2009-08-28

Direct cell seeding on collagen-coated silicone mandrels to generate cell-derived tissue tubes

Kshama J. Doshi
Worcester Polytechnic Institute

DIRECT CELL SEEDING ON COLLAGEN-COATED SILICONE MANDRELS TO GENERATE CELL-DERIVED TISSUE TUBES

A thesis submitted to the faculty of Worcester Polytechnic Institute in partial fulfillment of the requirements for the Degree of Master of Science

August 2009

Submitted by

Kshama Jitendra Doshi
Department of Biomedical Engineering

_____

Approved by:

_____
Glenn Gaudette, PhD
Assistant Professor
Department of Biomedical Engineering
Committee member

_____
Kristen Billiar, PhD
Associate Professor
Department of Biomedical Engineering
Committee member

_____
Marsha Rolle, PhD
Assistant Professor
Department of Biomedical Engineering
Thesis advisor

Acknowledgements

I would like to express gratitude to my research advisor Dr. Marsha Rolle for her endless support and enthusiasm while walking me through this project. I would also like to thank my committee members Dr. Kristen Billiar and Dr. Glenn Gaudette for all their feedback and their continued guidance in my education.

This project would have been impossible without the help of Tracy Gwyther right from the beginning of the project. Sharon Shaw has also been a great asset for all the help with the histology. I would like to thank Neil Whitehouse and Dr. Elizabeth Ryder whose assistance was vital in the completion of this project. A special thanks to the RET teachers Tom Oliva and Cecilia Gray and to Alex Christakis for building the vessel test device. Thank you to all the members of the BEI CRBE and Gateway Park for making this the most memorable experience of my life. I am also very grateful to all the Rolle lab members including Carlos Donado, Jason Hu, Zoe Reidinger and Stephanie Kaszuba for helping me and keeping a positive work environment.

A special thanks to my beloved Darshan Parekh for his love and support when most needed. And finally, I dedicate this thesis to my family, my parents Jitendra Doshi and Pallavi Doshi and my sister Kshiti for their encouragement and undying support.

Abstract

The large number of patients suffering from cardiovascular diseases has led to a high demand for functional arterial replacements. A variety of approaches to vascular graft tissue engineering have shown promise, including seeding cells onto natural and synthetic scaffolds or by culturing cell sheets which are subsequently rolled into a tube without exogenous scaffolds. The goal of this project is to develop and characterize cell-derived, fully biological small diameter tissue engineered tubes by seeding and culturing cells directly on tubular supports. Rat aortic smooth muscle cells were seeded onto collagen-coated silicone mandrels and cultured for 14 days. Cells proliferated on the mandrels to form tubes (1.19 mm inner diameter, 1.68±0.1 mm outer diameter and 230±63 μm thick; n=72). Histological analysis of the developed tissue tubes demonstrated circumferential alignment of smooth muscle cells, abundant glycosaminoglycan production and some amount of collagen production. On inflating at a constant rate, it was observed that the tissue tubes dilated to an average burst pressure of 256±76 mmHg; (n=11). In order to observe the effects of addition of soluble factors on extracellular matrix synthesis and mechanical properties, tissue tubes were grown in culture medium supplemented with 50 μg/ml sodium ascorbate. A significant decrease in outer diameter and wall thickness in the treated groups (1.57±0.02 mm and 189±10 μm; n=6 respectively) was observed as compared to the untreated control groups (1.66±0.06 mm and 234±32 μm; n=6; $p<0.05$). A slight increase in collagen production was observed by visual assessment of histological images of the ascorbate-treated tissue tubes. This suggests that by using a direct cell seeding approach, it is possible to develop completely biologic small diameter cell-derived tissue tubes that can withstand handling, and it may also possible to modulate matrix synthesis by optimizing cell culture conditions.

Table of Contents

Table of Figures

Table of Tables

Chapter 1: Introduction

The vascular system is the body's network of blood vessels. It includes the arteries, veins and capillaries that carry blood to and from the heart. Problems of the vascular system can be serious, since all tissues require normal flow of blood for adequate supplementation of oxygen and nutrients [1]. Some common problems in the vascular system include blockage of blood flow to the heart or brain due to blood clots, causing ischemia or stroke. Blockages or disruptions in the normal blood flow could result in improper functioning of organs like the heart, brain or kidneys.

Current methods of treatment for such vascular blockages include thrombolysis or stenting. In severe conditions, a bypass surgery needs to be performed in order to return adequate blood flow through the arteries and to minimize the risk of occlusion. In a bypass surgery, the blocked artery is replaced by a healthy vascular graft. The common sources of healthy native grafts are the internal mammary artery, radial artery or the saphenous vein [2, 3]. However, the lack of availability of these autologous grafts in certain patients has driven attention to alternative vascular grafts made from synthetic materials such as Dacron [4, 5] or Polytetrafluroethylene (PTFE) [6, 7]. While these grafts have shown to be well suited in large diameter applications, their patency is generally proved unfavorable as small-caliber (<6 mm) arterial substitutes [4, 5, 7, 8]. Therefore, there is still a need for an alternative vascular graft, which has redirected our attention to tissue engineered blood vessel substitutes for small diameter vessels.

Previously published reports in vascular tissue engineering have focused on four main approaches to create vascular conduits that recapitulate a healthy native blood vessel: (1)

biodegradable synthetic polymers as scaffolds such as polyglycolic acid (PGA) [9-13] (2) decellularized tissues as scaffolds [14-17] (3) cell populated biopolymer gels [18-22] and (4) the cell sheet-based tissue engineering method [23-27].

Tissue engineered blood vessels (TEBVs) developed by seeding cells on biodegradable synthetic polymers have resulted in strong TEBVs, however, postimplantation these TEBVs have often lead to aneurysms, intimal hyperplasia or inflammation due to rapid degradation rates of the polymer and biocompatibility issues [28]. Decellularized tissues have the advantage of biocompatibility for being entirely composed of natural extracellular matrix (ECM) [29], however, it has been shown that decellularization can adversely impact the tissue, resulting in graft failure [30]. TEBVs synthesized from cultured cells seeded in biopolymer gels is another notable approach that has several advantages such as tissue remodeling capability, reduced foreign body reaction and ease of manufacturing [20, 22, 23, 31, 32]. However, despite several attempts so far [15, 19, 33, 34], TEBVs made using this method have not been able to meet the appropriate mechanical properties to serve as vascular conduits for implantation in the arterial system.

More recently, a completely biologic TEBV was fabricated entirely from human cells and the endogenous ECM using a sheet-based tissue engineering method [24]. These vessels exhibited burst pressures exceeding those of human saphenous vein and remained patent as arteriovenous fistulas in dialysis patients for over 21 months [27, 35]. However, the limitations associated with this method include the possibility of cell sheet delamination at high blood flow rates [27], difficulty in scaling up the process at a commercial level, manipulation involved with rolling cell sheets around tubular mandrel and long culture times of up to 3 months [36, 37].

These limitations demonstrate the need to explore alternative solutions to bridge the gap between tissue engineered vascular conduits and native blood vessels.

Thus, the overall goal of this thesis project was to develop a method that generates fully biologic blood vessel grafts using a simple and minimally manipulative direct cell seeding approach. To do so, cells were directly seeded and cultured on a collagen-coated tubular mandrel as shown conceptually in Figure 1. The cells were expected to proliferate, produce endogenous ECM and form a tubular construct around the mandrel. Once a tissue tube was developed, it was harvested from the mandrel and analyzed for structural and biomechanical properties.

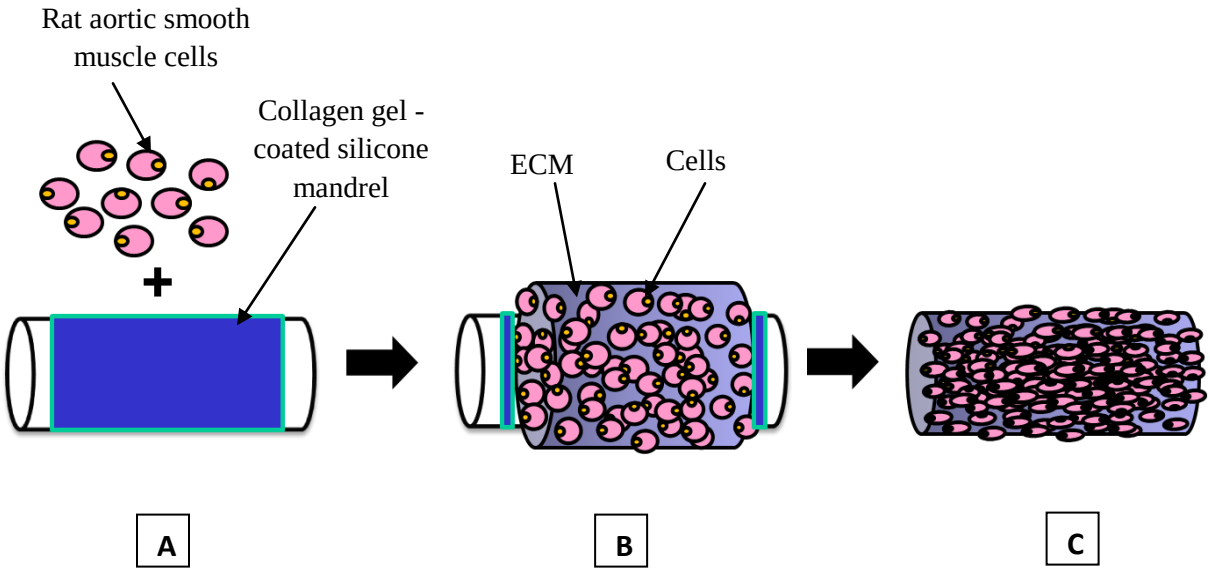

Figure 1: Schematic of direct cell seeding approach. A. Direct cell-seeding of rat aortic smooth muscle cells on a cell-adhesive tubular collagen gel-coated silicone mandrel. B. Cell-derived extracellular matrix (ECM) production during culture period on the tubular mandrel. C. Formation of a cohesive cell-derived tissue tube that can be harvested from the tubular mandrel.

The potential advantages of this method include likelihood of formation of a cohesive tissue tube that is morphologically more similar to native vessels, ease of production and minimal manipulation, as well as the ability to alter mechanical properties by addition of biochemical and biomechanical factors. This would facilitate design and assessment of a better

solution to TEBV production. With the potential advantages of a direct cell seeding approach, this project specifically aimed at achieving the following goals:

Specific Aim 1: To develop a technique to directly seed and culture cells on tubular mandrels to generate cell-derived small diameter tissue tubes

We hypothesize that by directly seeding and culturing cells on tubular mandrels, a cell-derived tissue tube can be generated. To do this, we seeded rat aortic smooth muscle cells on collagen gel-coated silicone mandrels by pipetting a cell suspension into a circular cell seeding assembly and inverting it to allow the cells to contact the coated mandrel. Following seeding, the cells were cultured on these coated mandrels for 14 days after which the developed tissue constructs were harvested. During the culture period, the tissue growth was monitored by serially measuring tissue tube thickness along the length of the tube using a non-invasive digital image acquisition system.

Specific Aim 2: To characterize the structural and biomechanical properties of the cell-derived tissue tubes

After 14 days of culture, the cell-derived tissue tubes were characterized for their structural properties, including wall thickness, uniformity and composition. Paraffin-embedded sections of the tissue tubes were stained with H&E, Movat's Pentachrome and Picrosirius Red/Fast Green to observe structure and ECM production. The burst pressures of the tissue tubes were measured to assess their mechanical properties.

Chapter 2: Background

In this project, an alternative approach for generating fully biologic cell-derived tissue tubes has been studied. This section briefly reviews the current approaches to engineer blood vessels and illustrates the rationale for using the direct cell seeding approach as an alternative.

2.1 Blood vessels: structure and function

The circulatory system is composed of a network of blood vessels including the arteries, veins and capillaries. The main function of a blood vessel is to transfer oxygenated blood from the heart to all of the body tissues and to return deoxygenated blood with high carbon dioxide content from the tissues to the lungs where it gets re-oxygenated. In order to fulfill its role, the vascular system must ensure delivery of blood to all tissues, be flexible and adaptable in order to vary the blood flow according to the metabolic requirements, to convert a pulsating blood flow in the arteries into a steady flow in the capillaries and to return blood to the heart.

The structure of the blood vessels varies in different parts of the body and is directly related to the function of each type of vessel. The three functional layers of a blood vessel as shown in Figure 2 are:

- Tunica intima: The intima is composed of a confluent monolayer of endothelial cells. The function of this layer of cells is to ensure hemocompatibility and anti-thrombogenicity of the vessel.
- Tunica media: The media is composed of smooth muscle cells (SMC) aligned concentrically along with collagen, elastin fibers and proteoglycans. The media presents

the majority of the mechanical and the contractile properties to the blood vessels and is the major component of muscular arteries.

- Tunica adventitia: The adventitia is mainly composed of collagen and fibroblast cells. The function of the adventitia is to provide anchorage with connective tissues and to maintain nutrient supply and vascularization to the internal layers of the vessel.

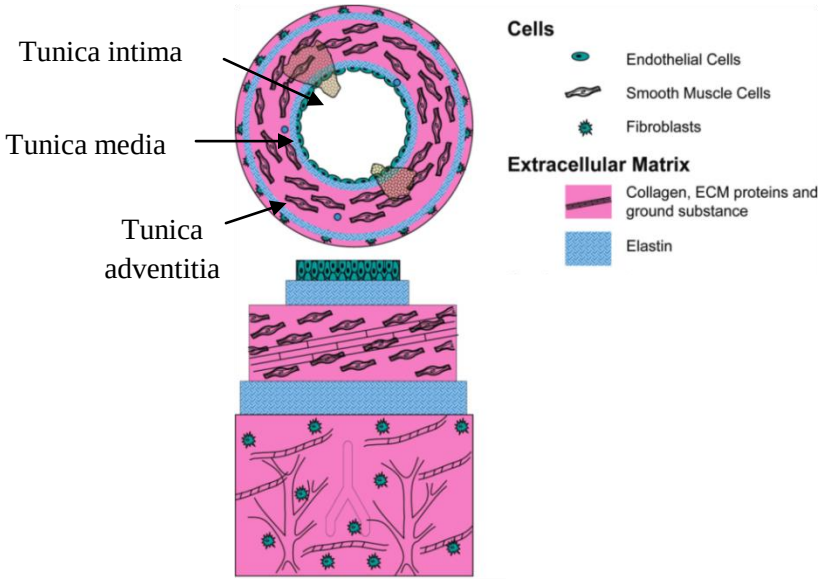

Figure 2: Structure of a blood vessel. Schematic of a blood vessel showing the three functional layers: Tunica intima, Tunica media, Tunica adventitia [38].

2.2 Vascular diseases

Approximately 80,000,000 people suffer from some form of cardiovascular disease in the United States [1]. When not treated, these diseases affect the heart, brain and other crucial organs (e.g the kidneys). Vascular diseases take many forms including coronary or peripheral vascular diseases such as atherosclerosis or aneurysm.

Atherosclerosis is characterized by the accumulation of lipids, cells and ECM in the vessel wall known as plaque [39]. When these plaques grow large enough, they significantly

reduce the blood flow through an artery or rupture to form emboli that break off and travel to other parts of the body. If this happens in a blood vessel that feeds a peripheral organ like the limbs, it might lead to difficulty in walking or gangrene. If a clot occurs in a major organ such as the heart or brain, it leads to heart attack or stroke. Often, cases of atherosclerosis are not detected until complete occlusion resulting heart attack or stroke.

2.3 Clinical need for tissue engineered blood vessels

Current therapies for treating such vascular diseases focus on medication or surgery or remove a plaque and repair the blood vessel wall. However, in many cases, pharmaceutical treatments are not sufficient and more drastic measures like stenting (to expand the narrowed arteries) or bypassing the blocked arteries with healthy native arteries must be taken in order to return adequate blood flow through the arteries. In 2006, more than 448,000 coronary artery bypass surgeries were performed [1]. In bypass surgeries, the patients' own vessel like the internal mammary artery, the radial artery or saphenous vein is used as the conduit [2, 3]. However, in 24% of the cases, autologous vessels were not available as a result of trauma, vessel disease or previous surgery [5]. Also, many patients do not qualify for this dual surgery procedure due to age or other health conditions. Thus, there is a need for an alternative source of reliable vascular grafts that can replace the diseased vessel.

Another major application of these vascular grafts is for creating arteriovenous fistulas for kidney dialysis. The number of patients undergoing dialysis has reached 506,256 as of the end of the calendar year in 2006 [40]. In some cases of kidney dialysis, it is essential to widen the access vein in order to make the needle insertion easier and to enable the blood to flow faster. This widening is done by creating an arteriovenous (AV) fistula between an adjoining vein and

artery in the arm. In some cases, an AV fistula takes as long as 24 months to develop, if the patients have small veins [41]. This presents the need for a vascular access that connects an artery to a vein using a graft that can be used repeatedly for needle placement and blood access during hemodialysis.

2.3.1 Current sources of artificial vascular grafts

To date, synthetic grafts are the only widely clinically available alternatives to autologous vessels. These grafts are made from synthetic materials such as PTFE [6, 7] and Dacron [4]. Replacement of large vessels using this method is feasible, but they are unsuitable for applications in surgeries in which smaller vessels are needed (<6 mm), because the low blood flow velocities in synthetic grafts of this size lead to thrombosis and occlusion. In addition, the materials that are commonly used lack growth potential and long-term results have revealed several material-related failures, such as stenosis, thromboembolization, calcium deposition and infection [4, 5, 8]. This issue of compliance mismatch can also often lead to anastomotic intimal hyperplasia [42, 43].

Thus, the lack of availability of small diameter vascular grafts for artery bypass applications coupled with the need to find a better solution for small diameter graft replacement, has drawn our attention towards the alternative option of engineering artificial blood vessels in vitro using various techniques.

2.4 Criteria for a successful vascular replacement

The “ideal” vascular graft would be characterized by its structural and biological resemblance to healthy native blood vessels, mechanical attributes and long-term postimplantation patency.

In order to mimic the structure of a native artery, the engineered blood vessels need to have a thick, highly developed medial layer composed almost entirely of smooth muscle cells as illustrated in Figure 2. This layer must possess abundant elastic fibers gathered together in sheets arranged in concentric layers. The elastic fibers run in bands around the circumference throughout the thickness of the media in order to counteract the distention of the vessel during systole. Interposed between the elastic layers should be smooth muscle cells and some collagen. The smooth muscle cells are usually arranged circumferentially at right angles to the long axis of the vessel. Therefore, an evaluation of the structural properties of TEBVs, including the measurement of circumferential alignment of smooth muscle cells, assessment of ECM production and measurement of wall thickness and uniformity of the tubular structure are critical to determine its potential as a blood vessel substitute. These structural properties can be evaluated using histological analysis and biochemical assays.

The structural properties of a blood vessel in turn translate into the mechanical strength of the blood vessels which enables them to withstand physiologic pressures. Thus, along with structural resemblance, mechanical strength is also a paramount issue for grafts placed in the arterial circulation. TEBVs must be capable of withstanding these long-term hemodynamic stresses without material failure. One of the measurements for estimating the patency of the developed TEBVs in physiologic environments is burst pressure. A native human artery is able to withstand about 2,031–4,225 mmHg pressure before it bursts [25]. Furthermore, simplicity of handling to minimize operating time and risks as well as expense is also important factors to be considered in order to estimate the mechanical integrity of the developed TEBVs.

Finally, postimplantation, the graft should be resistant to both thrombosis and infection and, should be completely incorporated by the body to yield a neovessel resembling a native

artery in structure and function. Biological alternatives such as the patient's own cells help in eliminating such problems due to immune response.

2.5 Vascular tissue engineering

In order to engineer a vascular graft that imitates the complex structure of a native blood vessel, various approaches have been investigated over the past three decades, including (1) use of synthetic biodegradable polymer scaffolds (2) decellularized tissue scaffolds (3) cell populated biopolymer gels and (4) cell sheet-based tissue engineering. Many of these approaches have resulted in tubular constructs that have met the prerequisites for implantation; however, long term patency has not been achieved in most cases. Although each method has its benefits, there are also problems associated with each approach.

2.5.1 Synthetic biodegradable polymers

Essentially, this approach involves seeding cells on a synthetic degradable scaffold that supports tissue growth and remodeling. Synthetic biodegradable polymers such as poly glycolic acid (PGA), poly lactic co-glycolic acid (PLGA) and poly lactic acid (PLA) are examples of commonly used scaffold materials in this approach of vascular tissue engineering. The advantage of using a synthetic polymer is that its microstructure, mechanical properties, and degradation rates can be controlled simply by altering the chemical composition and manufacturing conditions. The scaffolds provide initial mechanical function for the graft until the cells synthesize sufficient amounts of ECM. Also, the synthetic polymers are expected to be slowly reabsorbed in vivo or in vitro, leaving only the tissue generated by the cells.

In a study by Hoerstrup and colleagues [9] non-woven PGA fibers were seeded with myofibroblasts from ovine carotid arteries. At the end of a 28 day culture period under pulsatile flow, these grafts showed burst strengths of only 326 mmHg and suture retention strength of 64.3

g, indicating that the grafts were not strong enough to withstand physiologic pressures. In another study by Opitz [12] ovine vascular smooth muscle cells (vSMCs) were seeded on poly-4-hydroxybutyrate (P-4-HB) scaffolds and conditioned in a bioreactor for 14 days. On implanting these TEBVs in the descending aortas of juvenile sheep, it was observed that after 24 weeks, the grafts were able to withstand physiologic pressures and remained patent, however, the TEBVs dilated due to degradation of the scaffold material before sufficient amount of new tissue was developed. Also, the histology of the implanted grafts revealed absence of elastic fibers in the ECM layers [44].

