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Design of a Four Channel Pulsatile Perfusion Bioreactor for ex-Vivo Study of Vascular Grafts

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ABSTRACT

Cardiovascular diseases are the leading cause of death in the United States. Atherosclerosis in peripheral arteries is a major contributing factor. Autologous saphenous vein grafts are the most common bypass grafts for treating peripheral artery diseases. A quarter of these grafts fail within a year, and around half have failed within 10 years of the initial surgery. Graft failure is attributed to the development of intimal hyperplasia indicated by the migration and proliferation of vascular smooth muscle cells, fibroblasts, and the deposition of extracellular proteins.

Increases in flow, pressure, the pulse frequency, and the differential of pressure that model an arterial environment have been shown to trigger intimal hyperplasia. These tend to occur at the sites of flow disruption most often associated with the anastomotic sites. Turbid blood flow due to flow separation and flow reversal promote positive factors for intimal hyperplasia.

Perfusion bioreactors have allowed researchers to study vein grafts ex-vivo, creating a better understanding of vein remodeling. The goal of this project was to emulate the pressure, flow, and pulse frequency of the peripheral arterial environment. We developed a lab-built four-channel pulsatile flow perfusion bioreactor, which has the unique design features for testing varying vascular grafts and grafting techniques using optical coherence tomography and particle imaging velocimetry.
DEDICATION

I would like to dedicate this work to my family who have loved and supported me throughout my time here in Clemson. We’ve been through so much the last few years and even when things were looking down, they continued to provide me with great enthusiasm and joy for what I love to do. I feel like I’ve missed so much while being so far away them, and I hope the next chapter in my life brings me closer. To my mother, Lauri, you’ve been so strong, loving, and supportive especially these last few years, if I am half as strong as you, I know I will be ready to face anything that comes my way. To my father, Mike, I appreciate all you’ve done for our family, no one has been more loyal and more dedicated than you, it is truly inspiring. To my sister, Katie, you’ve been the best big sister I could have ever asked for, and I hope to always try and be the best little brother you could ever want. To my brother-in-law, Sean, I didn’t grow up with a brother, but I also kind of did. I will always look up to you. To my niece, Millie, you can’t read this yet, but your future is bright, and you have a fantastic family who will support whatever your endeavor. Finally, to my grandfather, Papa, best friends come in many different forms, and I’m so glad that I’ve been able to spend these summers with you, my best friend.
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CHAPTER ONE
INTRODUCTION AND BACKGROUND

Cardiovascular disease is the current leading cause of death for men and women in the United States killing 659,000 people on average every year (American Heart Association 2021). People who suffer from a host of diseases and lifestyle choices such as diabetes, obesity, addiction to alcohol, addiction to tobacco smoking, and physical inactivity are all at increased risk of suffering from cardiovascular disease. It is important to understand both the cardiovascular anatomy, as well as the pathology of its most prominent diseases.

1.1. Blood Vessel Anatomy and Physiology

Understanding the anatomical structure and the physiologic mechanisms involved in blood vessels help to better understand the pathology of diseases that affect blood vessels. As well, this information can be crucial in the development of solutions to cardiovascular diseases.

1.1.1. Arteries and Veins

Arteries and veins are the highway system of the human body, transporting oxygenated red blood cells, nutrients, and chemicals to tissue, as well as transporting de-oxygenated red blood cells, waste, and chemicals away from these tissues. Fluid is always flowing in a loop around the body centering at the heart, the acting pump.

In comparison to veins, arteries have a thicker wall meaning that veins store most of the blood in the body, around 60%. Veins also contain one-way valves to prevent
backflow due to low pressure and to fight the forces of gravity. Flow through arteries is very different from veins. Seen in figure 1, both the pressure and pulse frequency in arteries are significantly greater than in veins. This succeeds in creating two separate environments. The arterial environment which, depending on the order of artery, has an average pressure of around 100 mm Hg ± 20 mm Hg and a frequency of pulse ranging between 60 bpm and 120 bpm at rest. Comparatively, veins only average around 10 mm Hg and no pulse frequency.

Figure 1.1 Blood pressure following the order of flow (Waxman, 2010)

1.1.2. Structure of the Vessel Wall

The three basic parts that make up the wall of a blood vessel are the adventitia, media, and intima. The area where blood flows through is called the lumen. The adventitia is made up of collagen and elastic tissue. The media consists of smooth muscle cells and connective tissue. The intima is primarily made up of endothelial cells and a supporting
layer of tissue. The vessel walls of arteries are much thicker than veins due to the greater thickness of the media layer and the greater amount of smooth muscle cells in that layer.

1.1.3. Hemodynamics

Blood flow is a result of the pumping action of the heart and is driven by the differences in pressure in a unidirectional, laminar flow. It often can be simplified as a fluid flowing through a pipe, where the flow is in a gradient of parallel streamlines, reaching the maximum velocity in the middle and the minimum at the boundaries, seen in figure 2.

![Figure 1.2. Fluid flow through a pipe with shear forces shown](image)

Flow through a pipe is considered laminar when the Reynold’s number is less than or equal to 2100. The Reynold’s number can be defined as the inertial forces over the viscous forces:
The shear stress distribution of the fluid within the pipe is proportional to the velocity gradient and is defined as:

\[ \tau = -\mu \left( \frac{du}{dr} \right) \]

This is to say that the blood flow is driven by pressure creating a variation in velocity that exerts a defined shear stress along the wall of the blood vessel. Steady, laminar flow shear stress is a mechanical signal to endothelial cells to inhibit coagulation as well inhibit the migration of leukocytes and the proliferation of smooth muscle cells. (Pollock et al. 2021; Traub & Berk, 1998)

**1.2. Disease Pathology**

**1.2.1. Peripheral Artery Disease**

Atherosclerosis is the narrowing of the arteries that is indicated by the accumulation of fatty plaques in arteries that obstruct blood flow. During disease progression, plaque buildups, known as atheromatous plaque or atheroma, may burst, triggering acute occlusion (Rafieian-Kopaei et al. 2014). Pathogenesis of atherosclerosis is dependent on a multitude of factors such as the migration of inflammatory response cells such as
monocytes and platelets, proliferation of smooth muscle cells, cell apoptosis, and the formation of fibrous structures.

