Design and Testing of a Novel Cell Seeding and Bioreactor System for the In-Vitro Growth of Scaffold-Free Tissue Tubes for Tissue Engineered Blood Vessels

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Abstract

During this project we developed a new and more efficient system for creating tissue engineered tubes for small diameter blood vessel implants. Our objectives were to find a method for seeding cells in a tubular shape and provide nutrients and waste removal throughout growth. We achieved these objectives by designing a custom bioreactor system that provides a sterile environment for tissue tube growth. The outcome of this project provided proof that scaffold-free tissue tubes can be created using our method.

Acknowledgements

We would like to thank Lisa Wall for her continuous support acquiring necessary lab materials and equipment. Thanks are also due to Tracy Gwyther, for her guidance and aid in determining a suitable method for safely removing our tissue tubes. Many thanks to Rob Lowry for his assistance designing and manufacturing the automatic rotational aspect of the final prototype. We would also like to thank Russell Morin for producing the rapid prototypes for the tube cartridge, and Adriana Hera for her guidance and knowledge of the FLUENT program. Finally, we would like to thank Zoe Reidinger for introducing us to cell-seeding methodology and Jason Hu for cell culture assistance.

Authorship

The final version of this paper was written in equal parts by all group members. All group members were involved in editing all sections of the paper.

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1.0 Introduction

Coronary heart disease is a major malady facing the population of the United States which causes approximately one in every five deaths. In 2006 alone, over 16 million Americans suffered from some manifestation of coronary heart disease, including myocardial infarction, acute coronary syndrome and angina pectoralis. The American Heart Association predicts that this number will have risen by 785,000 victims in 2009 alone. In 2006, 448,000 coronary artery bypass graft (CABG) procedures were performed, with a mean cost of approximately $100,000 per procedure.[1] There is currently a critical need for a suitable small diameter (<6mm outer diameter) coronary artery analog for CABG procedures and, to a lesser extent, vascular replacement in the lower extremities.[2] Currently, there are three standard sources which can be used to replace these vessels. These sources are: autologous grafts, synthetic blood vessel replacements, and tissue engineered blood vessels.

The present state of the art for CABG procedures is the use of autologous vessels directly from the patient[2]. These vessels are typically extracted from either the internal mammary artery in the chest, or the saphenous vein in the posterior lower leg.[2] The major advantage of using autologous vessels is that, since they are retrieved directly from the patient, there is no possibility of immune rejection. The major limiting factors for this method of blood vessel replacement are the availability of blood vessels and the age of the patient. Since many patients who receive one bypass graft will eventually return for a second procedure,[1] there is a limited length of blood vessel which can be retrieved autologously. Additionally, the majority of patients who experience coronary artery diseases are in their mid-to-late 60's[1] and therefore the available autologous vessels are typically afflicted with atherosclerosis. These atherosclerotic blood vessels do not have sufficient elastic properties to resist the pressures a bypass graft would be subjected to in vivo.[3]

A second method used for synthetic grafts within the body is completely synthetic blood vessels. These grafts have been successfully applied to large diameter applications, such as in the aorta, but have shown poor performance in small diameter applications due to their high susceptibility to thrombosis.[2] As a result, many researchers have focused their studies on non-synthetic materials for small diameter replacements.

The third small diameter artery analogs that are being explored are tissue engineered blood vessels (TEBV's).[2,4] Several approaches have been taken to produce viable blood vessels using tissue engineering techniques. Two distinct approaches that have been studied are scaffold-based and scaffold-free tissue engineered blood vessels. A scaffold is a material on which cells are seeded in order to provide structural and mechanical support for the cells. The scaffold may or may not degrade over time and has been successfully used to form a variety of cell types into blood vessels.[5] Scaffold-free approaches to developing tissue engineered blood vessels are a more recent, though increasingly popular, topic of research in the field of tissue engineering. Examples of scaffold-free approaches include bioprinting,[6] tissue-sheet rolling,[4] and growth on collagen matrices.[7] The benefits of scaffold-free blood vessel engineering may include improved mechanical properties, stronger cellular adhesion within the tissue, and the elimination of byproducts from the degradation of a scaffold. These approaches, along with other methods of tissue engineering will be examined in detail in the literature review.

Currently, scaffold-free tissue engineering is resource intensive, expensive and requires a large amount of direct manipulation by researchers.[8] In this project we aim to alleviate the amount of time and effort required to grow tissue engineered blood vessels. Additionally, we will attempt to increase the rate at which viable blood vessels can be grown for either clinical or research applications.

The overall goal of this project is to design a system that efficiently and reproducibly grows rat aortic smooth muscle cells (RSMCs) into a viable cylindrical construct on a tubular mandrel. In order to achieve this goal, we have divided our project into three separate objectives. The first objective is to design a tubular mandrel on which we can seed cells. The mandrel must both promote cell adhesion and allow for easy removal of the final tubular construct once the cells have proliferated. The second objective is to develop a method by which we can stimulate cells to attach to the mandrel and proliferate. The third and final objective is to incorporate our cell seeding process and mandrel design into a bioreactor which can be used to grow viable tissue engineered blood vessels with limited user effort.

In order to accomplish the goals of our project, we began by approaching our three objectives individually. We started by devising conceptual designs by which we could accomplish the three top objectives of our project. After a period of extensive brainstorming and

idea development, we began to consolidate our concepts into complete preliminary designs. In the end we developed four preliminary designs that we decided warranted further testing. We then devised a set of quantitative metrics, which identified for us what would define a design that met our objectives. Each of our four designs was compared to these metrics and rated on how well we believed it would meet them based on preliminary testing. Additionally, for these four designs, we devised simple experiments that tested the efficacy of various important aspects of the design. Over time we determined that different designs were not feasible and eliminated designs to narrow our focus. In the end, we determined that one of our designs was the most promising based on our objectives, constraints and initial tests and chose to pursue that design further. We also continued testing a secondary design to ensure that we had a failsafe if our main design was unsuccessful. In the final stage of our project, we tested our entire apparatus to show that it produces tissue tubes that fall into our predefined metrics, design constraints, and objectives.

2.0 Background

This section will describe the background and current state of the art in the literature for various aspects of our project. We will begin by discussing the clinical significance of small diameter vascular grafts. Next we will discuss the general anatomy of a blood vessel and the molecular mechanisms necessary for cell attachment and later release of completed tissue tubes. Finally, we will discuss currently marketed small diameter vessels, the state of the art for tissue engineered blood vessels and the various types of bioreactors that are used in the lab to date.

2.1. Clinical Significance and Blood Vessel Pathology

Diseases affecting blood vessels are a prominent cause of medical problems throughout the world and, since 1997, have been determined to be the most common cause of death in the United States.[1] There is a wide range of diseases that can affect the blood vessels in the body, one of the most common being atherosclerosis. Atherosclerosis is a condition in which blood vessels are partially blocked by plaque buildup, which disrupts the flow of blood to parts of the body. The initial symptoms may not be obvious to patients or doctors; however a symptomless patient with atherosclerosis can rapidly degenerate into myocardial infarction or stroke. Atherosclerosis can cause coronary artery disease, carotid artery disease, and peripheral arterial disease, which are all problematic diseases as explained below.

Peripheral artery disease occurs when blood vessels that supply blood to the legs, arms, and pelvis become blocked. This condition can cause numbness, pain, and occasionally infections of the body. Carotid artery disease is caused when the carotid artery becomes blocked. A problematic condition that can arise due to carotid artery disease is a stroke, which involves cutting off blood supply to the brain and is the third most common cause of death in the United States each year. Finally, coronary artery disease is a blockage of the coronary artery and this is the main cause of myocardial infarction, leading to ischemic death of a portion of heart muscle.

According to the National Heart, Lung and Blood Institute, Coronary Artery Disease (CAD) was the leading cause of death in the United States in 2005.[1] There were nearly 500,000

deaths in the United States in 2005, making up about one fifth of all deaths in the US. Additionally, 16,800,000 people were victims of angina (chest pain due to coronary artery disease), heart attack, or CAD in general. This represents approximately 8.8% of the male population and 9.0% of the female population in the United States.[1]

Based on the high percentage of the population affected by coronary artery disease and the severe, and sometimes fatal, problems relating to CAD, treatment options will be of critical importance in the future of medicine in the United States. A current solution to this problem is coronary artery bypass grafting, (CABG), in which vascular grafts are surgically implanted to bypass the blockage and restore flow. Graft sources include synthetic, autologous, or tissue engineered grafts. In order to understand the various aspects of these grafts, we must first examine the anatomy and physiology of blood vessels. This project will focus on the field of tissue engineering, though a firm understanding of current technology is important in understanding the relevance of the project as a whole.

2.2. Structure and Function of Blood Vessels

Blood vessels are tubular structures that supply blood to all of the tissues and organs of the body. Blood vessels have an important physiological role and are responsible for the transport and regulation of blood flow throughout the body. Although blood vessels can be divided into several different vessel types, all blood vessels are composed of the same three cellular layers. In this section, we will first discuss the basic blood vessel anatomy and the different physiological characteristics of each cell layer. Next, we will discuss the different blood vessel types and how they differ. Finally, since our project will focus on smooth muscle cells, we will discuss the physiology behind the contraction of smooth muscle.

2.2.1. Blood Vessel Structure

All vascular tissue is composed of three anatomically distinct cellular layers. These layers are, from innermost to outermost, the tunica intima, the tunica media and the tunica adventitia. Each of these layers has specific structural and functional characteristics which allow the blood vessels to effectively carry out their role.

The tunica intima is the only layer of vascular tissue which comes into contact with blood. The tunica intima is formed exclusively of endothelial cells which are only one layer thick. These cells function to allow nutrient flow into and out of the lumen of the vessel and prevent blood clotting factors from coagulating and stopping blood flow.

The tunica media is composed of many layers of smooth muscle cells (SMCs). These cells provide the primary mechanical support for the vessel and help to prevent rupture due to blood pressure. Additionally, this layer allows the vessel to be contracted or dilated by the nervous system to alter blood flow to different areas of the body. This gives the brain precise control over what parts of the body are receiving the most nutrients and oxygen at any given time.

The final vascular layer, the tunica adventitia, is responsible for connecting blood vessels to the surrounding tissue and providing additional mechanical support. This layer is composed primarily of loosely woven collagen fibers and can also contain small capillaries to provide nutrients to the outer layers of the tunica media.

2.2.2. Blood Vessel Types

Although all types of blood vessels have the same three basic layers, the prominence of different layers differs between vessel types. Vasculature can be divided into five different blood vessel types: arteries, arterioles, capillaries, venules, and veins. During somatic circulation, the arteries and arterioles are the first vessels to receive blood from the heart and thus have a thick tunica media to resist bursting. These vessels have thick elastic walls which can withstand high pressures. The veins and venules, on the other hand, receive blood at the end of the circulatory cycle and thus have lower internal pressures. As a result, veins and venules have much thinner walls then arteries. Finally, capillaries are the thinnest vessels and are only composed of the tunica intima. Capillaries facilitate the transfer of nutrients and gases into body tissues and rely on surrounding tissues to support them structurally

2.2.3. Smooth Muscle Physiology

Since our project focuses specifically on growing the tunica media layer of the blood vessel, it is very important that we understand the physiological mechanisms controlling smooth muscle contraction. When smooth muscles contract, an electrochemical action potential is propagated through the cell membrane and into the sarcoplasmic reticulum; resulting in a

chemical pathway which activates cell contraction. In vivo, this pathway is typically activated by the sympathetic nervous system.[11] During sympathetic activation, a neurotransmitter activates the electrochemical action potential in the muscle cells. Previous studies have also found that muscle contraction can be activated by solutions with concentrated potassium ions. This process works because the high extracellular concentration of potassium ions causes the spontaneous depolarization and contraction of the smooth muscle cells.[12] The contractile nature of smooth muscle cells can be used in applications for vascular replacement.

2.3. Vascular Replacements

There are three prevalent vascular grafts that are currently being used or researched. These three main groups are synthetic grafts, autologous grafts, and tissue engineered grafts. Synthetic grafts are made of materials that are not naturally found in the body. Autologous grafts are blood vessels harvested from other areas of the patient's body and used to replace diseased vessels. In contrast, tissue engineered grafts are grown in a variety of ways by utilizing current cell seeding methods and creating an in vitro environment similar to that of the body. The current approaches for vascular grafts each have their own advantages and disadvantages as will be discussed in the following sections.

2.3.1. Autologous Grafts

Autologous grafts are currently the "gold standard" for vascular grafts and are almost always used in situations where a CABG procedure is required. The main advantage of an autologous graft is that they inherently possess the properties necessary for use as a blood vessel. Furthermore, since the graft is directly from the patient, there is little risk of rejection of the blood vessel. One disadvantage of an autologous graft is that patients who require bypass grafts may not have a blood vessel suitable for transplant. Another disadvantage is that this procedure requires a secondary surgery to remove the donor blood vessel, resulting in increased hospitalization time and risk of complications for the patient.

2.3.2. Synthetic Grafts

Synthetic vascular grafts are one option for patients that do not have a blood vessel available for transplantation. These grafts consist completely of materials that are not naturally

found in the body, which can lead to clotting, infection, and rejection by the body's immune system. Although they have been proposed as an alternative to autologous grafts, synthetic grafts are not used extensively for small diameter applications because of their high susceptibility to clotting.[13]

2.3.3. Tissue Engineered Vascular Grafts

There are a variety of methods that have been researched in order to produce tissue engineered blood vessels (TEBV). Some of these methods include the rolling of tissue sheets to form a tubular structure, the growth of cells on a scaffold, and the growth of cell tubes without the use of a scaffold. The rolling method involves culturing cells as a sheet, and once they have reached a sufficient thickness, removing the sheet and rolling it into a tube form. Scaffolds are structures that provide support to growing cells and are advantageous because they encourage the growth of cells into three dimensional tissues, allowing the cells to be manipulated into a variety of structures. Scaffolds may degrade as the cells proliferate in order to eventually have a completely cell based graft. Ideally, the cells will grow and synthesize extracellular matrix (ECM) at approximately the same rate as the polymer scaffold is resorbed, thus creating a structurally stable tissue tube.[14] Finally, scaffold-free mandrel based designs have also been researched and will be the primary focus of this study. A mandrel, in this application, is a cylindrical structure on which a tissue engineered blood vessel will be grown and/or supported. Mandrels may be made of a variety of materials depending on their exact application. Because the tissue engineered blood vessel will be supported by the mandrel, the outer diameter of the mandrel directly determines the inner diameter of the TEBV that is grown.

This section will examine the advantages and disadvantages of different methods of growing tissue engineered vascular grafts as described in literature to date. The two main categories examined are scaffold-based and scaffold-free grafts. Scaffold materials and designs currently in use can be broadly divided into three separate groups: natural scaffold materials, permanent synthetic scaffolds and resorbable synthetic scaffolds. Additionally we will discuss scaffold free methods in detail, as this method is the primary focus of our project.

Scaffold Based Grafts

The use of scaffolds in tissue engineering has proven to be a successful method for growing many different types of tissue. Various research groups have used scaffolds to culture cells and create multilayer tissue engineered blood vessels. One challenging aspect of using a scaffold is determining its degradation rate and matching it to an appropriate cellular growth rate. This is important because without the support of the scaffold, the blood vessel may burst under arterial pressures in vivo.

Biological Scaffold Materials

Biological scaffold materials have been used in many studies to date. These scaffolds can either consist of decellularized ECM harvested from natural sources,[15] or be composed of natural protein scaffolds that have been grown in vitro.[14]

Decellularized ECM is advantageous in comparison to other materials simply because it is harvested directly from biological sources and therefore does not typically cause fibrotic encapsulation.[16] Problems exhibited by these materials include: structural degeneration, breakdown of vascular walls over time, and overall insufficient mechanical properties.[15] Additionally, these scaffolds produce vessels that can cause infections, form aneurisms, or induce thrombosis.[14]

Scaffolds composed of natural proteins, such as collagen, fibrin, or fibronectin are ideal for cell adhesion because they are naturally synthesized by cells as part of ECM structures in the body. Historically, the first adhesive gels were made from collagen.[17] Collagen gels have been shown to yield a high percentage of circumferentially aligned cells, which closely resembles alignment in natural blood vessels.[14] Additionally, since collagen gels have been FDA approved for implantation in other applications, they can more easily be approved for similar applications. In some collagen studies, it has been shown that the resultant blood vessels cannot withstand vascular pressures that are found in the body.[18] In an attempt to improve these results, collagen has been cross linked with elastin providing the vessels with a greater elasticity and better mechanical properties. Although the collagen/elastin gels did provide better structural characteristics than pure collagen gels, they still could not withstand the pressures experienced by small diameter arteries.[14]

In more recent studies, collagen gels have been replaced with fibrin gels.[18] Fibroblasts, when implanted in fibrin gels, produce more ECM than in collagen. This has resulted in a more mechanically stable structure. In one study, blood vessels grown on fibrin scaffolds were successfully implanted in the jugular veins of sheep[19]. Despite the increased mechanical integrity, fibrin gels have still fallen short of providing the mechanical properties necessary for small diameter artery implantation without extensive mechanical conditioning.

Synthetic scaffolds

To date, many studies have examined the use of synthetic scaffolds for the growth of arterial substitutes. For these vessels, a biodegradable scaffold is used which is designed to be implanted with the vessel and degrade over time. More recently, some studies have begun to examine methods for growing vessels on bioinert materials, which do not integrate with the tissues or vessels that are grown.

Like natural scaffolds, synthetic scaffolds have been developed which support smooth muscle cell growth and ECM production. These arterial substitutes have shown great promise as viable arterial replacements.[15] Niklason et al. used poly glycolic acid (PGA) scaffolds in a pulsatile pressure bioreactor for 8 weeks.[20] The vessels resulting from this work were able to withstand burst pressures of greater than 2000 mmHg. In a similar study, Shum-tim et al. grew a mixture of smooth muscle and endothelial cells on a copolymer of PGA and polyhydroxyalkanoate (PHA).[21] The resulting vessels were implanted into lamb aortas with a 100% patency rate. The major problem facing this area of research is the long preparation time necessary for vessel growth if the scaffolds are allowed to grow cells prior to implantation. Due to the urgency of most clinical cases that would require a tissue engineered blood vessel, a more than 2 month waiting period from cell harvest to blood vessel implantation is not plausible.[15]

Scaffold Free Vascular Grafts

In the scaffold free approach, bioinert synthetic mandrels are typically used as cylindrical structures around which different cellular constructs are grown. This process differs from using synthetic scaffolds in that a mandrel made of a synthetic material provides support to the cells and tissues but does not integrate with the cells. L'Heureux et al., the pioneers in the field of scaffold free vessel engineering, used a poly tetra-fluoro-ethylene (PTFE) mandrel wrapped in a

smooth muscle sheet then an outer fibroblast layer. After the cells had generated the two tissue layers, the PTFE was removed and the lumen of the vessel was seeded with endothelial cells. The vessels were then implanted in animal models. These initial vessels only resulted in about 50% patency rates after one week.[22]

Other researchers have used a novel approach to growing scaffold free tubes. Chue et al. have grown blood vessels in the bodily cavities of dogs with some success. In this method, several different inert polymer materials were arthroscopically implanted into either the peritoneal or pleural cavities of dogs.[23] These tubes were then explanted 3 weeks later, and found to be coated in a sheet of living tissue. After removal, the tissue tubes were tested for burst pressure strength and found to burst at greater than 2500 mmHg, a value similar to that of a canine femoral artery. Despite its apparent success, this approach to tissue engineering blood vessels presents several problems. First and foremost, the blood vessels must be implanted in the patient's body for three weeks before they can be used—requiring a second surgery. Secondly, despite their phenomenal burst strength, the vessels grown in this method are not composed of smooth muscle, and therefore do not respond to medications or hormones in the same way as natural blood vessels.[23]

Another novel approach to scaffold free tissue engineering that has recently been published is the work of Norrotte et al.[6] In this work, several lines of cellular spheroids are "printed" by a mechanical device in a three dimensional agarose gel. As these cellular spheroids grow, they form into a tubular construct which is composed entirely of cellular matter. The resulting blood vessel produced by bioprinting has not yet been shown to have the mechanical properties necessary for implantation.[6]

Researchers such as L'Heureux et al. have rolled cell sheets to form tissue engineered blood vessels that have adequate mechanical properties.[4] As a result, current methods being used specifically for engineering cell sheets have the potential to be applied to tissue engineering of blood vessels.

Another application of rolled cell sheet tissue engineering is Cytograft's tissue engineered blood vessel. Cytograft's process involves taking a small dermal tissue sample from the patient and culturing these cells in sheet form, then using the sheets to mold into a tube. Researchers from Cytograft have shown that the rolled blood vessels have comparable, if not

stronger, strength than natural blood vessels, despite not using the SMC's typically seen in blood vessels. Possibly the most valuable attribute of this type of tissue engineering is that the TEBVs are made completely of the patient's own cells and therefore will not be rejected. The main disadvantage of this system is the length of time taken to grow the actual blood vessel. With a six to nine month growth time before implantation is possible, Cytograft's product is not an "off-the-shelf" product.[24]

2.4. Current Practices

Current practice in the Rolle Lab involves coating a silicone mandrel with pre-polymerized collagen in order to promote cellular adhesion.[7] This is accomplished by injecting collagen into a custom-made poly-tetrafluoroethylene (PTFE) shell using a syringe. This process is shown in Figure 2-1. The collagen coated silicon mandrel is then seeded with cells by suspending it by its two ends from a silicon washer. The tube is then placed upside down in a hanging drop of media. The entire cell seeding process is depicted in Figure 2-2. Next, the collagen mandrel and cell assembly is incubated until the cells proliferate enough to form a confluent tube. Removal of the vessel from the mandrel is accomplished by manually stretching the silicon tube and sliding the vessel off of the mandrel. This method leaves much to be desired by way of vessel wall uniformity, and mechanical integrity of the vessel upon removal. Additionally, this method is extremely time and resource intensive.

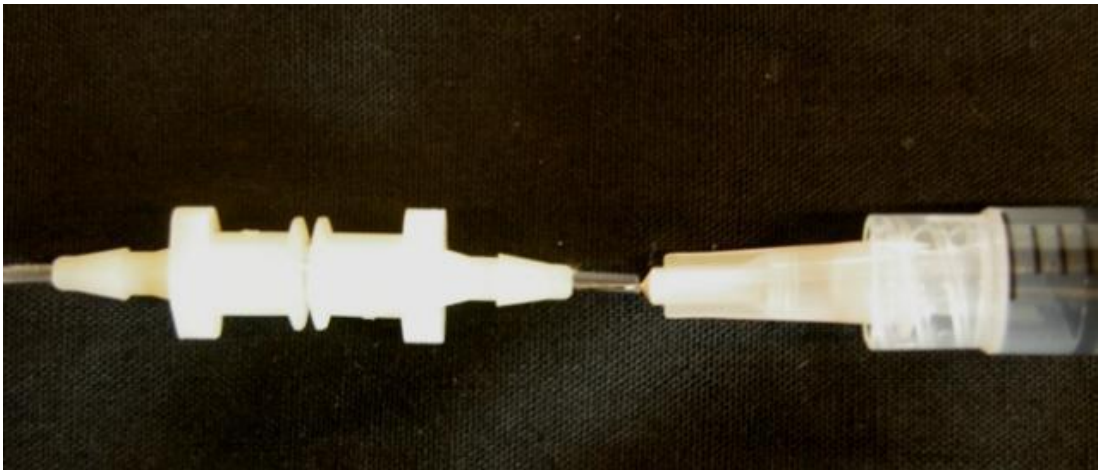

Figure 2-1: A picture of collagen being injected into a custom made PTFE shell to form a silicon coating. This process is part of the current method for creating TEBVs.[7]

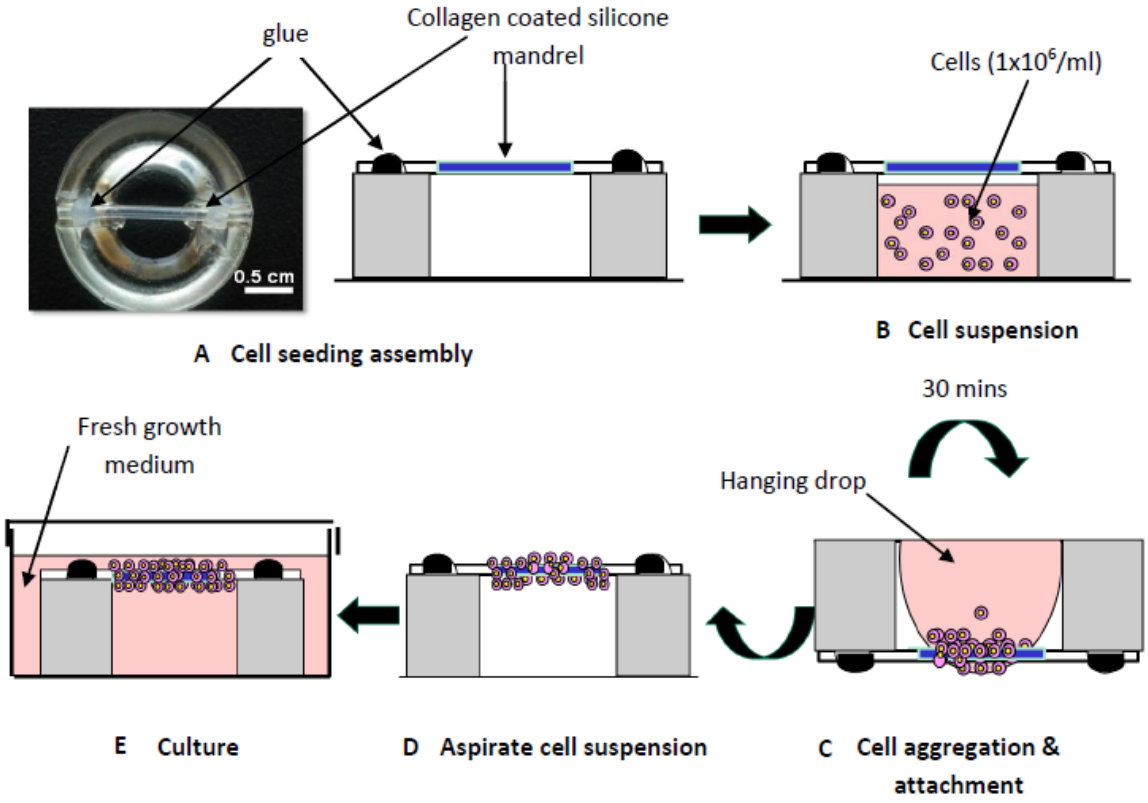

Figure 2-2: Pictorial depiction of the hanging drop method.[7]

2.5. Bioreactors

A bioreactor is any device that supports a biologically active environment. In the culture chamber, environmental conditions such as pH, temperature, pressure, nutrient supply, and waste removal, are tightly controlled to maximize culture growth and viability. Bioreactors are used in many applications such as industrial fermentation processing, wastewater treatment, food processing, the pharmaceutical industry and tissue engineering.

In the field of tissue engineering, bioreactors are used as a means for the production of tissue constructs. Worldwide costs for the substitution of organs are estimated to be 350 billion USD.[24] Successfully engineered tissue constructs could one day replace allograft materials such as bone or cartilage. The tissue engineered constructs would eliminate the inherent risk of pathogenic infections or graft rejection from the allograft materials.[25] Tissue engineered

constructs could also replace artificial implants made from synthetic materials which have problems with lifespan, biocompatibility, and inflammation.