2.5.2 Decellularized tissues

In this approach, native blood vessels including canine carotid artery [16], porcine iliac vessels [17], porcine aortas [45] are decellularized using chemical or mechanical treatments, to generate a naturally-derived scaffold consisting of native ECM molecules. These decellularized ECM-based tissue tubes are then used as scaffolds and populated by host cells in vivo post-implantation or seeded and cultured prior to implantation.

In a study by Cho and colleagues, decellularized canine carotid arteries seeded with bone marrow derived cells (BMCs) showed significant amounts of collagen and elastin synthesis and remained patent for up to 8 weeks [16]. However, after 8 weeks, occlusion due to thrombus formation was noted. Also, it is unclear if collagen and elastin production was cell-derived or already present in the decellularized tissue. In another study by Schaner et al., canine jugular veins were decellularized and implanted in vivo in a canine vein graft model [46]. The study revealed burst pressures of 2480±470 mmHg and suture-holding strength of 185±30 g. After 2 weeks of implantation, the remained patent without any observation of graft dilation, rupture or aneurysms. However, with these data it is not possible to predict the long-term durability of these

grafts. Another widely used decellularized tissue for developing vascular grafts is small intestine submucosa (SIS). SIS is a biomaterial composed primarily of acellular collagen, proteoglycans, glycosaminoglycans, glycoproteins and fibronectin [29]. It has been shown to promote tissue remodeling [47]. However, the patency results from the application of SIS in generating microvessels [14] and other larger diameter vessels [48] are inconsistent in terms of the thrombogenic response.

Therefore, decellularized matrices may be more appropriate as scaffolds for vascular grafts than synthetic polymeric matrices in terms of biocompatibility, mechanical strength and compliance, due to the presence of abundant amounts of ECM proteins in the decellularized tissues. However, the issues of availability of healthy native vessels for decellularization, the risk of the chemical treatments adversely impacting the tissue which results in chronic inflammatory response [30] and the long-term durability of these grafts in vivo still remain unsettled in this approach.

2.5.3 Cell populated biopolymer gels as scaffolds

Cell-populated biopolymer gels are generated by suspending cells in a solubilized protein matrix such as collagen or fibrin which can be molded into any desired shape while the scaffold gels are being made. Cells are immobilized in the gel and they rely on receptors for the proteins used in such hydrogels to bind and remodel. Since 1986, when Weinberg and Bell first reported their attempt to develop a completely biological vascular graft made from fibroblasts and SMCs in collagen, several similar attempts have been made to develop TEBVs using this biopolymer gel concept [22].

In 1993, an all human tissue engineered blood vessel was developed using the biopolymer gel concept wherein the medial layer was formed by contraction of a tubular collagen gel with vascular smooth muscle cells around a mandrel. Then an adventitia-like tissue was added around the media by embedding fibroblasts into a collagen gel after which endothelial cells were seeded intraluminally [23]. This vascular model exhibited structural alignment of the smooth muscle cells in the medial layer, however, burst pressures of <120mmHg were obtained. Several others studies attempted to develop TEBVs by varying collagen gel concentration [49] or alignment of collagen fibrils [50] however, the mechanical strength was still compromised in these TEBVs. In 2000, Nerem et al. investigated the effect of dynamic mechanical conditioning to improve mechanical properties of cell populated collagen gels. Cyclic strain was applied to the tubular constructs at a frequency of 1 Hz for 4 and 8 days. Results indicated increased gel contraction, circumferential orientation of SMCs and increased material modulus and ultimate stress. However, parameters of the dynamic culture are still being optimized before in vivo implantation.

Later, in an attempt to address the shortcomings of collagen based TEBVs, Grassl et al. [19] investigated the use of fibrin gel as an alternative to collagen. Here, a suspension of neonatal rat aortic SMCs in fibrinogen was mixed with thrombin solution and injected into a tubular mold and the constructs were cultured with optimized concentrations of transforming growth factor (TGF-ß1) and insulin in the culture medium. The entrapped cells compacted and aligned the fibrin gel scaffold and the synthesized collagen fibrils in a circumferential direction leading to a six-fold increase in collagen production [51].

Thus from literature, it is evident that the advantages of using biological alternatives are (1) ease of manufacturing biopolymer gels, (2) high degree of circumferential fibril and cell

alignment, (3) direct cellularization of the constructs, (4) tissue remodeling and (5) ease of manipulating the mechanical strength of the constructs by biochemical or biomechanical treatments. Hence, although the biopolymer gel based constructs are still limited by their poor mechanical properties and have not been suitable for arterial implantation, the efforts made at improving their mechanical properties are accelerating.

2.5.4 Sheet based tissue engineering

In 1998, L'Heureux et al. presented a radically different approach to TEBV production. This method was based on the use of cultured human cells, without synthetic or exogenous scaffold material. In this study, smooth muscle cells (SMC) and fibroblasts were cultured in culture medium supplemented with 50µg/ml of sodium ascorbate for 30 days to produce cell sheets. These sheets were then wrapped around a cylindrical support to produce a tube composed of concentric sheet layers. After a 1 week maturation period in a bioreactor, the cell layers adhered firmly to one another. Later, a sheet of fibroblasts was rolled around the vascular media to provide an adventitia which was matured for 7 weeks. The inner support mandrel was then removed and luminal endothelial cell seeding was done which was performed and constructs were cultured for another week [24].

This innovative approach took advantage of the presence of ascorbic acid in the culture medium to induce abundant synthesis of ECM by cells. This TEBV displayed histological organization, ECM composition, and physiologically relevant mechanical properties. These constructs demonstrated burst pressures of 3490±892mmHg which was significantly higher than that of human saphenous veins (1599±877 mmHg) [35]. Short term implantation in a canine model demonstrated a 50% patency rate after 1 week implantation. Postimplantation the grafts did not show any signs of rupture, degradation, dilation or thrombus formation [24]. The results

also showed that this graft can be sutured by using conventional surgical techniques and that it does not tear or dilate when exposed to clinical grafting conditions for a week [24].

Moving forward, human TEBVs (1.5mm ID) were developed using the same technique and implanted in nude rats and overall patency was 85% up to 225 days [25]. A stable diameter and no mechanical failures were observed in the Doppler-ultrasound evaluation. Also, following implantation histological assessment revealed that the TEBVs were nonthrombogenic and mechanically stable for 8 months.

More recently, these fully biologic TEBVs were implanted in 10 patients undergoing kidney dialysis [26]. The grafts were implanted as AV shunts and observed for 3 months, after which they were used as a hemodialysis access. These results demonstrated that a completely biological and clinically relevant TEBV can be assembled exclusively from a patient's own cells. The structural and mechanical properties of the TEBVs were analyzed beyond 21 months as an A–V shunt without intervention. Results demonstrated that this approach consistently produced vessels with mechanical properties (burst pressure: 3490±892 mmHg; compliance: 8.8±4.2 %/100mmHg; suture retention strength:152±50 gmf) which is similar or superior to those of native vein [35].

2.5.5 Limitations of the sheet based method

Although the vascular grafts by L'Heureux, have proved to be very successful as compared to any method of developing TEBVs so far, it is too early to conclude that the excellent results seen in the patients who advanced to hemodialysis access will be replicated across a broader range of patients [27].

The other limitations to this approach include the chances of delamination of the cell sheets in high blood flow regions. In the recent study by McAllister et al. [27], the histological sample for one of the patients showed an absence of the inner lining of endothelial cells. It was postulated that, the high postoperative flow had delaminated the innermost sheet resulting in unwinding of the rolled sheet. Another issue associated with this method is scaling up this model to the size needed for vascular reconstructions (i.e., ≈15 cm for coronary bypass and 15 to more than 30 cm for peripheral reconstructions) [24]. This scaling up either involves overlapping cell sheets or using larger culture containers to produce tubes from a single sheet and consecutively a relatively large number of cells from the initial biopsy. This brings up the critical factor of the time needed for TEBV production. With a culture period of at least 3 months to produce one TEBV, this type of engineered autologous tissue is not suitable for emergency surgeries.

2.6 Summary

When all these attempts are reviewed in detail, it is clear that cell-derived materials may offer several advantages over synthetic materials, including mechanical, structural and biochemical properties. This is due to the provision of appropriate signals by the cell-synthesized matrix and the 3-dimensional structure, which enables tissue remodeling and ECM synthesis [47]. However, the ideal blood vessel substitute has not been realized yet. Thus, the aim of this project is to address the need to develop an alternative approach for generating fully biologic blood vessels which require less time and manipulation for fabrication.

We propose to achieve this using a direct cell seeding approach wherein cell-derived tissue tubes can be manufactured by directly seeding and culturing cells on a tubular mandrel, similar to the method used by Neumann et al. for fabrication of perfused microvessels [52]. We hypothesize that by using this method uniform cell attachment and cohesive tissue tube

formation by the cells can be achieved. This will avoid the need for cell sheets and eliminate the likelihood of tissue sheet delamination. Also, by allowing the cells to form a tissue tube, it is expected that a more morphologically similar vascular graft can be developed. Furthermore, it may be possible to translate this method for creating larger or longer conduits by simply varying the mandrel size.

Chapter 3: Materials and Methods

This chapter describes the technique used to develop a completely cell-derived tissue tube using the direct cell seeding approach introduced in Chapter 1. The rationale behind using the direct cell seeding approach is to generate cell-derived tissue tubes using a simple and minimally manipulative process. For this, we considered seeding and culturing cells directly on a tubular mandrel instead of rolling flat sheets of cells. These cells were expected to attach uniformly on the tubular mandrel, proliferate and produce ECM to form a tubular tissue construct.

Briefly, rat aortic smooth muscle cells were seeded on a collagen gel-coated tubular silicone mandrel. After 14 days the developed tissue tube was harvested from the mandrel and its structural and biomechanical properties were characterized. Each of the procedures involved in manufacturing these cell-derived tissue tubes are discussed in detail in the following sections.

3.1 Fabrication of collagen gel-coated silicone mandrels

A custom mold was used to coat the silicone mandrels with collagen gel. The mold was made by using 1.58 mm I.D. Teflon tubing (PTFE tubing, Item# 06605-28, Cole Parmer), a 1.19 mm O.D. silicone tube (Silastic Laboratory tubing, Cat# 508-003, Dow Corning) and a pair of nylon connectors (Cat# LCN-F093-C, Small Parts Inc.) as shown in Figure 3A. Briefly, these molds were assembled by cutting approximately 3 cm long pieces of the Teflon tubing into two halves through the center using a scalpel blade. The two halves were then coupled together using two nylon connectors. Then, a 5 cm long piece of silicone tubing was inserted into the Teflon tubing through the connectors to form the final coating mold shown in Figure 3B.

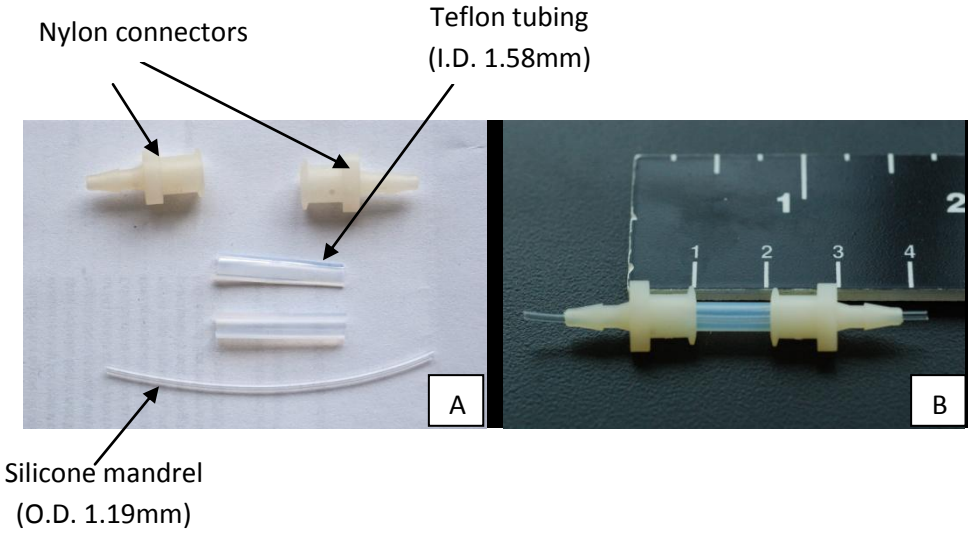

Figure 3: Collagen gel coating mold. A: Arrows indicate the parts of a collagen gel coating mold including the teflon tubing for the outer shell, two nylon connectors to hold the entire assembly and the silicone tubing mandrel for the collagen gel coating. B: Fully assembled coating mold.

A 1mg/ml collagen gel solution was prepared by mixing the 5X DMEM solution with 1 N sodium hydroxide (Cat# 5062-8576, Agilent Technologies), 0.1 N acetic acid (glacial acetic acid, Cat# 42322-5000, Acros) and rat tail collagen gel type I (Cat# 354236, BD Biosciences), on ice. A 5X Dulbecco's Modified Eagles Medium (DMEM) solution was made by mixing DMEM powder, (Conc. 13.49 g/L, Cat# 50-003-PB, CellGro, Mediatech Inc) with sodium bicarbonate (Conc. 3.7 g/L, Cat# S-5761, Sigma Aldrich) in distilled water. The solution was sterile-filtered using a 0.2 µm syringe filter (Acrodisc 13 mm syringe filter, Cat# 2012-03, Pall Corporation) before use.

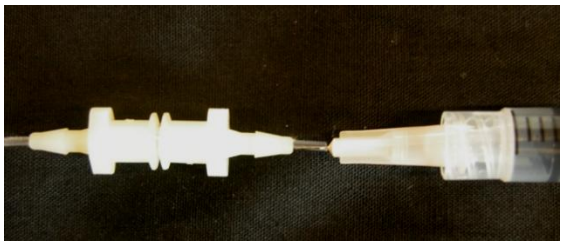

Figure 4: Collagen gel coating procedure. Collagen gel solution is loaded into a 1 ml syringe. The gel solution is injected into the coating mold using a 30 G hypodermic needle through the space between the nylon connector and the silicone mandrel.

Coating was achieved by injecting the prepared collagen gel solution into the custom molds using a 30 gauge hypodermic syringe needle (PrecisionGlide needles, Cat# 305106, BD) as shown in Figure 4. The collagen gel solution was allowed to polymerize for 1 hour at 37°C in an incubator. After 1 hour of polymerization, the gelled collagen-coated silicone mandrels were removed from the molds and dialyzed in sterile distilled water overnight to remove salts from the gel. Following dialysis, the coated silicone mandrels were air-dried overnight to form a dehydrated film of collagen around the silicone mandrel. After dehydrating the collagen gel coating on the silicone mandrel, its presence was confirmed in a subset of samples by staining the coated mandrels with Trypan blue solution (0.4% w/w, Cat# 25-900-CI, CellGro, Mediatech Inc.) for 10 mins. Trypan blue is a vital stain and it binds to connective tissue elements like collagen, reticulin and elastic fibers [53].

3.2 Fabrication of cell seeding assembly

Polydimethylsiloxane (PDMS), a silicon based organic polymer was used to fabricate a silicone support ring for the cell seeding assembly. PDMS was prepared by mixing 1:10 curing agent to elastomer base (184 Sylgard silicone ealstomer base kit, Dow Corning). This solution was then transferred into stainless steel washers (#12 Finishing washers, Item# 3273, Home Depot) using a liquid dropper as seen in Figure 5A. Then, a 1.6 mm O.D. Teflon tube used as a

placeholder (Cat# SLTT-16, Small Parts) was positioned along the diameter of the washer to create grooves for the collagen-coated silicone mandrels to fit (Figure 5B).

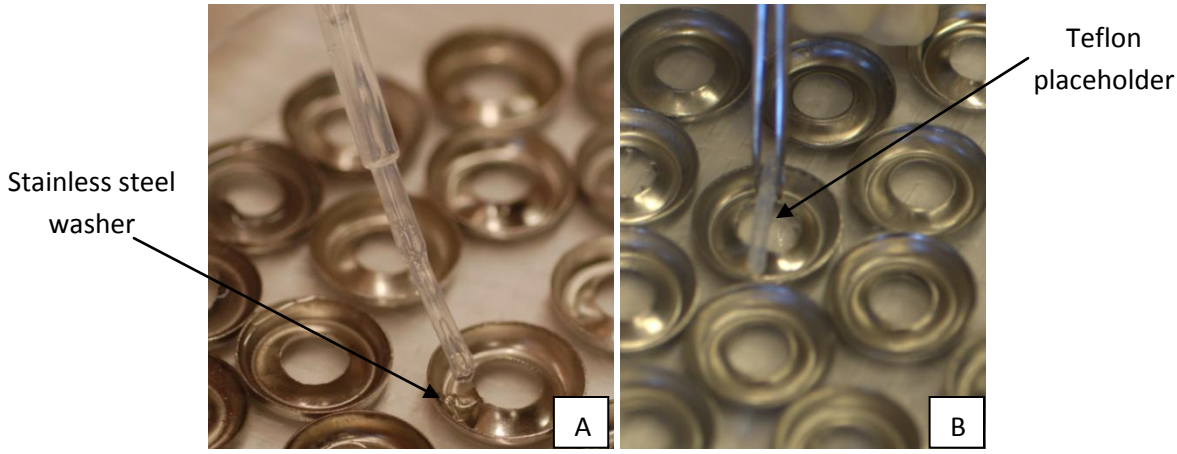

Figure 5: Casting silicone support rings. A: PDMS solution being poured into stainless steel washers (arrows) using a liquid dropper. B: A Teflon tube being positioned along the diameter of the stainless steel washer as a placeholder (arrows) to create grooves on the silicone support ring.

After 1 hour of polymerization at 60°C, the molded silicone support rings and the Teflon placeholders were removed from the stainless steel washers as seen in Figure 6A-B. Individual support rings were then autoclaved for 1 hour at 121°C and 19 psi pressure. After autoclaving, each of the support rings were glued into wells of a standard 6-well plate (Multiwell, 6 well tissue culture dish, Cat# 3026, BD) using a 3 ml syringe filled with sterile silicone glue (Silastic Silicone Medical Adhesive Type A, Dow Corning), as seen in Figure 7.

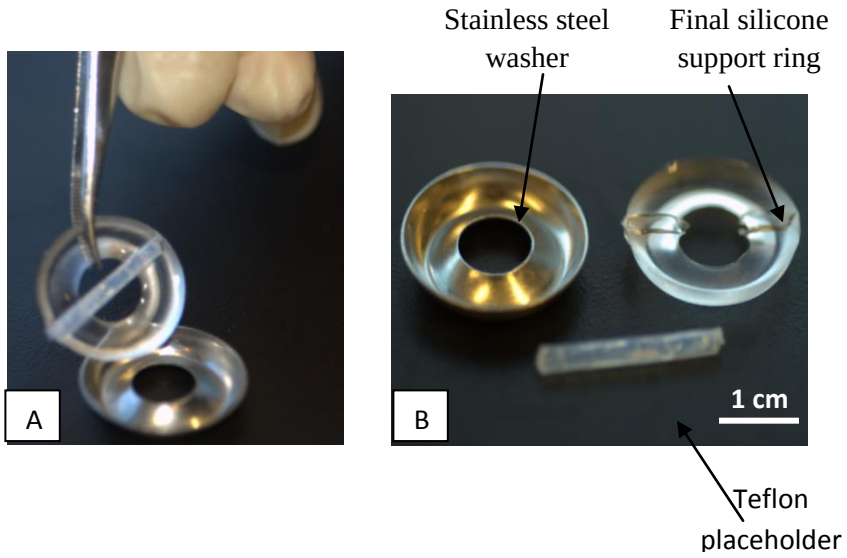

Figure 6: Molded silicone support ring. A: A molded silicone support ring removed from the stainless steel washer using a pair of forceps. B: The arrows indicate the final silicone support ring removed from the steel washer with grooves and the teflon placeholder and stainless steel washer individually.

To prepare for seeding, the collagen-coated silicone mandrels were glued into the grooves created by the Teflon placeholder on the silicone support rings using silicone glue as seen in Figure 8A. The glue was allowed to air dry for 24 hours.

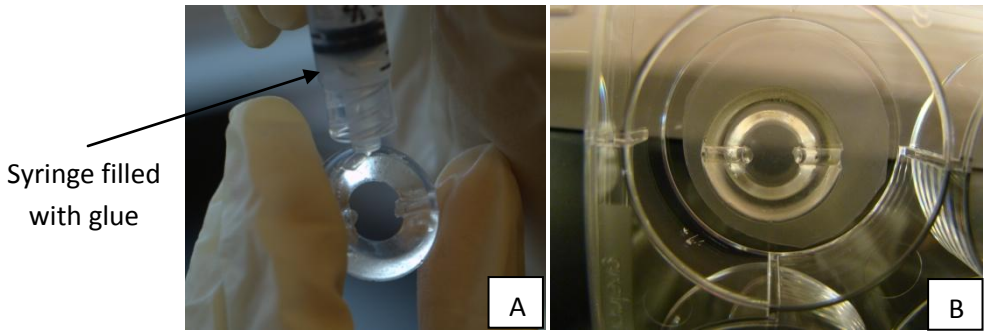

Figure 7: Fabrication of cell-seeding assemblies. A: Silicone adhesive being applied onto the bottom edge of the silicone support ring using a 1 ml syringe. B: A silicone support ring glued into a well of a standard 6- well plate.

