ADAPTING A MOCK CIRCULATORY LOOP FOR USE IN
THE STUDY OF TISSUE ENGINEERED HEART VALVES

A Thesis in
Bioengineering

by
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ABSTRACT

A mock circulatory loop is a tool that has been used in research of many cardiac therapies, such as ventricular assist devices and mechanical replacement valves. This equipment mimics the conditions in the circulatory system and is used in conjunction with blood analogue fluids that mimic the mechanical properties of blood, but allow for imaging and measurement of the fluid flow in the system. Using mock circulatory loops in the development of tissue engineering therapies is desirable, as it provides an environment very similar to the in vivo for testing of tissue engineered heart valves, without requiring test animals and surgery.

The principle challenge in adapting a mock circulatory loop for tissue engineering use is developing blood analogues to be used in the loop that allow for both imaging and valve construct conditioning studies, while maintaining viability of the cells in the tissue engineered valve. Traditional blood analogues, for example, an aqueous mixture of glycerol, xanthan gum, and sodium iodide, do not support cell growth, so new fluids must be developed and tested.

Using cell culture media as a base, we have developed two new blood analogues for use in mock circulatory loops. Both blood analogues have the viscous behavior of blood, but have the optical and cell-enrichment properties required for short-term (up to 8 hours) imaging and long-term (up to 4 weeks) conditioning of tissue engineered aortic valves. Coupled with our specially-designed loop that allows for imaging of the aortic valve position, these fluids will allow for fluid flow imaging around tissue-engineered valves and conditioning of these valves in vitro.

Using these new blood analogues and the specially designed mock circulatory loop, studies can be conducted to improve the design of tissue engineered aortic valves.
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Chapter 1

INTRODUCTION

1.1 Clinical Relevance

The heart, in its place at the center of the circulatory system, is an extremely important organ. Without blood pumping to the body’s organs, irreversible damage and death will occur in a matter of minutes due to a lack of oxygen. Unfortunately, there are many things that can go wrong with a heart including congenital abnormalities, blocked arteries, and dysfunctional valves. If even one of these failures occurs, the damage to nearly every system in the body is catastrophic. Thus, cardiac therapy is an area receiving a lot of attention in the research community.

Heart disease is one of the leading causes of death in the United States and the world, contributing to 1 in 6 deaths in the United States last year [1]. While heart disease is often manifested in myocardial infarction, problematic heart valves also cause a large number of heart disease-related deaths [2]. The valves in the heart control the flow of blood. They ensure that the blood flows in only one direction through the circulatory system. A poorly functioning heart valve can cause serious problems with the entire circulatory system. Heart malformations are present in 6 of every 1000 live births [3], with 30% of these being valve malformations [4]. The most common aortic valve problem is a bicuspid valve, where the valve has two leaflets instead of the usual three,
occurring in 1-2% of births [5]. Aortic valves of this type can function for years before becoming problematic.

There are currently two major types of therapies for pathologic heart valves. These are replacement of the valve or surgical repair of the valve [6]. Each therapy has its own positives and negatives, and is best suited to a particular type of patient.

Replacement of the valve can be done using a tissue or a mechanical valve. Tissue valves, commonly called bioprosthetic valves, can be homografts (a valve donated by a human patient recovered after he or she dies) or xenografts (usually porcine valves). In the porcine case, the valve is often decellularized before implantation to prevent immune rejection. Homografted valves and porcine valves usually have similar durability. Another aortic valve replacement option is called the Ross procedure (also called a pulmonary autograft), in which the patient’s own pulmonary valve is used as a replacement for the aortic valve, with a pulmonary homograft (donated pulmonary valve from a cadaver) used in the pulmonary position [7]. This procedure is primarily used in children, as it allows the new aortic valve to grow with the child. Bioprosthetic valves have a tendency to undergo calcification and structural degradation [8], leading to a
product lifetime of about 10-15 years or less. Because of this, patients with bioprosthetic valves require repeat surgery with greater frequency than those with mechanical valves [9]. The incidence of valve related reoperation among patients with bioprosthetic valves is 3.7 events/100 patient-years, while the incidence for mechanical valves is 0.5 events/100 patient-years, according to a 15-year study [10].

Mechanical valves are manufactured out of polymers and metals. These valves are designed to last much longer than tissue valves, or even the lifetime of the patient. Recently, a man was admitted to a hospital in Japan with heart failure, and his two mechanical replacement valves were replaced after he had had them for 27 years. The explanted mechanical valves showed no signs of degradation [11]. Mechanical valves do, however, increase the probability of blood clots forming around the valve, so patients with mechanical replacement valves are required to be on blood thinning medications, making them more at risk of bleeding [6]. Bioprosthetic valves are usually used in physically active patients, due to the lower dependence on blood thinners, but this reduces their lifetime to less than 10 years in most cases. However, antibiotics, vasodilators, and inotropic agents are often prescribed to patients with either type of valve replacement.