Bioreactors have been used for thousands of years for the fermentation of alcoholic beverages. While yeast and bacteria are encased within a tough cell wall and are able to withstand environmental stresses, mammalian cells are much more delicate. In particular, mammalian cells require a complex nutrient medium for growth and cannot withstand strong shear stresses. The physiological shear stresses in vascular tissue can vary widely. For example, the shear stresses in large arteries away from branches experience from 2-8 Pa. Vein capillaries on the other hand experience shear stresses between 0.1-0.6 Pa.[25] Most mammalian cells also require some sort of substrate surface to attach to in order to proliferate. [23]

2.5.1. Types of Bioreactors

Bioreactors can be classified by the type of flow produced or the mixing characteristics within the culture chamber. The two main groups of reactors are the stirred tank reactor and the tubular flow reactor. In the stirred tank reactor, shown in Figure 2-3, nutrient medium is pumped into the inlet, stirred by some sort of mixer, and then pumped out of the exit. Ideally, the concentration of nutrient medium, gases and other substrates would be uniform throughout the entire tank. This means that any cell within the chamber would always have the same concentration of nutrients and gases as other cells regardless of the position within the chamber. There would also be no “dead pockets” where there is a lack of nutrients or oxygen or any solid aggregates on the surface.

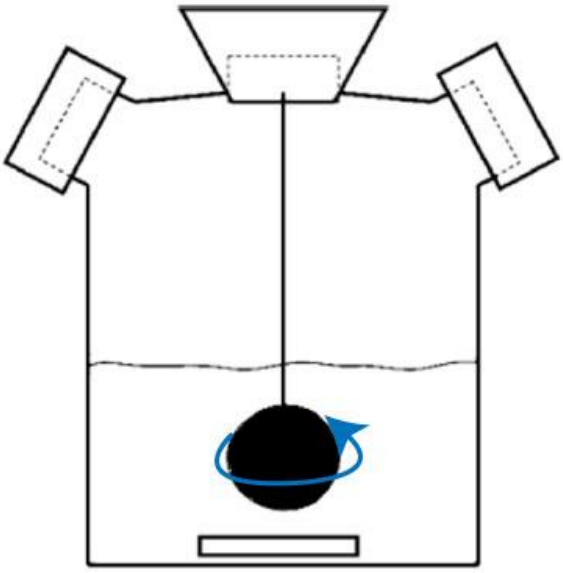

Figure 2-3: A schematic example of a stirred tank bioreactor. This design constantly stirs the media solution to keep nutrients evenly concentrated throughout the culture chamber[26]

A different type of bioreactor, known as the tubular flow reactor, is shown in Figure 2-4. In the tubular flow reactor, nutrient medium is pumped into the inlet, agitated by a rotor, and then pumped out through the exit. Many tubular reactors are used in the production of proteins by mammalian cells. Unlike the stirred tank reactor, the concentration levels of nutrients in the medium vary throughout the length of the reactor. The concentration of nutrients and gases will be high at the inlet and low at the outlet, while the concentration of metabolites will be low at the inlet and high at the outlet. The exact concentrations of nutrients and gases depends on the length of the reactor, metabolic activity of the cells, amount of cells and the initial concentrations of the medium. For this reason, the length of a tubular reactor is limited as the concentration gradient of nutrients, gases, and metabolites inhibit cell growth towards the end of the culture chamber.[26]

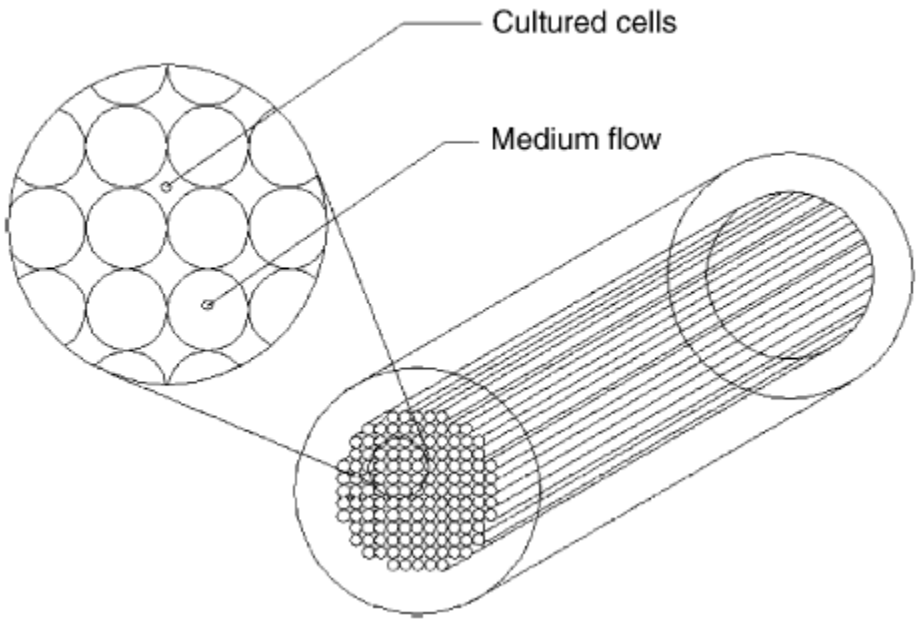

Figure 2-4: A schematic example of a tubular flow bioreactor. In this reactor, the medium flows linearly through the reactor from end to end at a constant rate[26]

One specific type of tubular reactor is the rotating wall vessel bioreactor, as shown in Figure 2-5. The rotation rate of the bioreactor is computed to balance out the gravitational force F_g, the drag force F_d, and the centrifugal force F_c. This creates a micro-gravity like environment in which cells undergo free fall as the bioreactor chamber rotates. The specific example shown in Figure 2-5 is the high aspect ratio vessel (HARV) developed by NASA's Johnson Space Center. In this reactor, the vessel walls rotate at a rate of 12-15 rpm. Nutrient medium is constantly pumped through the inlet and then flows down the center of the chamber and diffuses outward. The rotation of the chamber also serves as a way to agitate and mix the nutrient solution. The cells within the culture chamber undergo very small shear stresses, while the nutrient medium has a high mass transfer rate and high oxygen concentrations. As a result, cells grown in rotating wall vessel bioreactors grow in concentrations that are denser compared to cells grown in conventional stirred-tank bioreactors.[26]

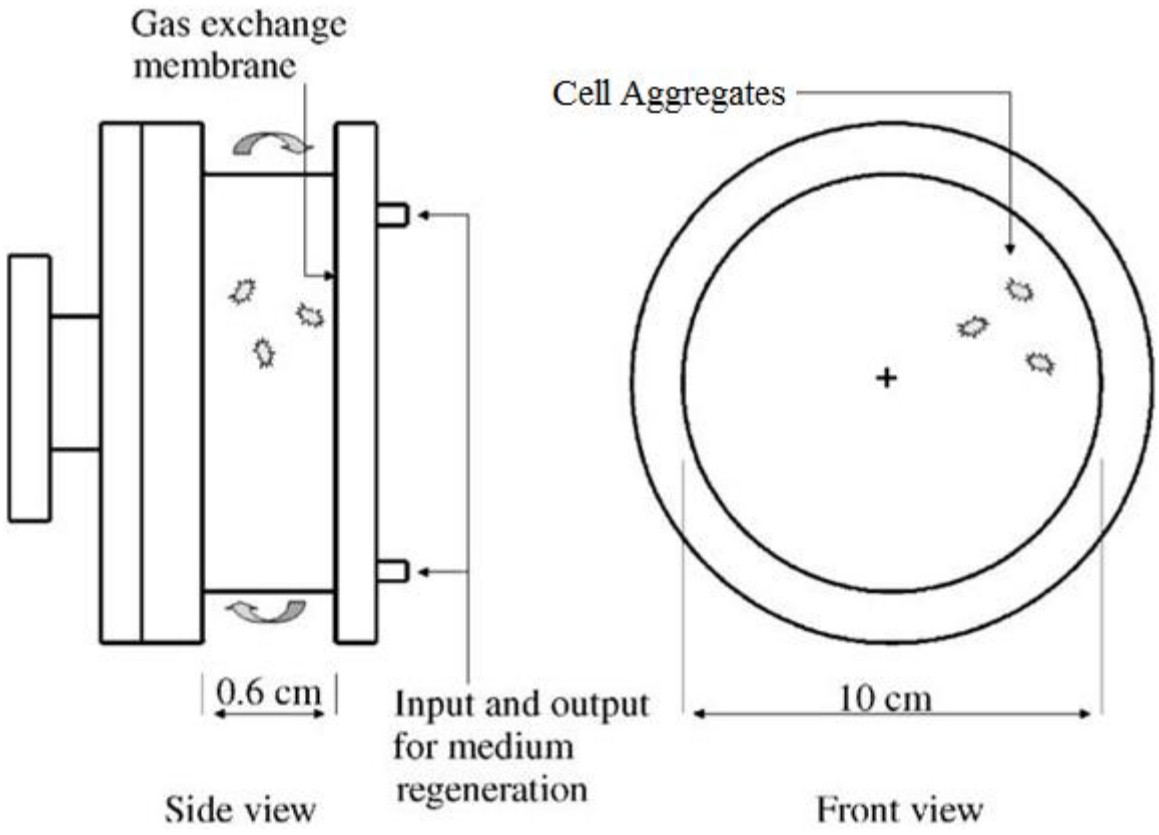

Figure 2-5: Rotating Wall Vessel Bioreactor. In this bioreactor, rotation puts cells into a state of constant free fall causing them to form aggregates[27]

2.5.2. Conditions Inside Bioreactors

The environment within a bioreactor culture chamber must be tightly controlled to produce viable and healthy cells. The temperature, pH and salt levels of the medium must be maintained at optimal levels for cell proliferation. The culture chamber must be sterilized to prevent outside contaminants from entering the culture chamber. Bacteria, fungi, and possibly even pathogens can easily destroy a culture of interest if allowed entry in the chamber. Antibiotics are usually added to prevent growth of unwanted organisms. Growth factors are sometimes added to induce differentiation of certain cell types. The nutrients within the medium such as glucose must be maintained at high enough concentrations to supply the proliferating cell cultures with nourishment. Metabolites such as lactase must also be controlled to prevent high concentrations from inhibiting cell growth.[26]

In addition to the environmental conditions within a culture chamber, other problems with bioreactors must be addressed to create optimal levels of growth and successful cell cultures. In the human body, the majority of cells are within 100 μm of a capillary.[26] These capillaries are able to supply the cells with their nutrient needs and additionally to remove cellular waste. This is a major problem in bioreactors, as the lack of natural vascularization of tissues within cell cultures greatly limits growth and aggregate density. Without proper vascularization for the mass transfer requirements of the cells, necrotic centers form within the cell aggregates once their diameters become too large.[27] Hypoxic conditions of the media also lead to necrotic damage since mammalian cell cultures require high levels of oxygen. Therefore the critical thickness for mammalian cell cultures is 100-200 μm due to the limitations of oxygen diffusion.[27]

2.6. Related Patents

To date, there are several patents which have been developed that are relevant to our project. The patents in this section were chosen for their relation to bioreactor design and as representatives of the current industry standards. We will discuss the various patents and describe the novel concepts that they present.

2.6.1. US Patent 7,510,866 B2

Patent US 7,510,866 B2 is a hybrid bioreactor for cell culture that is able to apply both compressive strain and shear strain. Application of compressive strain promotes the proliferation of cells while shear strain promotes the differentiation of cells. This would be helpful in any bioreactor design. The device, shown in Figure 2-6, has multiple reactor tube assemblies to grow multiple cultures simultaneously. The shear stress is applied by rotating the tube assemblies while the compressive stress is applied by continuously moving the assemblies vertically. The reactor tube assemblies are also able to support a porous culture mandrel that is able to grow cells that adhere to the culture mandrel. If a non porous culture mandrel is not used, then the reactor is also able to grow a culture of cells in suspension instead.[28]

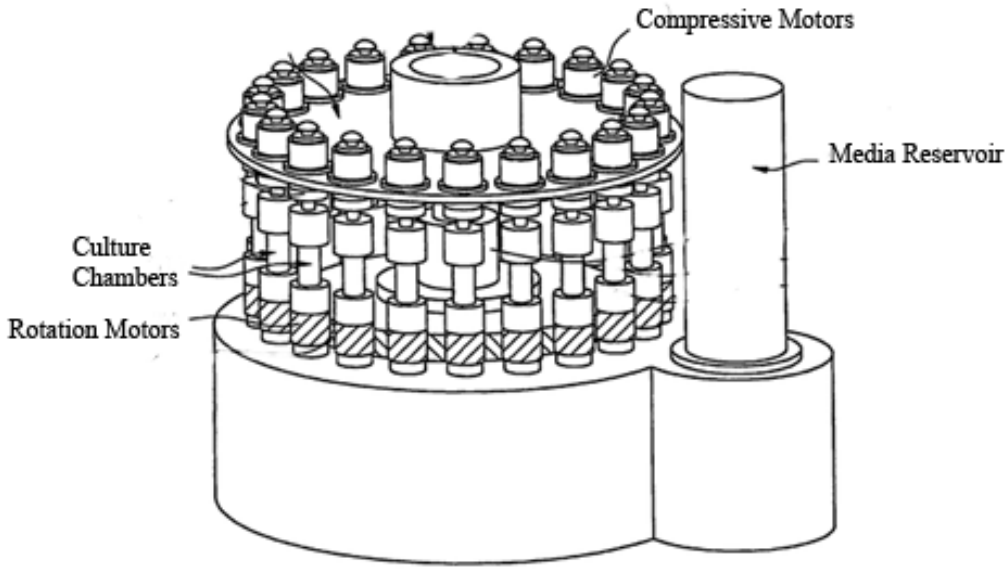

Figure 2-6: Hybrid Reactor for cell culture consisting of multiple rotating culture chambers connected to a single media reservoir[28]

2.6.2. US Patent 7,456,019

US patent 7,456,019 is a three-dimensional cell to tissue development process that is able to grow living mammalian cells with minimal damage from shear stress. The culture chamber, as shown in Figure 2-7, the vessel wall, labeled 27, rotates around the horizontal axis at variable rotation rates to negate gravitational flow. The mandrel material, labeled 23, also rotates around the horizontal axis. The process also includes applying a varying electromagnetic force that is driven by a pulsed square wave exerting magnetic field that ranges from 0.05 to 0.5 gauss. The time varying electromagnetic force is able to significantly increase cell growth and differentiation.[23] The very slow fluid shear stress in the device allows the cell cultures to grow in high densities and into higher order 3-dimensional multi-cellular tissue-like structures compared to other culturing processes. The patent contains additional means to maintain the environment of the culture chamber, as shown in Figure 2-8, such as introducing oxygen, removing wastes, and others.[29]

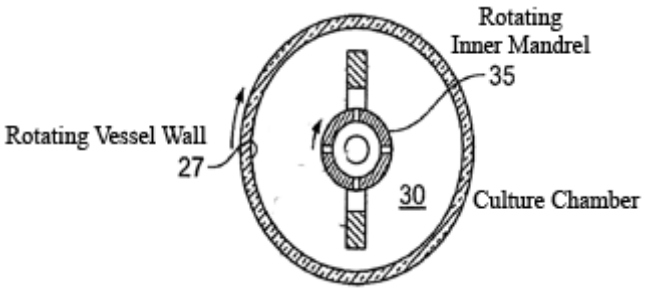

Figure 2-7: Vertical Cross-section of Culture Chamber for Tubular Flow Bioreactor. Both the inner mandrel and outer vessel rotate to negate gravitational flow.[29]

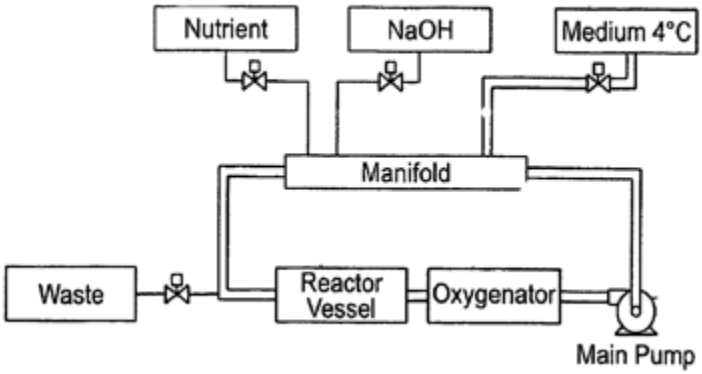

Figure 2-8: Flow Diagram for US 7,456,019 showing the major components which connect to the reactor vessel and the tissue culture chamber[29]

2.6.3. US Patent 7,144,727

Patent 7,144,727 titled "culture chamber for biologicals" is a culture chamber, shown in Figure 2-9 that is composed of one or more membranes that prevent flow of high molecular weight substances. Nutrient medium is pumped through the membrane into the chamber while the metabolites are transported away from the chamber though the membrane and out of the chamber outlet. The molecular weight cut-off membranes allow the nutrients and metabolites to pass through while keeping the culture cells within the chamber. Any other compounds passing through the chamber must have a molecular weight lower than the molecular weight cut-off for the membrane. The device allows for the possibility of having reusable chambers or a chamber housing that contains disposable bioreactor bags. The chamber is attached to a roller drive that rotates the culture chamber around its axis, producing a micro-gravity like environment within the culture chamber.[30]

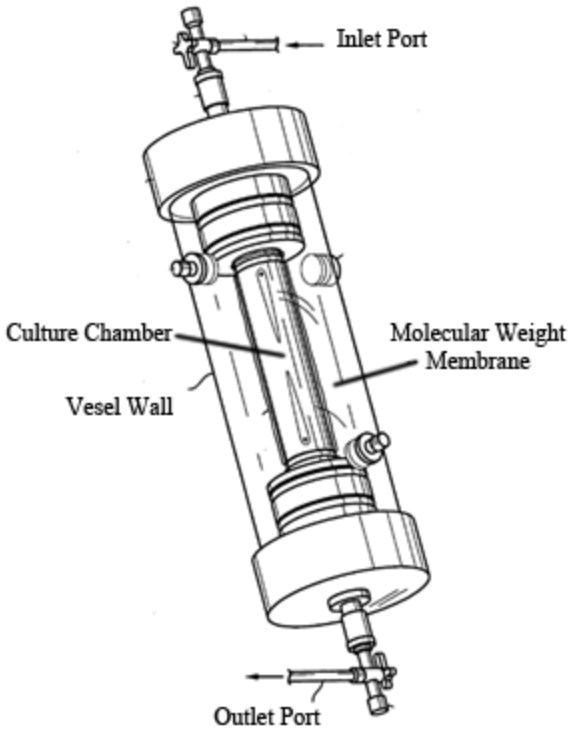

Figure 2-9: Culture chamber for a tubular flow reactor. A molecular weight membrane is used to keep the cells within the inner chamber while media solution can flow out.[30]

2.7. Gaps in the Current Technology

Current technology used for the creation of tissue engineered blood vessels is highly labor intensive and inefficient. It is the aim of this MQP to devise a more efficient and less labor intensive approach to forming tissue tubes without a scaffold. This approach should be more efficient and effective than the method currently used in the Rolle Lab. By paying specific attention to the mandrel material, it may be possible to seed the cells on the mandrel safely and evenly, and ultimately remove the mandrel so as not to damage the vessel once it is ready for mechanical conditioning or testing. Ideally, the process will involve minimal labor from the user and be partially or fully automated. Our primary focus lies in promoting cellular adhesion to a mandrel, creating a confluent tissue tube, and controlling the detachment of the tube from the mandrel at will.

3.0 Project Strategy

This Chapter will outline the strategy we used to approach our project. We will begin by presenting the initial client statement that we received at the onset of the project. Next we will discuss the methods we used to devise reasonable project objectives and eventually the revised problem statement that we developed based on our objectives. At the end of this chapter, we will describe the overall approach we took in completing this project.

3.1. Initial Client Statement

The initial client statement was given to the design team by the client, Dr. Marsha Rolle, at the beginning of the design process. The initial statement is vague and needed revisions in order to help the team develop a device that best met the client's needs and wants for her laboratory. The initial, unrevised client statement is as follows:

The goal of your project is to design a device that will allow seeding and culture of cells on tubular mandrels to generate tissue engineered blood vessels. Silicone tubing has been used as a mandrel material due to its flexibility, which is useful for cyclic mechanical loading to condition tissue constructs prior to transplantation. Our lab has used a "hanging drop" method to facilitate cell seeding[7], but this method is time consuming and inefficient. Others have shown that implanting silicone tubing in the peritoneal cavity of recipient animals ("in vivo bioreactor") results in cell adhesion and tissue encapsulation of mandrel materials, and the resulting tissue is strong enough to be implanted in the recipient's vascular system. Your task is to design an "in vitro bioreactor" that will achieve a similar result.

Through client interviews, the selection of specific objectives, and the weighting of these objectives, the design team was better able to understand which functions and qualities were specifically important to the client.

3.2. Objectives and Constraints

In order to create a measurable description of the development of the design, a thorough list of objectives was created. Due to the wide scope of the project, the objectives were divided into three categories: the mandrel, the cell seeding method, and the bioreactor. Additionally, a list of constraints was developed; if the device does not meet any of the constraints it cannot be used.

3.2.1. Constraints

Since the use and success of the device relies heavily on the constraints, concern for them is crucial in the design process. The team identified a few constraints for this design. The first constraint is cost as the team was given a budget of $624.00. This limitation includes all costs including design costs and final product costs. The next constraint is time. The design development, production, and testing must be completed by Project Presentation Day, which is on April 22nd 2010. In regards to the device itself, materials must be biocompatible and the must be able to be sterilized. Finally, the device must be safe for the users of the device and all possible risks must be eliminated or at least minimized.

3.2.2. Objectives of the Mandrel

The development of an appropriate mandrel is imperative to the success of this design. Since the mandrel will be in direct contact with the cells, it's material and structural properties are very important. The bulleted list below shows the objectives that were developed for the mandrel:

- Cells stick to mandrel material
- TEBV can be easily removed after growth
- Keeps TEBV ends open
- TEBV grows on mandrel but does not integrate with mandrel material
- Minimize material costs

These objectives are all important for the success of the mandrel itself. Cells must be able to stick to the mandrel in order to form a confluent tube of cells. However, cells cannot be integrated into the mandrel and must be easily removed after growth in order to provide a viable

tubular structure after removal. The ends must remain open in order to enable fluid flow through the vessel. Finally, the material costs should be minimized to ensure that the final design will be within budget.

3.2.3. Objectives of the Cell Seeding Method

There is a wide variety of cell seeding methods that could be used in this design in order to ensure that cells will stick to the mandrel and to make a usable tissue tube. These objectives can be seen below:

- Cells must stick evenly to all sides of mandrel
- Cells must grow and support themselves with ECM
- High success rate for adequate cell seeding

The objectives above are all important in relation to promoting cell adhesion to the mandrel. In order to create a mechanically functional tissue tube, cells must adhere evenly to the surface of the mandrel so there are no weak areas in the engineered blood vessel. The cells must be able to proliferate and produce extracellular matrix (ECM) in order to provide the mechanical properties of a natural blood vessel. Finally, there must be a high success rate of cells adhering to the mandrel. It would not be efficient or effective to place millions of cells into the device and only have 10% of the cells actually adhere to the mandrel and grow into a blood vessel.

3.2.4. Objectives of the Bioreactor

The final category of objectives determined by the team was the objectives of the bioreactor. In order to make the final product easy to use, the team would like to develop a more automated process to ultimately grow tissue engineered blood vessels. These objectives are listed below:

- Supply media to the cells, while removing wastes
- Small enough to fit in an incubator
- Maintain physiological environment

The main goal of the bioreactor is to culture the cells in a physiological environment. This can be achieved by making the bioreactor small enough to fit in an incubator, which will control the temperature along with other conditions, such as pH and humidity. This will allow

cells to survive and proliferate. The bioreactor will also simplify the process of feeding the cells by supplying media to the cells and removing the waste the cells produce.

3.2.5. Weighted Objectives

While all of the objectives are important in the development of this device, some objectives are of higher priority than others. The objectives were weighted by both the design team and the client using pair wise which can be seen in Appendix A.

As mentioned above, the objectives were divided into three primary categories: the mandrel objectives, the cell seeding objectives, and the bioreactor objectives. The importance of these groups of objectives was weighted before the sub-objectives were weighted. The mandrel material was found to be the most important aspect of the project. This is due to the poor performance of current mandrel materials in the field of scaffold-free tissue tube engineering. The cell seeding method was the next most important aspect of the project to the client. This is important because ensuring that the cells are seeded into the device properly will allow for better properties upon the growth of a tissue tube. Finally, the bioreactor is the least important aspect of the project to the client. The automated feeding is not necessary to the success of the design; however it will increase the ease of the design. The decision-making process used by the team will be described for each of these categories.

The most important objective for the design of the mandrel structure was ensuring that the cells would not integrate with the mandrel. This is important because the motivation for this project is to design an approach to scaffold-free blood vessel engineering. If the cells were to integrate with the mandrel, this will not meet the objective of eliminating foreign materials in the TEBV. The next most important objective was determined to be the biocompatibility of the mandrel material. Selecting a biocompatible mandrel material ensures that the cells will not die due to contact with the mandrel. Other less important objectives included a mandrel that can be sterilized easily, keeps the tissue tubes open at the end, and can be easily removed after tissue tube growth. Finally, the two least important objectives were that the cells are able to stick to the mandrel material and the minimization of material costs. The adherence of cells to the mandrel is not an overly important objective because the mandrel can be coated to promote cell adhesion or the cells may not need to initially adhere to the mandrel. Alternative options for cellular adhesion

to the mandrel will be discussed in the Alternative Designs chapter. The material cost was not of great concern to the client and was therefore ranked lowest. These weighted objectives ultimately aided the design team in prioritizing and focusing efforts on the most important aspects of the mandrel.

The second group of objectives to be evaluated was the cell seeding method. The most important objective of the cell seeding method was that the cells were able to grow and support their own extracellular matrix. This is very important to the success of the design because the tissue tubes will not have a scaffold to support them; therefore the support of the ECM is necessary in providing the structure the tube needs. The next most important objective was that the cells stick evenly to the mandrel. A uniform coverage is crucial in the engineering of tissue tubes due to the high pressures found in these vessels. If one portion of the tube wall is significantly thinner than another portion, this could lead to aneurysm formation or vessel rupture. Finally, the objective for cell seeding that was ranked lowest was the success rate of the process. This objective was likely ranked lowly because the current process is already inefficient and any improvement seen in the new approach will be of use to the client. A novel cell seeding method is necessary in this research in order to create a complete device that will be both successful and user friendly.

The final group of objectives that was weighted by the team involved the bioreactor. The most important objective was found to be the bioreactor's ability to maintain a physiological environment. This is clearly important in ensuring the growth and survival of the cells. The next most important was the capacity of the bioreactor to supply media to the cells. The least important objective of the bioreactor was the small size of the device. Ideally the device should be small enough to fit in an incubator in order to create proper cell culture conditions. Our client did not believe the size of the device was overly important as long as it met the other objectives and constraints set forth. By meeting these weighted objectives, the design team will better meet the needs of the client and will create a more successful device.

3.3. Revised Client Statement

After considering the constraints and objectives developed by the team, the client statement was revised to provide a clear, specific description of the needs of the client. The revised client statement is as follows:

The goal of this project is to design a device that will use tubular mandrels to produce tissue engineered blood vessels composed of cultured cells and a cell-derived extracellular matrix. An appropriate mandrel material should be used to allow for the efficient seeding of cells and removal of the blood vessel after a given cultivation period. Current methods being utilized involve coating a silicone mandrel with an adhesive biomaterial. Ideally the process would not involve any coatings. The generated blood vessels should be within 0.5 – 4 mm in inner diameter and 3 – 5 cm in length. The system should be easily modified to support a variety of vessel sizes. The device will incorporate a bioreactor that will simulate a physiological environment conducive for cell growth by maintaining temperature and pH, supplying nutrients and oxygen, removing waste, and preventing contamination.

3.4. Project Approach

Once the objectives and constraints for the three different sections of the device were decided upon, ranks were assigned to each of the three sections. Different methods and materials were brainstormed for each portion of the design as can be seen in our function means trees in Appendix B. After functions-means trees were created, the feasibility of various combinations of means were discussed and combined to develop four preliminary designs. These four designs were then conceptually tested for their ability to meet our objectives and constraints.