This is important since the silicone adhesive releases acetic acid vapors in the initial 24 hours of application, which is toxic to cells (Product data sheet, Silastic Silicone Medical Adhesive

Type A, Dow Corning). Preparation of seeding assemblies was performed in the biosafety cabinet.

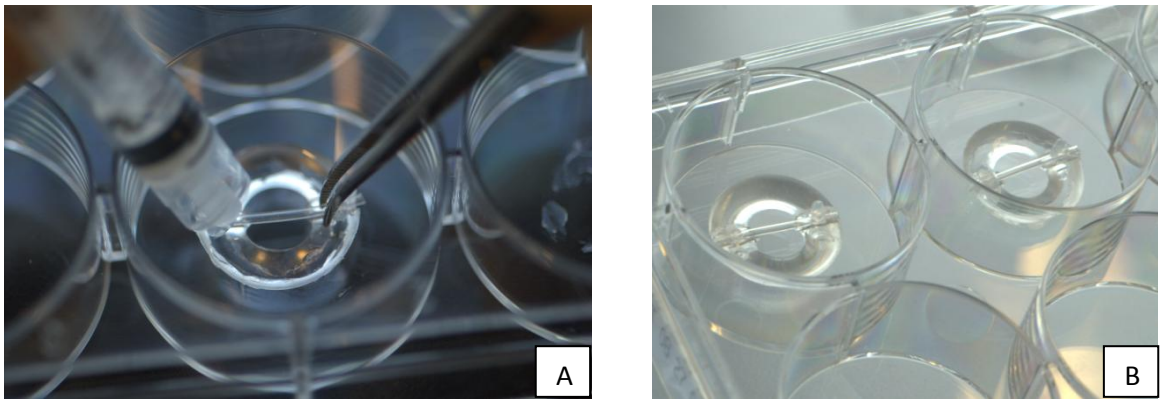

Figure 8: Final cell-seeding assembly. A: A collagen gel-coated silicone mandrel being glued into the grooves of the silicone support ring using a 1ml syringe filled with medical silicone adhesive. B: Final cell seeding assembly with the silicone support ring and the collagen gel coated mandrel ready for cell seeding.

3.3 Cell culture

Adult rat aortic smooth muscle cells (a generous gift of Dr. Thomas Neumann) [52] were plated at a density of 6,500 cells/cm^2 in a 150 mm Petri dish (Cellstar tissue culture dishes, Cat# 639160, Greiner Biosciences) and cultured in growth medium consisting of 1X Dulbecco's Modified Eagle Medium (1X DMEM, Cat# 15-017-CV, CellGro, Mediatech Inc.) supplemented with 10% fetal bovine serum (FBS, Cat# A15-201, PAA Laboratories), 1% non-essential amino acids (NEAA, Cat# 25-025-CI, CellGro, Mediatech Inc.), 1% Glutamax (Cat# 25-015-CI, CellGro, Mediatech Inc.) 1% sodium pyruvate (Cat# 25-000-CI, CellGro, Mediatech Inc.) and 1% penicillin/streptomycin (Cat# 30-002-CI, CellGro, Mediatech Inc.) until they reached 80% confluency. The cells were then trypsinized, counted and resuspended at a concentration of 1 million cells/ml in growth medium.

3.4 Cell seeding and culture on collagen-coated silicone mandrels

After preparing the cell seeding assemblies (Figure 8B), and prior to seeding, the collagen-coated silicone mandrels were rehydrated with 400 µl of standard growth medium for 30 minutes. For cell seeding, 280 µl of the prepared cell suspension (280,000 cells total) was pipetted into the center of each silicone support ring as seen in Figure 9. The culture dishes were then inverted and incubated at 37°C, 5% CO_2 for 30 minutes. The cell suspension forms a droplet and the cells aggregate at the bottom of this droplet which allows them to contact the surface of the coated silicone mandrel. After 30 minutes of incubation in this inverted position, the culture dish was turned upright and the samples were rinsed twice with 1X PBS to remove the unattached cells.

After rinsing with PBS, 6ml of fresh growth media was added to each well and the samples were allowed to culture at 37°C, 5% CO_2. Following seeding, cell attachment on collagen-coated silicone mandrels was verified by taking phase contrast images using a Leica DM IL inverted microscope. The images of the cell-seeded mandrels were compared with the unseeded silicone mandrels immediately after 30 minutes of seeding (See Results Section 4.2). This observation tested the necessity of collagen gel coating in tissue tube formation by direct cell seeding method.

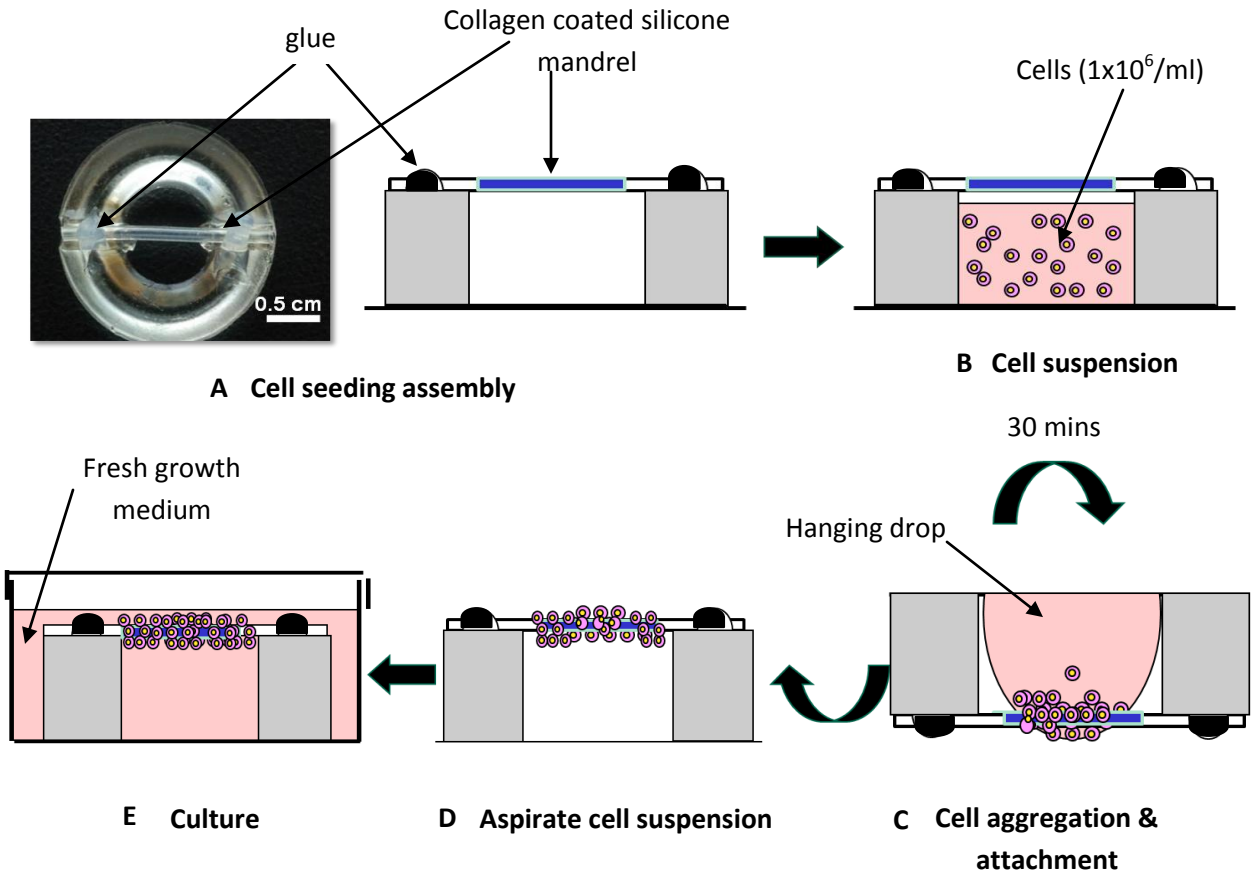

Figure 9: Schematic of the hanging drop cell seeding method. A: Top view photograph and side view schematic of the final cell seeding assembly. B: RASMC cell suspension pipetted into the center of the seeding assembly. C: Seeding assembly flipped upside down for cell-seeding using the hanging drop technique. D: Cell suspension aspirated and samples rinsed in 1X PBS twice. E: Cell-seeded samples cultured in media for 14 days.

3.5 Verification of cell attachment and growth with Hoechst

Cell attachment was monitored over time by staining the cell-seeded samples with Hoechst dye at days 1, 5, 8, 11 and 14.

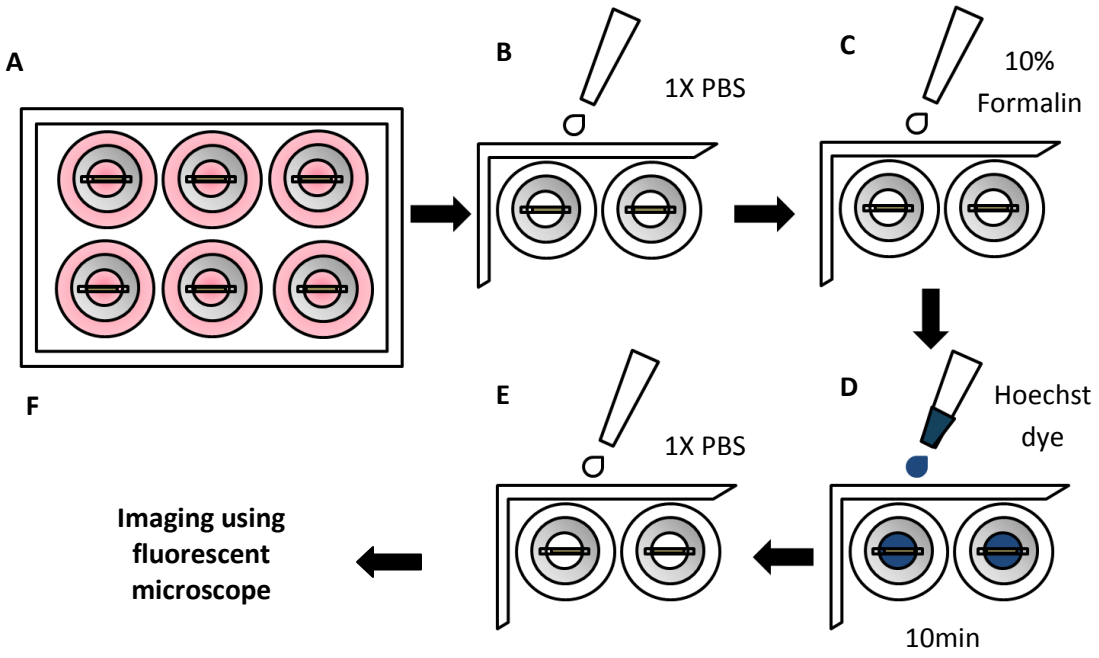

Figure 10: Hoechst staining on cell-seeded samples. A: Media was aspirated from the cell-seeded samples. B: Samples were rinsed with 1X PBS, twice. Figure 10C: Samples were fixed in 10% neutral buffered formalin for 30 mins. D: Hoechst dye was added to the fixed samples for 5 mins. E: Stained samples were rinsed with 1X PBS, twice. F: Samples were observed under a fluorescent microscope and the cell attachment was verified by detecting the presence of blue nuclei.

At each time point, media was aspirated and the samples were washed with 1X PBS twice for 5 minutes. The samples were fixed in 10% neutral buffered formalin for 30 minutes. After fixation, the samples were stained for 10 minutes with Hoechst dye (Hoechst 33342, Cat# H350, Invitrogen, Molecular Probes) diluted 1:300 in distilled water to a final concentration of 1.5µg/ml. Following staining, the samples were once again rinsed in 1X PBS twice and were observed under a Leica DM IL inverted fluorescent microscope. Images were obtained with a 4X objective.

3.6 Observation of vessel growth over time

Growth of cells on the silicone mandrels was monitored by measuring the increase in outer diameter (O.D) of the cell-seeded silicone mandrels over time. This enabled us to calculate the wall thickness as a function of time as:

$$Wall\ thickness = \frac{(Measured\ O.D - Known\ O.D.of\ silicone\ tube)}{2}$$

The outer diameter of the cell-seeded silicone mandrels was measured using the machine vision system as shown in Figure 11. This system consists of a CCD camera (DVT Series 600, Cognex) controlled by image acquisition software (Framework 2.0, Cognex).

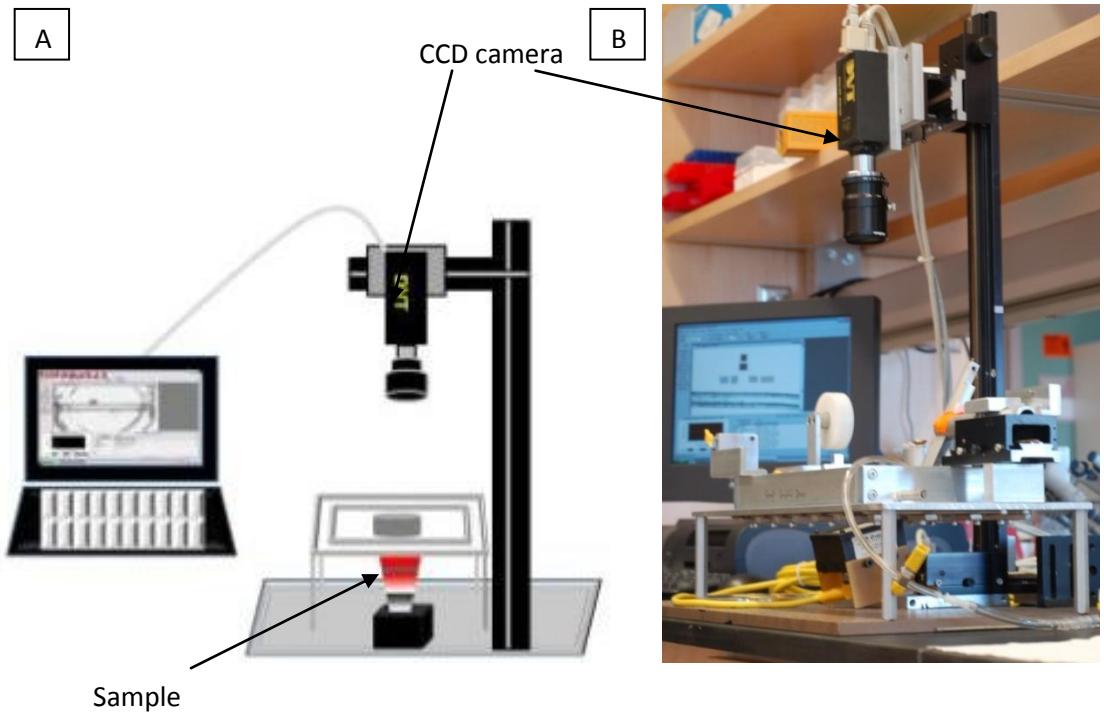

Figure 11: Machine vision system. A: Schematic of the machine vision system. B: Photograph of the actual machine vision system. Arrows indicate the CCD camera (DVT Series 600)

As the images were being acquired, two edge detection sensors were used to locate the outer edges of the silicone mandrel based on pixel intensity differences at an edge. Once the outer edges were located, another image sensor was used to measure the distance between the two edge detection sensors. The distance between the edge detection sensors was in pixels, thus, a static scaling factor was used to convert the pixel value to millimeters. Briefly, this was done by using an uncoated unseeded silicone mandrel (1.19 mm outer diameter), glued onto a silicone support ring in a standard 6-well plate as a reference standard Figure 12.

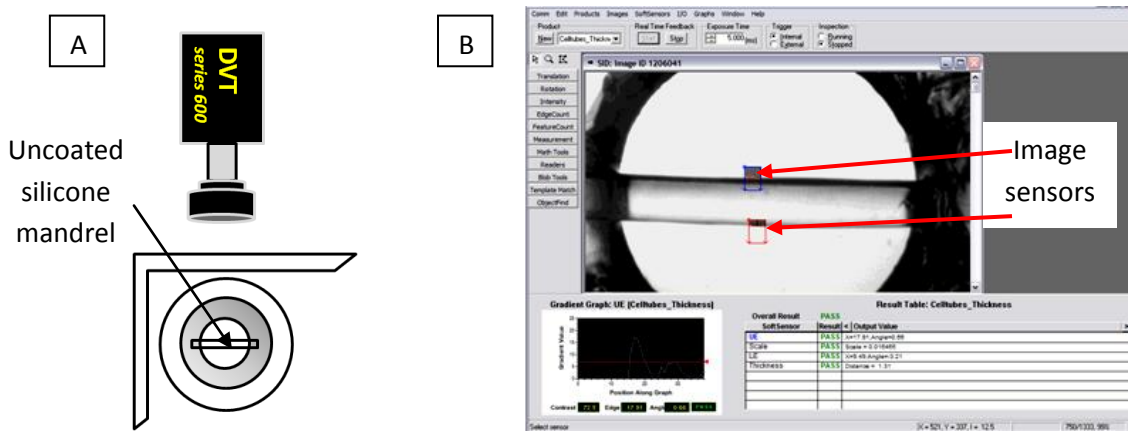

Figure 12: Calibration of the DVT system. A: A schematic of the placement of the unseeded uncoated silicone mandrel under the DVT camera for calibration. B: Screen snapshot of an unseeded silicone mandrel under the camera. Arrows indicate placement of image sensors to detect the edges of the silicone mandrel.

Once the pixel conversion factor was entered, cell-seeded samples were removed from the culture incubator and the media was aspirated from each well. The outer diameter of the cell-tube samples was measured at ten locations along the length of the sample using the image sensors as seen in Figure 13. The outer diameter of each sample was measured at each time point. Prior to taking measurements on the cell-seeded samples, the machine vision system was calibrated each time to the uncoated, unseeded silicone mandrel as mentioned above.

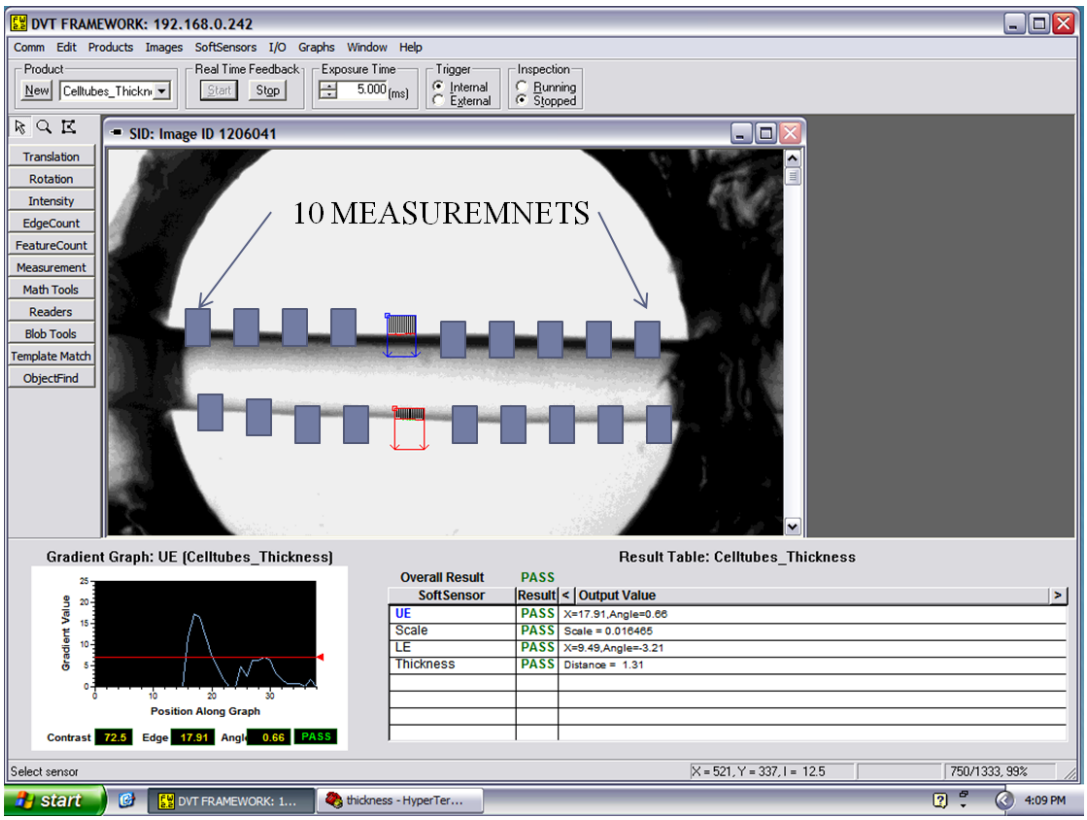

Figure 13: Lengthwise outer diameter measurement. A screen snapshot to indicate the placement of image sensors at 10 different locations along the length of the cell-seeded silicone mandrel.

3.7 Cell-derived tissue tube harvest

After 14 days in culture, engineered tissue tubes were harvested fresh or after fixing in 10% neutral buffered formalin (30 mins) from the mandrel support for purposes of biomechanical testing and histological analysis, respectively. As shown in Figure 14A, the silicone mandrels were first removed from the silicone support ring using a scalpel . Then, both the ends of the silicone mandrel were stretched in opposite directions using a pair of forceps to detach the tissue tube from the mandrel (Figure 14B). The tissue tube was then effortlessly slid off the silicone mandrel for further characterization (Figure 14C).

