

# A Method for the Tubular Self-Assembly of Cells to Create Tissue Engineered Blood Vessels

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1. blood vessel
2. bioreactor
3. self-assembly



## Table of Contents

Authorship .....	v
Acknowledgements.....	vi
Abstract.....	vii
Tables of Tables.....	viii
Tables of Figures.....	ix
Chapter 1- Introduction.....	1
Chapter 2 - Background.....	5
Clinical Need .....	5
Physiology of Blood Vessels .....	8
Current Vascular Grafting Techniques .....	9
Autograft.....	9
Allograft.....	11
Synthetic Grafts .....	11
Tissue Engineered Blood Vessels.....	12
Tissue Engineered Blood Vessels with an Exogenous Scaffold.....	13
Completely Cell-Based Biological Models.....	17
The Tissue Engineering Business Model.....	20
Our Method.....	21
Chapter 3 - Project Strategy.....	22
Initial Client Statement .....	22
Objectives .....	22
Constraints .....	25
Functions and Specifications .....	26
Revised Client Statement.....	28
Chapter 4 – Design Alternatives .....	29
Design Alternatives.....	29
Needs Analysis.....	37
Feasibility Studies.....	40
Cellular Adhesion Testing .....	40
Vortex .....	51

Radial Flow .....	52
Vacuum .....	55
Cell Mold .....	67
Rotating Mandrel .....	69
Centrifugal Force .....	79
Final Design Decision.....	89
Chapter 5 - Design Verification.....	91
Experiment # 1: One Day Culture.....	91
Experiment #2: 1 Day versus 5 Day Culture .....	91
Experiment #3: Screw vs Fitted Interface Design and Variable Voltage .....	95
Experiment #4: Increased Cell Number.....	97
Experiment #5: Fix Tubes after Spinning.....	98
Design of Vertical Incubation Chamber Culture System.....	99
Experiment #6: Membrane Paper and Vertical Culture System .....	100
Design of Horizontal Culture System .....	102
Experiment #7: Horizontal Culture.....	103
Experiment #8: Polycarbonate and Variable Speed.....	103
Experiment #9: Original setup with Polycarbonate .....	106
Experiment #10: Spinning in Culture (Polycarbonate).....	106
Experiment #11: Spinning in Culture (Acrylic).....	108
Experiment #12: Membrane Paper Cytotoxicity Assay.....	108
Experiment #13: Smaller Diameter Polycarbonate.....	110
Experiment #14: Variable Time Testing.....	110
Rotations per Minute (rpm) Testing.....	111
Centrifugal Force Calculation.....	114
Experiment #15: Variable Speed Testing .....	115
Chapter 6 - Final Design & Validation .....	118
Manufacture .....	118
Tube Creation Process .....	120
Validation.....	122
Chapter 7 - Discussion .....	128
Design For.....	131
Chapter 8 - Conclusions and Recommendation .....	137

Recommendations.....	137
References.....	140
Glossary .....	144
Appendix A: Pairwise Comparison Charts .....	147
Appendix B: Supplemental Procedures .....	148
Trypan Blue Staining .....	148
Cell Counting Assay .....	148
Cell Viability Assay.....	149
PDMS Mold Production .....	149
Appendix C: Manufacturer Information .....	150
Cell-Adhesion Testing Materials .....	150
Final Design Bill of Materials.....	150

## Authorship

All members contributed equally to the writing and editing of this report. In addition, all members performed important laboratory tasks to move the project forward and were instrumental in achieving the results of the final design.

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## Abstract

The purpose of the project was to develop a method to induce the self-assembly of cells into a tubular construct for use as a blood vessel substitute. The primary objectives of this method were to create a tube in less than 7 days, be reproducible (similar length and diameter tubes), involve limited human manipulation, and enable easy harvesting of the tubes. To do this, we utilized the concept of centrifugal force. Cells were inserted into a non-adhesive hollow polycarbonate mandrel. The mandrel was then spun for 15 minutes, forcing the cells to distribute themselves against the inner wall of the mandrel. After spinning, the mandrel was placed in a bioreactor and incubated for 3 days. During incubation, the cells aggregated and contracted off the wall of the mandrel, forming a tubular construct. This cellular tube was then removed from inside the mandrel using forceps. Histology was used to examine tissue structure and distribution of cells. The harvested tubes exhibited dense aggregates of cells indicative of a cohesive tissue.

## Tables of Tables

Table 1: Weighted objectives.....	25
Table 2: Morphological chart.....	29
Table 3: Numerical evaluation matrix .....	38
Table 4: Numerical evaluation matrix of top ranked alternative designs .....	40
Table 5: Potential mandrel materials. ....	41
Table 6: Results of the competitive cell adhesion assay .....	43
Table 7: The results for the non-competitive cell adhesion assay.....	46
Table 8: Results of the cell adhesion assay.....	47
Table 9: Fitted versus screw design results.....	92
Table 10: Screw vs fitted design and 3 vs 9 volts results.....	96
Table 11: Rotational speed of spinning tubes with and without media .....	112
Table 12: Rotational speed of spinning tubes at original speed and with a resistor. ....	113
Table 13: Tubular construct results from Design Experiment # 14. ....	116



## Tables of Figures

Figure 1: Preliminary sketch of vacuum pressure design .....	30
Figure 2: Preliminary sketch of electrostatic force design.....	31
Figure 3: Preliminary sketch of magnetic force design .....	32
Figure 4: Preliminary sketch of radial flow design.....	32
Figure 5: Preliminary sketch of vortex design.....	33
Figure 6: Preliminary sketch of intraluminal flow design.....	34
Figure 7: Preliminary sketch of centrifugal force design.....	35
Figure 8: Preliminary sketch of rotating mandrel design.....	35
Figure 9: Preliminary sketch of mold design .....	36
Figure 10: Preliminary sketch mold design with porous paper.....	36
Figure 11: Preliminary sketch of blow up tube design .....	37
Figure 12: Results of the competitive cell adhesion assay.....	43
Figure 13: Schematic of PDMS molds .....	45
Figure 14: Results of the non-competitive cell adhesion assay .....	46
Figure 15: Graph of the three tests of the cell adhesion assay.....	49
Figure 16: Graph showing the average cell adhesion for each of the materials tested. ....	50
Figure 17: Cell vortex in graduated cylinder .....	52
Figure 18: Radial Flow Complete Setup.....	53
Figure 19: Radial Flow Design using a pump.....	55
Figure 20: Vacuum conceptual testing setup with tubing.....	56
Figure 21: Recirculating media system.....	60
Figure 22: Modified recirculating system.....	61
Figure 23: Results of vacuum test with coffee filter and pepper .....	63
Figure 24: System with second pump added. ....	64
Figure 25: Prototype setup for vacuum design with new peristaltic pump.....	67
Figure 26: Cell mold prototype.....	68
Figure 27: PETG mandrel with indicating line for determining cellular adhesion.....	71
Figure 28: PETG control mandrel with indicating line for determining cellular adhesion.....	71
Figure 29: PETG mandrels after staining with Trypan blue .....	72
Figure 30: Garolite mandrel after staining with Trypan blue .....	72
Figure 31: Garolite mandrel with visible cellular adhesion on the outside.....	73
Figure 32: PETG mandrel with visible cellular adhesion on the outside.....	73
Figure 33: Schematic of rotating mandrel concept. ....	75
Figure 34: Results from rotating mandrel Experiment #5 .....	76
Figure 35: Results from rotating mandrel Experiment #5 .....	76
Figure 36: Results from 15 hour culture for Experiment #6.....	78
Figure 37: Setup for centrifugal force conceptual testing, Experiments 4-7. ....	81
Figure 38: Less transparent tube after spinning for 10 minutes.....	82
Figure 39: Trypan blue stain of the tube after spinning and fixing cells .....	82
Figure 40: Tube in chuck on drill.....	84
Figure 41: Stabilization device designed to reduce vibration in the spinning tube.....	85

Figure 42. New motor/tube interface .....	87
Figure 43: Two tests of cells spun for 15 minutes, fixed, and stained with Trypan blue. ....	88
Figure 44: Trypan blue staining of Tube A for Experiment #2 .....	93
Figure 45: Trypan blue staining of Tube C for Experiment #2.....	94
Figure 46: Trypan blue staining of Tube F for Experiment #2.....	94
Figure 47: Results of spinning Tube E for Experiment #2 .....	95
Figure 48: Vertical incubation chamber.....	99
Figure 49: Removal of membrane paper after three days of culture.....	100
Figure 50: Possible contamination seen in the culture chamber .....	101
Figure 51: Horizontal Culture Chamber .....	102
Figure 52: Schematic of circuit with 10 ohm resistor .....	102
Figure 53: Circuit with 10 ohm resistor. ....	105
Figure 54: Trypan blue staining of polycarbonate after 15 minutes spinning with 25 million cells.....	105
Figure 55. Polycarbonate tube cultured vertically with no co-culture insert .....	107
Figure 56: Graph of membrane paper cytotoxicity assay results .....	109
Figure 57: Cell coverage after 5, 10, 60 minute spinning times .....	111
Figure 58: Motor secured in stand clamp with tube attached .....	119
Figure 59: Incubation chamber assembly .....	120
Figure 60: Method for insertion of cell suspension into tube. ....	121
Figure 61: Hoechst staining at 20x showing cell density in tubular construct.....	123
Figure 62: Trichrome stain at 20x showing density of cells and cell alignment.....	124
Figure 63: Trichrome staining at 10x showing a portion of the tubular construct.....	125
Figure 64: Trichrome staining at 40x comparing material and rotational speed.....	126
Figure 65: H&E staining at 40x showing cohesive tube structure.....	127
Figure 66: A tubular construct of cells formed a tube after 3 days in culture.....	128

## Chapter 1- Introduction

Cardiovascular disease is among the leading causes of death in the developed world. In the United States alone, 80 million people are affected annually; treatment cost in 2008 reached \$475.3 billion dollars (American Heart Association, 2009). Coronary heart disease comprises about 50% of cardiovascular disease cases. This disease is caused by the build-up of fats, cells, and cholesterol inside the coronary artery, thus leading to the formation of plaque, effectively blocking the flow of blood.

Coronary heart disease leads to heart failure, as the coronary arteries can no longer provide the heart muscle with nutrients. A lack of sufficient nutrients and oxygen cause cardiomyocytes, the contractile cells of the heart, to die. This is known as a myocardial infarction, more commonly referred to as a heart attack. In the majority of cases, a stent is implanted to push the clot against the vessel wall and clear the path so blood can flow unobstructed once again. Unfortunately, this is not a permanent solution, as 25% of stented vessels form clots again after surgery (Dangas, 2002). Often, in severe cases, coronary artery bypass grafts (CABG) must be used to shunt blood around the blocked area in the coronary artery. To do this, surgeons perform an initial surgery to harvest a blood vessel, usually the saphenous vein, from another location in the body and then a secondary surgery to implant the harvested autologous vessel as a bypass graft. This procedure is performed approximately 500,000 times each year (Mitchell, 2003). Though this is the gold standard, patients often require multiple bypass operations in a lifetime and subsequently need additional sources of vessels. One third of patients requiring CABG do not have suitable vessels available for an autograft, due to previous harvest or poor vascular health in general (Wang, 2007). An easy solution would be to implant a synthetic vessel; however, at the small diameter necessary for a bypass vessel, these

have been shown to fail due to poor patency rates caused by the body's reaction to foreign, exogenous material. A tissue engineered blood vessel derived from autologous cells could fill this need as it has limited immune response, low thrombogenicity, and sufficient mechanical properties. Furthermore, a vessel with no exogenous scaffold, made only of cells and extracellular matrix, would enhance these properties and provide surgeons with an option for bypass surgery that is equivalent to—and potentially better than—an autograft.

Current tissue engineering technologies are not ideal for clinical applications as no process has been developed to make such tissue engineered blood vessel quickly, with adequate mechanical properties, or with good endothelial layers to prevent clotting. It takes long periods of time to grow cells from a biopsy and form them into functional tissue. The only completely cell-derived small diameter tissue engineered blood vessel to reach clinical trials so far requires approximately three months to produce (L'Heureux, 2007) and therefore can only be used for AV-fistula applications. To avoid heart failure, patients requiring CABG need a bypass vessel rapidly, leaving autografts as the most practical option.

The goal of this project is to develop a process that will allow for the assembly of a cellular tube in less than seven days. This will be enough time to force cells to arrange into a tubular shape and retain that shape long enough for them to create extracellular matrix interactions, giving the tube mechanical strength. This process should be repeatable, such that each time you use a certain cell number the end result will be a tube with no significantly different dimensions.

In order to achieve the final goal of the tube, the process to make it must meet certain functions. It must hold the cells in a tubular shape, it must provide them with adequate nutrients to create extracellular matrix, and it must be removable from the device. To keep the cells in

tubular shape a device must force them into a construct without injuring the cells to ensure that they will be healthy and retain their native-like properties. Ideally, the shape must be kept long term so it can be cultured in media in order to allow the cells to obtain nutrients and create extracellular matrix, which will strengthen the tube. If the cells do not create extracellular matrix, then they will not form a cohesive tissue and will simply be individual cells stuck in a tube shape, which will likely fall apart with little disturbance. The device must allow for the removal of the tube in the end to prove that it is truly a tissue and not just a cluster of cells. Due to the difficulty of measuring each of these individual functions without the other two, the ability of the bioreactor to meet its functional requirements was judged on in depth experimental testing and the end result of tube formation.

The design concept was derived from laboratory technology that has been used extensively for decades, the centrifuge. A centrifuge is used to separate a cellular suspension into two parts: cells and media, where the cells are forced into a pellet at the bottom of a test tube by centrifugal force. Our design utilizes centrifugal force by spinning a tube filled with a cell suspension around its central lengthwise axis. This forces the cells to form the same kind of pellet against the wall of the tube as they do at the bottom of a centrifuge tube. This structure can then be immersed in media and cultured to allow for the creation of extracellular matrix, resulting in a stand-alone tubular construct.

After extensive experimentation and optimization, the results of this process showed that a cohesive tubular construct of cells can be created in less than four days. The tubes were found to contract off of the mandrel wall into a freestanding entity. The tubes we produced are similar in length and outer diameter; however, their contraction into these dimensions was not controlled. The tubes were also examined histologically and it was found that no collagen was

present after three days. Future research using this process may want to investigate different culture conditions (i.e. longer incubation time) to promote collagen production, as well as methods to control the contraction of the tubes to obtain desired dimensions. It may also be worthwhile to modify the initial set up for easier cell suspension input and enhance the user interface to allow more controlled speed and duration of spinning.

## Chapter 2 - Background

There is a vast clinical need for small diameter blood vessels to treat cardiovascular disease. Unfortunately, the current methods for bypassing blocked arteries involves vascular grafts which have limited supply, poor mechanical properties, or take a long time to produce. Our method will improve upon the current attempts to create high quality tissue engineered blood vessels by providing a method for rapid production with limited human intervention.

### Clinical Need

Cardiovascular disease is the leading cause of death in the United States, claiming approximately 2,400 lives per day, which amounts to nearly 1 million per year (American Heart Association, 2009). An estimated 80 million people in the United States are affected by some form of cardiovascular disease. The prevalence of cardiovascular disease increases with age, with over 70% of the population over the age of 60 affected. (American Heart Association, 2009) In 2008, the estimated cost of cardiovascular disease was \$475.3 billion dollars.

Coronary heart disease accounts for more than 50% of deaths from cardiovascular disease. The percentage of the population with coronary heart disease increases with age, especially in men (American Heart Association, 2009). Coronary heart disease occurs when arteries become narrow due to the buildup of plaque, which is known as atherosclerosis. The plaque is made of up deposits of fats, cholesterol and other substances. Plaque buildup can restrict blood flow through arteries, which can cause chest pain. This is one of the main precursors to a myocardial infarction—a heart attack, which results in myocardial cell death. While heart diseases can be hereditary, plaque buildup and other detrimental events can be caused by smoking, an unhealthy diet, or a lack of physical activity.

It is extremely important to replace damaged blood vessels with substitutes in a timely manner, so that the region of the body that is vascularized by the diseased vessel will continue to receive a steady supply of blood. If sufficient blood flow is not maintained to any given region of the body, then the tissues in that region will eventually necrose due to lack of oxygen and other nutrients. In some conditions, such as an occluded coronary artery, the blood-deprived heart could experience a decrease in functionality since some of the tissue has died. In more severe cases, the organ could fail completely, which would present a lethal problem for the suffering patient. Incidences such as this can often prove fatal in very short periods of time. Due to the serious nature of the implications that can arise due to poorly functioning vasculature, it is pertinent to restore functionality as quickly as possible.

Stenting and angioplasty procedures are often performed to treat coronary heart disease caused by blockage to the coronary artery, which supplies the heart muscle with its own blood supply (American Heart Association, 2009). The procedure involves inflating a balloon at the site of the blockage to widen the artery, followed by the insertion of a stent in 70% of cases, which opens the vessel to relieve the narrowing caused by plaque buildup. Approximately 1 million angioplasty procedures are completed each year in the United States. This is a commonplace, relatively low-risk procedure which may offer only temporary relief, as approximately 25% of stented vessels will suffer from restenosis within 5 years (Dangas, 2002). A more effective treatment, albeit a higher-risk procedure, is a coronary artery bypass graft (CABG) operation. More than 500,000 bypass operations are performed each year in the United States (Mitchell, 2003). CABG is a revascularization procedure in which a healthy small diameter artery or vein is taken from another location in the patient's body and attached to the blocked artery, bypassing the blockage. Grafts for CABG are typically taken from the saphenous



vein in the leg or internal mammary artery in the chest. Unfortunately, approximately one-third of patients needing an arterial bypass do not have suitable veins or arteries available due to disease or previous surgeries (Wang, 2007). Therefore, there is a clinical need for a blood vessel substitute for arterial bypasses for patients suffering from coronary heart disease.

Vascular grafts are also needed for treatment of vascular trauma, aneurysms, organ transplantation, limb revascularization, and arteriovenous fistula placement (Wang, 2007). Peripheral bypasses are necessary for limb revascularization in patients with peripheral vascular disease (PVD), which afflicts 8 million Americans (American Heart Association, 2009). PVD can affect the veins, or more commonly, the arteries in the legs or pelvis.

Renal failure is another example of a situation where vascular grafts are needed. The number of patients with end-stage renal disease is increasing by 9% each year (D'Agata, 2000). The state of the art treatment for this disease is hemodialysis. As of 2007, approximately 1.6 million patients require dialysis in the United States (Lysaght, 2008). This process involves rerouting the patient's blood through a dialysis machine, which utilizes diffusion to remove waste products before returning the blood to the patient. To access the blood, arteriovenous (AV) fistulas are used (US Dept of Health and Human Services, 2008). The AV fistula consists of an artery connected directly to a vein which allows for greater blood flow into the vein. A synthetic graft or catheter is also used to create the AV fistula, especially in patients with unhealthy or small veins. In 2003, 35% of dialysis patients in the United States received AV fistulas, while the use of grafts and catheters remains more common (Schon, 2007). Another purpose of the fistula is to provide mechanical strengthening and widening to the vein, so it will be able to handle the higher pressure of the blood coming from the dialysis machine without the risk of aneurysm formation.

There is a significant clinical need for vascular grafts due to coronary heart disease, peripheral vascular disease, and AV fistula placement for dialysis. There are several disadvantages to the current sources of vascular grafts, and therefore an artificial blood vessel substitute is needed. In order to create a replacement graft, it is important to understand the physiology of blood vessels.

### **Physiology of Blood Vessels**

The function of blood vessels is to transport blood cells, nutrients, as well as other circulatory fluid from the capillaries of the lungs to the extremities of the body (Cliff, 2009). In effect, the vessels bring oxygen and nutrients to the body and return cellular byproducts to the lungs, kidney, and liver for neutralization and removal. Without this biological piping system, our own cells would poison us with their waste. In addition to gas exchange, the circulatory system acts as a transport system for the immune system and helps regulate body temperature. The human body contains approximately 50 trillion cells, and in order to supply each and every one with the necessary nutrients for survival, 60,000 miles of vasculature is necessary (O'Neil, 2009). The importance of the vasculature system becomes obvious when it stops functioning properly. Diseases such as tissue necrosis, ischemia, myocardial infarction, and stroke can result from prolonged reduced blood flow (Krasnow, 2004).

Blood vessels are made up of three layers: the tunica intima, the tunica media, and the tunica adventitia. Each layer is comprised of a different combination of cells and plays a specific role in the transport of blood. The tunica intima is a uni-cellular layer of endothelial cells that are in direct contact with blood flow (Cliff, 1976; O'Neil, 2009). This layer helps reduce turbulent flow and prevents objects or other cells from binding to the vessel wall. The function of the intima is particularly important in preventing occlusion that can result in ischemia. The tunica

media, located in the middle of the vessel wall, is comprised of smooth muscle cells and elastin. In arteries, larger diameters correspond to greater elastin content. This relates to the increased pressure and pulsatile action of vessels closer to the heart. These large arteries expand with each beat of the heart and therefore must be able to return to their original shape; the presence of elastin allows for this. The tunica adventitia is the outermost layer of the blood vessel comprised of collagen. This outer layer stabilizes the vessel using nearby bodily structures to hold it in place (Cliff, 1976; O'Neil, 2009).

The mechanical properties of blood vessels vary depending on their size and proximity to the heart (Vogel, 2004). Large arteries with a thick tunica media layer have a greater burst pressure than arteries that are smaller. Generally, the burst pressure varies between 1500 to 3000 mmHg (Cliff, 1976). Some arteries can experience strains of up to 10 percent, an important factor to consider when determining the desired mechanical properties of a tissue engineered blood vessel or any vascular graft (O'Neil, 2009).

When a blood vessel becomes obstructed, tissue downstream of the vessel no longer receives nutrients and oxygen. This can lead to necrosis and a loss of organ function. This is particularly detrimental in the heart, which has high metabolic demands. Restoring blood flow is critical to preventing tissue damage and beginning the wound healing process. Current techniques that have been developed to accomplish this include angioplasty, stenting, and vascular grafts.

## **Current Vascular Grafting Techniques**

### **Autograft**

There are currently several treatment options available for patients whose vasculature has been damaged by cardiovascular disease, trauma, atherosclerotic plaque build-up, or a myriad of

other causes. As previously stated, the state of the art involves removing an artery or vein from one part of the body and using it to shunt blood flow around a closed region of the target vessel, such as in a coronary artery bypass graft (CABG). The grafted vessel is attached to either end of the site of poor functionality. The most common graft vessel candidates are the saphenous vein in the lower leg and the internal mammary artery in the thoracic cavity. While the saphenous vein is more commonly used, both vessels are being compared for long-term patency analysis. Regardless of which vessel is utilized, numerous problems are associated with their use. Firstly, a large portion of patients needing vascular grafts do not have suitable autologous vessel harvest sites to begin with, usually due to their cardiovascular disease or age (Isenberg, 2006). Other patients lack suitable grafts because the best options have been previously harvested for past operations (Isenberg, 2006). Atherosclerosis (plaque buildup on the walls of arteries which reduce blood flow) and varicosis (elongation and dilation of superficial veins) alone are conditions which result in approximately 30% of patients not having suitable autologous vessels (Aper, 2007). Next, if a vessel is taken from an arm, leg, or other region of the body to be placed somewhere else, a second surgery will be necessary. This harvesting operation increases the chance of an infection or other detrimental surgical complications. In the case that the internal mammary artery is used—or other vessel in the thoracic cavity—the time that the chest is open during surgery will be prolonged, which increases the risk of patient death during surgery. Another disadvantage to using the saphenous vein, or any other superficial vein, is that they contain one-way valves, which prevent blood from back-flowing on its way back to the heart. Arteries do not contain valves, and so it becomes necessary to remove the valves in venous grafts. This is done with a device called a valvulotome (Connolly, 2005). The valvulotome is inserted into the vein, and retractable blades are manipulated in order to sever the valves. Not

only does this procedure prolong the length of the overall surgery, the cutting of the valves can damage the fragile endothelial layer of the vessel. Injury of the endothelial layer can cause uncontrollable hyperplasia of the intimal vessel layer, or fibrous scar tissue formation, both which could occlude the vessel, rendering it useless as a vascular graft (Connolly, 2005).

### **Allograft**

Another source for vascular grafts is an allograft, which is a tissue or organ transplant from one person to another. Cadaver tissues and organs are commonly used for transplant surgery. While these vessels are similar to autografts, in that they have the same mechanical properties as the native vessel, they are far from ideal. The donor grafts are not of the same genotype as the host, increasing the likelihood of rejection (Biedermann, 2008). This is known as graft versus host disease. This is due to the host's immune response, in which specialized leukocytes recognize the foreign antigens being expressed on the cell surface of the grafts, and attack them. Once enough of the graft's cells have been destroyed, the graft will fail, leaving the patients in need of a new transplant. One way to reduce the risk of graft versus host disease is to place the patient on powerful immunosuppressant drugs (Biedermann, 2008). These drugs inhibit the body from recognizing and neutralizing foreign invaders. Not only are these drugs expensive, but they can reduce a patient's ability to fight off even commonplace colds or infections, which could result in the death of the patient.

### **Synthetic Grafts**

In the event that an autologous vessel cannot be harvested, synthetic grafts made of woven polymer fibers, such as Dacron or ePTFE, have been used instead. Synthetic grafts are much less ideal for use in vascular bypass surgery. Studies have shown that while large diameter (>6mm) synthetic grafts have had some success as lower aortic grafts, small diameter synthetic grafts (<6mm) have an extremely poor patency rate, often less than 50% after six months and

typically less than 10% after a full year in CABG and peripheral vasculature grafts (Pasic, 1996). Unfortunately, these materials do not mimic natural materials in the body, resulting in compliance mismatch and an adverse response in the clot formation cascade. The primary reason why these types of grafts fail are because of the foreign body responses they elicit. Such responses include fibrotic encapsulation and platelet adhesion, which can result in thrombosis of the affected vessel (Ravi, 2009). Due to the lack of an endothelial layer on most synthetic grafts, blood proteins and platelets are free to adhere to their inner surface. If enough of these elements collect inside the graft, then it could become occluded, rendering it useless (Pasic, 1996).

While these methods have been shown to be successful in certain circumstances, there are still many instances where all of them are unsuitable. In the case of autografts, the patient may have already used the desired replacement vessels for previous bypass surgeries or the vessels may be weak due to age and disease. Allografts often result in immune and inflammatory responses and are of limited supply. Synthetic grafts have been shown to be effective in large diameter applications, but have shown very poor patency in small diameter applications. Noting these complications, there is still a need for a mechanically stable, immune-resistant substitute for small diameter vessels.

### **Tissue Engineered Blood Vessels**

Tissue Engineered Blood Vessels (TEBVs) are theoretically the ideal solution to the need for small diameter blood vessels. If conditioned properly, they can exhibit mechanical properties, physiology, and immunogenicity closer to native blood vessels than synthetic grafts. If an effective method for creating TEBVs is found, they can also be made more readily available than autografts or allografts without requiring extensive second surgeries. However, a complex system for the incubation and creation of these tissue engineered constructs is necessary.

In order to create tissue engineered vascular grafts, a common approach is to seed cells onto a scaffold to create a tissue construct. Traditionally, static cell culturing has been used to seed polymer scaffolds (Nasseri, 2003). In one example, polymer tubes are coated with collagen and put in a suspension of cells for the cells to adhere to the polymer scaffold. This has been successful in creating tubular constructs, but there are some associated problems when using this method. Human or mechanical error may result in imperfect TEBVs if cells are not seeded on the scaffold evenly. This can cause clot formation, especially in cases where there is not a confluent anti-thrombogenic endothelial layer. Therefore, bioreactors are now more commonly used to culture the vascular grafts. Bioreactors can facilitate directed cell position and attachment to scaffolds by controlling physiological conditions, providing mechanical forces to mimic a more native environment, and providing sufficient nutrients and oxygen. Newer techniques that have been developed to seed cells onto a tubular construct use forces such as rotation, flow, electric fields, magnetic fields, and vacuums (Nasseri, 2003; Narita, 2004; Shimizu, 2007; Soletti, 2006).

There are two general methods of developing TEBVs. The first method is to seed a biodegradable, synthetic or ECM scaffold with cells. Another promising method is entirely cell-based tissue engineering using an inert mandrel so the TEBV can be removed after culture, rather than implanting a synthetic or biological scaffold. The resemblance of cell-derived grafts to native tissue and the absence of an exogenous material make them more desirable than other methods. These properties reduce inflammation and immune response, as well as undesirable blood interactions such as coagulation and hemolysis.

#### **Tissue Engineered Blood Vessels with an Exogenous Scaffold**

Currently, the most common method of obtaining a tube made of cells is to create a polymer scaffold in a tube shape and seed the cells onto it. Mirensky, et al. used a degradable poly-L-lactic acid scaffold seeded with SMCs and endothelial cells to create a TEBV (Mirensky,

2009). This was implanted in mice with good patency and no evidence of stenosis after one year. There was slight dilation of the blood vessels at about the midpoint of the study that was repaired by the natural cellular remodeling process. However, this suggests that these vessels did not exhibit ideal mechanical properties. Another side effect of most biodegradable polymers is that there can be localized pH changes from the degradation of the scaffold, which is harmful to the native tissues, making them far from ideal for small arterial substitutes.

Weinberg and Bell were the first to attempt to create a tissue-engineered vascular graft, which they did using a collagen scaffold (Weinberg, 1986). They created an adventitia layer from fibroblasts and collagen, a media layer from SMCs and collagen, and an endothelial monolayer. The tubular structure was encased in Dacron to enhance mechanical strength, without the support of Dacron, burst pressure of the collagen graft was less than 10 mmHg. This suggests that the exogenous material, Dacron, was providing the majority of the mechanical strength and structure.

Soletti et al. created a bioreactor using a vacuum in order to seed tubular structures (Soletti, 2006). A cell suspension is flowed through the inside of a biodegradable poly(ester urethane)urea scaffold by use of a syringe pump. Three different cell types were tested, including murine muscle-derived stem cells (MDSCs), rat bone marrow derived progenitor cells (rBMPCs), and bovine aortic endothelial cells (BAECs). After the cells are inserted into the bioreactor, a vacuum force is applied to the outside of a porous poly(ester urethane)urea (PEUU) scaffold to suction the cells onto the lumen of said scaffold. The cells get trapped in the pores while the media continues flowing through. The scaffold is also continuously rotated to aid in distributing the cells uniformly. The results of the study showed that the cells were homogeneously distributed circumferentially and longitudinally on the tubular scaffold.



However, the scaffolds that achieved cell uniformity were limited in length to 2 cm. Also, this method uses a biodegradable polymer scaffold which could cause adverse side effects if it is implanted in the body.