4.0 Alternative Designs

In order to ensure that our design was the most promising design that we could complete within our constraints, we first had to create, analyze, and compare various design concepts. In this section we will begin by discussing the target benchmarks for our tissue tubes along with the functions of our device and the means by which we can accomplish them. We will then discuss our four preliminary designs, show our models for each design, and discuss the benefits and limitations of each design. Finally, we will discuss the feasibility that each of our designs will be able to achieve our project objectives while remaining within budget and time constraints.

4.1. Needs Analysis

In order to better understand our project and how we should approach it, we had to first analyze our needs. Based on our revised problem statement and meetings with our client, we developed a set of benchmarks that define the physical properties of a successful tissue tube. Table 4.1 displays our benchmarks and their respective target values. After setting benchmarks for our designs, we reexamined our objectives and developed a list of specific functions that our design must perform.

Table 4.1: Physical Benchmarks of Tissue Tubes

Benchmark	Target Value
Tissue Tube Inner Diameter	0.5-4mm
Tissue Tube Length	3-5cm
Mechanical Integrity	Able to be manipulated without falling apart

Additionally, for each of our functions, we developed a list of means by which our design could achieve that function. These means, along with their respective functions were placed into functions-means trees. The functions-means trees are shown in Appendix B. The functions we devised and how they relate to our design concepts will be discussed in further detail in later in this chapter.

4.2. Conceptual Designs

Various methods were devised to seed cells effectively onto a tubular mandrel. Active methods include using centripetal force, gravity or a vacuum to force the cells to coat the mandrel. Passive methods include using materials with adhesive properties such as collagen, fetal bovine serum, or Poly-(N-isopropylacrylamide) (NIPAAM) to coat the mandrel. Some designs used a rotating mandrel in order to get the cells to coat the mandrel surfaces evenly. Many of our alternative designs, described below, incorporate more than one of these methods to seed the cells effectively and evenly onto the mandrel.

The mandrel material was designed to promote adhesion of cells onto its surface while they grew into a tissue tube. Some designs required media to flow down the center and through the material. Using a hemocytometer, the size of the cells was estimated and most RSMCs were between 30 and 50 µm. This requires that the mandrel material chosen contains pores smaller than 30 µm. This would ensure that media flows through the mandrel, but the majority of cells would remain on the outside. Finally, once the tissue tube grew around the mandrel, it would need to be detached from the mandrel in a relatively easy manner. This could be accomplished through use of a bioinert material or an exertion of force such as a pressure. Bioinert materials that could be used for the mandrel are listed below in Table 4.2.

The bioreactor segment of the design was intended to automate the process of feeding the cell cultures while maintaining an optimal physiological environment. Many of these functions, such as maintaining temperature, oxygen, and CO_2 levels, would be carried out by the incubator where the device would be placed. The main constraint of the bioreactor is that it would have to fit into the incubator. The bioreactor would also need to circulate nutrient media, keep contaminants out, and maintain pH and salt concentrations. This would be accomplished through the use of a peristaltic pump that would continuously circulate media through the culture chamber and a media reservoir. Additionally, the reservoir would be large enough to contain enough media to maintain salt and nutrient concentrations throughout the growth period. The pH of the media would be controlled through pH buffers and CO_2. The media reservoir would be changed once the concentrations of salts and media became too low or if the concentration of

metabolic wastes became too high. This can be determined based on color changes within the media.

Table 4.2: Possible bioinert mandrel materials

Type of Material	**Material Options**					
Polymer	PTFE	PEG	Polystyrene	Polysulfone	UHMWPE	Polypropylene
Ceramic	Alumina	Zirconia	Glass			
Metal	Stainless Steel	Co-Cr Alloy	Ti-Al-V Alloy	Gold		

4.3. Preliminary Designs

After developing the various means for our designs, we combined our concepts for mandrels, cell seeding methods and bioreactors into several preliminary designs. Next, we created four preliminary designs that we chose to pursue as possible candidates for our final device. This section will outline each of our four preliminary designs including: advantages, limitations, and a detailed description of each design.

4.3.1. Vacuum Design

One of the bioreactor designs featured a porous inner mandrel material that utilizes a vacuum force for cell seeding. A schematic of the design is shown below in Figure 4-1. In this design, the mandrel is placed within the bioreactor chamber and immersed in a media and cell solution. A pump is used to drain the media from the inside of the mandrel where it is circulated and then returned through inlet tubes from the outside of the chamber. Four inlet tubes are used to allow for attachment of a motor shaft to rotate the chamber. The size of the pores of the mandrel would be smaller than 30 µm. This would ensure that media can be drawn through the mandrel, while the cells remain stuck on the surface of the mandrel. A preliminary CAD drawing showing the major components is seen below in Figure 4-2. A series of inlet tubes feed media to the chamber, while media is drawn out from a tube that is connected to the inner portion of the mandrel, causing a vacuum force from outside to inside of the mandrel. Included with the chamber is a rotation motor that is used to disperse the cells in the media and evenly coat the surface of the mandrel. A media reservoir is used to contain the media as it circulates. A large

media reservoir ensures that the solution is buffered properly and contains enough nutrients to sustain the cells throughout the growth period.

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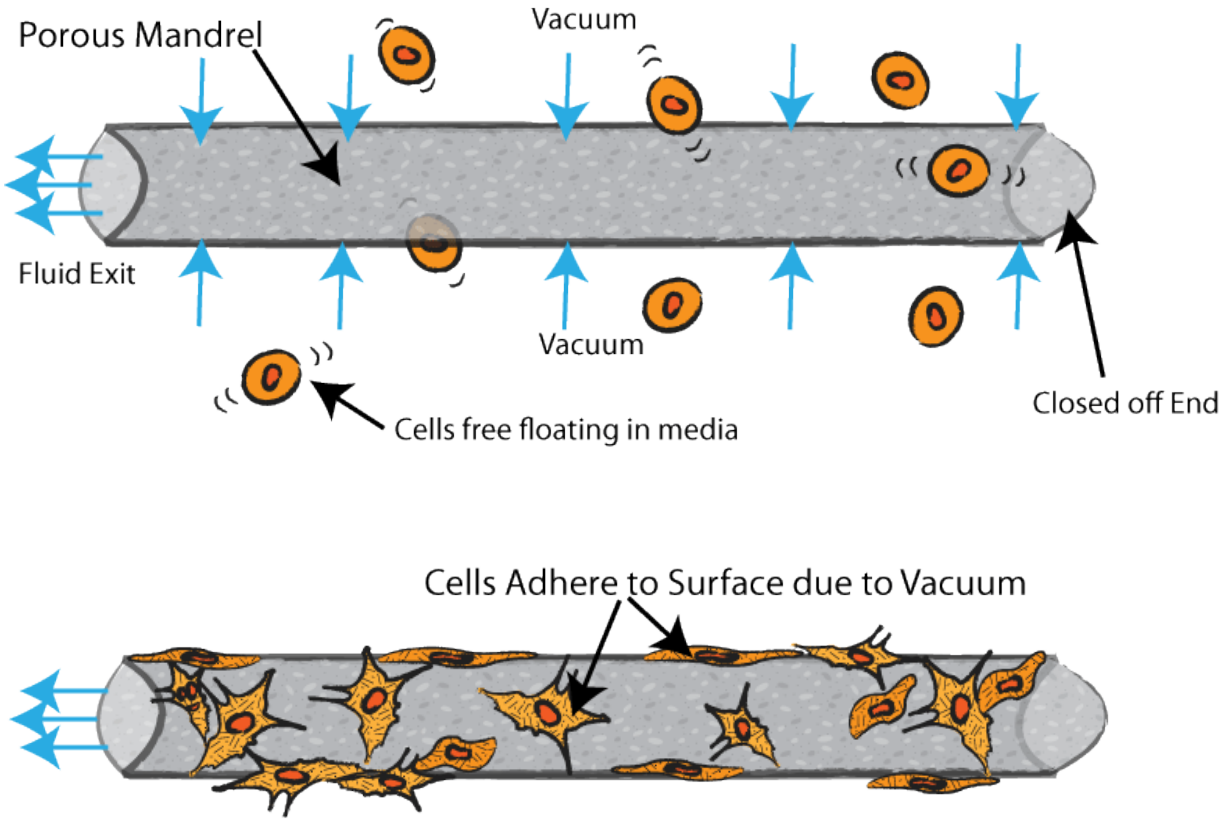

Figure 4-1 Porous Mandrel and Cell adhesion due to Vacuum

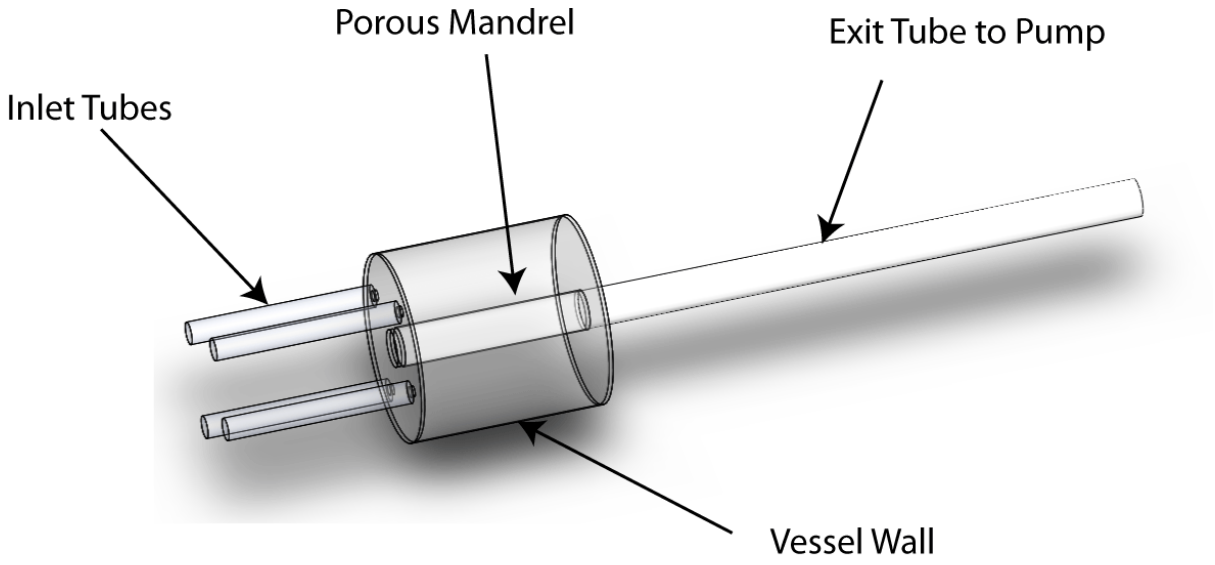

Figure 4-2: CAD drawing of vacuum design

Advantages for this design include a simple mechanism for cell seeding. This design evenly coats mandrels because as cells stick to the surface of the mandrel and cover up pores, they would impede the vacuum force of the covered pores. This would result in the cells being forced to stick to the open pores, thereby coating the mandrel evenly. Once the cells have grown to confluency around the mandrel, they could be removed by reversing the pump, which would apply a pressure to force the tissue off of the mandrel surface. The shear stress from the vacuum could also be an advantage by mechanically stimulating the growing cells. However, the unknown shear stresses may be too strong and destroy the cells. Another disadvantage of this approach is the complexity of the design compared to the other designs. This would require many moving parts and would be harder to manufacture.

4.3.2. Washing Machine Design

Another approach to seeding cells onto a mandrel uses a rotating inner mandrel surrounded by a finned outer tube as seen in Figure 4-3. Cells are injected into the open area between the mandrel and outer tube. Similar systems have been developed by NASA to keep cells in constant freefall and create cellular aggregates.[31] Based on this design's appearance, we've named it the washing machine design.

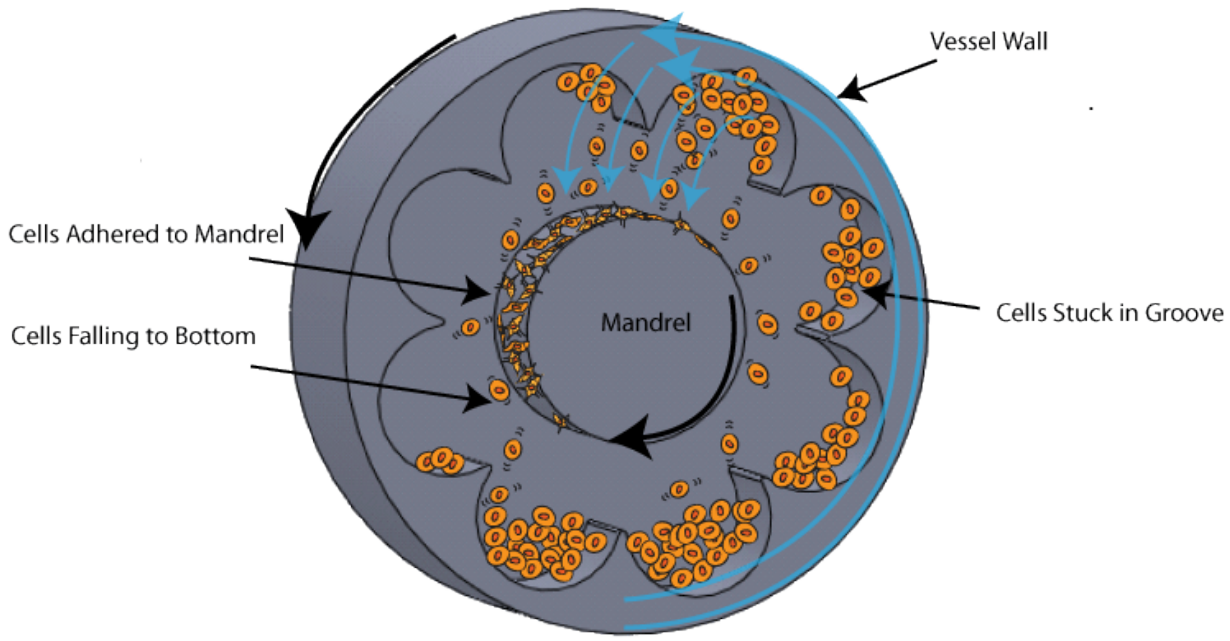

Figure 4-3: Schematic of Washing Machine

This design will have an inner mandrel composed of a material that allows cell adhesion. The material must be porous enough to allow fluid flow to the chamber and be bioinert. A porous material is necessary to allow fluid flow through the lumen of the tissue tube in order to ensure adequate nutrient perfusion throughout the tube. To optimize this design we could control the rotational speed. As the vessel chamber is rotated, the cells are captured by the grooves in the fins and are carried to the top of the chamber where they naturally fall due to gravity. The cells then have a chance of sticking to the inner mandrel, which rotates the opposite direction to maximize even cell seeding of the inner mandrel. The design of the fins can be varied to optimize the number of cells that are directed towards the inner mandrel.

There are a few advantages to this design: mechanical stresses on the cells are likely to be low due to the slow rotation rate, ensuring the cells' safety in the bioreactor and the porous inner mandrel allows feeding inside the lumen of the tissue tube. The constant fluid motion also lends itself to even media and nutrient distribution. Additionally, this design is cost-effective and is relatively simple to manufacture.

4.3.3. Agitation Design

This preliminary design takes advantage of gravity in order to seed cells onto one of many mandrels. The design consists of a box with one removable side as illustrated in Figure 4-5. There are numerous mandrels attached to the side of the box that is opposite from the removable side. On the removable side there are holes that correspond to each of the mandrels to provide support once the entire box is assembled. The assembled box is shown in Figure 4-6. Finally, there are injection sites at the top of the box, one is used to inject the cells into the device and the other allows for air that is originally in the device to exit when cells and media are injected. Both holes can be sealed using medical grade silicone glue. The device is injected with cells and media and then attached to the motor, which agitates the entire device allowing the cells to contact and adhere to any of the mandrels. The device is left in this position until most of the cells that have not attached have hit the bottom of the chamber. The device is then flipped 180 degrees and the process is repeated in the opposite direction. The seeding process is illustrated in Figure 4-4.

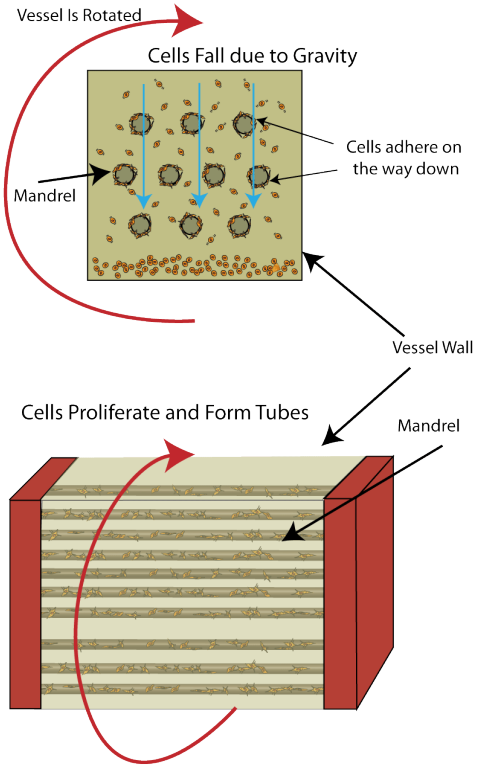

Figure 4-4: Schematic of Agitation Design

The main advantage of this device is that multiple tissue tubes can be made at once. Additionally, the unit is entirely self-contained, which is advantageous because once sterilized, the risk of contaminating the device is very low.

The major disadvantage of this design is that it relies on random freefall to seed cells onto mandrels. Other disadvantages include the precision needed for successful seeding and the wasted materials from unsuccessful seeding. The speed of both agitation and rotation must be carefully assessed to find optimal speeds for each motion. Finally, cells that either don't adhere to a mandrel or adhere but do not completely form a tube around a mandrel will be wasted. More waste will occur because the size of this bioreactor is larger than other designs since it contains numerous mandrels, therefore more media will be required to fill the bioreactor and ensure that the cells are being adequately fed.

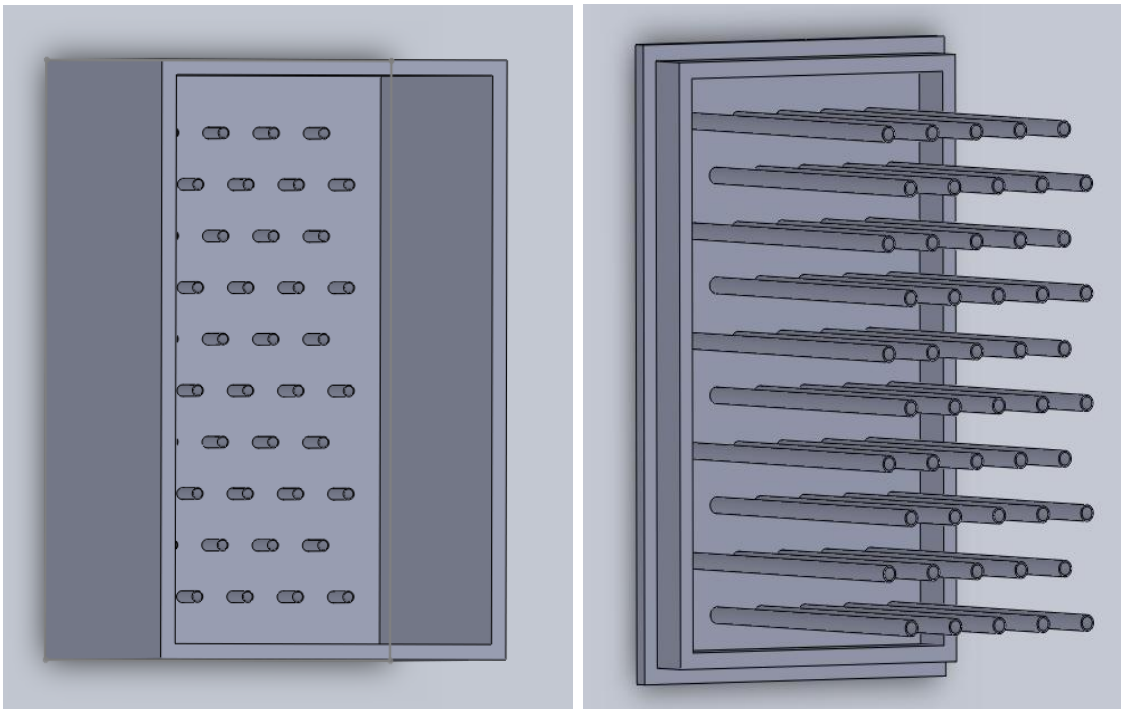

Figure 4-5: The main box-like part of the agitation design is seen on the left and the cover to the box is seen on the right. Fifty individual mandrels are dispersed on the cover and fifty corresponding attaching pegs can be seen inside the box.

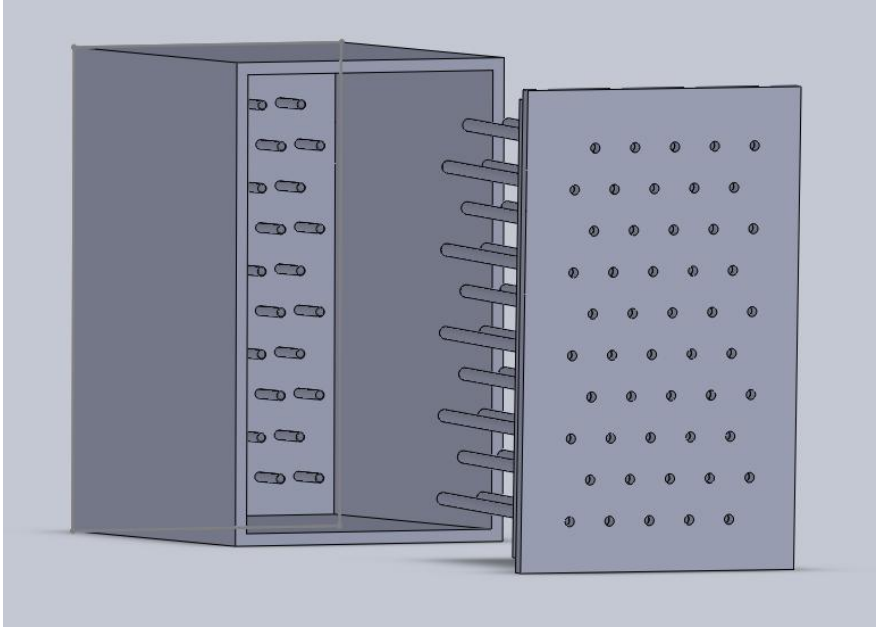

Figure 4-6: An exploded assembly of the entire agitation device. The box and the cover structure fit together and the mandrels and their corresponding pegs align and connect.

4.3.4. Teacup Design

Another of our preliminary designs, which we called "the teacup", takes an entirely different approach to cell seeding in comparison to our other designs. In this design, illustrated in Figure 4-7, we attempt to seed the cells on the inner surface of a glass tube rather than the outer surface of a mandrel. This design provides a unique advantage in that simply rotating the mandrel at a slow rate will allow the cells to settle on the seeding surface due to the force of gravity. There are four major components necessary for this design: the outer seeding mandrel, the inner mandrel, the mandrel supports, and the bioreactor/rotation mechanism.

The initial concept for this design used a thin coating of the thermo-responsive polymer NIPAAM on the inner surface to allow for cell attachment. NIPAAM is a hydrogel that exhibits phase separation when lowered below its lower critical solution temperature of 32^{o}C. Below the LCST, NIPAAM exists as hydrophilic coils of single chains. Above the LCST, NIPAAM undergoes a sharp coil-to-globule transition and as a result forms into a hydrophobic aggregation.

This polymer coating can then be released by lowering the system to below its LCST, thus detaching the cell tube from the outer mandrel. If correctly constructed, this system would make

the removal of the cell tube from the mandrel easy and efficient. The outer mandrel will likely be composed of either plastic or Pyrex glass that can be easily coated with NIPAAM.

The inner mandrel of the teacup design concept functions mainly to "catch" the tissue tube once it has been released from the outer mandrel and to prevent the tissue tube from closing once it has been released. The initial design called for the inner mandrel to be composed of a porous material that would prevent cellular ingrowth but allow for fluid flow through the lumen of the tissue tube. As our studies progressed, however, we have realized that by supplying fluid through the tube at low physiological pressures (80mmHg), we should be able to keep the tissue tube open without using an inner mandrel. Another possible replacement or change to the inner mandrel design is the use of a thin fiber in place of the mandrel that will simply act as a deterrent to the tissue tube closing in addition to the fluid pressure.

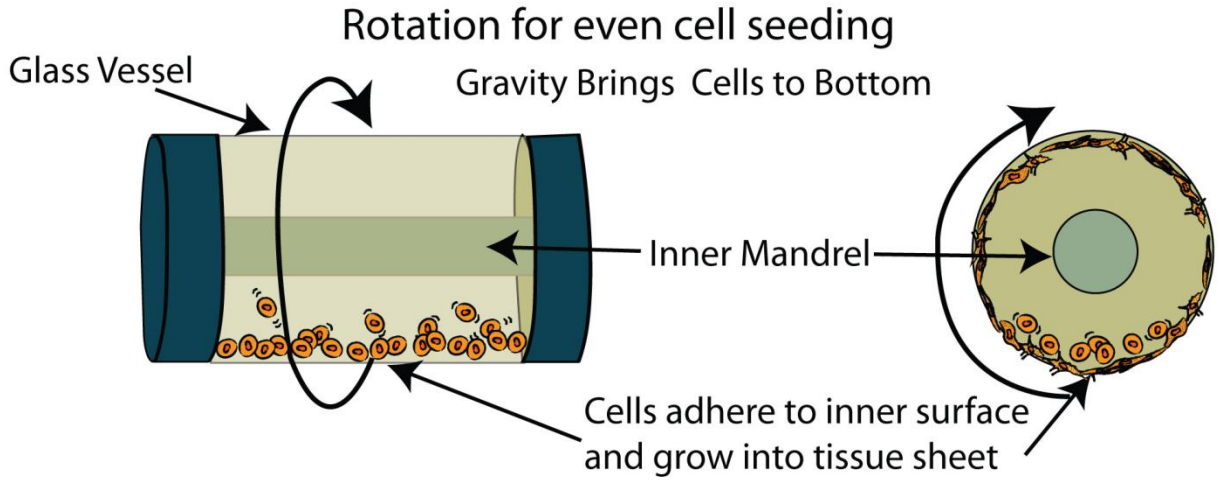

Figure 4-7: Schematic of Teacup Design utilizing rotation for even cell seeding of inner vessel surface

The mandrel supports, shown as a close-up in Figure 4-9, in the teacup design will need to perform several different functions. First and foremost, these supports must seal the outer mandrel from the outside environment and prevent transmittance of bacteria or other infectious agents into the bioreactor system. Second, the supports must easily connect to the bioreactor system and allow for fluid flow into and out of the polymer tube. Third, the outer mandrel supports must interface with a gear or pulley system on one end of the mandrel to allow for the continuous and slow rotation of the mandrel. Finally, the mandrel supports must have a built in mechanism for "catching" the open ends of the tissue tube once it has been released. In our first

design for this concept, this catching method is composed simply of hook-like protrusions which will capture the cell sheet as it is released from the outer mandrel.

Figure 4-8 shows a solid model of the entire teacup preliminary design. The inner and outer mandrels are held in place by the mandrel supports and one of the mandrel supports includes a gear to allow for rotation of the entire mandrel system. Both the inlet and outlet of the design will be connected to a peristaltic pump using a syringe. Figure 4-9 shows a close-up of one of the two mandrel supports for the "teacup" design. The important entities shown in Figure 4-9 are the clear glass outer mandrel, the inner mandrel, the geared mandrel support and the syringe tip. Also of significant importance are the smooth hooked protrusions on the tip of the mandrel supports. These protrusions are designed to "catch" the tissue tube when it is released from the outer mandrel.

