proliferation when compared to an only endothelial cell seeding methodology.

5. Mechanical Testing of SF Grafts

5.1. Methods

5.1.1. Materials

A Harvard Apparatus Pump 11 Elite syringe pump (Holliston, MA), 2 mL VWR serological pipettes (Radnor, PA), plastic three-way stopcocks, plugs, and Masterflex tubing (Vernon Hills, IL) were purchased from their respective providers. A 0-100 psi digital pressure gauge with a built-in pressure sensor was obtained from McMaster-Carr (Elmhurst, IL). Quick-Sil Two-Part RTV Molding Putty was obtained from Castaldo (Franklin, MA). Electrical tape and BD Liquid Dispensing Syringes (30 mL, 30cc) without needles were ordered from Amazon (Seattle, WA). A DMA Q800 was from Texas Instruments (Dallas, TX). 3D printed clips were generously donated from the Optics Biotechnology lab run by Dr. Giuliano Scarcelli of the University of Maryland, College Park.

5.1.2. Preliminary Work

5.1.2.1. Burst Pressure Test

Dipped SF grafts, rather than electrospun SF grafts, were used to develop the initial design for the burst pressure test setup. Briefly, the set-up involved a system to pump air into the graft and a sensor to detect the increase in pressure inside the vessel. The entire graft was submerged under water so that the presence of bubbles can be used to indicate that the graft has burst. In order to measure the change in pressure, it was essential to create an airtight system that can sustain a constant flow rate. Various products were tested in regards to their ability to create a seal between the graft and the tubing supplying the air that is generating internal pressure. The products tested were glue (Vetbond), molding putty, binder clips, and electrical tape. To evaluate if the system was airtight, the sealed graft was placed in a beaker and air was pushed through it

manually using a syringe. Video footage was used to identify the exact location at which the seal broke and bubbles were released. After much trial and error, we discovered that electrical tape was the best option for providing an airtight seal.

In the final experimental model, a digital gauge pressure was used in conjunction with a syringe pump to measure the pressure values over time. Electrical tape was used to connect the graft to a serological pipette that carried air supplied by a syringe pump, and a second pipette tip was used and secured with tape to seal off the other end of the graft.

5.1.2.2. Longitudinal and Circumferential Tensile Strength

The Longitudinal Tensile Strength (LTS) is a test that measures the vascular graft's resistance to the internal, long-term longitudinal stresses that would occur in the body after implantation.[97] To determine LTS, a segment of a graft with a known cross-sectional area was mounted in a tensile testing device, such as a dynamic mechanical analyzer (DMA).[48] The machine then pulled the graft segment with a known amount of force on the long axis of the graft until it broke, at which point the peak force value was recorded to calculate the LTS.[99] However, the efficacy of this instrument was dependent upon the size and shape of the sample graft; if these characteristics were not uniform and convenient to clamp, then results from the instrument were rendered imprecise.

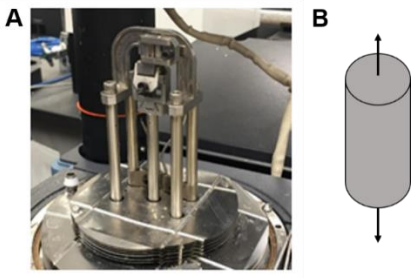


Figure 5.1.2.2.1. (A) An image of the DMA machine used for this study. (B) An illustration of the graft, represented by the cylinder, where the arrows indicate the direction that the clamp on the machine pulls the graft.

5.1.3. Burst Pressure Test

The burst pressure method used to test electrospun grafts was adapted from Marecik et. al (2006) and the method used at the Krieger lab at Children's National Medical Center, Sheikh Zayed Institute for Pediatric Surgical Innovation.[103] The air tight system was formed using serological pipette tips, tubing, and three-way stopcocks secured by electrical tape as shown in Figure 5.1.3.1. The grafts were hydrated in PBS and then attached to the closed system at each end, with a length of ~2.3-3.5 mm between each secured end. Then, the grafts were submerged in 1 L of water. Using a syringe pump and a 30 mL syringe, air was pumped continuously at a rate of 2 mL/min into the system, until a leak was observed or for a maximum of 20 minutes. Pressure was measured and recorded throughout this process using a digital pressure gauge. Six grafts were evaluated in this manner.

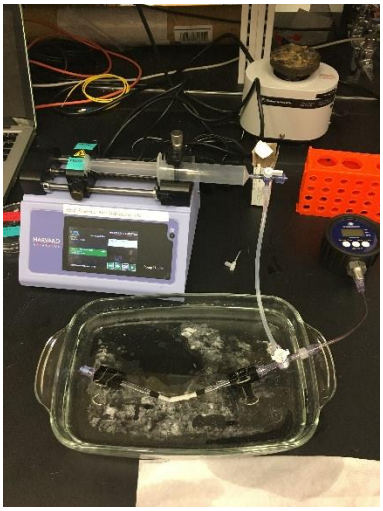


Figure 5.1.3.1. The burst pressure setup consists of clips that closed off the ends of the grafts, a syringe pump to supply air, and a pressure gauge to measure the burst pressure.

5.1.4. Longitudinal and Circumferential Tensile Strength

As a result, various other methods were researched to inspire new testing techniques. A manually developed tensile test was decided to be the best course of action. In this method, two

small-sized binder clips would act as clamps holding the graft on each end. A small weight was hung to one side of the binder clip and gradually, weights were added to this side until a tear in the graft was observed. This entire process was video recorded and later analyzed in ImageJ to measure the stress-strain rate so that the breaking point could be appropriately captured. Despite this method working in theory, the binder clips failed to grip the graft tightly enough in practice. Therefore, this method was deemed ineffective.

This test was reperformed using a 3D printed lens stretcher in hopes of attaining accurate and reliable tensile strength data. Though lens stretchers are normally used to mount and stretch the accommodation apparatus in *ex vivo* eyes, it was incorporated into our project as another alternative to the DMA Q800 technique. Measurements of a manufactured lens stretcher were taken and proportionally enlarged to create enough space for the graft to fit in. After insertion, the contraption would be rotated to slowly tighten the graft until tearing. Again, this process was video recorded and processed using ImageJ software. Still, it was difficult to obtain data due to difficulty in clamping the grafts down tightly enough. Also, manual rotation inherently introduces errors such as inconsistencies in tension, the key component of this test. Therefore, this third method was deemed ineffective for tensile testing.