The use of flow in bioreactors to seed cells on polymer scaffolds for vascular grafts has been used in several different ways, including pulsatile flow, convective flow, and transmural flow. It has been shown that replicating a native cell environment is beneficial to culturing cells when creating a vascular graft. The first bioreactor showing the beneficial use of pulsatile flow to condition vascular grafts was built by Niklason et al. (1999). They developed vessels from smooth muscle cells and endothelial cells derived from a vascular biopsy that could withstand high rupture pressure (2000 mmHg) due to 50% collagen content. They later showed that the pulsatile flow is directly related to collagen and MMP-1 production, which increases the mechanical strength of the vessel (Solan, 2001). Therefore, pulsatile flow has been used in more recent bioreactors to condition cells (Narita, 2004). Yuji Narita et al. used a balloon pumping system to replicate systolic and diastolic blood pressure in a bioreactor which also used a compliance chamber and resistant clamps. The bioreactor with pulsatile flow created tissue constructs on a PLA scaffold with widely distributed cells and higher cell numbers and protein content than constructs cultured under static conditions. Bjork and Tranquillo created several bioreactors to evaluate how different methods of flow affect oxygen delivery to the cells (Bjork, 2009). The results show that a combination of transmural and axial flow provide the best uniformity of nutrient and oxygen delivery.

Several studies have used rotational forces to create tubular constructs of cells. In a study by Nasser et al., ovine myofibroblasts were seeded onto polyglycolic acid/poly-4-hydroxybutyrate scaffolds coated with collagen (Nasser, 2003). The tubular scaffolds were

placed in a suspension of cells and rotated around a central axis at five rotations per minute in a hybridization oven to allow for rotational seeding and culture. After five days, cells had attached to the inner and outer surfaces of the scaffold and were aligned circumferentially, similar to native vessels. The hybridization oven does not allow gas exchange, and thus involves daily human intervention to exchange the media for oxygenation, which increases the time and complexity of producing a vessel.

Magnetic force has also been researched as a potential method for forming tubular constructs for vascular tissue engineering. In a 2007 study by Shimizu et al, cationic liposomes which contain magnetite nanoparticles were used to magnetically label fibroblasts. Decellularized porcine carotid artery was used as the scaffold, and a cylindrical magnet was placed inside. The scaffold was placed in a suspension of the magnetically labeled cells. The cells successfully attached to the scaffold with 99% efficiency. However, one disadvantage to this method is that the magnetic liposomes remain in the cells and could have potentially harmful effects if implanted in the body.

Extracellular matrix proteins have an important role in the body, including blood vessels; therefore, several proteins have been used as scaffold materials for tissue engineered blood vessels including collagen and fibrin (Weinberg, 1986; Swartz, 2004). Collagen is the most abundant protein in the body and can act as a substrate for cell attachment and cell signaling. Hirai and Matsuda used this approach by creating a solution of collagen and SMCs, which was then warmed into a gel in the shape of a tube (Hirai, 1996). One of the main roles of collagen in blood vessels is providing good mechanical properties. As a gel, collagen does not have a high tensile strength and is not suitable for the blood vessel application. However, research is being

conducted to determine if cells can be used to remodel the collagen and increase its mechanical properties.

Perhaps the most promising ECM protein for a TEBV scaffold is fibrin. A 2004 study by Swartz et al. embedded a fibrin scaffold with ovine SMCs (initially) and endothelial cells (3-10 days before implantation) (Swartz, 2004). The TEBV was implanted in lamb venous systems and showed remarkable tissue remodeling with the presence of collagen and elastin, resulting in adequate mechanical properties. The authors noted that fibrin can facilitate cell adhesion and migration similar to collagen, but can also have controlled degradation and be obtained from autologous sources. However, the vessels required 2-3 weeks of maturation before implantation.

### **Completely Cell-Based Biological Models**

Completely cell-based biological tissue engineered blood vessels have high value. They lack synthetic materials which may increase inflammation, infection, immune response, and overall graft rejection. They also provide a familiar environment for cellular matrix remodeling because the matrix was produced by the same cells. Instead of seeding cells onto a scaffold and then implanting the entire composite, completely cell-based TEBVs arrange cells into a tubular shape, sometimes with the assistance of a scaffold, but then remove the finished tissue from the scaffold and implant it alone. These methods may take more time, however, the end products have shown to be more biocompatible, have adequate mechanical properties, and show little thrombosis (Konig, 2006; Norotte, 2009;). The largest gap in the biological model is that there is currently no technology to simply and quickly force cells to aggregate in a tubular construct.

Norotte, et al. have developed a method of engineering TEBVs using no exogenous scaffolds that utilizes bioprinting technology (Norotte, 2009). This group is able to create spheroids of cells and then arrange these into small rod structures. These structures can then be stacked into a tube using agarose rods as fillers around and inside the tube to act scaffold-like as

the cell spheroid rods fuse together. Since cells do not adhere to agarose, the tube can be easily removed from the scaffold-like construct. However, no mechanical tests were performed to determine if these specific TEBVs could be a viable implant. This procedure also requires much time for cell culture. Each spheroid contains 8,000-15,000 cells and is roughly 300  $\mu\text{m}$  in diameter. To make rods that are as short as 3 cm this would require billions of cells, which take time to grow, especially if obtained from a patient biopsy.

Frangos and Sobolewski invented an approach to creating TEBVs without any exogenous scaffold called convective flow tissue assembly (Frangos, 2008). La Jolla Bioengineering Institute was granted a patent for their method on October 21, 2008 (U.S. Patent #7,439,057). This involves assembling cells on a porous mandrel by using the convective flow of culture medium. The mandrel is later removed once cells form a tubular construct so there is no exogenous scaffold in the implantable graft. Transmural flow is used to provide the cells with the necessary nutrients and oxygen.

The convective flow is created in a bioreactor by creating a lower pressure on the luminal side of the mandrel than the rest of the chamber to create a transmural pressure gradient and radial flow (Frangos, 2008). The cells distribute uniformly on the mandrel by fluid drag forces. The cells do not actually attach to the mandrel, but the fluid forces the cells to form a tubular shape. The cells then make their own extracellular matrix to form a tubular tissue construct. The invention would enable the creation of multi-layered vessels by adding different cell types at different times into the culture. The vessel is endothelialized after the other layers have been formed by removing the mandrel and reversing the pressure gradient and flow direction. In a study by the inventors, DSF-3 dog skin fibroblast cells were used with a porous polyethylene mandrel. Culture media had to be changed every three days, and more cells were added every 6

days. The length of culture varied between 7 and 14 days, with a 1 mm thick vessel obtained after 2 weeks.

The idea behind the only completely biological TEBV currently in clinical trials was invented in the late 1990's by Nicolas L'Heureux et al. (L'Heureux, 1998). Their vessel features dermal fibroblast cells obtained from skin biopsy, to avoid multiple surgeries and problems with obtaining three different cell types from a patient. These vessels have also shown adequate mechanical properties and earned a patent (U.S. Patent #7,112,218) on September 26, 2006 for "Tissue engineered blood vessels and apparatus for their manufacture," (Konig, 2009). To develop the TEBV in clinical trials, the group first created a sheet of autologous dermal fibroblasts, which was wrapped around a mandrel and then dehydrated to create a decellularized layer for endothelial cell seeding and for blocking cell migration into the lumen of the vessel. Then, more dermal fibroblasts were cultured into a sheet in a flask, which was subsequently wrapped around the decellularized layer (L'Heureux, 2006). It is the first tissue engineered construct to provide mechanical strength (specifically burst pressure 3490 +/-892 mmHg) comparable to natural blood vessels that are currently used as autologous implants (saphenous vein 1599 +/-877 mmHg, internal mammary artery 3196 +/-1264 mmHg) (Konig, 2009). Finally, endothelial cells were seeded on the inner lumen of the vessel. These three steps simulate the structure of a natural blood vessel and result in mechanical strength and burst pressure strong enough to withstand mechanical stresses within the body. The term "sheet-based tissue engineering" has been coined for this type of method.

In addition to the TEBV in clinical trials, L'Heureux et al. have created another completely biological, tri-layered blood vessel. This product is constructed in a manner similar to the vessel in clinical trials. This vessel also utilizes the initial dehydrated fibroblast layer as

described above. Smooth muscle cells are then cultured into a sheet and wrapped around an inert mandrel. A sheet of fibroblasts is then wrapped around this smooth muscle cell layer. Finally, endothelial cells are seeded in the lumen (L'Heureux, 1998)

However, this method also has its limitations. A significant amount of time is needed to create TEBVs in this manner. Between the culturing of the SMC and fibroblast sheets and the maturation of the blood vessel once the layers are in place, approximately 3 months of work are required to create the TEBV, with more time required for the complete transition from biopsy to implant (L'Heureux, 1998). This is not ideal, especially if autologous tissue is available. Furthermore, the use of cell sheets requires that they be physically rolled into a tubular construct rather than being developed already in a tube. This requires time and the manipulation of the sheets to deliver them into a tube-rolling device.

### **The Tissue Engineering Business Model**

In 2002, two of the largest tissue engineering companies, Organogenesis and Advanced Tissue Sciences, filed for bankruptcy (Bouchie, 2002). Creating tissues in a lab is not as easy as it was initially proposed to be and it is not as clinically viable as anticipated. One significant gap separating engineered tissues from the clinic is time. For a limited immune response, a patient's own cells must be taken in a biopsy and grown in a lab until they can be re-implanted as a tissue. However, the number and growth rate of cells obtained in biopsy are not ideal for quick development of tissues. It takes complicated processes and several months to force cells to grow in a desired shape or orientation. If a simple, rapid process could be designed, it would decrease the overall production time of tissue engineered constructs making them more viable for clinical applications.

## Our Method

Despite the improvement in the field of small arterial substitutes, there are significant gaps in technology that inhibit the fast production of a reliable vessel for blood flow. Synthetic polymer grafts have been shown to have low patency. Cell-seeded polymer scaffolds can have harmful side effects and be unreliable. The production of completely biological TEBVs is promising, yet still requires long production times and often results in poor mechanical properties, with one exception. Still, no bioreactor has been created that can have an input of cells and a quick output of a tubular construct.

We propose that an ideal small arterial substitute should be a completely biological TEBV created using the patient's own cells. We have developed a method to do this in a shorter production time than current methods. Our bioreactor is able to create an entirely self-assembled, biological tubular construct with minimal human intervention..

## **Chapter 3 - Project Strategy**

Given only a client statement to begin the design process with, we had to learn more about tissue engineered blood vessels. After conducting extensive background research pertaining to that topic, in addition to the structure of blood vessels, current state of the art technologies for vascular grafting procedures, and bioreactors, we were prepared for the next stage of the design process. Determining objectives and functions turned out to be an unexpectedly challenging task. However, through the use of pairwise comparison charts, client interviews, and further research, we were able to clearly define not only the objectives and functions of our major qualifying project, but also the constraints and specifications. With these parameters set, we were able to make our client statement much more specific.

### **Initial Client Statement**

The initial statement provided by our client, Marsha Rolle, Ph.D., Assistant Professor of Biomedical Engineering at Worcester Polytechnic Institute, was “Develop a bioreactor to assemble cells into tubular structures to create a tissue engineered blood vessel”. The client statement was expanded and refined through several interviews with the client and extensive background research as we determined our objectives, constraints, and functions.

### **Objectives**

After receiving our client’s statement, we set forth to determine the objectives that our bioreactor must be capable of achieving. Through discussions and client interviews we were able to narrow an extensive list of potential objectives to seven primary objectives. Below lists the primary objectives of our bioreactor design:



- Form a Tubular Assembly of Cells
- Repeatability
- Short Production Time
- Easy to Remove/Harvest
- Limited User Manipulation
- Manufacturability
- Monitoring of Construct

The most important objective was that the bioreactor be able to manipulate cells to self-assemble into tubular constructs that could be used as vascular grafts. This addresses the client statement, and without meeting this objective, the project cannot be successful.

Another important objective is repeatability. This means that the bioreactor should create vessels of uniform length, outer diameter, wall thickness, and mechanical strength (an optional, supplemental objective). The properties of the cultured tubes must be predictable in order to be used in a clinical setting. If a batch of vessels does not have similar properties, they cannot be relied on. A single outlier could prove deadly to a patient if its wall thickness or burst pressure is insufficient. A surgeon must be aware of the dimensions and mechanical properties of the product he or she will be implanting in order to properly prepare for surgery.

The third objective is for a bioreactor that facilitates short vessel production time. Many patients who need vascular graft surgery often have little time to wait for a vessel to grow, which is why autologous grafts are used whenever possible. Therefore, a product that can be produced at a fast rate is extremely desirable.

Next, the bioreactor must allow for simple harvesting of the finished blood vessel without the risk of compromising its integrity. With the potential for clinical application, the cultured

tubular construct must be removed without causing any imperfections. Additionally, cell viability could be compromised if the vessel is exposed to an unsterile environment for too long. Obviously, if the vessel is ripped or damaged in any way during the harvesting process, then its integrity will be compromised, thus rendering it useless as a vascular graft.

By limiting human intervention, we can minimize the inherent human error factor. If the bioreactor technician has minimal responsibilities concerning the operation of the device once vessel culturing has been initiated, then there is less of a chance that the technician will commit a mistake, and thus the overall quality of the produced vessels will improve. It can help limit the risk of contamination as well. It will also be an improvement over the state of the art sheet-based tissue engineered blood vessels, which require multiple manipulation procedures during production, such as rolling cultured cell sheets into a tubular shape.

In terms of manufacturability, by making the bioreactor from cheap, commercially available, easily accessible materials, assembly can be minimal in terms of time and labor requirements. This would allow repairs or modifications to easily be made by a technician, rather than by a specialist in bioreactor repair who would otherwise have to use customized parts. The bioreactor construction process should be easy to follow and replicate, so that different laboratories can create vessels of the same quality and homogeneity.

Lastly, a minor objective of the bioreactor design would be to allow visual monitoring of the progress of tube construction. If the tube can be viewed due to transparency of the bioreactor, then users would be able to view the tube periodically to ensure that a tube is forming. If, after an appropriate amount of time, tube formation cannot be verified, then this might indicate that proper protocols were not followed, thus allowing the user to restart the process.

These objectives were evaluated by using pairwise comparison charts that were given to each of the four designers, in addition to the client and potential users. The pairwise comparison charts can be seen in Appendix: Pairwise Comparison Charts. After averaging the results of these charts, we were able to arrange the objectives in order of importance. The seven weighted objectives can be seen in Table 1.

**Table 1: Weighted Objectives**

<b>Objectives</b>	<b>Weight</b>
1. Tubular assembly	28.6%
2. Repeatability	21.4%
3. Short Production Time	19.0%
4. Limited Human Interaction	11.9%
5. Manufacturability	9.5%
6. Easy to Remove	7.1%
7. Monitoring of construct	2.4%

## **Constraints**

Early in the design process we realized the importance of determining our project's constraints. If our bioreactor was unable to comply with constraints, then it would indefinitely fail.

Our first and most obvious constraint was the timeframe in which we had to complete our project. The project must be completed by MQP presentation day, which will be held on April 22<sup>nd</sup>, 2010.

Another constraint that was immediately made obvious to us was our limited budget. \$156 dollars had been appropriated to each member of the team, amounting to a total of a \$624 dollar budget. Any additional expenses above the budget limit will be the responsibility of the team members. Because of the small budget, the purchase of bioreactor components must be

methodical and well thought out. There is little room for error when it comes to purchasing unnecessary components.

The bioreactor must be safe. It must not present any reasonable possibility of harming the user or cells inside of it. This means that the bioreactor should have no sharp edges, should not become hot enough to cause burns or tissue damage, and should be non-toxic.

The bioreactor must be benign, in that it must not hamper the culturing of the cells, or their assembly into a tubular construct. The bioreactor must not present any unnecessary stresses to the developing cells/construct, and it must be sterile and easily sterilizable so that it does not facilitate the introduction of contamination to the culture system.

## **Functions and Specifications**

After assigning objectives and constraints, we worked on creating a list of functions that would define exactly what our bioreactor would have to do in order to accomplish our objectives.

The functions are as follows:

- Provide access to vessel for harvesting
- Allow culture of vessel
- Manipulate cell position or move cells
- Provide nutrients to cells
- Remove waste
- Permit addition of cells

In accordance with our primary objective, cellular assembly into a tubular construct, the most important function of our bioreactor is to actively manipulate the position of cells so that they form a tube. Based on production times of the current state of the art and client demands, we determined that a vessel assembly time of approximately seven days would give our design a

sizable advantage over current bioreactors. This would be a significant improvement over the production time of 3 months of the current state of the art TEBVs of L'Heureux et al. Other specifications include that the tubular construct must retain its structure in media, and, upon completion, must stand alone as a tube without any support, such as a mandrel or chemical fixation. Also, since our bioreactor will be designed to create blood vessels for implantation into human subjects, the tubular construct should be 5-10 centimeters in length and 2-4 millimeters in inner diameter (common dimensions of vascular grafts).

In compliance with objectives and constraints, was the function which was to provide a mechanism for access to the tubular construct for harvesting once culturing was complete. This is important because the vessel must be harvested from the bioreactor before it can be used. The harvesting mechanism must not tear or damage the construct in any way. The tubular construct must be removed in one piece, and the process of removing it should be less than five minutes, in order to minimize the chance of contamination from the external environment outside of the bioreactor.

Incubating the cells in an in-vivo like environment is crucial to cell viability and their ability to proliferate, which will affect their ability to self-assemble into the desired construct. If cells are not kept at 37 degrees Celsius, with an atmospheric carbon dioxide content of 5%, then they will either die or suffer from severely decreased biological function. The bioreactor must fit in an incubator with a shelf 18 inches wide by 18 inches deep by 8 inches high.

The bioreactor's ability to feed cells and remove waste is another necessary function. The cells must have regularly replenished nutrient supply, and waste must not be allowed to accumulate. This will be accomplished through media renewals.

Lastly, the bioreactor must be capable of taking in an initial cell suspension with which to begin the tubular construction process, in addition to the possibility of adding supplemental cell suspensions for the purpose of expediting the culturing process.

### **Revised Client Statement**

We expanded on our initial client statement by performing extensive background research, detailing reactor objectives and creating comprehensive functions means trees. Our revised client statement is as follows:

“The goal of this project is to design a novel bioreactor that will allow cells to self-assemble into a tubular construct that contains no exogenous material. The time frame for production should be less than or equal to one week, and the product should be robust enough to be handled without being damaged. Harvesting must be a simple process and pose little threat to the integrity of the tube. The produced tubular constructs should be homogenous in terms of dimensions and cellular components. The bioreactor must be small enough to fit into a three shelf incubator and should provide a biomimetic environment.”

By designing a device that performs the aforementioned functions, we hope to simplify current techniques of culturing blood vessels. This is a necessary part of addressing the large need for effective vascular grafts. By comprehensively defining our objectives and functions, we hope make a simple product that can easily and quickly grow vessels, while in turn addressing key issues, such as the scaling up of the entire production process.

## Chapter 4 – Design Alternatives

After completing our list of objectives, functions, and constraints, we developed preliminary designs that would fulfill these parameters. Through further research and brainstorming, we were able to compile a set of plausible alternative designs. These ideas were then experimentally evaluated to determine their feasibility.

### Design Alternatives

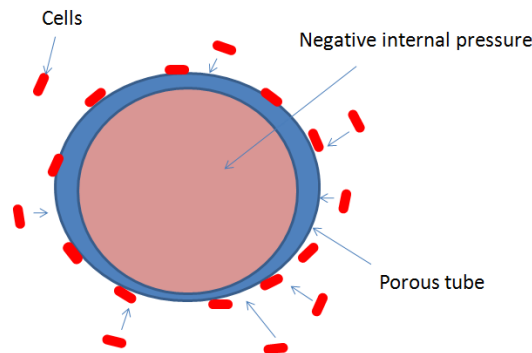
In order to come up with our conceptual designs, the team brainstormed various means to meet our functions. We first created a morphological chart as seen in Table 2. Since our primary objective was to create a tubular construct from cells, we focused first on means to achieve that function.

**Table 2: Morphological Chart**

<b>Function</b>	<b>Means</b>				
<b>Tubular construct</b>	centrifugal force	radial flow	mold	vacuum	Electrostatic forces/charge
	rotating mandrel coating	cell vortex	intraluminal flow/rotation	magnet	blow up tube
<b>Harvesting</b>	Pull it off	vacuum	trypsin coating	collapsible mandrel	opening cylinder
	stretchable mandrel	enzyme degradation			
<b>Feed cells</b>	media inlet	pump	porous mandrel		
<b>Remove waste</b>	aspiration tube	closed media loop	filter		
<b>Add cells</b>	injection port	seed cells			
<b>Incubation</b>	filter cover	open hole			

### *Vacuum*

By utilizing negative pressure around a porous mandrel, we theorize that cells would be suctioned to its inner surface based on previous literature as stated in the background. The pores in the mandrel should be small enough to allow media to flow through, but small enough to prevent cells from migrating through it, as seen in Figure 1. The theory behind this design is that after forcing cells into the shape of a tube via the suction force of the vacuum, they will begin to create their own extracellular matrix. Once the matrix is sufficient enough to hold the cells together, the vacuum pressure could be removed. Research with umbilical smooth muscle cells has suggested this can take 5-7 days (Norotte 2009). The cellular tube could then be removed from the bioreactor.



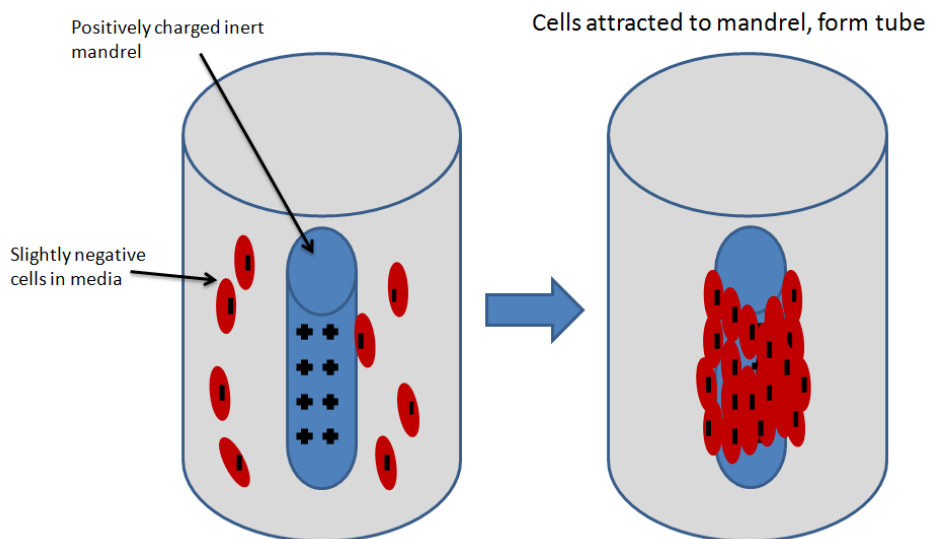
**Figure 1: Preliminary sketch of vacuum pressure design showing how a negative internal pressure can be used to force cells to move towards a mandrel without going through it due to pore size**

### *Electrostatic Forces*

In order to use electrostatic forces to create a tubular construct, a solid mandrel would be placed in the center of the bioreactor. The mandrel would be charged by some means, such as an electrical current, in order to create a polar surface. Since cells hold a small negative charge, they would be attracted to the mandrel, as seen in Figure 2 (Hjortso, 1995). Once the cells adhered around the mandrel, the charge would be removed and the cells would be allowed to further



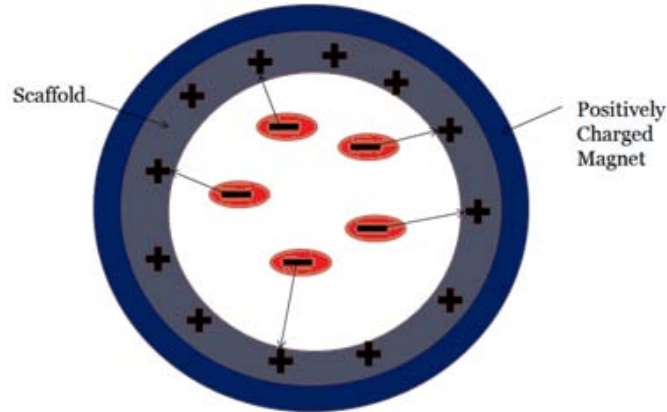
culture, before removing the formed tube from the mandrel. A potential problem with this design is that the proteins and other biological molecules in the media might also be negatively charged and attracted to the mandrel. Additionally, since the electrostatic forces are very weak, they may not be strong enough to hold the cells in place while they create their own extracellular matrix.



**Figure 2: Preliminary sketch of electrostatic force design showing that negatively charged cells will be attracted to a positively charged mandrel**

### *Magnetic Force*

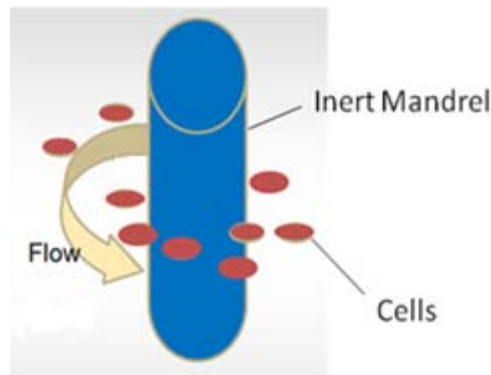
Similar to the electrostatic force idea, this magnetic force strategy would involve surrounding a mandrel with a magnet, or multiple magnets, as shown in Figure 3. This would take advantage of the negative charge in cells, drawing them towards the inner surface of a mandrel, where they would attach, proliferate, and create an extracellular matrix. However, the electrostatic force of the cells is very weak, and can be decreased by the presence of ions in the media (Hjortso, 1995). Previous studies have loaded the cells with magnetic particles in order to have them drawn to a magnet (Shimizu, 2007). It is possible that without any additional forces being introduced to the system, the electrostatic force of the cells won't be strong enough to draw them towards a magnetized mandrel.



**Figure 3: Preliminary sketch of magnetic force design showing how a positively charged magnet could influence cells to move towards a mandrel from the inside**

*Radial Flow*

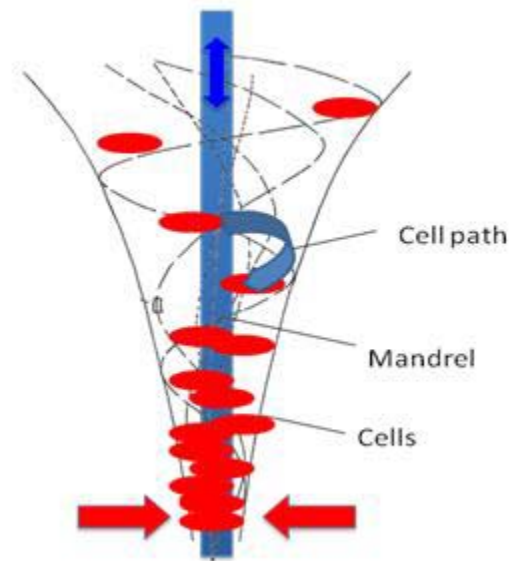
In order to create a tubular construct, radial flow could be applied around a centered, vertical, inert mandrel. The cell suspension would be circulated around the mandrel by means of a media pump. The drag forces would cause the cells to go towards the mandrel. While the cells would not adhere to the mandrel, they would be held in place around the mandrel by the force of the media while they created their own extracellular matrix (see Figure 4). The flow could be stopped after some time to allow further culture of the cells and eventually the tubular construct could be removed from around the mandrel.



**Figure 4: Preliminary sketch of radial flow design showing how the forces involved in circular fluid flow can drive the cells toward a central mandrel**

### *Vortex*

This design is a variation of the radial flow concept. This design will create a vertically oriented, constant, uniform, spinning vortex of cells and media. The motion will force the cells to the bottom and center of the vortex where they could attach to the mandrel there, as seen in Figure 5. While the concept of using circularly flowing water to direct the movement of cells is the same as the radial flow design, the vortex will direct the cells to a specific location on the mandrel at the bottom of the vortex, rather than cells being spread out along the entire length of the mandrel as in radial flow. The speed of the vortex could be manipulated, to raise or lower the media level and allow the cells to attach to all regions of the mandrel.

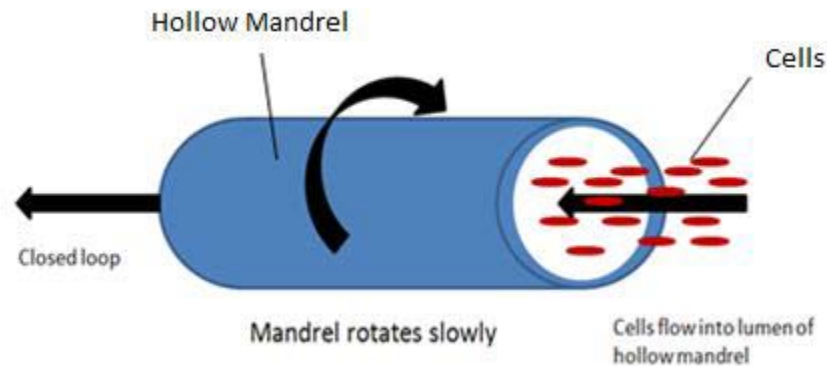


**Figure 5: Preliminary sketch of vortex design showing how material in a vortex is forced to the bottom center and how this could be utilized by inserting a mandrel and allowing cells to attach**

### *Intraluminal Flow/Rotation*

To create a tubular construct using intraluminal flow, a hollow mandrel could be placed horizontally in a bioreactor. A cell suspension would be forced to flow through the inside of the mandrel while the mandrel rotated (see Figure 6). Cells would adhere to the edge of the mandrel,

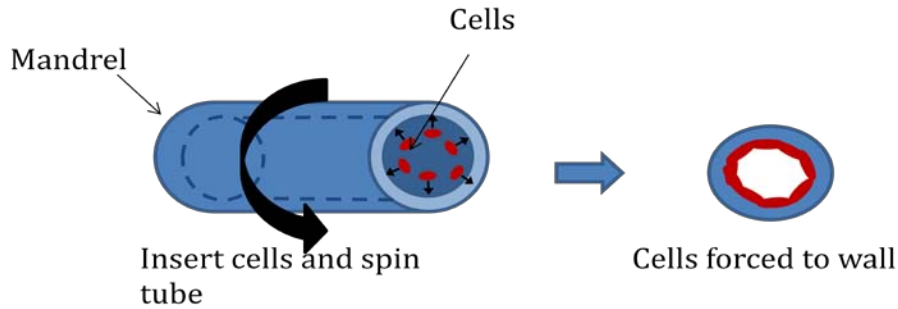
making a vessel like shape. After the cells had formed a tubular construct, the flow and rotation could be stopped so the tube could be removed.