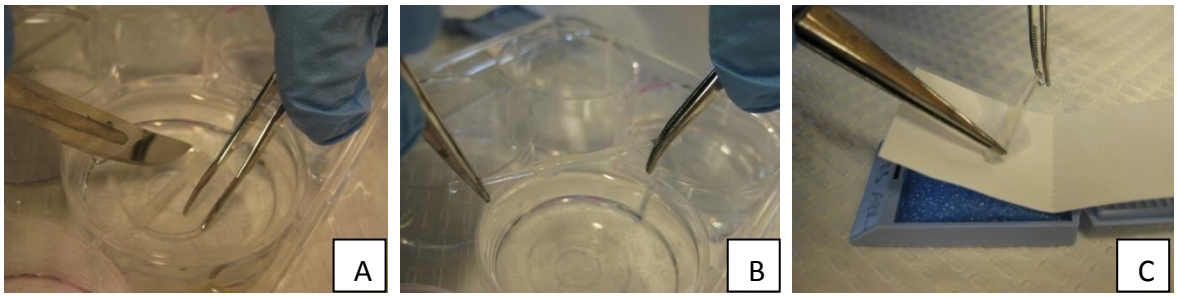

Figure 14: Cell-derived tissue tube harvest. A: A tissue tube sample on the silicone mandrel being cut from the silicone support ring using a scalpel blade. B: Silicone mandrel being pulled in opposite directions using a pair of forceps to release the cell-derived tissue tube. C: Sliding off the cell-derived tissue tube from the silicone mandrel.

3.8 Structural characterization of vascular tissue tubes

For structural characterization, the vascular tissue tubes were fixed in 10% neutral buffered formalin for 30 minutes after 14 days of culture. The fixed samples were removed from the tubular mandrels as described in Section 3.6, cut into two halves at the centre, processed and embedded vertically in paraffin. After embedding, the tissue tubes were sectioned using a microtome (5 µm) and mounted on glass slides (Cat # 48311-703, VWR). The slides were then baked at 60°C for 30 minutes.

3.8.1 Histological analysis

Tissue sections were stained with H&E, Movat's Pentachrome and Picrosirius Red/Fast Green to observe tissue morphology and ECM synthesis. Briefly, slides were deparaffinized in xylene and graded series of ethanols. For Movat's Pentachrome, slides were submerged in Bouin's solution at 80-90°C in a water bath for 10 minutes. Slides were cooled for 10 minutes in room temperature water and rinsed under running water for another 10 minutes. After rinsing, the slides were immersed in 1% Alcian Blue solution for 25 minutes and rinsed in running water for 5 minutes. Following Alcian Blue staining, the slides were dipped in

preheated alkaline alcohol at 60°C for 10 minutes. Following another rinsing step, the slides were immersed in Weigert's solution for 15 minutes and then differentiated in 2% aqueous ferric chloride for 3 minutes. After rinsing in running water, slides were immersed in sodium thiosulfate for 1 minute, then immersed in crocein scarlet/acid fuchsin for 1.5 minutes and rinsed in water for 5 minutes. Slides were then submerged in 5% phosphotungsitic acid for 5 minutes and transferred directly into 1% acetic acid for another 5 minutes. Following another rinsing step, the slides were dehydrated for 1 minute each in 95% EtOH, 100% EtOH and 100% EtOH, followed by staining in alcoholic saffron for 60 minutes. After rinsing in two changes of absolute EtOH for 1 minute each, the slides were cleared in xylene and mounted using aqueous mounting medium (Cytoseal, Cat#81678, Richard-Allan Scientific).

In order to gauge collagen production, the tissue sections were also stained with Picrosirius Red/Fast Green. Briefly, the slides were deparaffinized and rehydrated in xylene and graded series of ethanols. The slides were then stained in a Fast green/Picrosirius Red solution for 30 mins. After staining, the slides were dehydrated in a graded series of ethanol, cleared in xylene and mounted using aqueous mounting medium (Cytoseal, Cat#81678, Richard-Allan Scientific).

3.8.2 Wall thickness measurements

In order to measure the wall thickness of the cell-derived tissue tubes, 10 images of H&E stained cross sections were taken at 10 different locations circumferentially. Each image was taken at 20X magnification using the Leica DM LB2 light microscope. These images were then transferred into Image J for analysis. Using a set scale, line measurements were taken for wall thickness on each of the ten images per sample (Figure 15). Engineered tissue tubes were

compared to a native rat carotid artery with respect to wall thickness measurements and inner diameter.

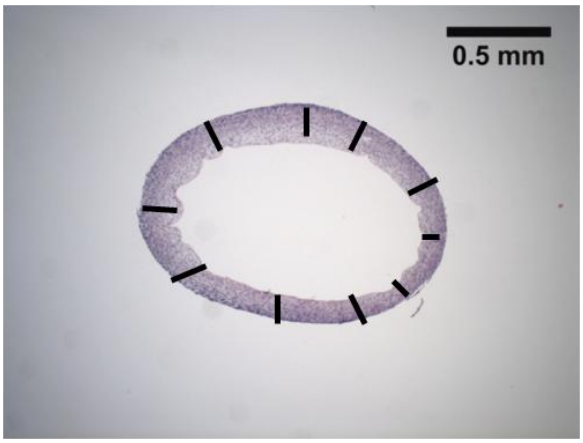

Figure 15: Wall thickness measurements. H&E stained image of a cross section of a cell-derived tissue tube after 14 days in culture at 5X magnification. Lines indicate the approximate locations where the wall thickness measurements were taken using ImageJ.

3.9 Biomechanical characterization of vascular tissue tubes

Engineered tissue tubes were tested for burst pressure with a custom-made vessel test device shown in Figure 16. The device consists of three main modules, the graft mounting assembly, a programmable syringe pump and a pressure transducer assembly connected to a panel meter for digital readout.

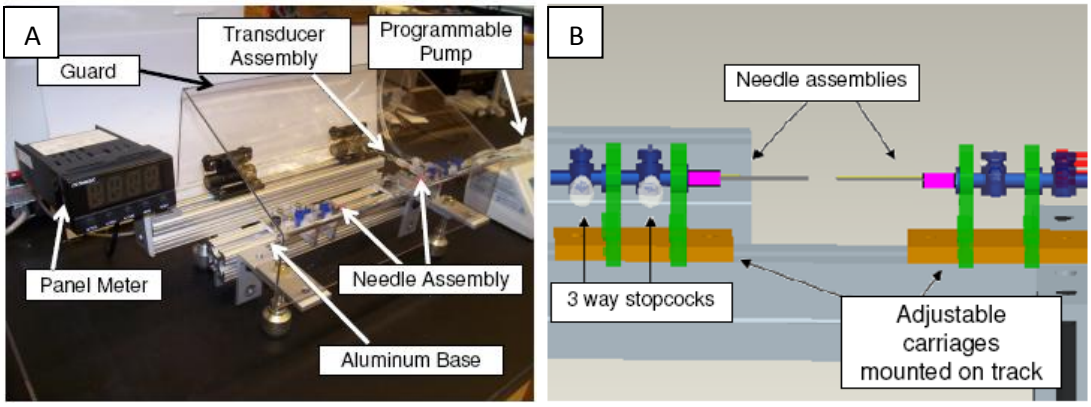

Figure 16: Vessel test device and graft mounting assembly. A: Photograph of the vessel test device. Arrows indicate the parts of the system: Needle assembly, programmable syringe pump, transducer assembly, panel meter, aluminum base and guard B: Schematic of the needle assembly on which the cell-derived tissue tubes are mounted for burst pressure testing.

Briefly, samples cultured for 14 days were harvested from the silicone mandrels and rinsed with 1X PBS. The samples were then mounted on two 22 gauge blunt needles (Cat# NE-21PL-C, Small Parts) on the custom burst test device. The samples were secured on the needles with a 4-0 silk suture (Cat# S-A53, Lukens Medical Corporation) using simple surgical knots as seen in Figure 17.

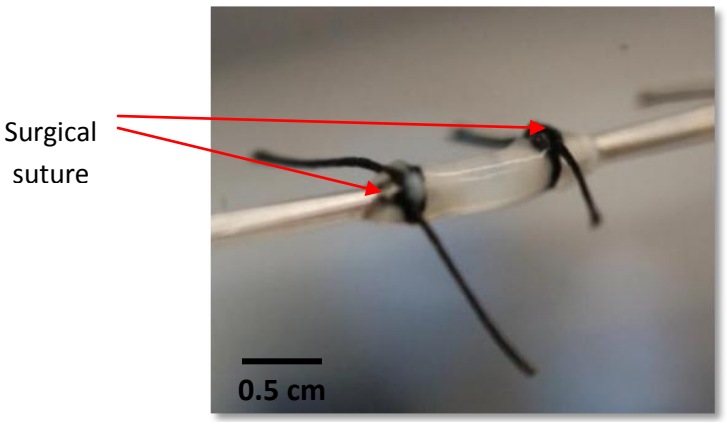

Figure 17: Sample mounted on vessel test device. Cell-derived tissue tube mounted on the 22G blunt needle assembly of the vessel test device using simple surgical suture knots (arrows)

The apparatus was then connected to a programmable syringe pump (SEP-10 Plus, AITECS) that infused 1X PBS into the tissue constructs at constant rate of 0.5 ml/min until failure. A pressure transducer (PX26-100, Omega) connected to a digital panel meter (PX300-15GV, Omega) was used to determine the peak pressure.

3.10 Statistical analysis

3.10.1 Evaluation of tissue growth over time

In order to determine if there was a significant increase in tissue tube outer diameter as a function of time in a particular batch, a one way Analysis of Variance (ANOVA) was performed with culture period as the factor of interest using SigmaPlot 11.0 software . A post hoc analysis was done using the Holm-Sidak method of comparison to determine exactly which time point was statistically different from the others ($p<0.05$).

3.10.2 Evaluation of lengthwise uniformity of cell-derived tissue tubes

In order to evaluate the lengthwise uniformity of the developed tissue tubes, O.D. measurements were taken at 10 locations along the length using the machine vision system (See Section 3.6). These measurements were grouped as per the measurement position (1 through 10) for all the samples for the batch of interest. A one way ANOVA was performed with measurement position as the factor. Pairwise comparisons were made using the Holm-Sidak method to isolate the measurement position significantly different from the rest.

Using the O.D. measurements a uniformity index was also calculated to quantify the lengthwise uniformity of the thickness of the developed tissue tubes in each batch. The uniformity index was calculated using the percent co-efficient of variance at each measurement position as:

$$Uniformity\ index = \left(1 - \left(\frac{Standard\ error\ of\ mean\ \sigma}{Mean\ \mu}\right)\right) * 100$$

3.10.3 Evaluation of batch to batch variation from O.D. measurements

In order to estimate the efficiency of the direct cell seeding technique, in terms of batch to batch reproducibility, we performed a Kruskal-Wallis one way ANOVA on ranks on the average values of the ten outer diameter measurements of each sample in all the batches. Post hoc analysis was done using the Dunn's method to isolate the group or groups that differ from the others using pairwise multiple comparisons. To quantify the maximum difference in the mean O.D. of all the samples across all the batches, a percent difference between the batches was determined using the average minimum (min O.D.) and maximum (max O.D.) outer diameter measurement as:

$$\%\ Difference = \frac{(\max O.D. - \min O.D.)}{\min O.D}$$

In order to evaluate the batch to batch reproducibility in terms of lengthwise uniformity of the developed tissue tubes along the length of the silicone mandrels, the 14 days O.D. measurements of the tissue tubes were taken at 10 locations along the length using the machine vision system. These measurements were grouped as per the measurement position (1 through 10) for all the samples across all the batches. A two way ANOVA was performed with batch and measurement position as the two factors for the O.D. measurements grouped by measurement position. Again, to quantify the uniformity of the developed tissue tubes across all the batches, uniformity index was calculated as illustrated in Section 3.10.2.

3.10.4 Evaluation of batch to batch variation from histological wall thickness measurements

In order to estimate the consistency in wall thickness in terms of batch to batch reproducibility, we performed a Kruskal-Wallis one way ANOVA on ranks on all the samples in all the batches. Post hoc analysis was done using the Dunn's method to isolate the group or groups that differ from the others using pairwise multiple comparisons. The difference in the mean histological wall thicknesses of all the samples across all the batches was calculated as a percent difference between the batches was determined using the average minimum (min W.T.) and maximum (max W.T.) wall thickness measurement as:

$$\%\ Difference = \frac{(\max W.T. - \min W.T.)}{\min W.T.}$$

3.10.5 Evaluation of circumferential uniformity using histological wall thickness measuremets

In order to evaluate the feasibility of uniform tissue growth around the circumference of the silicone mandrel, wall thickness measurements were taken at 10 locations along the circumference of a histological section of all the paraffin-embedded tissue samples at the end of the 14 day culture period as described in Section 3.7.2. The 10 wall thickness measurements for each of the samples were arranged in an ascending order. The minimum and the maximum wall thickness measurements were then grouped as thinnest and thickest measurements, respectively. A paired t-test was performed on this data to determine if there was a significant difference between the thickest and thinnest measurements.

Chapter 4: Results

This section describes the results of direct cell-seeding on collagen coated silicone mandrels, quantification of amount of tissue growth as a function of time, structural properties of the developed tissue tubes and the mechanical integrity of the constructs.

4.1 Seeding mandrel

In our first attempt to generate fully biologic TEBVs, adult rat aortic smooth muscle cells were seeded onto plain silicone tubing. The cell seeded tubes were monitored under a Leica DM IL microscope for a period of 7 days. It was observed that there was no cell attachment on the plain silicone tube as seen in Figure 18. This indicated that silicone alone did not serve as a good substrate for seeding cells to generate tissue tubes and that the surface properties of the silicone mandrel need to be modified to facilitate cell attachment.

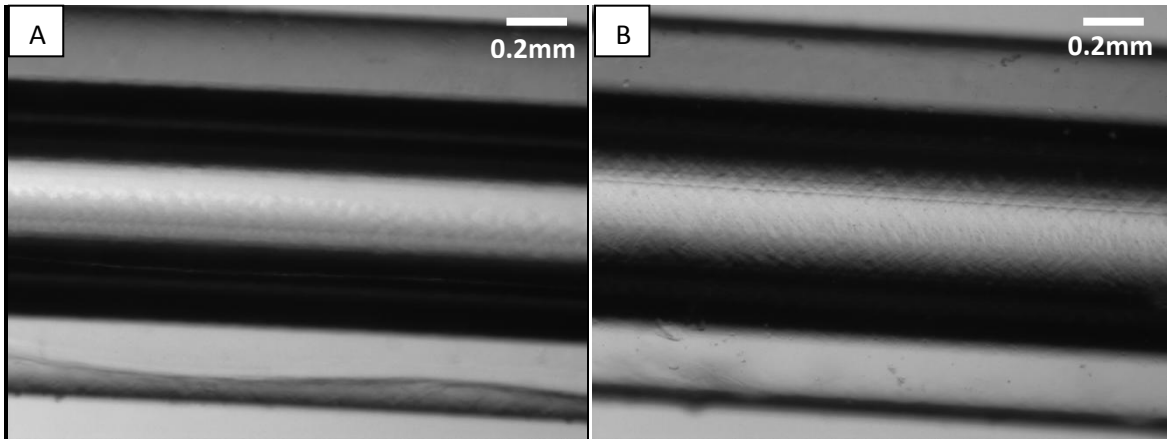

Figure 18: Cell attachment on plain silicone mandrels. A: Image of a cell-seeded plain silicone mandrel after 30 mins of cell seeding. B: Image of an unseeded silicone mandrel.

4.2 Fabrication of collagen gel-coated silicone mandrels

Following one hour of incubation, the collagen solution polymerized to form collagen gels in the shape of the PTFE outer shell around the silicone mandrel. The coated silicone mandrels were then gently removed from the molds with the gelled coating intact on the mandrel surface. Figure 19A shows the collagen gel coating on the mandrel resting on a PDMS washer immediately after removing the coating mold and dialyzing in distilled water. Following dialysis, the coated mandrels (Figure 19B) were glued on silicone support rings and air-dried overnight to form seeding assemblies (Figure 19C).

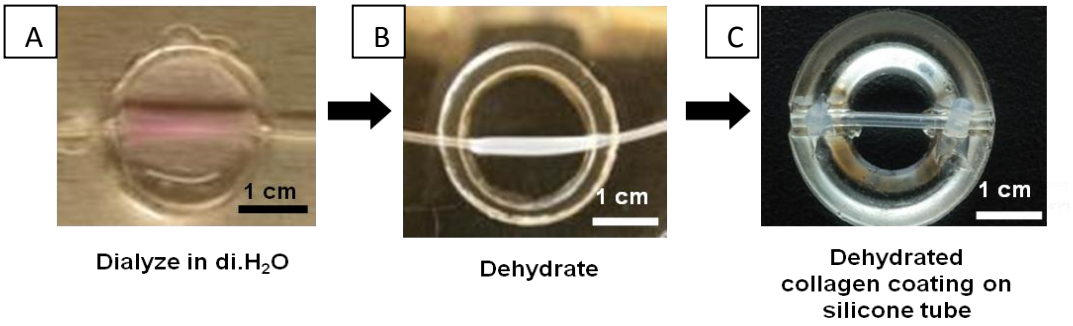

Figure 19: Collagen gel coating on silicone mandrel. A: Collagen-gel coating on the silicone mandrel after 1 hr of polymerization. B: Collagen coated silicone mandrel after 24 hrs of dialysis. C: dehydrated collagen-coated silicone mandrel glued into the cell seeding assembly.

On staining the dehydrated layer of collagen coating with Trypan blue dye a blue film is seen in the coated region of the silicone mandrel (Figure 20). Also, the uncoated regions of the silicone mandrel are not stained blue, indicating that the Trypan blue dye binds only to collagen and not plain silicone.

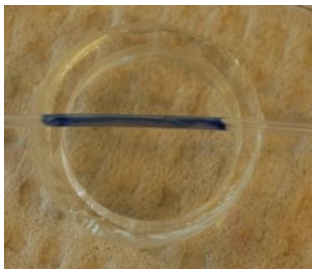

Figure 20: Trypan blue staining

4.3 Cell seeding on coated silicone mandrels

In order to confirm the feasibility of direct cell seeding on collagen coated tubular mandrels, following the 30 minute hanging drop, phase contrast images of these samples were taken to observe cell attachment. As seen in the image on the right in Figure 21B, cell attachment is clearly seen on the cell-seeded collagen-coated silicone mandrel while there are no cells on the unseeded silicone mandrel at Day 0 (Figure 21A).

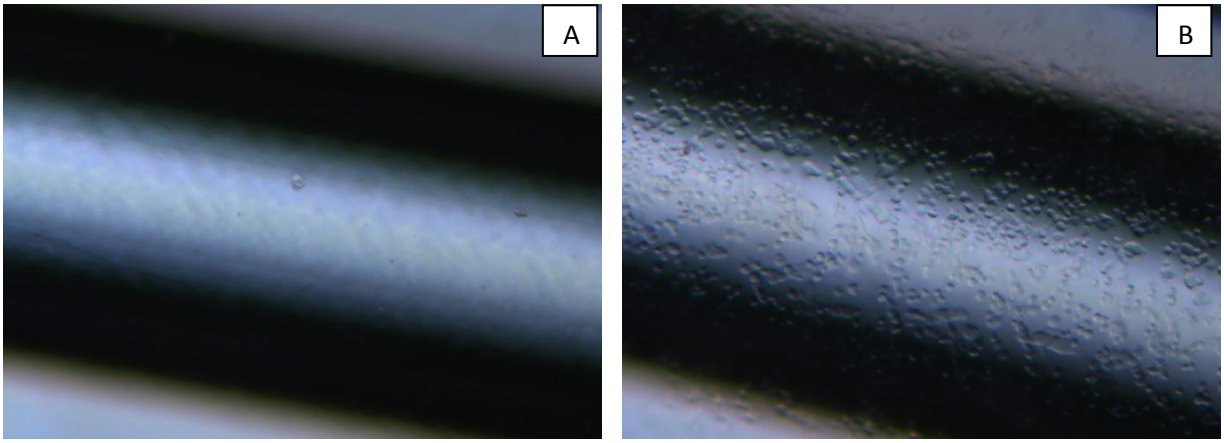

Figure 21: Cell attachment on collagen gel-coated silicone mandrels. A: Image of an unseeded silicone mandrel. B: Imgae of a collagen gel-coated cell-seeded silicone mandrel after 30 mins of seeding.

4.3.1 Verification of cell attachment and growth with Hoechst

Hoechst is a cell permeable nucleic acid stain that fluoresces blue when excited with xenon, mercury-arc lamp or with a UV laser. Hoechst is most widely used to confirm cell seeding by visualizing cell nuclei. Figure 22 shows the phase contrast and Hoechst-stained images of a sample after 1 day of cell seeding compared to an unseeded mandrel at the same timepoint. The cells appear to attach and cover the silicone mandrel after 1 day, and then between days 5 and 14, cells appear to start forming multiple layers on top of each other. The amount of cell growth on the silicone mandrels was quantified using the machine vision system as a function of time.