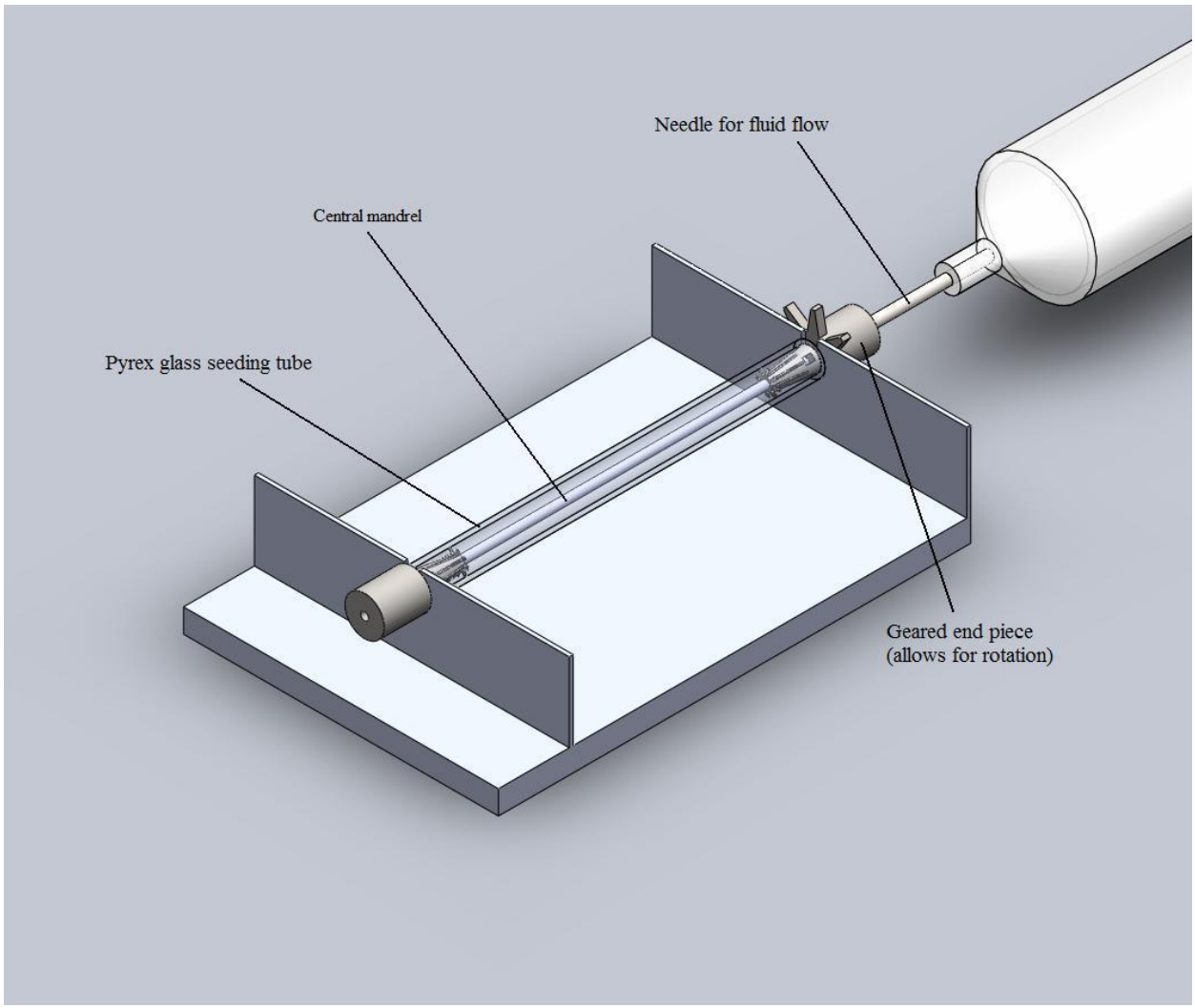

Figure 4-8: Solid model of the "teacup" design. This design will interface with a fluid flow bioreactor using two standard 18 gauge syringes (only one is pictured) and includes both an inner and outer mandrel which are held in place by two mandrel supports. One mandrel support allows the model to be geared to a motor.

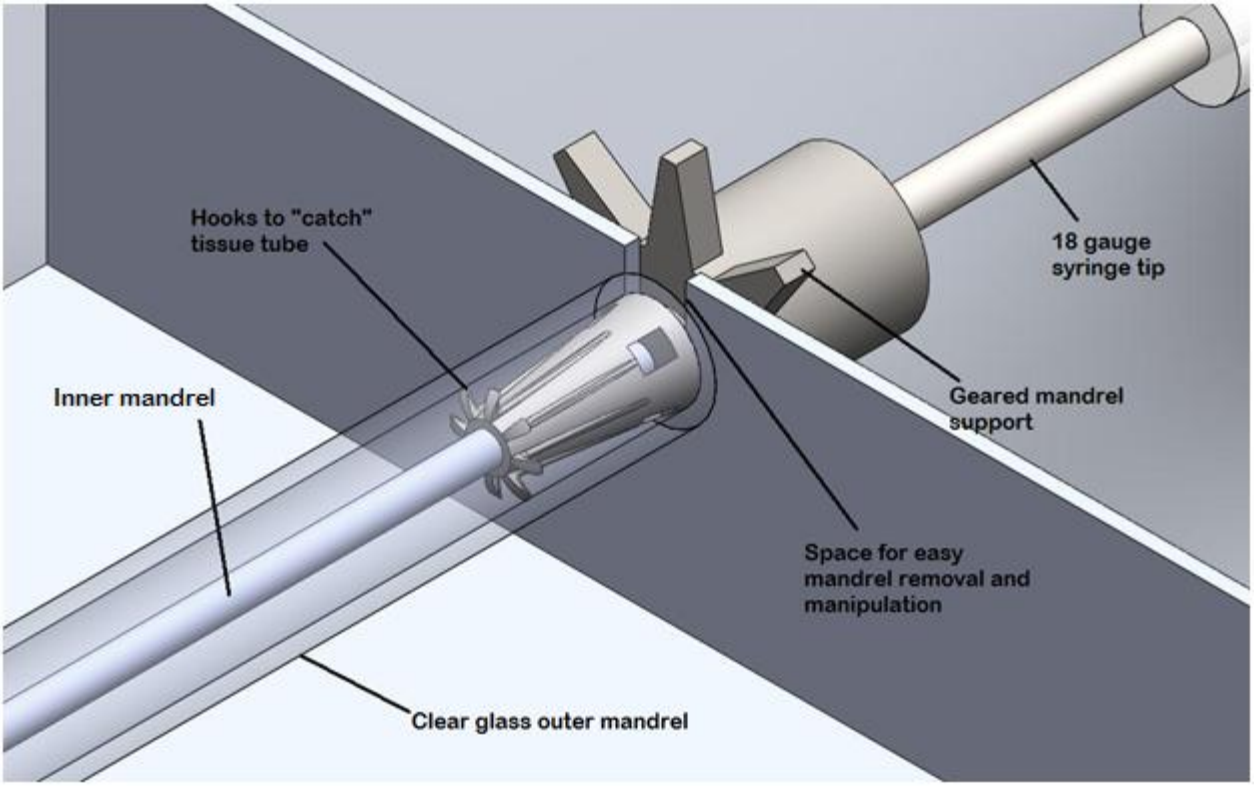

Figure 4-9: Close-up view of a mandrel support.

Compared to our other designs, the teacup design has several key features that make it attractive. First and foremost, since it seeds the cells on the inside of a mandrel, the teacup design does not require an inner mandrel that is either porous or promotes cell seeding. This solves the major issue of finding and testing mandrel materials that meet strict design criteria. Second, based on our preliminary experiments, the teacup design may be possible to use without the use of NIPAAM or other coatings. This eliminates the need to develop a method of coating mandrel surfaces.

The teacup design concept includes several assumptions that must be proven before it can be considered a viable design. The first assumption is that we can form a NIPAAM polymer coating on the inner aspect of a glass or polymer tube. To our knowledge, this has not been done in any scientific literature to date. This has, however, been successfully accomplished on the outer aspect of glass tubes.[32] Based on our preliminary experiments, we may eliminate this assumption. The second assumption is that the cells will contract when released from the outer mandrel and remain a contiguous tissue tube. Based on our preliminary experiments thus far, we

have found that smooth muscle cells that are grown on tissue culture plates will spontaneously release in a tissue sheet when they reach a certain cell density. As such, we believe it is reasonable to expect that a similar phenomenon will occur when the cells are released from a NIPAAM coated tube and possibly from a tube without a NIPAAM coating.

Despite its promise, the teacup design does present with several limitations. First and foremost, the teacup design does not provide an easy method for increasing production of blood vessels. Since the design is such that a single blood vessel is made in its own self contained environment, manufacturing of multiple blood vessels simultaneously would require that a new self contained vessel reactor to be built for each additional vessel.

4.4. Feasibility Study

An evaluation matrix was generated to distinguish advantages and disadvantages between each alternative design. The scores were based on data from the conceptual testing regarding how well each design suits the necessary functions as described by our constraints and objectives. Each score was given out of 100 by the design team. The final scores were based on our objective weights from the pair wise comparison charts in Appendix A.

. The final ratings are expressed as a percentage. These objectives have also been given weights correlating to their significance for a successful overall design. Designs were evaluated for individual performance, then scaled based on importance of each function and for each sub-objective, unless the design must be ruled out due to lack of compliance with constraints.

The constraints specified that the resulting bioreactor must meet several conditions. It must be composed of biocompatible materials, easy to sterilize, allow for safe removal of a completed tube, allow for seeding of SMCs, and be sized to fit into an incubator. As each design operates within these constraints, analysis is next governed by a design's ability to complete device objectives. Major objectives were subdivided to provide sufficient discriminatory detail among designs. The major objectives, by order of decreasing weight were mandrel support functions, cell-seeding functions, and bioreactor functions.

Table 4.3: Conceptual Design evaluation matrix

OBJECTIVE	OBJECTIVE WEIGHT	SUBOBJECTIVE	SUBOBJECTIVE WEIGHT	Teacup	Vacuum Design	Pinball	Washing Machine
MANDREL	2	Cells adhere to mandrel	2	85	80	60	75
		TEBV easily removed	3	100	100	85	100
	Totals:			**0.94**	**0.92**	**0.75**	**0.9**
CELL SEEDING	3	Cells adhere uniformly	2	95	85	70	90
		High Success seeding rate	1	95	95	95	90
		Grow and support own ECM	3	100	90	100	85
	Totals:			**0.975**	**0.892**	**0.8917**	**0.875**
BIOREACTOR	1	Media Circulation	3	100	85	90	100
		Maintain physiological environment	2	100	100	100	100
	Totals:			**1**	**0.91**	**0.94**	**1**
	Overall Total:			**96.8%**	**90.4%**	**85.3%**	**90.4%**

5.0 Design Verification

As mentioned in the previous chapter, four preliminary designs were created to achieve the necessary goals and objectives as dictated by the client statement. Each design took different approaches to the same goal of ultimately growing a tissue tube. This chapter describes in detail the preliminary tests and results for each conceptual design. The specific testing protocols can be found in Appendix C.

5.1. Vacuum Design

The vacuum design featured a porous inner mandrel and utilized a pump to create a negative pressure within the mandrel, which would seed cells onto its outer surface. To test this concept, two mandrel types were created from different materials.

The first experiment used nylon mesh which contained 34 µm pores. It was estimated through a hemocytometer that the size of a rat aortic smooth muscle cell (RSMC) is between 30µm and 50µm. Therefore the pore size was small enough to capture the RSMCs. The nylon mesh was fixed into a conical shape and glued using medical grade silicon as shown in Figure 5-1.

Figure 5-1: Nylon Mesh configured into a conical shape

The wider ends of the nylon cones were plugged with Polydimethylsiloxane (PDMS). These cones were placed in a cell solution containing 90% Dulbecco's Modified Eagle Medium (DMEM), 10% Fetal Bovine Serum (FBS) and 1% of the antibiotic combination

penicillin/streptomycin. Vacuum forces were created by using a micropipette with a 1mL tip which would theoretically pull cells and force them to adhere to the outer surface of the nylon cones. It was found that this approach was an ineffective method for producing vacuum forces as the micropipette tips did not form a strong seal with the ends of the nylon cones. The first of the nylon cones was sacrificed at day 3, and another was sacrificed every day afterwards. It was found that no cells were able to adhere and grow onto the nylon cones as shown below in Figure 5-2 (left). This image is shown in comparison to Figure 5-2 (right) which is a control nylon cone that did not undergo cell seeding.

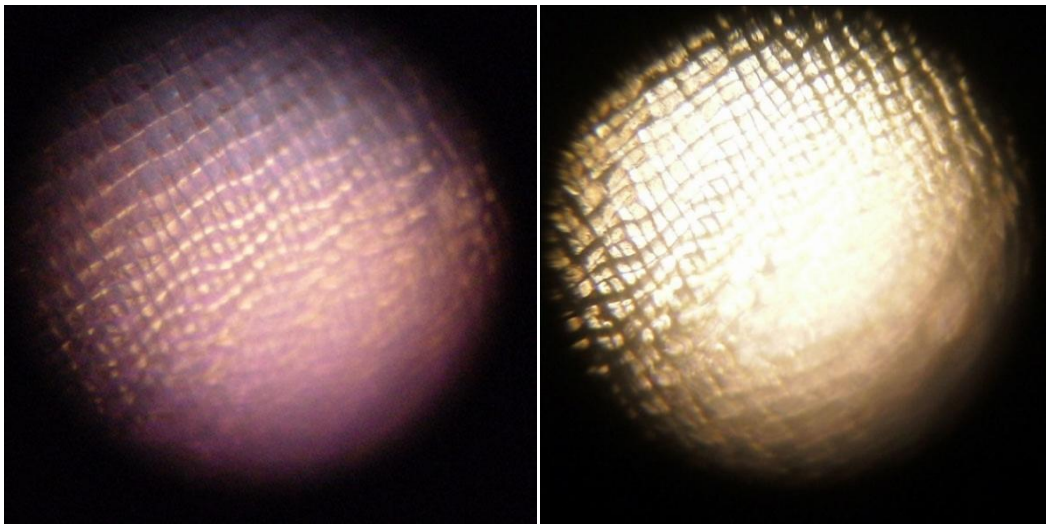

Figure 5-2: Nylon Mesh under a 10X Optical Microscope after cell seeding using vacuum forces(left) and without being seeded (right)

The second experiment for the vacuum design used polysulfone as a mandrel material as shown below in Figure 5-3. The polysulfone mandrel was clamped down on one end, and a needle and syringe were used to create a vacuum. Solution media was aspirated through the middle of the mandrel. It was found that creating a vacuum through the polysulfone mandrel was difficult and the needle easily pierced through the mandrel. The mandrel material could not be viewed under a microscope due to its thickness, and trypan blue staining yielded inconclusive results as the material absorbed the dye. However, after 14 days, the nutrient medium remained the same color suggesting no metabolic activity of any kind and therefore it was unlikely that cells were present.

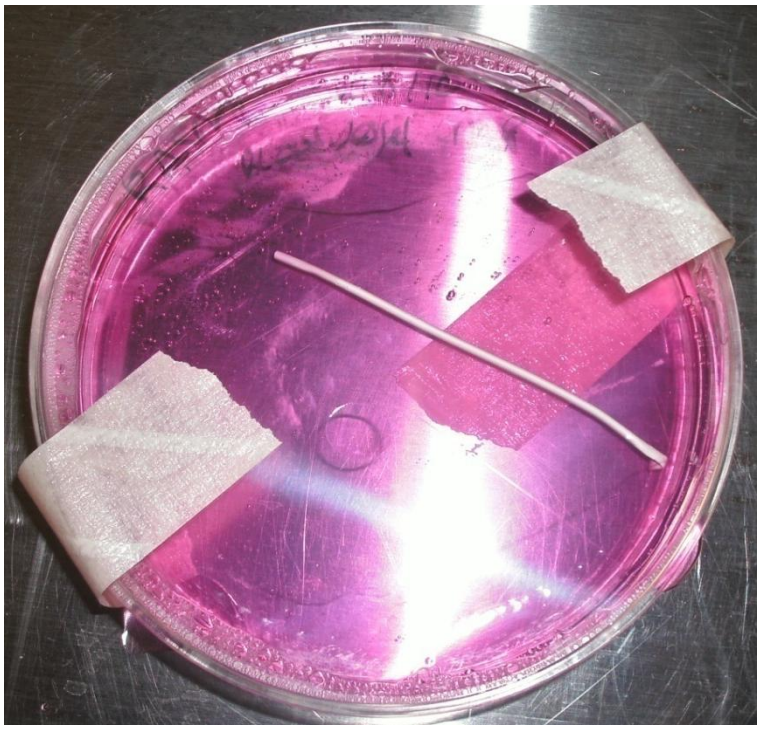

Figure 5-3: Photograph of Polysulfone Mandrel in Nutrient Medium

These preliminary experiments suggest that the available materials would not work effectively with this design. Furthermore, suitable manufactured porous mandrels could not be found for purchase, and manufacturing the mandrels in-house was not feasible due to time constraints, equipment, and expertise required.

5.2. Washing Machine Design

The washing machine design took advantage of convective motion to direct cells towards an inner mandrel. Testing of this model involved anticipating fluid pressures and velocities to predict the particle path within the vessel. This testing was best achieved by using computational fluid dynamics and several models were created and testing using the FLUENT fluid modeling program. The various parameters of the models including fin shapes, sizes, and vessel rotational speeds are described in Appendix C.

Figure 5-4 is a graphical representation of fluid velocity and direction in one of the washing machine vessel options. At a rotation speed of 2.0 rad/s, the fluid velocities in m/s can be seen in the right hand side of the diagram.

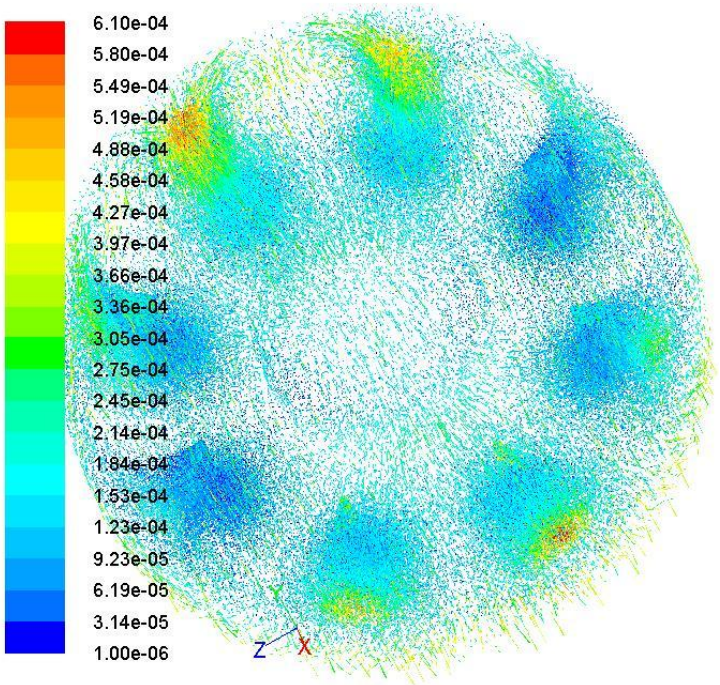

Figure 5-4: FLUENT Model of a concave fin Washing Machine Design

It can clearly be seen that the majority of the fluid is directed towards the outer portions of the vessel due to the centripetal force from rotation. This would cause cells to congregate in pockets near the fins. If rotational speed is decreased, gravity overcomes the rotation and the cells aggregate at the bottom of the chamber. If the rotational speed is increased, the centripetal forces hold the cells on the outer surface and do not allow them to drop on the mandrel. As a result, the washing machine design would not have efficient cell seeding of the inner mandrel due to the majority of cells aggregating on the outer fins.

5.3. Agitation Design

This preliminary design takes advantage of gravity in order to seed cells onto one of many mandrels. To conceptually test this design, a model was built using a Petri dish for the vessel, silicone tubing for the mandrels, and pepper to represent cells. A photograph of the model can be seen below in Figure 5-5. By rotating the model, the interactions between the pepper particles (cells) and silicone mandrels could be visualized. It was found that the rotation caused a "snow globe" effect, and the pepper particles were able to evenly "coat" the mandrels.

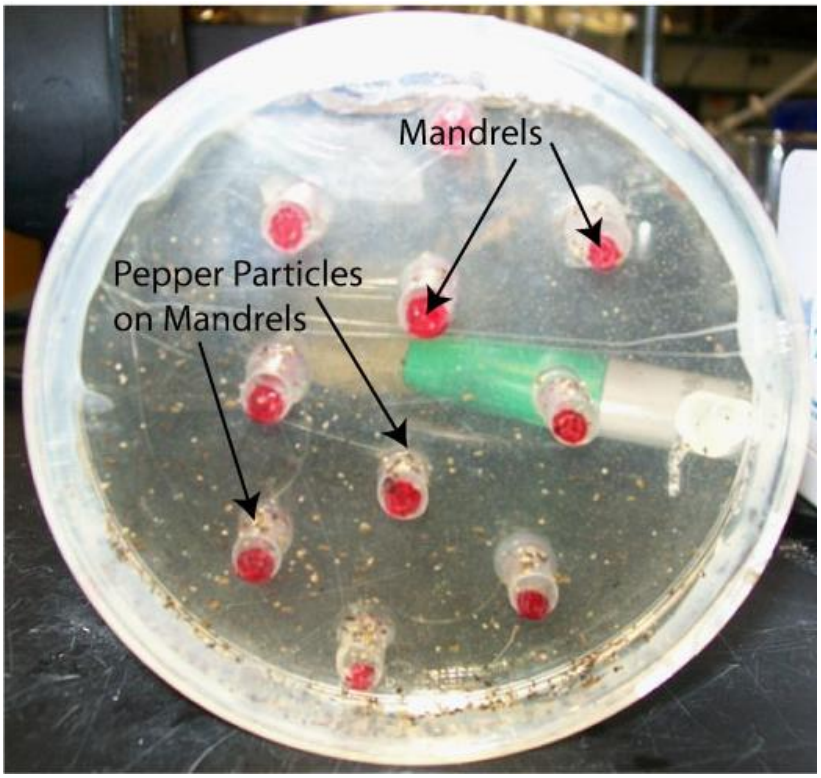

Figure 5-5: Model of Agitation design with Pepper Particles to visualize fluid flow

Due to the successful results of even coating of the conceptual agitation design model, a prototype of the agitation design was built and tested with actual RSMCs. The prototype was similar to the model, but in this case, the silicone mandrels were coated with fetal bovine serum (FBS) in order to adhere to the cells. This was achieved by soaking the mandrels overnight at 25°C and allowing it to dry; thereby creating a coating of adhesive proteins on the silicone mandrels. A cell solution containing approximately 20 million cells was injected into the vessel and cultured for 5 days. After the fifth day, the prototype was fixed with 70% ethanol, and stained with trypan blue. A photograph of the stained prototype is shown below in Figure 5-6.

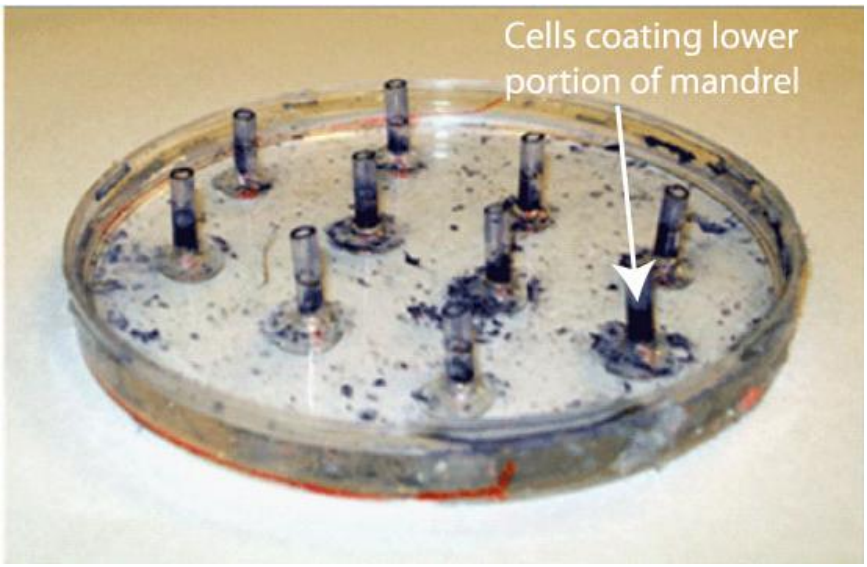

Figure 5-6: Agitation Prototype Stained with Trypan Blue

The stained prototype shows uneven adhesion of the cells onto the mandrels. The bottom portion of the mandrels received an even coating of cells, however, there was little to no adhesion of cells on the top half. In addition, a large amount of cells adhered to the vessel wall itself. Due to these results, this design was not pursued further.

5.4. Teacup Design

In this conceptual design, Rat Aortic Smooth Muscle Cells (RSMCs) are seeded onto the inner surface of a glass tube rather than the outer portion. The glass tubes were scored and snapped using a triangular file and the ends were plugged using PDMS. The PDMS plugs had to be cured in an oven at 60^{O}C for approximately 1 hour. A cell solution was then injected using sterile syringes and 20 gauge needles. It was found that a cell seeding density of 250,000 cells per tube achieved the best coverage. The tubes were marked evenly into eight sections circumferentially, and were rotated $1/8^{th}$ of a turn every 10 minutes for 2 hours after the initial cell seeding. The cells were then fed with 3.0 mL of fresh DMEM every day until they were ready for harvest. It was found that leaving the needle tips in the PDMS plugs with filtered micropipette tips plugging the syringes allowed for sufficient gas exchange.

Due to the curvature of the tube it was difficult to view the tubes under a microscope. Cell adhesion was determined by fixing the cells in 70% ethanol and staining with Trypan Blue.

A picture of a stained tube and a control tube is shown below in Figure 5-7. It can be seen that there is generally uniform coverage around the inner diameter of the tube. However, there are some areas with little cell adhesion. The unstained region near the top of the tube is most likely due to the feeding process. Injecting fresh media through a 20 gauge syringe most likely sheared off cells in that region. In addition, small air bubbles found in the tubes would kill cells near the center after a prolonged amount of time. These problems could potentially be resolved through the use of an automated feeding system using a pump and a reservoir.

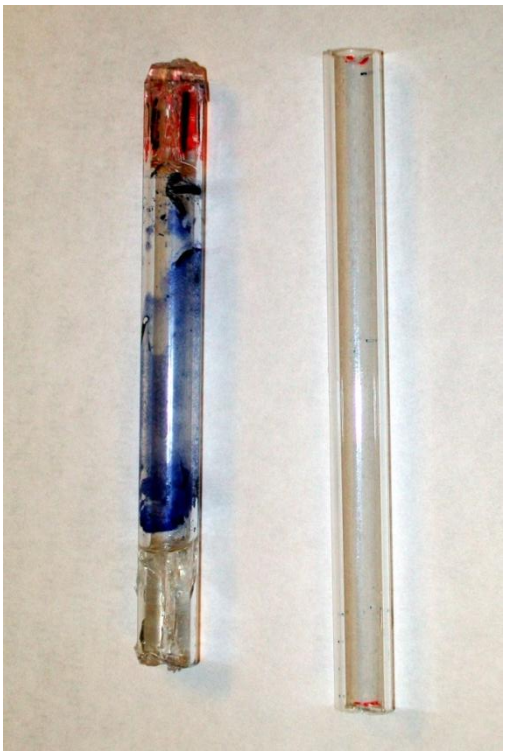

Figure 5-7: Teacup tube stained with trypan blue (left) in comparison to unseeded tube (right).

5.4.1. Tissue Contraction Tests

Once the cells were seeded onto the tubes of the teacup design, a procedure is required to remove the tissue sheet from the tubes. Earlier experiences with tissue culture found that RSMCs would contract off tissue culture dishes spontaneously if they are 100% confluent after a complete media change. It was thought that this contraction could be used to harvest completely formed tissue sheets from the inner diameter of tubes. Once contracted, they can be captured and continually cultured for proper mechanical strength and tissue tube thickness.