**Figure 6: Preliminary sketch of intraluminal flow design showing how cells flowing through a tube could result in them attaching to a mandrel**

### *Centrifugal Force*

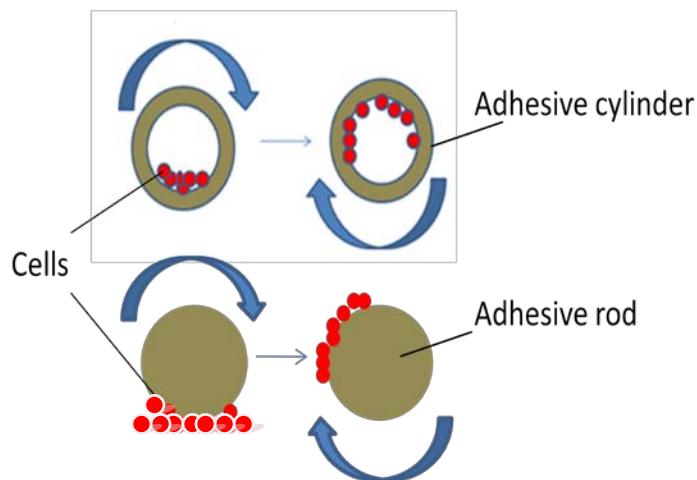
The basic design concept was derived from laboratory technology that has been used extensively for decades, the centrifuge. A centrifuge is used to separate a cellular suspension into two parts: cells and media, where the cells are forced into a pellet at the bottom of a test tube by centrifugal force. The outward seeking force created when a particle is spun circularly could be used to align cells or pellet them around the inner diameter of a tube. A hollow cylinder with cells and media enclosed within it could be spun at a rate great enough to force the cells against the walls, as seen in Figure 7. The cells will be kept there until they form an extracellular matrix after which the cylinder will be stopped and the tube removed.



**Figure 7: Preliminary sketch of centrifugal force design showing how, similar to a centrifuge, centrifugal force can be used to force the cells in an outward direction toward an inert mandrel**

*Rotating Mandrel*

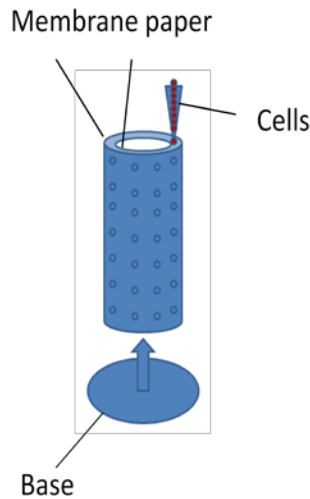
The principle of cell adhesion is utilized in this design. A mandrel is either placed in a well of cells or a hollow tube is loaded with cells and placed in media. The cells are allowed time to adhere to the surface after which the mandrel is rotated to allow new cells to attach to a bare area, as shown in Figure 8. After the cells have coated the surface of the mandrel or hollow tube, the process would be continued to allow for cell to cell adhesion. The cell-mandrel adhesion will have to be compromised without disrupting the cell-cell interactions in order to allow for harvesting to take place without damaging the tubular construct.



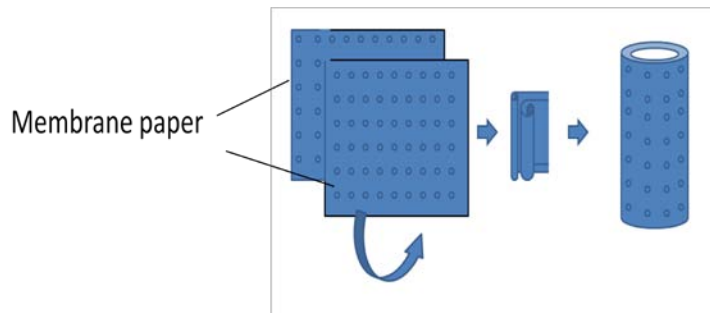
**Figure 8: Preliminary sketch of rotating mandrel design showing how an adhesive hollow or solid rod can be used to attach cells in a tubular shape**

### *Mold*

In this design, we would develop a mold by placing two inert mandrels concentric with each other, which would allow cells to flow through in a closed loop (Figure 9). The outer layer of the mandrel will be porous for the media and nutrients from the outside (not part of the cell & media loop) to reach the cells. Ideally the cells will adhere to the inner walls of the mandrel and uniformly fill the cross sectional space with a self constructed extracellular matrix, creating a tube. Alternatively two pieces of membrane paper could be wrapped into concentric cylinders and cells cultured in the inter-membrane space (see Figure 10).



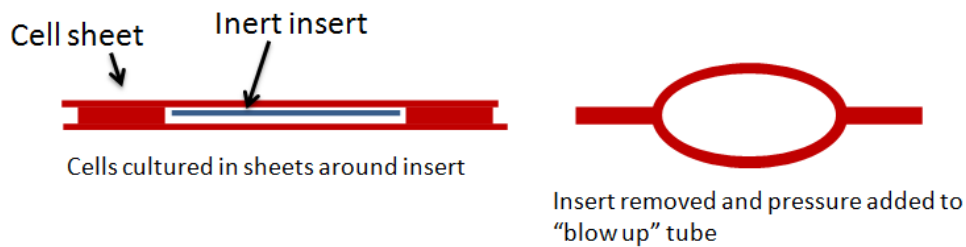
**Figure 9: Preliminary sketch of mold design showing how cells would be inserted between two tubes**



**Figure 10: Preliminary sketch mold design construction showing how two porous pieces of membrane paper could be rolled concentrically to form a mold**

### Blow up Tube

In this design, we would have a membrane separating two sheets of cells. There will be two small areas on either side of the cell sheet that cells can adhere to, but the area in between them will be inert, as shown in Figure 11. This will allow the cells to attach to each other but only attach to the surface at desired points. After the sheets are confluent the membrane between the layers can be removed and a tool can be used to blow up the non-attached areas into a tube shape using media. This could be removed by cutting the attached regions enough to pull it off or by locally trypsinizing the attached regions.



**Figure 11: Preliminary sketch of blow up tube design showing how a sheet of cells could be cultured with a central, inert material between two layers to create an area that could be “blown up” into a tube**

### Needs Analysis

To help us narrow down our list of design alternatives we realized that our designs must be based on the needs of our client. To effectively use our client’s desires to choose our alternative design we used a Numerical Evaluation Matrix, which is shown below in Table 3 for all of our design alternatives. To use the matrix, the designs first had to be evaluated in terms of our constraints (shown in yellow). After that, the matrix allowed us to rank our designs by the weighted objectives determined from our pairwise comparison charts. The rankings on a 0.1-1.0 scale were determined based on our speculation of the design’s ability to achieve that objective and our research on similar ideas in the literature. For example, if the cells are growing inside a

metal mandrel, then it will be hard to see the cells and when they had finished forming a tube. This would subtract from that design's "Transparency" score.

**Table 3: Numerical Evaluation Matrix**

Numerical Evaluation Matrix												
	Weight	Centrifugal Force	Vacuum	Electrostatic Forces	Magnetic Forces	Cell Vortex	Mold	Cell Mold	Radial Flow	Intralumen Flow	Rotating Mandrel	Blow Up Tube
no scaffold												
time												
sterilizable												
size												
<b>tubular assembly</b>	<b>28.6</b>	0.8	0.8	0.4	0.8	0.7	0.8	0.9	0.8	0.7	0.8	0.4
O1 score		22.9	22.9	11.4	22.9	20.0	22.9	25.7	22.9	20.0	22.9	11.4
<b>Limited Interaction</b>	<b>11.9</b>	0.8	0.8	0.8	0.8	0.7	0.8	0.8	0.8	0.8	0.8	0.7
O2 score		9.5	9.5	9.5	9.5	8.3	9.5	9.5	9.5	9.5	9.5	8.3
<b>Manufacturability</b>	<b>9.5</b>	0.7	0.5	0.8	0.4	0.4	0.5	0.4	0.9	0.7	0.7	0.5
O3 score		6.7	4.8	7.6	3.8	3.8	4.8	3.8	8.6	6.7	6.7	4.8
<b>Short Time</b>	<b>19.0</b>	0.8	0.8	0.6	0.8	0.6	0.8	0.8	0.7	0.7	0.4	0.6
O4 score		15.2	15.2	11.4	15.2	11.4	15.2	15.2	13.3	13.3	7.6	11.4
<b>Repeatability</b>	<b>21.4</b>	0.7	0.7	0.8	0.8	0.6	0.8	0.8	0.6	0.6	0.8	0.8
O5 score		15.0	15.0	17.1	17.1	12.8	17.1	17.1	12.8	12.8	17.1	17.1
<b>Easy to Remove</b>	<b>7.1</b>	0.8	0.8	0.9	0.8	0.6	0.6	0.5	0.8	0.7	0.7	0.7
O6 score		5.7	5.7	6.4	5.7	4.3	4.3	3.6	5.7	5.0	5.0	5.0
<b>Transparent</b>	<b>2.4</b>	0.4	0.4	0.9	0.9	0.7	0.4	0.4	0.8	0.4	0.5	0.9
O7 score		1.0	1.0	2.2	2.2	1.7	1.0	1.0	1.9	1.0	1.2	2.2
<b>TOTALS</b>	<b>99.9</b>	<b>75.9</b>	<b>74.0</b>	<b>65.6</b>	<b>76.4</b>	<b>62.3</b>	<b>74.7</b>	<b>75.9</b>	<b>74.7</b>	<b>68.3</b>	<b>69.9</b>	<b>60.2</b>

Based on the outcomes of our numerical evaluation matrix, we began to eliminate some of the alternative designs. The mold and cell mold ideas were combined into a single design, as they were very similar. The intraluminal flow idea was discarded after we learned that cells had to be in contact with a surface for a given period of time before they could adhere to it. Therefore, we decided that if cells were going to be flowing through a mandrel, they would not be able to adhere to it, and thus would not form a tube. Furthermore, we determined that because the idea required cells adhering to the inside of a tube, it would be difficult to remove the cellular construct afterwards, so the idea was not pursued. The electrostatic forces conceptual design was also eliminated because it received a low score in the matrix. This was due to uncertainty that it would form a tubular construct. It is possible that proteins and ions in the media would decrease the electrostatic force between the cells and the mandrel, and the electrostatic forces of cells is already a very weak force. The blow up tube design received a low score as well, because when



two sheets are blown up, it may not form a tubular shape. There would also not be a strong connection where the two sheets are bound together, and the design may lack overall mechanical stability. While the magnetic force idea received one of the higher scores in the matrix, we decided not to pursue this idea because similar ideas were found in the literature showing that additional charges need to be added to the cells besides their natural force in order to create a strong enough charge between the cells and a magnet to manipulate their movement towards a magnetized mandrel (Shimizu, 2007). Rotating mandrel and cell vortex each received low scores in our numerical evaluation matrix, however, we decided to include these ideas for further testing. The rotating mandrel idea received a low score because it would take a longer time than the other designs for the cells to form a tubular construct because they need to adhere to the mandrel. However, since we are unsure exactly how long it would take for cells to adhere to the mandrel or how long the other designs will take for the cells to form a tube and produce their own ECM, we decided to test this idea. The cell vortex received a low score for manufacturability, time, and repeatability. We were uncertain of the difficulty to build this conceptual design, since it would involve varying rotational speeds to adjust the height of the vortex in order to coat the whole mandrel. The repeatability of this process is also uncertain based on the ability to create a device that automatically adjusts vortex height over time, and creates a similar vortex with even distribution of cells. However, we decided the concept would be simple to test and we would reevaluate its scoring in the matrix after further testing.

Overall, we decided to pursue six of our conceptual designs ideas based on the numerical evaluation matrix and reasons stated above. These top ranked designs include centrifugal force, vacuum, cell vortex, cell mold, radial flow, and rotating mandrel. The numerical evaluation matrix of the six designs we decided to further pursue is shown in Table 4 below.

**Table 4: Numerical Evaluation Matrix of Top Ranked Alternative Designs**

Numerical Evaluation Matrix							
	Weight	Centrifugal Force	Vacuum	Cell Vortex	Cell Mold	Radial Flow	Rotating Mandrel
no scaffold							
time							
sterilizable							
size							
<b>tubular assembly</b>	<b>28.6</b>	0.8	0.8	0.7	0.9	0.8	0.8
O1 score		22.9	22.9	20.0	25.7	22.9	22.9
<b>Limited Interaction</b>	<b>11.9</b>	0.8	0.8	0.7	0.8	0.8	0.8
O2 score		9.5	9.5	8.3	9.5	9.5	9.5
<b>Manufacturability</b>	<b>9.5</b>	0.7	0.5	0.4	0.4	0.9	0.7
O3 score		6.7	4.8	3.8	3.8	8.6	6.7
<b>Short Time</b>	<b>19.0</b>	0.8	0.8	0.6	0.8	0.7	0.4
O4 score		15.2	15.2	11.4	15.2	13.3	7.6
<b>Repeatability</b>	<b>21.4</b>	0.7	0.7	0.6	0.8	0.6	0.8
O5 score		15.0	15.0	12.8	17.1	12.8	17.1
<b>Easy to Remove</b>	<b>7.1</b>	0.8	0.8	0.6	0.5	0.8	0.7
O6 score		5.7	5.7	4.3	3.6	5.7	5.0
<b>Transparent</b>	<b>2.4</b>	0.4	0.4	0.7	0.4	0.8	0.5
O7 score		1.0	1.0	1.7	1.0	1.9	1.2
<b>TOTALS</b>	<b>99.9</b>	<b>75.9</b>	<b>74.0</b>	<b>62.3</b>	<b>75.9</b>	<b>74.7</b>	<b>69.9</b>

## Feasibility Studies




Before we can produce our final design, we must first justify our choice by performing a number of tests to prove that it is likely to be successful. Preliminary testing is a critical step towards determining a final design, and proving whether or not the concept of each individual design is feasible. From these tests we can rate the designs and see which may or may not meet our benchmarks in order to narrow down our design alternatives to the most promising methods. Finally, once the final design is chosen, we will do further testing to ensure that the chosen design meets each of our required benchmarks.

## Cellular Adhesion Testing

All of our designs require the use of either a cellularly adhesive or non-adhesive mandrel. In order to fulfill this need, research was conducted to correlate material properties with cellular binding. Based on this research, mandrels of various materials were ordered for our prototypes. In order to characterize the adhesive properties of the mandrels, cellular adhesion testing was completed for each of the materials. Table 5 below shows a list of the materials tested in the

cellular adhesion assays. This testing is necessary to make the proper material choices to match the conceptual theory behind each design. Through the use of experimental testing, we were able to make informed decisions on the choice of material for the mandrel in each of our designs.

**Table 5: List of the 8 different materials tested in the cellular adhesion assays.**

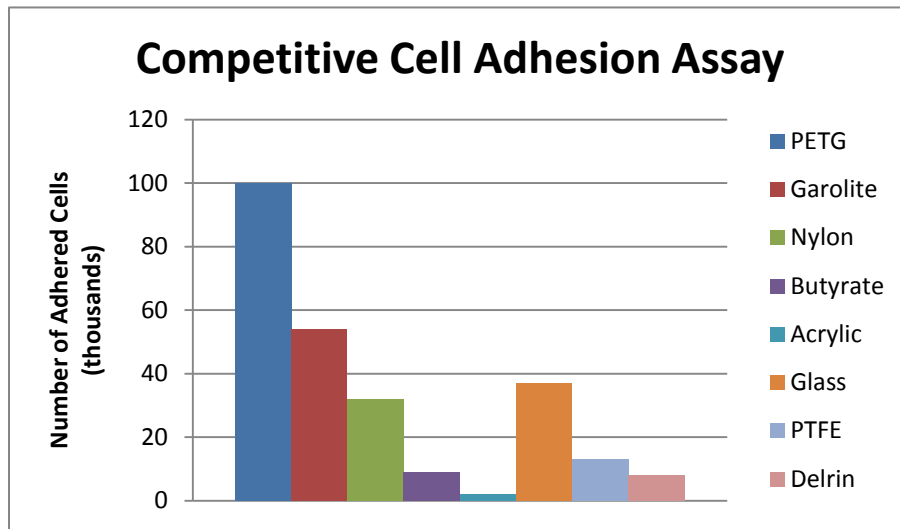
Materials Tested	Outer Diameter	Inner Diameter	Illustration
PETG	3/8"	1/4"	
Garolite	3/8"	1/4"	
Nylon	3/8"	1/4"	
Butyrate	3/8"	1/4"	
Acrylic	3/8"	1/4"	
Glass	6 mm = 0.236"	5 mm = 0.197"	
PTFE	3/16"	N/A	
Delrin	1/4"	N/A	

### *Competitive Cell Adhesion Assay*

Rods/tubes of the material to be tested were cut, using the hack saw and clamp, into 2 cm sections. The ends of the tubes were blocked with duct tape. Sections were placed in a bath of 70% ethanol to be sterilized for five minutes. Rat smooth muscle cells were trypsinized for 5 min and spun down at 1000 rpm for 5 min. The cell pellet was re-suspended in 1 mL of media and counted using a hemocytometer and microscope. In a sterile field, the prepared mandrel sections were removed from the ethanol solution, allowed to dry for five minutes, and then placed in the bottoms of the T-25 flasks. After 10 minutes, 20 mL of cell suspension were added to the upright flask effectively introducing 1 million cells to the outer surface of the mandrels. The flasks were then placed in an incubator for an hour at 37C. Media was aspirated, the material was rinsed with PBS, and then trypsin was pipetted into the flask. After five minutes the mandrels were removed with forceps and an equal volume of 10% FBS DMEM was added. Media was poured over the mandrels to shear cells from the material. Next, this solution was centrifuged at 1000 rpm for 5 min. The supernatant was aspirated and the cell pellet resuspended in 0.5 mL of media. Cells were counted with a hemocytometer and microscope according to the procedure in Appendix B: Supplemental Procedures. The results of this experiment are summarized in the Table 6 and represented in Figure 12 below.

**Table 6: Results of the competitive cell adhesion assay showing the number of adhered cells for each of the tested materials. The shaded boxes represent materials that were not the same dimensions (inner/outer diameter) as the other materials and had a different seeded surface area.**

Material	Number of Adhered cells	Seeded Surface Area (in <sup>2</sup> )
PETG	54000	0.927
Garolite	100000	0.927
Nylon	32000	0.927
Butyrate	9000	0.927
Acrylic	2000	0.927
Glass	37000	0.583
PTFE	13000	0.464
Delrin	8000	0.618



**Figure 12: Results of the competitive cell adhesion assay showing the number of adhered cells for each material. The last three materials (glass, PTFE, and Delrin) had a smaller surface area than the rest, so the results are not comparable.**

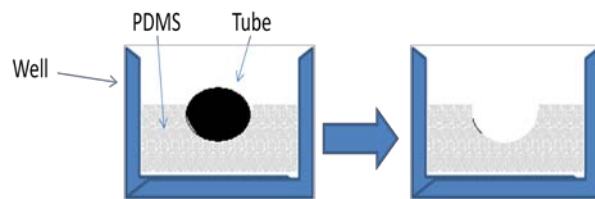
Garolite was found to be the most adhesive material, while acrylic was the least adhesive. However, garolite is an opaque material and we needed a transparent adhesive mandrel for the rotating mandrel design. For this we used PETG, which was the second most adhesive material. Though this assay provided us with insight for choosing materials for designs, we realize that it was not a completely fair comparison of materials. Due to the fact that three of the mandrels (glass, PTFE, and Delrin) did not have the same dimensions as the other 5 materials (the surface area for attachment was different) the results for number of adhered cells cannot be accurately

compared. Instead, the results serve as guidelines for the relative adhesiveness of these three materials based on the surface area.

Another important issue we noted was that the containers in which we performed the assay were cell adhesive, creating a competitive environment for cell adhesion. We determined that we should investigate further and create a more conclusive non-competitive cell adhesion assay.

### *Cell Adhesion Assay 2*

After completion of the first cellular adhesion assay modifications were made in order to make the protocol more accurate. The most pressing issue that was addressed was the container that the cells and test material were housed in. In the first experiment, cell culture flasks were used; this material, polystyrene, is cellular adhesive. Therefore, cells may be interacting with the flask and not the material being tested. This could have altered the results of the above competitive assay. In order to overcome this limitation, custom seeding wells made of PDMS, a non-adhesive elastomer, was used. PDMS is made from two liquids and prior to curing, can be poured and molded. The protocol to make this material can be found in Appendix B: Supplemental Procedures. BD Falcon six-well culture plates and 2 cm sections of ½ inch diameter tubing were used to make the molds. First, PDMS was poured into the plates until they were filled half way. Then, the 2 cm tubing was placed in the PDMS such that half of the tube was immersed leaving space between the bottom of the flask and the bottom of well (Figure 13).



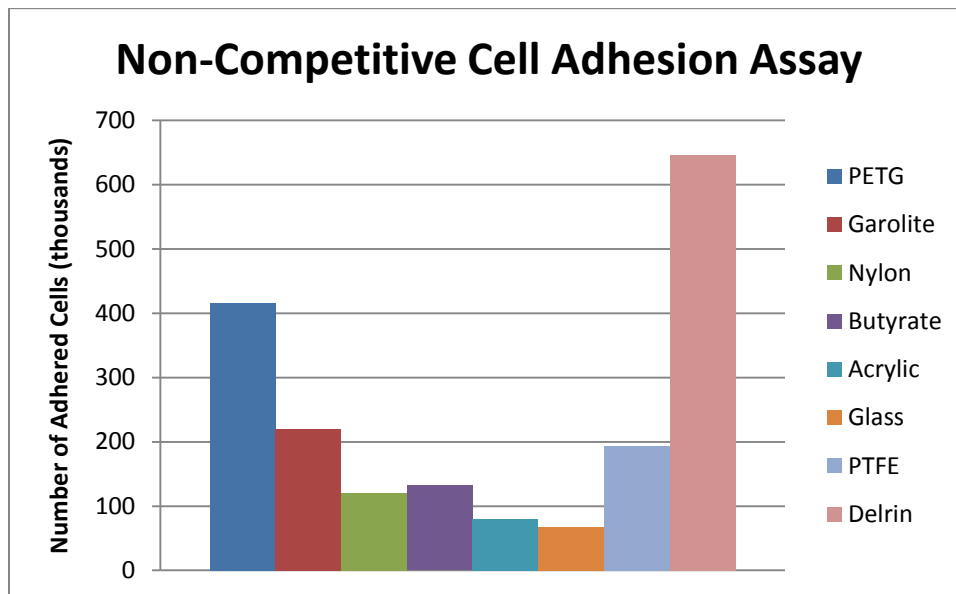
**Figure 13: Schematic of PDMS molds created for non-competitive cell adhesion assay.**

As shown in the figure above, the tube was removed leaving space for the tubes being tested to be inserted along with a cell suspension. A total of 8 molds were cast in order to test our 8 different materials.

After the molds were allowed to dry for 24 hours at room temperature, they were sterilized in 70% ethanol for 10 minutes. The ethanol was then aspirated and the molds were placed in the hood for 5 minutes to ensure complete evaporation. A cell suspension of 600,000 cells in 1 mL of media was added to each well. Subsequently, 2 cm sections of the materials were placed in the wells and incubated for 45 minutes. After incubation, the mandrels were removed and trypsinized, and the cells were counted using the cell counting assay described in Appendix B: Supplemental Protocols. The number of adhered cells for each material is shown below in Table 7.

**Table 7: The results for the non-competitive cell adhesion assay, showing the number of adhered cells for each material. The shaded boxes represent materials that were not the same dimensions (inner/outer diameter) as the other materials.**

Material	Number of Adhered cells	Seeded Surface Area (in <sup>2</sup> )	Cells/Area
PETG	416000	0.927	449000
Garolite	220000	0.927	237000
Nylon	120000	0.927	129000
Butyrate	132000	0.927	142000
Acrylic	80000	0.927	86000
Glass	68000	0.583	117000
PTFE	194000	0.464	418000
Delrin	646000	0.618	1045000



**Figure 14: Results of the non-competitive cell adhesion assay showing the number of adhered cells for each material. The last three materials (glass, PTFE, and Delrin) had a smaller surface area than the other materials. (n=1)**

The results of this experiment were consistent with those of the first cellular adhesion assay. Garolite and PETG showed high adhesion characteristics while acrylic was the least adhesive. This makes garolite and PETG good candidates for use in the rotating mandrel design



as cellular adhesion is required. Based on the results acrylic is the best mandrel for the other designs, which institute the use of a non-adhesive mandrel.

### *Cell Adhesion Assay 3*

In order to better quantify the relative adhesiveness of tubing materials, we repeated the cell adhesion assay with more materials, a higher sample number (n=3), and tubes of uniform dimensions. Tubing (3/8" OD, 1/4" ID) was cut into 2 cm sections and the ends were sealed with silicone. The tubes were placed in a Petri dish and secured in place with duct tape to prevent them from rolling around. Three tubes of each material were tested including: garolite, butyrate, acrylic, nylon, PTFE, polycarbonate, and PETG. Approximately 1 million cells were added per tube. The tubes were incubated for 30 minutes, then the silicone was removed and the media was aspirated. The tubes were then washed with PBS and trypsinized. Then the adhered cells for each material were counted (see Table 8 for results).

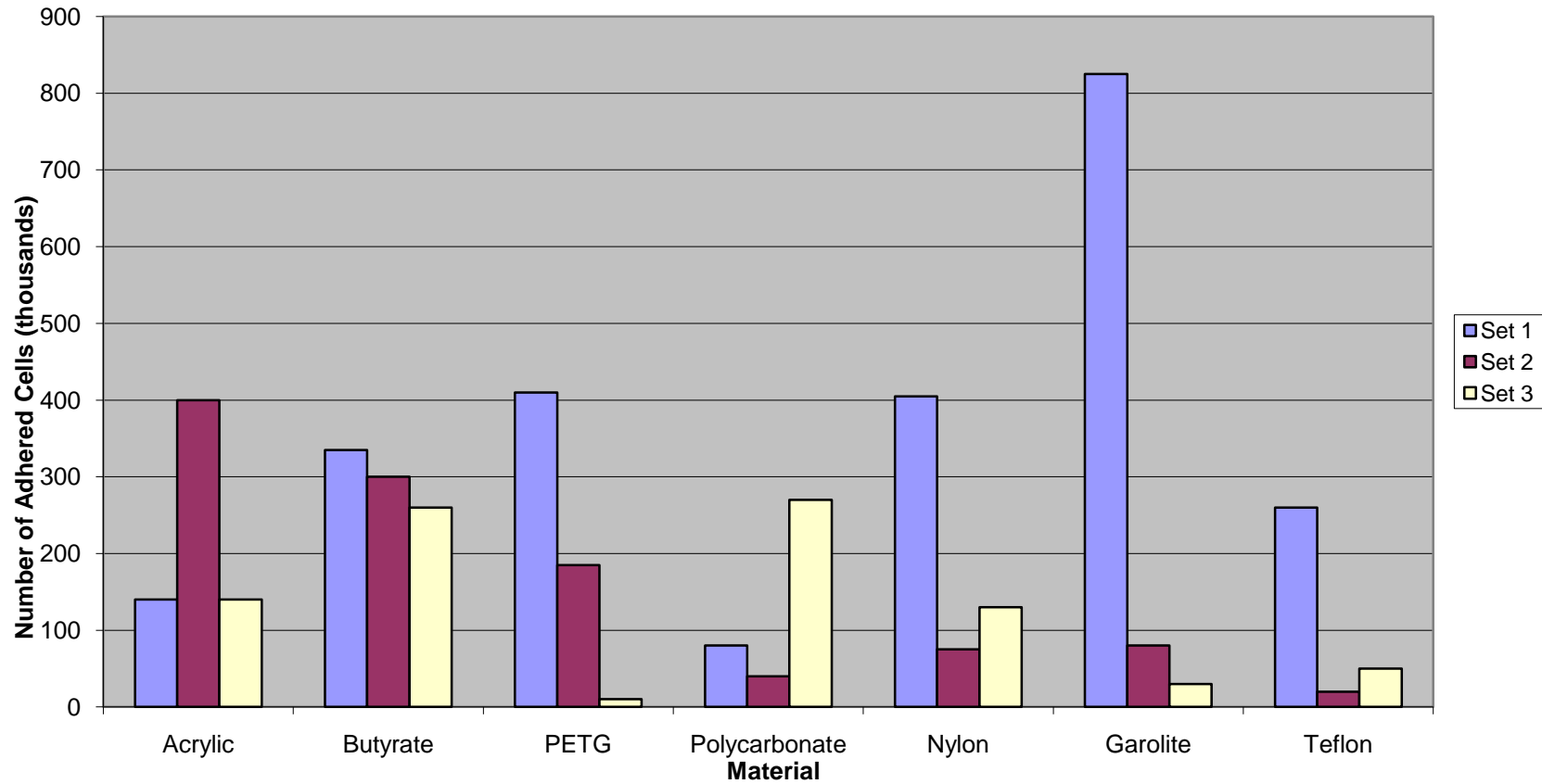
**Table 8: Results of the cell adhesion assay (n=3).**

Material	Number of Adhered Cells (Thousands)			Average (n=3)	Standard Deviation
	Test 1	Test 2	Test 3		
Acrylic	140	400	140	226.67	150.11
Butyrate	335	300	260	298.33	37.53
PETG	410	185	10	201.67	200.52
Polycarbonate	80	40	270	130.00	122.88
Nylon	405	75	130	203.33	176.80
Garolite	825	80	30	311.67	445.26
PTFE	260	20	50	110.00	130.77

The number of adhered cells also decreased for each of the three tests. The cells had been sitting in trypsin while they were prepared to be counted. The cells may have been aspirated when preparing the slides, disturbing the cell count. Overall, the results from the first adhesion assay most closely mimic the initial cell adhesion results obtained previously, with acrylic being relatively non-adhesive and garolite being adhesive. Polycarbonate had very low cell adhesion in two of the three tests.

However, as shown in the graphs below, there was no consistency in the results across tests (Figure 15 and Figure 16). A one-way ANOVA (analysis of variance) test was completed using Minitab v. 15 in order to statistically compare the cellular adhesion of the materials tested. A p-value of 0.883 is much greater than the confidence interval of 0.05, indicating that there is no statistical difference between any of the materials tested. Therefore, from this adhesion assay we can conclude the cellular adhesiveness of all the materials is similar, so due to the availability and cost of polycarbonate, we decided to test this material with the centrifugal force design to see if we can replicate the results with acrylic. Acrylic is a more costly material and its properties make it difficult to sterilize. Polycarbonate is easily sterilized by autoclaving or other methods.