Peripheral artery disease (PAD) is a debilitating, atherosclerotic disease of the lower limbs that affects between 8 and 12 million people in the U.S. every year (Allison et al., 2007). Blood flow to the leg is restricted and when blood flow demands are less than the ability to supply areas of the leg, patients can experience leg cramping and pain. Intermittent claudication (IC) is a stage of disease progression which is leg cramping and pain during normal physical activity such as walking and can be alleviated through rest. IC is associated with quality-of-life concerns, and increased disease progression could lead to critical limb ischemia (CLI) or acute limb ischemia (ALI). CLI results in symptoms of limb pain, development of ulcers and sores, skin infections, and gangrene while ALI results in symptoms of limb pain, tingling sensation in the legs, pallor, and paralysis. CLI and ALI are both very critical conditions of PAD which are usually treated surgically (Thukkani and Kinlay, 2015).

1.2.2. Surgical Solutions

The goal of surgical solutions for critical peripheral artery disease is prolongment of life and limb salvation. As with most surgical problems, the deciding factor of which solution to choose is based on the case. For example, catheter deployed self-expanding stents are often used in treatment of infrainguinal arteries. Longer segments of atherosclerotic lesions, especially in the femoral artery, are treated using bypass graft surgery, primarily because stents longer than 200 mm have a higher risk of restenosis. These decisions are made by the surgeon, and it is important to note that peripheral artery
bypass grafting is still a relevant surgical solution to PAD (Thukkani and Kinlay, 2015; Slovut and Lipsitz, 2012)

Autologous great saphenous veins are the best conduits for infrainguinal artery bypass surgery (Thukkani and Kinlay, 2015; El-Sayed, 2012; Macario et al., 2008). The great saphenous vein continues to have the highest patency rate among grafting options for PAD and is most often used to bypass the infrapopliteal arteries. This procedure allows for surgeons to salvage limbs and prolong the survival of patients (El-Sayed, 2012).

Figure 1.4. Various surgical solutions to Peripheral Artery Disease (Luck, 2019)
1.2.3. Graft Failure

The flaw of bypass grafting is that saphenous vein grafts under arterial loads can occlude and fail within a period from a few weeks to decades after a patient’s bypass surgery (Grondin et al., 1984). Other issues may arise because some patients may have limited availability of viable saphenous vein tissue (Jafarihaghighi et al., 2020). Without other options, patients may end up having a limb amputation to prolong their lives.

Disease progression starts with migration of smooth muscle cells into the intima and the development of extracellular fibrosis of the intima in a process called interstitial hyperplasia (Saucy et al., 2010). This leads to a stiffening of the graft preventing proper remodeling to occur and eventually leading to the development of thrombosis and atherosclerotic lesions.

Mechanically, the source of these issues can be traced to disruption of blood flow including flow reversals and flow separations which occur at anastomotic sites. Without the high shear, laminar flow of the normal arterial environment, endothelial cells release factors and express surface molecules that favor the development of atherosclerotic lesions, thrombosis, and SMC migration (Traub & Berk, 1998).

Figure 1.5. Effects of positive laminar shear stress and low turbulent shear stress on endothelial cells. *Adapted from Traub & Berk, 1998*
Disruption of blood flow is prevalent at the anastomotic sites where blood flow is disrupted during changes in direction or widening of the lumen (Greenwald 2000). In end-to-side anastomosis, flow reversal and flow separation can be seen creating areas of low flow, low shear stress next to areas of high flow, high shear stress. In end-to-end anastomosis, flow separation is mostly seen at the anastomotic site where the differences in the lumen sizes creates a widening.

Figure 1.6. End to side and end to end anastomosis and the accompanying flow disruption points Adapted from Greenwald and Berry, 2000

1.3. Pulsatile Perfusion Bioreactors

Perfusion bioreactors are effective tools for emulation of arterial blood flow, providing both a steady laminar flow along with a pulsatile pressure and flow pattern modeled after normal arterial blood flow (Jafarihaghghi et al. 2020).

1.3.1. Usage

Perfusion bioreactors are useful in the emulation of arterial blood flow by providing both a steady flow between 100 – 450 mL/min as well as the ability to add a pulsatile
blood flow between 1 – 2 Hz. Providing both a high laminar shear stress environment along with cyclic wall deformation is important for mechanical stimulation and remodeling of saphenous veins in \textit{vitro} \cite{Tosun2015,Saucy2010}.

These bioreactors have applications in tissue engineering through in \textit{vitro} decellularization of veins and arteries into an extracellular scaffold. These scaffolds can then be used as a template for different cells to migrate to and grow on \cite{Hakansson2021}.

Other applications include in \textit{vitro} remodeling of saphenous vein tissue which occurs during the vessel walls exposure to arterial pressure and flow conditions \cite{Saucy2010}. Often these bioreactors are used in lab experiments which look to show the biochemical pathways involved in vessel wall remodeling, or in experiments designed to see the physical changes in the vessel wall over the days remodeling is occurring.

1.3.2. \textbf{Requirements of Pulsatile Perfusion Bioreactors}

Pulsatile perfusion bioreactors (PPBs) come in many different designs that serve different functions specific to the independent researcher. Still, there are some basic rules that almost all PPBs must be designed to follow. The most important requirements include sterility, fluid perfusion and pulse, closed loop system, and the ability to pressurize the lumen. Other requirements depend on the use of the specific bioreactor. For tissue culture, the bioreactor should be able to exchange media every few days, as well, the bioreactor should be able to house inside of a cell culture incubator. Also, the bioreactor should allow for gas exchange to maintain pH balance.
CHAPTER TWO

PROJECT APPROACH

2.1. Project Overview

Saphenous vein bypass grafting is currently the most effective surgical option for peripheral artery disease (PAD) in long lesions. Issues still arise that lead to graft failure, and, in some patients, failure can occur soon after initial surgery. This puts more people into the operating room, increasing morbidity and mortality of PAD. The goal of this project was to design, build, and test a four-channel pulsatile perfusion bioreactor that emulates pressure and flow profiles while maintaining graft viability for the duration of two to four weeks. This bioreactor is designed to be incorporated into a custom optical coherence tomography imaging system.