To take advantage of the contractile properties of the cell as described above, other methods for forcing cell contraction were researched. It was found that the tissue sheets can be forced to contract at will by completely replacing the medium with 60 mM Potassium Phosphate Saline Solution (K-PSS) at 37^{o}C. A conceptual picture describing the steps of inducing tissue contracting using K-PSS is shown in Figure 5-8. The tissue sheet will then contract within 2 hours. The requirements of this procedure are that the cells must be nearly 100% confluent and must have enough contractile proteins in their extra cellular matrix. This can be achieved by seeding the cells at a low density and allowing them to grow for a sufficient amount of time to produce contractile proteins. Figure 5-9 shows a tissue sheet that has contracted into an inner mandrel.

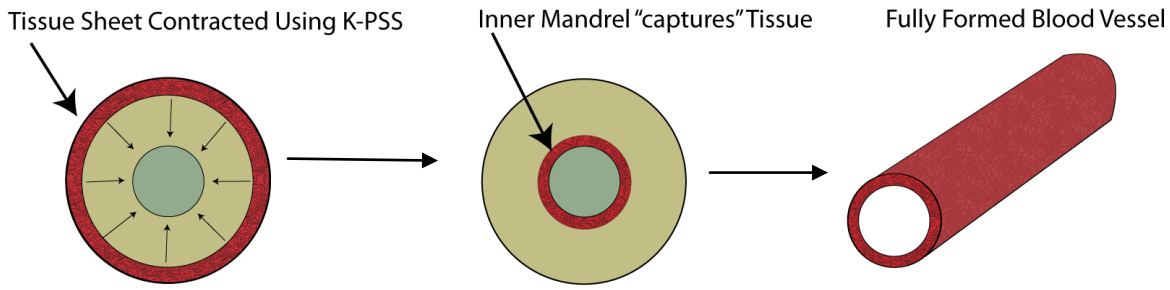

Figure 5-8: Conceptual representation of K-PSS contraction off of a glass tube

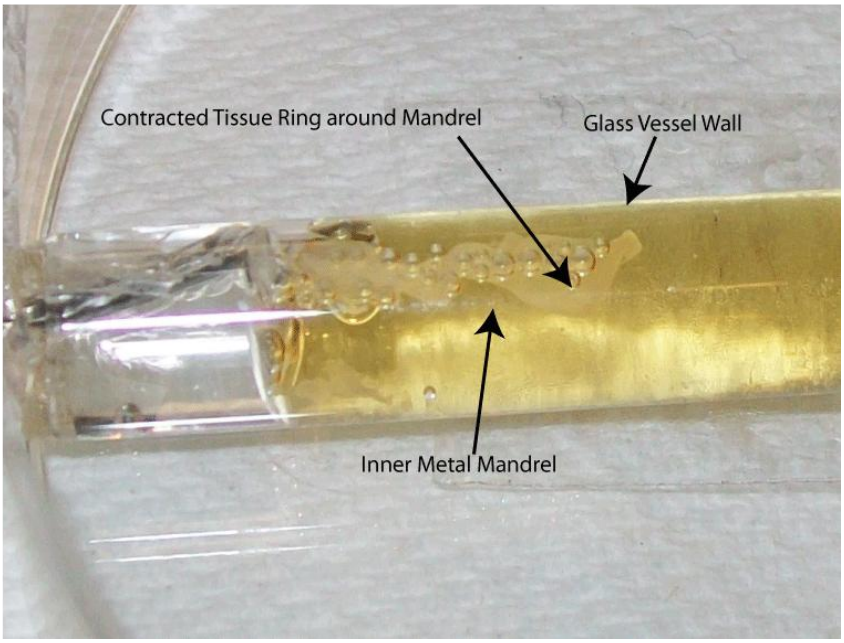

Figure 5-9: Tissue Sheet Contracted Around Inner Mandrel. Yellow media indicates an acidic pH.

As shown by the preliminary test results for the Teacup design, uniform cell adhesion to the inner diameter of glass tubes can be achieved. It was determined that the tissue can be harvested using K-PSS by contracting the tissue onto an inner mandrel. Once the tissue has been harvested, it can continue to be grown until the cylindrical tissue has sufficient mechanical properties for testing. Due to these successes, we chose to purse the Teacup design as our final design.

6.0 Discussion

The following section will summarize and evaluate the major implications of our preliminary testing. This testing was used in order to select a final design to further pursue.

6.1. Vacuum

The first preliminary tests of the vacuum design showed that cells were unable to adhere to the nylon mesh as was intended. Although the size of the pores was chosen to be less than the diameter of the RSMCs, cells were still unable to adhere to the outside of the mesh mandrel. This may be due to the viscoelastic properties of cells, as their ability to deform may have allowed them to slip through the pores of the mandrel. Although smaller-sized mesh material could be purchased or manufactured, these materials would add greatly to manufacturing costs or would otherwise require knowledge of the limit to which cells may pass through an opening, which may require further testing. It was assumed that the cells would behave as solids, but because they passed through a mesh that was smaller than their diameter, they clearly must be capable of deforming in response to pressures.

Additional testing, involving the use of polysulfone tubing yielded different testing results. Polysulfone was used as a mandrel in previous MQPs[33] and is both porous and elastic, conducive to media circulation, gas exchange and vessel removal. However, for the purposes of this project, the tubes were ineffective at seeding cells without using an adhesive coating. The smaller pores of the polysulfone tubing seemed small enough to block the cells from entering the mandrel, but would still allow for media circulation to the surrounding cells. Unfortunately, the small size of the pores made the pressure too high to effectively seed the mandrel with cells. These are not the convenient cell seeding conditions that the group intended to achieve. Additionally, the polysulfone absorbed the trypan blue dye, leaving cell staining tests inconclusive. It appeared that the material simply absorbed the dye, although it is possible that all of the cells stained were localized within the mandrel. In case of complete cell absorption, the tubes were allowed to culture for an additional two weeks, but judging by media color as an indication of nutrient depletion, there was little to no metabolic activity. Therefore this process was deemed ineffective at supporting cells for tissue culture.

Both tests proved that this design, as tested with the available materials and within project constraints, is incompatible with the objectives for both the mandrel and cell seeding which assert that cells must stick to the mandrel without integrating with it.

6.2. Washing Machine

Testing of the washing machine effectively demonstrated that the design would not be the optimal for this project's purposes. Velocity vectors visualized using FLUENT, a computational fluid dynamics analysis program, indicated that particles would be directed along the outer wall of the vessel. This result was directly opposite of the goal to force cells on the mandrel. Multiple fin-mandrel configurations were tested including convex waves, concave waves, and a control model of concentric cylinders rotating at the same angular velocity. However, none of these models yielded desirable results.

Accurately modeling the washing machine system was limited by the user's ability to describe the environment in terms that the program could compute. Although several values and options were explored, simulations showed results that could not be interpreted when attempting to use the Macroscopic Particle Model, (MPM). Due to its predicted insufficient ability to successfully seed cells uniformly onto the mandrel, this design was abandoned as ineffective and other designs were pursued.

6.3. Agitation

The successful initial testing of the agitation method made it a strong candidate for the final design. The initial testing of the agitation model using pepper to represent cells showed that the probability of cells adhering to each mandrel in the model was promising enough to follow through with an experiment using live cells. The design required only seeding of cells and a single manual 180 degree rotation of the housing structure making it simple to use and requiring only a short amount of time to operate. It satisfied all of the team's objectives for a mandrel which cells could adhere to, as well as for uniform cell seeding and maintaining a physiologically favorable environment for cells. These results encouraged the group to further explore this design's capabilities with live cells.

The second phase of testing confirmed that the agitation method was a viable design for a vascular graft bioreactor. Using a similar Petri dish setup with FBS-coated silicone tubes, live cells were seeded into the agitation prototype. The prototype was rotated 180 degrees every half hour for two hours. It was then allowed to culture for five days. The results showed consistent coating patterns on all mandrels. The individual mandrels were not, however, coated evenly. Trypan staining of cells showed that seeding of each individual mandrel was only about 50% successful, as cells adhered almost exclusively to the lower half of the mandrel, likely due to the force of gravity.

The effective seeding of cells onto multiple mandrels and its capabilities for media circulation made it a highly attractive improvement to the current challenges of cell seeding. Unfortunately this design was also ultimately ruled out due to the low success rate of cell seeding, and also because it was limited by its ease of manufacture. Although having 50 mandrels in one design greatly increases its rate of tube production, manually configuring each device took far too long and greatly decreased the ease of operation for the user, which takes precedence over the number of vessels produced.

6.4. Teacup

The unique concept of seeding cells onto the inside of a tube proved to be the innovation that propelled the project towards pursuing this design. Seeding the cells onto the inner tube essentially creates a continuous surface upon which cells may accumulate and form adhesions to one another. In this way, the vessels may be devoid of seams that can be produced by seeding cells onto different parts of the mandrel at different times and can leave tissue susceptible to mechanical failure. By taking advantage of gravity to direct cells towards the bottom of a cylindrical tube, and simply rotating the tube, cells will contact the wall of the tube at every point and are far more likely to form a vessel of uniform wall thickness. Determining the optimal cell concentration and frequency of tube rotation would provide sufficient information to produce tissue tubes. Ultimately these conceptual experiments show that this design concept could potentially meet our design objectives as set forth in Chapter 3.

The method of removal devised solidified the teacup's place as the conceptual design to pursue as a prototype. Taking advantage of the contractile nature of RSMCs allowed the tissue

tube to be contracted onto the mandrel by administering K-PSS and returning the vessel to an incubator for 30 minutes. This method of removal lessens the risk of tearing the tissue as compared with manual removal.

The teacup design satisfied all of the defined objectives for cell seeding, physiologically supporting cells, and the mandrel-tissue interactions. There were adequate success rates for cell seeding and uniformity, no need for adhesive or any artificial matrix, and the cells remained viable throughout the culture process. The mandrel was designed to provide structural support to the tube once contracted, while not interacting with cells or hindering tube removal in any way. The design effectively circulates media and uniformly distributes cells to form a confluent, continuous sheet. All materials chosen were biocompatible and easily sterilized, and the assembly is fit for use in an incubator.

7.0 Final Design and Validation

Based on the testing and validation of the four conceptual designs, we were able to develop the design that held the most promise and to apply our time and effort to improving the design. During preliminary testing, the teacup design showed the most promise, therefore, the final design uses many of the features seen in the teacup design. Despite this, several aspects from other conceptual designs were incorporated into the final design. In this chapter we will first describe the different parts used to build the final prototype, and then discuss the methods we used to construct and test the design with cells, and finally, we will discuss the methods we used to verify that our final design meets the design objectives set forth at the beginning of the design process.

7.1. Part Acquisition and Modification

In order to build our final design within our time and budget constraints, acquiring materials from various sources was necessary. After acquisition, these materials were modified to meet the specific design specifications of each aspect of our project.

7.1.1. Octagonal Stand

The only custom part that was built specifically for our final design is the octagonal stand. The stand, shown in Figure 7-1, was designed in Solidworks and manufactured out of Acrylonitrile Butadiene Styrene (ABS) plastic using rapid prototyping. Two stands are needed in order to seed and grow eight tubes. The primary function of the stand is to hold the glass cell seeding tubes and the steel inner mandrels in place throughout cell seeding. The large indentation seen in Figure 7-1 inset is designed to hold the glass seeding tubes while the hole centered in the indentation is designed to hold the inner mandrel. This design allows for both mandrels to be held firmly in place throughout cell seeding. The additional off-center holes in the indentations are designed to allow 20 gauge syringe access to the tubes for easy cell seeding and media exchange. The octagonal shape of the tube holder is designed to allow for metered manual rotation. This shape allows the stands to hold the tubes in eight different positions with respect to gravity, allowing for even coating of cells on the glass tubes. This process is shown schematically in Figure 7-2. The square central hole in the stands was designed to allow the

stands to interface with and be rotated by a rotisserie system, which allows for the automatic rotation during the cell seeding process. We used the manual seeding process during our earlier tests before we were able to build an automatic rotation system.

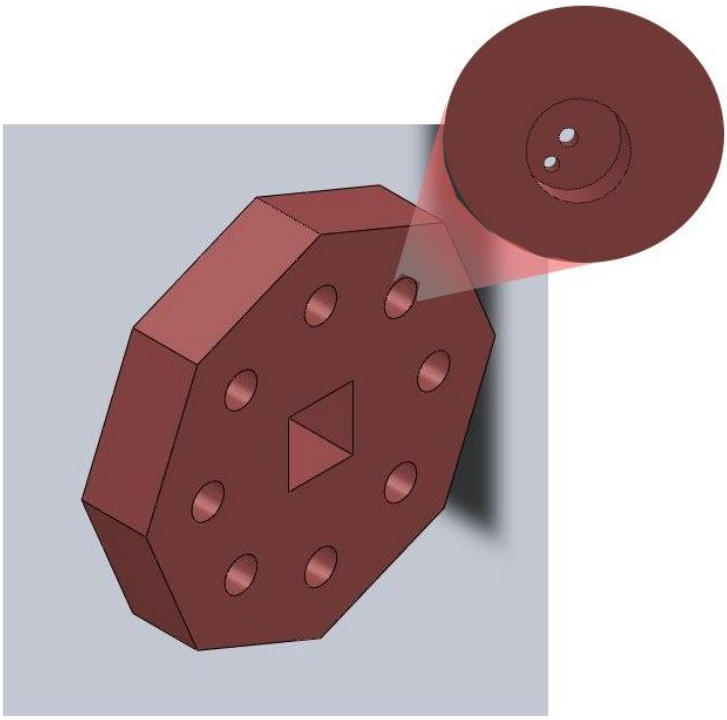

Figure 7-1: Octagonal stand. This part can be used to hold up to 8 mandrels and cell culture tubes. This design allows for seeding of multiple tubes in limited space. The inset shows an indent designed to hold a glass cell seeding tube, a centered hole to support an inner mandrel and a hole for cell injection and media flow.

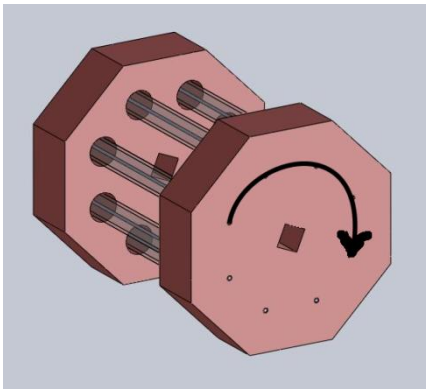

Figure 7-2: Schematic showing the manual rotation of the octagonal stand

7.1.2. Cell Seeding Tube

The functional component of the cell seeding aspect of our design is the Pyrex glass tube. These tubes were derived from standard 5mL Pyrex glass pipettes. The pipette tips are scored every 9cm using a triangular file and snapped by hand. A solid model of one of these tubes is shown in Figure 7-3. The tubes are secured to the octagonal stand using PDMS. The octagonal stand also serves as a mold for the PDMS ensuring that the inner lumen of the tubes is sealed from the outside environment.

7.1.3. Inner Mandrel

The inner mandrel was included in our final design to "catch" tissue tubes after they contracted off of the cell seeding tube. The inner mandrels are derived from a small stainless steel tube that was scored and broken by hand to span the distance between the two tube holders. The inner mandrel is shown in Figure 7-3 in the middle of the cell seeding tube. The inner mandrels are secured to the tube holding stand using PDMS.

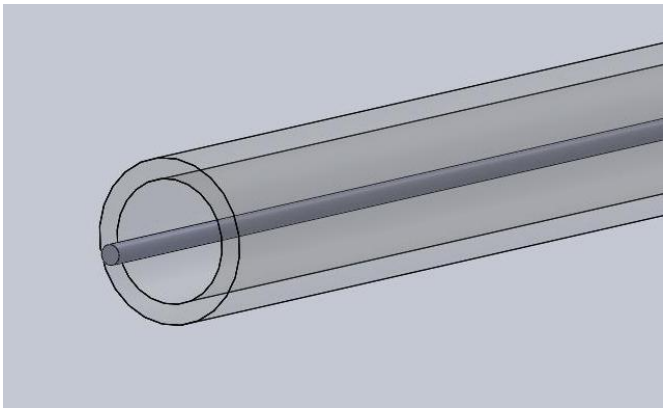

Figure 7-3: Solid model showing the inner mandrel and cell seeding tube

7.1.4. Automated rotation device

An automated rotation system was built to increase the uniformity of cell seeding and decrease the amount of operator time and effort. A Mr. Flame™ rotisserie was purchased from Son of Hibachi™ and incorporated to rotate the entire tube cartridge system. The Mr. Flame rotisserie is firmly attached to a metal base such that there is enough clearance under the rotisserie bar to support the tube cartridge. The final automated rotation system is shown below in Figure 7-4. Since the original rotation rate of the rotisserie was about 6 rotations per minute, a Vex gearing system was used to reduce the rotation rate of the cartridge to the experimentally determined rate of one rotation per hour. This system, constructed of three pairs of gears with 7:1 gear ratios, provides a total ratio of 343:1. This ratio converts the average rotation rate of the cartridge to about one rotation every 55 minutes. The seeding cartridge is designed to slide directly onto a removable axle, making attachment or removal of the cartridge quick and easy.

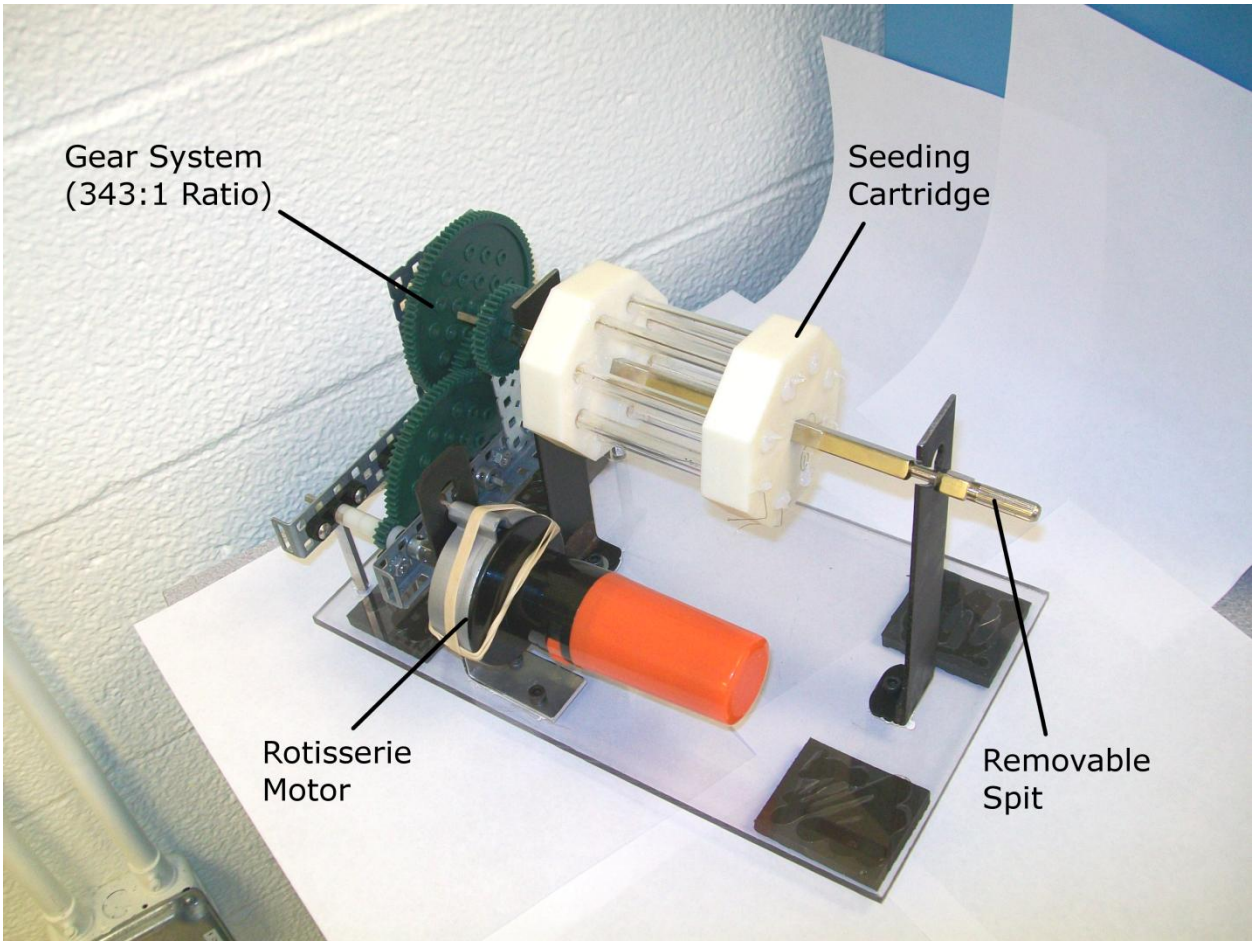

Figure 7-4: Automated rotation system of the tube stands using a rotisserie motor. This setup rotates the stand approximately one turn every hour.

7.1.5. Peristaltic pump

A low flow peristaltic pump was acquired from the Rolle Lab to provide fluid flow to all of the tissue tubes. A peristaltic pump was optimal for our application because we need to keep the inside of our tubing sterile to prevent contamination of our cells. A low flow pump was chosen to prevent shearing of cells off of the walls of the tubes. The peristaltic pump used for our design is shown in Figure 7-5.

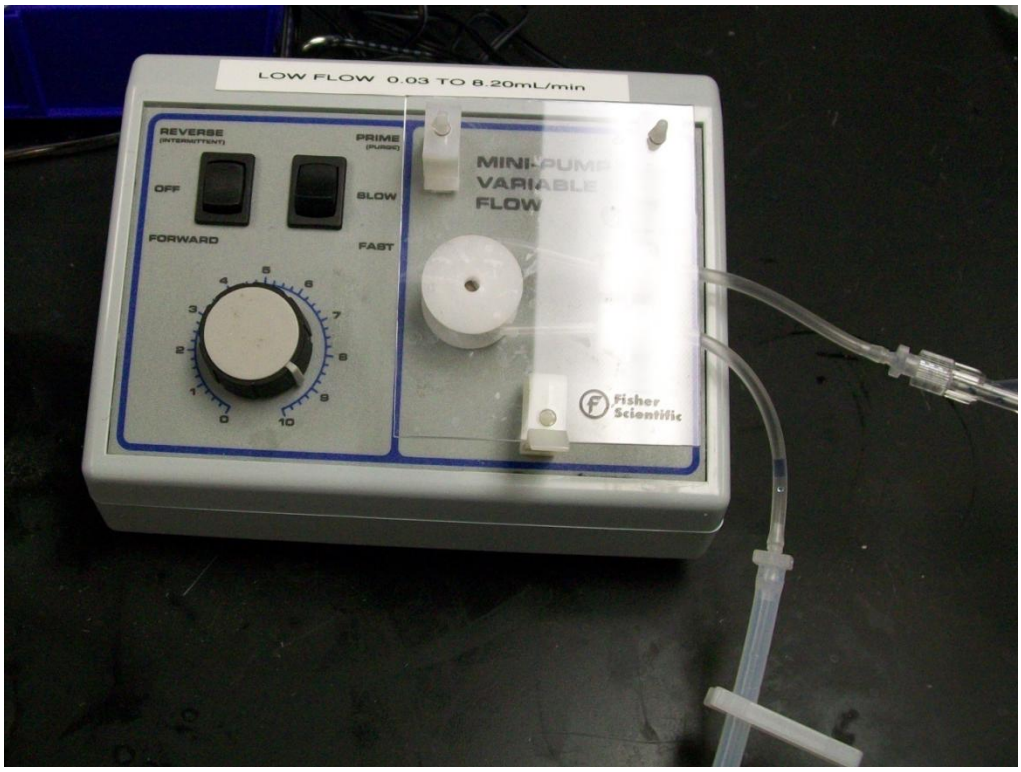

Figure 7-5: The peristaltic pump used for fluid flow. This pump is designed to pump sterile media without contamination.

7.1.6. Media reservoir

A media reservoir was built from a Pyrex bottle and two 1mL glass pipettes. These materials were chosen because they are easily sterilized and attached to our tubing system. The completed media reservoir is shown in Figure 7-6.

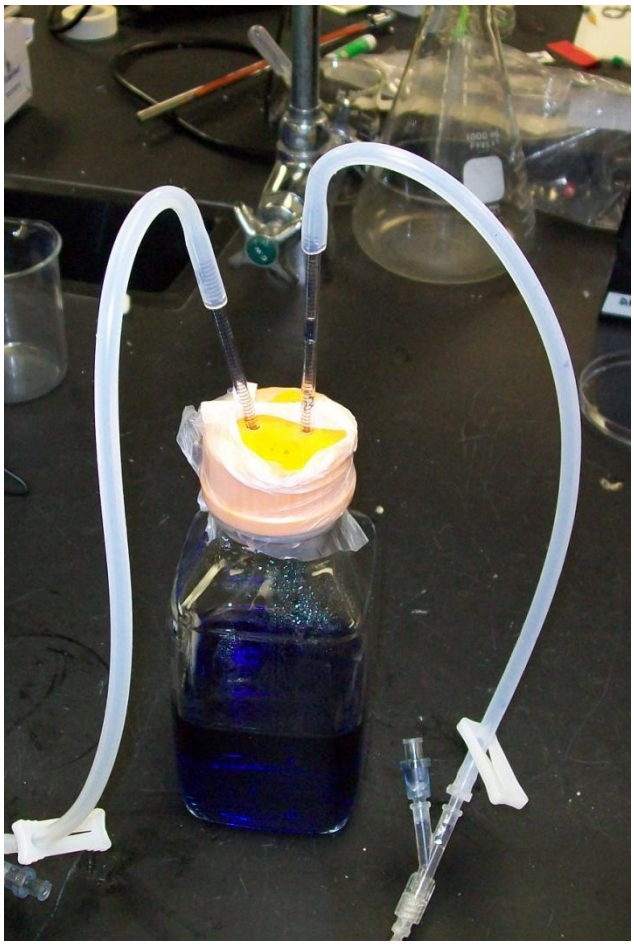

Figure 7-6: The media reservoir that was used for our final design. Two holes were made in the cap of this reservoir to allow for connection to the fluid flow system.

7.1.7. Gas exchange coil

A coil of silicone tubing was attached to the tubing system to increase the overall surface area of media exposed to silicone. Since silicone allows is gas permeable, this created gas exchange for our media. The gas exchange coil is shown in Figure 7-7.

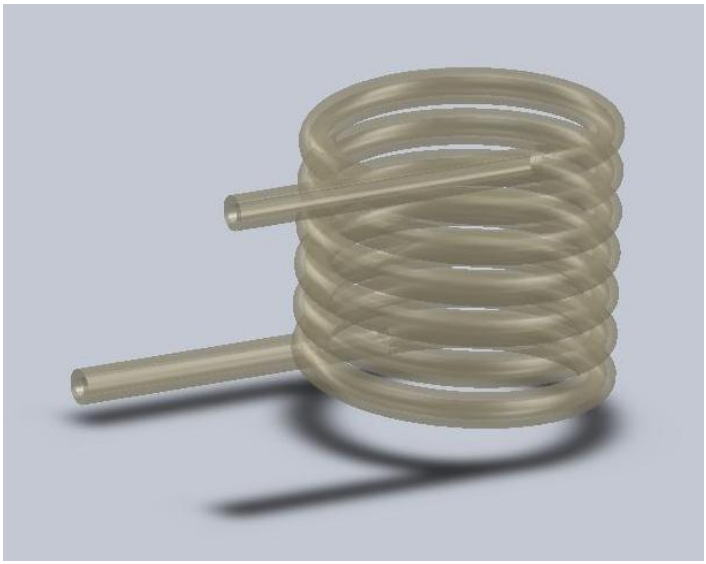

Figure 7-7- A gas exchange coil made from silicone tubing to provide gas exchange to our media. The coil is about 50 inches long.