5.2. Results and Discussion

5.2.1. Burst Pressure Test

To verify that the burst pressure set up is capable of measuring up to the established physiological pressure for a human saphenous vein, we measured the pressure increase of a closed system without a graft attached over a seven minute interval, as shown in Figure 5.2.1.1. The maximum pressure reached during this time was 40.7 psi, about 2100 mmHg, which is above the average pressure associated with a human saphenous vein (1300 +/- 500 mmHg).[12]

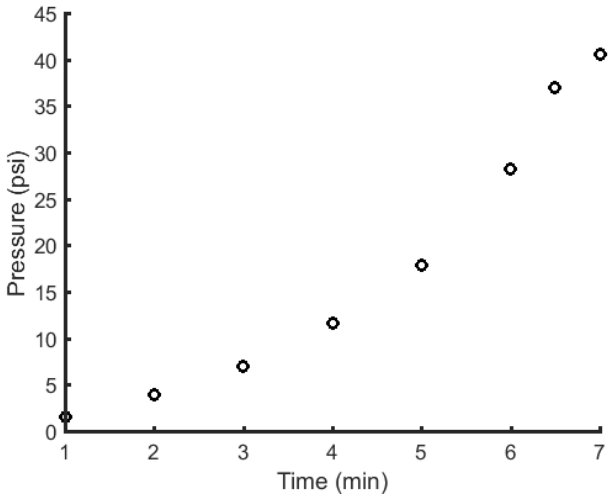


Figure 5.2.1.1. Closed system test for measurement of maximum pressure capacity in psi over time.

Burst pressure testing was successfully performed for four cylindrical grafts: two grafts failed because holes in the material inhibited proper inflation, and these were not counted. The pressure versus time plots for the four grafts tested successfully are shown below in Figure 5.2.1.2. Burst pressure values ranged from 2.5-3.9 psi, with an average burst pressure of 3.3 ± 0.6 psi. These values correspond to values of 129-202 mmHg, with an average of 169 ± 33 mmHg.

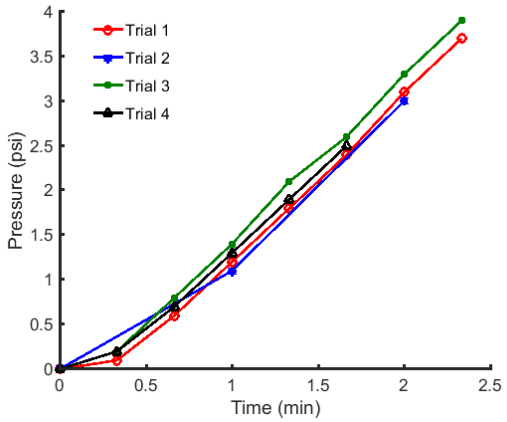


Figure 5.2.1.2. Pressure versus time plots for burst pressure test of four cylindrical electrospun silk grafts. The legend distinguishes each plot line by showing the construction date of each graft.

The tested grafts were shown to withstand the lower range of physiological pressures in the human body. A single pulse pressure in small arteries typically ranges from 80 to 120 mmHg.[101] All grafts could consistently withstand this range, but failed at pressures above ~120 mmHg. This is less than the pressure other experimental grafts were able to withstand and lower than the upper limit of physiological pressures in the body. Therefore, additional layers would substantially reinforce the grafts, and lead to higher burst pressure capacities. Improving the homogeneity of the graft would also likely improve burst pressure capacity. Failure was observed more frequently at portions of the graft that were slightly thinner. Although low burst pressure was observed, the measurements were consistent overall, indicating that the method was moderately precise.

The setup used for burst pressure testing was designed to secure the small-diameter electrospun silk grafts and inflate them at a constant rate until rupture occurred. When initially designing the setup, adhesive material was necessary to create an airtight seal in the slick, rehydrated silk fibroin. The main problem was ensuring that the grafts were securely connected at both ends to the plastic pipettes that deliver air to and from the graft. Additionally, every attachment in the system had to withstand more pressure than the graft, otherwise the system would fail before the graft.

A moderate amount of difficulty was met in ensuring no air escaped from the graft, either from the sealed ends or from holes in the middle region of the graft. To avoid the latter occurrence, grafts were handled with great care during construction of the setup.

5.2.2. Longitudinal and Circumferential Tensile Strength

As a preliminary test, the DMA Q800 was used to test the mechanical strength of dipped grafts in order to establish a working procedure for future testing. The results from the tensile

testing was compiled as a stress-strain curve (Figure 5.2.2.1), indicating the point of rupture at which the material achieves maximum strength before breaking. For this particular tensile test, the rate was set to 2.5% strain per minute and measured between a range from 0 to 25% strain. The graft exhibited its point of rupture, failing at about 17% strain. This result is lower than the literature values for the gold standard (i.e. native human blood vessels), which showed an ultimate stress of 6.3 MPa and a 83% strain at the failure point.[53]

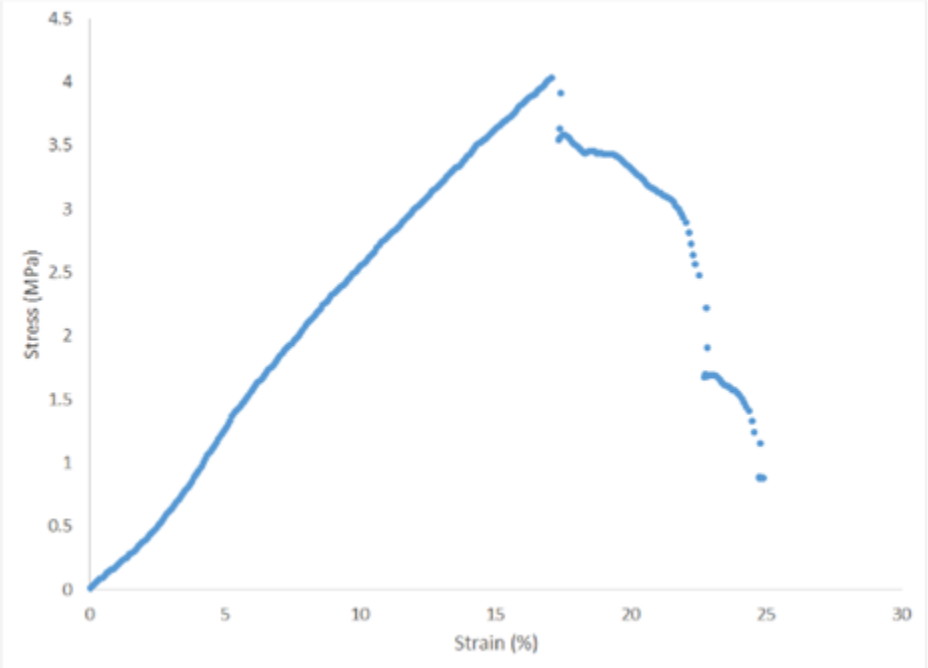


Figure 5.2.2.1 Tensile testing of a dipped graft that resulted in an ultimate stress of about 4 MPa and 17% strain at the failure point.