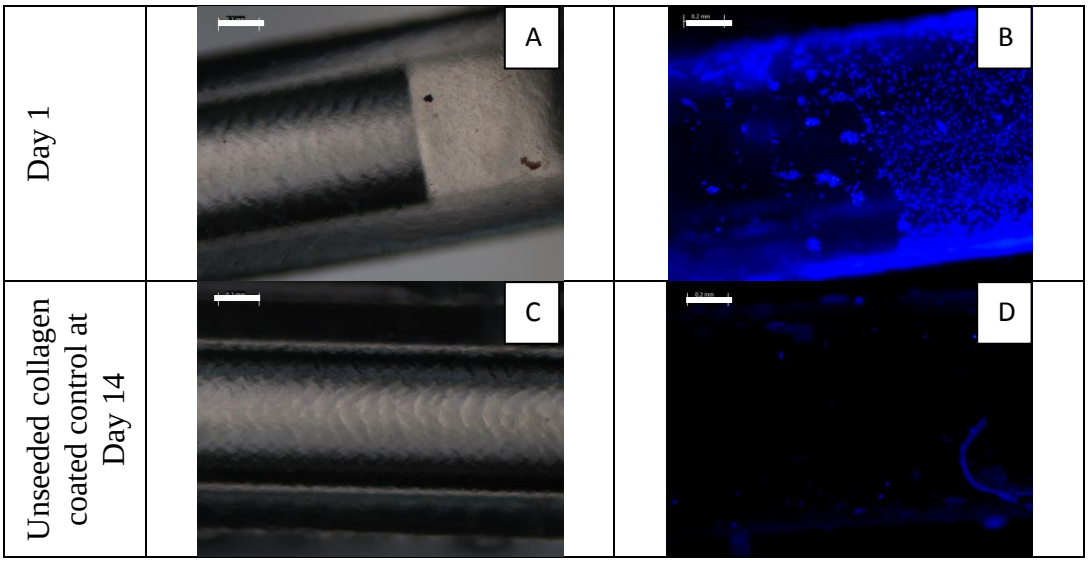

Figure 22: Confirmation of cell attachment. A-B. Phase and Hoechst images of a cell-seeded sample at day 1 of culture period. C-D: Phase and Hoechst images of an unseeded silicone mandrel at day 1 of culture period. Scale bar= 0.2 mm

4.4 Observation of vessel growth over time

Growth of cells on the silicone mandrels was monitored by measuring the increase in O.D. of the cell-seeded silicone mandrels over time using the machine vision system. Images of

cell seeded silicone mandrels at day 5 and day 14 of the culture period are shown in Figure 23. These images show a clear increase in the outer diameter of the silicone mandrel.

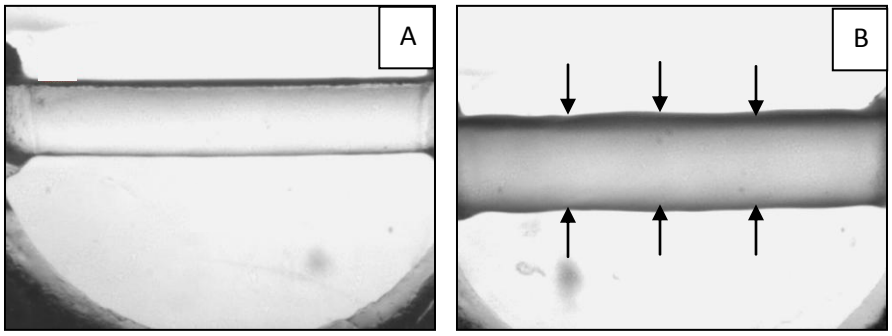

Figure 23: Cell-seeded sample under the machine vision system. A: Cell seeded sample at Day 4. B: Cell seeded sample at day 14. Arrows indicate outer edges of the tissue tube growing on the silicone mandrels.

4.4.1 Evaluation of tissue growth over time

Figure 24 shows a graph of tissue growth over time for one of the representative batches. For this batch, O.D. measurements were taken at 2, 5, 9, 11 and 14 days of culture. An average O.D. of 1.74±0.1 mm (mean±SEM; n=3) was obtained at 14 days. Based on this data, the average wall thickness of the tissue tubes in this batch was calculated as 276±50 μm (mean±SEM; n=3). On performing one way ANOVA and post hoc analysis using the Holm-Sidak method on the average O.D measurements of all the samples at each time point, it was observed that there was a significant increase in O.D. from day 5 through 14 and from day 9 through 14. There was no significant increase between days 2 and 5 and also between days 11 and 14.

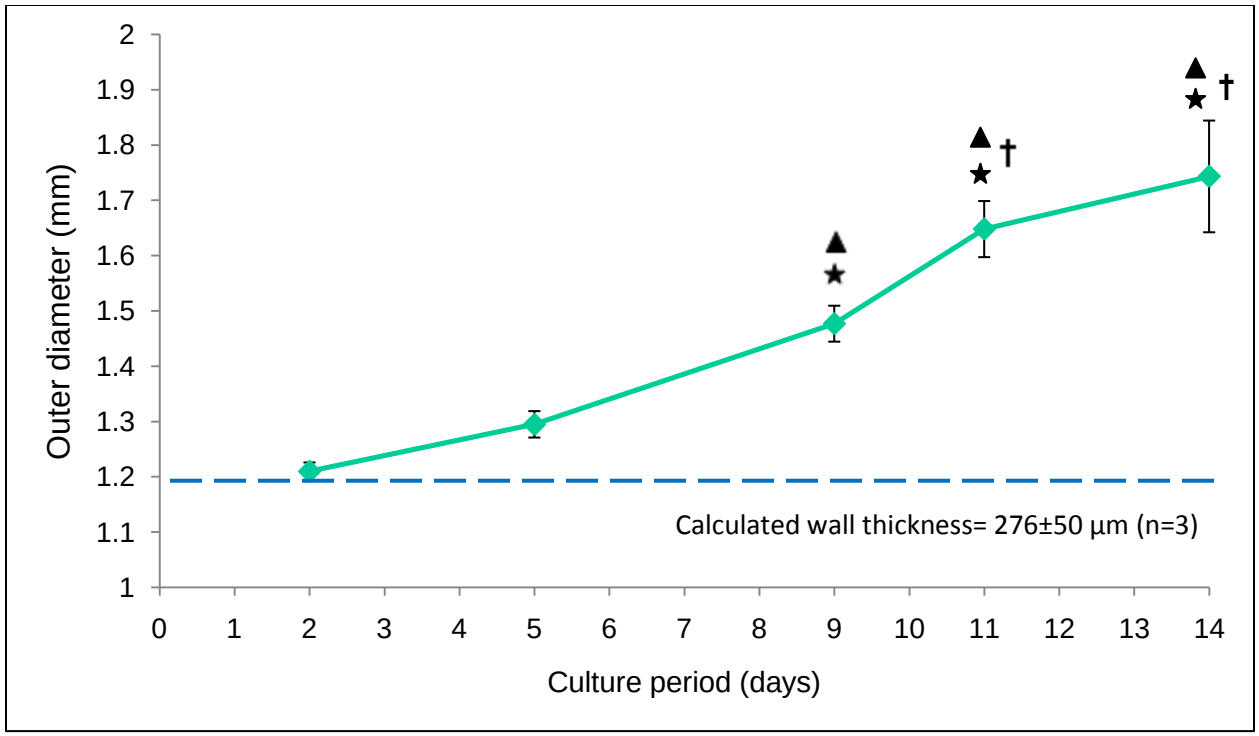

Figure 24: Vessel growth over time. A graph of outer diameter measurements from one representative batch over time. Dotted blue line indicates outer diameter of the unseeded silicone mandrel. O.D. measurements taken at 2, 5, 9, 11 and 14 days. ★p<0.05 from Day 2. ▲p<0.05 from Day 5. † p<0.05 from Day 9. n=3

4.4.2 Evaluation of lengthwise uniformity

In order to estimate lengthwise uniformity of all the samples in this representative batch average O.D. measurements at each measurement position for all the samples were plotted (Figure 25). On performing one way ANOVA and a Holm-Sidak post hoc analysis with measurement position as the factor of interest, it was observed that there was no significant difference between any of the O.D. measurements at each measurement location. This indicated that all the samples in a particular batch were uniform along the length.

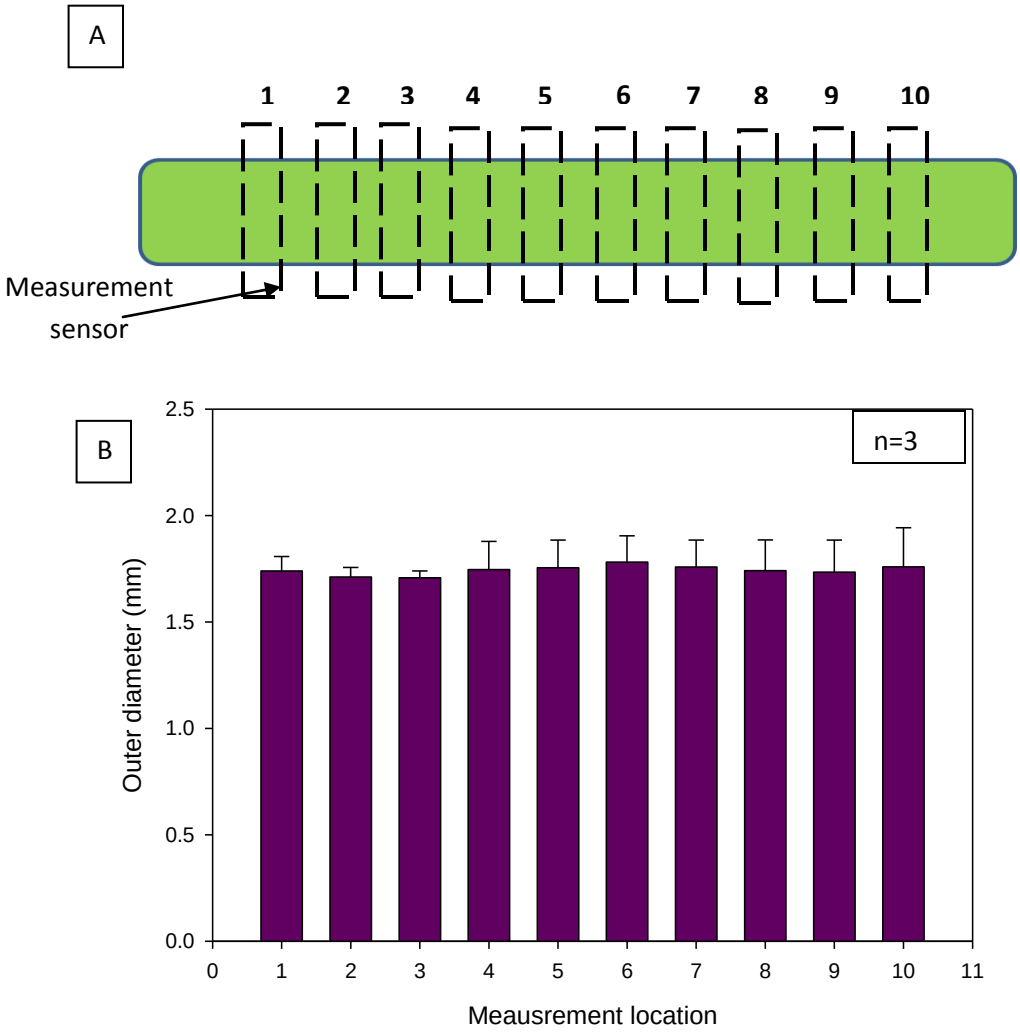

Figure 25: Lengthwise uniformity. A: A schematic of the cell-derived tissue tube and the locations at which O.D. measurements are taken. Dotted rectangles indicate measurement sensor location. B: A graph of mean O.D. measurements of all the samples in one representative batch with respect to the measurement location. n=3

In order to quantify this lengthwise uniformity, the co-efficient of variation was calculated, and used to calculate the index of uniformity (see Section 3.10.2). For this batch, the lengthwise uniformity index ranged from 89.5-98.1%.

4.5 Cell-derived tissue tube harvest

At the end of the 14 day culture period all cell-derived tissue tube samples were strong enough to be removed from the silicone mandrel without any difficulty. Some samples were

fixed and processed for histological assessment or harvested fresh for burst pressure testing. In Figure 26, an open lumen of a tissue tube fixed in 10% neutral buffered formalin can be seen clearly.

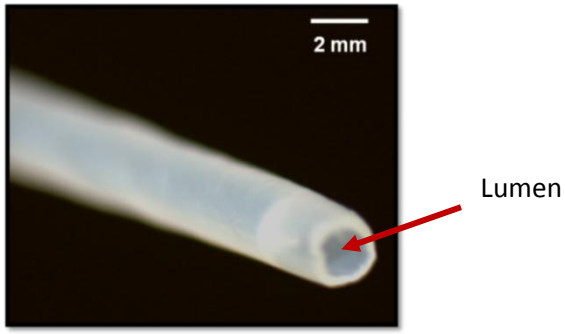

Figure 26: Harvested cell-derived tissue tube. Image of a cell-derived tissue tube fixed in 10% neutral buffered formalin and harvested from the silicone mandrel at 14 days. Arrow indicates open lumen.

4.6 Structural characterization of vascular tissue tubes

Characterization of structural properties includes an evaluation of cell and tissue morphology, ECM synthesis and wall thickness.

4.6.1 Histological analysis

The H&E, Movat's Pentachrome and Picrosirius Red/Fast Green stained images of the tissue tube sections are shown in Figure 27. As seen in the H&E stained images (Figure 27A-B), the cellular alignment appeared to assume circumferential orientation along the outer edge of the tubes based on visualization, however this was not evaluated quantitatively. The Movat's Pentachrome (Figure 27C-D) stains nuclei and elastic fibers black, collagen yellow, and glycosaminoglycans (GAGs) blue and allows visualization of the composition and organization of ECM in the tissue sample. From the Movat's Pentachrome image, it is evident that the cells produced abundant amounts of GAGs indicated by the blue color (Alcian Blue staining). Also,

the distinct yellow edge of the collagen gel coating on the side of the lumen of the tissue tube indicated that the collagen coating still remained after the 14 day culture period. The presence of the coating layer was confirmed by the Picrosirius Red/Fast Green staining (Figure 27E-F), where collagen stains red. Picrosirius red staining also indicated cell-derived collagen production in the tissue tube at the 14 day culture time point.

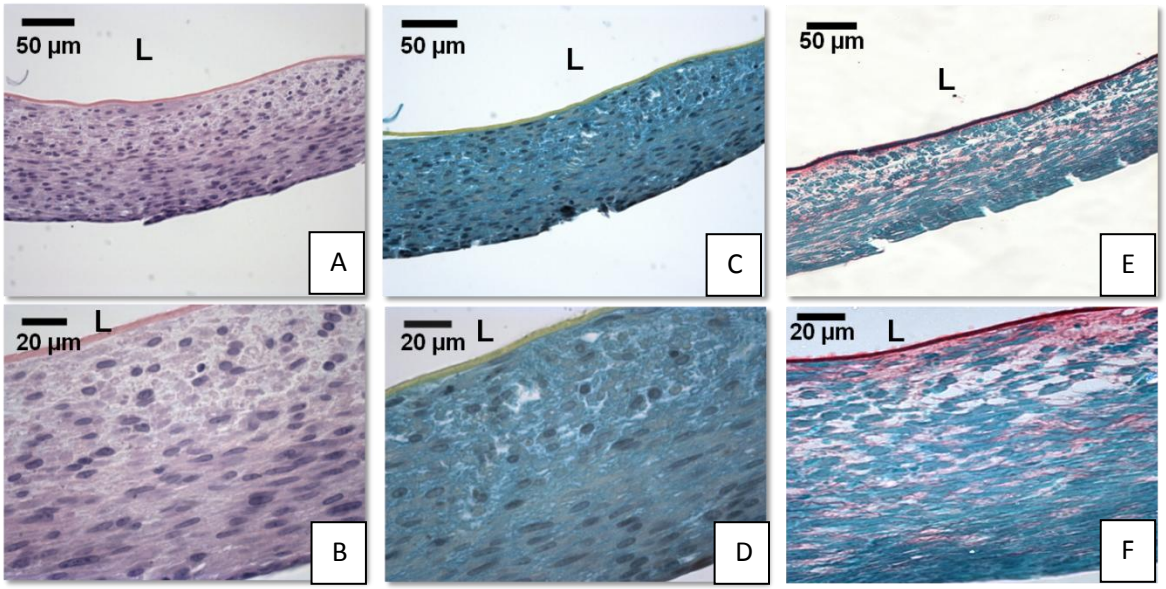

Figure 27: Histological images of the cell-derived tissue tube sections. A-B: H&E stained images of cell-derived tissue tube sections.Purple=nuclei, Pink=cytoplasm. C-D: Movat's Pentachrome stained images of tissue section. Blue=glycosaminoglycans, Yellow=collagen. E-F: Picrosirius Red/Fast Green. Red=collagen, Green= tissue counterstain. L indicates lumen.

4.6.2 Wall thickness measurements

The image of a cross section of the cell-derived tissue tube is a representative image of all the samples across all the batches (Figure 28A). The measured average wall thickness of the cell-derived tissue tubes from the histological assessment using ImageJ at the end of 14 days for this

batch was 160±35 μm (n=3). For comparison, wall thickness measurements were also made on a rat carotid artery, as presented in Table 1.

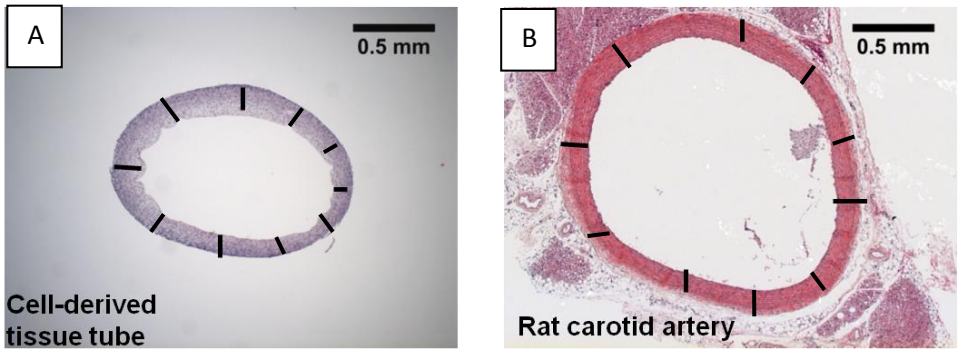

Figure 28: Wall thickness measurements. A: H&E stained cross section of a cell-derived tissue tube. B: H&E stained cross section of a rat carotid artery for comparison of wall thickness measurement. Lines indicate the position at which wall thickness measurements were taken.

Table 1: Comparison of wall thickness of cell-derived tissue tubes and rat carotid artery

	Measured wall thickness (μm)
Cell-derived tissue tube	160±35 (n=3)
Rat carotid artery	137±15 (n=3)

4.7 Biomechanical characterization of vascular tissue tubes

All samples were sufficiently thick and strong at the end of the 14 day culture period to be handleable and sutured onto the mechanical testing device. As seen in

Figure 29A, once the samples were successfully mounted onto the vessel test device and inflated at a constant flow rate, there was an increase in the pressure inside the tissue tube. Inflation continued until the samples burst at the peak pressure they could withstand. The peak pressure recorded by the pressure transducer averaged to 256±73 mmHg for 11 samples tested.

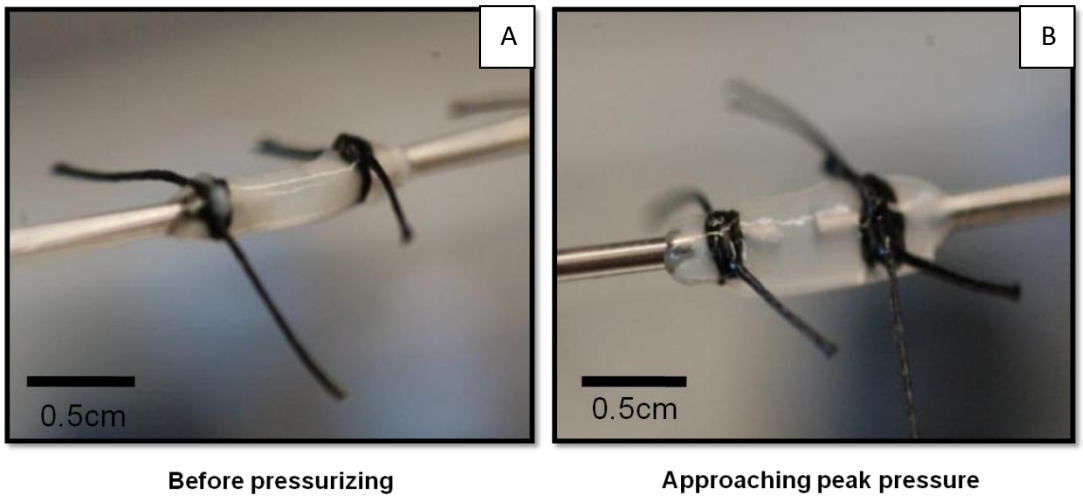

Figure 29: Burst pressure testing on cell-derived tissue tube. A. A photograph of a cell-derived tissue tube sample successfully mounted on the needle assembly of the vessel test device with a pair of surgical knots using a 4-0 silk suture. B. A photograph of the tissue tube almost approaching peak pressure (the pressure before it bursts) while being inflated at a constant rate with 1X PBS using a programmable syringe pump.

4.8 System validation/Batch-Batch reproducibility

To develop the direct cell seeding method, several batches of collagen-coated mandrels were seeded, cultured and analyzed. Table 2 below gives a summary of the number of batches made and the number of samples analyzed in each batch. The pooled data represents data from 12 batches with a total of 72 analyzed samples. As seen in Table 2, the mean O.D of all the samples was 1.68±0.04 mm (mean±SEM; n=72) with a minimum O.D. of 1.55±0.03 mm and maximum O.D. of 1.90±0.11 mm. The average calculated wall thickness from the machine vision system was 230±63 μm (mean±SEM; n=72) while the average measured wall thickness from the histological images was 177±19 μm (mean±SEM; n=30).