### Cell Adhesion Assay



**Figure 15: Graph of the three tests of the cell adhesion assay showing the number of adhered cells for each material. As shown by the graph, there are no trends between the tests and the results overall are inconclusive.**

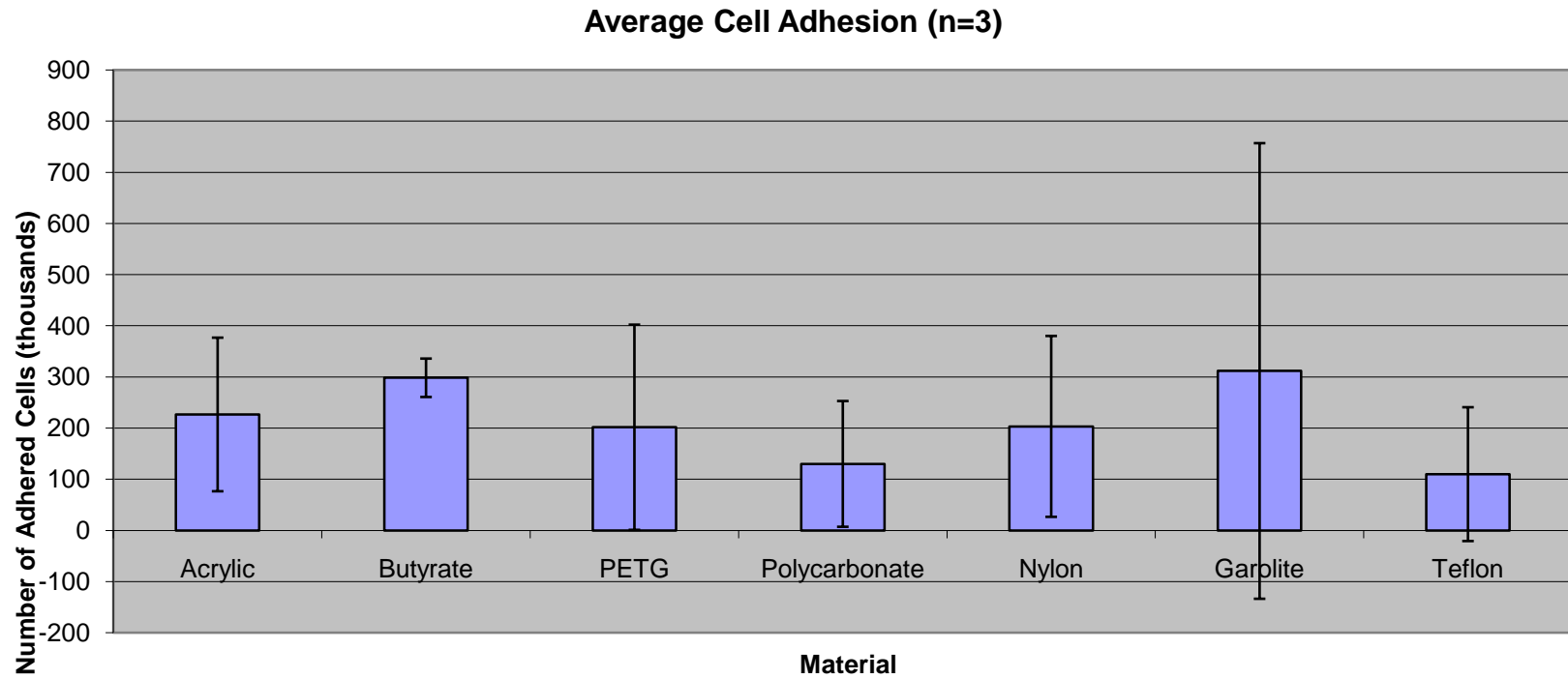
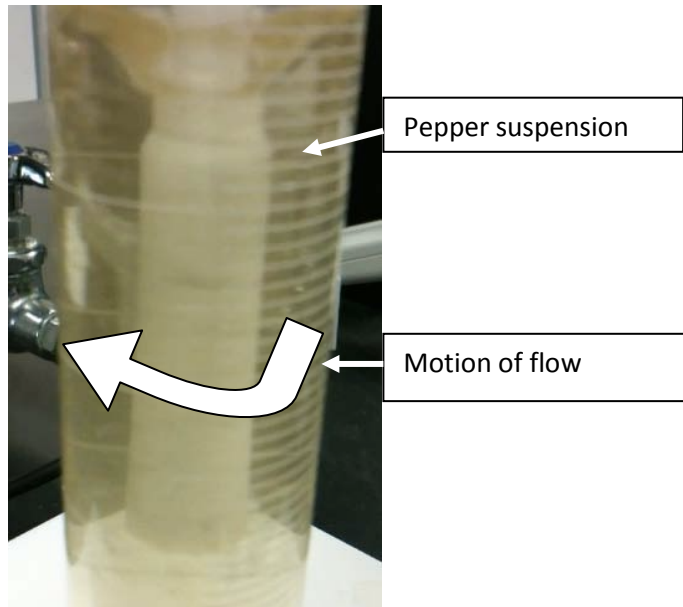


Figure 16: Graph showing the average cell adhesion for each of the materials tested. There is a large standard deviation for most of the materials.

## Vortex

To test the concept of the vortex design, we added 15 mL water and pepper to a 20 mL beaker. Pepper was chosen as a model cell substitute because it forms a suspension in water similar to cells. The suspended pepper particles allow for visual inspection of the forces being generated in the solution. The beaker was placed on a magnetic spin plate and a magnetic spinner was placed at the bottom of the beaker. We also tested this using a 150 mL beaker (at 50, 100, 150 mL water) and 50 mL graduated cylinder (at 20 and 40 mL water). The experiment was repeated with changes in pepper concentration and speed of spinning (200-1600 RPM) for each sized container.

We found that a lower concentration of pepper allowed for better visibility of flow. Also, we noted that high speeds resulted in a large vortex while low speeds failed to create much of a vortex. Overall, the spinning speed desired was dependant on the volume used and should be determined on a case by case basis. The pepper flow observed in the beaker appeared the same independent of different volume vortexes. We noted that the pepper spun in homogenous solution for the most part, not just in the center as we expected. However, there was slightly higher concentration in the center. This was even more apparent in the graduated cylinder due to its smaller diameter. We held a mandrel in the central location of flow and could see pepper contacting it, suggesting that some cells might attach, however, this seemed to be too fast of a motion (Figure 17).



**Figure 17: Cell vortex in graduated cylinder**

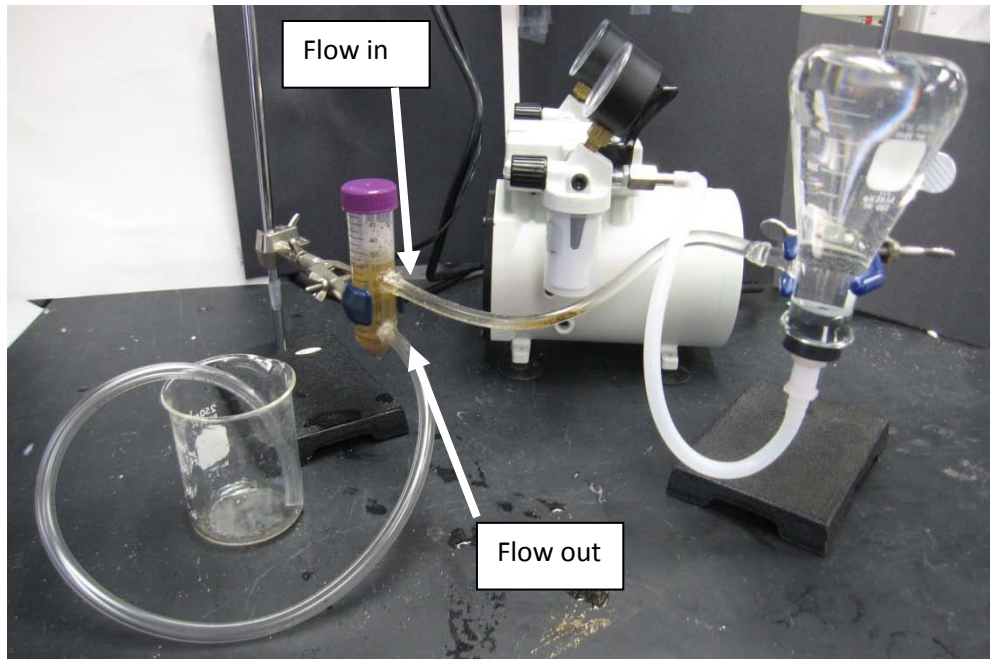
Based on our observations and the poor manufacturability of the design with the difficulty of trying to alter the location of the vortex center on the mandrel, we determined that it was simply a more complicated version of the radial flow design and should no longer be pursued.

## **Radial Flow**

### *Experiment 1*

To test the radial flow design concept, we first created a pumping system to create liquid flow into a vessel culture chamber. The vessel culture chamber is composed of a modified 50 mL conical tube. A 4-inch length glass mandrel (OD 1/4") was attached, centered, to the inside of the conical tube cap using silicone glue. A hole was drilled around the middle (25 mL line) of the conical tube to allow the insertion of tubing for flow in, which was sealed in place using silicone glue. Another hole was drilled near the bottom of the conical tube to allow the insertion of another tube for flow outward, which was also sealed in place using silicone glue. This tube was

covered with 0.4 micron porous membrane paper to act as a filter to prevent pepper flow out, but allow water to leave. The inlet tubing was connected to an aspirator flask filled with a water and pepper solution. The outlet tubing drained into a waste container. The aspirator flask was subsequently connected to an air pump using similar tubing. The entire setup can be seen below in Figure 18.



**Figure 18: Radial flow complete setup**

The aspirator flask was inverted to allow more water to exit through the culture chamber inlet tubing. The pump was then turned on to pump air into the flask, pushing the water and pepper solution out and into the culture chamber. Unfortunately, this increased the pressure in the aspirator flask causing the top to burst off, spilling the water. We determined that the pores in the membrane paper must be too small to allow rapid water flow through. This resulted in an input but no output, increasing the pressure within the system and causing it to fail.

### *Experiment 2*

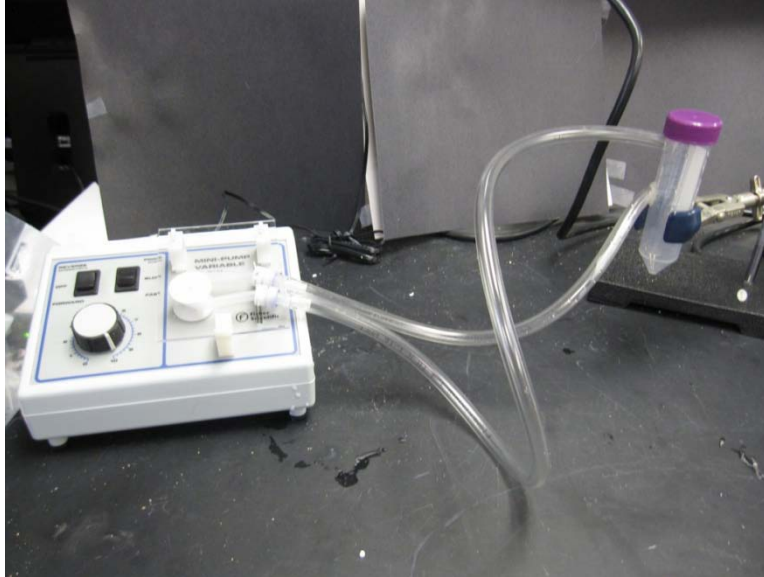
For this experiment, we used a similar set up as the previous one. The major change was that we used a paper towel to cover the outlet tubing instead of membrane paper because we assumed that it would be more permeable to water.

We observed some radial-like flow of the pepper particles around the mandrel in the culture chamber. We could visualize some pepper particles bouncing off the mandrel, suggesting that the flow forced them in a central direction, towards the mandrel. However, the paper towel quickly ripped and allowed liquid and pepper to exit the culture chamber quickly. Because the outlet tube was larger than the inlet tube, this resulted in the water level decreasing to the height of the outlet tube. In order to accurately test this conceptual design, we need to utilize a peristaltic pump for a closed loop system that will allow the water level in the conical tube to remain constant.

### *Experiment 3*

We redesigned the radial flow system by connecting a Fisher Scientific Peristaltic Pump. One end of the tubing was connected to the top of the conical tube for outflow of water, while the same diameter tubing was connected to the middle of the conical tube for in flow. The inflow port was angled in order to push the water around the centered mandrel.





**Figure 19: Radial flow design using a pump to control the flow of cells around a mandrel**

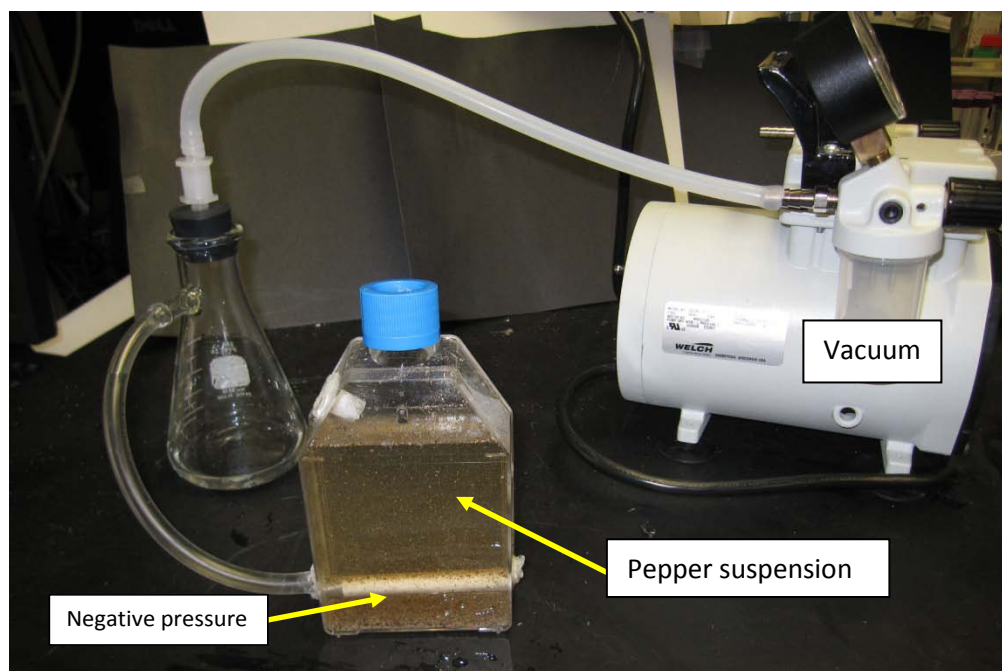
After turning on the pump, pepper was added to the system. The speed of the pump was adjusted so that we could visualize the effect of high and low water flow around the mandrel. However, neither setting allowed the pepper to be pushed towards the mandrel. The pepper appeared to move freely in the conical tube. There were no forces being created to push the pepper towards the middle. Therefore, after this testing it appears this design is not feasible in producing a tubular construct.

## **Vacuum**

### *Experiment 1*

In order to test the alternative design idea of a vacuum bioreactor, a porous, hollow mandrel was constructed by punching out holes with a thumbtack in a 10 cm long piece of flexible silicone tubing, with an I.D. of 0.25 inches and an O.D. of 3/8 inches. Two holes (5/16 inch) were drilled across from each other in sides of a T-25 flask, approximately 1.5 cm from the bottom. Western blot membrane paper with pores of 0.4 microns was wrapped around the porous mandrel and secured with superglue. The tubing was placed through the holes in the flask and secured in place with silicone adhesive. The flask was filled with water, and pepper was added.

One side of the porous tubing was sealed off with silicone adhesive, while the other side was attached to an Erlenmeyer flask, to act as a reservoir to catch all the water being suctioned out. The tube was attached to a glass tube that was placed through a hole in the center of a rubber stopper. The rubber stopper was placed on top of the flask to close the top. Another piece of flexible silicone tubing was attached to the side port on the flask and to a vacuum pump. The setup can be seen in Figure 20 below.



**Figure 20: Vacuum conceptual testing setup showing the tubing used to form a vacuum**

The pump was turned on, and it was observed whether or not the vacuum is capable of attracting the pepper to the mandrel, how much pepper is attracted, and the length of time it took to fully cover the mandrel. The setup was unsuccessful, as there was very little suction being produced at the porous mandrel. The holes from the thumbtack just closed back up due to the force of the suction.

### *Experiment 2*

The experiment was repeated using a piece of acrylic tubing since it is more rigid than the silicone tubing (1/4" I.D., 3/8" O.D.). Holes were drilled through the tubing that were 1/16 inches in diameter. The Western blot paper was again wrapped around the tube and secured with superglue on the edges. The same setup was repeated as in Experiment 1 with pepper as a cell substitute. While the holes were now drilled out in rigid tubing so that the vacuum could not suction them shut, the experiment was still not successful. There was only a small amount of water sucked through the paper and tubing, and there was no effect on the pepper. The pore size of the paper must be too small to suck the water through readily, even with the force of the vacuum. Therefore, further testing was planned using a piece of acrylic tubing that has larger holes drilled out, and an overall higher porosity. Also, the western blot membrane paper (0.4 micron pore diameter) must be replaced with another filter that has a larger pore size. The size of the cells would need to be calculated so that the pores are large enough to allow media diffusion, but small enough to prevent cell migration.

### *Experiment 3*

In this experiment, a vacuum pump was attached by means of polypropylene tubing to a water collection reservoir, which was connected to a T-75 flask. This test involved trial runs designed to monitor the ability of the vacuum to pull water through two different nylon mandrel designs. The first mandrel (A) was drilled out with a 3/64 in drill bit, while the second (B) was hollowed with a 1/16 in drill bit. The first mandrel was more porous than the second as a result of the larger quantity of holes that could be drilled using the smaller bit. The tubes were then wrapped with coffee filters. The two flasks were filled with 275 mL of water and 1.07 g of pepper.

Using mandrel A, the vacuum was not strong enough to suction water through the coffee filter-covered mandrel. Very little water was collected in the reservoir. Pepper appeared to be stuck to the top portion of the mandrel; however, we determined that this was simply due to gravity, as opposed to vacuum pressure, as a manual disturbance of the flask caused the pepper to fall off the mandrel. Additionally, a slight leak in the tubing interface with the mandrel could have caused a pressure decrease significant enough to prevent the pepper from being forced towards the mandrel. The surface tension of the water around the coffee filter may hinder its ability to pass through the pores in the mandrel. A more porous mandrel may be necessary. A stronger vacuum pressure may be necessary. Previously reviewed literature showed that a setup similar to ours worked, however, the mandrel materials used in that experiment are beyond our \$628 budget constraints (Frangos, 2008).

In the experiment with mandrel B no pepper was added to the system, yet the vacuum was still unable to pull water through the coffee filter-cover mandrel. The rubber stopper on the water collection reservoir was found to be clogged with a rubber fragment, which we determined was restricting the vacuum pump's ability to pull water through the mandrel. The trial was restarted, this time with pepper, and this time all of the water was quickly pulled into the reservoir (< 3 seconds). Most of the pepper in the flask was suctioned to the mandrel. This design proved successful, and more testing will be conducted to determine its efficacy in producing a blood vessel.

#### *Experiment 4*

The previous test was repeated three times, with slight variations in each. Test 1 substituted membrane paper for the coffee filters, as we believe that the reason for the failure of Test A in the previous experiment (due to the clogged rubber stopper) has been resolved. Test 2

involved using membrane paper, but water was replaced with DMEM in order to ensure that media would flow through the membrane paper readily. Test 3 was the same as Test 2, but with the addition of  $13 \times 10^6$  rat smooth muscle cells. The mandrel would be stained after the media was vacuumed out in order to determine if the cells had been forced to it.

In Test 1, the vacuum was strong enough to quickly suction all of the water from the flask into the water trap. When this was repeated with media in Test 2, the same results were found and the vacuum pulled the media quickly through the membrane paper.

In Test 3 the vacuum sucked the media/cell solution towards the mandrel. The media passed through the membrane paper, and we then removed the mandrel and stained it with Trypan blue. We had expected to see a pattern of dots on the membrane paper showing where the cells had been suctioned to the numerous pores in the mandrel, however, we discovered that the membrane paper was simply absorbing the dye, like a sponge, and visualizing cell distribution about the mandrel was impossible.

#### *Experiment 5: Closed Loop Design*

In order to build a more effective bioreactor (not requiring as massive media reserve or media changes every few seconds), we decided that a recirculating system would be necessary. The setup can be seen below in Figure 21.

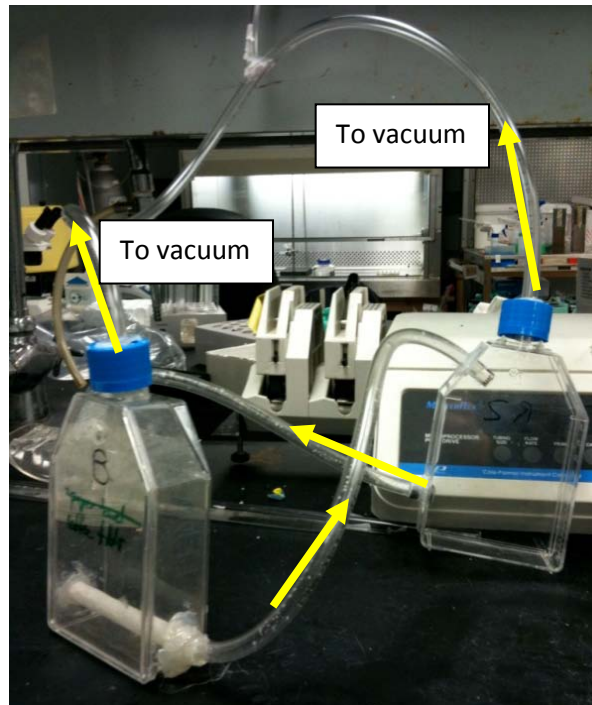


**Figure 21: Recirculating media system.**

Water circulated in the system; however, the rate at which water entered the reservoir was not equal to the rate that water entered the reactor chamber flask, resulting in the reservoir overflowing.

#### *Experiment 6: Closed Loop Design 2*

A new closed loop was designed in an attempt to overcome the problem of the overflowing reservoir. The setup can be seen below in Figure 22.



**Figure 22: Modified recirculating system**

This design proved to be even more ineffective than the first design, as there was no water movement in the system. We added pepper to the water to try to see if there was at least slight flow, but the pepper just settled to the bottom of the flasks. A steady stream of bubbles coming from the mandrel/flask interface seemed to indicate a leak, which could have affected the circulation by weakening the vacuum force (sucking external air rather than creating vacuum pressure inside the system). The vacuum was also creating pressure voids in both flasks simultaneously; therefore we determined that this was the primary reason why no circulation was noted.

### *Experiment 7*

#### Test 1

The initial closed loop system was reused in order to try to replicate the initial results. The initial results of circulating water couldn't be reproduced to any extent. Bubble streams



indicated new leaks. Various vacuum strength levels, ranging from lowest to highest, made no difference in achieving circulation. A possibility for this failure is that there was a siphon effect. If the tubes were full of water, a slight amount of suction could maintain a flow that appeared to be caused by vacuum force, as may be the case in the original closed loop testing results. This was confirmed when the vacuum was shut off, because the water continued to trickle for several minutes without aid.

#### Test 2

A new system was developed in which a larger reservoir was suspended above the bioreactor flask. The thought behind this was that gravity would cause the water in the reservoir to flow downwards into the bioreactor flask. This would ideally force the vacuum to suck water through the exit port in the bioreactor flask and into the reservoir. The results were the same as in the previous test. The vacuum pressure was too strong and did not allow water to flow from the reservoir into the bioreactor flask, even at the lowest pressure setting.

#### Test 3

One final attempt was made with this design using the same setup as in the previous test, but without the mandrel. This would enable us to determine if the mandrel itself is creating too much resistance for the vacuum to suction water back to the reservoir.

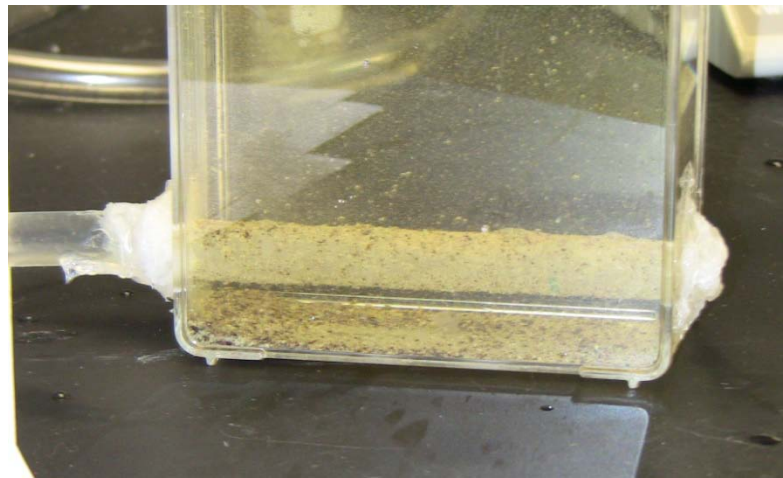
Water was observed to be suctioned from the bioreactor flask into the reservoir due to the vacuum. Therefore, a new design is needed to actively force water from the reservoir back into the bioreactor flask, possibly by using a pump.

### *Experiment 8*

A Fisher Scientific Mini Pump with variable flow rates (0.4 - 85 mL/min) was attached to the bioreactor flask with the largest available diameter tubing. In order to test the pumping force



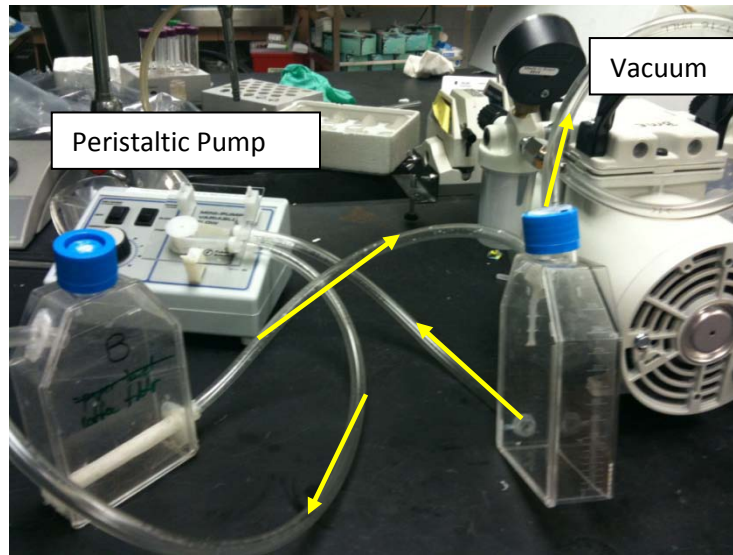
of the peristaltic pump, the vacuum was not used in this setup. Tubing was attached to mandrel inserted into the flask, wrapped around the pump, and attached to the top of the flask to pump the water back in that had been pumped out through the mandrel. A water/pepper solution was inserted in the bioreactor flask to determine if the pump would be able to apply enough force to cause the pepper to be suctioned to the mandrel (coated with a coffee filter). After approximately 30 seconds, pepper was observed to be coating the entire mandrel, though mostly on the top portion (Figure 23).



**Figure 23: The results of experiment #8 showing the pepper adhered to the coffee filter after the vacuum was applied.**

### *Experiment 9*

In order to enable the flow of water/media back from the reservoir into the flask, we attached a peristaltic pump to the system. As seen in Figure 24 the pump was attached between the reservoir and flask, to push the water against the suction of the vacuum back into the flask to create a closed loop system.



**Figure 24: System with second pump added.**

The pump was a Fisher Scientific Variable Flow Mini-pump with Medium Flow (4.0 to 85 mL/min). In the first test with this setup, we tried a slotted tube design instead of drilled holes, in order to try to increase the area for vacuum suction. The tube was wrapped in western blot paper as before, and the system was filled with water and pepper. The pump was first turned on to release air from the tubing in the system. Then the vacuum was turned on its lowest setting, while the pump was on its highest setting. The water levels in the flask and reservoir were monitored.

The pump was able to help reverse the vacuum force and successfully pumped water from the reservoir into the flask. Some pepper was suctioned to the tube, but the vacuum force was not very strong. The slotted tube was a poor design, and collapsed under the pressure of the vacuum, sucking the membrane paper inwards through the slots. We were unable to get the water levels to be constant as the flow was not in equilibrium. The vacuum force was stronger than the pump, and the water was not being replenished in the flask. The system needs to have a stronger pump to compensate for the vacuum force. The vacuum force needs to remain strong in order to suction the pepper/cells to the tube.

### *Experiment 10*

The same setup in experiment 9 was repeated, using a porous mandrel with holes drilled instead of the slotted design. At first, only water was added to the system and no pepper. The flow of water between the containers was observed. With the pump on the highest setting, and the vacuum power slightly higher than the lowest setting, the flow of the water was able to be in equilibrium. The water levels in the flask and reservoir remained constant for the 5 minute test period. Next, pepper was added to the system to test if the vacuum is strong enough to suction it to the mandrel. The vacuum strength decreased upon addition of pepper, and the flask filled up with water. It was concluded that the pepper was clogging the membrane paper, and therefore was not a sufficient substitute for cells. In order to conceptually test our loop system design, we replaced the membrane paper with a coffee filter. The filter has much larger pores and will not become clogged by the pepper. The water levels were able to remain constant when the vacuum was on the lowest setting, but the force was not strong enough to suction the pepper to the mandrel. When the vacuum was on the highest setting, the pepper was suctioned to the mandrel. Therefore, the design conceptually works and cells could be suctioned to the mandrel through the vacuum force, and a loop system could be used to replenish the media. In order to test the design with cells, we will need to increase the speed of the pump.