The difference in values may have been due to the use of a dipped graft as opposed to the electrospinning method, which was later on implemented as the primary technique to develop experimental grafts. In addition, there were multiple inconsistencies in experimental conditions that rendered the data unusable. For example, the graft was being held between two metal clamps on the tensile testing machine. Throughout this lengthy testing process, the graft would change in consistency from hydrated to dehydrated. As a result, the graft would have to be carefully

rehydrated while still mounted within the DMA Q800; however, it is difficult to assess the consistency using this method. Furthermore, the dipped graft was not materially uniform. It exhibited breaks in consistency and did not appear to be entirely longitudinal. The DMA Q800 was not used for the electrospun graft due primarily to its lack of availability due to mechanical repairs. However, the machine was also avoided in anticipation of the electrospun grafts experiencing similar disadvantages as the tested dipped grafts. Due to these experimental flaws, several other testing methods were considered, including the manually developed test and a 3D printed lens stretcher (results not shown). Data could not be collected using the 3D printed lens stretcher, as a result of an inability to properly and consistently clamp the hydrated graft down without it dehydrating throughout the experimental process. Results were unattainable due to inconsistencies in the both DMA Q800 and manual testing.

5.3. Conclusions

The grafts were tested for their mechanical strength including burst pressure strength and tensile capacity. Results from the burst pressure testing indicate the grafts are not able to withstand human physiological conditions with the current method of graft construction. While the material is pliable enough to give with the pressure, the layers of the graft are too thin to prevent failure. By examining this data and comparing specific features of the grafts that were more uniform and had a higher burst capacity, we hope to create a more fortified graft that will not fail during testing. Tensile testing showed that the ultimate stress was nearly comparable to that of the autologous gold standard for bypass surgery, the internal mammary artery and the saphenous vein. That being said, the strain was lower, possibly due to drying as the sample was tested. Overall, while the grafts were not completely equivalent to the gold standards in

mechanical properties, continued experimentation should be able to increase both their strength and elasticity.

6. Concluding Remarks

6.1. Summary & Contributions to the Academic Community

There is currently a clinical need for viable, off-the-shelf, engineered, small diameter vascular grafts for use in peripheral arterial bypass surgery.[110] As the above research has noted, there is much potential for the use of SF as a biomaterial in vascular grafts. There have been positive results from previous electrospinning of SF; however, additional research must be conducted in order to fix the shortcomings of using electrospun SF for vascular grafts. A suitable graft must mimic the properties of the native vessel, carrying out the functions for which the blocked vessel is responsible until it is replaced by natural tissue.[11,15,110] Silk-based grafts have the potential for fulfilling this purpose, as silk exhibits excellent mechanical strength and biocompatibility.[21] Although silk has demonstrated many of the necessary characteristics of a good biomaterial, silk-based grafts still need improvement before they can be implemented clinically. Our work aimed to address this need for improvement.

The herein detailed project plays an informative role in the tissue engineering community. While the given research is not yet at a state ready for translation, it provides information to the academic community about how vascular endothelial cells respond to physical changes in their scaffold, as a mimic for changes in the ECM structure. We also demonstrated that cells could proliferate on the graft. Furthermore, with Matrigel, we were able to increase adhesion of endothelial cells on the graft in a dose-dependent manner. Using a co-culture between vascular endothelial cells and smooth muscle cells improved the proliferation of endothelial cells on the grafts, resulting in a closer model to a native vascular environment. Although the electrospun grafts were not able to withstand the same burst pressure as the

saphenous vein, they were able to withstand the pressure that would be exhibited in that part of the vasculature.

6.2. Future Work

Future work will both expand upon the current graft modifications, and other factors may also be tested. Further experiments with Matrigel will seek to understand its dose-dependence. Furthermore, the key individual growth factors from Matrigel will be tested individually to understand which factors are the most key to proliferation of cells on the graft. Experiments will further seek to understand if Matrigel or its subset growth factors can not only adhere cells, but also recruit them in a dynamic tissue culture. Other factors that are key to recruiting endothelial cells to the graft, such as anti-CD34 or anti-VE-cadherin, could also be explored as routes to increase cell proliferation on the silk fibroin scaffolds. They have been studied on grafts made with other biomaterials, but never on SF grafts to the best of our knowledge.[111–113] In addition, there are many other electrospinning parameters that could be further explored to analyze the effect on the SF fibers and the cell growth on the scaffolds.

Additionally, our work can be further validated by using an *in vivo* model. While *in vitro* assays can give an idealistic estimate of the biocompatibility of these scaffolds, the complex dynamic environment of the organism can greatly complicate the grafts' success. As such, this validation is necessary. Grafts would be implanted into a rodent model. A study would be conducted looking at histological slices of the grafts at various time points. The slices from the treated grafts could also be compared to those that do not have biochemical modification. This would allow visualization of both the scaffold degradation over time and the tissue growth on the graft. Additionally, it would allow detection of intimal hyperplasia, which is an issue that currently plagues many small-diameter vascular grafts. These studies would also be necessary to

confirm that the grafts are non-toxic. Finally, it would allow understanding of the immune response or lack thereof to the graft. Overall, testing the grafts in an *in vivo* model would be the next step towards application of small-diameter electrospun SF vascular grafts to bypass surgery. *In vivo* models would allow direct comparison between our graft and the current commercial standards for vascular prostheses, providing invaluable information to the medical community. Finally, future work will consider scale-up factors to demonstrate why silk has advantages in manufacturing large quantities of grafts.

References

(1) Sell, S. A.; McClure, M. J.; Garg, K.; Wolfe, P. S.; Bowlin, G. L. Electrospinning of Collagen/Biopolymers for Regenerative Medicine and Cardiovascular Tissue Engineering. *Advanced Drug Delivery Reviews* **2009**, *61*, 1007–1019.

(2) Lloyd-Jones, D.; Adams, R. J.; Brown, T. M.; Carnethon, M.; Dai, S.; Simone, G. D.; Ferguson, T. B.; Ford, E.; Furie, K.; Gillespie, C.; *et al.* Heart Disease and Stroke Statistics—2010 Update A Report From the American Heart Association. *Circulation* **2010**, *121*, e46–e215.

(3) Writing Group Members; Rosamond, W.; Flegal, K.; Furie, K.; Go, A.; Greenlund, K.; Haase, N.; Hailpern, S. M.; Ho, M.; Howard, V.; *et al.* Heart Disease and Stroke Statistics—2008 Update. *Circulation* **2008**, *117*, e25–e146.

(4) About Peripheral Artery Disease (PAD) http://www.heart.org/HEARTORG/Conditions/VascularHealth/PeripheralArteryDisease/About-Peripheral-Artery-Disease-PAD_UCM_301301_Article.jsp#.WNFipGfavIU (accessed Mar 21, 2017).

(5) Fowkes, F. G. R.; Rudan, D.; Rudan, I.; Aboyans, V.; Denenberg, J. O.; McDermott, M. M.; Norman, P. E.; Sampson, U. K.; Williams, L. J.; Mensah, G. A.; *et al.* Comparison of Global Estimates of Prevalence and Risk Factors for Peripheral Artery Disease in 2000 and 2010: A Systematic Review and Analysis. *The Lancet* **2013**, *382*, 1329–1340.