7.2. Design assembly

Our final design assembly can be divided into two separate design components. The first component is the blood vessel growth cartridge. This component is responsible for providing the necessary environment for cell growth and tube formation. The second component is the media exchange system. The media exchange system is responsible for supplying fresh media and effective gas exchange to all vessels throughout growth of the cell tubes. The media system also contains the capability to inject K-PSS or other liquids directly into the cell tubes to cause tube contraction.

7.2.1. Vessel growth cartridge

The vessel growth cartridge is built from two octagonal stands, eight cell seeding tubes and eight inner mandrels. During construction, the back of the two tube holders are first covered with duct tape to block the feeding and mandrel holes and prevent leakage. One octagonal stand is then placed on its back and eight inner mandrels and eight cell seeding tubes are then inserted

into their respective holes in the tube holder. Polydimethylsiloxane (PDMS), an easily used and relatively cheap rubber polymer is then used to seal the tubes in place. PDMS is mixed and poured in and around each cell seeding tube and mandrel. The tube holder is then heated at approximately 60°C for one hour to cure the PDMS. The second tube holder is then placed on its back on a table and the free ends of the mandrels and tubes are placed in the second tube holder. PDMS is once again poured around the tubes to form a seal. The PDMS is cured at 60°C for approximately 1 hour. The final result of this process is a seeding apparatus that can hold up to eight tissue tubes throughout the process of cell seeding and tissue growth. The entire system can be autoclaved before use to ensure sterility. A graphical depiction of the construction of the vessel growth cartridge is shown in Figure 7-9 and a picture of the actual constructed cartridge is shown in Figure 7-8

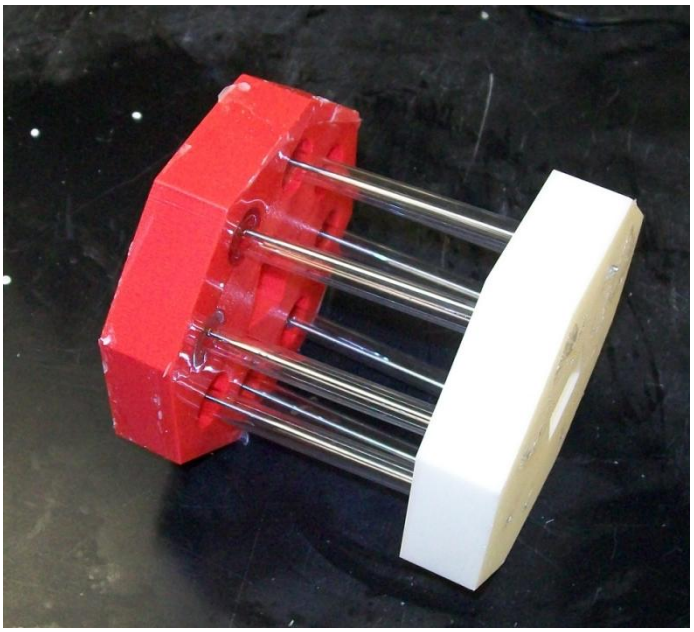

Figure 7-8: The completed vessel growth cartridge.

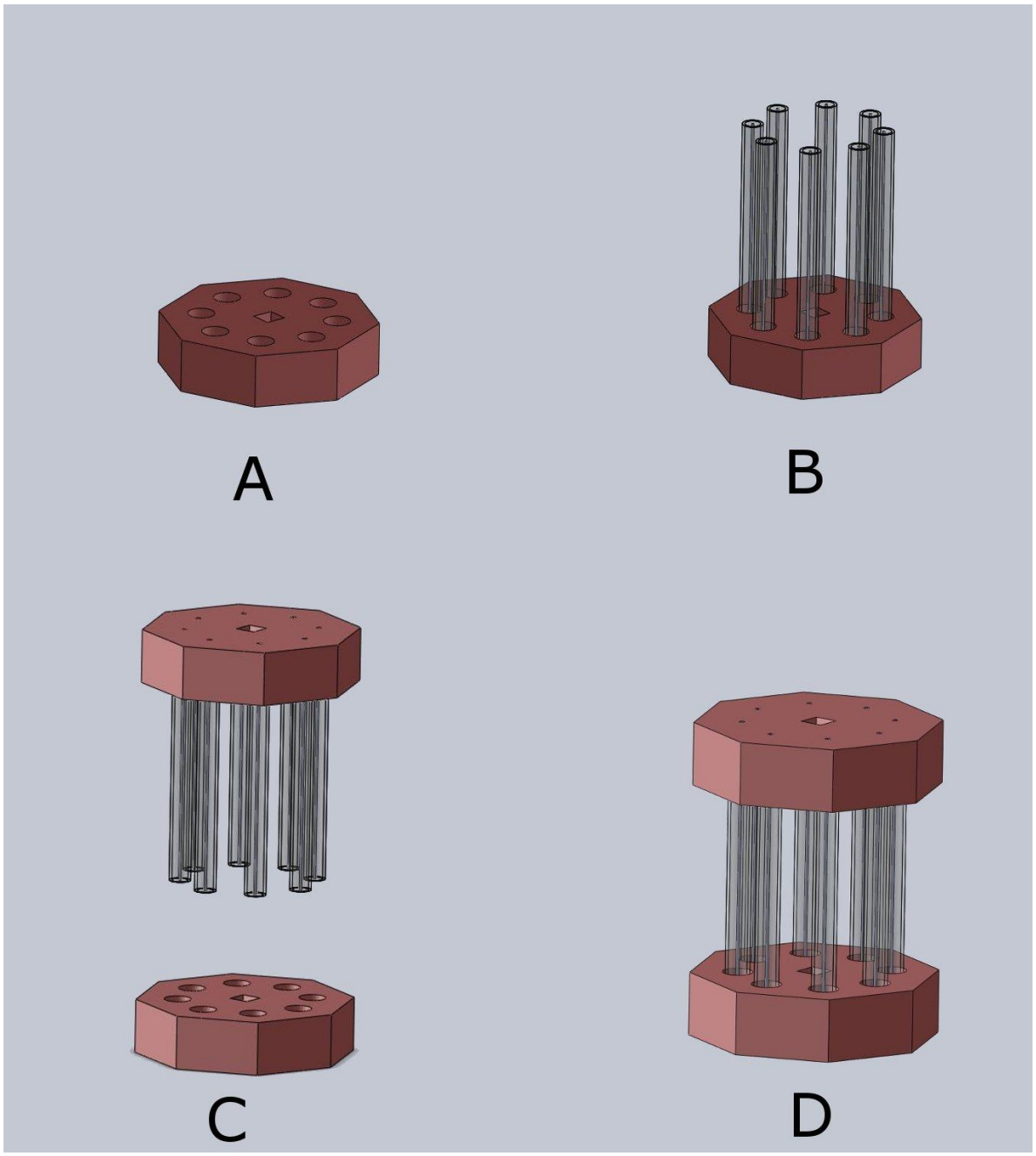

Figure 7-9: Construction of the vessel growth cartridge. (A) Duct tape is applied to the back of a tube holder and it is placed face up on a lab bench. (B) The glass cell seeding tubes and inner mandrels are placed in their respective holes in the tube holder. PDMS is prepared and poured around the tubes and the PDMS is cured. (C) The second tube holder is seeded and placed face up on the bench top. The free ends of the tubes are placed in the second mandrel and sealed with PDMS. (D) The final vessel growth cartridge is ready to be autoclaved.

7.2.2. Media exchange system

The media exchange system is composed of a media reservoir, a gas exchange coil, a peristaltic pump, 16 twenty gauge needles, a variety of luer-lock tubing accessories and a length of 1/8 inch silicone tubing. The entire tubing portion and media reservoir of the media exchange system can be autoclaved before use to ensure sterility. The final media exchange system is shown and described in detail in Figure 7-10.

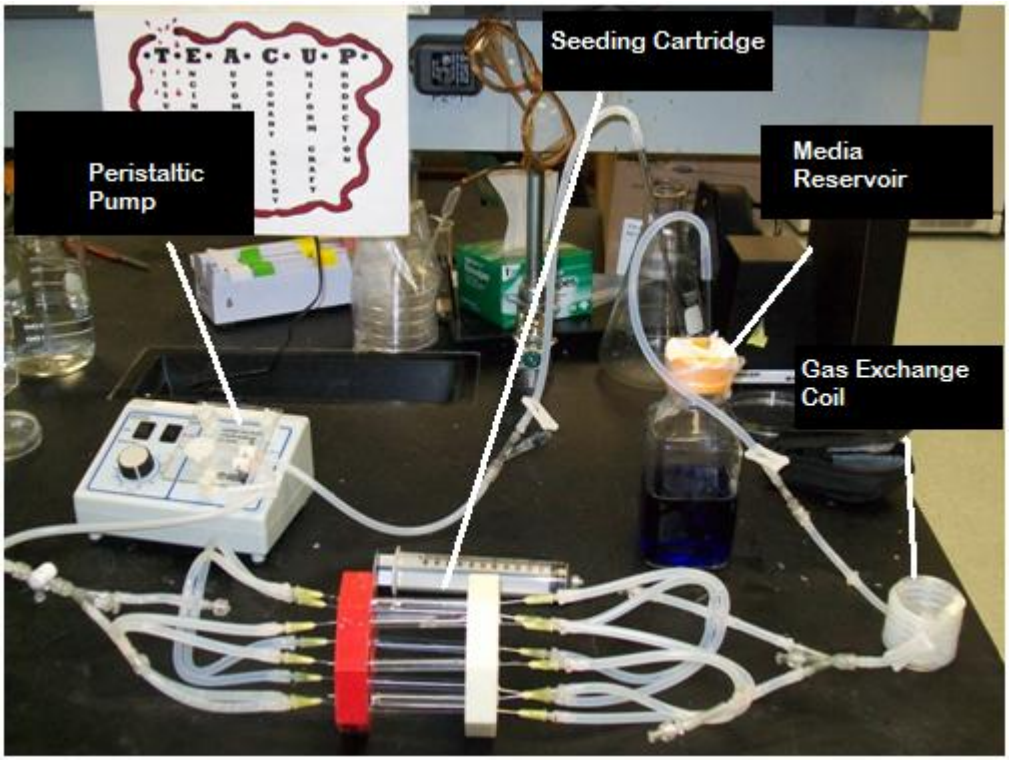

Figure 7-10: Media exchange system. Media is pumped from the media reservoir and through the gas exchange coil where it reaches each of the glass vessel culture chambers. From the culture chambers the media is returned to the reservoir for recirculation. Included in the tubing are ports where K-PSS solution can be injected into the media tubing system.

7.3. Growth of tissue tubes

In order to maximize the efficiency and efficacy of our final design, we endeavored to reduce the number of steps involved in seeding and feeding the tubes while minimizing material expenditure and maintaining a sterile cellular environment. As a result, the steps involved in using our design can be broadly divided into three major steps. The first of these steps is cell

seeding, in which the goal is to evenly coat the glass tubes with cells at an ideal seeding density. The second of these steps is the growth phase during which we aim to provide optimum physiological conditions to the growing tubes. The final step is the tube contraction step. During tube contraction, we aim to force the confluent tissue tubes to contract off of the seeding tubes and close around the inner mandrel. During this period, we minimize the period of time that the cells are placed in a purely K-PSS solution.

7.3.1. Cell Seeding

All of the steps in this method were completed under sterile conditions in a laminar flow hood. To begin cell seeding, we first spun down and counted rat aortic smooth muscles from our cell culture flasks. We then diluted the cells to a target value and thoroughly mixed the cells in a conical tube. A sterile 20 gauge needle was placed into the needle port of one of the cell seeding tubes in the sterile vessel growth cartridge. While holding the cartridge vertically, with the aforementioned needle at the top, a volume of cell solution containing the target number of cells was slowly injected into the opposite end of the tube using a second 20 gauge needle and 3.0 mL syringe. Next, approximately 1.5 mL of sterile media was injected in the same manner into the tube until media began to flow out of the upper needle. Both needles were then carefully removed to minimize the formation of bubbles in the seeding tube. These steps were then repeated for the remaining seven seeding tubes. The seeding process is shown schematically in Figure 7-11.

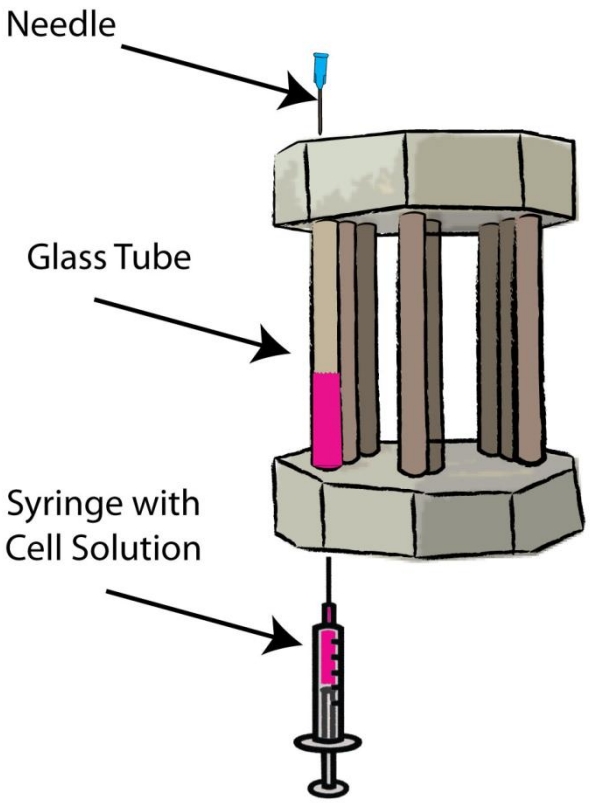

Figure 7-11: Schematic showing the seeding of a Pyrex Tube. This process is repeated for each of the eight tubes

7.3.2. Tissue Growth Phase

After the cells are seeded on the glass tubes, it will be necessary to provide constant media and gas exchange to optimize cellular growth. To accomplish this, we will attach the vessel growth cartridge to the media exchange system. To ensure sterility, the entire system and tubing ends will be placed in a sterile laminar flow hood to make the connections. A sterile 60mL syringe will be used to inject media into the tubing upstream of the peristaltic pump in order to prime the pump and remove any potential air bubbles. Sterile 20 gauge needles will be inserted into each of the 16 needle ports on the vessel growth cartridge. The syringes will then be attached to the pump system as shown above in Figure 7-10. Once the entire tubing system is sterilely connected, the entire apparatus will be placed in the incubator and fluid slowly pumped through the system.

7.3.3. Tube Contraction

The third and final step was to contract the cultured tissue from the seeding tubes. After 72 hours, K-PSS was drawn into a 25mL syringe and injected into the seeding tubes. The tubes were left in the incubator and monitored until they had visibly detached from the seeding tubes and contracted around the inner mandrel. This process typically took less than 20 minutes. The tubes were then ready for removal or continued growth. The contraction process is shown in Figure 7-12.

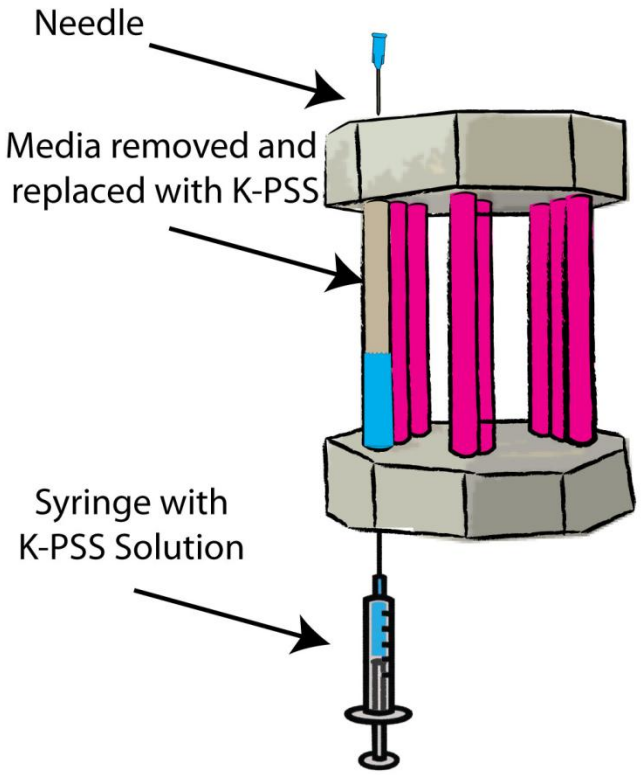

Figure 7-12: After the cell seeding phase, the media is removed from the tube and replaced with K-PSS to induce contraction.

7.4. Design Verification

The overall goal of our design was to create a viable tissue tube from completely cellular components using minimal operator time and resources. To verify that our design achieved this goal, we tested several components of our design separately to show that our design will work in its intended manner. The major parts of our design verification were to show that we could evenly coat a seeding tube with smooth muscle cells, to show that we could cause the smooth

muscle cells to spontaneously contract off of their substrate and to show that our bioreactor could provide even media flow to all eight tubes that were connected to it. This section will describe the methods and results of our design verification experiments

7.4.1. Even cell coatings

To test even coating of cells on the Pyrex glass seeding tubes, cells were manually injected into four sealed tubes and manually rotated by 1/8 of a rotation every 10 minutes for 2 hours. Two of these tubes were fixed with ethanol after 24hrs and two were fixed with ethanol after 72 hours. The cells were stained with nuclear fusion red, which stains the nuclei of the cells a reddish color. This was accomplished by immersing the tissue in nuclear fusion red for five minutes and rinsing with distilled water. The distilled water was aspirated and replaced with ethanol for long term storage.

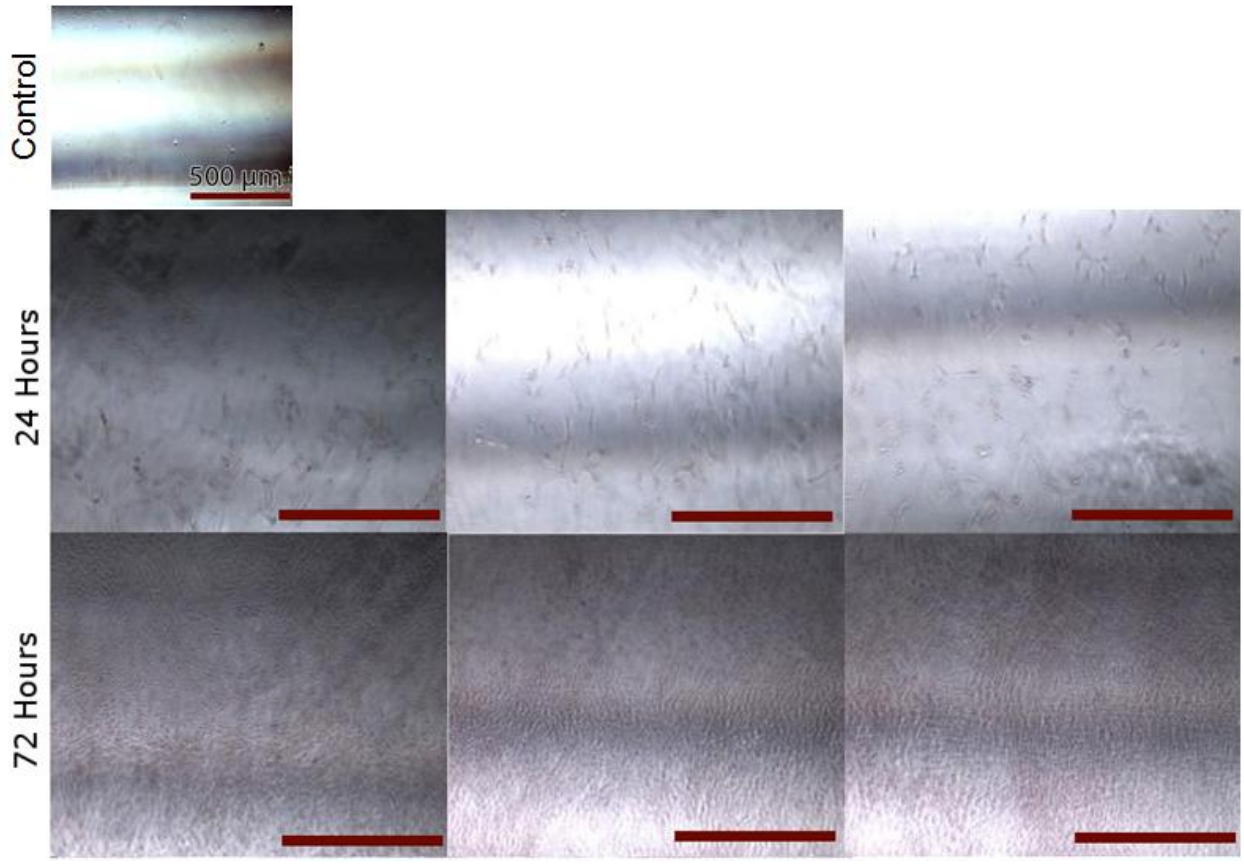

Figure 7-13: Images showing confluency of cell seeding at 24 and 72 hours. The top three panels show different portions of a tube 24 hours after cell seeding. The bottom three images show tubes imaged 72 hours after cell

seeding. These images are representative of all sections of tubes. Scale bars are 500 μm.

7.4.2. Cell contraction

During our preliminary testing, we found that sheets of smooth muscle could be induced to contract off of tissue culture plastic using K-PSS. In order to optimize this process, we used 75 cm^2 flasks and seeded them at either high cell density (16 million cells) or low cell density (2 million cells) for 1, 2, 3 or 4 days. During the cell growth period, we replaced the media as needed. At the end of the growth period, we replaced the media with K-PSS and observed to see if the cell sheets contracted off of the plates. Through these experiments, we determined that the optimal density was 2 million cells / 75cm^2 with the addition of K-PSS after 72 hours. This resulted in the contraction of essentially the entire sheet off of the tissue culture plastic. Once the tissue sheet was contracted, the flask was fixed using 70% ethanol and stained with Trypan Blue. Figure 7-14 is an image of a stained flask after induced tissue sheet contraction using K-PSS. It can be seen that most of the tissue sheet in the majority of the flask was contracted, with only a patch of tissue around the neck region of the flask remaining.

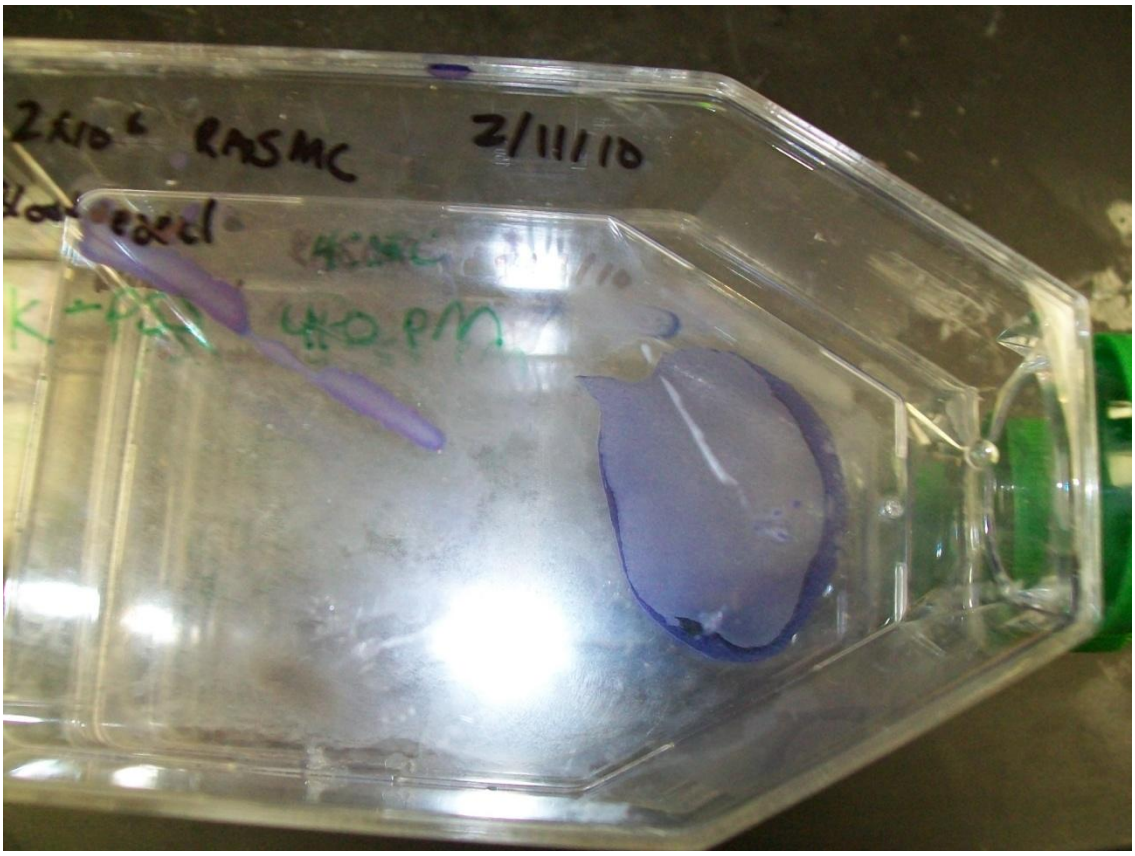

Figure 7-14 Flask stained with Trypan Blue after tissue sheet contraction using K-PSS

To translate these results to our cell tubes, we determined the approximate cell seeding surface area of our tubes and converted the cell density accordingly. This density, 250,000 cells/tube was then injected into several cell culture tubes and rotated by hand as previously described. At 48 hours, the media in the tubes was replaced and the tubes were replaced in the incubator to continue growing. After 72 hours, the media in the tubes was replaced with K-PSS and the tubes were incubated until contraction occurred. It was found that the K-PSS caused tissue to contract off of all tubes within 30 minutes. In particular, we found that a portion of one tube contracted radially and actually attached to the inner mandrel to form a small tissue tube as shown in Figure 7-15. This result shows that our cell density experiments have found a correct density and seeding period that can form tissues through cell contraction with K-PSS.

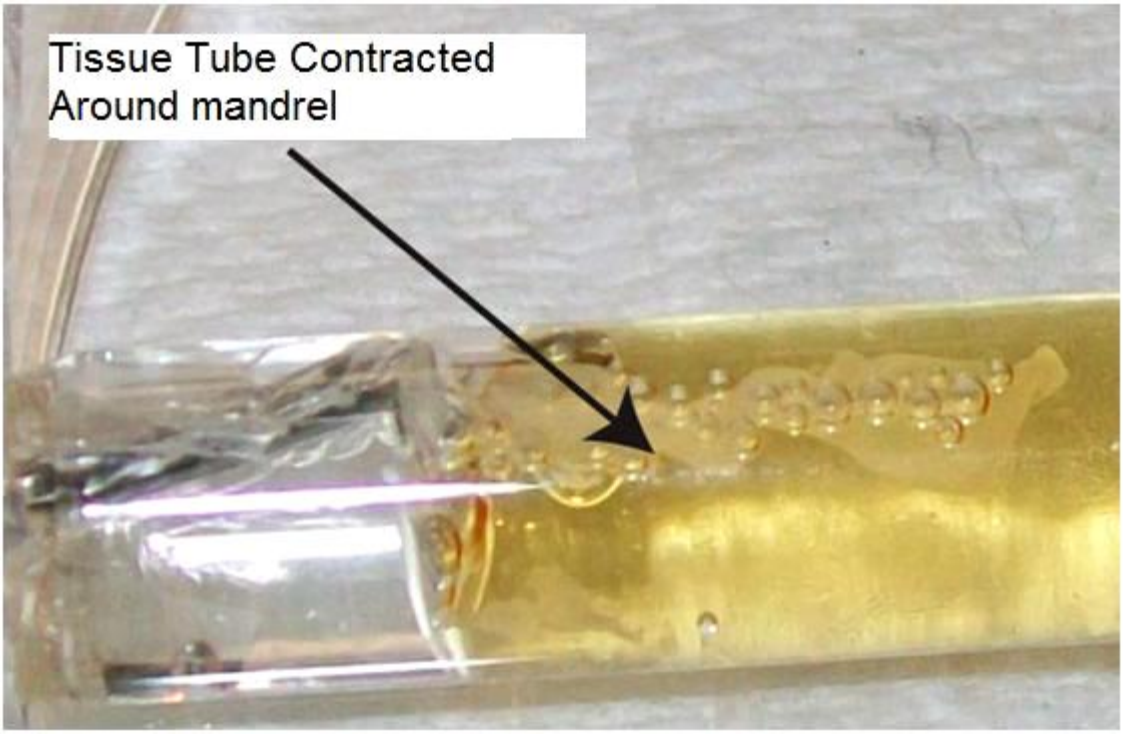

Figure 7-15 Tissue tube contracted around entire diameter of central mandrel

Using these results, the final design was assembled and tested. Each tube was seeded with a density of 250,000 cells/tube and the cell culture stand was rotated approximately 1 revolution/hour for 2 hours using the rotation system. The entire device was placed within the incubator as shown in Figure 7-17.After two hours, the rotation system was separated from the cell culture stand and removed from the incubator.