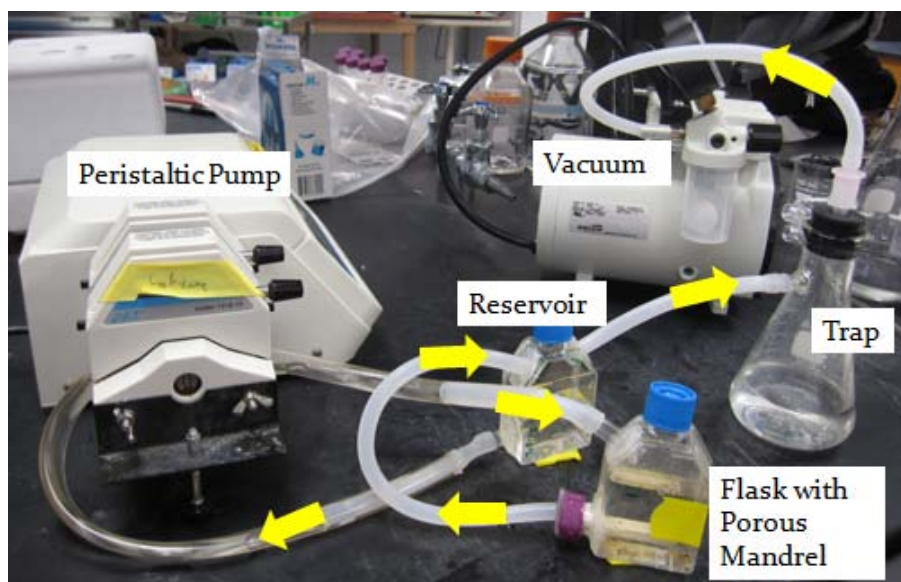
### *Experiment 11*

The setup in the previous experiment was recreated using T-25 flasks for the reservoir and flask containing the mandrel, along with a shorter mandrel. The mandrel was wrapped in membrane paper, and only water was added to the system. We were able to reproduce the original results and the flow was in equilibrium when the vacuum was on the lowest setting, and pump was on its highest. Therefore, we decided to test the system with media and see if the flow

rates would still remain constant. With the same vacuum and pump settings, the flow was even between the containers. It was also noted when the vacuum was shut off and the pump was still running, the flow rates were still held constant. The media was dripping into the reservoir from the flask due to a suction effect from the pump. Therefore, with the vacuum on the lowest setting, it is essentially not contributing to the movement of the media through the system. A stronger pump is needed to further test this design.

### *Experiment 12*

The Fisher Scientific pump was replaced with a higher flow rate peristaltic pump, the Cole Parmer Instrument Masterflex microprocessor pump drive. An additional trap was added to the system between the reservoir and the vacuum as a precaution to prevent any media from reaching the vacuum. The vacuum system was set up with the T-25 flasks and porous mandrel wrapped in western blot membrane paper (See Figure 25 below for the setup). First, the system was tested with water. The flow rate was even when the pump was on high and the vacuum was slightly increased. Therefore, the pump was not significantly stronger than the previous pump, and the flow still needs to be increased. We replaced the peristaltic pump tubing with larger diameter tubing (1 cm inner diameter) in order to increase the flow rate of the pump. The flow was able to be in equilibrium, but only when the vacuum was still on a low setting.



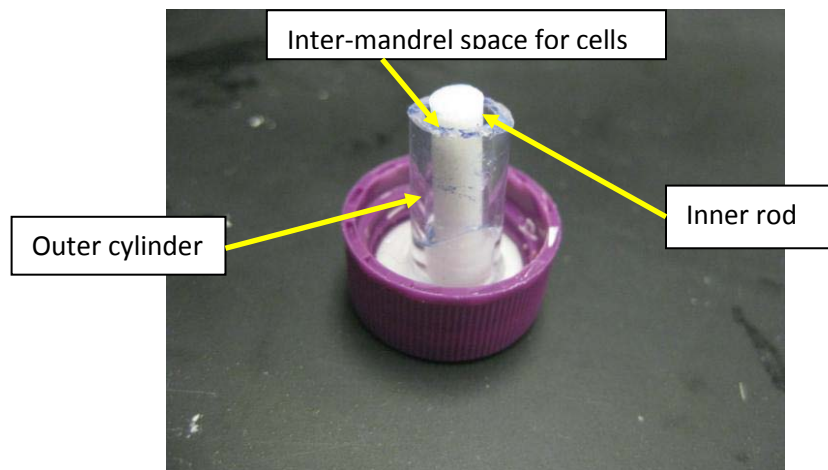
**Figure 25: Prototype setup for vacuum design with Cole Parmer peristaltic pump (Model #7524-10)**

The vacuum alternative design cannot be further tested due to the constraints of the project including time and budget. Without access to a stronger pump, the closed loop vacuum system cannot be tested with cells and media. The vacuum would need to be on a high setting to create a high enough force through the western blot paper to suction the cells to the mandrel. However, without a stronger pump the flow rate of the media in the system would not be constant. From all the prior testing, the alternative design conceptually shows promise. In the future, research should be continued on this design in order to test its ability to create tubular constructs with cells.

### Cell Mold

To create a model of a cell mold we first obtained the cap of a 15 mL conical tube and placed it flat side down. This was filled with enough PDMS to cover the bottom. A 2 cm length of clear acrylic tube (OD 5/16", ID 1/4") was embedded with one end in the PDMS. A 2 cm length of Teflon rod (OD 3/16") was placed inside the acrylic tube and centered as best as

possible by hand so it would also be embedded in PDMS. The structure was left to cure for 24 hours and is shown below in Figure 26.



**Figure 26: Cell mold prototype.**

The structure was sterilized in 70% ethanol for 5 minutes. It was then left to dry. Once dry,  $8 \times 10^6$  cells were injected into the space between the acrylic and Teflon. This was incubated for 24 hours. After 24 hours the cells were fixed in 70% ethanol and stained with Trypan blue to observe location.

Cells had formed a small ring inside the bottom of the mold and had some slight, unexpected adherence to the walls of the mold according to the blue stain. We injected  $8 \times 10^6$  cells, however, according to our calculations of the mold volume and cell volume, it would require over  $55 \times 10^6$  cells to fill the mold entirely.

We determined that the cell mold concept would require a large number of cells. Culturing this amount, especially if cells are to be from an autologous source, would take excessive time; this is contrary to one of our highest order objectives. It also has poor manufacturability, as it would require a method of media exchange between cells a chamber outside the mold, which would exceed our budget. Therefore, we have decided not to pursue the cell mold concept any further.

## Rotating Mandrel

### *Experiment 1*

A 5 cm section of a garolite rod was cut using a hack saw and clamp. This section was sterilized by submerging it in 70% ethanol. The ethanol soaked rod was then placed in a Petri dish in a sterile field to allow for the ethanol to evaporate. After 10 minutes one end of the tube was capped with a Millicell PTFE co-culture insert with 0.4  $\mu\text{m}$  pores (Model #PICM01250). A cell suspension of 1.7 million cells in 1 mL of media was prepared and pipetted into the cylinder. This process was difficult as a few drops fell from the tube in the process. The other end of the tube was then capped with the second co-culture insert. The assembly was placed on the Petri dish and the top of the tube was marked with a Sharpie marker. Before placing the Petri dish with the tube assembly in the incubator, DMEM media was added to submerge the cylinder. After one hour in the incubator, the rod was rotated 180 degrees and then allowed to incubate for a second hour. After incubation we attempted to saw the mandrel in half with a hack saw in order to compare the two halves of the tube. We intended to trypsinize each individual half, but the thick blade of the hack saw was not able to accurately cut the small tube into halves. Much of the tube was pulverized in the sawing process tainting any results that could have been attained. To avoid this problem in the next experiment each half of the tube will be submerged in trypsin for 5 minutes, while the other remains dry. This will allow us to analyze cell adhesion on each side without having to saw the tube in half.

### *Experiment 2*

The second experiment with the rotating mandrel followed the same protocol as the first, with the exceptions being that a PETG mandrel was used in place of garolite because it is an adhesive material that is clear unlike garolite, and the incubation time before the rotation was 45 minutes instead of 1 hour.



We observed what appeared to be a layer of cells on part of the mandrel. If they were cells, then that would imply that the cells had fully adhered to the mandrel before the mandrel was rotated. If this is true (to be determined by future tests), then we will consider the possibility of injecting more cells after the first mandrel rotation.

### *Experiment 3*

The third experiment with the rotating mandrel design involved testing a total of 4 mandrels, two each of garolite and PETG. One of each material was tested with cells placed within the hollow portion of the mandrel, while the remaining two mandrels were placed in a bath of cells to determine adhesion to the outer surface. The experimental procedure for this experiment follows the same protocol as the first two, with the following changes: 600,000 cells were injected into each mandrel, and the mandrels were rotated 180 degrees once after 45 minutes and then allowed to culture for one day before cell fixing and counting. Additionally, the mandrels were placed in media inside of our PDMS molds, shown previously in Figure 13.

After staining the cells with Trypan blue, it was obvious that they had only adhered to the first side of the mandrel that they were exposed to. This led us to the conclusion that the cells must take less than 45 minutes to adhere to both PETG and garolite. The counting of unadhered cells showed that just over 670,000 cells had not adhered to the inner surface of the garolite mandrel, while approximately 1.38 million cells had not adhered to the inner surface of the PETG mandrel. We decided that the only way that more cells could have been counted than had initially been inserted was that they must have proliferated. Therefore, we cannot determine the percentage of cells that had attached to the mandrels. As a visual aid, a green line was drawn on the surface of the PETG mandrel, shown below in the left frame of Figure 27. After the mandrel was rotated 180 degrees, it is shown that the green line is obscured by a layer of cells that had adhered to the inner surface of the mandrel (see the right frame of Figure 27).





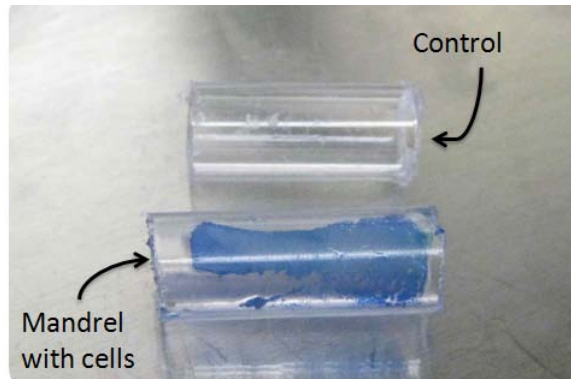
**Figure 27: PETG mandrel with indicating line for determining cellular adhesion (left image). The image on the right is the same mandrel, rotated 180 degrees.**

Control tests were run with PETG mandrels in a media bath devoid of cells. We used this control to show that proteins in the media were not responsible for the clouding of the mandrel. The control mandrel can be seen below in Figure 28. As shown on the right, after the tube has been rotated 180 degrees, the green line is still clearly visible, unlike in the PETG tube that was cultured with cells.



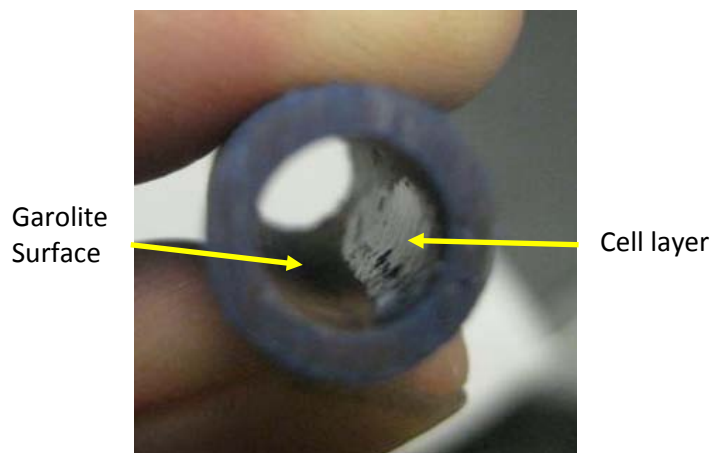
**Figure 28: PETG control (unseeded) mandrel with indicating line for determining cellular adhesion (left image). The image on the right is the same mandrel, rotated 180 degrees.**

After the cells had been fixed in 70% ethanol, the mandrels were stained with Trypan blue, along with the unseeded controls. Figure 29, shown below, depicts the results of the staining. The mandrel that had been placed in a media bath with cells shows a distinct, confluent patch of cells. This patch of cells was larger prior to being photographed; however, it was damaged during a removal attempt.



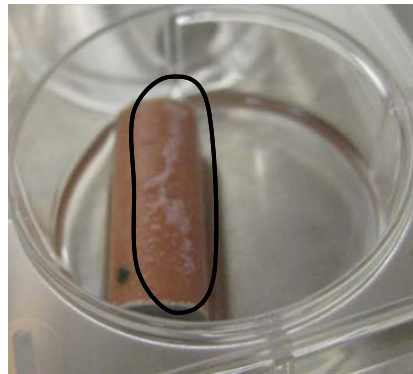
**Figure 29: PETG mandrels after staining with Trypan blue showing that cells did adhere to the tube in a fairly even distribution.**

The garolite mandrel, after being stained with Trypan blue, is shown below, in Figure 30. Similar to the PETG mandrel, a confluent layer was seen on the inner surface of the garolite mandrel. An unforeseen problem in determining cellular adhesion occurred when the Trypan blue stain was seen on the outer surface of the mandrel and on the ends. We attribute this to the rough edges created through sawing the mandrel, and to the fact that garolite is not completely smooth. This obviously affects the ability of the Trypan blue stain to give us an accurate qualitative examination of cellular adhesion, and will need to be addressed in future tests.



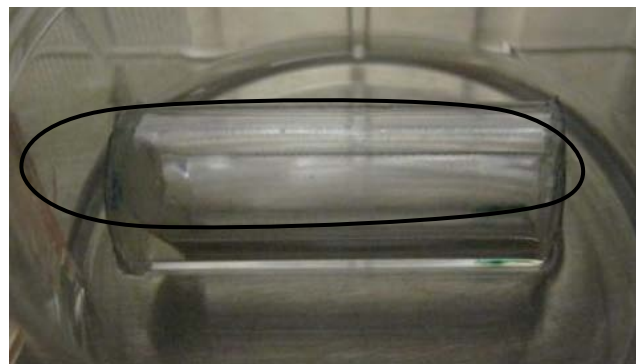
**Figure 30: Garolite mandrel after staining with Trypan blue showing the inside where cells were attached and where they were not.**

The result of the garolite mandrel that was placed in a media/cell well to determine if the cells will adhere to the outside of a rotating mandrel can be seen in Figure 31 . A thin line of cells is visible on the outer surface of the mandrel, indicating that the cells settled to the bottom of the well prior to adhesion. Because this method resulted in a lower percentage of the mandrel being covered with cells, the first method, which involves injecting cells and media inside the mandrel and capping off the ends, appears more promising.



**Figure 31: Garolite mandrel with visible cellular adhesion on the outside after culture with cells.**

Similarly, the PETG mandrel that was placed in a media/cell well showed only a thin line of adhering cells, as seen below in Figure 32.

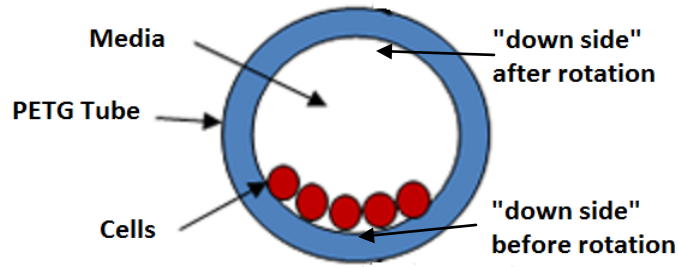


**Figure 32: PETG mandrel with visible cellular adhesion on the outside after culture with cells.**

Based on these results from conceptual testing of the rotating mandrel design, coating of the inner side of the tube proved to be more effective in producing a homogeneous layer of cells. Cells that were adhered to the outside of the tube tended to form clumps as shown in the above figures. Another important observation was that only one side of the tube was coated with cells. The side that was coated was the bottom portion of the tube which was seeded with cells initially. This means that once the tube was rotated, the majority, if not all, of the cells had already attached to the first side. Subsequent experiments will be conducted altering the rotation time or adding more cells after rotation in an attempt to form a uniform tube.

#### *Experiment 4*

In previous tests using this design, results showed cell coverage that was not uniform and only appeared in patches on the area of the tube that the cells were first seeded on. Due to this, we determined that we will need to rotate the tube and then add more cells for each rotation in order to allow the cells to attach on the other areas of the tube. To test this concept, we decided to attempt to achieve cell attachment on two opposite sides of the tube. To do this, we initially added 500,000 cells to one PETG tube (3/8" outer diameter, 1/4" inner diameter) cut to a 2 cm length and capped with co-culture inserts as before, and allowed them to attach to the "down side" for 30 minutes, while in the incubator. After this, we rotated the tube approximately 180 degrees and injected 500,000 more cells so they could attach to the new "down side" while incubating for 30 minutes. A schematic of this procedure is show in Figure 33 below.



**Figure 33: Schematic of rotating mandrel concept, where cells will settle on the inside of the tube and attach, and then the tube will be rotated and more cells will be added.**

After the second 30 minutes, the tube was fixed in 70% ethanol, stained with Trypan blue to observe cell location, and washed with PBS to remove excess Trypan blue. Results showed that about 60% of the tube was covered with cells. As expected, the majority of this coverage was seen on the two areas of the tube that were the “down side.” However, we saw very extensive gaps on areas of the tube that were vertical during both rotations of the test.

### *Experiment 5*

Based on the results from the previous experiment with this design, we hypothesized that the solution to getting more complete cell coverage of the tube would be to use more cells and more rotations. Using the same 2 cm length PETG tubes, we added 1 million cells per rotation and rotated three times, such that every 90 degrees would be the “down side” at the time of a 30 minute incubation for cell attachment. We did this with two tubes.

One tube was fixed in 70% ethanol, stained with Trypan blue, and washed with PBS immediately after all four sides had been incubated. This tube had incomplete coverage with the majority of the cells covering 2/3 of the tube (Figure 34).



**Figure 34: Results from rotating mandrel Experiment #5, showing Trypan blue staining of tube (left) and control (right).**

We believe that this may have resulted from excessive manipulation and disturbance of the tube caused by rotating and moving the apparatus back and forth from the incubator. The second tube was left horizontally to be incubated for approximately one day to observe if continued cell growth on the tube could result in better coverage. However, after 70% ethanol fixing, Trypan blue staining, and a PBS wash, there was still not full coverage and one side had barely any blue staining.



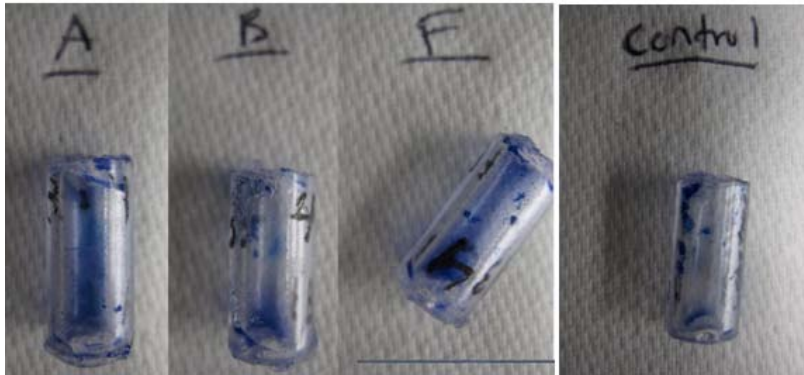
**Figure 35: Results from rotating mandrel experiment #5. Cultured tube stained with Trypan blue after 1 day, showing patchy cell coverage.**

We believe that gravity may have forced cells to move or fall from the top side of the tube because this was the area that had barely any coverage. We anticipate that rotations during culture may help to solve this problem.

### *Experiment 6*

After observing incomplete coverage and possible cell movement due to gravity in previous tests, we determined that we will need more cells and more rotations. This way all sides of the tube would be exposed to the same amount of gravitational force during incubation. To test this we used the same PETG tubes cut to 2 cm length and blocked the ends with silicone glue caps for easier cell injections. We added 1.5 million cells into 8 tubes, then incubated them for 30 minutes in empty 6 well plates, then rotated 90 degrees and repeated the process until all four “sides” had cells injected for a total of 6 million cells. We added dabs of silicone glue to the bottoms of the 6 well plates to act as barriers to prevent the tubes from rolling when moved to and from the incubator. After this we kept two tubes as controls and did not rotate them, but let them incubate for the same amount of time as the test tubes. For the 6 test tubes, we rotated each one 90 degrees every hour until we had reached 15 hours. After 4 hours we removed the silicone caps and filled the 6 well plates with DMEM, supplemented with 2.5% FBS. Reducing the serum concentration has shown, through experience, to decrease cell proliferation and to promote ECM production. By removing the caps, the cells will be provided with more nutrients than the 1 mL of media inside the PETG tube.

After 15 hours we took 3 test tubes and 1 control and fixed them in 70% ethanol, stained with Trypan blue, and washed with PBS. The results showed that there were little to no cells attached to the walls of the tube for the control or the test tubes (see Figure 36).



**Figure 36: Results from 15 hour culture for Experiment #6, showing Trypan blue staining of tubes A, B, F and control.**

We then trypsinized the bottoms of the 6 well plates they were in and counted the cells. There were cells present, but not the entire 6 million. We determined that the cells must have been agitated and detached when the media was added and silicone removed. Another possibility is that when we removed media from the tube to add an additional 1.5 million cells each rotation, we were actually removing cells as well and they had not attached as securely as we had anticipated.

We allowed the remaining 3 test tubes and 1 control to incubate untouched for 5 days. After 5 days the results were the same as for the 15 hour tubes. There was no visible cell coverage on any of the remaining tubes. The cells must have come out of the tubes into the media after the initial rotations.

It has been difficult to repeat our original results for this design. In all of the experiments we have obtained cell adhesion only in patches or not at all. It is difficult to determine what might be causing this difference. We hypothesize that the act of removing the silicone to allow media exchange resulted in the cells leaving the tubes, because they were not tightly attached to the mandrel. Throughout all of our testing we have been unable to obtain complete, uniform cell



coverage of the tube. For this reason, we believe that pursuing this design further is not in the best interests of our objectives.

## **Centrifugal Force**

### *Experiment 1*

In order to test the preliminary centrifugal force conceptual design, a hollow, glass tube with a 5 mm inner diameter was connected to a gear hobby motor (1.5-3VDC Metal Gear Motor, RadioShack Model# 273-258) to spin the tube. The tube was secured in a hole through the middle of a rubber stopper, while the other side of the stopper was glued around a gear on the motor. The motor was connected to a 9 volt battery and a bread board with a potentiometer to be able to vary the speed of the motor to determine the speed which best forces the cells against the inner wall. The glass tube was filled with water and pepper was added as a cell substitute. Visual observation was used to determine if the pepper was pushed outward. We also observed how evenly distributed the pepper is against the inner wall of the tube. The pepper appeared to be pushed outward, though the distribution was difficult to determine.

### *Experiment 2*

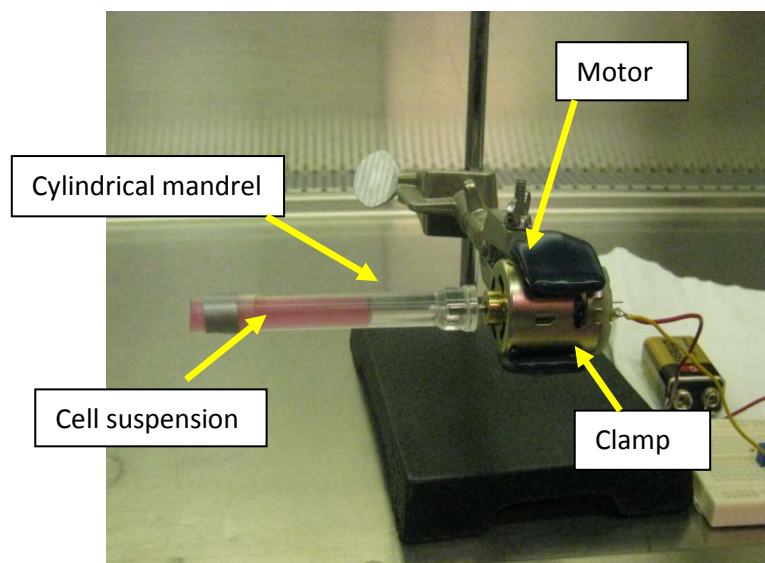
The experiment was repeated using a 2 cm long piece of acrylic tubing (1/4" I.D. and 3/8" O.D.) attached to the motor through the use of the rubber stopper. The acrylic tubing had been previously determined to have relatively low cell adhesion properties, and for this design, it is necessary that the cells do not adhere to the tube so a formed tissue tube can be removed. A plastic cap was placed over the end of the tube once it was filled with media and  $6 \times 10^6$  rat aortic smooth muscle cells. The tube was spun using the same motor and bread board setup as before. It was determined that the rubber stopper was not a sufficient connection between the motor and the tube, because the tube was not spinning evenly.

### *Experiment 3*

Due to the uneven spinning of the tube, it was determined that a better connection was needed between the tube and motor besides the rubber stopper. A 6 cm long piece of acrylic tubing was filled approximately a quarter of the way with PDMS. A hole was then drilled into the center of the PDMS in order to insert the shaft of a motor. For this testing, we used a different hobby motor (super speed 9V DC, RadioShack Model #273-256) since it did not have the gear. It was believed that a straight shaft would be more easily secured into the PDMS, and it was glued in place. The motor was again connected to a 9 volt battery and bread board with a potentiometer to slow the speed. The setup was tested with water and pepper. The top of the tube was secured with a plastic cap. The new connection was successful and the tube was spinning very evenly. It also appeared that there was a fairly even distribution of the pepper during spinning.

### *Experiment 4*

Since the initial testing with pepper in Experiment 3 had promising results, we repeated the experiment with cells in media. The same materials and setup was used. The tube was sterilized first, and then 16.5 million cells and media were added. The end of the tube was capped with a piece of parafilm and duct tape. The motor was secured in a stand clamp in order to hold the tube horizontal. The setup is shown in Figure 37 below.

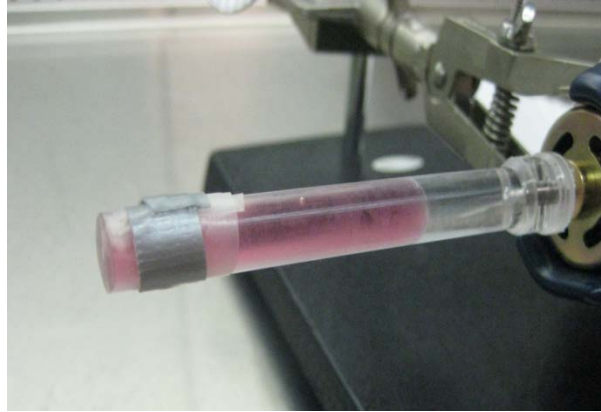


**Figure 37: Setup for centrifugal force conceptual testing, Experiments 4-7.**

The tube was spun for 5 minutes. However, the tube was not spinning uniformly. A plastic holder was placed around the tube in an effort to reduce the wobbling and straighten out the spinning. After 5 minutes, it was observed that there were still cells in suspension, although a small pellet had formed. A viability assay was performed, and it was calculated that 7.1% of the cells had died during the experiment. It could not be determined if this was from the spinning since in order to break up the pellet, the media was titrated vigorously, which may have caused damage to the cells.

### *Experiment 5*

In Experiment 4, the tube was not spinning evenly after being clamped in place. We found that the pressure from the clamp on the motor caused the erratic spinning. Therefore, we repeated Experiment 4 using the same setup and materials, but did not secure the motor as tightly in the clamp. For this experiment, 3.6 million cells were added to the sterilized acrylic tube. The tube was spun for 10 minutes, and during the entire experiment the tube was spinning evenly. After 10 minutes, the spinning was stopped, and there were cells along the inner surface of the tube as visible by the diminished transparency (see Figure 38).



**Figure 38: Less transparent tube after spinning for 10 minutes, due to the opaque presence of cells on the inner walls of the tube.**

However, after a few seconds, a significant portion of the cells fell off the sides of the tube. The media was aspirated, and the cells were fixed with 70% ethanol, and then stained with Trypan blue. The results can be seen in Figure 39 below. The results appear to be promising, as there were cells adhered to the sides of the plastic tube, although they were easily removed due to the lack of cell adhesion properties of the acrylic tubing. In order to test if the cells will be able to form a tube, we decided to test the idea again using more cells, and a shorter piece of tubing. This will ensure a higher cell density and may give better results for cell distribution and adhesion.



**Figure 39: Trypan blue stain of the tube after spinning and fixing cells**

### *Experiment 6*

The testing procedure in Experiment 5 was repeated. However, the length of acrylic tubing was first cut in half to decrease the surface area that the cells will be forced against. Approximately 2 cm of tube length was left above the PDMS level in the tube. To further increase the cell density, 5.7 million cells were added. We also increased the spinning time to 20 minutes to allow further time for the cells to form a tube around the inner surface. Unexpectedly, the tube was not spinning evenly even though the clamp was not secured tightly. It is believed that sawing off half of the tube may have disrupted the connection between the PDMS and the motor. After 20 minutes, the cells were fixed, and stained with Trypan blue. There was very little blue visible since almost no cells had adhered to the sides of the tube due to the uneven spinning.

### *Experiment 7*

Experiment 6 was repeated with paper towels placed around the motor before it was clamped in place. We hoped that the paper towels would act as a cushion to reduce the force that the clamp was placing on the motor, and allow it to spin more evenly. The motor was also duct taped in place because the vibrations caused it to slide around in the clamp. After 10 minutes the spinning was stopped because the tube was still not spinning evenly. Thus, it was determined that the connection between the PDMS and motor must have been the reason for the uneven spinning in experiments 6 and 7, since it was not due to the clamp pressure on the motor. Therefore, we planned to repeat this testing once we have rebuilt the connection between the motor and the tube.

### *Experiment 8: Chuck Interface*

The purpose of this test was to evaluate the concept of using a chuck to spin an acrylic tube filled with cells. To accomplish this, an electric power drill was used, shown in Figure 40 with a chuck large enough to accommodate a 3/8" O.D. tube.

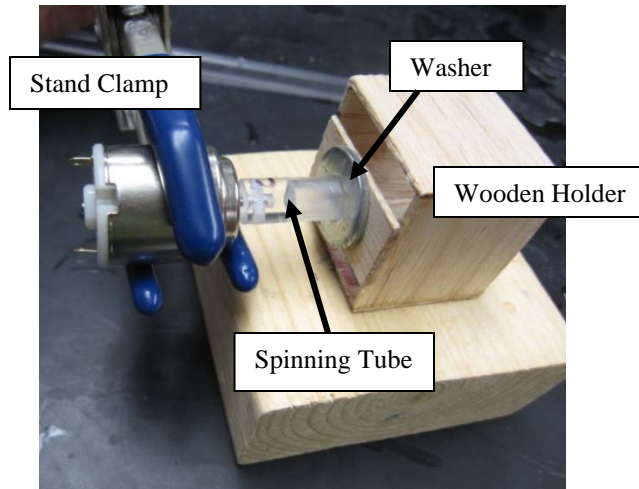


**Figure 40: Tube in chuck on drill.**

A 2 cm acrylic tube with a silicone cap was filled with 2.6 million cells by using a syringe and needle. The drill was then held in the “on” position for 2 minutes. After the two minute test period the silicone cap was removed and the tube submerged in 70% ethanol, washed, and then stained with Trypan blue. The staining did not show significant cell coverage. This may be credited to a crack that was observed during pre-test tightening of the chuck. Another source of error was that the drill was handheld and not clamped in place. This could have resulted in erroneous movements that do not support tube formation.