(6) Allison, M. A.; Ho, E.; Denenberg, J. O.; Langer, R. D.; Newman, A. B.; Fabsitz, R. R.; Criqui, M. H. Ethnic-Specific Prevalence of Peripheral Arterial Disease in the United States. *American Journal of Preventive Medicine* **2007**, *32*, 328–333.

(7) Mahoney, E. M.; Wang, K.; Cohen, D. J.; Hirsch, A. T.; Alberts, M. J.; Eagle, K.; Mosse, F.; Jackson, J. D.; Steg, P. G.; Bhatt, D. L. One-Year Costs in Patients With a History of or at Risk for Atherothrombosis in the United States. *Circulation: Cardiovascular Quality and Outcomes* **2008**, *1*, 38–45.

(8) Kullo, I. J.; Rooke, T. W. Peripheral Artery Disease. *New England Journal of Medicine* **2016**, *374*, 861–871.

(9) Chi, Y.-W.; Jaff, M. R. Optimal Risk Factor Modification and Medical Management of the Patient with Peripheral Arterial Disease. *Cathet. Cardiovasc. Intervent.* **2008**, *71*, 475–489.

(10) Belch, J. J. F.; Topol, E. J.; Agnelli, G.; Bertrand, M.; Califf, R. M.; Clement, D. L.; Creager, M. A.; Easton, J. D.; Gavin, J. R.; Greenland, P.; *et al.* Critical Issues in Peripheral Arterial Disease Detection and Management: A Call to Action. *Arch Intern Med* **2003**, *163*, 884–892.

(11) Thomas, A. C.; Campbell, G. R.; Campbell, J. H. Advances in Vascular Tissue Engineering. *Cardiovascular Pathology* **2003**, *12*, 271–276.

(12) Liu, S.; Dong, C.; Lu, G.; Lu, Q.; Li, Z.; Kaplan, D. L.; Zhu, H. Bilayered Vascular Grafts Based on Silk Proteins. *Acta Biomaterialia* **2013**, *9*, 8991–9003.

(13) Foster, E.; Kranc, M. Alternative Conduits for Aortocoronary Bypass Grafting. *Circulation* **1989**, *79*, I34-9.

(14) Motwani, J. G.; Topol, E. J. Aortocoronary Saphenous Vein Graft Disease Pathogenesis, Predisposition, and Prevention. *Circulation* **1998**, *97*, 916–931.

(15) Vara, D. S.; Salacinski, H. J.; Kannan, R. Y.; Bordenave, L.; Hamilton, G.; Seifalian, A. M. Cardiovascular Tissue Engineering: State of the Art. *Pathologie Biologie* **2005**, *53*, 599–612.

(16) Allaire, E.; Bruneval, P.; Mandet, C.; Becquemin, J.-P.; Michel, J.-B. The Immunogenicity of the Extracellular Matrix in Arterial Xenografts. *Surgery* **1997**, *122*, 73–81.

(17) Martin, R. S.; Edwards, W. H.; Mulherin, J. L.; Edwards, W. H.; Jenkins, J. M.; Hoff, S. J. Cryopreserved Saphenous Vein Allografts for below-Knee Lower Extremity Revascularization. *Ann Surg* **1994**, *219*, 664–672.

(18) Vartanian, S. M.; Conte, M. S. Surgical Intervention for Peripheral Arterial Disease. *Circulation Research* **2015**, *116*, 1614–1628.

(19) Pérez-Rigueiro, J.; Viney, C.; Llorca, J.; Elices, M. Mechanical Properties of Single-Brin Silkworm Silk. *J. Appl. Polym. Sci.* **2000**, *75*, 1270–1277.

(20) Zhang, X.; Reagan, M. R.; Kaplan, D. L. Electrospun Silk Biomaterial Scaffolds for Regenerative Medicine. *Adv Drug Deliv Rev* **2009**, *61*, 988–1006.

(21) Altman, G. H.; Diaz, F.; Jakuba, C.; Calabro, T.; Horan, R. L.; Chen, J.; Lu, H.; Richmond, J.; Kaplan, D. L. Silk-Based Biomaterials. *Biomaterials* **2003**, *24*, 401–416.

(22) Vepari, C.; Kaplan, D. L. Silk as a Biomaterial. *Progress in Polymer Science* **2007**, *32*, 991–1007.

(23) Qi, Y.; Wang, H.; Wei, K.; Yang, Y.; Zheng, R.-Y.; Kim, I. S.; Zhang, K.-Q. A Review of Structure Construction of Silk Fibroin Biomaterials from Single Structures to Multi-Level Structures. *International Journal of Molecular Sciences* **2017**, *18*, 237.

(24) Yang, M. Silk-Based Biomaterials. *Microsc Res Tech* **2017**, *80*, 321–330.

(25) Li, D.; Xia, Y. Electrospinning of Nanofibers: Reinventing the Wheel? *Adv. Mater.* **2004**, *16*, 1151–1170.

(26) Agarwal, S.; Greiner, A.; Wendorff, J. H. Functional Materials by Electrospinning of Polymers. *Progress in Polymer Science* **2013**, *38*, 963–991.

(27) Hillis, D.; Sadava, D.; Hill, R.; Price, M. *Principles of Life*; 2nd ed.; W. H. Freeman, 2014.

(28) Reece, J.; Urry, L.; Cain, M.; Wasserman, S.; Minorsky, P.; Jackson, R. *Campbell Biology*; 9th ed.; Benjamin Cummings: Boston, 2011.

(29) Alberts, B.; Johnson, A.; Lewis, J.; Raff, M.; Roberts, K.; Walter, P. *Molecular Biology of the Cell*; 5th ed.; Garland Science: New York, 2008.

(30) Sarkar, S.; Schmitz-Rixen, T.; Hamilton, G.; Seifalian, A. M. Achieving the Ideal Properties for Vascular Bypass Grafts Using a Tissue Engineered Approach: A Review. *Med Bio Eng Comput* **2007**, *45*, 327–336.

(31) Eble, J. A.; Niland, S. The Extracellular Matrix of Blood Vessels. *Curr. Pharm. Des.* **2009**, *15*, 1385–1400.

(32) Buschmann, I.; Schaper, W. Arteriogenesis Versus Angiogenesis: Two Mechanisms of Vessel Growth. *Physiology* **1999**, *14*, 121–125.

(33) Kurobe, H.; Maxfield, M. W.; Breuer, C. K.; Shinoka, T. Concise Review: Tissue-Engineered Vascular Grafts for Cardiac Surgery: Past, Present, and Future. *Stem Cells Trans Med* **2012**, *1*, 566–571.

(34) Dionigi, B.; Fauza, D. O. Autologous Approaches to Tissue Engineering. In *StemBook*; Harvard Stem Cell Institute: Cambridge (MA), 2008.

(35) McKee, J. Autograft or Allograft for ACL Reconstruction? *AAOS Now* **2012**.

(36) Eastlund, T. Bacterial Infection Transmitted by Human Tissue Allograft Transplantation. *Cell Tissue Banking* **2006**, *7*, 147–166.