2.2. Project Justification

Pulsatile perfusion bioreactors come in many different forms for different uses throughout the world of research. Often these bioreactors are used for experiments focused on tissue culture and the physical traits associated with vascular remodeling and the development of intimal hyperplasia. However, there is currently a gap in research, specifically in live imaging of vascular grafts, that this bioreactor design aims to fill. This design is adapted to a custom-built optical coherence tomography imaging system as well as a scanning microscope stage. The combination of the three should work to further the understanding of the biomechanical forces involved in vascular remodeling and the development of intimal hyperplasia.
2.3. Project Aims

The first aim of this project is to design and build a pulsatile perfusion bioreactor that can accommodate four sample grafts while keeping in mind the requirements and restrictions of the labs OCT imaging system. The bioreactor should emulate natural, human pressure wave forms at varying frequencies between 1 Hz and 2 Hz, and varying pressures between 60 mm Hg and 180 mm Hg. It should also emulate natural, human flow wave forms ranging from 100 mL/min to 450 mL/min. Both factors need be monitored in real time and the system should have the ability to easily adjust parameters such as pressure, pulse rate, and flow. All parts must be able to be sterilized either by steam sterilization (preferred), or by ethylene oxide (increases cost). The bioreactor should be consolidated and easily transportable, preferably contained on one plane of height. The bioreactor should allow for gas exchange and transport of nutrients to the sample grafts. Media change should be easy to do without risking contamination. The entire bioreactor system should fit within a cell culture incubator. For this bioreactor, grafts should be kept in individual cells that allow for the application of drugs to the ablumen without applying those drugs to the entire system. This will be restricted by the depth of diffusion of the drugs to prevent mixing into the lumen. Finally, a procedural protocol should be developed to maximize efficiency in both graft anchoring and inclusion in the bioreactor loop.

The second aim of this project is to test and validate the bioreactor. The initial validation is applying and maintaining pressure, as well as creating a pressure waveform that emulates natural, human pressure waveforms of the femoral artery. The second
validation is emulating the flow wave form of human femoral arteries using particle image velocimetry and turbine flow sensors. The final validation is maintaining tissue viability and sterility of the bioreactor over a two-week period. Both sensor calibration and the following of proper sterility procedures is crucial for this aim.
CHAPTER THREE

REVIEW OF PULSATILE PERFUSION BIOREACTORS

Perfusion bioreactors are effective tools for emulation of arterial blood flow, providing both a steady laminar flow along with a pulsatile pressure and flow pattern modeled after normal arterial blood flow (Jafarihaghighi et al. 2020). This review will look at five separate experimental perfusion bioreactors to show the current state of the technology, and what exactly goes into building a perfusion bioreactor.

3.1. Bioreactor Designs

Dummler et al. used an experimental bioreactor to study the expression of the MMP-2 protein after exposing human saphenous vein grafts (HSVG) to arterial conditions over a two-week period. Small portions of HSVGs were acquired from 35 patients (average age 71.4 ± 7.7 years, 9 females, 26 males) undergoing a coronary artery bypass surgery and stored on ice until mounted within the perfusion flow system. The perfusion flow system consisted of a ISMATEC S2 roller pump, a custom vessel chamber, a syringe pump, two pressure sensors fixed at the inlet and outlet of the vessel chamber, and a PC to control the pumps and monitor pressure. This was all contained within a Styrofoam insulated incubator at 37°C. The HSVGs were perfused during various time periods in either venous conditions (flow = 5 ml/min, pressure = 10 mmHg, n = 12) or arterial conditions (flow = 50 ml/min, Pressure = 100 mmHg, n = 12). The pressure model used ranged between 90 and 105 mmHg at 1 Hz. This experiment found that the protein MMP-2 is upregulated during HSVG exposure to arterial blood flow conditions which has been linked to the process of remodeling. It also determined that HSVGs supported the arterial
environment for one week, but after 12 days, the viability of the cells within the graft began to decrease (Dummler et al. 2011).

Saucy et al. utilized a perfusion bioreactor to study the relationship between the arterial environment and intimal hyperplasia. Human saphenous veins segments were acquired from nine patients (average age 67.3 ± 0.5 years, 3 females, 6 males) undergoing peripheral bypass surgery. Parts of each segment were kept as control, half stored in formal, half frozen in liquid nitrogen. The other segments were perfused in an experimental perfusion bioreactor that was made up of a peristaltic pump to provide a 4 to 420 ml/min steady flow, a gearing pump to provide a pulsatile flow and pressure waveform, a vessel chamber made of acrylic, a media cylinder to provide capacitance, pressure and flow transducers, and a computer to control and monitor flow and pressure. The media cylinder and the vessel chambers were kept inside an incubator. Vessels were attached to stainless steel tube fittings using sutures. Perfusion experiments were run for 7 and 14 days. After seven days it was found through histomorphometry that intimal thickness had increased and had reached its maximum thickness within seven days. Also, plasminogen activator inhibitor 1 was increased in samples perfused over 7 days. After 14 days the same intimal thickness could be seen as well as a similar amount of plasminogen activator inhibitor 1. This group successfully accomplished the goal of this experiment which was to test the viability of their experimental perfusion system for the remodeling of human saphenous veins (Saucy et al. 2010)
Figure 3.1 Above shows the increasing intimal thickness from the control segments to the perfusion segments from 0 days to 14 days. Intima thickness increased over the first 7 days and stayed constant from day 7 to day 14. (Saucy et al. 2010)