### *Experiment 9: Wooden Holder*

This test was done to examine the potential of using a stabilizing device around the tube to prevent vibration. A washer with the same inner diameter as the outer diameter of the tube was embedded in a piece of wood (see Figure 41).



**Figure 41: Stabilization device designed to reduce vibration in the spinning tube.**

Using the PDMS motor interface previously described, an acrylic tube was attached to an electric motor. The tube was then placed inside of the washer and spun for 6 minutes with 2.6 million cells. Staining revealed a faint layer of cells surrounding the tube. The results from this test are inconclusive as it did not fail to make a tube, but the layer of cells was faint, not nearly as strong as observed previously.

#### *Experiment 10: Wooden Holder 2*

This test was done to expand upon the results of the previous centrifugal force testing in which a tube stabilizer was used, to have a higher sample number as well as test different spinning durations. Due to poor cell attachment in the previous experiment, we decided to test another material that may improve these results. Four tests were performed, 2 with butyrate tubing and 2 with acrylic tubing. Based on the results of our adhesion assay, butyrate was also relatively non-adhesive, like acrylic. Tubes were 3 cm in length, and filled with 1.5 cm of PDMS, leaving the tube length exposed to cells at 1.5 cm. Holes were drilled into the PDMS in order to attach the shaft of the electric motor. The open end of the tube was sealed with silicone, which was allowed to cure overnight. A total of 1.5 million cells were injected through the

silicone into the tube in a total media suspension of 0.5 mL. The time points tested were 5 minutes and 10 minutes, one of each material for each time point.

The butyrate tube spun at five minutes experienced changes in accelerations, potentially caused by a poor connection with the battery. The test was forced to end after three minutes of spinning as the battery disconnected. Trypan blue staining was similar to the control and showed little to no cell adhesion. The acrylic tube spun for five minutes gave the same results. The ten minute tests did not yield better results. It was noted during the tests that rattling could be heard from the holder. This most likely means that the tubes were vibrating against the washer which would prevent cell adhesion. Initially, testing was also planned for a time period of 30 minutes, but because of this rattling we felt it was not worth going forward. Based on the results of this experiment the use of a holder to stabilize the spinning mandrel was abandoned.

#### *Experiment 11: Chuck Interface 2*

This experiment was done to further explore the possibility of using a chuck to spin the tubes. As the previous experiment with a drill, Experiment #8, was done while holding the drill we decided to clamp it down for stability. Acrylic tubes filled with PDMS and capped with silicone as previously described were used for this experiment. Three tests were run, two at three minutes and one at five minutes. A cell suspension of 1.5 million cells in 0.5 mL of media was injected into each tube and subsequently tubes were hand tightened into the chuck of a Craftsman Professional 4 amp 3/8" drill. The drill was then stabilized with a clamp in the biosafety cabinet and turned on for the desired amount of time. All three of the tests yielded little to no cell adhesion. A viability assay revealed that the cells were still alive and healthy, but were not attached. The high spinning rate of the drill compared with the motor may be responsible for the poor results. As a result of this test the idea of using a drill was abandoned.



### *Experiment 12: Solid Rod Design*

Previous centrifugal force testing showed that vibration from a poor motor/tube interface was a hindrance to tubular self assembly. The interface was unstable due to material properties of the PDMS causing the motor shaft to be able to wobble when inserted. To fix this problem, we came up with a new connection (See Figure 42). Instead of using a hollow cylinder we decided to start our design with a solid rod. A 2 cm rod to insert and spin the cells in was lathed to be the desired diameter of  $\frac{1}{4}$  inch and  $\frac{3}{4}$  inch deep. A much smaller hole on the other end of the rod was drilled to be slightly smaller than the diameter of the motor shaft.



**Figure 42. The previous mandrel fabrication involved PDMS casting into a tube, while the new method involved drilling a hole into a rod creating a much more stable connection to the motor**

The rod was then forced onto the shaft of the motor ensuring a tight fit. Preliminary spinning of the rod revealed this new interface to spin much better than the previous set up. A cell test with 6 million cells in 0.633 mL showed a thin layer of cells surrounding the tube. Trypan blue staining confirmed this as a notable difference existed between the control and the spun tube. This initial testing showed that the new design has promise.

### *Experiment 13: Solid Rod 2*

This test was done to expand upon the preliminary results of Experiment #12 using the new interface design. Acrylic tubes were manufactured in the same fashion as previously described by lathing a hole in 2 cm sections of rods. Two tests were run, both with 7.5 million cells in 0.633 mL of media. The tubes were spun for 15 minutes and then stained with Trypan blue to observe cell tube formation. Both tests yielded a uniform cell layer around the inside of the tube, as seen in Figure 43, verifying the fitted acrylic motor shaft/rod connection enabled uniform cell spinning.



**Figure 43: Two tests of cells spun for 15 minutes (right), fixed, and stained with Trypan blue reveal remarkable cell coverage next to a control (left).**

### *Membrane Paper Adhesion Assay*

This test coincides with our centrifugal force design alternative. We believe that a promising method for removing our final cell tube from the acrylic tube it was spun in is to use membrane paper. We hope to fold a small sheet of membrane paper so it creates a layer flush against the inner wall of the acrylic tube to act as a removable barrier. This means that when the cells are spun they will be pelleted against the membrane paper instead of the acrylic. After culturing we will then be able to pull the membrane paper from the acrylic tube easily and the

cell tube will pull out with it. We can then detach the cell tube from the membrane paper because the cell-cell interactions will be stronger than the cell-membrane paper interactions. However, for this idea to work we will need to use a membrane paper that is not cellularly adhesive.

We cut a circular piece of membrane paper that would fit into the bottom of a 35 x 10 mm circular Petri dish. We added 1 million cells in 3 mL of media on top of the membrane paper and followed the same methods in a Petri dish with no membrane paper as a control. The cells incubated for 1 hour and 20 minutes. The membrane paper was then removed from the dish it was incubated in and put into a new dish, where it was soaked in trypsin for 5 minutes and also extensively washed with trypsin by spraying it directly onto the membrane paper with a 1000  $\mu$ L pipette. The cells in the trypsin washed from the membrane paper were then counted. The control was also trypsinized and counted. The control had approximately all the cells it started with according to the cell count with a hemocytometer and the membrane paper had only 1% of the cells it started with. Due to these results we determined that the membrane paper is non-adhesive for cells.

### **Final Design Decision**

Based on the construction and testing of our prototypes, the centrifugal force design has showed the most potential to meet our original design objectives. It was the only design to repeatedly create a cohesive cellular tube, our top ranked objective. Although the rotating mandrel was able to create half cylinders, it was unable to make complete tubes. The cellular mold failed to assemble the cells into a three dimensional construct, instead making a ring of cells. The other designs never advanced far enough to be tested with cells. In addition to tubular construction, the centrifugal force design met other high level objectives. Time required to make the tube was relatively short (minutes) compared to hours with rotating mandrel or cell mold.

In addition to meeting most of our high level objectives, the centrifugal force design does not conflict with our constraints. The parts required are relatively inexpensive, assembly is not time intensive, and the process is relatively safe for both the user and the cells.

Based on these results we have chosen to pursue the use of centrifugal force to self assemble cells into tubes. This concept will be the basis of our final design. The biggest obstacle that must be overcome is harvesting the tubes from the mandrel, as this is the one high level objective that initial design alternative testing was not able to accomplish.

## **Chapter 5 - Design Verification**

In this chapter we discuss the experiments that were conducted to mature our centrifugal force design concept into a final design that meets as many of our objectives as possible. Some of the major obstacles we overcame were keeping the cells against the wall of the mandrel during culture, developing adequate systems to deliver new media to the cells, and removing the cultured tissue from the mandrel after incubation.

### **Experiment # 1: One Day Culture**

The purpose of this test was to culture assembled tubes in order to allow ECM formation in an effort to remove the tube from the lathed rod. Three acrylic rods were set up as previously described and 7.5 million cells were injected. Rods were spun for 15 minutes. Trypan blue staining was performed on one of the rods to confirm uniform cellular distribution. Based on the positive results of this staining, the other two mandrels were placed in the incubator and cultured for 24 hours. After culture, the silicone caps were removed from the tubes and forceps were used to try to remove the cellular tube. The layer of cells proved to be too thin to grab and broke apart when pulled. In doing this experiment we noted that after spinning, the rod was securely stuck to the motor shaft. A large amount of force was needed to pull it off, which could have had an effect on cell distribution, and may have knocked some cells off the walls of the tube. As a result, we decided to investigate and develop a new interface between the motor and the rod.

### **Experiment #2: 1 Day versus 5 Day Culture**

For this test, we wanted to keep the same motor set up as we had used previously. We used a stand clamp to hold the 3 volt hobby motor above the surface of a tissue culture hood. We then used alligator clips to connect the motor to a 9 volt battery. Our acrylic rods (3/8" outer

diameter) were machined to have a ¼” inner diameter using a lathe. The lathe was also used to drill a smaller hole in the opposite side of the rod for the attachment to the motor. A different motor attachment was developed for this test, because in previous tests with fitted tubes we found it difficult to remove from the motor shaft with little disturbance of the cells. We created 6 tubes that had a slightly larger hole than the size of the shaft. A second set of holes were made perpendicular to this hole and were tapped to allow screws to be inserted. This would allow us to tighten the screws to ensure the tube is secure to the motor and then also easily remove the tube without disturbing the pellet of cells by unscrewing the taps. We kept all other variables the same, so we could determine which motor interface resulted in superior cell coverage and ease of use. Each tube received between 7 and 10 million cells injected through a silicone glue cap and were spun for 15 minutes. Observations during and after spinning for each individual tube can be seen in Table 9.

**Table 9: Table showing a summary of the testing and results from comparing the fitted versus screw design for the motor shaft/tube interface.**

SAMPLE A	SAMPLE B	SAMPLE C	SAMPLE D	SAMPLE E	SAMPLE F
10 million cells	10 million cells	8 million cells	8 million cells	7 million cells	7 million cells
Cultured 1 day	Cultured 5 days	Cultured 1 day	Cultured 5 days	Cultured 5 days	Cultured 1 day
Partial coverage	Partial coverage	Complete coverage	Complete coverage	Complete coverage	Complete coverage
Cell suspension sat in tube for 5 minutes before spinning	Cell suspension sat in tube for 5 minutes before spinning	Screws not tight. Stopped spinning at 10 minutes.			
	Motor interface hole not centered				

We believe that the partial coverage observed in tests A and B were caused by the fact that the cell suspension sat horizontally for 5 minutes allowing the cells to sink to the bottom of

the tube. Due to the nature of centrifugal force, these cells would then be spun in the position in which they started rather than evenly distributed in the tube. Test B also had a greater amount of vibration and wobbled spinning because the hole interfaced with the motor was not centered on the tube. Test C stopped at 10 minutes because the screws were not tightened enough and eventually the motor shaft continued to spin, but the lack of connection between the motor and tube resulted in the tube not spinning. Tests D, E, and F were completed without any adverse observations.

After letting tubes A, C, and F incubate horizontally in a Petri dish for approximately one day, they were fixed for approximately 2 hours in a 1:1 solution of formalin and 30% sucrose in distilled water injected directly into the acrylic tube after silicone cap and media removal. Cell coverage could be observed by the naked eye. Test A had a layer of cells similar to that observed initially and little to no coverage elsewhere. It had also seemed to have clumps of cells that had contracted (Figure 44).



**Figure 44: Trypan blue staining of Tube A for Experiment #2**

Test C appeared to have a thin layer of cells but with 1-2 mm large patches of no cells (Figure 45).



**Figure 45: Trypan blue staining of Tube C for Experiment #2**

Test F had a complete layer of cells with no breaks; however, there were areas that had visibly denser cell coverage (Figure 46).



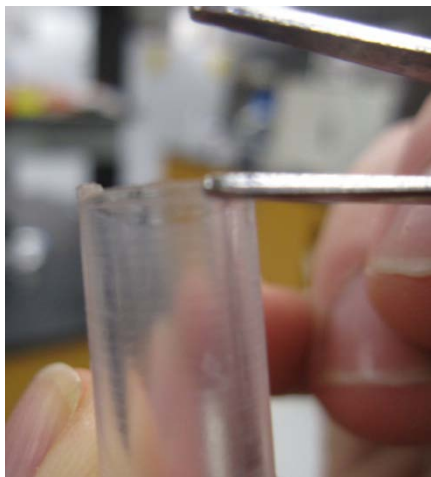
**Figure 46: Trypan blue staining of Tube F for Experiment #2**

It was also a notably thinner layer of cells than those stained immediately after spinning in previous tests. The incubation period may have had the same gravitational effects that were seen with the rotating mandrel design. None of these tubes were stable or thick enough to remove from the acrylic tube scaffolds.

Tests B, D, and E had the media changed after day 1. Media was changed by putting a syringe and an open needle through the silicone to remove media and add new media, similar to how cells are added initially. After culture for 5 days, the three tubes were fixed by the same method as A, C, and F. Tests B and D had visibly more opaque sides of the tube that were face down during culture, once again due to gravity, and were much too thin to harvest. Test E had a homogenous layer of cells, with a small pellet on the bottom end of the tube. While there was a



visible difference between where cells were and were not (where silicone cap was), this tube was also too thin to harvest (Figure 47).



**Figure 47: Results of spinning Tube E for Experiment 2, showing thin layer of cells visualized by a change in opacity below where the silicone cap was attached.**

### **Experiment #3: Screw vs Fitted Interface Design and Variable Voltage**

In order to create a secure connection between the motor and tube, two designs were created: screws and fitted. For the fitted design, a hole is drilled in the tube that is slightly smaller than the motor shaft, as in Experiment #1. The screw design consists of a hole slightly larger than the shaft, with two screws tapped into the sides of the tube to help tighten the tube on the shaft, as in Experiment #2. An additional parameter that was tested was the voltage of the power source. The motors for testing are 1.5-3 volt motors. We previously had been using a 9 volt battery for power, but after a motor stopped working and we investigated the problem, we realized the motors were rated for less voltage. Therefore, we tested out spinning tubes with 9 volt battery and 2 AA (1.5V each) batteries, to ensure that the 3 volt power source will still produce the original results and not affect the spinning of the tubes. The table below summarizes the testing (Table 10). Approximately 8 million cells were added to each tube, and the tubes were spun for 15 minutes. After spinning the tubes were cultured for 5 days.

**Table 10: Summary of testing parameters for Experiment #3 with screw vs fitted design and 3 vs 9 volts.**

<b>Tube</b>	<b>Power Source</b>	<b>Tube/Motor Connection</b>	<b>Observations</b>
G	3V	Fitted	N/A
H	9V	Screws	N/A
I	3V	Screws	Cells sat in tube for few minutes before spinning
J	9V	Fitted	Cells sat in tube for few minutes before spinning
K	3V	Screws	N/A
L	9V	Fitted	Started spinning slower at 10 min (battery died)

After 5 days of culture, the tubes were fixed in formalin and 30% sucrose solution for 2 hours. Initial observation showed that there was only a thin layer of cells in all the tubes, but the cells uniformly coated the inner surface of the tubes. We did not attempt to harvest the tubes as the cell coating appeared as thin as in the previous experiment in which harvesting was not possible. We determined that we needed to spin the tubes with more cells in order to try to increase the thickness of the cell layer in the tubes. We also learned that decreasing the percentage of FBS to 2.5% in the media can increase the crosslinking and the extracellular matrix formation by the cells. This should make a more cohesive cell and ECM layer that would be easier to harvest. We also determined that the screw tubes were slightly complicated and difficult to deal with and the fitted tubes were still difficult to remove from the motor shaft.

## Experiment #4: Increased Cell Number

In order to try to increase the thickness of the cell layer in the tubes, we tested spinning the acrylic tubes with more cells. Based on the conclusions from the previous test, these tubes had a loose fit to the motor (slightly smaller hole than the motor shaft). The motors were hooked up to a 3 volt battery source and held in a stand clamp. The loose fit tubes spun very well in the stand clamp without the use of any putty or tape to prevent vibrations of the motor. Five tubes were spun in total for 15 minutes each. Tubes A and B had approximately 7 million cells which replicates the setup used in previous tests, Tubes C and D had 28 million cells, and Tube E had 56 million cells. After spinning, tubes C, D, and E appeared to have much thicker cell coverage on the walls than previously observed. To help the cells in crosslinking and producing more collagen, the tubes were cultured in 2.5% FBS rather than the normal 10% that was used in all previous centrifugal force tests. The culturing system remained consistent with previous testing where the mandrel (still closed with silicone glue) is removed from the motor and placed horizontally in a culture dish in the incubator.

Upon changing the media after day 1, tubes C, D, and E experienced some disturbance from the method of media change. By the drop by drop addition of media through a syringe, the force of the drops disturbed some of the cells that had not adhered. The tubes with higher cell counts were probably disturbed more due to the thicker layer of cells. Tube E was moved to a container filled with media to allow more nutrients for the large number of cells. The silicone was removed and a co-culture insert was used to cover the top for media exchange during vertical culture of the tube. After three days in culture, the cell construct in Tube E began to fall apart, possibly due to gravity from being incubated vertically. Also, the container in which the tube was being incubated held about 10 mL of media. The media turned yellow (indicating a

drop in pH) after only a few hours due to the high cell count. After 5 days of culture, the tubes were fixed with 30% sucrose and formalin solution. Tubes A and B had uniform but thin cell coverage as observed in the previous experiments and the cell layer was too thin to harvest from the tube. Tubes C and D with 28 million cells produced very similar results even though they had 4 times as many cells. The tubular cell layer was too thin to harvest. Tube E had thick cell coverage on the acrylic tube, but the coverage was inconsistent. Parts of the cell tube had fallen off the acrylic tube wall. From this testing it was concluded that a better method of incubating and changing of media was necessary in order to reduce disturbance to the cell construct. It was also determined that 56 million cells might be too many due to the deterioration of the tube and 7 million is too few.

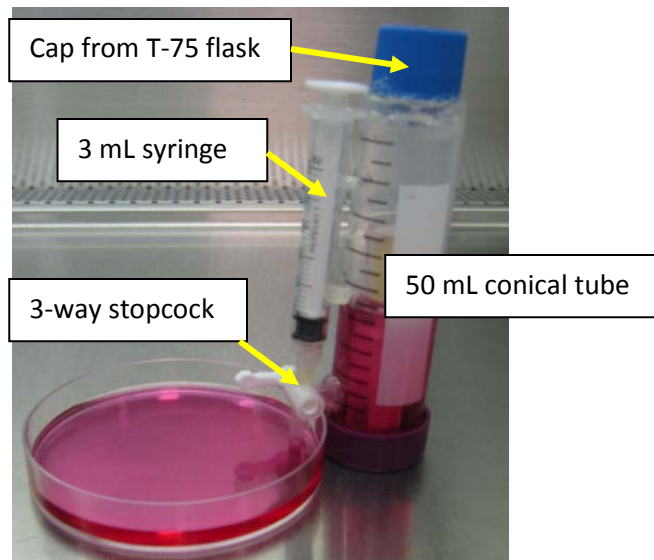
### **Experiment #5: Fix Tubes after Spinning**

In this test, we wanted to observe the thickness and uniformity of the cell layer in the spun tubes immediately after spinning to compare the results to the tubes that had been cultured in previous experiments. The same setup was used as in the previous experiment. Two tubes were spun with approximately 24 million cells. After 15 minutes of spinning, they were fixed in 30% sucrose and formalin solution. Both tubes had thicker cell coverage than observed previously when using 7 million cells. The cell coverage was also completely uniform, unlike the tubes that had been cultured in previous experiments. The increased cell number helped form thicker tubes as expected; however, we were still unable to harvest the tubes. Part of the problem was the use of forceps that were too large. Parts of tube near the top were able to be pushed off the walls of the acrylic tubing, but the tissue ripped apart from the rest of the tube when trying to remove it. Since harvesting of the cellular tubes from the acrylic tubes is a problem, we next

tested the use of membrane paper inside the tubes to help with their removal. We also noted that something must be happening in our culture process to deteriorate these uniform and thick tubes.

### Design of Vertical Incubation Chamber Culture System

One cause of tube deterioration and cell detachment from the mandrel wall could be a lack of nutrients. In order to allow better media exchange to the cells in the tubular constructs, we designed a vertical culture system (Figure 48). This bioreactor system will enable media change with less disruption to the cells. The system consists of a 50 mL conical tube with a filter cap attached to the end. A stopcock is attached to the bottom of the conical tube to allow for drainage of the media. A syringe is attached to the stopcock in order to allow a slow, controlled way to add media to the system without disturbing the cells. Media is inserted into the syringe, and the plunger is slowly pushed downwards to push the media into the conical tube. The mandrel is fitted snugly into a cryovial that is glued to the top of the conical tube.



**Figure 48: Vertical incubation chamber to allow easy media exchange for tubular construct.**

## Experiment #6: Membrane Paper and Vertical Culture System

In this next experiment, the same setup as described previously was used, but pieces of rolled up membrane paper were inserted into the acrylic tubes. Four tubes were spun for 15 minutes with approximately 25 million cells per tube. Two of the tubes did not have membrane paper to act as a control. Tube A had membrane paper and after spinning had an apparent cell layer. Tube B also had membrane paper but was not spinning uniformly during the test which may disrupt the results. All four tubes were incubated in the vertical culture bioreactors for 3 days after the silicone caps were removed and replaced with co-culture inserts. However, after 1 day a problem arose in which the conical tube cap was not screwed on completely to the conical tube for control Tube D. Most of the media had drained out, so the media had to be replaced and the cap was tightened.

After culture in vertical media chambers, media was drained through the valve into a Petri dish and all tubes were removed from holders. Tubes A and B did not form strong cellular tubes. When the silicone was cut from the tubes with a scalpel immediately after spinning, it was glued on to the top of the membrane paper, which caused some disruption and may have compromised the tubular assembly of cells. After three days of culture, when we pulled the membrane paper from the acrylic tubes we could see the clumps of cells falling out and back into the media in the tubes as seen in Figure 49.



**Figure 49: Cells disturbed and detached upon removal of membrane paper after three days of culture.**

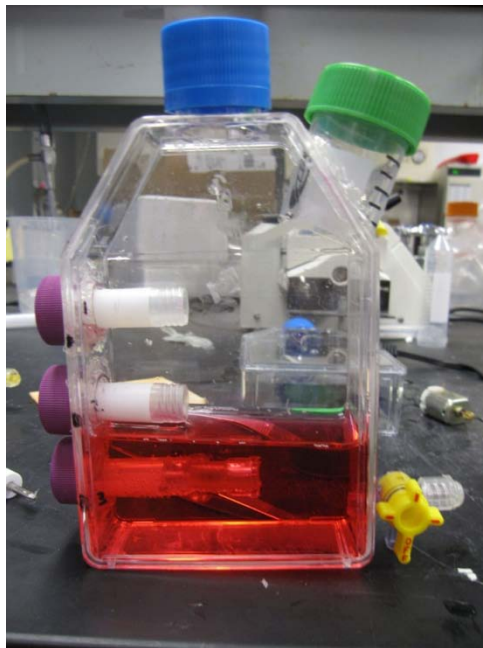
We also noted that the media inside the acrylic tubes was yellow, while the media inside the vertical media chamber was pink, suggesting that media and nutrients were not diffusing through the co-culture insert between the tubes and the chambers. After culture, Tube C looked slightly more opaque than a tube with no cells; however the media in this tube was also yellow, while the chamber was pink. There was also a vertical crack the entire length of the tube resulting from an unknown cause. For Tube D, we could obtain no results. After one day in culture the media had begun to drain from the vertical media chamber because the cap was not screwed on tight enough. We replenished the media; however at day three of culture we noted that the media in this chamber was unhealthily yellow where the others were pink. We noted some white-yellow gooey residue on the cap, as seen in Figure 50, and believe that it was bacteria that began growing in the chamber from some sort of contamination resulting from the previous leak. The tube did have a visible layer of cells, but when the tube was dipped in media the entire structure fell apart.



**Figure 50: Possible contamination seen in the culture chamber of Tube D**

## Design of Horizontal Culture System

Due to the poor media exchange observed in the vertical culture system, we decided to design a better system. We concluded that one reason for limited media exchange between the tubes and the conical tube chamber could be a pressure gradient. Tension from the media and also gravity could be preventing the diffusion of media into and out of the tubes. By culturing the tubes horizontally instead of vertically, we hoped to reduce some of these effects and increase media exchange. A T-75 flask was used with three ports on the side for inserting spun tubes, as seen in Figure 51. Another port with screw cap was attached to the top of the flask to allow the insertion of a pipette to add media to the flask. Like the vertical system, a stopcock was attached to the bottom of the flask to allow for media drainage.



**Figure 51: Horizontal Culture Chamber that allows three tubes to be incubated and for easy media change**



## **Experiment #7: Horizontal Culture**

We were unsure if it would be more ideal to culture cellular tube assemblies in a vertical or horizontal orientation because both have disadvantages. In vertical culture, gravity would pull the cells down the sides and likely into a clump in the bottom. In horizontal culture, gravity would pull the cells from the top and sides to form a simple line of cells on the bottom edge. We wanted to test both methods to see if one worked.

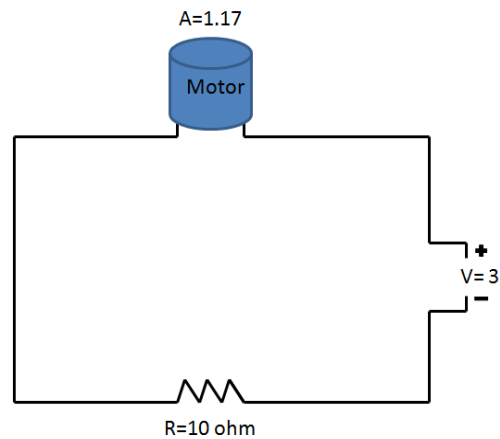
Using the same set up as previous recent testing, we spun two tubes (A and B) for 15 minutes, with membrane paper inside, and injected 28 million cells. The same problem with the silicone glue sticking to the membrane paper was seen in this test, which may have resulted in the disruption of the cells during removal of the silicone after spinning. These were then cultured in the horizontal chamber for approximately 2 days. After culture the membrane paper was removed from the tube and placed in a dish of media, still rolled in shape. The paper was then unrolled using tweezers. We could visibly see a cloud of cells float into the media upon manipulation of the membrane paper. This suggests that the cells were not coherent with the membrane paper, possibly from the silicone glue or from possible yellow media inside the tube. The same results were seen for both tubes.

## **Experiment #8: Polycarbonate and Variable Speed**

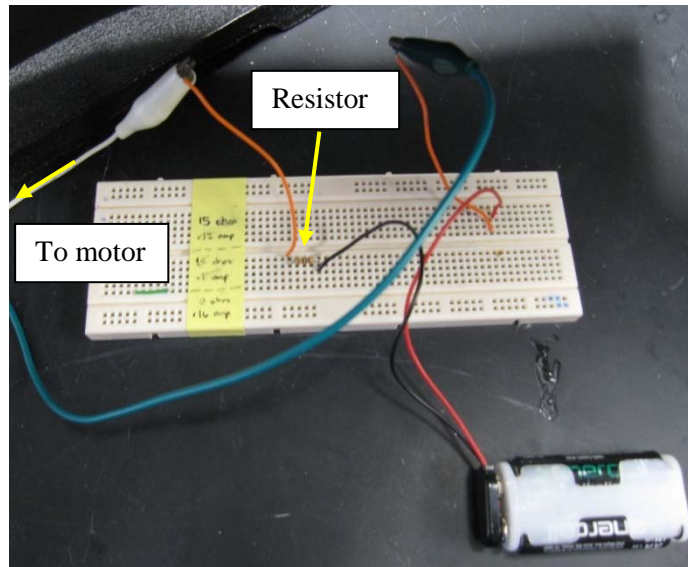
After much consideration of materials, we decided to perform some tests using polycarbonate tubes instead of acrylic tubes. Acrylic cracks easily under chemical stress induced by ethanol and will melt in an autoclave, leaving us with limited methods of sterilization. Polycarbonate can be autoclaved, is a much less expensive material, and has been used in the construction of bioreactors previously. However, we wanted to ensure that polycarbonate would

give us the same results acrylic did when spun. The polycarbonate tubes were manufactured the same way as the acrylic tubes.

At this time we also wanted to test the effect of motor speed on tube formation. To do this we created two circuits. For Test 1 (tubes A and B) we used the circuit we have used previously, with just the motor attached to a 3 volt battery. This system allows approximately 0.16 amps through the motor. For Test 2 (tube C and D) we used a breadboard and a 10 ohm resistor in series with the motor to allow 0.12 amps through it (Figure 52 and Figure 53). All tests were spun for 15 minutes with 25 million cells in each tube.



**Figure 52: Diagram of circuit for centrifugal force testing with resistor to decrease the speed of the motor.**



**Figure 53: Circuit used to decrease spinning speed of motor using a 10 ohm resistor.**

Tube A was fixed in 70% ethanol and stained with Trypan blue to determine if polycarbonate had initial spinning results similar to acrylic. We saw dense cell coverage, with a slight break near the top where a pipette tip scraped the side of the tube while adding the ethanol (Figure 54).



**Figure 54: Results of Experiment 5 showing Trypan blue staining of polycarbonate after 15 minutes spinning with 25 million cells.**

Overall, the spinning of polycarbonate was concluded to be adequate for our applications. Tubes, B, C, and D were cultured for 1 day in horizontal culture chambers. After culture, all tubes had yellow or orange media, while the media in the remainder of the chamber had pink media. This suggests that the cell culture inserts we have been using to cover the tubes in culture do not allow media exchange as we hoped they would. All cells had sunk to the bottom edges of

the tubes after one day, so the culture was terminated. We cannot be sure if this was caused by disturbance of tubes as they were put into the culture chamber, the lack of nutrients from yellow media, or polycarbonate being less adhesive for long term culture than acrylic.

### **Experiment #9: Original setup with Polycarbonate**

Since we had only introduced polycarbonate as a possible tubing material when we introduced the horizontal culture system, we decided to repeat the original promising results with the acrylic. The original setup cultured the tubes with the silicone end caps in place, and changed the media by inserting a needle and syringe through the silicone into the tube. This test will give us more information regarding the adhesiveness of polycarbonate compared to acrylic. We tested 2 polycarbonate tubes prepared the same way as the original acrylic tube. We spun the tubes for 15 minutes, and cultured them for 5 days. Tube A had 7 million cells, and tube B had 28 million cells, the two different cell numbers we had previously tested with acrylic.

After one day in culture, the cells in tube B had mostly all detached and fallen to the bottom in yellow media. At this point tube A's cells were still attached in a tubular shape, but by day 5 they had also all fallen to the bottom. We anticipate that this was due to lack of nutrients and pH change due to quick media use.