(37) McAllister, D. R.; Joyce, M. J.; Mann, B. J.; Vangsness, C. T. Allograft Update: The Current Status of Tissue Regulation, Procurement, Processing, and Sterilization. *Am J Sports Med* **2007**, *35*, 2148–2158.

(38) Balsly, C. R.; Cotter, A. T.; Williams, L. A.; Gaskins, B. D.; Moore, M. A.; Jr, L. W. Effect of Low Dose and Moderate Dose Gamma Irradiation on the Mechanical Properties of Bone and Soft Tissue Allografts. *Cell Tissue Bank* **2008**, *9*, 289–298.

(39) Cooper, D. K. C. A Brief History of Cross-Species Organ Transplantation. *Proceedings (Baylor University. Medical Center)* **2012**, *25*, 49–51.

(40) Committee on Xenograft Transplantation: Ethical Issues and Public Policy; Institute of Medicine. *Xenotransplantation: Science, Ethics, and Public Policy*; National Acadamy Press: Washington D.C., 1996.

(41) Weiss, R. A. Xenotransplantation. *BMJ* **1998**, *317*, 931–934.

(42) Roll, S.; Müller-Nordhorn, J.; Keil, T.; Scholz, H.; Eidt, D.; Greiner, W.; Willich, S. N. Dacron® vs. PTFE as Bypass Materials in Peripheral Vascular Surgery – Systematic Review and Meta-Analysis. *BMC Surgery* **2008**, *8*, 22.

(43) Chan, B. P.; Leong, K. W. Scaffolding in Tissue Engineering: General Approaches and Tissue-Specific Considerations. *Eur Spine J* **2008**, *17*, 467–479.

(44) Hoerstrup, S. P.; Zünd, G.; Sodian, R.; Schnell, A. M.; Grünenfelder, J.; Turina, M. I. Tissue Engineering of Small Caliber Vascular Grafts. *Eur J Cardiothorac Surg* **2001**, *20*, 164–169.

(45) Yarlagadda, P. K. D. V.; Chandrasekharan, M.; Shyan, J. Y. M. Recent Advances and Current Developments in Tissue Scaffolding. *Bio-Medical Materials and Engineering* **2005**, *15*, 159–177.

(46) Rathore, A.; Cleary, M.; Naito, Y.; Rocco, K.; Breuer, C. Development of Tissue Engineered Vascular Grafts and Application of Nanomedicine. *WIREs Nanomed Nanobiotechnol* **2012**, *4*, 257–272.

(47) Ratcliffe, A. Tissue Engineering of Vascular Grafts. *Matrix Biol.* **2000**, *19*, 353–357.

(48) Vrana, N. E.; Annabi, N.; Pinar Zorlutuna. *Scaffolds for Tissue Engineering: Biological Design, Materials and Fabrication*; Pan Stanford Publishing Pte. Ltd, 2014.

(49) Gilbert, T. W.; Sellaro, T. L.; Badylak, S. F. Decellularization of Tissues and Organs. *Biomaterials* **2006**, *27*, 3675–3683.

(50) Orive, G.; Hernández, R. M.; Gascón, A. R.; Calafiore, R.; Chang, T. M. S.; Vos, P. D.; Hortelano, G.; Hunkeler, D.; Lacík, I.; Shapiro, A. M. J.; *et al.* Cell Encapsulation: Promise and Progress. *Nat Med* **2003**, *9*, 104–107.

(51) Chevalier, E.; Chulia, D.; Pouget, C.; Viana, M. Fabrication of Porous Substrates: A Review of Processes Using Pore Forming Agents in the Biomaterial Field. *Journal of Pharmaceutical Sciences* **2008**, *97*, 1135–1154.

(52) Catto, V.; Farè, S.; Cattaneo, I.; Figliuzzi, M.; Alessandrino, A.; Freddi, G.; Remuzzi, A.; Tanzi, M. C. Small Diameter Electrospun Silk Fibroin Vascular Grafts: Mechanical Properties, in Vitro Biodegradability, and in Vivo Biocompatibility. *Materials Science and Engineering: C* **2015**, *54*, 101–111.

(53) Hasan, A.; Memic, A.; Annabi, N.; Hossain, M.; Paul, A.; Dokmeci, M. R.; Dehghani, F.; Khademhosseini, A. Electrospun Scaffolds for Tissue Engineering of Vascular Grafts. *Acta Biomaterialia* **2014**, *10*, 11–25.

(54) Stekelenburg, M.; Rutten, M. C. M.; Snoeckx, L. H. E. H.; Baaijens, F. P. T. Dynamic Straining Combined with Fibrin Gel Cell Seeding Improves Strength of Tissue-Engineered Small-Diameter Vascular Grafts. *Tissue Engineering Part A* **2008**, *15*, 1081–1089.

(55) Williams, D. F. On the Mechanisms of Biocompatibility. *Biomaterials* **2008**, *29*, 2941–2953.

(56) Ahmed, F.; Dutta, N. K.; Zannettino, A.; Vandyke, K.; Choudhury, N. R. Engineering Interaction between Bone Marrow Derived Endothelial Cells and Electrospun Surfaces for Artificial Vascular Graft Applications. *Biomacromolecules* **2014**, *15*, 1276–1287.

(57) Yao, Y.; Wang, J.; Cui, Y.; Xu, R.; Wang, Z.; Zhang, J.; Wang, K.; Li, Y.; Zhao, Q.; Kong, D. Effect of Sustained Heparin Release from PCL/Chitosan Hybrid Small-Diameter Vascular Grafts on Anti-Thrombogenic Property and Endothelialization. *Acta Biomaterialia* **2014**, *10*, 2739–2749.

(58) Chen, Q.-Z.; Harding, S. E.; Ali, N. N.; Lyon, A. R.; Boccaccini, A. R. Biomaterials in Cardiac Tissue Engineering: Ten Years of Research Survey. *Materials Science and Engineering: R: Reports* **2008**, *59*, 1–37.

(59) Seal, B. L.; Otero, T. C.; Panitch, A. Polymeric Biomaterials for Tissue and Organ Regeneration. *Materials Science and Engineering: R: Reports* **2001**, *34*, 147–230.

(60) Kundu, B.; Rajkhowa, R.; Kundu, S. C.; Wang, X. Silk Fibroin Biomaterials for Tissue Regenerations. *Advanced Drug Delivery Reviews* **2013**, *65*, 457–470.

(61) Mondal, M.; Trivedy, K.; Nirmal Kumar, S. The Silk Proteins, Sericin and Fibroin in Silkworm, Bombyx Mori Linn., - a Review. *Caspian Journal of Environmental Science* **2007**, *5*, 63–76.

(62) Jin, H.-J.; Kaplan, D. L. Mechanism of Silk Processing in Insects and Spiders. *Nature* **2003**, *424*, 1057–1061.

(63) Murphy, A. R.; Kaplan, D. L. Biomedical Applications of Chemically-Modified Silk Fibroin. *Journal of Materials Chemistry* **2009**, *19*, 6443.