Tosun and McFetridge experimented with a perfusion bioreactor to look at the effects of varying pulse frequencies on vSMC phenotype switching during remodeling. The pulse rate of an individual was measured throughout a 12-hour period and was used to model the pulse frequencies of the perfusion bioreactor. A control was set at a constant pulse frequency of 1.3 Hz. The experimental pulse rate was then used in a 12-hour varying, 12-hour constant model, and a 24-hour varying model, repeating the 12-hour sample with no constant frequency. The varying frequencies ranged from less than 1 Hz to 1.7 Hz. Human vSMCs were sourced from umbilical arteries and seeded onto acellular scaffolds derived from human umbilical veins. These were perfused under different
conditions using an experimental perfusion bioreactor. This perfusion bioreactor was made up of peristaltic pumps (model # EW-68332-06, Cole Parmer, Vernon Hills, IL) which were linked in series to three vessel chambers. Two separate media chambers were used for the perfusion of the lumen and the recycling of media within the ablumen. A computer program was used to increase or decrease the RPM of the peristaltic pumps to vary the frequency of pulses. Pressure transducers were placed at the outlets of the last vessel chamber. Variability of pulse frequency showed to have a profound effect on vSMC seeding and remodeling driving cells to a synthetic state. The graft also showed increased tensile strength, increased stiffness, and less vasoactivity 24-hour variation of the pulse frequency showed the highest tensile strength and young’s modulus, with little to no vasoactivity, representative of development of intimal hyperplasia and resulting occlusion of the vein graft. Overall, this experiment shows the necessity of varying pulse frequencies to accurately emulate arterial blood flow conditions in vitro (Tosun and McFetridge 2015).
**Figure 3.2** Diagram of the perfusion bioreactor used in the Tosun and McFetridge experiment. Media for the lumen and ablumen were kept separately and recycled separately. Vessel chambers were linked in series. *(Tosun and McFetridge 2015)*

Prim et al. developed an experimental perfusion bioreactor to test porcine replacements of commonly used human bypass graft sources. These included the great saphenous vein, the internal thoracic artery, and the radial artery. Graft tissues were harvested from freshly slaughtered pigs (~200 kg; 3 years old) and stored in a modified Moscona’s saline solution. The perfusion bioreactor consisted of a MasterFlex L/S Easy-Load II roller pump, two custom vessel chambers, a pinch valve, and a pressure transducer all contained inside of an incubator at 37°C and 5% CO₂. All grafts were perfused using their native environmental conditions as well as a set coronary-like arterial condition. The coronary perfusion was done at a pressure of 102 mmHg +8.4 with a pulse of 1.33 Hz and a flow rate of 0.52 +0.13 mL/s. After 7 days of perfusion, the internal thoracic artery had significantly lower intimal and medial cell proliferation compared to the perfused radial artery and great saphenous vein. Gene expression was similar in all graft models and the radial artery and saphenous vein seemed to experience pathological remodeling that was not seen in the internal thoracic artery or the in-situ graft models. Molecular pathways linked to tissue remodeling were activated as quickly as 6 hours after introduction to the arterial environment *(Prim et al. 2018).*
Hahn et al. utilized a pulsatile perfusion bioreactor to test the effects of biochemical and biomechanical stimuli on vascular cells over a 7-week perfusion period. In this study, vascular cells were grafted onto a poly (ethylene glycol) hydrogel scaffold and perfused at both pediatric heart rates (2 Hz), and adult heart rates (1 Hz), with a min diastolic pressure of 80 mmHg and a maximum systolic pressure of 120 mmHg. The perfusion system consisted of a MasterFlex peristaltic pump, a 100 mL glass compliance chamber, CellMax pulsatile pumps, check valves before and after the pulsatile pump, the vein graft chamber, a pressure transducer, and a media chamber. The system was housed in a tissue culture incubator at 5% CO$_2$ and 37°C. Pulsatile flow perfusion had a positive impact on collagen content as well as the elastic moduli and tensile strength of the grafts when compared to static culture. As a proof of concept, these results supported their hypothesis.
that a pulsatile flow perfusion system could be used to improve tissue engineered vascular grafts (Hahn et al. 2006).

**Figure 3.4** (a) Bioreactor schematic as well as (b) the pressure versus time waveform, and (c) pictures of the bioreactor in use. *(Hahn et al. 2006)*
Piola et al. developed a pulsatile perfusion bioreactor to study the conditions in which saphenous vein grafts fail. Their system is designed to cyclically load and unload vein grafts over defined periods of time. The loads were designed to mimic physiological venous conditions with constant low pressure (5 mm Hg) as well as arterial conditions with higher pressure (80 mm Hg – 120 mm Hg). The design was not intended to emulate flow, but merely to allow for cyclic recirculation of media in the vessel lumen. A roller pump in conjunction with an electronic pinch valve created the loading, pulsatile loading, and unloading phases. Experiments were run for validation that looked at pressure versus volume change within the vessel, tissue viability evaluation using MTT staining, morphological and immunofluorescence assessments, and morphological measurements. They found that their bioreactor successfully provided the necessary cyclic loading and unloading for their desired experiment (Piola et al. 2013).

![Bioreactor Design and SVG Anchoring](image)

**Figure 3.5** Shows the bioreactor design as well as the pictures for the anchoring of the SVG procedure. *(Piola et al. 2013)*
### 3.2. Current State of the Art

<table>
<thead>
<tr>
<th>Reference</th>
<th>Bioreactor Type</th>
<th>Tissue Type(s)</th>
<th>Flow Driver And Pulse Actuator</th>
<th>Pressure Parameters (mmHg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dummler et al. 2011</td>
<td>Pulsatile Perfusion Bioreactor</td>
<td>Human Great Saphenous Vein</td>
<td>Ismatec S2 Roller Pump, NA</td>
<td>Venous emulation: 10 (n=12)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Arterial Emulation: 100 (n=12)</td>
</tr>
<tr>
<td>Prim et al. 2018</td>
<td>Pulsatile Perfusion Bioreactor</td>
<td>Porcine coronary artery (LAD), internal thoracic artery (ITA), radial artery (RA), and great saphenous vein (GSV)</td>
<td>Cole-Parmer Masterflex L/S Easy-Load II Roller Pump Head + Cole-Parmer Masterflex L/S Roller Pump, NA</td>
<td>In Situ: LAD = 102 ± 8.4, ITA = 100 ± 9.5, RA = 94 ± 2.3, GSV = 15 ± 1.9, Coronary: P = 102 ± 8.4</td>
</tr>
<tr>
<td>Tosun and McFettridge 2014</td>
<td>Pulsatile Perfusion Bioreactor</td>
<td>Human Umbilical Vein (Decellularized)</td>
<td>Peristaltic Pump, NA</td>
<td>Dual Perfusion Acellular Control: 120/80, Dual perfusion high pressure: 80/120, Single perfusion low pressure 30-50, Single perfusion high pressure 80/120</td>
</tr>
<tr>
<td>Piola et al.</td>
<td>Pulsatile Perfusion Bioreactor</td>
<td>Human Great Saphenous Vein</td>
<td>Watson Marlow 323D Peristaltic Pump, Solenoid Pinch Valve (s305-09, SIRAI Elettromecanica)</td>
<td>Coronary Artery: Hypotension = 75 ± 15, Normal = 100 ± 20, Hypertension = 120 ± 20, Venous: 5 mmHg</td>
</tr>
</tbody>
</table>