Table 2: Summarized analysis of all cell-derived tissue tubes

	Batch no.	No. of samples analyzed (n)	Mode of analysis	Average outer diameter from DVT analysis	Average wall thickness from histological analysis
	1	3	Histology (n=3)	1.74 ± 0.101 (n=3)	149 ± 20 (n=3)
	2	3	Histology (n=3)	1.65 ± 0.100 (n=3)	191 ± 10 (n=3)
	3	4	Histology (n=4)	1.74 ± 0.048 (n=4)	233 ± 82 (n=4)
	4	5	Histology (n=5)	1.73 ± 0.089 (n=5)	183 ± 24 (n=5)
	5	4	Histology (n=2)	1.71 ± 0.032 (n=4)	181 ± 10 (n=2)
	6	3	--	1.71 ± 0.092 (n=3)	--
	7	13	Burst pressure (n=5)	1.68 ± 0.084 (n=13)	--
	8	2	Burst pressure (n=2)	1.74 ± 0.009 (n=2)	--
	9	16	Histology (n=4); Burst pressure (n=4)	1.63 ± 0.050 (n=16)	173 ± 19 (n=4)
	10	3	Histology (n=3)	1.60 ± 0.016 (n=3)	156 ± 9 (n=3)
	11	10	Histology (n=3)	1.67 ± 0.051 (n=10)	171 ± 5 (n=3)
	12	6	Histology (n=3)	1.66 ± 0.065 (n=6)	182 ± 15 (n=3)
Total	**12**	**72**	Histology (n=30); Burst pressure (n=11)	1.68±0.04 (n=72)	177±19 (n=30)

4.8.1 Evaluation of batch to batch variation using O.D. measurements

In order to estimate batch to batch variability in terms of the O.D. measurements, a one way ANOVA was performed on the average O.D. measurements of all the samples across all the batches. Results indicated a significant difference between the batches. However, the Holm-Sidak post hoc analysis was unable to isolate the batches that were different from the rest. This may be attributed to the failed normality tests and a highly variable samples size in all the batches (which ranged from n=2 per batch to n=16 in the largest batch). To accommodate the effect of varying sample sizes a Kruskal-Wallis one way ANOVA on ranks was performed with

a Dunn's post hoc test. However, this test was also unable to isolate the groups specifically different from the rest. In order to gauge the difference between the batches percent difference between the minimum and maximum O.D. was calculated. A maximum difference of 22.8% was reported between the batches.

For estimation of lengthwise uniformity as a function of measurement position in all the samples across all the batches a graph of the mean O.D. measurements at each of the measurement locations was plotted (Figure 30). A two way ANOVA was performed with batch and measurement position as the factors of interest. It was observed that the difference in the mean values among the different levels of measurement position was not statistically significantly different ($p=0.127$). Also, the effect of different levels of batch did not depend on what level of position was present, indicating that there was no statistically significant interaction between batch and position ($p=1.000$). However, the difference in the mean values among the different levels of batch was statistically significant ($p<0.05$).

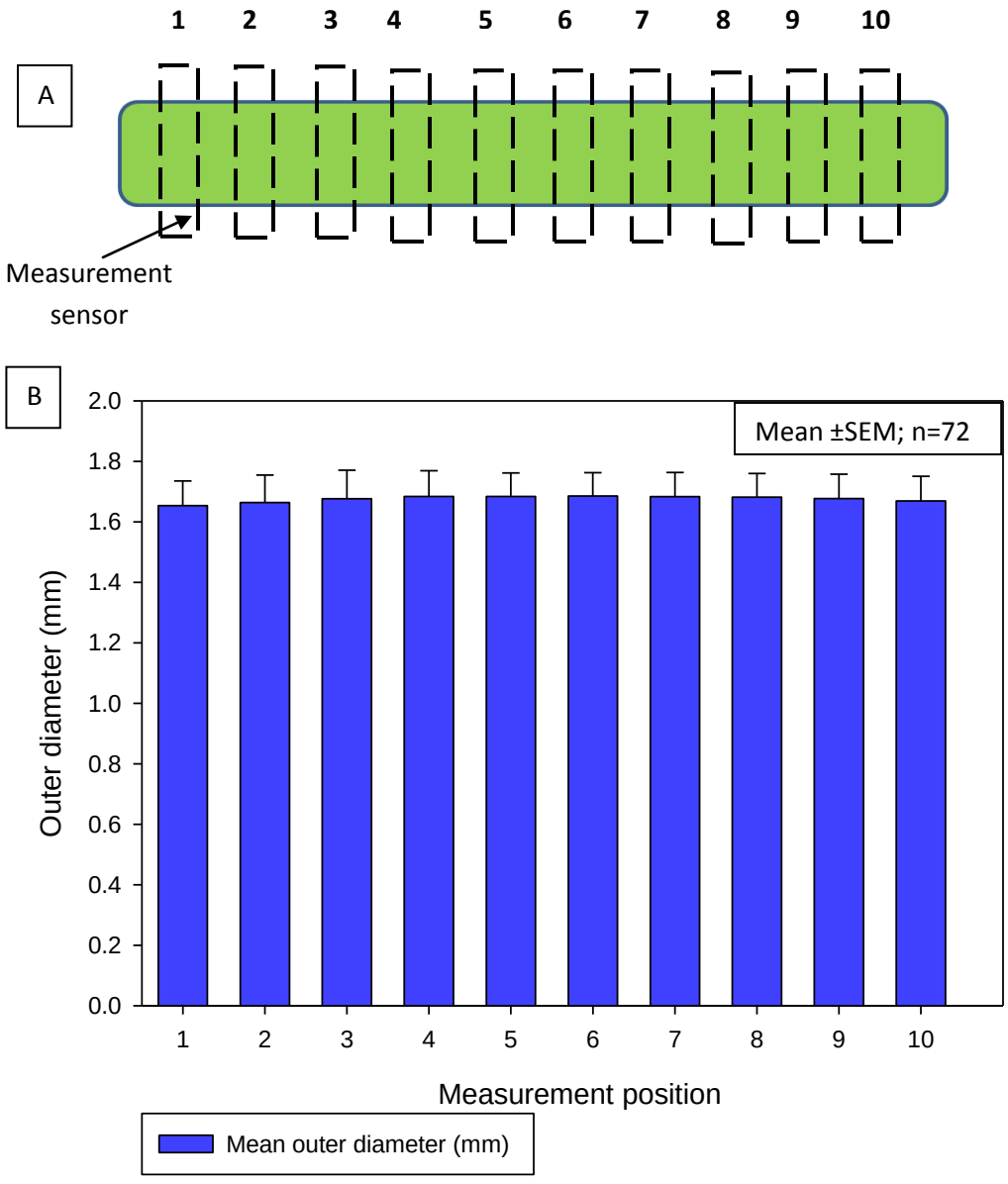

Figure 30: Assessment of lengthwise uniformity on tissue tubes. A: A schematic of the cell-derived tissue tube and the locations at which O.D. measurements are taken. Dotted rectangles indicate measurement sensor location. B: A graph of mean O.D. measurements of all the samples across all the batches with respect to the measurement location. n=72

In order to quantify this lengthwise uniformity, the uniformity index was calculated using the co-efficient of variation. For this batch, the uniformity index ranged from 93.2-99.3% for all the samples across all the batches.

4.8.2 Evaluation of batch to batch variation using histological wall thickness measurements

In order to determine if there was a batch to batch variability in the wall thickness measurements of the cell-derived tissue tubes, a Kruskal-Wallis one way ANOVA on ranks was performed on the average wall thickness of all the samples from each batch measured using histological images (see Section 3.7.2). Results indicated no significant differences in wall thickness measurements between all the batches. A 39.6% variation was reported between the batches.

4.8.3 Evaluation of circumferential uniformity using histological wall thickness measurements

The other major observation from visual assessment was that certain regions in the tissue section appeared thinner compared to the rest of the wall of the tube. This phenomenon was present in almost all the samples from all batches. Thus, it was required to analyze the wall thickness of the engineered tissue tubes and confirm the uniformity of tissue growth around the circumference of the tube. As seen in Figure 31, the results from the paired t-test between the thinnest and the thickest wall thickness measurements indicated that there is a statistically significant difference between the thin and the thick regions of the tissue tube sections ($p<0.05$). This revealed the circumferential non-uniformity of the cell-derived tissue tubes which needs to be addressed.

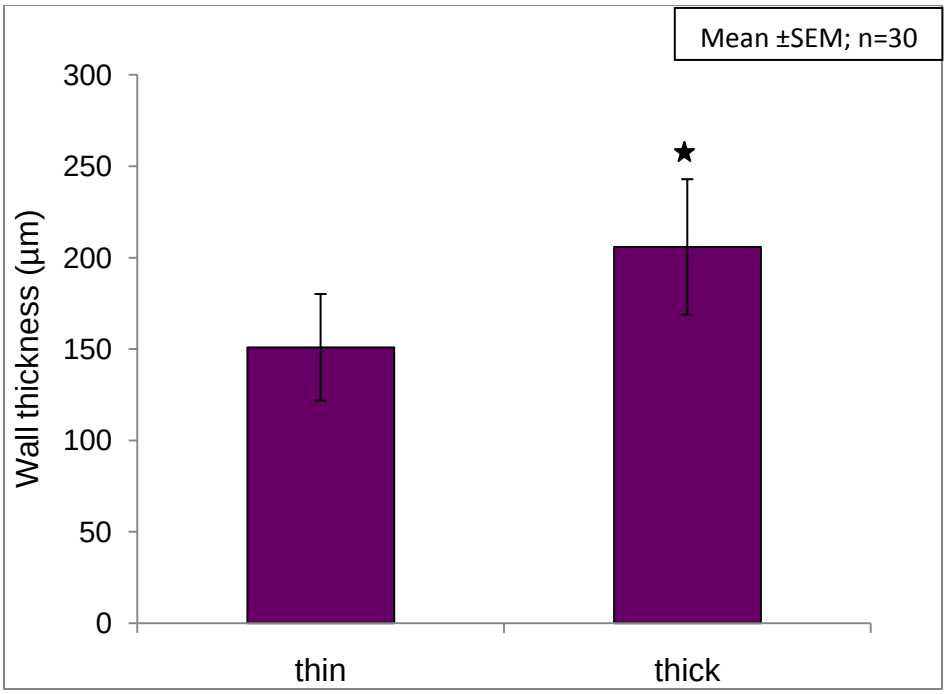

Figure 31: Assessment of circumferential uniformity. A graph comparing the thinnest and the thickest wall thickness measurements made using the ImageJ software. ★ Indicates significant difference in the thick regions of the samples as compared to the thin regions (Paired t-test $p<0.05$; n=30)

Chapter 5: Effect of ascorbic acid treatment on cell-derived tissue tubes

From the previous chapters, it is demonstrated that the method of direct cell seeding on collagen-coated silicone mandrels accomplishes the goal of generating a handleable tissue tube. However, cell-derived tissue tubes developed using this method show fairly low amounts of collagen production, resulting in extremely low burst pressures. In this chapter we investigate the effect of addition of sodium ascorbate to the culture medium on collagen production, toward the goal of improving tissue tube mechanical strength.

5.1 Rationale

Culture conditions can modify the composition of the ECM of cultured smooth muscle cells. One of way of observing changes in ECM synthesis is the addition of soluble factors. Ascorbic acid is one such soluble factor that has been extensively investigated for its role in collagen synthesis.

5.1.1. Role of ascorbate in cell culture

It has been reported that deposition of collagen occurs upon ascorbate supplementation in cultured aortic smooth muscle cells (SMCs) and with increased time of exposure collagen becomes the dominant protein of the ECM (greater than 80%) [33]. Collagen accumulation follows a sigmoidal time-course, and microfibrillar proteins and increased amounts of proteoglycans and fibronectin also accumulate concurrently with collagen [33]. Also, there is evidence that ascorbate protects SMCs from apoptosis/necrosis [54]. On human skin fibroblasts, collagen synthesis increased approximately 8- fold due to ascorbate addition [55].

5.1.2. Role of ascorbate in tissue engineered blood vessels

With an increase in collagen production, the mechanical integrity of tissue constructs increases as well. Several studies in vascular tissue engineering have taken advantage of this phenomenon to increase the tensile strength of the engineered conduits. In a recent study by Ogle et al., the mechanical properties of TEBVs made using biopolymer gels were improved by manipulating ECM synthesis [34]. Briefly, human aortic SMCs in collagen gels were treated with 0.1mM retinoic acid and 0.3mM ascorbic acid. A two fold increase in elastin and collagen synthesis was observed after 30 days of incubation. It was also observed that the TEBV strength (ultimate stress) increased and stiffness decreased compared to untreated TEBV controls as a function of time. This data provides evidence that changes in ECM composition due to soluble factors may play an important role in the development of tissue mechanical properties [34]. Swartz et al generated fibrin-based small diameter functional and implantable TEBVs in culture medium supplemented with ascorbic acid which attained considerable mechanical strength and vasoreactivity after only 2 weeks in culture [56]. When implanted into 12-wk-old lambs, fibrin-based TEBVs exhibited remarkable remodeling with considerable production of collagen and elastin and significantly increased mechanical strength [56]. More recently, L'Heureux et al derived their fully biologic blood vessels by culturing fibroblasts in ascorbic acid-supplemented media. These vessels exhibit burst pressures exceeding those of human saphenous vein and have remained patent as A-V shunts for more than 21 months [27, 35].

Based on this, we hypothesized that the addition of ascorbic acid to the culture media will result in an increase in the collagen production and in turn increase the mechanical integrity of the engineered tissue tubes developed using the direct cell seeding method.

5.2 Methods

5.2.1 Cell seeding on collagen-coated silicone mandrels

After preparing the seeding assemblies and prior to seeding the coated silicone mandrels were rehydrated with 400 µl of standard growth medium for 30 mins. For cell seeding, 280 µl of the prepared cell suspension (280,000 cells) was pipetted into the center of each silicone support ring as seen in Figure 32. The culture dishes were then inverted and incubated at 37°C, 5% CO_2 for 30 mins, to allow the cells to contact the coated mandrels. After incubation, the culture dish was turned upright and the samples were rinsed twice with 1X PBS to remove the unattached cells.

In order to assess the effect of addition of ascorbic acid, 50µg/ml of sodium ascorbate was added to the medium. A stock solution of 50mg/ml of ascorbic acid was made by adding ascorbic acid (L-ascorbic acid, Cat# 255564-100G, Sigma Aldrich) to 50ml of 1X DMEM. This stock solution was stored at 2-6 °C. A working solution of 50 µg/ml of ascorbic acid in growth medium was prepared by adding 40 µl of this 50mg/ml ascorbic acid stock solution to 40ml of standard growth medium. After rinsing with 1X PBS, 6 ml of this fresh ascorbic acid-supplemented growth medium was added to each well and the samples were allowed to culture at 37°C, 5% CO_2 for 14 days. Media was changed every 24 hours for 14 days. Each time a fresh working solution of ascorbic acid supplemented growth medium was prepared from the stock solution, prior to feeding.

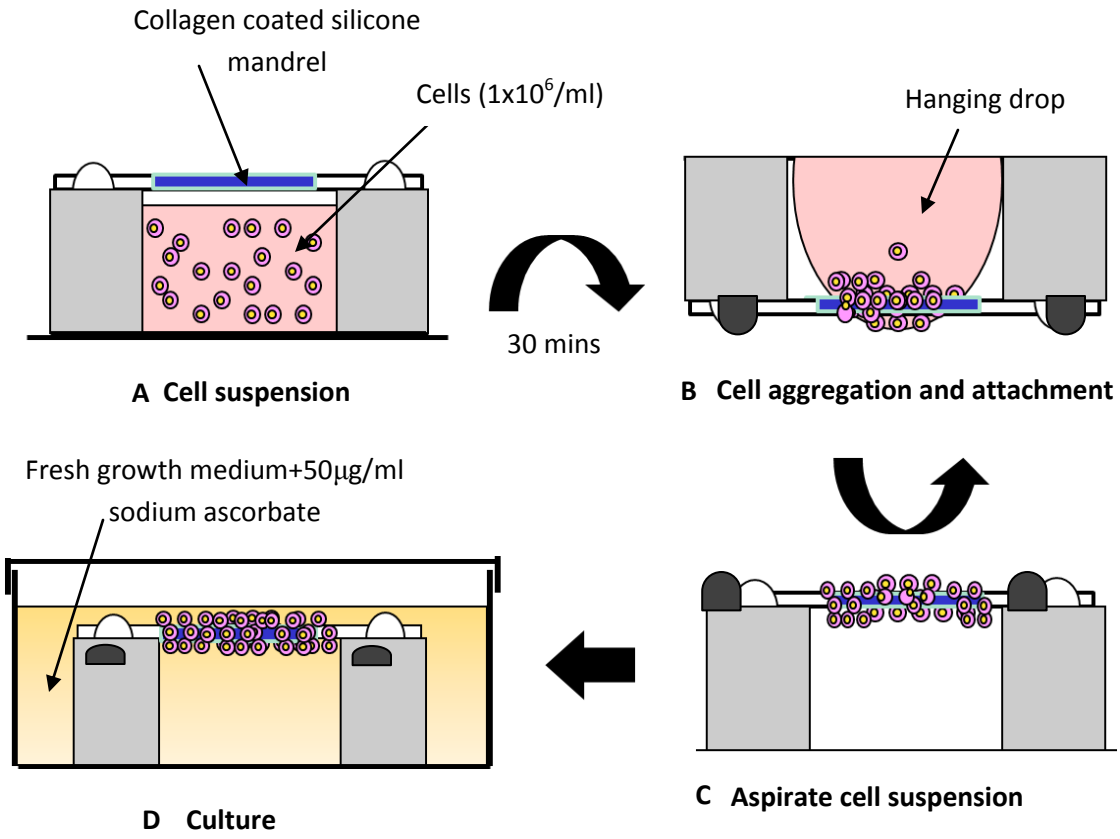

Figure 32: Sodium ascorbate treatment to cell-derived tissue tubes. A: RASMC cell suspension pipetted into the center of the seeding assembly. B: Seeding assembly flipped upside down for cell-seeding using the hanging drop technique. C: Cell suspension aspirated and samples rinsed in 1X PBS twice. D: Cell-seeded samples culture in media supplemented with sodium ascorbate (50µg/ml) for 14 days.

5.2.2 Analysis of effect of ascorbic acid

All the methods used for quantification of tissue growth over time, histological assessments, wall thickness measurements and burst pressure testing were followed exactly as described in Chapter 3.

Cell attachment on the collagen-coated silicone mandrels was verified by observing the samples under a Leica DM IL light microscope immediately after seeding and 24 hours after seeding.

The outer diameter of the treated and untreated tissue tubes was measured using the machine vision system, for both the treated and the untreated groups. In order to determine if there was a significant increase in tissue growth as a function of outer diameter over time in the ascorbate treated tissue tube samples, one way ANOVA with culture period as the factor of interest, was performed on the average of ten outer diameter measurements for each sample at each time point. For pair wise multiple comparisons between culture periods, the Holm-Sidak method of comparison was used which enabled us to determine exactly which culture period was statistically different. In order to determine if there was a significant difference in the O.D. of the ascorbate treated samples and the untreated samples, a t-test was performed on the mean O.D. measurements of all the samples in both the groups at each time point in the culture period. For estimation of lengthwise uniformity, using the lengthwise data a uniformity index was also calculated for each group (See section 3.10.3).

After the 14 day culture period, the cell-derived tissue tubes were harvested for biomechanical testing and histological assessments. The structural properties were characterized using histological staining with H&E and Picrosirius Red/Fast Green. Wall thickness measurements were also made on these histological sections. In order to evaluate circumferential uniformity wall thickness measurements were taken at 10 locations along the circumference of the tissue samples as described in Section 3.7.2. The 10 wall thickness measurements for each of the samples were arranged in an ascending order. A paired t-test was performed on the thinnest and thickest measurements for all the samples in both the treatment and control groups to determine if there was a significant difference.

5.3 Results

The data presented in this chapter is the outcome of one preliminary experiment consisting of a total of 12 samples; (6 treated with sodium ascorbate and 6 untreated).

5.3.1 Observation of vessel growth over time

Growth of cells on the silicone mandrels was monitored by measuring the increase in O.D. of the cell-seeded silicone mandrels over time using the machine vision system for both the treated and untreated samples. Figure 33 shows a graph of tissue growth over time for all the samples in this batch. Outer diameter measurements were taken at 4, 8, 12 and 14 days of culture. At 14 days, an average O.D. of 1.57±0.02 mm (mean±SEM; n=6) and 1.66±0.06 mm (mean±SEM; n=6) was obtained for the treated and untreated groups, respectively. Based on this data, the average wall thickness of the tissue tubes was calculated as 189±10 μm (mean±SEM; n=6) for the treated group and 234±32 μm (mean±SEM; n=6) for the untreated controls. On performing a one way ANOVA on the O.D. measurements with culture period as the factor of interest, a significant increase in O.D from day 4 to each time-point, and from Day 8 to each time-point was observed. There was no difference in the O.D. at days 12 and 14.