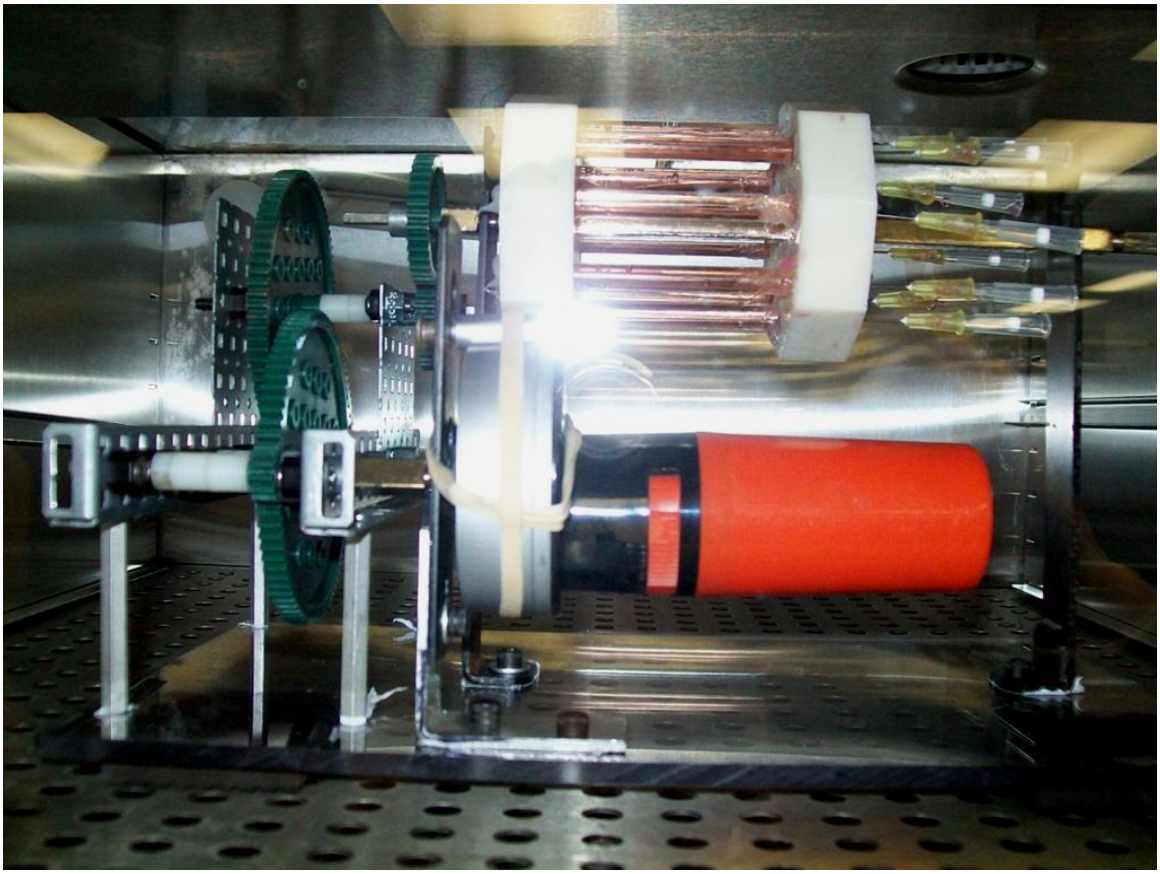

Figure 7-16 Tissue culture stand placed on automatic rotation inside the incubator

Due to difficulty in containing leaks, the pump system was not utilized and cells were fed manually using syringes at 48 hours. At 72 hours the sheets were harvested by completely replacing the media via injecting K-PSS into the culture chamber. Within 30 minutes, the majority of the tissue had contracted off of the glass tube wall. However, rather than contracting as a continuous sheet, the tissue sheet tore into smaller pieces and contracted off the tube as individual "flakes". Figure 7-17 shows an image of a "flake" of tissue contracting off of the glass tube. This outcome is most likely due patches of dead cells caused by air bubbles that formed within the glass tube chamber. These dead patches cause stress concentrations due to uneven sheet thickness resulting in tearing during contraction. Furthermore, an uneven distribution of contractile proteins within the cell sheet would also result in non-continuous sheet contraction.

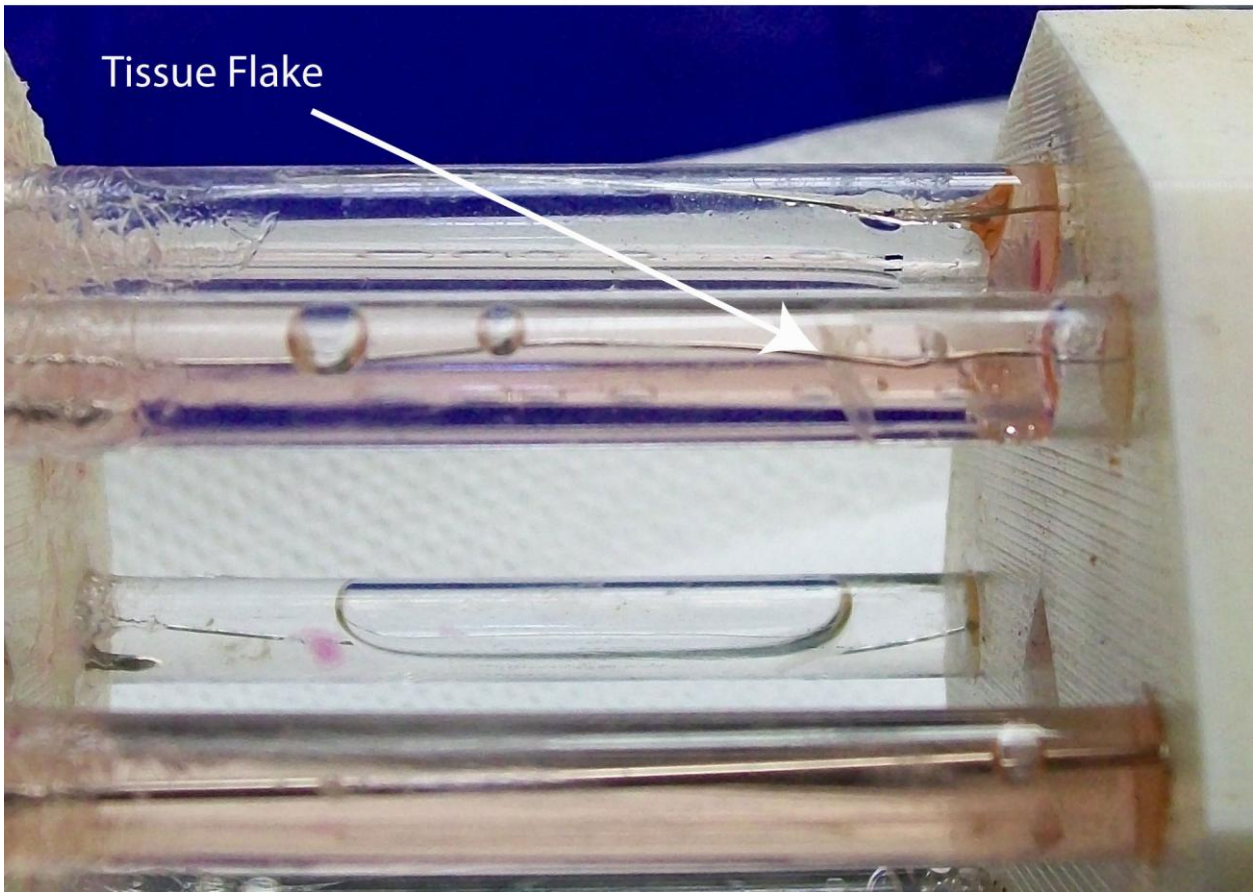

Figure 7-17: Tissue flake after tissue contraction using K-PSS solution.

7.4.3. Fluid flow

Fluid flow testing used the media flow system described earlier in this section. The tubing system and manifolds used to divide media input to each cell culture tube were tested to ensure even fluid flow. A dyed water solution was used to better visualize the fluid flow. This solution passes from the Pyrex bottle, which acts as the media reservoir, through the tubing structures and a peristaltic pump. Eventually the fluid reaches a set of two manifolds that divide the tubing into eight separate pathways. Each pathway leads to a 15 mL conical tube to collect the output solution and then observed for equal rates and quantities. The final volumes in the conical tubes were similar at the end of the experiment, although not perfectly equal.

Ideally, the fluid flow would be even to prevent any differences in flow between tubes, however uniform media flow to each cell culture tube was difficult to achieve. Even flow is

critical when contracting the tissue sheets off of the outer tubes because the K-PSS is injected into the tubing directly upstream of the glass seeding tubes. If unequal amounts of K-PSS reach the tubes, some tubes may contract fully while others only contract partially or not at all.

The design team experienced considerable difficulty creating even fluid flow through each of the glass culture tubes. This problem was likely due to the air that initially was in the tubes before flow begins. The air bubbles seem to prevent fluid flow down a tube and therefore some tubes receive a large volume of fluid while others receive little. In order to overcome this problem, the team attempted to completely fill the tubing with fluid before activating the pump. Media was systematically injected into segments of the tubing blocked off by tubing clips and stop-valves. In this way, the team attempted to ensure that the tubing and cartridge were filled to capacity with media, instead of estimating the volume of media the entire system contains and filling it all at once. Additionally, this allowed the team to observe any problems encountered by the fluid flow in each segment, providing an opportunity to make any adjustments necessary. The team also tried forcing fluid down each of the tubes while withdrawing air to create a vacuum, such that the air can be removed as the volume is replaced with media; however these methods were largely ineffective at avoiding leaks and air bubbles trapped in the tubing during media flow.

While each of these methods improved on the results of fluid flow, neither was able to create the flow through all of the tubes. It may be possible that the process of piercing through the PDMS of the cell culture cartridge, or the ports to the media reservoir which introduce a breach in the system that should be otherwise airtight. In this case, an alternate method of sealing the glass culture tubes onto the vessel or sealing the tubes leading into the media reservoir may be effective at eliminating fluid flow issues in the future.

8.0 Conclusions and Recommendations

Although each individual component of our device was conceptually shown to work, there is still room for improvement through future research and development. Conclusions made from our research, as well as future recommendations for the advancement of our device will be discussed in this chapter.

8.1. Design Considerations

As with any design project, the larger societal impact of this project and its outcomes must be examined and discussed. In this section we will analyze the impact of this project on various aspects of the world around us.

8.1.1. Economic Impact

If the device is successful in growing tissue tubes, a large amount of lives could be saved by using the products of this device to replace native diseased arteries. Heart disease remains the leading cause of death in the United States. If a successful graft is grown, people would be healthier longer and be able to remain in the workforce longer creating a positive economic impact.

With FDA approval, autologous grafts would not be needed which would minimize the cost of a second surgery. Any complications and extended stay from a second surgery would also be eliminated. Furthermore, the number of heart transplants would be reduced due to diseased arteries being replaced before any cardiac event happens. This would save money on the costly procedure of a heart transplant and any immunosuppressant drugs that would be needed for the rest of the patients' life.

The device could also generate jobs in Tissue Engineering research, product manufacturing and in sales. Since there is essentially no product on the market except for the Lifeline graft, the growth of this market could be immense.

8.1.2. Societal Impact

Based on the aging population and the high instance of coronary artery disease in the population today, tissue engineering has a large opportunity to impact society. With the

development of a completely tissue engineered blood vessel, an increase in life expectancy would likely be seen. The tissue tubes could be used to restore blood flow to infarct areas of the heart after heart attacks. Based on this information, the potential societal impact is substantial with the development of a tissue engineered blood vessel.

8.1.3. Ethical Impact

Due to its recent growth in public awareness, the field of tissue engineering is increasingly becoming a subject for debate on ethics.[34] To some, Tissue Engineering is a step in the right direction in that it attempts to solely use cells derived from nature. Others, however, identify the use of cells outside of the body to create "body parts" as a direct violation of nature, and therefore unethical. Although we have only used somatic cells derived from rats thus far in the design and testing of our project, we must address the fact that the eventual intent of this project is to produce tissue tubes from human cells. This practice, which is considered morally suspect by many religious groups[35], would make the use of our project controversial if it were ever used in a clinical setting.

To support those who disagree with tissue engineering, while not denying the benefits of our project from those who accept it morally, we recommend that patients receiving the benefits of our project receive informed consent.[35,36] This concept, which has been supported by previous studies[35], requires that patients are informed of the origin of all tissues that are implanted in them prior to receiving surgeries. This approach simply and effectively appeases both sides of the issue and gives patients the power to make decisions based on their own ethics and beliefs.

8.1.4. Health and Safety

With regards to health and safety, it is imperative that the device be readily sterilized to ensure that the vessel produced is free from contaminates. As such, it would be wise that the bioreactor be composed of a number of parts that may be disassembled to allow for cleaning. Also, these parts must be composed of materials that can be cleaned without being damaged. It would also be important for the bioreactor to be controlled for media circulation and gas exchange to allow minimum exposure to elements outside of the desired of the system.

Finally, the design must ensure that the user interface provided minimizes contact with the vessel or media and does not pose any bodily threat to the user, (infection, electrocution, burns etc.)

8.1.5. Manufacturing

In order to design for manufacturing, the device will the composed of parts that are widely commercially available as well as economical. Each part of the device will be chosen to be as mechanically simple as the design will allow, while minimizing the number of parts used in total. The device will be assembled in a manner that is comprehensive and reproducible for future replication.

8.1.6. Environmental Impact

With respect to the environment, all parts used will be of materials that are minimally hazardous to the environment and of materials that do not produce any toxic waste-products. To minimize environmental impact, we have chosen materials that can be sterilized and reused through several seeding cycles. The device was designed such that all materials are conserved and eventually disposed of in a suitable manner by following OSHA and EPA regulations for biohazard disposal. Any power necessary for running the device will electric, and may produce by-products known to be harmful to the environment depending on the source of the electricity. Depending on the manufacturing company contracted to build and distribute the device, large-scale production of the device may be responsible for greenhouse gas emissions due to industrial and transporting processes.

8.2. Conclusions

The preliminary testing of a novel method for three-dimensional cell culture shows that tissue tubes may be produced by seeding cells into a cylindrical tube and contracting the cultured tissue sheet onto an inner mandrel. This method is superior to previous methods in that the tubular device allows for adequate cellular adhesion without the use of a coating, which has been difficult and time consuming in the past. Additionally, due to the contractile nature of smooth muscle cells and the cellular response to K-PSS buffer, the vessel produced may be contracted onto a mandrel once confluence has been achieved. This would allow researchers to create tubes

of various wall thicknesses based on culture time and seeding density, in order to optimize tissue properties. Following tissue contraction, the vessel can be easily removed from the inner mandrel and conditioned for desired mechanical tissue properties.

This method of culturing cylindrical tubes offers many advantages over the current hanging drop method which is limited to seeding cells onto only one side of a mandrel at a time. Vessels produced in this manner are often non-uniform in cell thickness axially, as this method is by and large a two-dimensional approach. Inconsistent wall thickness results in uneven stress concentrations in the vessel wall, which compromises the vessel's mechanical integrity. Seeding cells into a cylindrical tube and rotating the tube applies the same concept of directing cell attachment but approaches it in a three-dimensional sense. By allowing cells to adhere to one another freely along a continuous inner surface, one can produce a tissue sheet cylindrical in shape without the uniformity issues associated with the hanging drop method.

The hanging drop method also requires the use of a mandrel coated with collagen, which promotes cellular adhesion to the mandrel. The procedure of coating the mandrels used in the hanging drop method is especially labor-intensive and often produces mandrels with imperfections, such as air bubbles, rendering the products unusable. The method developed by the current research produces grafts that are free of artificial adhesives. This greatly decreases the time required to set up a device that is able to grow tissue engineered blood vessels. Additionally, the reproducibility of viable tubes is greatly increased due to the decrease of possible imperfections in the tubes produced.

The success and simplicity of this method allows the entire process to be altered in a few ways to better meet the client's needs. First of all, the device can be "scaled-up" and may produce several scaffold-free tissue tubes at once. Initially the device could only produce one tube at a time. The current design, however, allows for up to eight tubes to be grown at a time. This increases the likelihood that viable tubes will be produced with each "batch" that is seeded. Secondly, the completely contained fluid flow system provides many advantages in this design. For example, the fluid flow rates in the bioreactor can be adjusted based on the pump speed and the tubing setup. This allows for a sufficient and equal amount of media to be supplied to each tube. This ensures that any differences in the tissue tubes produced are not due to insufficient media supply. The complete pumping system is also an added benefit because it helps to

automate the process, which decreases the work required by the operator. It is completely self-contained, which provides a sterile environment within which cells may be seeded and cultured. Ports are also provided in the bioreactor so that at the appropriate time K-PSS may be administered without disrupting the sterility of the media flow system. The entire assembly is small enough and compatible for use directly inside an incubator.

Based on the results of the conceptual testing described above, this device shows great promise in producing tissue tubes with the potential for use as vascular grafts. While more research is needed to perfect this approach, we believe that the device has the potential to impact the future of tissue engineered vascular grafts for use in arterial bypass surgeries. As described in previous sections of this chapter, the impact of this device may have implications reaching far beyond the field of tissue engineering.

8.3. Recommendations

Given more time and a larger budget, this design could be improved in several ways. The following sections include some of the suggestions the design team has for future researchers using or expanding upon our device.

8.3.1. Device Construction

Although our device is more time and labor efficient than the method currently used in the Rolle lab, we believe that our design could be further optimized to decrease the labor and time required in its construction. The main change we think could be making the seeding tubes more easily removable from the seeding cartridge. This change would allow the tubes to be more easily viewed under the microscope throughout the seeding process. Additionally, we would recommend replacing PDMS connections with a more easily disassembled connector that still retains the sterility of the tubes.

8.3.2. Cell Seeding Optimization

In our early cell seeding experiments, we compared low concentration with high concentration cell seeding. During these experiments we determined that seeding our cells at a lower concentration for three days produced more robust tissue sheets than seeding at higher concentrations for a shorter period of time. Given more time and resources, we would have liked

to have tested more concentration and seed time alternatives to further deduce the most effective concentrations. Additionally, altering culture conditions, such as the concentration of fetal bovine serum in the media, could improve the structural properties and mechanical functions of the tubes being produced.

8.3.3. Fluid Flow System

During our project, we designed a low flow pump and tubing system to provide constant media exchange to our cell seeding cartridge. Although this system worked conceptually during non-sterile tests for even flow, we found that trying to use this system during an actual tube seeding process was not feasible. Due to space constraints in a cell culture hood and incubator, and time constraints required to keep our cells at optimal temperatures, we found that the system was hard to connect and manage. As a result, we recommend that a new system be developed to provide the bioreactor portion of our design. Specifically, to increase the effectiveness of this system, the cell culture tubes should be redesigned to connect directly to the pump tubing.

8.3.4. NIPAAM

Based on the ineffective tissue tube removal methods described previously, this device could be greatly improved by using a consistent and deliberate tube removal method. One possible approach that could be taken in the future is the use of NIPAAM (N-isopropylacrylamide), which is a thermo-responsive polymer that can be used to coat cell culture surfaces. NIPAAM is hydrophilic at 37°C (the temperature used for incubation during cell culture) but becomes hydrophobic when the temperature drops below 32°C. Therefore, if cells are cultured on a NIPAAM coated surface, they will adhere to the surface, proliferate, and eventually form a confluent sheet across the surface area. Then, once the temperature is lowered below 32°C, the surface becomes hydrophobic and the cell sheet releases from the surface and remains in sheet form due to the cell derived extracellular matrix. A schematic of this process is seen below in Figure 8-1.

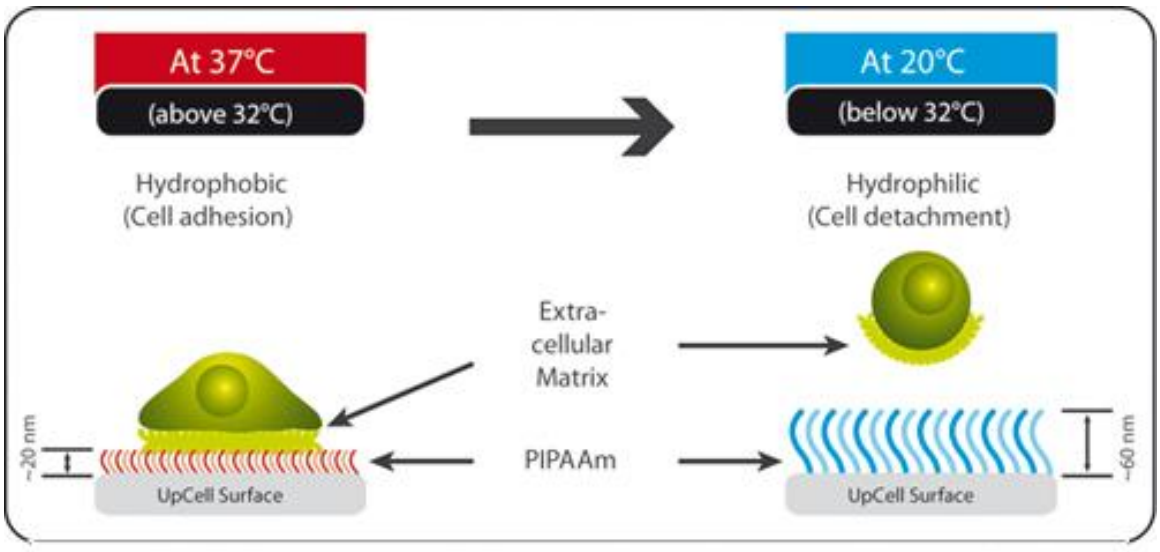

Figure 8-1: Schematic representation of the thermo-responsive polymer NIPAAM used as a surface for cell seeding. As seen above, a temperature change from 37°C to 32°C will release the cells from the seeding surface.[37]

The team completed preliminary testing of NIPAAM using an UpCell 24-well plate made by Thermo Scientific. As described in Appendix D, the wells were seeded with fifteen thousand, thirty thousand, or sixty thousand cells and allow to culture for 72 hours. The plate was then left at room temperature for 30 minutes while being observed. At this time, there was no noticeable change in the appearance of the tissue sheets. Therefore it was cooled using an ice bath. After being cooled for 5 minutes, it was noticeable in some areas that the tissue sheet had released from the surface. A picture taken during this time is seen below in Figure 8-2.

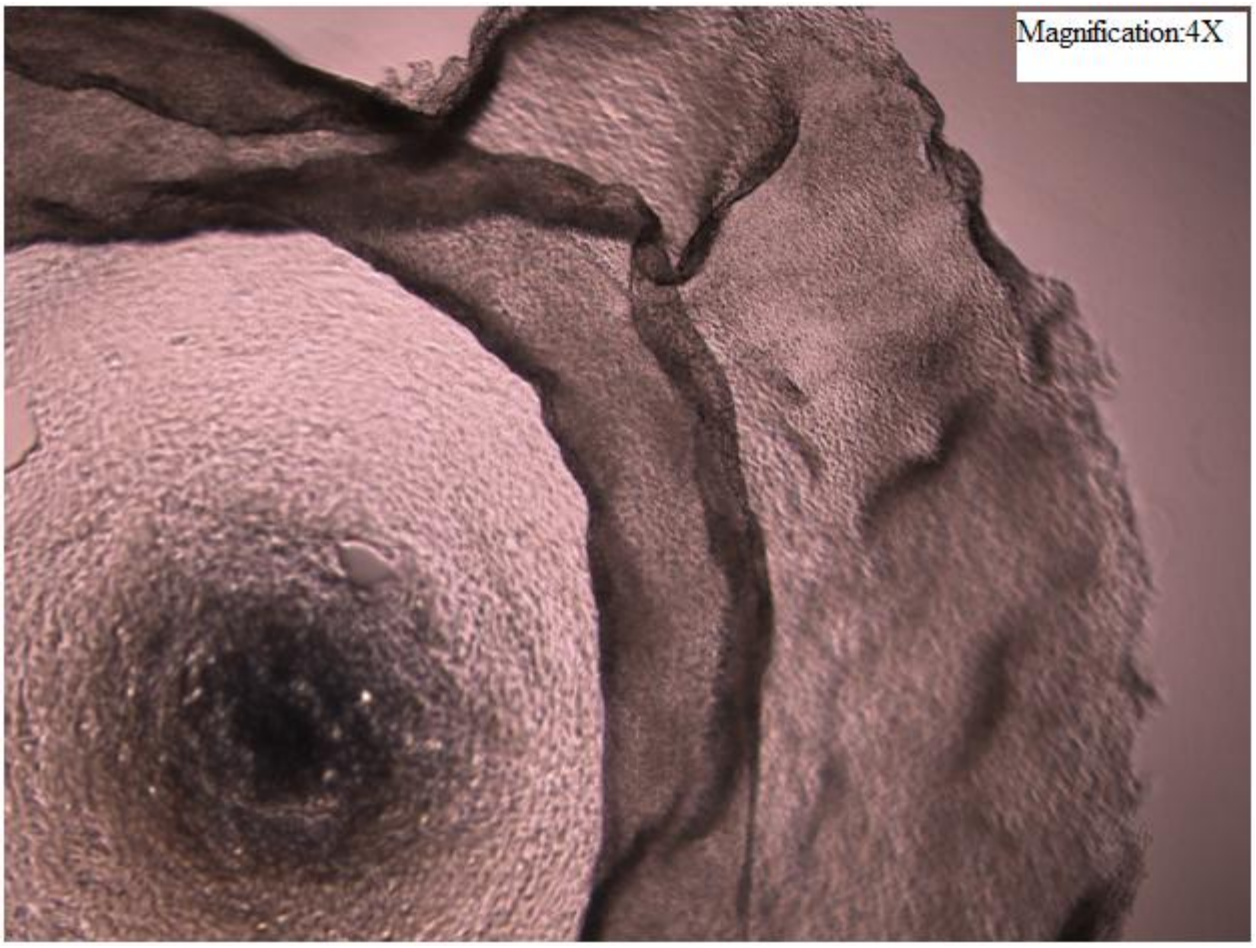

Figure 8-2: A contracting cell sheet on an UpCell NIPAAM coated plate. This well was seeded with sixty thousand cells and allowed to culture for 72 hours.