Table 3.1. Current state of the art of pulsatile perfusion bioreactors.
Table 3.1. (continued) Current state of the art of pulsatile perfusion bioreactors.

Current devices being used in research focus on tissue engineering and remodeling using genetic, biochemical, or histological analysis. No pulsatile perfusion bioreactors in the literature were integrated within an OCT imaging system and none of the papers included OCT imaging as a methodology for studying graft remodeling. There is need for a pulsatile perfusion bioreactor that can be integrated within an OCT imaging system and can also be actively imaged during graft perfusion. This should allow our group to study
the biomechanical forces involved in physiological and pathological remodeling of vascular grafts.

3.3. Tissue Culture Media

<table>
<thead>
<tr>
<th>Source</th>
<th>Primary Media</th>
<th>Serum</th>
<th>Additives</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dummler et al. 2011</td>
<td>DMEM</td>
<td>10% FCS</td>
<td>2 nM Glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin</td>
</tr>
<tr>
<td>Saucy et al. 2010</td>
<td>RPMI-1640 + Glutamax</td>
<td>30% FCS</td>
<td>2% 70-kDa dextran, 10,000 U/ml penicillin G, 10,000 µg/ml streptomycin sulfate, 25 mg/ml Amphotericin B, 0.5 µg/ml gentamycin</td>
</tr>
<tr>
<td>Tosun and McFetridge 2014</td>
<td>DMEM</td>
<td>10% FBS</td>
<td>1% L-glutamine, 1% penicillin streptomycin</td>
</tr>
<tr>
<td>Prim et al. 2018</td>
<td>DMEM</td>
<td>10% FBS</td>
<td>1% penicillin streptomycin, 1% amphotericin B, 1% streptomycin</td>
</tr>
<tr>
<td>Hahn et al. 2006</td>
<td>MEM</td>
<td>10% FBS</td>
<td>10 ng/ml TGF-B1, 10 µg/mL insulin, 50 mM L-ascorbic acid, 10 µg/ml ciprofloxacin, 100 µU/ml penicillin, 100 mg/L streptomycin, 0.25 µg/mL fungizone</td>
</tr>
<tr>
<td>Piola et al. 2013</td>
<td>DMEM</td>
<td>10% FBS</td>
<td>1% L-Glutamine, 1% penicillin streptomycin</td>
</tr>
</tbody>
</table>

*Table 3.2.* List of media used in reviewed papers.
CHAPTER FOUR
DESIGN OF A PULSATILE PERFUSION BIOREACTOR

Pulsatile perfusion bioreactors are designed to provide the necessary amount of nutrient and gas exchange to cardiovascular tissue, while also modeling the physical environments of the cardiovascular system (Jafarihaghighi et al. 2020). Small diameter vascular grafts are important to study, and a bioreactor designed specifically for saphenous vein grafts would increase the current understanding of grafting strategies and graft failure.

4.1. Design Needs

A bioreactor is a device that provides a controlled environment that maintains certain conditions conducive for the development of biological and biochemical processes ex vivo. These conditions include pH, temperature, pressure, delivery of nutrients, and the transport of wastes. Perfusion bioreactors accomplish this through the movement of fluids over or through cells/tissues (Martin et al. 2004). A pulsatile perfusion bioreactor also adds the extra element of varying pressure and flow at a given frequency.

Maintaining these conditions is vital for the survival of the cells being studied. Both pH and temperature can be maintained in a cell culture incubator. Literature shows the most ideal temperature to be 37 Celsius and the CO₂ concentration to be 5% to maintain pH. Bioreactors must also be designed with gas exchange in mind, either through the use of silicone tubing or air filters for gas exchange.

Sterility is crucial to maintain during assembly as well as during operation. The biggest risk of sterility compromise is during the assembly process. Assembly should be
conducted under a sterile, laminar flow biological hood using proper PPE and aseptic techniques. Media should also be sterile and media change should be done underneath the bio-hood. Finally, the system needs to be sealable to prevent any exposure to the open air, and place where gas exchange occurs should be done through a sterile air filter.

The design should consider the delivery of nutrients, oxygen, and the transport of waste to and from the tissue. In the case of a pulsatile perfusion bioreactor, this accomplished through a media flow loop, powered by a peristaltic pump. This creates a flow of media through the lumen of the tissue preventing stagnation and a constant supply of gas and waste exchange. A second actuator mechanism is required to change the pressure and flow of the loop for pulsatile flow. This can be accomplished using physical actuators, roller pumps, pneumatic pressure systems, or electronic pinch valves. Finally, media should be changed every 2 to 3 days to optimize nutrient and gas exchange.

Fluid pressure and flow measurement is also prioritized in the bioreactor design. This is both for validation and control of the pressure and flow waveforms. Flow and pressure measurements should be conducted with the least amount of impact on the experiment. As well, pressure and flow readings should be instantaneous and easily accessed through the bioreactors programming. Finally, sensors need to be accurate enough to give reliable measurements of pressure and flow, and sensors should be calibrated to their specifications.

Finally, the design should have a defined form which serves to decrease the complications and fragility of the individual parts. Having four channels, this bioreactor
has many parts which increase the risk of misplacement and sterility compromise. The overall form of the design should allow for the best possible ease of assembly and transport. This form should also consider the internal dimensions of the desired cell culture incubator. Total cost to build shouldn’t exceed more than $5000.