### **Experiment #10: Spinning in Culture (Polycarbonate)**

We next decided to test spinning tubes for an extended period of time, in hopes that the cells would be better adhered to the tubing material and able to stay attached to the tubing long enough to form cell-cell adhesions. A stand clamp was placed in the incubator so we could spin the cells for a period of time that was significantly longer than in the previous tests (5-15min), while providing them with an environment conducive to cell survival and growth. Polycarbonate tubing was used with an added 25 million cells. The end of the tubing was sealed with silicone in

order to hold the cells and media in the tubing while spinning. While this may prevent gas exchange to the cells, we lack a system to spin the cells in an open environment. The testing was initially planned for 12 hours, however, after 4 hours it was noted that the media had already turned yellow due to the high cell density. Therefore, we decided to end the test early, as 4 hours was still much longer than our typical 15 minute tests. We removed the silicone cap from the tubes and placed them in the vertical bioreactor for media exchange. We did not place a co-culture insert on the end of the tube due to previous experiments showing that they do not permit sufficient media exchange. It is believed that the initial spinning of the cells will have caused them to adhere to the walls strongly enough to not float out into the media. The system was set up to culture for 3 days. We chose to use the vertical culture chamber over the horizontal because we anticipated that the effect of gravity would have a more serious effect in the horizontal culture based on results from centrifugal force and rotating mandrel designs.

After 3 days of culture, there was a thick, uniform cell layer visible inside the polycarbonate tube and the media in the mandrel was pink, suggesting that the cells were getting adequate nutrients where they were not in previous cultured experiments (Figure 55).



**Figure 55: Polycarbonate tube cultured vertically with no co-culture insert showed fresh media exchange and a thick cell layer**

Tweezers were used to grab an edge of the tube and try to remove the cell construct from the polycarbonate tubing. However, the piece that was grabbed broke off from the rest of the construct. When the tubing with the rest of the cell construct was immersed in media, the cells fell out and became a cloud in the media. It is possible that the lengthened spinning time may have resulted in the cells' inability to form intracellular bonds with one another.

### **Experiment #11: Spinning in Culture (Acrylic)**

Due to the fact that the cells detached from the polycarbonate walls during harvest in the previous experiment, we decided to repeat Experiment #10 using acrylic tubing instead. Previous results showed that the cells adhered to acrylic stronger than desired after spinning and are difficult to remove from the tubing even after a few days of culture. We wanted the cells to adhere to the tube enough so that they would not fall off during submersion in media, but they also need to have a weak enough attraction to the tube so they can be easily removed.

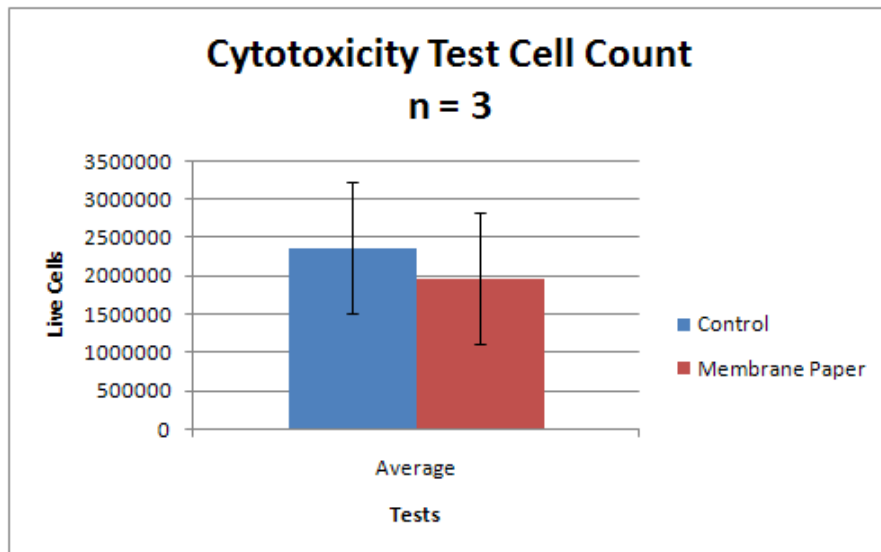
As in Experiment #10, the cells had formed a thick layer around the walls of the acrylic tubing. Using tweezers, we were able to grab on to the end of the tube. After applying pressure, the piece of tube being held broke apart from the rest of the tube. However, in contrast to the results with polycarbonate, the cells remained adhered to the acrylic tubing after immersion in media and attempted harvesting.

### **Experiment #12: Membrane Paper Cytotoxicity Assay**

Based on the results from spinning in culture (Experiments #10-11), we wanted to repeat the same setup except using membrane paper to line the tubes. Both experiments produced cellular constructs that were thick and uniform, however, they were not harvestable from the tubing. Lining the tubing with membrane paper would enable us to remove the cellular construct

from the tubing by simply pulling out the membrane paper. Before we conducted this testing, we wanted to test the cytotoxicity of the membrane paper to see how the paper affects the cells, and ensure nothing harmful will leach from the paper. We cut 1 x 1 cm sections of western blot membrane paper and placed it in a 35 mm diameter by 10 mm deep tissue culture dish. We then placed 2 million cells and 3 mL of media in three of the prepared dishes and three dishes without membrane paper as controls. We cultured all six dishes for 3 days, which is the length of time we had been using previously to culture our spun cells.

The cells were trypsinized and counted after three days of culture. Looking at the cell suspension under the microscope in a hemocytometer showed much debris in both the control samples and the membrane paper samples. Cell counts can be seen in Figure 56 below. A student's t-test was conducted to compare the cell counts for the two materials, and the resulting p-value was 0.559. A p-value greater than 0.05 suggests that with 95% confidence the membrane paper is no more toxic to rat smooth muscle cells than tissue culture plastic is.



**Figure 56: Graph of the average live cell counts of 3 tests cultured with membrane paper and 3 tests cultured without membrane paper (p = 0.559)**

### **Experiment #13: Smaller Diameter Polycarbonate**

While waiting on the results of the membrane paper adhesion assay, we proceeded to test other variables which may help in forming stronger tubes to enable easier harvesting. We decreased the diameter of the tubing to 1/5 inch, and still filled it with 25 million cells, thus increasing the cell density. We used polycarbonate tubing and spun the tubes (n=2) for 4 hours. The tubes were then cultured in vertical culture chambers for 3 days.

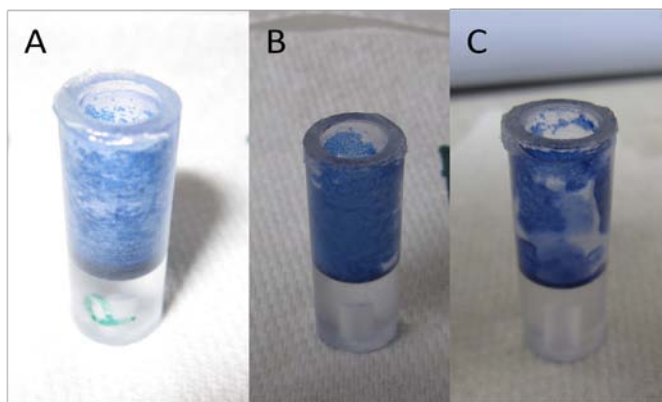
After culturing, we tried to harvest the cellular constructs that had formed in one of the two tubes. The first tube had a cell layer that was still too thin to harvest, even though more cells were used in this experiment. When placed in media after the attempted harvesting, some of the cells fell out of the tube indicating the harvesting disrupted their adhesion. Since the second tube also only had a thin cell layer, we did not attempt to harvest it initially, and fixed the tube in a formalin/sucrose solution. After fixing for 1 day, we attempted to harvest the second tube, but we could not grab hold of the tube with tweezers because the cell layer was too thin and too adhered to the walls. Therefore, it was concluded that with the setup used for extended culture, increasing the cell number does not enable a stronger tube or easier harvesting.

### **Experiment #14: Variable Time Testing**

To test the distribution of cells after spinning for different periods of time we decided to perform an experiment of spinning polycarbonate tubes for 5, 15, or 60 minutes and fix them immediately after, rather than culturing them. Part of this test would also be to count the number of cells that were still suspended in the media inside the tube after spinning. Each tube had 25 million cells and all other variables remained constant. After spinning, the media was pipetted out without disturbing the sides of the tube. The tubes were fixed in 70% ethanol and stained with Trypan blue to observe cell coverage. A cell count of the removed media for each tube



suggested that the number of cells attached to the wall is consistent regardless of spinning time. The tube spun for 5 minutes tube had sporadic cell coverage, due to a rough inner surface from manufacturing, and 475,000 cells in media. The 15 minute tube had uniform cell coverage and 455,000 cells in the media. Finally, the 60 minute tube showed sporadic coverage and 515,000 cells in media. This suggests that long term spinning might be too rigorous for the cells and cause poor cell distribution. The respective cell coverage of the tubes can be seen in Figure 57.



**Figure 57: A) Cell coverage after 5 minute spin. B) Cell coverage after 15 minute spin. C) Cell coverage after 60 minute spin.**

### **Rotations per Minute (rpm) Testing**

Due to the lack of strength and cohesive structure in the formed tubular constructs, we hypothesized that the fast spinning of the motor may be causing stresses on the cells, which are inhibiting their ability to form strong cellular bonds with one another. We completed testing to determine the speed in rotations per minute (rpm) that the tubes were being spun at in order to compute the forces on the cells. We tested the speed of the spinning tubes when spun with a 3 volt power source and no resistance, as conducted in the previous tests. The speed was also calculated when using a 10 ohm resistor to reduce the current and speed of the motor. This lower speed would be used in the following test.

The same centrifugal force setup as the previous tests was used, with the motor clamped in a stand with a 2 cm hollow rod attached. A reflective marker was adhered to outside surface of the tube. A Neiko Laser Photo Tachometer (model #20713A) was placed in line with the reflective marker to measure the rotational speed. Testing was first completed at original speed using a 3 volt battery source for a tube with and without media. The tube was filled with media to better represent the weight of the tube during testing, as the weight will affect the rotational speed. Five tests were conducted for each setup to determine if the media affected the spinning of the tube. The results are shown below (Table 11).

**Table 11: Rotational speed of spinning tubes in RPM with and without media (n=5)**

Setup	Test #	Rotations per Minute (rpm)
With Media	1	11300
	2	10700
	3	9800
	4	10200
	5	10000
Without Media	1	11100
	2	11300
	3	11400
	4	11400
	5	11400

A student's t-test was completed in Minitab v.15 as shown below. The p-value was 0.029, which is less than the confidence level of 0.05, indicating that the two tests are statistically

different. Therefore, the weight of the media does contribute to the rotational speed of the tubes.

Further RPM testing needs be conducted with media added to the tubes.

**Two-Sample T-Test and CI: With Media, Without Media**

Two-sample T for With Media vs Without Media

	N	Mean	StDev	SE Mean
With Media	5	10400	604	270
Without Media	5	11320	130	58

Difference = mu (With Media) - mu (Without Media)

Estimate for difference: -920

95% CI for difference: (-1687, -153)

T-Test of difference = 0 (vs not =): T-Value = -3.33 P-Value = 0.029 DF = 4

Rotational speed testing was conducted with three different tubes filled with media to determine the average rotational speed of the spinning tubes with and without a resistor added to the circuit.

Five tests were completed for each tube, and the motor was disconnected from the battery in

between each test. The results are shown below (Table 12).

**Table 12: Rotational Speed of spinning tubes in RPM at original speed and with a resistor.**

Setup	Test #	Rotations per Minute (rpm)	
		Original Speed	10 Ohm Resistor
Tube 1	1	11300	5100
	2	10700	5200
	3	9800	5300
	4	10200	5300
	5	10000	5300
Tube 2	1	11100	5500
	2	11300	5500
	3	11400	5600

	4	11400	5600
	5	11400	5600
Tube 3	1	10400	5000
	2	10500	4900
	3	10500	5000
	4	10500	5000
	5	10500	5000
Mean		10733	5260
Standard Deviation		543	253

The average rotational speed of the tubes when no resistor is used is  $10,733 \pm 543$  rpm.

When a 10 ohm resistor is used, the average rotational speed is  $5260 \pm 253$  rpm.

### Centrifugal Force Calculation

To determine the stress that cells experience during spinning in this design we decided to calculate the amount of force exerted on the cells when spun at 11,000 rpm at the inner radius of our mandrel (0.3175 cm). To do this we obtained the equation to calculate force from the user manual of the BHG Hermle Z320 Laboratory Centrifuge used to pellet cell suspensions.

$$g = 11.18 \times r \times \left( \frac{n}{1000} \right)^2$$

g = relative centrifugal force (RCF),  
 i.e. relative to the acceleration  
 of the earth which has the constant  
 value  $g = 9.81 \text{ m/s}^2$

r = spinning radius in cm

n = revolutions per minute (RPM)

Inserting the radius of 0.3175 cm and the revolutions per minute of 11,000 we obtained a value of 429.5 g-force as the amount of force experienced by the cells in our spinning method. When we spun at the slower speed of 5,000 rpm the cells experienced a force of 88.74 xg (times gravity), which is almost 21% of the force experienced at 11,000 rpm. We believe that this force is not strong enough to harm the cells because the centrifuge mentioned above with a 16 cm radius spun at 4,000 rpm will give a resultant force of 2,860xg, which is much higher than the force applied in our method.

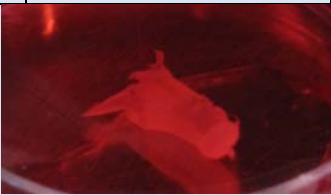
### **Experiment #15: Variable Speed Testing**

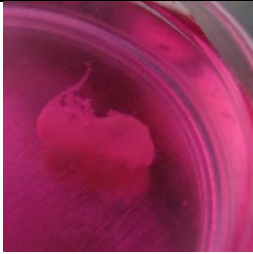



In order to test the effectiveness of spinning the cells at a lower speed we used the 10 ohm resistor which, as shown above, reduces rotational speed by approximately half and force by 80%. We tested 3 polycarbonate and 3 acrylic tubes, added 25 million cells, and spun for 15 minutes. One of each tubing material was spun at the original speed with a 3 volt power source and no resistance, which is about 0.16 amps of current, and two at the lower speed. After spinning, the tubes were cultured in the vertical chambers with open ends for 3 days.

After culturing for 3 days, the 6 tubes were removed from the incubator. In 4 of the tubes, the cells lining the tube had contracted and pulled away from portions of the tube wall. The

contraction of the cells formed a tubular construct that was only adhered to the wall on one side, and was much stronger than any of the previous tubes as it was able to be removed by pulling with tweezers. The results are summarized below along with pictures (Table 13). The tubes that were harvested were fixed in formalin/sucrose in order to be histologically examined. We believe that the combination of spinning for 15 minutes and culturing vertically in an open ended tube was the ideal combination that resulted in coherent, removable tubular constructs.

**Table 13: Summary of results from Experiment # 14.**

<b>Tube Material</b>	<b>Rotations per Minute</b>	<b>Tube Formed?</b>	<b>Removal Method</b>	<b>Observations</b>
Polycarbonate	10,800	No	N/A	<ul style="list-style-type: none"> <li>Media had leaked out of culture system and was below level of tube</li> <li>Media was not exchanged in tube and was yellow</li> <li>Some tissue had contracted, but was dissociated and globular</li> </ul>
Polycarbonate	5,200	Yes	<ul style="list-style-type: none"> <li>Grabbed edge of tube with tweezers and pulled it away from the side</li> </ul>	<ul style="list-style-type: none"> <li>One end of the tube was torn</li> <li>Removal from the wall of the tube may have caused the damage</li> <li>Approximately 8 mm long, 3 mm O.D.</li> </ul>
				
Polycarbonate	5,200	Yes	<ul style="list-style-type: none"> <li>Tube construct was adhered to bottom of tube</li> <li>Placed tweezers around construct, squeezed, and pulled away</li> </ul>	<ul style="list-style-type: none"> <li>Even after squeezing the tube, it retained its tubular shape</li> <li>Approximately 6 mm long, 2 mm O.D.</li> </ul>

				
Acrylic	10,800	Yes	<ul style="list-style-type: none"> <li>• Tweezers were able to be inserted into the tube construct</li> <li>• Pulled tweezers away from wall of tube and removed</li> </ul>	<ul style="list-style-type: none"> <li>• Approximately 7 mm long, 4 mm O.D.</li> </ul>
				
Acrylic	5,200	Yes	<ul style="list-style-type: none"> <li>• Grabbed edge of tube with tweezers and pulled it away from the side</li> </ul>	<ul style="list-style-type: none"> <li>• Approximately 6 mm long, 3 mm O.D.</li> </ul>
				
Acrylic	5,200	No	N/A	<ul style="list-style-type: none"> <li>• Tissue had not contracted</li> <li>• Nothing to grab a hold of with tweezers to harvest</li> </ul>
				

## Chapter 6 - Final Design & Validation

The final design to create a cohesive, completely cell-derived tubular construct is a simple and relatively quick process. First, cells are loaded into a mandrel, which is spun around its central axis by a motor to force the cells to form a uniform layer on the wall. This mandrel is then moved into a culture chamber where it is provided with nutrients and media to aid the cells in the creation of ECM and the transformation into a tissue.

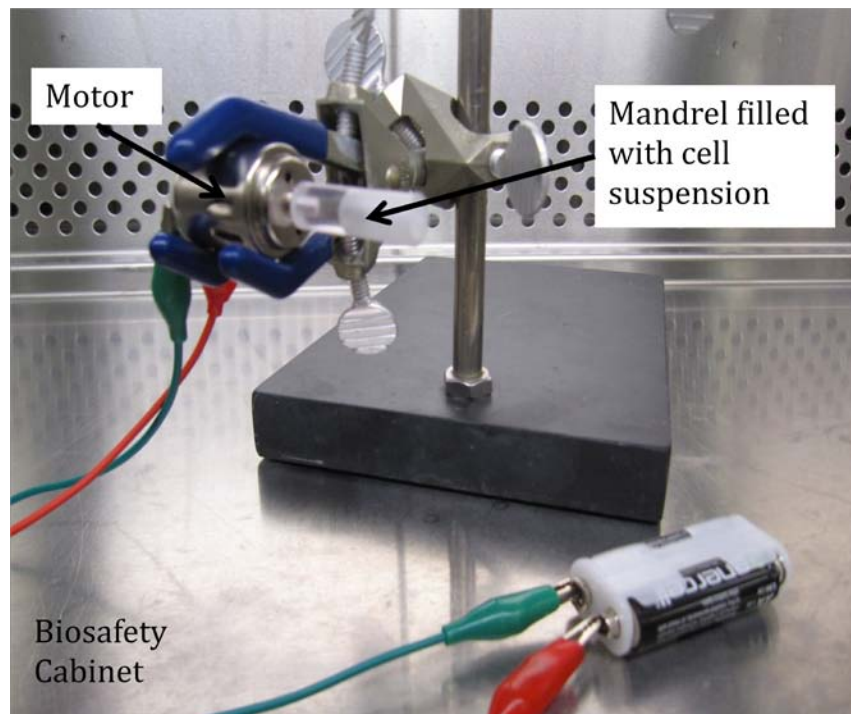
### Manufacture

The final design consists of three parts: the mandrel, the motor assembly, and the incubation chamber.

First, the mandrel is manufactured from a 3/8 inch diameter acrylic or polycarbonate rod cut to a length of 3 cm. A lathe is used to drill a 1/4 inch diameter hole down the central axis of the rod which extends 3/4 inch deep into one end. On the other end of the rod, a 1 mm diameter hole is lathed out to a depth of 1/4 inch. The larger hole of the tube is then covered with silicone glue to completely seal the space inside and is allowed to cure for 24 hours. The curing process is required to ensure that the glue is fully dried.

The second part of the final design is the motor assembly and clamp interface (Figure 58). A 3 volt hobby motor is clamped to a ring stand using a three point clamp. Two double AA batteries are placed in a battery pack. Two alligator clips are used to hook up the battery pack to the motor to complete a circuit.

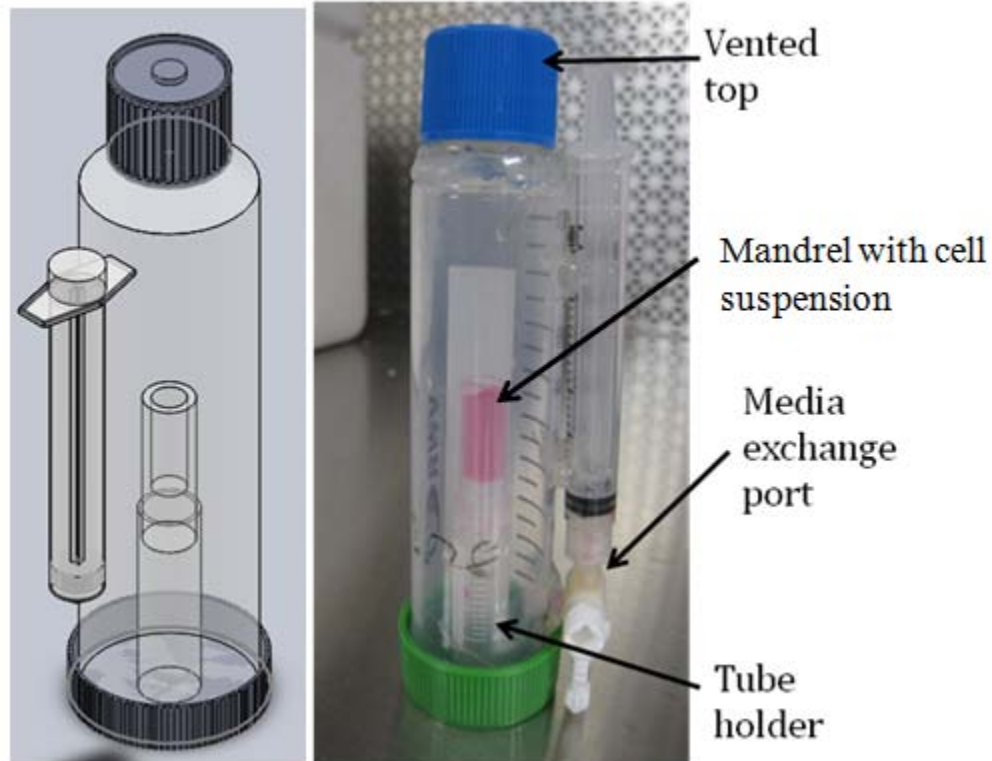




**Figure 58: Motor secured in stand clamp with tube attached.**

The third part of the final design is the incubation chamber. The incubation chamber is made by combining a 50 mL conical tube, a T-75 gas exchange screw top, a three-way stopcock, and a 5 mL syringe. A hole is drilled into the bottom of the conical flask after which the top of the T-75 flask is fitted and sealed to the hole. This is to allow gas to diffuse through the cap while preventing contaminating agents from entering the system. A smaller hole is drilled in the side of the tube just below the top of the tube (around the 45 mL mark); subsequently, one end of the stop-cock is hot glued into the hole. Once dry, the 5 mL syringe is screwed into the stop cock such that it runs parallel to the long axis of the conical tube. The opening of the syringe should be pointing towards the bottom of the conical tube. The purpose of this syringe-stopcock assembly is to create an access port where media can be added and removed without exposing the inside of the conical tube to the ambient environment. Lastly, a cryovial (or any tubular structure) with a 3/8 inch inner diameter is glued to the underside of the conical tube cap. This

piece is intended to hold the mandrel in place during culture. It is important to note that the incubation device is intended to be used with the conical tube resting on its cap and the bottom end facing up as seen in Figure 59 below.



**Figure 59: Incubation chamber assembly built around an inverted 50 mL conical tube to allow incubation and ECM production of cells after they are spun into a tubular shape.**

### Tube Creation Process

Using these three pieces, the following protocol is followed to create a tube. First, cells are trypsinized (media aspirated and 2 mL trypsin added) from of several T-75 flasks and counted using standard cell culture protocols. The cells are then spun down in a centrifuge for 5 minutes at 1000 rpm, and the supernatant is aspirated off. The cells are resuspended to a desired concentration of 25 million cells per 0.633 mL (the volume of the inside of the mandrel). Then, 0.633 mL of the cell suspension is loaded into a syringe. It is important to note that the cell suspension is drawn into the syringe prior to the attachment of the needle. This way, damage to

cells due to shearing can be minimized. The syringe is then used to inject the suspension into the mandrel through the silicone—a second needle is needed to allow air to escape (Figure 60). Once the absence of bubbles is confirmed visually, the mandrel is fitted onto the axis of the motor. The alligator clamps are then hooked up to the motor and battery and the mandrel is spun for 15 minutes.



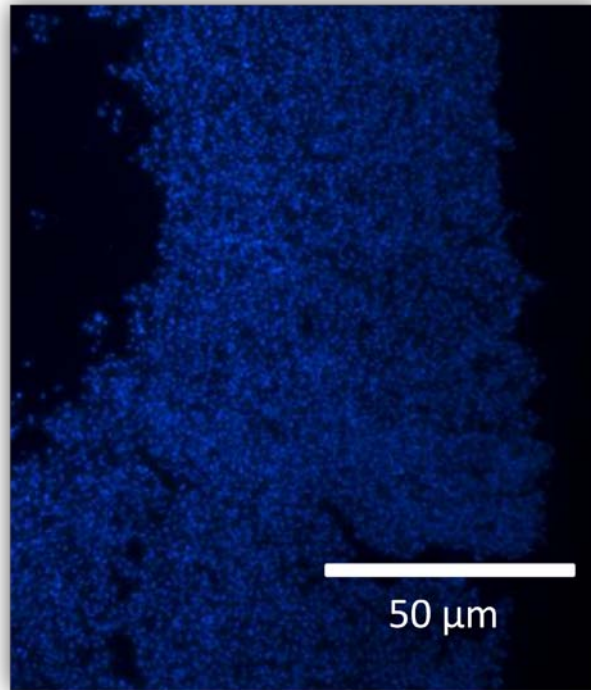
**Figure 60: Method for insertion of cell suspension into tube.**

After the appropriate spinning time has been allowed, an alligator clamp (it does not matter which one) is removed to stop the motor. The mandrel is then removed from the motor and the silicone cap is delicately removed using a scalpel. The mandrel is then placed in the cryovial holder on the cap of the incubation chamber. The tube is then screwed into the cap over the holder. Using the syringe port, the chamber is filled with DMEM media supplemented with 2.5% FBS, such that the mandrel is fully immersed. The incubation chamber with the mandrel is then placed in the incubator (37 degree Celsius, 5% CO<sub>2</sub>) and allowed to culture for 3 days. Media is changed prior to the end point only if noticeable yellow-orange discoloration occurs.

After incubating for 3 days, the mandrel can be removed. The incubation chamber is drained by opening the stopcock, and the conical tube is unscrewed from the cap. The mandrel is then exposed and can be removed from the cryovial. Small tweezers are used to pull the cellular tube from the mandrel and into the drained media bath, by gently removing the last remaining adhesive portion from the mandrel. The blood vessel is now ready for conditioning or testing.

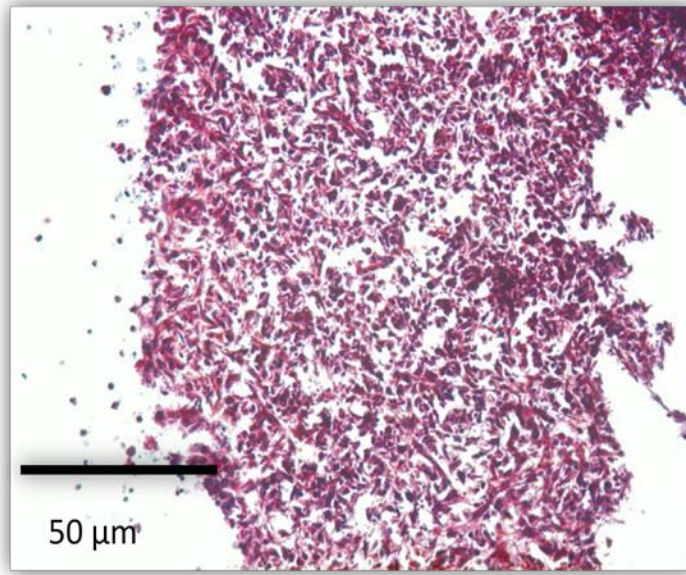
## **Validation**

In order to examine the biological composition of the newly formed tissue, histology and immunohistochemistry was conducted. To fix the tubes they were placed overnight in a 50/50 solution of paraformaldehyde and 30% sucrose. After fixation the tissue was submerged in liquid OCT and frozen to embed it. While still frozen, the tissue-contained blocks of OCT were cut into 8  $\mu\text{m}$  thick sections and placed on slides to be stained. The first staining done was a fluorescent nuclear indicator, Hoechst. This staining causes the nucleus of the cell to emit blue light when excited. The purpose of doing this staining is to examine cellular density and distribution. As smooth muscle cells are mono-nucleated, each nucleus corresponds to one cell and therefore the number of nuclei directly correlates to the quantity of cells. Figure 61 below shows the tube stained with Hoechst at 20x. This image shows that the nuclei are tightly packed, inferring the tissue has a high cellular density. A break in the tube can be seen in the lower right hand corner of the picture; this may be a result of processing.



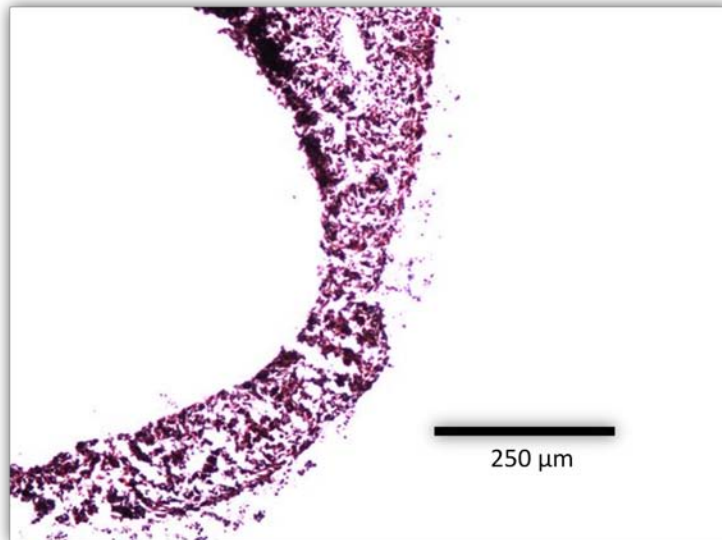
**Figure 61: Hoechst staining at 20x shows cell density in tubular construct**

In order to visualize the cytoplasm of the cell as well as extracellular collagen, Masson's Trichrome staining was performed. This procedure stains the nuclei black, the cytoplasm red, and collagen blue. The picture below is a Trichrome-stained section imaged at 20x (Figure 62). The distribution of black staining coincides with the previous image of the Hoechst staining. Based on the shape of the cytoplasm, the orientation of the cells appears random. Cell distribution is mostly even across the tube with some areas of higher density at the edges, however, the density is relatively high overall. There is no significant amount of positive collagen signal, represented by blue. This was an expected result as these tubes were cultured for only three days, which is insufficient time for considerable collagen production.