(64) Zhou, C.-Z.; Confalonieri, F.; Jacquet, M.; Perasso, R.; Li, Z.-G.; Janin, J. Silk Fibroin: Structural Implications of a Remarkable Amino Acid Sequence. *Proteins* **2001**, *44*, 119–122.

(65) Rockwood, D. N.; Preda, R. C.; Yücel, T.; Wang, X.; Lovett, M. L.; Kaplan, D. L. Materials Fabrication from Bombyx Mori Silk Fibroin. *Nat Protoc* **2011**, *6*.

(66) Kim, U.-J.; Park, J.; Li, C.; Jin, H.-J.; Valluzzi, R.; Kaplan, D. L. Structure and Properties of Silk Hydrogels. *Biomacromolecules* **2004**, *5*, 786–792.

(67) Wang, Y.; Rudym, D. D.; Walsh, A.; Abrahamsen, L.; Kim, H.-J.; Kim, H. S.; Kirker-Head, C.; Kaplan, D. L. In Vivo Degradation of Three-Dimensional Silk Fibroin Scaffolds. *Biomaterials* **2008**, *29*, 3415–3428.

(68) Lovett, M. L.; Cannizzaro, C.; Daheron, L.; Messmer, B.; Vunjak-Novakovic, G.; Kaplan, D. L. Silk Fibroin Microtubes for Blood Vessel Engineering. *Biomaterials* **2007**, *28*, 5271–5279.

(69) Wang, J.; Wei, Y.; Yi, H.; Liu, Z.; Sun, D.; Zhao, H. Cytocompatibility of a Silk Fibroin Tubular Scaffold. *Materials Science and Engineering: C* **2014**, *34*, 429–436.

(70) Liu, H.; Li, X.; Zhou, G.; Fan, H.; Fan, Y. Electrospun Sulfated Silk Fibroin Nanofibrous Scaffolds for Vascular Tissue Engineering. *Biomaterials* **2011**, *32*, 3784–3793.

(71) Marelli, B.; Achilli, M.; Alessandrino, A.; Freddi, G.; Tanzi, M. C.; Farè, S.; Mantovani, D. Collagen-Reinforced Electrospun Silk Fibroin Tubular Construct as Small Calibre Vascular Graft. *Macromol. Biosci.* **2012**, *12*, 1566–1574.

(72) Soletti, L.; Hong, Y.; Guan, J.; Stankus, J. J.; El-Kurdi, M. S.; Wagner, W. R.; Vorp, D. A. A Bilayered Elastomeric Scaffold for Tissue Engineering of Small Diameter Vascular Grafts. *Acta Biomaterialia* **2010**, *6*, 110–122.

(73) Dahl, S. L. M.; Kypson, A. P.; Lawson, J. H.; Blum, J. L.; Strader, J. T.; Li, Y.; Manson, R. J.; Tente, W. E.; DiBernardo, L.; Hensley, M. T.; *et al.* Readily Available Tissue-Engineered Vascular Grafts. *Sci Transl Med* **2011**, *3*, 1–11.

(74) Konig, G.; McAllister, T. N.; Dusserre, N.; Garrido, S. A.; Iyican, C.; Marini, A.; Fiorillo, A.; Avila, H.; Wystrychowski, W.; Zagalski, K.; *et al.* Mechanical Properties of Completely Autologous Human Tissue Engineered Blood Vessels Compared to Human Saphenous Vein and Mammary Artery. *Biomaterials* **2009**, *30*, 1542–1550.

(75) L'heureux, N.; Pâquet, S.; Labbé, R.; Germain, L.; Auger, F. A. A Completely Biological Tissue-Engineered Human Blood Vessel. *FASEB J* **1998**, *12*, 47–56.

(76) Ding, X.; Zou, T.; Gong, X.; Ren, C.; Kang, H.; Xu, P.; Liu, H.; Fan, Y. Trilayered Sulfated Silk Fibroin Vascular Grafts Enhanced with Braided Silk Tube. *Journal of Bioactive and Compatible* **2016**, *31*, 613–623.

(77) Pawar, H. S.; Francis, N. K.; Rameshbabu, A. P.; Dhara, S. 2,5-Dihydro-2,5-Dimethoxyfuran Crosslinked Silk-Chitosan Blend Tubular Construct for Vascular Graft Application. *Materials Today Communications* **2016**, *8*, 139–147.

(78) Yin, A.; Luo, R.; Li, J.; Mo, X.; Wang, Y.; Zhang, X. Coaxial Electrospinning Multicomponent Functional Controlled-Release Vascular Graft: Optimization of Graft Properties. *Colloids and Surfaces B: Biointerfaces* **2017**, *152*, 432–439.

(79) Tan, Y.; Richards, D. J.; Trusk, T. C.; Visconti, R. P.; Yost, M. J.; Kindy, M. S.; Drake, C. J.; Argraves, W. S.; Markwald, R. R.; Mei, Y. 3D Printing Facilitated Scaffold-Free Tissue Unit Fabrication. *Biofabrication* **2014**, *6*, 024111.

(80) Lovett, M.; Eng, G.; Kluge, J. A.; Cannizzaro, C.; Vunjak-Novakovic, G.; Kaplan, D. L. Tubular Silk Scaffolds for Small Diameter Vascular Grafts. *Organogenesis* **2010**, *6*, 217–224.

(81) Bhardwaj, N.; Kundu, S. C. Electrospinning: A Fascinating Fiber Fabrication Technique. *Biotechnology Advances* **2010**, *28*, 325–347.

(82) Cui, W.; Zhou, Y.; Chang, J. Electrospun Nanofibrous Materials for Tissue Engineering and Drug Delivery. *Sci. Technol. Adv. Mater.* **2010**, *11*, 014108.

(83) Sill, T.; von Recum, H. Electrospinning: Applications in Drug Delivery and Tissue Engineering. *Biomatrials* **2008**, *29*, 1989–2006.

(84) Gaumer, J.; Prasad, A.; Lee, D.; Lannutti, J. Structure–function Relationships and Source-to-Ground Distance in Electrospun Polycaprolactone. *Acta Biomaterialia* **2009**, *5*, 1552–1561.

(85) Powell, H. M.; Boyce, S. T. Fiber Density of Electrospun Gelatin Scaffolds Regulates Morphogenesis of Dermal–epidermal Skin Substitutes. *J. Biomed. Mater. Res.* **2008**, *84A*, 1078–1086.

(86) Sisson, K.; Zhang, C.; Farach-Carson, M.; Chase, D. B.; Rabolt, J. Fiber Diameters Control Osteoblastic Cell Migration and Differentiation in Electrospun Gelatin. *Journal of Biomedical Materials Research* **2010**, *94A*.

(87) Tschoeke, B.; Flanagan, T. C.; Koch, S.; Harwoko, M. S.; Deichmann, T.; Ellå, V.; Sachweh, J. S.; Kellomåki, M.; Gries, T.; Schmitz-Rode, T.; *et al.* Tissue-Engineered Small-Caliber Vascular Graft Based on a Novel Biodegradable Composite Fibrin-Polylactide Scaffold. *Tissue Eng Part A* **2009**, *15*, 1909–1918.