4.2. Design Phases

When designing anything, it is important to consider and follow a basic design structure. For designing our bioreactor, we used the structure seen in figure 4.1.

Figure 4.1. Bioreactor design protocol pentagon
4.2.1 Research and Review

Extensive literature research of pulsatile perfusion bioreactor designs was conducted to pinpoint what exactly was needed to build our own. These bioreactors have already been covered and discussed in section three. From these experimental designs we created a list of necessary components.

![Figure 4.2. List of components gathered from literature review.](image)

The first component necessary to the design is a mechanism for driving flow, most cases used a peristaltic pump. Our design would use a MasterFlex L/S digital peristaltic pump.
The second component, the pulse actuator, had the most variability in form and function. Some designs used roller pumps (Dummler et al., Saucy et al., Prim et al.), one used a Berlin Heart (Heise et al. 2003), and other designs used pinch valves (Piola et al.). Each was different in their approach, but the overall goal was to create a pulsatile pressure wave form. In our case, our goal was to decrease system noise as much as possible while also retaining a simplistic design. It was decided to use a pneumatic design similar to that of the Berlin Heart that could be 3D printed and easily assembled. This design would use a polydimethylsiloxane (PDMS) membrane as the barrier between the fluid and the air.

The third component important to the design is the vessel chamber. This varies depending on the needs of the experiment, but the same requirements hold true for most. The vessel chamber is where grafts are mounted, often done using surgical sutures. The design of the chamber should allow for media to completely cover the outside of the graft and allow for cyclic change of that media. Some designs incorporated perfusion of the ablumen space as well (Tosun and McFetridge 2015), but for the purposes of this experiment that won’t be necessary. Some experimenters used sealed containment areas while other simply covered their containers, the difference seems to be the sterility of the environment in the cell culture incubators. All vessel chambers were made from clear, biocompatible materials such as glass, polyethylene terephthalate (PET), polystyrene, or acrylic. Often these containers were custom made adaptations of common lab containers used in cell culture. The reproducibility of these containers was thus expensive or time
consuming. Our design would be a container which would be 3D printed in an autoclavable material such as polyether ether ketone (PEEK) or a photopolymer resin.

The fourth component is the compliance chamber, which is used to reduce noise as well as provide the minimum diastolic pressure. Almost all of the experiments used compliance chambers, and most were either syringe tubes or glass bottles. Syringe tubes were often used with electronic syringe pumps to adjust the minimum diastolic pressure during the cycle. For our bioreactor we will use custom glass bottles which will be pressurized using one-way pressure bulbs from sphygmomanometers.

Finally, at least one check valve is needed to keep flow unidirectional during perfusion. Most experiments used one way check valves, those that didn’t were using roller pumps that wouldn’t have any reversal in flow. In this experiment, one-way valves will be necessary.
4.2.2. Form Design

With the understanding of what basic components are needed from our literature review, we were able to create a schematic design to work from, seen in figure 4.3.

**Figure 4.3.** Schematic of the pulsatile perfusion system using both a peristaltic pump and pneumatic pressure pump

This design would use the MasterFlex L/S digital peristaltic pump to provide a steady continuous flow media into a pneumatic pressure pump. This pressure pump would be controlled with a solenoid valve to provide increased air pressure from the house system. Media would then flow through a flow straightener and split into four channels into the bioreactor. Finally, media would flow back into the compliance chamber where the peristaltic pump would begin the cycle again. Pressure and flow sensors would be placed before and after the vessels.
4.2.3. Functional Design

Using the form design, we created a design with detailed parts which can be seen in figure 4.4.

![Functional design with detailed parts](image)

**Figure 4.4.** Functional design with detailed parts

This bioreactor would use 3D printed parts for the vessel containment as well as the pressure box, labeled diaphragm box in figure 4.4. An Arduino mega would be used to control the solenoid valves as well as collect voltage using analog read to measure the pressure both in and out of the sample grafts. The vessel chamber would be one solid piece with four wells for each graft and would be enclosed with a clear acrylic lid. Vessels would be mounted to metal barbes and held in the center of the wells using 3D printed scaffolds. Finally, two capacitance bottles would be used before and after the pressure box to reduce noise. The bottles would be fitted with a glass barb for the outlet, and custom, 3D printed GL45 caps with barbs for the inlet media and the air pressure.
4.2.4 Build

Parts were ordered and gathered based on a list of parts. Custom made parts were created in SolidWorks 3D modeling program and printed using a CreatBot F430 filament 3D printer. As a draft, parts were initially printed in PLA and then final drafts were printed with PEEK.

Figure 4.5. SolidWorks 3D model of the vessel container system.

Two “keys” would be used along with support pieces to form the graft anchoring scaffold. The scaffolds would be mounted to the vessel container keeping the vessels submerged in media.
Figure 4.6 SolidWorks 3D model of the pressure pump assembly

The pressure pump assembly above is designed to fill with media and is sealed with a layer of PDMS. The PDMS membrane is spun at around 300 – 400 rpms for 60 seconds to reach a thickness of 300-400 microns. Norgren solenoid valves control the air pressure entering and leaving the pressure pump. The pulse frequency is controlled by sending a signal from the Arduino to the solenoid valves to open or close.
Figure 4.7. Solenoid Valve from Norgren

Figure 4.8. Circuitry diagram of the solenoid control using an Arduino Uno and 12V power supply
Parts were printed and assembled using the functional design diagram as a guide. In place of sample grafts, 1/8” silicone tubing was used. In place of media we used water to identify points of failure and receive feedback on this design.

4.2.5 Feedback

After building and testing of the bioreactor, several flaws in the initial design were identified. These included flaws in the material, as well as the overall form of the design. Here we will discuss those flaws.

Filament 3D printing was initially chosen due to its accessibility and ease of custom part building. However, filament 3D printing proved to be too difficult to work with at this scale. First, this form of 3D printing limits the geometric detail especially in places of overhangs. Larger details can be scaffolded and still come out without flaw, but smaller details tend to warp or deform during scaffolding. As well, due to the layering process, solid parts under pressure leaked through the material. In a system designed to be under 80 mm Hg to 120 mm Hg of pressure this flaw was extremely detrimental. A different material and printing method would need to be sourced to create the parts we desired.