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Appendix A: Pairwise Comparison Charts

Top Objectives

	Bioreactor	Cell Seeding	Mandrel Material	Score
Bioreactor		0	0	0
Cell Seeding	1		0	1
Mandrel Material	1	1		2

Bioreactor

	Automatically Supply media	Fits into incubator	Can be sterilized	Physiological Environment	Score
Automatically Supply media		1	1	0	2
Fits into incubator	0		0	0	0
Can be sterilized	0	1		0	1
Physiological Environment	1	1	1		3

Cell Seeding

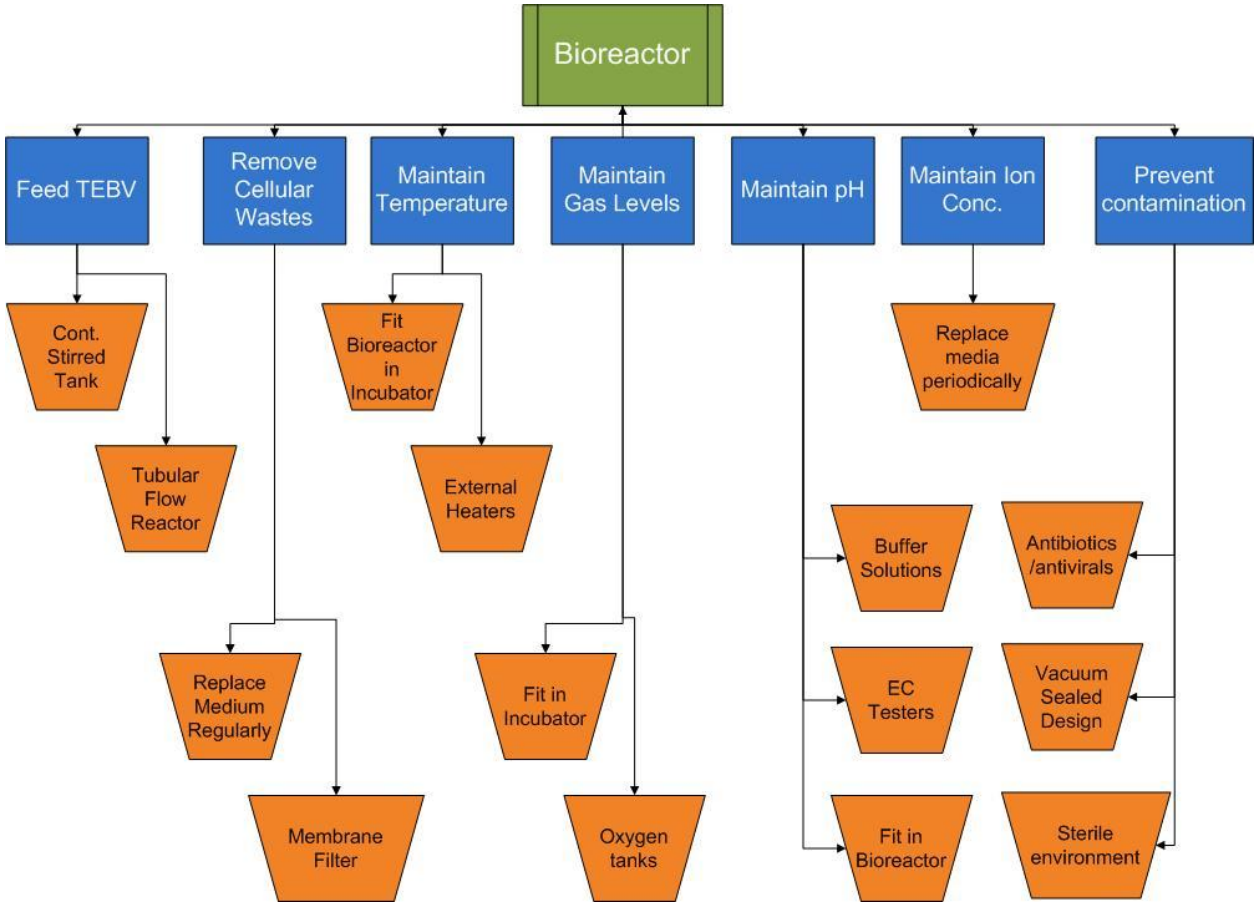
	Stick evenly to mandrel	Grow and support own ECM	High success rate	Score
Stick evenly to mandrel		0	1	1
Grow and support own ECM	1		1	2
High success rate	0	0		0

Mandrel

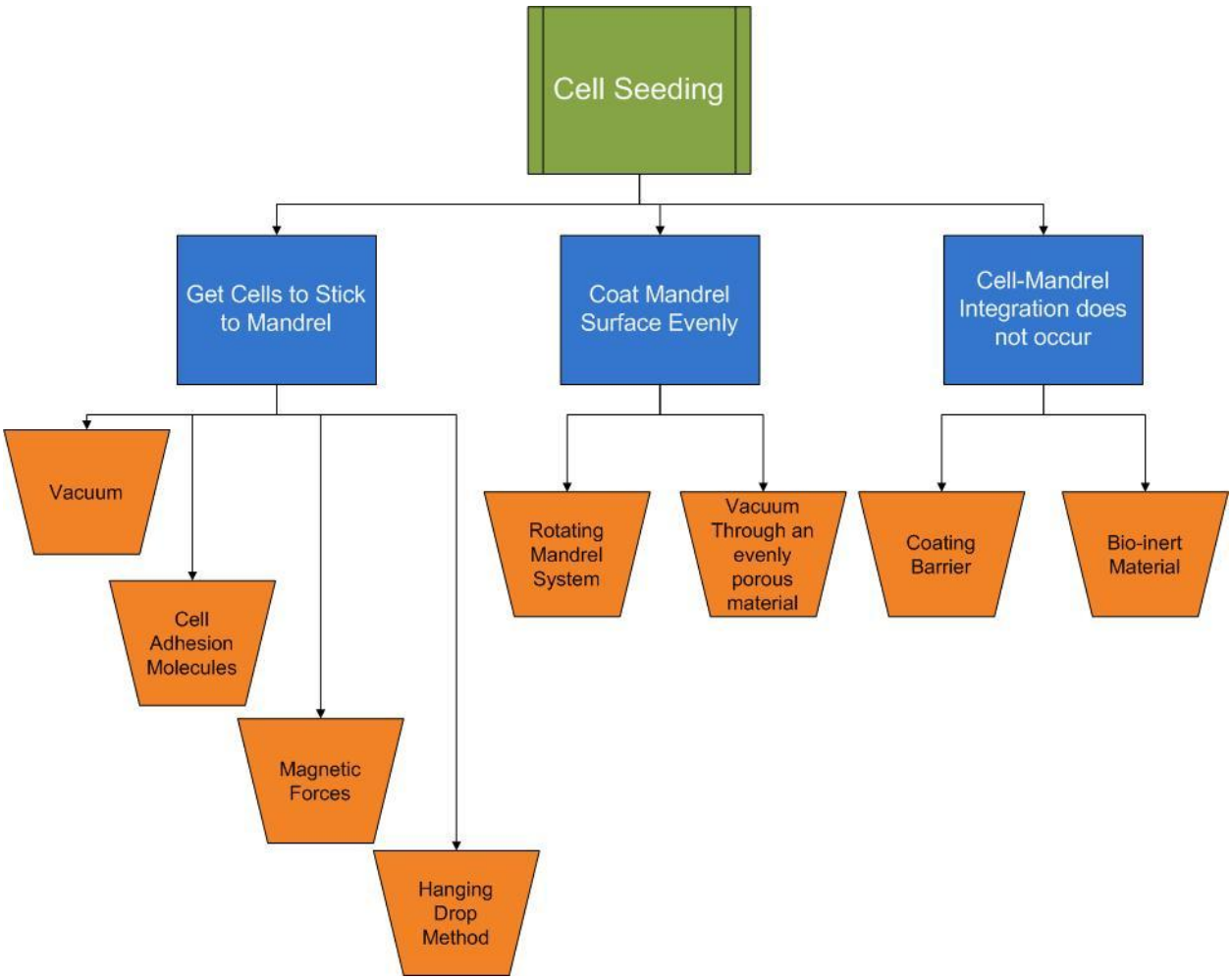
	Cells Stick to mandrel material	Mandrel can be removed from TEBV	Keeps TEBV ends open	Can be Sterilized	Cells don't integrate with mandrel material	Minimize material costs	Biocompatible Material	Score
Cells Stick to mandrel material		0	0	0	0	1	0	1
Mandrel can be removed from TEBV	1		0.5	0	0	1	0	2.5
Keeps TEBV ends open	1	.5		1	0	1	0	3.5
Can be Sterilized	1	1	0		0	1	0	3
Cells don't integrate with mandrel material	1	1	1	1		1	1	6
Minimize material costs	0	0	0	0	0		0	0
Biocompatible Material	1	1	1	1	0	1		5

Appendix B: Functions-Means Trees

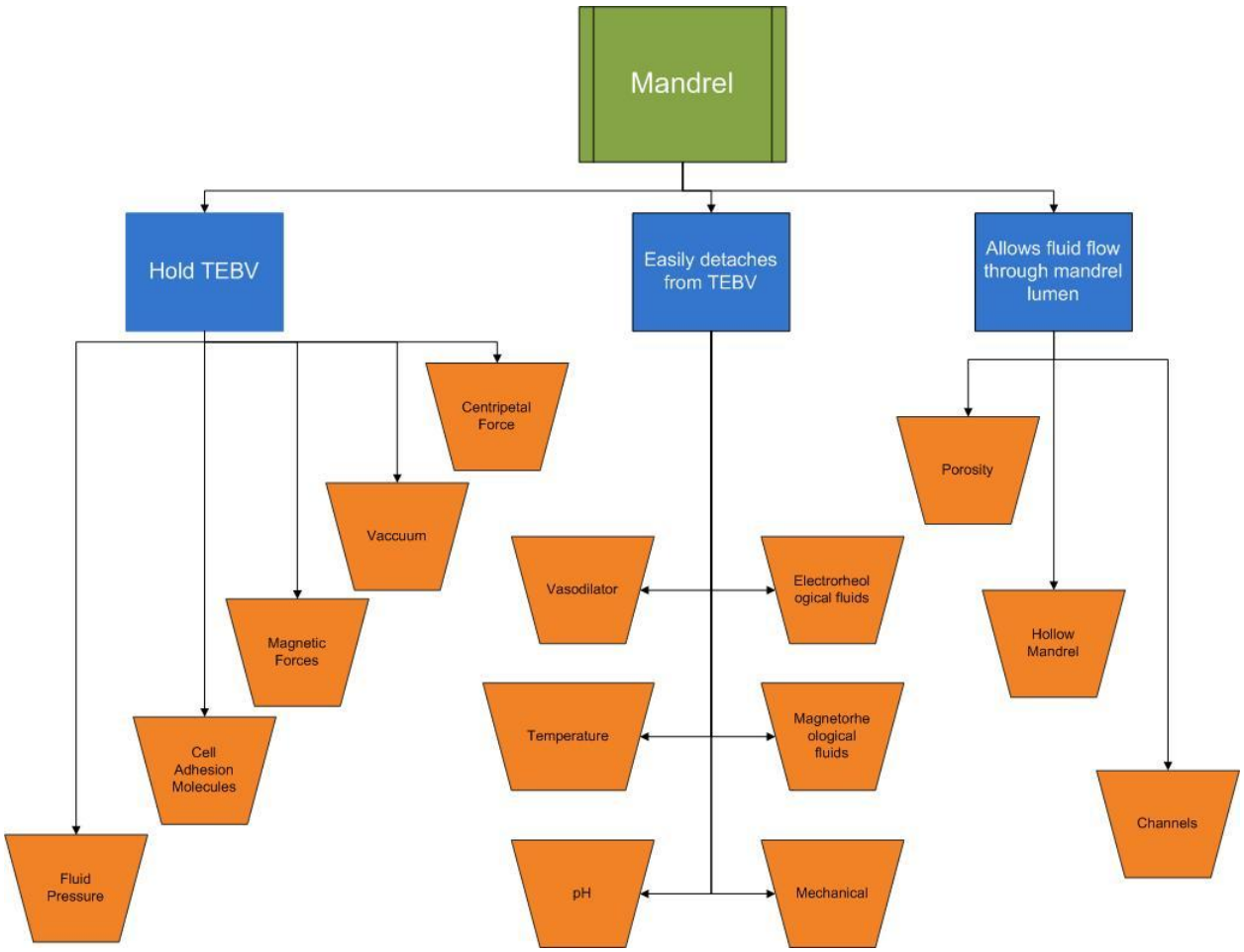
Bioreactor



Cell Seeding



Mandrel Material



Appendix C: Preliminary Experimental Protocols

Testing Protocols:

I. Tissue-sheet culture

1. Optimizing serum concentration

Materials-

Tissue culture flasks

FBS, 2% and 10%

DMEM-solution

Ethanol

Trypan blue

hemocytometer

Procedure-

1. Fill flasks with media.
2. Inject flasks with cells.
3. Allow to culture for 4 days and were fed with fresh DMEM as necessary, (based on metabolic rate assessed by media color).
4. After 4 days, cells were fixed with ethanol and stained with trypan blue.
5. A 40 µm sample was loaded onto hemocytometer and counted.

II. Mesh Mandrel and Vacuum Testing

1. Constructing Mesh Tubing

Materials-

Mesh tubing, (34 µm pores)

Micropipette tips

Silicone glue

Procedure-

1. Roll mesh sheet onto micropipette tip.
2. Apply silicone glue to side of tube.

3. Press tube wall together until glue sets.
4. Repeat steps 2-3 until mesh layers are securely adhered.
5. Repeat steps 1-4 for each tube.

*Note: Bending mesh to make pre-formed creases helps secure layers.

2. Constructing PDMS Plugs

Materials-

Dow Corning® Sylgard 184 Silicone Elastomer kit:

PDMS

Curing agent

Syringe

96 well plate

Procedure-

1. Mix PDMS and curing agent at 10:1 ratio.
2. Syringe into 96 well plate.
3. Place item to be "plugged" into PDMS well.
4. Place in incubator at 60^{O}C and allow setting for an hour.
5. Repeat steps 3-4 for each plug.

3. Procedure for Mesh Experiment

Materials-

Mesh tubes

PDMS

RSMC-solution

syringe

Procedure-

1. Plug one end of mesh tube with PDMS.
2. Seed 1 mL, (9,825,000 cells) of cell solution into tube with syringe.
3. Incubate for 24 hours.

4. Vacuum design

Goal: Determine if vacuum will increase cell adhesion on mandrel.

Control: Mandrel without vacuum.

Materials-

Polysulfone mandrels

Hypodermic needle

3 mL syringe

Petri dish

Media

70% EtOH

Procedure-

1. Sterilize materials with 70% EtOH and place in biosafety cabinet
2. Fill petri dish with media
3. Manually pinch on end of mandrel and insert needle, and draw media in through mandrel by pulling back on the syringe
4. Allow to incubate for 1-2 weeks, checking and feeding as necessary
5. Verify cell adhesion by submerging and staining with Trypan blue. Observe results under microscope.

- Sterilize materials (chamber, pump, mandrels)
- Hook up pump to the mandrel via silicone tubing and clamp down
- Clamp down one end of mandrel to create vacuum
- Circulate medium by putting inlet of pump into chamber
- Add ___cells to ___ml of medium
- Add media to culture chamber
- Turn on pump for ___ml/min
- Put bioreactor into incubator
- After ___ hours, take chamber out
- Count cells via stripping off mandrel, or adding fluorescent Hoechst dye to mandrel itself.

III. Glass Tube Reactors

1. Optimizing cell density and culture time

Materials-

5 mL glass pipettes

Metal file

RSMC-solution

Syringe

DMEM

Procedure-

1. Pipettes were scored and broken into tubes of approximately 7.0 cm.
2. Plug ends with PDMS.
3. Inject tubes with RSMC-solution, (16 million cells/ 75 cm^2, or 0.402 million * L) and allow to culture for 4 days.
4. Tubes were rotated ¼ of a turn every hour.
5. Cells were fed with 3.0 mL of fresh DMEM each day.

*This experiment was repeated with various cell densities, culture times, and rotational frequency.

2. Pyrex Tube Rotational Seeding

Materials-

5 mL pyrex glass pipette tube
Steel file
16 millions cells/75 cm^2 cell solution
Media prepared with 10% FBS
petri dish
PDMS
Hypodermic needle
3 mL syringe
Autoclave
scissors

Procedure-

1. Prepare 9 cm glass tubes by scoring with steel file and snapping them.
2. Plug ends with PDMS as described on pg. 87, (PDMS plugs)
3. Create stand for tubes by cutting edge of petri dish.
4. Sterilize tubes in autoclave and use hypodermic needle to inject with cell solution to capacity. Mark tubes every ¼ length of circumference.

5. Place in petri dish and allow to culture for 2 hours. Turn tube a quarter turn every ½ hour.
6. Allow tube to culture for additional 48 hours. Remove from incubator and observe for confluency.

IV. Pinball cell-seeding probability

1. Cell motion visualization

Materials-

Petri dish

Pepper

Water

Silicon tubes

Silicon glue

Procedure-

1. Cut silicon tubing short enough to fit within closed Petri dish.
2. Glue tubing to bottom of Petri dish.
3. Fill with water and pepper.
4. Place lid on dish, seal with silicon glue. Allow to set for an hour.
5. Shake disk, observe pepper-motion.

2. Cell seeding success rate

Materials-

Petri dish

RSMC-solution

Silicon tubes

Silicon glue

Trypan blue

DMEM

Fetal bovine serum, (FBS)

Ethanol

syringe

Procedure-

1. Cut silicon tubing to height of Petri dish.
2. Glue to bottom of dish with silicon glue.
3. Create hole in lid of dish and plug with PDMS.
4. Spray with ethanol and place in UV light to sterilize.
5. Coat Petri dish lid with FBS.
6. Seal Petri dish with silicon glue, allow setting for an hour.
7. Inject cell solution through PDMS plug with syringe and culture for 5 days.
8. Cells were fed with fresh DMEM twice during the culture period.

* Experiment was repeated and tested for the various cell densities.

V. Washing Machine FLUENT modeling

1. Fin-shape evaluation

Materials-

ANSYS Suite, FLUENT modeling program

Lab PC

Procedure-

1. Model fin array in FLUENT.
2. Generate mesh.
3. Specify solution conditions:
   - Solver = pressure-based
   - Velocity formulation = absolute
   - Time= steady
   - Gravity = -9.81 m/s^2 on the y-direction
   - Multiphase model- Eulerian with 1 phase
     - Parameter=Dense Discrete Model Phase Model, 1 phase
   - Volume fraction= implicit
   - Discrete phase = on
   - Fluid= serum
     - Density= 1000 kg/m^3
     - Viscosity = 0.385 kg/m^3

     - Temperature reference= 298.15 K

Solid= aluminum

Density = 2719 kg/m^3

Inert particle= anthracite

Density= 1550 kg/m3

4. Initialize and run calculation for 100 interations.
5. View results and analyze velocity vectors.

VI. Tissue Tube contraction in the cell culture vessel

1. KCl contraction

Materials-

Pyrex cell culture vessels
Media prepared with 10% FBS
Media prepared with 2% FBS
KCl solution with 10% FBS
Hypodermic needles
3 mL syringe
Petri dish stand

Procedure-

1. Prepare pyrex tubes by plugging with PDMS, as previously described
2. Inject culture vessel with cell solution and media and place in petri dish stand
3. Rotate vessel 1/8 of a turn every 10 minutes for 2 hours in incubator. Allow to culture for an additional 48 hours.
4. Observe for confluency and feed cells as needed.

VII. K-PSS contraction

1. Observing contraction rates of tissue sheets at different cell densities

Materials-

2- 75 cm^2 flasks

Media with 10% FBS

2 mL of 1 million cells/mL solution in each

60 mM K-PSS solution

60 mM PSS solution

Procedure-

1. Allow to culture in flasks in incubator for approximately 72 hours or until confluency is observed.
2. Aspirate media from flasks and rinse with 10 mL PSS, as a control.
3. Return flask to incubator and check every 20-30 minutes for contraction.
4. Repeat Steps 2-3 for K-PSS.

2. K-PSS contraction in the cell culture cartridge

Materials-

3 mLof 1 million cells/mL solution

Media prepared with 10% FBS

Cell cu lture cartiridge

automated rotation system

hypodermic needles

3mL syringe

Biosafety cabinet

70% EtOH

Procedure-

1. Hold cartridge vertically and inject culture vessel with media and cell solution from bottom of chamber. Pierce through PDMS using hypodermic needle. Repeat for each culture vessel.
2. Place cartridge onto gear system, place device into incubator, and start rotation. Allow cartridge to rotate in incubator for 2 hours.
3. Remove device from incubator, and remove cartridge from system. Replace cartridge to incubator and allow to incubate for an additional 72 hours.

4. Remove cartridge from incubator and place in sterile biosafety cabinet.
5. Sterilize hypodermic needles syringe and media containers.
6. Aspirate media from each culture chamber and fill with K-PSS.
7. Return to incubator and check for contraction every 20-30 minutes.

VIII. Final Design Assembly

1. Timing the rotisserie system

Materials-

Mr. Flame rotisserie system

Cell culture cartridge

Rotational Force transducer

Rubber band

LabView computational program

Lab PC

D-battery

Procedure-

1. Setup LabView for data acquisition of rotation force transducer using lab PC
2. Slide cell culture cartage onto rotisserie spit and plug shaft into Mr. Flame motor. Be sure that motor is supplied with a D-battery.
3. Wrap a rubber band around the spit and around the smaller radius knob on the force transducer
4. Start rotation, and begin data acquisition.
5. Interpret rotational speed from angular position graph.

2. Assembling the gear system

Materials-

Vex gears and system components:

Screws

Collars

Motor shaft

Sheet metal supports

Plastic washers

Allen wrench

Mr. Flame rotisserie stands

Vertical band-saw

Metal file

Polycarbonate sheet

Rubber grip

Procedure-

1. Use 3 sets of 84-tooth to 12-tooth gear ratios to create 343:1 gear ratio
2. Place small gear on Mr. Flame motor output shaft. Use small gear to drive a large gear with another smaller gear on the same shaft.
3. Allow smaller gear to drive another larger gear, with another smaller gear on the same shaft.
4. Allow smaller gear to drive another larger gear.
5. Place smaller gear on rotisserie spit and align stands such that the gears make adequate contact and the spit can be still be removed.
6. Affix gear system to sheet metal supports using screws and tightening with an allen wrench. Place plastic collars on gear shafts before securing so that gears with rotate smoothly.

7. Use vertical band saw to cut Mr. Flame stands to appropriate height and file edges until smooth
8. Cut rubber grip material into four squares and glue to bottom corners of polycarbonate sheet to increase traction on bench surface and reduce slip.
9. Secure gear system and stand to polycarbonate sheet using hot glue and secure gear system with screws

3. Assembling the Cell Culture Cartridge

Materials-

Rapid prototyped octagonal stands

Rapid prototyped "table" component

Duct tape

PDMS

Silicon glue

8- 9 cm stainless steel mandrels

8- Pyrex glass cell culture tubes

IsoTemp oven

Procedure-

1. Block injection holes with duct tape
2. Score and cut pyrex tubes as previously described.
3. Score and snap 8- 9 cm stainless steel mandrels from original length received
4. Place pyrex tubes and mandrels into each designated hole in the octagonal stand. Use the table to hold and align the mandrels.
5. Fill each space surrounding the tubes with PDMS and seal with silicon glue. Allow to cure in IsoTemp oven at 60°C for approximately 1 hour.

6. Invert structure and align tubes and mandrels into designated holes on other octagonal stand.
7. Repeat Step 5 and sterilize in autoclave prior to use.

4. Assembling the Fluid Flow System

Materials-

1 L pyrex container

5 mL pipette tips

3- Tubing stop-clips

Luer fittings:

2- Injection port fitting

2- 4-way manifolds

4- 6-way manifolds

3-way stop-valve

4- Stop-fittings, (plugs)

Silicone tubing

50 mL beaker

Tape

Low-flow peristaltic pump

Media

60 mL syringe

Procedure-

1. Pierce cap of Pyrex bottle and insert 2- 5 mL glass pipette tubes. Seal with PDMS to act as media reservoir.
2. Slide stop clip onto tubing and attach to pipette tube in reservoir.

3. Attach injection port fitting directed away from the media reservoir.
4. Attach an additional length of tubing and run it through the peristaltic pump
5. Attach end of tubing to 4-way manifold and plug center vessel with luer stop-fitting.
6. Attach lengths of tubing to each remaining vessel pathway and attach each end to a 6-way manifold. Plug vertical vessel pathways with stop-fittings. Connect remaining vessels pathways to segments of tubing leading to the cell culture cartridge.
7. Repeat Steps 2-3 for remaining pipette tip of the media reservoir.
8. Wrap lengthof tubing around 50 mL beaker and secure with tape to act as gas exchange coil. Connect one end to remaining injection fitting.
9. Slide a stop-clip onto free end, and attach to 3-way stop valve.
10. Connect stop-valve to additional segment of tubing and repeat Steps 5-6.
11. Use injection portals, stop valves, and tube clips to systematically fill segments of the system with media using the 60 mL syringe.

5. Seeding cells in the cell culture cartridge with the automated rotation system

Materials-

Modified rotation system

Cell solution

Media prepared with 10% FBS

Hypodermic needles

3 mL syringe

70% EtOH

biosafety cabinet

Procedure-

1. Sterilize gear system and materials using 70% EtOH and place in biosafety cabinet
2. Use hypodermic needle and 3 mL syringe to seed cell culture vessels in cartridge as previously described.
3. Place cartridge onto rotation shaft and onto rotation system
4. Place rotation device and cell seeding assembly in incubator and run rotation for 2 hours
5. Remove from rotation system from incubator and return cell seeding cartridge to incubator and allow to culture an additional 48 hours.

IX. NIPAAM experiment

1. Goal: Evaluate cell-growth on NIPAAM

Experimental group: NIPAAM coated TCPs, (UpCell 24-well plates)

Materials-

24-well UpCell plate

15,000 cell/well solution

30,000 cell/well solution

60,000 cell/well solution

Micropipetter

Incubator

Icebath

Procedure-

1. Cell concentrations of 15,000 cells/well, 30,000 cells/well and 60,00 cells/well were prepared.
2. Cell solutions were pipetted into 8 wells each and incubated for 72 hours.
3. Cells were observed under a microscope for confluency.
4. Cells were placed in an icebath at 2.7° C for 5 minutes and then moved to a microscope to observe contraction.

Appendix D: Bill of Materials

Stand

Part Number	Part Description	Units	Company	Cost
Custom	Octagonal Stand	1 (two pieces)	WPI Mechanical Engineering Dept. - Rapid Prototyping	$41.40
	5mL Pyrex Glass Pipettes cut to 9cm sections	8 pieces per device	WPI Biomedical Engineering Dept.	
309603	5mL Syringes	2 per seeding	B-D	
305176	Hypodermic Needles	16 per device	B-D	
364116-000010	Silicon Glue	1 tube	Dow Corning	
(400)00010 4511207	PDMS Kit	1 kit	Dow Corning	
B000HZX47O	Stainless Steel Centerless Ground Rod	1 pack of 72inches	Small Parts, Inc.	$9.40

Rotation System

Part Number	Part Description	Units	Company	Cost
53767	Mr. Flame Rotisserie	1	Aqua Superstore	$22.99
Varies based on desired rotation speed	Vex Gears	5 (various sizes)	WPI Electrical and Computer Engineering Dept.	
Scrap Piece	Polycarbonate Base	1	Plastics Unlimited	

Pump System

Part Number	Part Description	Units	Company	Cost
13-876-1 Model 3385	Peristaltic Pump	1	Fisher Scientific	
Various	Luer Fittings	Various	WPI Biomedical Engineering Dept. – Luer Fittings Kit	
6PTT230-6	Six-port Thru Flow Manifolds	100/pack	Value Plastics, Inc.	$45.00
13162-100	Male Luer Fitting 1/8”ID	100/pack	World Precision Instruments, Inc.	
Custom	Media Reservoir	1	Materials from WPI Biomedical Engineering Dept.	
B000FN1I9Y	Silicone Med-X Tubing	1 pack (50 ft.)	Small Parts, Inc.	$57.45