(88) Xue, L.; Greisler, H. P. Biomaterials in the Development and Future of Vascular Grafts. *Journal of Vascular Surgery* **2003**, *37*, 472–480.

(89) Nelson, S. R.; deSouza, N. M.; Allison, D. J. Endovascular Stents and Stent-Grafts: Is Heparin Coating Desirable? *CardioVascular and Interventional Radiology* **2000**, *23*, 252–255.

(90) Burgess, W. H.; Maciag, T. The Heparin-Binding (Fibroblast) Growth Factor Family of Proteins. *Annual Review of Biochemistry* **1989**, *58*, 575–602.

(91) Wise, S. G.; Byrom, M. J.; Waterhouse, A.; Bannon, P. G.; Weiss, A. S.; Ng, M. K. C. A Multilayered Synthetic Human Elastin/Polycaprolactone Hybrid Vascular Graft with Tailored Mechanical Properties. *Acta Biomater* **2011**, *7*, 295–303.

(92) Reinders, M. E. J.; Sho, M.; Izawa, A.; Wang, P.; Mukhopadhyay, D.; Koss, K. E.; Geehan, C. S.; Luster, A. D.; Sayegh, M. H.; Briscoe, D. M. Proinflammatory Functions of Vascular Endothelial Growth Factor in Alloimmunity. *Journal of Clinical Investigation* **2003**, *112*, 1655–1665.

(93) Moore, W. S. *Vascular and Endovascular Surgery: A Comprehensive Review*; 8th ed.; Elsevier Health Sciences, 2012.

(94) Hughes, C. S.; Postovit, L. M.; Lajoie, G. A. Matrigel: A Complex Protein Mixture Required for Optimal Growth of Cell Culture. *Proteomics* **2010**, *10*, 1886–1890.

(95) Soofi, S. S.; Last, J. A.; Liliensiek, S. J.; Nealey, P. F.; Murphy, C. J. The Elastic Modulus of Matrigel™ as Determined by Atomic Force Microscopy. *J Struct Biol* **2009**, *167*, 216–219.

(96) Zhang, X.; Wang, X.; Keshav, V.; Wang, X.; Johanas, J. T.; Leisk, G. G.; Kaplan, D. L. Dynamic Culture Conditions to Generate Silk-Based Tissue-Engineered Vascular Grafts. *Biomaterials* **2009**, *30*, 3213–3223.

(97) Kleinman, H. K.; Martin, G. R. Matrigel: Basement Membrane Matrix with Biological Activity. *Seminars in Cancer Biology* **2005**, *15*, 378–386.

(98) Truskey, G. A. Endothelial Cell Vascular Smooth Muscle Cell Co-Culture Assay For High Throughput Screening Assays For Discovery of Anti-Angiogenesis Agents and Other Therapeutic Molecules. *Int J High Throughput Screen* **2010**, *2010*, 171–181.

(99) Conti, J. C.; Strope, E. R.; Price, K. S.; Goldenberg, L. M. The High Frequency Testing of Vascular Grafts and Vascular Stents: Influence of Sample Dimensions on Maximum Allowable Frequency. *Biomed Sci Instrum* **1999**, *35*, 339–346.

(100) Nagiah, N.; Johnson, R.; Anderson, R.; Elliott, W.; Tan, W. Highly Compliant Vascular Grafts with Gelatin-Sheathed Coaxially Structured Nanofibers. *Langmuir* **2015**, *31*, 12993–13002.

(101) Guyton, A. C.; Hall, J. E. *Textbook of Medical Physiology*; Guyton Physiology Series; 11th ed.; Elsevier Saunders, 2006.

(102) *Encyclopedic Handbook of Biomaterials and Bioengineering: V. 1-2. Applications*; CRC Press, 1995.

(103) Marecik, S. J.; Chaudhry, V.; Jan, A.; Pearl, R. K.; Park, J. J.; Prasad, L. M. A Comparison of Robotic, Laparoscopic, and Hand-Sewn Intestinal Sutured Anastomoses Performed by Residents. *The American Journal of Surgery* **2007**, *193*, 349–355.

(104) Isaka, M.; Nishibe, T.; Okuda, Y.; Saito, M.; Seno, T.; Yamashita, K.; Izumisawa, Y.; Kotani, T.; Yasuda, K. Experimental Study on Stability of a High-Porosity Expanded Polytetrafluoroethylene Graft in Dogs. *Ann Thorac Cardiovasc Surg* **2006**, *12*, 37–41.

(105) LIVE/DEAD Cell Viability Assays https://www.thermofisher.com/us/en/home/brands/molecular-probes/key-molecular-probes-products/live-dead-viability-brand-page.html (accessed Mar 21, 2017).

(106) Dadwal, U.; Falank, C.; Fairfield, H.; Linehan, S.; Rosen, C. J.; Kaplan, D. L.; Sterling, J.; Reagan, M. R. Tissue-Engineered 3D Cancer-in-Bone Modeling: Silk and PUR Protocols. *BoneKEy Reports* **2016**, *5*.

(107) Wallace, C. S.; Truskey, G. A. Direct-Contact Co-Culture between Smooth Muscle and Endothelial Cells Inhibits TNF-α-Mediated Endothelial Cell Activation. *Am J Physiol Heart Circ Physiol* **2010**, *299*, H338–H346.

(108) Korff, T.; Kimmina, S.; Martiny-Baron, G.; Augustin, H. G. Blood Vessel Maturation in a 3-Dimensional Spheroidal Coculture Model: Direct Contact with Smooth Muscle Cells Regulates Endothelial Cell Quiescence and Abrogates VEGF Responsiveness. *FASEB J* **2001**, *15*, 447–457.

(109) Zhang, X.; Baughman, C. B.; Kaplan, D. L. In Vitro Evaluation of Electrospun Silk Fibroin Scaffolds for Vascular Cell Growth. *Biomaterials* **2008**, *29*, 2217–2227.

(110) Udelsman, B. V.; Maxfield, M. W.; Breuer, C. K. Tissue Engineering of Blood Vessels in Cardiovascular Disease: Moving towards Clinical Translation. *Heart* **2013**, *99*, 454–460.

(111) Yin, M.; Yuan, Y.; Liu, C.; Wang, J. Combinatorial Coating of Adhesive Polypeptide and Anti-CD34 Antibody for Improved Endothelial Cell Adhesion and Proliferation. *J Mater Sci: Mater Med* **2009**, *20*, 1513–1523.

(112) Lee, J. M.; Choe, W.; Kim, B.-K.; Seo, W.-W.; Lim, W.-H.; Kang, C.-K.; Kyeong, S.; Eom, K. D.; Cho, H.-J.; Kim, Y.-C.; *et al.* Comparison of Endothelialization and Neointimal Formation with Stents Coated with Antibodies against CD34 and Vascular Endothelial-Cadherin. *Biomaterials* **2012**, *33*, 8917–8927.