**Figure 62: Trichrome stain at 20x showing density of cells and cell alignment**

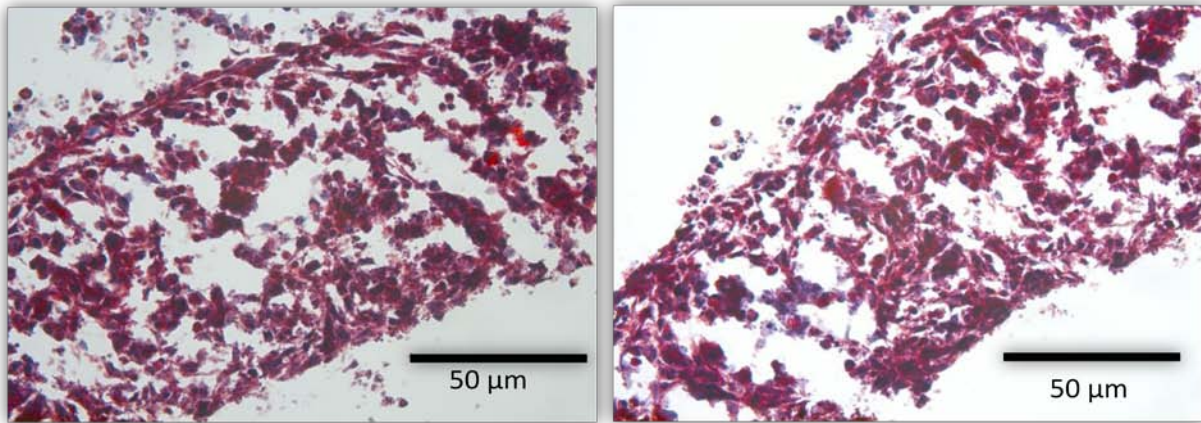
The 5x image below shows the general shape of the tubes (Figure 63). The thickness changes significantly and the tissue breaks at points. But, the circular shape is clear and the vessel is sufficiently thick.



**Figure 63: Trichrome staining at 10x shows a portion of the tubular construct displaying**

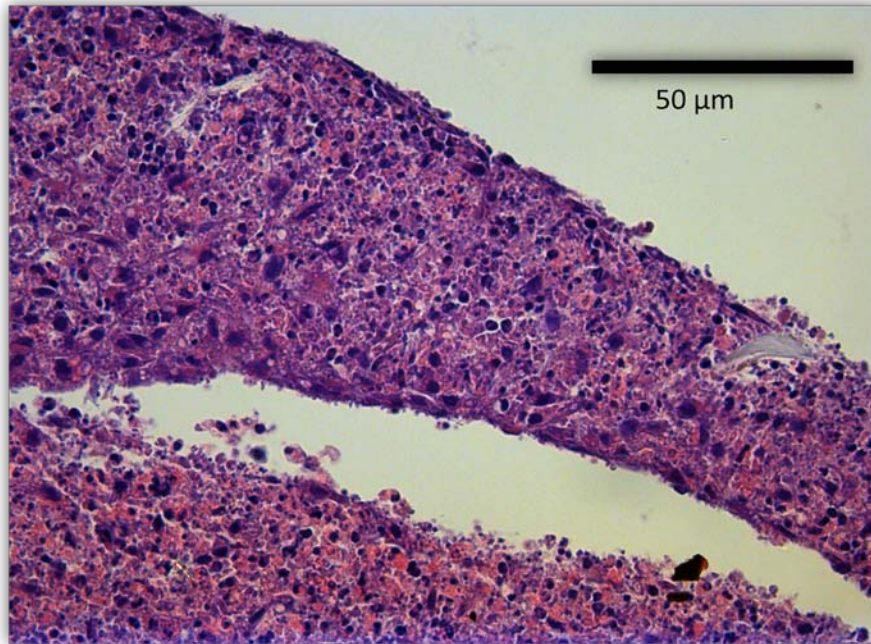


As seen in the 40x images below, an acrylic tube spun at 11,000 rpm and a polycarbonate tube spun at 5,000 rpm produced similar histology, indicating the repeatability of our method (Figure 64). Between the cells there are apparent white spaces, which were not anticipated since normal confluent tissue is continuous. These breaks could be a result of embedding or cutting of the tube sections.



**Figure 64: Trichrome staining at 40x showing an acrylic tube spun at 11,000 rpm (left) and a polycarbonate tube spun at 5,000 rpm (right). The similarities in the tubes show that our method is repeatable with different mandrel materials and rotational speeds.**

To determine if the incontinuity of the tubes was a result of tissue processing, we implemented an alternative embedding method. Instead of freezing and cutting the vessels, we embedded the vessels in parafin and cut them at room temperature. After, the slides were stained with hemotoxylin and eosin staining, a stain that presents nuclei as blue and ECM as red. As seen in Figure 65 the tissue prepared with parafin is continuous and does not have white space as the frozen sections do. This proves that our cell tube formation method does produce continuous tissue.



**Figure 65: Parafin embedded H&E staining (40x), showing the cohesive tubular structure.**

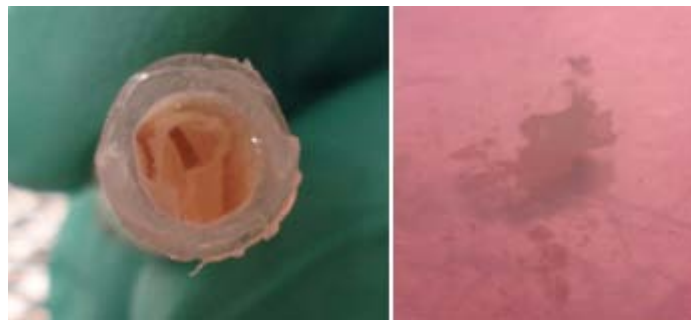
Based on the histological examination, collagen production must be increased in order to create a more mechanically robust tube. This can be accomplished by culturing the tubes for an extended period of time.

### *Validation Test*

The goal of this test was to replicate the results shown above, in which the tubes contracted off the mandrel, to show that our methods are repeatable. This test included a 15-minute spin time, using 25 million cells loaded into polycarbonate hollow rods. The rods were of the “loose fit” design and they were cultured upright in the vertical bioreactor for 3 days. No resistors were used this time, because with this sample number (n=4) results would not be enough to statistically compare variable resistances on the ability to form a tube. Instead, the test was designed simply to reproduce the formation of tubular constructs that contract from the mandrel wall.



The results of this test did not fully repeat the previous results of tubular constructs. Upon removal from the incubator it was clear that cellular constructs had contracted off the mandrel walls. However, they did not appear to be as tubular or as mechanically stable as seen previously. Tubes 1 and 2 were not strong enough to remove from their mandrels. They fell apart under the same removal technique that had worked in the previous experiment by gentle manipulation with forceps. The cells in tube 1 appeared more like a solid mass rather than a clearly visible, self-supporting tubular construct. The cells in tube 2 appeared similar to the constructs removed in the prior experiment; however, they had no structural integrity and fell apart upon attempted removal.



**Figure 66: A tubular construct of cells formed a tube after 3 days in culture (left), but was not as cohesive as previous results and fell apart upon removal (right).**

Tubes 3 and 4 had visible cellular constructs. Based on what happened when we attempted to remove the construct from tube 2, we decided to allow an additional culturing period of one day. After this was allowed, the cells in tube 3 appeared to have condensed into a clump of cells with no visible tubular shape. The cells in tube 4 remained in a tubular shape during incubation, but fell apart upon attempted removal. A portion of the tube was extracted, although it ripped, forming a sheet of cells rather than an actual tube or ring. Upon investigating why these results were inconsistent with the previous test, we found that the media was contaminated with bacteria.

## Chapter 7 - Discussion

The results we gathered through numerous tests allowed us to determine the combination of variables, such as rod material, motor speed, spin time, culture time, and culture setup, which allowed us to produce a cellular tube.

In order to make a completely cell-derived blood vessel (no exogenous materials present in the final product), we injected a cell suspension into a hollowed polycarbonate rod that was sealed with silicone glue at the open end. The rod was then attached to a small motor and spun rapidly for a period of 15 minutes. After spinning had concluded, the rod was placed upright in our novel culturing chamber. After a culturing period of only 3 days, we were able to harvest a tubular vessel that held its shape when placed in a media bath with no supporting materials.

The current state of the art in tissue engineered small diameter blood vessel development comes from the work of L'Heureux et al. Their technique involves growing the individual layers of a blood vessel in sheets, after which they are rolled around a mandrel and allowed to adhere to one another. While this produces vessels that have comparable mechanical properties to natural vasculature, the major limiting factor is the long culture time, approximately 3 months, required by this process. For many patients, waiting 3 months for a blood vessel, created by the methods of L'Heureux, is simply not an option. We believe that our tissue engineered blood vessels, which can stand alone after only 3 days in culture, may provide a faster method of supplying blood vessels to patients in need. While our blood vessels will certainly need additional culturing time in order to achieve the necessary physiological mechanical properties, we believe that our method of vessel production has the potential to ultimately provide transplantable blood vessels to patients at a faster rate. However, the rate of production using our method would be dependent on which cell type we use, the location of the biopsy, and how many cells can be obtained. Our

method is an improvement in that we do not use a scaffold to create our blood vessels. It is well known that the insertion of foreign materials into the body, as is the case with synthetic scaffolds, elicits a detrimental immune response, which can cause the failure of medical devices. Scaffolds made of ECM materials often exhibit weak mechanical properties (Hirai, 1996). Our method overcomes these issues by evenly layering cells about the inner surface of a hollow plastic tube, as evidenced by confluent Trypan blue staining, at which point they can proliferate and create their own extracellular matrix, completely free of exogenous materials. We anticipate that cell-derived ECM will have more native-like mechanical properties.

The question we asked ourselves repeatedly throughout the course of our project was, “how are we going to get cells to stick together in a tubular construct?” Cellular self-assembly is a challenge, as evidenced by the fact that it is extremely difficult to get cells to form complex three dimensional structures in an ex vivo environment. We know that cells require a combination of physiological conditions, signals, and nutrients in order to grow. In order to make blood vessels outside of the body, we need to mimic cells’ natural environment, and use additional variables to force cells into a tubular shape, at which point they would form cell-cell bonds and create ECM. Through initial research, and substantial experimentation, we found the right combination of those variables that allowed us to complete our project objective: Cells in, tube out!

We were able to comply with our objects and constraints by carefully analyzing each test that we ran. We often determined that some tests were not possible to complete because they would either not meet an objective, or would infringe upon a constraint. To create a tubular assembly of cells, our primary objective, we created a method that utilizes centrifugal force to assemble cells in a tubular shape, after which they were allowed to culture for 3 days, which was

sufficient time from them to form enough cell-cell adhesions to stand alone as a tube after harvesting. Histology of these tubes does not reveal significant ECM, specifically collagen production; however, we are confident that by 7 days in culture, our tubes will show increased levels of matrix proteins. Repeatability was achieved by adhering to strict step-by-step plans for each experiment. This allowed us to obtain reproducible results, necessary for validating our design. We completed and exceeded our objective of short production, 7 days, by creating tubes that could stand alone after only 3 days. Initially, we thought that harvesting the tubes would be a difficult task; however, we found that the cells contracted and actually pulled themselves off the mandrel, so that when it came time to remove them, we consistently found that only gentle manipulation with tweezers was required for extraction. We achieved minimal user handling by creating a 3-step handling process for tube creation. The user first inserts the cells into the tube and spins it with a small motor. Next, the user must carefully remove the tube and place it in the incubation chamber. Lastly, after the appropriate time in culture, the user simply removes the cellular construct from the rod. By using commonly available components, we met the manufacturability objective. Most labs would easily be able to re-create our system with minimal cost and time requirements. Lastly, progress of tube formation can easily be monitored through our transparent culturing system. If tube formation is not visibly confirmed after approximately 2 days, then the user would know that an error had occurred during the initial stages of vessel production.

The constraint that caused us the most trouble was that of our budget. We were unable to further investigate some of our design alternatives simply because the cost of materials would bankrupt our project. However, we did the best we could with our limited resources. Rather than purchasing pre-made hollow tubes, or paying a machinist to construct them for us, we simply

bought plastic rods and learned to use various pieces of machinery, such as band saws, drill presses, and lathes. When it came to producing alternative design prototypes, we were able to construct devices out of common lab materials, such as cell culture flasks, centrifuge tubes, rubber hosing, filter paper and adhesives. This allowed us to conserve our monetary resources for our final product design.

The major limitation of our data and testing were the sample numbers for each test we ran. We simply did not have the budget or time to create larger numbers of samples for our tests. Given more time to complete additional tests, which would have allowed for higher sample numbers, we could have better represented the ability of our methodology to repeatedly produce identical tissue constructs. We believe that outlying results—caused by problems between tests and human error—could have been eliminated. Additionally, in some cases it was difficult to determine whether or not results confirmed or rejected our experimental hypotheses, as we would occasionally find a wide range of results within a particular test. In order to overcome these limitations, we ran tests multiple times, and by achieving reproducible results, we concluded that those results were viable evidence that our methodology was conducive to creating a tube.

### **Design For...**

When faced with the design of a new medical product, there are many aspects to be considered that may not be readily apparent. In order to create a successful product, it must be designed for the least negative impact, in addition to improvement of patient health. Some important considerations that we took the time to design for in our product are societal influence, manufacturability, environmental impact, ethical concern, political ramifications, sustainability, economics, and, finally, health and safety issues.

## *Societal Influence*

The introduction of new and untested medical treatments can have an unexpected societal impact. In order to reduce any negative social implications, it is necessary to consider these issues in the design process. The potential benefits that society stands to gain from a new medical technology should far outweigh the possible risks associated with it. Additionally, any negative effects that our tissue engineered blood vessels may have would be no more serious than the current state of the art technologies for vascular grafting procedures mentioned previously.

The most significant and beneficial societal impact will be that our bioreactor allows bypass operations to become available to patients who otherwise might not have been able to have the procedure. This includes the elderly population, people who previously have had vessels harvested for vascular grafts, and people with poor vascular health due to diabetes or vascular disease, who may lack suitable veins and arteries for an autologous graft. This will mitigate the impact that cardiovascular disease can have on patients without suitable autologous vessels for their operation.

The willingness of insurance companies to support the use of our product is a large concern. If one or more insurance companies decide not to cover the use of our device, it could marginalize our user base and limit the access of this treatment to patients with lower income. For this reason, we must prove that our bioreactor can create vessels that will comply with FDA standards, and optimally, show a lower rate of negative implications associated with the current state of the art.

## *Manufacturability*

In order to make a fiscally successful medical device, large-scale production must be possible. In designing our bioreactor, we have tried to make manufacturability a key component in our objectives. We define ease of manufacturing by the amount of time and resources that must go into fabricating the final product. We are working to achieve this by using a minimal number of components that are also cheap, reliable, and commercially available.

In assessing the manufacturability of our product, we determined the availability of the required parts. Parts that must be custom machined significantly slow down the manufacturing process and make it more difficult for a third party to assemble the final product. Therefore, we chose to use only “off the shelf” parts in designing our device. Excluding common laboratory materials, the total cost of one device is less than \$10. The approximate prices for each of the components of the design are listed below:

- 3V Hobby Motor = \$3
- Polycarbonate Rod = \$0.19
- 2 AA Batteries = \$1
- Battery Holder = \$2
- 2 Alligator Clamps = \$2

Limiting the total number of parts is another criterion that we have aimed for in our design. The fewer the number of parts that are required, the easier the production process becomes. Limiting the total number of parts also decreases the likelihood that assembly errors will occur, and will make repairs less of a hassle. Making a product that can be assembled by an untrained technician is another goal of our project that will increase its appeal.

### *Environmental Impact*

In order to minimize any potential negative environmental effects, our spinning apparatus is designed to be sterilized and reused to make many vascular grafts. However, the parts used to make the tubular constructs will be synthetic and non-natural materials. Using plastics to build the bioreactor instead of natural materials will mean that toxic chemicals will be released into the environment during the manufacturing of the components, as well as when the bioreactor is eventually discarded. The length of time it takes for the materials to degrade will depend on the plastic used. The mandrels and bioreactor chambers will not be recyclable because they will be in contact with cells, so they would be considered a biohazard, and must be properly disposed of.

### *Ethical Concern*

There may be several ethical concerns about using a bioreactor to create tissue engineered blood vessels. Some people, based on personal or religious beliefs (for example, the Amish or Scientologists), feel that the use of medicine in general is wrong and not natural. Others do not believe in the use of non-autologous material inside the body, so there will be less concern if a patient's own cells are used to create a graft, which also contains no other exogenous materials. Another potential concern associated with the bioreactor would be if stem cells are used. Many people are opposed to the use of stem cells, especially embryonic stem cells, due to religious beliefs. Therefore, we used rat smooth muscle cells in order to eliminate this concern.

### *Political Ramifications*

This bioreactor could have the ability to spread tissue engineering to regions of the planet where the field was previously inaccessible. Because it uses commonplace components and requires minimal human intervention, the bioreactor could potentially be used by countries with



weaker economies and a less educated work force. Sharing medical technologies could prove to be a great tool in promoting countries to work together for the benefit of all.

### *Sustainability*

In order to determine the sustainability of the bioreactor, one must consider not just the energy cost required to power the device so that it may create vessels, but also the energy costs of making the device components, assembling the components, manufacturing the materials needed to make the components, shipping the components, etc.. We built our bioreactor with as few components as possible, and with components that are widely available.

We speculate that despite this vast assortment of energy expenses, our technology will actually help to decrease overall resource consumption. If people suffering from cardiovascular disease can receive vascular grafts created by our bioreactor, then they will no longer need as many medications, hospital visits, surgeries, and/or support devices to live a more satisfying life.

### *Economic*

Our device could have a significant economic impact because it is an innovation in the health care industry. Although this would likely been an expensive procedure, it would help people because they would not have to pay as many recurring charges for medication and multiple surgeries. However, due to the expense, if insurance is not granted for the procedure, then this device may not be beneficial to people in an unstable financial state.

This device could also provide the basis for a start-up company, which would create a small number of jobs in the industry. However, there would be competition with companies that make stents and artificial vascular grafts. Our device would likely be used in more severe cases, where stents would not solve the problem or where autologous graft harvesting is not an option.

It would also be better suited for CABG than a synthetic graft, because those types of grafts have typically only found success in large diameter applications, such as aortic replacements.

### *Health and Safety Issue*

Our device could potentially have many health and safety issues, as with any surgery. The graft itself may have issues with graft versus host disease, depending if autologous cells are used or not. There could also be graft failure, though ideally each graft produced will pass strict quality control specifications. Finally, there could also be variability in the longevity of the graft on a case by case basis.

There could also be safety issues during the implantation of the graft. Improper surgical handling of the graft, could cause bacterial contamination and result in infection or inflammation. Furthermore, there could be surgical complications, as with any procedure. However, these health and safety issues are likely to be rare, isolated incidents, and the device will enhance the quality and length of life greatly when successful. Also, we feel that our bioreactor would create vessels that ultimately would have no more of the potentially dangerous implications that are experienced with current state of the art technologies.

## Chapter 8 - Conclusions and Recommendation

Through our testing regimen we have developed conclusions pertaining to our final design and its ability to make tubes. Primarily, when spun inside a tube, cells will self assemble into a cylindrical shape. Even after spinning, cells retain this shape in culture. Additionally, it was determined that fifteen minutes of spinning was sufficient time to force the cells out of suspension and against the wall. By staining cells with Trypan blue after spinning we were able to determine cell viability is not affected by the forces experienced during high speed rotation. In summary, cells can be made into a healthy tube using centrifugal force.

### Recommendations

Due to time and resource constraints, we were unable to complete a range of tests that would support the viability of our methods in the creation of a tubular construct. Therefore, we recommend that follow-up testing is conducted in the future to further the development of our designed technology. Such testing should include: mechanical testing, mechanical conditioning, variable tube dimensions, other cell sources and types, and applications other than blood vessels.

Mechanical testing is an important aspect of tissue engineering, as tissue engineered replacements must have similar strengths to the natural tissue that is being replaced; otherwise it is likely to fail catastrophically. If the tissue engineered product is not mechanically tested, then there is no way of knowing if it will be able to endure the stresses associated with its intended use, and therefore the risk to a patient is far too great. By mechanically testing our blood vessels, their properties can be better characterized, and modifications can be made to our design and culturing processes to create stronger tubes if necessary.

By mechanically conditioning our tubes with pulsatile flow during the culture period, we believe that vessels could be created that would be better suited for transplant into the body. By introduction of the vessels to conditions similar to their native environment, they will be able to form the necessary organization and supporting structures, and thus would be better prepared compared to unconditioned vessels.

With our current methods, we are able to produce tubes about 1 cm in length and 2 mm in diameter. While these tubes are excellent proof that our methodology is effective in taking cells and producing a stand-alone construct, they are not particularly useful for use as vascular grafts. Therefore, we recommend attempting to create larger tubes (both in length and diameter). A length of 5-10 cm with a diameter of 4-6 mm would be more ideal. This would involve only slight modifications to our system, including the need to create longer rods, which may require that a stronger motor be used, and an extension of the incubation chamber.

A “one size fits all” tag is not practical when it comes to creating blood vessels for vascular grafting procedures. Therefore, our system must have an assortment of rods that will be capable of creating custom vessels on a patient-by-patient basis. Additionally, testing various diameter vessels for mechanical strength would allow us to determine if our system is capable of creating tubes with consistent properties, regardless of size.

Ultimately, we would like to test our system with human cells, as these will be the cells that are used in patients. While we would expect to see similar results as compared to the tests we performed with rat cells, the different contractile properties could significantly alter the formation of a tube. We would also recommend experimenting with the introduction of vascular endothelial cells to the culture system, so as to create an endothelial lining in our blood vessels, which has been shown to be important for improved patency and lessened occurrences of

stenosis. This would also be an excellent use of centrifugal force, as it would push endothelial cells out of suspension and onto the layer of smooth muscle cells by spinning in the same manner we created the tubes initially.

Blood vessels are not the only tubular structures found in the human body. We believe that with modifications to our system, it has the potential for creating numerous other tubular tissues and organs, such as the intestines, airways, and urinary tracts.

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## Glossary

Anastomosis – The joining of two tubular structures to make one.

Angioplasty – An operation in which a balloon is inflated in a clogged artery to widen it.

Atherosclerosis- Condition in which plaque builds up along the walls of blood vessels.

Autologous – Coming from one's self.

Biocompatible- A substance that does not instigate a negative immune or inflammatory response when put in contact with the body.

Biomaterial – Any material that interfaces with a biological system.

Cell-adhesion – The attachment of cells to a material.

Coronary heart disease – Blockage of the coronary arteries resulting in loss of heart function or death.

Cytotoxicity – The extent to which a substance harms cells.

Dulbecco's Modified Eagle Medium (DMEM)- Media containing amino acids and vitamins necessary for cell culture.

Exogenous – Any non-cellular material that is non-autologous.

Fetal Bovine Serum (FBS)- non-cellular component of fetal bovine blood.

Graft – A vessel, biological or synthetic, that is surgically implanted to hold and deliver blood.

Hemocytometer – A device for counting cells in suspension.

Hoechst – A fluorescent immuno-histochemical staining that binds to genetic material and fluoresces blue when excited.

Inert – A substance that elicits no response from the body.

Internal mammary artery – An artery located on the inside of the chest cavity. There is one on

each side.

Ischemia – A condition in which tissue receives an insufficient amount of blood for normal cellular processes.

Lathe- A machining device used to hollow out centered holes in rods.

Mandrel – A rod or hollow cylinder.

Masson's Trichrome – A histological staining that presents collagen as blue, nuclei as black, and cytoplasm as red.

Media – The combination of DMEM, Penicillin, Streptomycin, and fetal bovine serum, used to feed cells in culture.

Patency – The openness of a blood vessel.

Penicillin & Streptomycin – Anti-bacterial agents added to media.

Polydimethylsiloxane (PDMS) - a silicone elastomer that can be poured into a mold and set with the use of heat.

Restenosis – Chronic occlusion of the blood vessels.

Saphenous vein – The vein running down the thigh oriented near the skin on the inner thigh.

Smooth Muscle Cells (SMC) - Muscle cells that encapsulate tubular orifices in the body such as the intestine, esophagus or blood vessels.

Synthetic – Fabricated with non-biological material.

Tap – A machining device used to thread holes.

Thrombosis – The formation of a blood clot that is stationary in the circulatory system.

Trypan blue – A nuclear staining that tags the genetic material of dead cells blue.

Trypsin – An enzyme that digests the adhesive bonds of cells, used to place adhered cells into suspension.

Tubular Construct – An assembly of cells in the form of a hollow cylinder.

Vascular – Relating to blood vessels.

## Appendix A: Pairwise Comparison Charts

Objective	Tubular Assembly	Transparent	Limited Human Interaction	Manufacturability	Short Production Time	Repeatability	Easy to remove	Designers	Client	Designers	Client	Average
Tubular Assembly		1	1	1	1	1	1	6	6	28.6%	28.6%	28.6%
Transparent	0		0	0	0	0	0	0	1	0.0%	4.8%	2.4%
Limited Human Interaction	0	1		0	1	0	0	2	3	9.5%	14.3%	11.9%
Manufacturability	0	1	1		0	0	0	2	2	9.5%	9.5%	9.5%
Short Production Time	0	1	0	1		0	1	3	5	14.3%	23.8%	19.0%
Repeatability	0	1	1	1	1		1	5	4	23.8%	19.0%	21.4%
Easy to remove	0	1	1	1	0	0		3	0	14.3%	0.0%	7.1%

## Appendix B: Supplemental Procedures

### Trypan Blue Staining

The material or plate to be stained is first rinsed with PBS (phosphate buffered solution) to remove any unattached cells. A mixture of PBS and Trypan blue stain (Mediatech, Inc., Catalog Number: 25-900-CL) is made in a 1:1 volumetric ratio. The object to be stained is then submerged in the Trypan blue mixture for 5 minutes. After this step, the object is immersed in PBS repeatedly to remove any excess stain. The blue staining is absorbed by the dead cells and therefore not washed away during rinsing.

In order to complete the Trypan staining if the attached cells are still alive, first wash the unattached cells off using PBS. Next, soak the mandrel in a 70% ethanol solution for 5 minutes to fix the cells, killing them. Finally, stain with Trypan Blue and wash with PBS as described above. Pictures should be taken and the material should be examined for blue stain. In this case, the blue coloring corresponds to the presence of cells. Negative controls should be treated the same way to examine the material's reaction to staining, as materials with small crevices or fibers may trap the stain, resulting in a false positive for cell presence.

### Cell Counting Assay

In order to count the number of cells in a suspension, the mixture is spun down in a centrifuge for 5 min at 1000 rpm. The supernatant is aspirated using a Pasteur pipette and vacuum pump. Care should be taken to ensure that the cell pellet at the bottom is not disturbed. One mL of media is added to the pellet and a 1000  $\mu$ L pipette and tip should be used to triturate the pellet until the solution is homogeneous. Using a 10  $\mu$ L pipette and tips, 10  $\mu$ L of the cell suspension should be removed and placed in a 1 mL conical tube with 5  $\mu$ L of PBS and 5  $\mu$ L of Trypan blue. The total volume of this mixture will be 20  $\mu$ L. Each side of a hemocytometer is

loaded with 10  $\mu\text{L}$  of the cell stain solution. The hemocytometer should be examined under a microscope. In the hemocytometer there are two sets of five boxes. In a set of five boxes, the top one is counted first and then a clockwise rotation. To obtain an accurate cell count, at least 100 cells must be counted; however, the count cannot be stopped in the middle of a box. Cells that are stained blue should not be counted, as only dead cells absorb the stain. Finally, the following formula is used to determine the number of cells per mL.

$$\frac{\text{Cells counted}}{\# \text{ of boxes counted}} \times 2 \times 10^4 = \frac{\text{cells}}{\text{mL}}$$

### Cell Viability Assay

Using the techniques described in the cell counting assay, cells are put into a Trypan blue solution and loaded into a hemocytometer. The total number of cells, including stained and unstained, are counted for all of the five boxes on a side. Subsequently, count the number of blue dead cells in all of the five boxes. Divide the number of dead cells by the number of live cells and multiply by 100 to determine the percentage of dead cells. This number can be subtracted from 100 to arrive at the percentage of viable cells.

### PDMS Mold Production

To make PDMS, a mold must first be created that is the inverse of the desired PDMS shape. Then, the PDMS (Polydimethylsiloxane; Sylgard 184, Dow Corning, Midland, MI) must be mixed with curing agent at a weight ratio of 10:1. To make batches in bulk, 30 mL of PDMS (in a 50 mL conical test tube) and 3.5 mL of curing agent result in the desired weight ratio. After combining the two solutions, they must be mixed by vortex vigorously for at least five minutes. Inside a fume hood the tube can be opened and cast into the mold. The PDMS needs to cure at room temperature for 24 hours.

## Appendix C: Manufacturer Information

### Cell-Adhesion Testing Materials

Mandrel Material	Manufacturer	Catalog #
PETG	McMaster-Carr	9245K17
Garolite	McMaster-Carr	8527K132
Nylon	McMaster-Carr	8628K51
Butyrate	McMaster-Carr	8565K33
Acrylic	McMaster-Carr	8532K11
Glass	Small Parts	B001GOXNWG
PTFE	Small Parts	B000FMZ1NO
Delrin	Small Parts	B000FN1JT8

### Final Design Bill of Materials

Part	Specifications	Manufacturer	Part #
Acrylic Rod	3/8" O.D.	McMaster-Carr	8532K11
Polycarbonate Rod	3/8" O.D.	McMaster-Carr	8571K13
Silicone Adhesive	Premium Waterproof, Clear	GE	GE28490644
Hobby Motor	1.5-3 Volt DC Motor	Radioshack	273-0223
Battery Holder	2-AA	Radioshack	270-382
50 mL Conical Tube	Centrifuge Tube, Sterilized by Gamma Irradiation, Non-pyrogenic	CellTreat	229421
Syringe	3 mL, luer-lok	BD	309603
Three-way Stopcock	Sterile, single-use	Seamless Hospital Products	17-0060
Cryovial	Low Temperature Freezer Vials, Self-standing	VWR International	479-0821
T-75 Cap	75cm <sup>2</sup> Tissue Culture Treated Flask, Vent Cap, Sterilized by Gamma Irradiation, Non-Pyrogenic	CellTreat	229341