Another flaw in the initial design was over complication, which was better seen after building the bioreactor. Having three different tubing sizes (3/8”, 1/4”, and 1/8”) was unnecessary and created to many points where reduction fittings were needed. Also, many parts of the bioreactor were fragile and would need to be anchored to something to prevent the tubing from moving or knocking over these parts and breaking them. A new design that included an anchoring system would need to be developed to prevent movement of parts as well as allow for ease of transport in and out of the incubator.
Finally, a sealed vessel chamber with a smaller interior volume would be beneficial towards the end design. This would improve media changes, transport, and decrease risks of contamination.
4.3. Making Improvements

After identifying issues with the original design, a new design can be made in consideration of these needs. This design can be seen below in figure 4.9.

Figure 4.9. Perfusion system design with anchored parts and simplistic form.

This design is intended for part anchoring to a 12”x18” anodized aluminum breadboard with 1/4” screw holes spaced 1” apart in a grid pattern. Every loose part should be anchored to the board to prevent movement during transport. This also keeps the vessels on the same plane of height as the pressure sensors reducing inaccuracies in
the pressure readings. External anchoring parts are not shown, but they were created in SolidWorks and printed using PLA filament 3D printing. This new design also limits connections outside the incubator to only a few electrical wires.

4.3.1. Choosing a New Material

Considering the drawbacks of filament 3D printing, an improved method to develop our parts was needed. Resin cured 3D printing used liquid resin and a UV curing process to make smaller, more detailed parts with limited scaffolding. This technology is often used in the dental industry to make molds out of a biocompatible resin. Clemson’s Bioengineering department added a new Bioreactor Development Facility in the summer of 2021 specifically for the purpose of creating 3D parts using resin-based printing. With this new resource we were able to develop new parts. The printer used to make these parts is a Formlabs Form 3B+ resin curing printer.

4.3.2. Pressure Pump

![Figure 4.10. New pressure pump assembly printed in BIOE amber dental resin](image)
The new pressure pump design is similar to the original. The inner geometry is circular with an entrance and exit for the media. Barbed fittings are now used to ensure a proper seal for the 1/4” tubing. The air pressure ports are now parallel to the PDMS membrane to reduce noise caused by the flow of air over the membrane. A base was created with 1/4” holes for anchoring to an aluminum bread board.

4.3.3. Vessel Chamber

Figure 4.11. Vessel chamber with luer lock connections for reading pressure and changing media

This is a small vessel chamber design originally created by Dr. Lee Sierad which we modified to fit our needs. The vessel chamber consists of three parts, the base, the middle, and the top. The middle part can be seen above in figure 4.11. which contains all the complexities of the assembly. There are two O-ring tracks, on each side for silicone O-ring seals. There are 1/8” connectors at each end and block lock connectors for the placement of small O-rings that lock in vessels without the need for suture ties. Luer
connectors are on every corner, two of which are connected to the interior of the chamber for media change, and the other two which are connected to the interior of the tube for pressure measurements. This allows for the shortest possible distance from the pressure measurements and the ends of the graft. Six screw holes outline the structure to create a sealed object. The vessel chamber is clamped to the bread board using L-shaped PLA clamps.

Figure 4.12. Assembled vessel chamber with a porcine vein and DMEM media

Future experiments that will utilize this bioreactor require a different design for the vessel chamber, one that accommodates for a water dipping objective. This will be used during OCT imaging of graft segments. The adjustment being made is only to the top section of the vessel chamber. In this adjustment, an area is created to allow the objective to scan in both the x and y direction. As well, a microscope slide can be fixed underneath
the vessel chamber top acting as the only barrier between the fluid in the chamber and the microscope objective.

Figure 4.13. Modified vessel chamber top for use with a 24 x 60 mm microscope slide

4.4. Measuring Pressure and Flow

Measuring both pressure and flow in real time is necessary for calibration and validation of pressure and flow waveforms. Pressure can be measured at multiple points in our bioreactor. This includes the entrance and exit sites of each sample graft. Flow can be measured in two different ways, using particle image velocimetry, or using sterile turbine flow sensors. The advantage to using PIV to measure flow is that it isn’t invasive. The disadvantage of using PIV is that the measurements wouldn’t be live. A turbine flow sensor would give us the ability to receive live data and process that data into flow measurements.
4.4.1. Pressure

The bioreactor we designed is a four-channel system, which means that if we measured pressure at the entrance and exit of each graft, we will have a total of eight individual sensors. This creates an issue with space, so the sensors that are chosen must be small, compact, and easy to implement. One of the best ways to accomplish this is to use board mounted pressure sensors, which are both small, compact, and limited to only a few wires to connect.

For this bioreactor we chose to use Honeywell SSCDRRN005PDAA5 differential pressure sensors. These are directly mounted to the breadboard and are powered by the Arduino Mega prototyping board. An analog voltage signal is sent to the Arduino that can be converted to a pressure reading using LabView with LINX programming.

Sensors are calibrated using column pressure at known heights and known fluid density. The relationship between voltage and actual pressure is linear.

4.4.2 Flow

Flow velocity is measured using a Cole-Palmer turbine flow sensor catalog # EW-25507-44. The sensor is connected to a data acquisition device (DAQ) and binary data is used to read flow velocity. The flow sensor is pre-calibrated and specifications for system calibration are provided by Cole-Palmer.
4.5. Final Assembly

![Bioreactor assembly in the cell culture incubator.](image)

Custom 250 mL glass bottles make up the media well and capacitance chambers. The MasterFlex L/S peristaltic pump transfers fluid between the two bottles and drives flow in the system. Fluid is drawn and pumped using the custom pressure pump. A solenoid controls air pressure in the head space which applies a force to the fluid. A one-way valve ensures that the pressure pump doesn’t reverse flow. Y-splitters divide the flow into four channels that run through the vein. A pinch clamp creates a closure that increases resistance and can be used to control end diastolic pressure.