(113) Melchiorri, A. J.; Hibino, N.; Yi, T.; Lee, Y. U.; Sugiura, T.; Tara, S.; Shinoka, T.; Breuer, C.; Fisher, J. P. Contrasting Biofunctionalization Strategies for the Enhanced Endothelialization of Biodegradable Vascular Grafts. *Biomacromolecules* **2015**, *16*, 437–446.

(114) Hubbell, J. A. Bioactive Biomaterials. *Current Opinion in Biotechnology* **1999**, *10*, 123–129.

Glossary

Allogeneic: Of or relating to molecules derived from a donor individual that is of the same species as the recipient (compare to xenogeneic)

Allografts: A tissue graft derived from a donor individual that is of the same species as the recipient (compare to autologous graft and xenograft)

Angiogenesis: The growth of a new blood vessel

Anticoagulant: A type of molecule that prevents the clotting of blood

Arterial bypass surgery: A type of surgery that improves blood flow to the heart and peripheral vessels through the usage of a graft or healthy vessel to surgically bypass the blocked vessel (see coronary arterial bypass surgery and peripheral arterial bypass surgery)

Arteriogenesis: The process of increasing vessel diameter (compare to angiogensis)

Atherosclerosis: A disease in which plaque builds up in the arteries, narrowing the path for blood to flow and oxygenate the surrounding tissue (compare to coronary heart disease)

Autologous grafts: A tissue graft derived from the patient's own tissue (compare to xenografts and allograft)

Bioactivity: How a biomaterial interacts favorably with the host biological environment, often to produce a desired cellular response[114]

Biocompatibility: The co-existence of a biomaterial and its host biological environment without any adverse effects to either. Biocompatibility is application specific[55]

Burst strength: A strength property measuring the resistance to rupturing

Complement system: A group of proteins which move freely through the circulatory system which play a role in inflammatory response

Coronary arterial bypass surgery: A procedure used to reroute blocked arteries in the heart (see arterial bypass surgery)

Coronary heart disease: A disease in which plaque builds up in the coronary arteries narrowing the path for the heart to pump blood around the body (compare to atherosclerosis)

Crystallinity: The degree of structural order

Degradability: A material breaks down into its components or into smaller polymer chains due to various cleavage mechanisms

Degumming: Process by which the sericin, waxes and other materials are removed from raw silk to leave the silk fibroin

Diastole: The pressure exerted on the heart during relaxation

Elasticity: The ability to return to original shape after stretch or compression

Elastin: Elastic protein in connective tissue

Electrospinning: Electrostatic fiber fabrication technique

Endogenous cells: Cells from the native tissue

Endothelialization: The aggregation of endothelial cells

Engineered graft: A graft construct developed using a variety a techniques

Epidermal growth factor: A transmembrane that helps stimulate cell proliferation by binding to one of two receptor tyrosine kinases

Extracellular matrix: A microenvironment that provides structural support and anchorage to surrounding tissue

Flow rate: Rate of dispensed solution from the solution source

Glycosaminoglycans: Complex carbohydrates that participate in many biological processes through the regulation of their various protein partners

Hemodynamic: Relating to the dynamics of blood flow

Heparin: An anticoagulant protein

Hydrophilicity: The ability to readily absorb or dissolve in water

Hyperplasia: Increased cell production in a normal tissue or organ

Intimal hyperplasia: Thickening of the arteries often caused by a vascular surgery

Ischemia: Blood flow is decreased by a blockage in the heart's vessels, leading to tissue oxygen starvation

Pre-made porous scaffolds: A manufactured scaffold which contains pores

Matrigel: Microenvironment (ECM) similar to native tissue in order to stimulate cell growth

Neointimal formation: Scar tissue that forms in the blood vessel, often as a result of vascular surgery

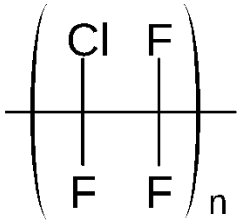
Neovascularization: Development of new blood vessels

Non-thrombogenicity: Resistance to the tendency of a material to cause clotting of the blood

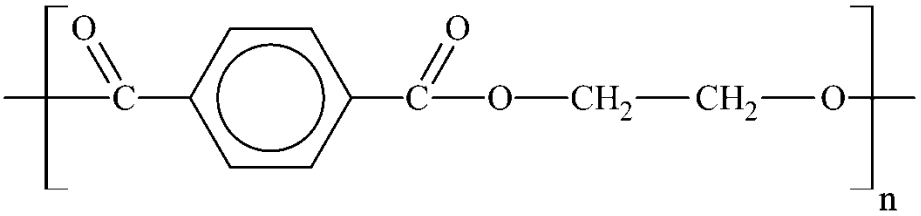
Occlusion: The closing of a blood vessel

Patency: The chance that a graft remains open after a given amount of time

Polytetrafluoroethylene (PTFE) (Teflon): A synthetic fiber commonly used in the production of engineered graft. Structure shown below (see Polyethylene Terephthalate)



Polyethylene Terephthalate (PET) (Dacron): A polyester fiber commonly used in the production of engineered grafts. Structure shown below (see Polytetrafluoroethylene)



Peripheral arterial bypass surgery: A procedure used to reroute blocked arteries in the peripheral cardiovascular system (see arterial bypass surgery)

Porogen: A pore forming agent that may burn away during firing or heating or dissolve in a solution leaving pores in the remaining material

Porosity: The varying degrees and sizes of the pores within the grafts

Regeneration: Return to original functionality

Saphenous vein: A prominent blood vessel along the side of the leg

Sensitization: Small (non-immune cell mediated) interaction between allergen/foreign materials and the host

Sericin: The protein of silk from *B. mori* that holds the fibers together

Silk fibroin (SF): The structural protein of silk from *B. mori*

Stenosis: Obstruction of blood flow

Systole: The pressure exerted on the heart during contraction

T-cell mediated hypersensitivity: A reaction to a foreign antigen that has a delayed response, as the T-cells respond over time, producing inflammation after some time has passed

Taylor Cone: A cone observed on the tip of the needle from the electrospinning process when the fiber solution is ejected

Tensile test: A mechanical test that subjects a material to controlled tension until destruction

Tissue scaffold: A structure capable of supporting cell growth and tissue formation

Thromboembolization: Formation of a clot in one blood vessel that breaks loose and obstructs another vessel

Thrombogenicity: The tendency of a material to cause clotting of the blood

Thrombosis: A localized clotting of blood within a blood vessel

Vascular Endothelial Growth Factor: A glycoprotein manufactured by T cells, macrophages, and endothelial cells that helps stimulate angiogenesis by binding to one of two receptor tyrosine kinases

Xenogeneic: Of or relating to molecules derived from a donor that is a different species from the recipient (allogeneic)

Xenografts: A tissue graft derived from a donor that is a different species from the recipient (compare to autologous graft and allograft)