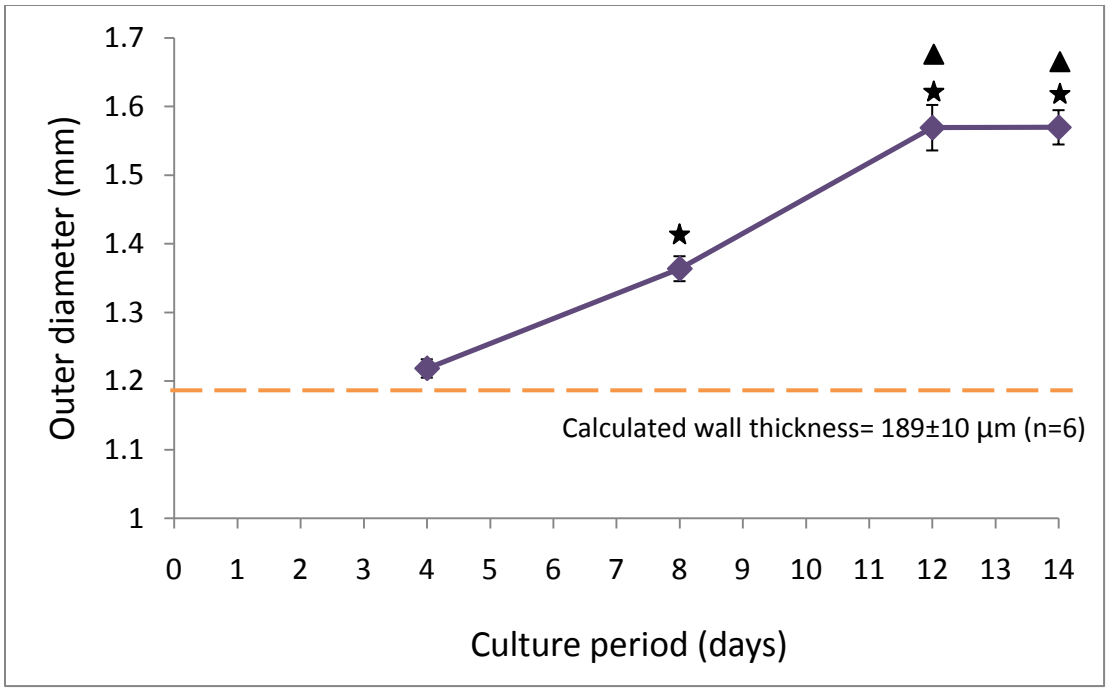

Figure 33: Vessel growth over time in ascorbate treated samples. A graph of outer diameter measurements from one representative batch over time. dotted orange line indicates outer diameter of the unseeded silicone mandrel. O.D. measurements taken at 4, 8, 12 and 14 days. ★$p<0.05$ from Day 4. ▲ $p<0.05$ from Day 8. n=6

On performing a t-test on the mean outer diameter measurements of the treated and untreated groups at each culture time point, it was observed that the there was a significant difference in the O.D. measurements of the two groups from day 8 up to day 14 (Figure 34). This indicates that sodium ascorbate treatment resulted in tissue tubes thinner as compared to the untreated controls.

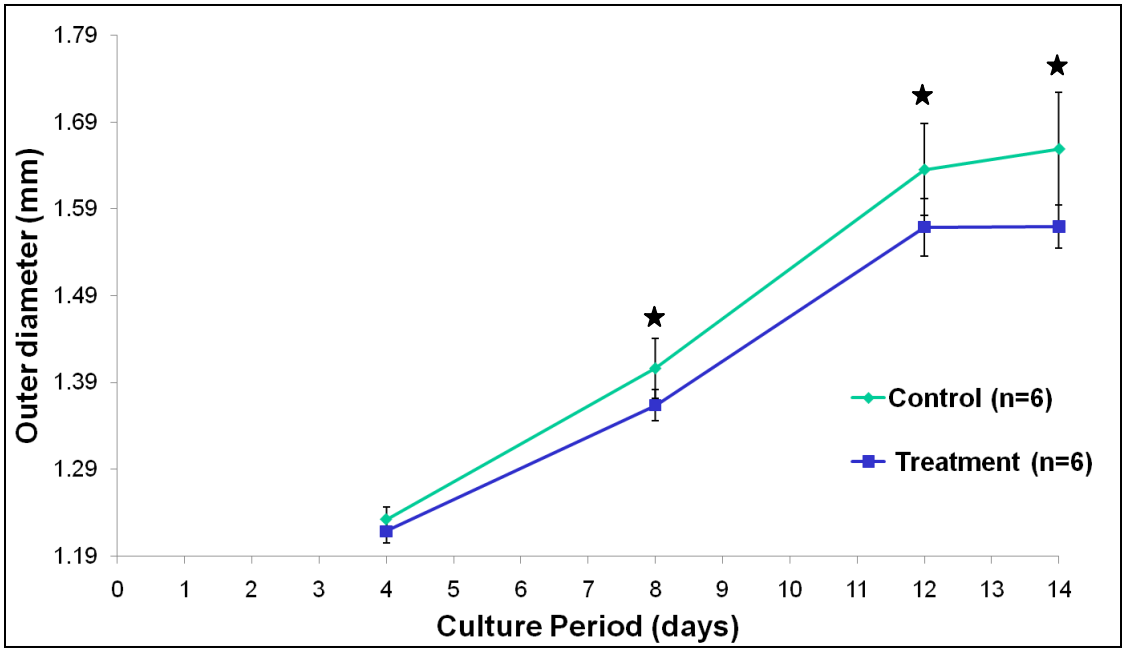

Figure 34: Vessel growth of treated and untreated tissue tubes over time. A graph comparing the outer diameter measurements of the treated and untreated groups at the 4, 8, 12 and 14 day time points. ★t-test $p<0.05$ between groups at that time point. n=6 for each group.

In order to determine lengthwise uniformity on the sodium ascorbate treated tissue tubes, one way ANOVA was performed based on the O.D. measurement of each sample at each measurement position. Results indicated that the differences in the mean values among the measurement positions are not great enough and there is not a statistically significant difference ($p=0.634$). Similarly, to test the lengthwise uniformity on the untreated controls, one way ANOVA was performed based on the O.D. measurement of each sample at each measurement position. Results indicated that the differences in the median values among the measurement positions are not great enough and there is not a statistically significant difference ($p<0.796$).

The lengthwise uniformity index of the tissue tubes based on measurement location of the samples treated with ascorbate at 14 days, was in the range of 97.1-98.4% (n=6), while that of the untreated group was 96.6-98.2% (n=6).

5.3.2 Histological analysis

The H&E and Picrosirius Red/Fast Green stained images of the tissue tube sections are shown in H&E stained images show the cellular morphology and alignment in both the treated and untreated groups Figure 34A-B. In addition to this, the tissue tubes exhibited collagen production as seen in the Picrosirus Red/Fast Green stained images (Figure 34C-D) in the sodium ascorbate treated group, as compared to the untreated group. Although this was not quantified using any biochemical assays, by visual observation the collagen production appears slightly higher in the sodium ascorbate treated images compared to the untreated controls.

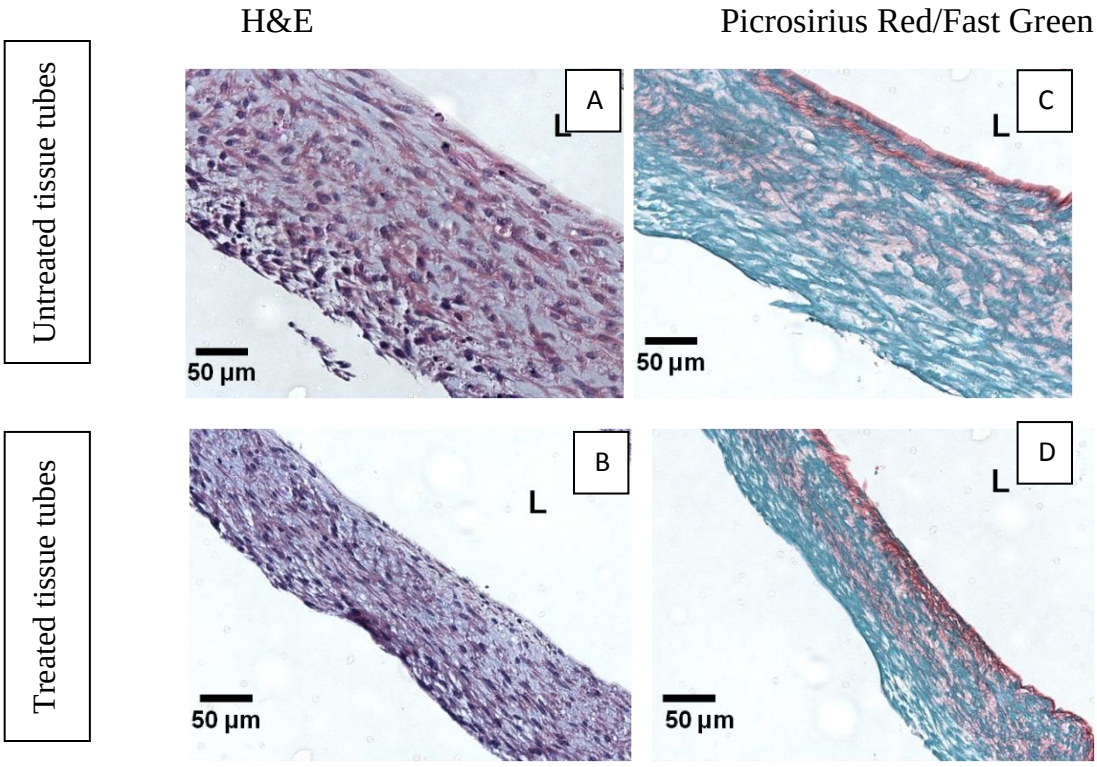

Figure 35: Histological comparison of treated and untreated tissue tubes. A-B: H&E stained images of cell-derived tissue tube sections.Purple=nuclei, Pink=cytoplasm. C-D: Picrosirius Red/Fast Green. Red=collagen, Green= tissue counterstain. L indicates lumen

5.3.3 Wall thickness measurements

The wall thickness of the treated cell-derived tissue tubes (Figure 36B) appeared smaller than that of the untreated control (Figure 36A) in all the samples.

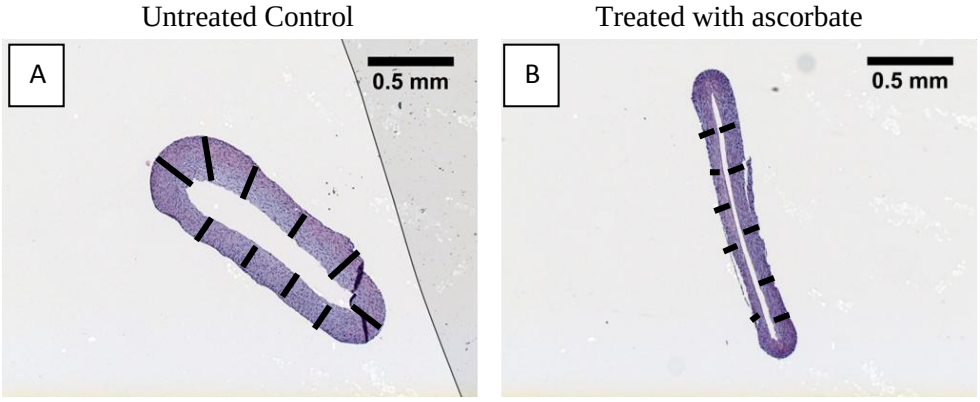

Figure 36: Wall thickness measurements on treated and untreated tissue tubes. A: H&E stained cross section of an untreated cell-derived tissue tube. B: H&E stained cross section of a cell-derived tissue tube treated with ascorbate for comparison of wall thickness measurement. Lines indicate the position at which wall thickness measurements were taken.

The measured average wall thickness of the treated cell-derived tissue tubes from the histological assessment using ImageJ at the end of 14 days for this batch was 132±29 (n=3) while that of the untreated tissue tubes was 182±15 (n=3).

Table 3: Comparison of wall thickness measurements on ascorbate treated versus untreated tissue tubes. † t-test, $p<0.05$; n=6 each

	Measured wall thickness (µm)
Untreated control †	182±15 (n=3)
Treated with ascorbate †	132±29 (n=3)

The wall thickness of the engineered tissue tubes was analyzed to assess the uniformity of tissue growth around the circumference of the tube. As seen in Figure 37, the results from the paired t-test between the thinnest and the thickest wall thickness measurements of the cell-derived tissue tubes on the treated ($p<0.05$) and on the untreated group ($p<0.05$), indicated that there is a statistically significant difference between the thickest and the thinnest regions of all the samples in the ascorbate treated and untreated groups respectively.

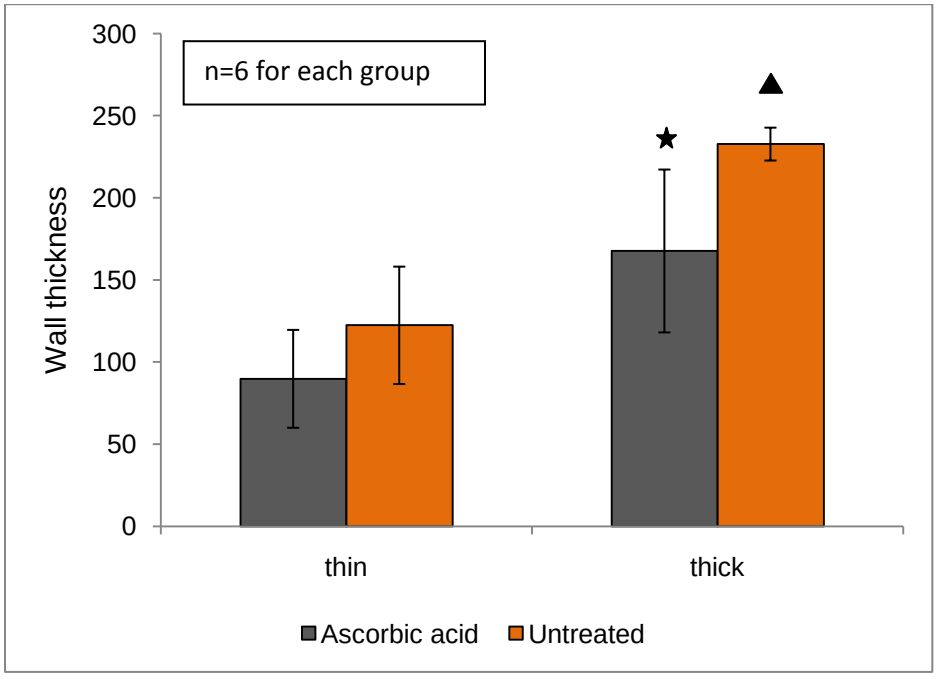

Figure 37: Circumferential uniformity in treated and untreated tissue tubes. A graph comparing the thinnest and the thickest wall thickness measurements on the treated samples (★ paired t-test $p<0.05$; n=6) and the thinnest and the thickest wall thickness measurements on the untreated samples (▲ paired t-test $p<0.05$; n=6)

Chapter 6: Discussion and Conclusions

In this study, we report the feasibility of generating fully biologic cell-derived tissue tubes using the direct cell seeding approach. In this section, the significance of all the methods and results presented in the previous chapters is reviewed and interpreted in relation to the overall goal of this project.

Silicone tubing was chosen as the cylindrical mandrel for seeding cells since it is a commercially available, autoclavable, medical grade tubing that is flexible. However, on directly seeding cells on plain silicone tubing, no cell attachment was observed even after 7 days of culture. This indicated the need to modify the surface properties of the seeding mandrel to facilitate cell attachment. Biopolymer gels like collagen and fibrin have been widely used to coat various substrates to facilitate cell attachment. Type I collagen is most often used for coating substrates for cell culture of epithelial cells [57], smooth muscle cells [58-60], endothelial cells [59] and fibroblasts [60-62] because it is easily obtainable from rat tails. Thus, we successfully coated silicone mandrels with collagen gel, and cultured rat aortic smooth muscle cells on the coated silicone mandrels. The images of Hoechst dye staining on cell seeded samples at the initial time point (Day1) in the culture period indicate that, using this approach it is possible to achieve uniform cell seeding on collagen-coated silicone mandrels.

The quantification of cell growth on seeding mandrels using the machine vision system, indicated that at Day 14, a tissue tube with an average outer diameter 1.68±0.04 mm (average of all the samples across all the batches; n=72) can be obtained. It was also observed that, over time, the outer diameter of the tissue tubes increased significantly across all the batches. In order to determine batch to batch reproducibility in terms of mean O.D. measurement of all the

samples from all the batches, a one way ANOVA was performed. On doing so the normality test failed and a Holm-Sidak post hoc test was performed to determine the batches with significantly different mean O.D. Although the results indicated a significant difference between the batches, the post hoc test was unable to isolate the specific groups. This may be due to the highly variable sample sizes in all the batches. Thus, to accommodate the effect of varying sample sizes a Kruskal-Wallis one way ANOVA on ranks had to be performed with a Dunn's post hoc test. However, this test was also unable to isolate the groups specifically different from the rest. Thus, an estimate of the batch to batch variability in average outer diameter was calculated using the percent difference in the minimum and maximum means of the samples. A batch to batch variability of 22.8% was reported. Considering that this is a completely cell-based system, this variability can be due to differences in nutrient consumption, media conditions, pH, cell proliferation rates, and vial to vial differences in freeze/thaw processes.

In terms of lengthwise uniformity of the tissue tubes, statistical analysis on the outer diameter measurements suggested no significant difference in the measurements along the length of the tissue tube for all the samples across all the batches. Also, a uniformity index ranging from 93.1-99.3% suggested that it is possible to develop tissue tubes that are uniform and consistent lengthwise using the direct cell seeding technique.

The feasibility of harvesting a homogenous structure in the form of a cohesive tissue tubes from the silicone mandrel provides evidence that these cell-derived tissue tubes were handleable after only 14 days of culture. The structural properties of a TEBV are very closely associated to its functional properties indicating that this is a very crucial investigation. Thus, once the tissue tubes were harvested, an analysis of the structural properties of the tissue constructs was done by staining tissue sections with H&E, Movat's Pentachrome and Picrosirius

Red/Fast Green. H&E stained images revealed that there is circumferential alignment of the smooth muscle cells along the tube. The Movat's pentachrome staining suggested that there is abundant proteoglycan or glycosaminoglycan production but very little collagen production. PicroSirius Red/Fast Green staining confirmed the presence of some amount of collagen synthesis in these constructs. It was interesting to note that the collagen gel coating was distinctly visible in all the samples. Thus, despite culturing in 10% fetal bovine serum and without any exogenous biochemical or growth factors to specifically control or optimize ECM synthesis, there still appeared to be some evidence of endogenous collagen production.

The wall thickness assessment of the tissue tubes using the histological images suggests that an average wall thickness of 177±19 μm (mean±SEM; n=30) was achieved at the end of 14 days. These data are significantly different from the wall thickness calculated using the outer diameter measurements of 230±63 μm (mean±SEM; n=72). However, dehydration of the tissue during processing in ethanol could be the possible artifact causing this difference in measurements. One more factor that can attribute to this issue is the resolution of the DVT Series 600 camera in the machine vision system. For this study, the camera was setup to give a resolution of 31 μm (measured using USAF 1951 resolution target).

The circumferential uniformity of the tissue tubes calculated using the wall thickness measurements demonstrated a slightly different story. Statistical analysis suggested that there is a significant difference between the thinnest and the thickest region of the tissue construct, indicating that the tissue tubes are not uniform. Since this pattern of thin versus thick region was observed in almost all the samples, one of the postulations to explain this occurrence is that, during the hanging drop seeding, the cell attachment on one side of the silicone mandrel is lower as compared to the other side (Figure 38). Due to this, it is possible that one side of the tissue

tube is significantly thinner than the other. In this study the possibility of improper cell-seeding with the hanging drop method was not quantified and confirmed. To do so, the tissue tubes need to be sectioned cross-sectionally throughout the length and adjacent sections compared.

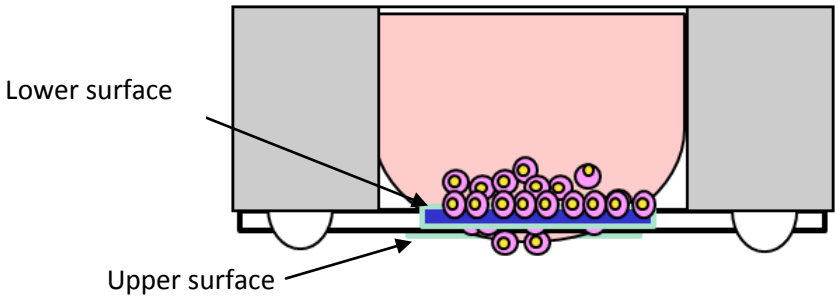

Figure 38: Possible problem associated with hanging drop seeding

The other concern associated with the wall thickness of the cell-derived tissue tubes was the diffusion of nutrients through the thickness of the construct to the cell layers adjacent to the silicone mandrel. This nutrient deficiency might result in apoptosis of the cells near the silicone mandrel. To quantify apoptosis, an immunohistochemical assay like Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) can be performed. Some preliminary staining on the tissue sections has been done so far, however an optimized protocol has yet to be developed. Nutrient diffusion may be improved using multiple options: (1) reducing the culture time, (2) reducing the serum concentration or (3) using a porous seeding mandrel that permits diffusion of media nutrients to the cells near the lumen.