**Figure 4.14.** Bioreactor assembly in the cell culture incubator.
4.6. User Interface

NI LabView 2020 is used to show pressure measurements as well as provide an interface for solenoid opening and closing.

**Figure 4.15.** Bioreactor control interface

Solenoid control is based on time in milliseconds. In this control format, time open and time closed are the only two inputs. Multiple inputs can be used to add more details to the pressure waveform if there is demand for that specificity.
CHAPTER FIVE

TESTING AND VALIDATION OF A PULSATILE PERFUSION BIOREACTOR

5.1. Pressure Validation

After bioreactor assembly, a validation of pressure function was conducted. This was done at 60 bpm sampling at a frequency of 20 Hz. End diastolic pressure was equal to 90 mm Hg and end systolic pressure was equal to 115 mm Hg. Solenoid valve opening time was 750 ms per cycle and closing time was 250 ms per cycle. Input air pressure was tuned to increase pressure from 90 to 115 mm Hg in the 250 ms timeframe.

![Pressure waveform at 60 bpm, 5 seconds](image)

**Figure 5.1.** Pressure waveform at 60 bpm, 5 seconds
Figure 5.2. Actual pressure waveform (purple, dashed) set at 60 bpm versus simulation pressure waveform of the femoral artery (orange, solid) at 60 bpm.
5.2. Compatibility Validation

System compatibility with vein graft segments can be confirmed using simple experiments based on graft remodeling.

5.2.1. Graft Collection and Preparation

Saphenous vein grafts of sacrificed pigs are collected from Clemson’s Godley-Snell research facility where we can assure that they are as fresh as possible. The vein is segmented into four parts 9 cm in length. Before any other manipulation occurs, fresh samples of 1 cm long rings are excised. These samples are rinsed in Hank’s Balanced Salt Solution (HBSS) and flash frozen in liquid nitrogen. The rest of the veins are immediately stored in a solution of HBSS supplemented with 20 U/mL heparin, 1% penicillin streptomycin, 1% amphotericin B, and 1% gentamycin. After transportation back to the lab, vein grafts are cut to size, mounted, and secured to the vessel chambers. Chambers are filled with media consisting of DMEM supplemented with 10% FBS, 1% penicillin streptomycin, 1% amphotericin B, and 1% L-Glutamine. Time is recorded from removal to implementation into the bioreactor.

5.2.2. Perfusion Cycle

The vessel chambers are mounted to the bioreactor, and perfusion of the vein grafts begins by removing all air bubbles within the system. This is done by applying pressure to the head capacitance bottle and leaving the tail bottle unsealed. Once all air bubbles can be visually confirmed, steady flow is started using the MasterFlex L/S peristaltic pump. Flow is set at 150 mL/min. Pressure is tuned to an end diastolic pressure of 80 mm
Hg with an end systolic pressure of 120 mm Hg, and a pulse rate of 60 bpm. Pressure and flow are monitored using the LabView user interface. The bioreactor is maintained inside the cell culture incubator at 37°C and a CO₂ concentration of 5%. Media is changed every 2 days. The perfusion of the vein grafts is done for 7 days. After perfusion is complete, 1 cm long samples are taken from the middle of the vein grafts and are set in OCT and frozen for histological analysis at a later time. Another 1 cm sample can also be taken from each graft to conduct MTT staining.

5.2.3. Histological Analysis

Samples taken before and after perfusion are fixed in OCT and sectioned with a microtome at a thickness of 5 µm. They are then stained with hematoxylin/eosin. Images of the stained sections are analyzed using ImageJ to calculate the average intimal thickness.

5.2.4. MTT Staining

MTT staining can be performed on the extra graft samples to test the viability of the vein graft segments after and before perfusion. Samples are added to a serum-free medium with MTT diluted to 0.5 mg/mL. They are then incubated at 37°C for one hour. MTT staining can be used by comparing the change in color. Viable cells will convert the yellow MTT solution into a purple product. Thus, viable tissue will show up stained purple and dead tissue will show up stained yellow.
CHAPTER SIX
CONCLUSIONS

The development of a pulsatile perfusion bioreactor was necessary for the testing of vascular grafts and grafting techniques. This bioreactor should have the capability to induce a flow and perfusion profile that emulates that of the arterial environment. Understanding the conditions that may affect the remodeling and the failure of saphenous vein grafts will hopefully contribute to the overall understanding of bypass grafting. The results above show that this completed pulsatile perfusion bioreactor has the following capabilities.

1. Emulation of physiological pressures, flow, and pulse frequencies which can be maintained over long periods of time.

2. The ability to provide gas and nutrient exchange in both the lumen and the ablumen for sample blood vessels.

3. A form that allows for the transport to and from various imaging systems and special adaptations to allow for optical coherence tomography imaging.

4. Methods of measuring flow and pressure in real time

5. A user interface which can display pressure and flow while allowing for solenoid opening control.

6. Reproducible custom parts which can be printed at a lower cost to alternative methods of part development.
CHAPTER SEVEN

RECOMMENDATIONS

1. The next step for this bioreactor is to complete validation experiments including compatibility and flow validation. This falls in line with the need to fine tune settings of pressure, flow, and pulse to the specific experiment.

2. Vessel chambers are currently only designed to accommodate a singular graft length and diameter. Adaptations to vessel chamber size are needed to include grafts of various length. Adapters are needed to include more vessel diameters.

3. Fine tuning and practice of bioreactor implementation into the lab’s optical coherence tomography imaging system should be conducted.

4. An experiment involving decellularization of saphenous vein tissue should be performed to test these bioreactors capabilities in recellularization of a tissue scaffold.

5. A lumped parameter network model including the parameters from the bioreactor should be created to assist in matching physiological and pathological patient data to the pressure and flow profiles being created by the bioreactor.

Improvement of this pulsatile perfusion bioreactor should be constant and based on the experimenter’s experience. This ensures that the bioreactor stays useful to the lab and that the versatility of the bioreactor increases over time.
Appendix A

Block diagram of bioreactor user interface
REFERENCES


Luck, J. C. (2019). Effects of peripheral revascularization on blood pressure and calf muscle oxygen saturation in peripheral artery disease.