Burst pressure testing is one of the most widely used tests for estimating the mechanical properties of a tissue construct. From literature, TEBVs generated by Hoerstrup et al. demonstrated burst pressures of 240 mmHg after pulsatile conditioning [9]. In another approach, TEBVs developed by mixing bovine aortic smooth muscle cells and Type I collagen showed a burst pressure <100 mmHg [63]. The burst pressure of TEBVs constructed by scaffolding the

cell-populated collagenous tube with polyurethane was also only about 240 mmHg [64]. Keeping in mind that these cell-derived tissue tubes were cultured without any growth factors or mechanical conditioning, there is significantly less amount of ECM production; specifically collagen and elastin by the tissue tubes. Thus, it is expected that the mechanical properties of these constructs are not at all close enough to physiological standards. Thus, when a comparison of TEBV burst pressures of these attempts is made with our recording of 256 mmHg, at the end of a 14 day culture period, it is probable that tissue constructs developed using the direct cell seeding approach will improve in mechanical properties with longer culture periods and optimized culture conditions

In an attempt to increase the mechanical strength of the tissue constructs developed using direct cell seeding, we investigated the effect of addition of sodium ascorbate on collagen production. The outer diameter measurements on the ascorbate treated tissue tubes increased with culture period. On comparison with the mean O.D. measurements from the untreated control samples, it was observed that the treated samples were significantly thinner than their untreated counterparts at 8, 12 and 14 days. At day 8, the difference between the O.D. measurements of each group is 42 μm. Although, there is a significant difference between the two measurements at this time point, it is important to consider the effect of resolution of the machine vision system (31 μm). However, when these data are corroborated with the wall thickness data from histological sections of the tissue samples at day 14, a similar difference in the treated and untreated groups is observed. This observed difference in the outer diameter and wall thickness measurements of the tissue tube treated with ascorbate can be attributed to the fact that possibly there is some amount of compaction due to the presence of sodium ascorbate and increased collagen synthesis as seen in the study by Seliktar et al. [15].

By visual observation of the histological images, there was a slight increase in collagen production due to sodium ascorbate. In order to quantify this increase in collagen production biochemical assays for measuring collagen production like SIRCOL or hydroxyproline assay should be performed. Also, a dose dependant study for various concentrations of sodium ascorbate in the media should be done in order to optimize the collagen production.. Overall, the results from this preliminary study suggested that it may be possible to manipulate the culture conditions slightly to obtain differences in ECM production.

Conclusions

The goal of this project was to explore an alternative approach to developing tissue engineered blood vessels using a simple and minimally manipulative method. Our results demonstrate that the direct cell seeding approach is a feasible method for generating fully biologic cell-derived tissue tubes, wherein the cells proliferate, produce ECM proteins and can be harvested and characterized for their structural and mechanical properties. This study also suggests the feasibility of manipulating the collagen production by addition of a soluble factor, ascorbic acid.

Chapter 7: Future work and Implications

7.1 Structural and biomechanical characterization of current system

The results from this study indicate that it is possible to develop fully biologic cell-derived tissue tubes using the direct cell seeding approach. However, in order to completely and thoroughly characterize this system, it is essential to perform some additional analytical procedures. In order to validate that the increased tissue growth is due to cell proliferation, future studies should include immunohistochemical analysis such as Ki-67 or BrDU staining. Apart from that, it is essential to quantify the ECM synthesis using biochemical assays like SIRCOL for collagen production. This assessment will be particularly important when culture conditions are modified by biochemical or biomechanical stimulation in order to determine the effectiveness of the applied treatment. Also, in order to determine if the developed constructs exhibit elastic properties similar to native vessels, it is essential to characterize the TEBV compliance.

7.2 Optimizing culture conditions

Although the engineered tissue tubes have not been able to accomplish sufficient burst pressure compared to reported approaches to tissue tube fabrication, there are a broad range of alternatives that can be explored to increase the mechanical strength.

7.2.1 Biochemical factors

The effect of culture environment on cellular responses has been widely studied. It has been observed that the effect of addition of exogenous factors and mechanical conditioning results in vascular constructs that more closely resemble native vessels. Several studies with the collagen-based TEBVs have attempted to increase ECM synthesis by addition of sodium ascorbate, retinoic acid, TGF- ß1, or insulin. In a study by Neidert and Tranquillo et al., TGF-ß1

and insulin [19, 51] are shown to affect collagen synthesis to the extent of a six-fold increase. Retinoic acid has been used to increase elastin production by Ogle et al. [34]. Sodium ascorbate has also been used extensively [24, 33, 34] in the literature to increase collagen synthesis and thus, mechanical strength.

7.2.2. Mechanical conditioning

Apart from these biochemical factors, mechanical conditioning regiments employed by Nikalson et al. induced ECM synthesis [10]. Seliktar et al., also showed that by dynamically conditioning the rat smooth muscle cells in collagen gels, it is possible to achieve highly organized collagen production and thus higher tensile properties [15]. The benefit of mechanical conditioning was also studied by Stegemann and Nerem in 2005, where they showed that conditioning in a bioreactor increased cell proliferation and gel contraction. In the same study, the combined effects of biochemical and biomechanical stimulation on compaction, cell proliferation were also observed [21].

The advantage of the direct cell seeding approach is that the entire system can be fairly easily accommodated to observe effects of biochemical factors as well as the effect of mechanical conditioning. Some preliminary work has been done to observe the effectiveness of addition of sodium ascorbate on the cell-derived tissue tubes, as mentioned in Chapter 5. Apart from that, some preliminary work was also done in order to develop a bioreactor system for dynamically conditioning the cell constructs (results not shown).

7.3 Application to human cells

In order to determine the feasibility of this method to develop fully biologic cell-derived tissue tubes, this project developed tissue tubes from rat aortic smooth muscle cells. However,

for clinical relevance, it is required to produce the tissue constructs using human cells. Human vascular smooth muscle cells [65], human dermal fibroblasts [65-67], or human mesenchymal stem cells [11, 68] are a few of the cell source alternatives that can be used.

7.4 Technical limitations of current system

7.4.1. Failure during development

Along the course of this study, in general the efficiency of each batch was around 60-70%. The mode of failure of a sample during culture was typically due to the detachment of the SMC tissue tube from one end of the tube resulting in contraction of the sample. Figure 39 shows an image of a sample at 14 days with a contracted tube of SMCs on one end. The approach that could be used to avoid this issue is the use of anchors to hold the SMCs in place during the culture period.

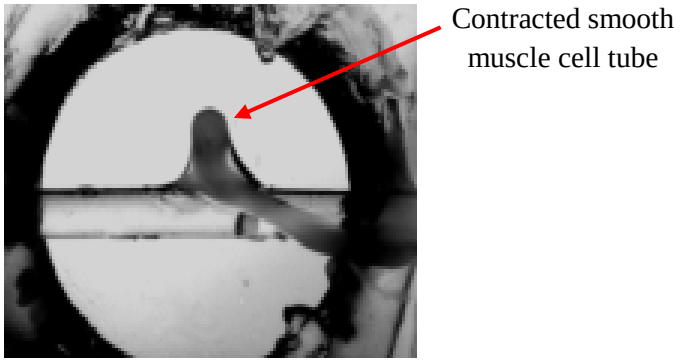

Figure 39: Mode of failure during development. Image of a contracted smooth muscle cell tube on silicone mandrel detached from the left side of the mandrel and contracted to form a peak indicated by the arrow.

7.4.2. Failure during testing

One of the other major technical limitations leading to failure during testing is related to the measurement of burst pressure using the custom vessel test device. The major issue with this

system is the difficulty in mounting tissue samples securely on the needle assembly. This is mainly due to the small length and diameter of the cell-derived tissue tube. The small length of the tissue tube does not provide sufficient room for properly suturing the vessel. Since tying the knot is a manual procedure, it is quite possible that the sutures may be too tight or too lose causing tear or leakage. This problem can be resolved by generating longer cell-derived tissue tubes using the same method, by simply changing the size of the seeding assembly.

Another technical limitation here is the alignment of the needles in the needle mounting assembly. The figure above is an image of the two needle ends of the needle assembly as seen under the machine vision system. Due to this misalignment, a number of tissue samples rupture or tear due to the needles. Due to these issues, only 11 out of 49 tissue tube samples could be tested for burst pressure. This can be resolved by re-designing the needle assembly.

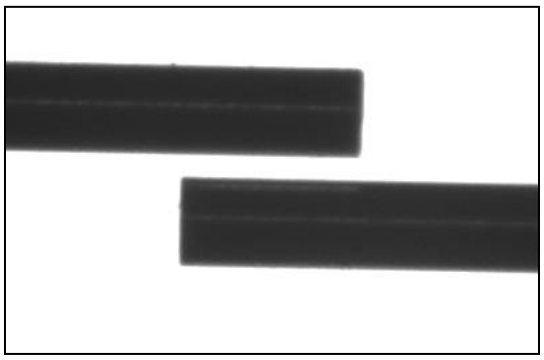

Figure 40: Technical limitation with the vessel test device. An image of the 22 gauge blunt needles seen under the machine vision system. Misalignment of needle assembly on vessel test device causes the samples to fail during testing.

7.5 Summary

The direct cell seeding approach for generating fully biologic cell and cell-derived ECM based tissue engineered blood vessels is a novel approach. In summary, this study showed proof-of-principle that a direct cell seeding technique may be used as an alternative technology for development of tissue engineered cell-derived vascular grafts. While our work was done to develop tissue tubes of 1.19 m I.D., this technology has the potential capability to accommodate any variation in either the length or the diameter of the developed constructs by simply varying the size of the silicone mandrel and adjusting the number of cells accordingly. This technique can also be used to determine the effect of addition of soluble factors (such as sodium ascorbate, retinoic acid, or insulin) on matrix synthesis and eventually mechanical properties. Additionally, by combining the incorporation of growth factors with a mechanical conditioning regimen in an automated seeding and culturing bioreactor, tissue tubes with improved structural and mechanical properties could be developed. Ultimately, this technology could further be applicable to primary cell isolates or human cells.

References

1. Jones, D.L., et al, *Heart diseases and stroke statistics-2009 Update.* Circulation American Heart Association, 2009. **119**: p. 1-161.
2. Goldman, S., et al., *Long-term patency of saphenous vein and left internal mammary artery grafts after coronary artery bypass surgery.* Journal of the American College of Cardiology, 2004. **44**(11): p. 2149-56.
3. Khot, U.N., *Radial artery bypass grafts have an increased occurrence of angiographically severe stenosis and occlusion compared with left internal mammary arteries and saphenous vein grafts.* Circulation 2004 **109** (17): p. 2086-91.
4. Baan, J., et al, *Vessel wall and flow characteristics after carotid endarterectomy: eversion endarterectomy compared with Dacron patch plasty.* Eur J Vasc Endovasc Surg, 1997. **13**(6): p. 583-591.
5. Lau J.M., et al, *Use of Substitute Conduits in Traumatic Vascular Injury.* The Journal of Trauma, 1977. **17**(7).
6. Demiri E.C., Iordandinis, S.L., Mantinaos CF., *Experimental use of prosthetic grafts in microvascular surgery.* Handchir Mikrochir Plast Chir, 1999. **31**(2): p. 102-106.
7. Harris, J.R, Seikaly, H. *Evaluation of polytetrafluoroethylene micrografts in microvascular surgery.* The Journal of otolaryngology, 2002. **31**(2): p. 89-92.
8. Conte, M.S., *The ideal small arterial substitute: a search for the Holy Grail?* . FASEB Journal, 1998. **12**: p. 43-45.
9. Hoerstrup S.P.,et al, *Tissue engineering of small caliber vascular grafts.* Eur J Cardiothorac Surg, 2001 **20**(1): p. 164-9.
10. Niklason L.E.,et al, *Functional arteries grown in vitro.* Science, 1999. **284**(5413): p. 489-93.
11. Gong, Z., Niklason, L.E. *Small-diameter human vessel wall engineered from bone marrow-derived mesenchymal stem cells.* The FASEB Journal, 2008. **22**: p. 1635-1648.
12. Opitz, F. et al, *Tissue Engineering of Ovine Aortic Blood Vessel Substitutes Using Applied Shear Stress and Enzymatically Derived Vascular Smooth Muscle Cells.* Annals of Biomedical Engineering, 2004. **32**(2): p. 212-222.
13. Opitz, F. et al, *Tissue engineering of aortic tissue: dire consequence of suboptimal elastic fiber synthesis in vivo.* Cardiovascular Research 2004. **63**: p. 719-730.
14. Prevel, C. D.et al, *Experimental evaluation of small intestinal submucosa as a microvascular graft material.* Microsurgery, 1994. **15**: p. 586-593.
5. Seliktar, D., et al, *Dynamic Mechanical Conditioning of Collagen-Gel Blood Vessel Constructs Induces Remodeling In Vitro.* Annals of Biomedical Engineering, 2000. **28**: p. 351-362.
16. Cho, S., et al, *Small-Diameter Blood Vessels Engineered With Bone marrow derived cells.* Annals of Surgery, 2005. **241**(3): p. 506-515.
17. Kaushal, S., et al, *Functional small-diameter neovessels created using endothelial progenitor cells expanded ex vivo.* Nature Medicine, 2001. **7**: p. 1035-1040.
18. Berglund, J.D., et al, *A biological hybrid model for collagen-based tissue engineered vascular constructs.* Biomaterials 2003. **24**: p. 1241-1254.
19. Grassl E.D., et al, *Fibrin as an alternative biopolymer to type-I collagen for the fabrication of a media equivalent.* Journal of Biomedical Materials Research, 2002. **60**: p. 607-612.
20. Hirai, J., Matsuda,T., *Venous reconstruction using hybrid vascular tissue composed of vascular cells and collagen tissue regeneration process.* Cell Transplant, 1996. **5**: p. 93-105.
21. Stegemann, J.P., et al, *Mechanical, biochemical, and extracellular matrix effects on vascular smooth muscle cell phenotype.* J Appl Physiol 2005. **98**: p. 2321-2327.
22. Weinberg, C.B., Bell, E., *A blood vessel model constructed from collagen and cultured vascular cells.* Science, 1986. **231**: p. 397-400.

23. L'Heureux, N., et al., *In vitro construction of a human blood vessel from cultured vascular cells: a morphologic study.* Journal of Vascular Surgery 1993. **17**: p. 499-509.
24. L'Heureux, N., et al., *A completely biological tissue-engineered human blood vessel.* FASEB Journal, 1998. **12**: p. 47-56.
25. L'Heureux, N., et al, *Human Tissue Engineered Blood Vessel For Adult Arterial Revascularization.* Nat Med., 2006. **12**(3): p. 361-365.
26. L'Heureux, N. and McAllister, T.N., *Tissue-Engineered Blood Vessel for Adult Arterial Revascularization.* New England Journal of Medicine, 2007. **357**(14): p. 1451-1453.
27. McAllister, T.N. et al, *Effectiveness of haemodialysis access with an autologous tissue-engineered vascular graft: a multicentre cohort study.* Lancet, 2009. **373** p. 1440–46.
28. Xue, L., Greisler, H.P., *Biomaterials in the development and future of vascular grafts.* Journal of vascular surgery 2003. **37**: p. 472-480.
29. Schmidt C.E., Baier, J.M., *Acellular vascular tissues: natural biomaterials for tissue repair and tissue engineering.* Biomaterials, 2000. **21**: p. 2215–2231.
30. Dahl S.L., et al, *Decellularized native and engineered arterial scaffolds for transplantation.* Cell Transplant, 2003. **12**: p. 659-666.
31. Birinyi, K.L., et al., *Increased resistance to bacteremic graft infection after endothelial cell seeding.* Surgery 1987. **5**: p. 193-197.
32. Bricker, D., Beall, A., DeBakey, M. *The differential response to infection of autogenous vein versus Dacron arterial prosthesis.* Chest, 1970. **58**: p. 566-570.
33. Schwartz, E., et al, *Changes in the Components of Extracellular Matrix and in Growth Properties of Cultured Aortic Smooth Muscle Cells upon Ascorbate Feeding.* The Journal of Cell Biology, 1982. **92**(2): p. 462-470.
34. Ogle B.M, Mooradian. D.L., *Manipulation of remodeling pathways to enhance the mechanical properties of a tissue engineered blood vessel.* J Biomech Eng, 2002. **124**(6): p. 724-33.
35. Konig, G. et al, *Mechanical properties of completely autologous human tissue engineered blood vessels compared to human saphenous vein and mammary artery.* Biomaterials, 2009. **30**: p. 1542-1550.
36. L'Heureux, N., et al., *Human tissue-engineered blood vessels for adult arterial revascularization.* Nat Med, 2006. **12**(3): p. 361-5.
37. L'Heureux, N., McAllister, T.N., de la Fuente, L.M., *Tissue-engineered blood vessel for adult arterial revascularization.* N Engl J Med, 2007. **357**(14): p. 1451-3.
38. Stegemann,J.P., Kaszuba, S.N., Rowe, S.L., *Review: Advances in Vascular Tissue Engineering Using Protein-Based Biomaterials.* Tissue Engineering, 2007. **13**(11): p. 2601-2613.
39. Reape, T.J., Groot, E., *Chemokines and atherosclerosis.* Atherosclerosis, 1999. **147** (2): p. 213-225.
40. USRDS 2008 Annual Data Report: *Atlas of Chronic Kidney Disease and End-Stage Renal Disease in the United States*. 2008, National Institutes of Health, National Institute of Diabetes and Digestive and Kidney Diseases: Bethesda.
41. *Vascular Access for Hemodialysis.* National Institutes of Health, 2008. **08–4554**
42. Dobrin, P.B. et al, *Mechanics of end-to-end artery-to-PTFE graft anastomoses.* Annals of Vascular surgery, 1998. **12**(4): p. 317-323.
43. AbuRahma, A.F. DeLuca, J.A., *Multiple nonanastomotic aneurysms in an external velour ringed dacron felnoropopliteal vascular prosthesis.* Annals of Vascular surgery, 1995. **9**(5): p. 493-496.
44. Opitz, F., et al, *Tissue engineering of aortic tissue: dire consequence of suboptimal elastic fiber synthesis in vivo.* Cardiovascular Research 2004. **63**: p. 719-730.
45. Amiel, G.E., et al, *Engineering of Blood Vessels from Acellular Collagen Matrices Coated with Human Endothelial Cells.* Tissue Engineering, 2006: p. 12(8): 2355-2365.
46. Schaner, P.J.et al, *Decellularized vein as a potential scaffold for vascular tissue engineering.* Journal of vascular surgery, 2004. **40**(1): p. 146-153.

47. Baier, C.E., *Acellular vascular tissues: natural biomaterials for tissue repair and tissue engineering.* Biomaterials, 2000. **21**(22): p. 2215-2231.
48. Sandusky, G.E., et al, *Histologic findings after in vivo placement of small intestine submucosal vascular grafts and saphenous vein grafts in the carotid artery in dogs.* American Journal of Physiology 1992. **140**: p. 317-324.
49. Hirai J., et al, *Highly oriented, tubular hybrid vascular tissue for a low pressure ciculatory system.* American society for artificial internal organs, 1994. **40**: p. 383-388.
50. Tranquillo R.T.,et al, *Magnetically orientated tissue-equivalent tubes: application to a circumferentially orientated media-equivalent.* Biomaterials, 1996. **17**: p. 349-357.
51. Neidert M.R.,et al, *Enhanced fibrin remodeling in vitro with TGF-beta1, insulin and plasmin for improved tissue-equivalents.* Biomaterials, 2002. **23**: p. 3717-3731.
52. Neumann, T., Nicholson, B.S., Sanders, J.E. *Tissue engineering of perfused microvessels.* Microvascular Research, 2003. **66**(1): p. 59-67.
53. King, L.S., *Vital staining of the connective tissues.* Journal of Experimental Medicine, 1938. **68** (1): p. 63-72.
54. Aguirre, R., *Inflammation in the vascular bed: Importance of vitamin C.* Pharmacology & Therapeutics, 2008. **119**: p. 96-103.
55. Murad, S., et al, *Regulation of collagen synthesis by ascorbic acid.* Biochemistry, 1981. **78**(5): p. 2879-2882.
56. Swartz, D.D., et al, *Engineering of fibrin-based functional and implantable small-diameter blood vessels.* Am J Physiol Heart Circ Physiol 2005. **288**: p. H1451–H1460.
57. Winder S.J., et al, *Characteristics of ruminant mammary epithelial cells grown in primary culture in serum-free medium.* . Journal of Dairy Research, 1992. **59**: p. 491-498.
58. Elliott J.T., et al, *Thin Films of Collagen Affect Smooth Muscle Cell Morphology.* . Langmuir, 2003. **19**: p. 1506-1514.
59. Weinberg, C.B., Bell, E., *A Blood Vessel Model Constructed from Collagen and Cultured Vascular Cells.* . Science AAAS, 1986. **231**: p. 397-400.
60. Elsdale T., *Collagen substrata for studies on cell behavior.* The journal of Cell Biology, 1972. **54**: p. 626-637.
61. Schor,S.L., *Cell proliferation and migration on collagen substrata in vitro.* Journal of Cell Science 1980. **41**: p. 159-175.
62. Feng Z., Nakamura T., *Measurements of the mechanical properties of contracted collagen gels populated with rat fibroblasts or cardiomyocytes.* . Journal of Artificial Organs, 2003. **6**: p. 192-196.
63. Hirai J, Matsuda, T., *Self-organized, tubular hybrid vascular tissue composed of vascular cells and collagen for low-pressure-loaded venous system.* Cell Transplant 1995. **4**(6): p. 597-608.
64. Kobashi T, Matsuda, T., *Fabrication of compliant hybrid grafts supported with elastomeric meshes.* Cell Transplant, 1999. **8**(5): p. 477-488.
65. Konig, G. et al *Mechanical properties of completely autologous human tissue engineered blood vessels compared to human saphenous vein and mammary artery.* Biomaterials, 2009. **30**: p. 1542-1550.
66. Berglund, J.D., et al, *A biological hybrid model for collagen-based tissue engineered vascular constructs.* Biomaterials 2003. **24**: p. 1241-1254.
67. Neumann, T. et al, *Tissue engineering of perfused microvessels.* Microvascular Research, 2003. **66**(1): p. 59-67.
68. O'Cearbhaill, E.D. et al, *Response of mesenchymal stem cells to the biomechanical environment of the endothelium on a flexible tubular silicone substrate.* Biomaterials, 2008. **29**: p. 1610-1619.