Surgical repair of a valve is a process used when part of the valve is salvageable, and thus total replacement is not required. Two major valve repair surgeries are valvuloplasty [12] and valvulotomy [13]. Valvuloplasty is used to repair a stenotic valve, or one with a narrowed opening due to stiffening of the valve from calcium buildup. In a valvuloplasty procedure, a balloon is placed on the stenotic valve and is then inflated to increase the opening area of the valve and improve blood flow. This procedure is not an
optimal one, and is typically used as an alternative to the preferred replacement therapy when it is not available. Valvulotomy is a repair method in which one or more incisions are made at the edges of the commissure formed between two or three valves with the goal of reducing the constriction caused by stenosis. Like valvuloplasty, this therapy is only used when the preferred therapy, valve replacement, is not available or not able to be performed.

As mentioned above, both replacement and repair options have drawbacks. The largest drawback of most of these therapies is that they are not intended for the long-term. This problem is especially present in tissue valve replacements and surgical valve repair. Valve replacements have finite lifetimes in the body and surgical repair of valves, especially in the pediatric case, often requires multiple repeat surgeries [14]. While mechanical replacement valves are designed to last longer than a patient could possibly need them, the dependency on blood thinning medications has a major effect on the lifestyle of the patient.

A relatively new therapy for the repair and replacement of problematic tissues and organs is tissue engineering. Tissue engineering involves seeding naturally or synthetically derived scaffolds with cells to form replacement tissues [15]. Tissue engineered heart valves are still in development at many universities and research centers [16,17]. The focus of this thesis is a new method to aid in the development of tissue engineered heart valves.
1.2 Cardiovascular Anatomy

The heart is composed of four chambers, two atria and two ventricles. The atria act as receiving chambers for blood and the ventricles act as discharging chambers in the two circulations of blood through the body: pulmonary and systemic. Deoxygenated blood flows through the right side of the heart, with the right atrium receiving blood from the systemic circulation and the right ventricle pumping blood into the pulmonary circulation. Oxygenated blood flows through the left side of the heart, with the left atrium receiving blood from the pulmonary circulation and the left ventricle pumping blood into the systemic circulation [18]. A simple schematic of this is shown in Figure 1-2.

![Figure 1-2: A simple diagram of the heart, showing pathways for systematic (sys) and pulmonary (pulm) circulations and oxygenated (oxy) and deoxygenated (deoxy) blood.]

A system of valves keeps the blood flowing in the proper direction. The atrioventricular (AV) valves are positioned between the atria and the ventricles and direct blood flow from the atria to the ventricles. These are called the mitral valve (connecting
the left atrium to the left ventricle) and the tricuspid valve (connecting the right atrium to the right ventricle). The semilunar valves connect the ventricles to arteries, and direct blood flow away from the heart. The pulmonary valve connects the right ventricle to the pulmonary artery that leads to the lungs where the blood is oxygenated. The aortic valve connects the left ventricle to the aorta that leads out to the body and all the other tissues (Figure 1-3). Because of its position, the aortic valve is very important. Problems with the aortic valve lead to blood leaking back into the heart, which decreases effective forward flow of blood out to the tissues of the body. Severe problems with the aortic valve can lead to congestive heart failure, or an inability of the heart to pump sufficient blood to the body.

Figure 1-3: The heart with all valves and chambers labeled. The aortic valve is near the middle (circled) (source: texasheartinstitute.org).
The aortic valve is a trileaflet valve consisting of right, left, and posterior semilunar cusps (Figure 1-4). Each leaflet contains three layers. The ventricularis, which is on the ventricular side of the leaflet, is composed of elastin-rich fibers aligned in the radial direction. The fibrosa, which is on the aortic side of the leaflet, is composed of fibroblasts and collagen fibers that are aligned circumferentially. In between these two layers, there is a loose layer of connective tissue called the spongiosa, which is made up of fibroblasts, mesenchymal cells, and a mucopolysaccharide matrix [19].

![Figure 1-4](image-url) (A) The aortic valve. Note trileaflet structure. (Source: Misfield and Sievers, Phil. Trans. R. Soc. B (2007) 362:1421) (B) The aorta cut open showing three leaflets (numbered).

After leaving the heart through the aorta, the blood moves through arteries that gradually reduce in size to capillaries, where the gas exchange takes place within the tissues, and then through a gradually increasing veins blood flows back to the heart through the vena cava and the right atrium. The primary resistance vessels are the medium-sized arteries, called arterioles. These reduce the pressure of the system for the capillaries and the venous system, where the pressure is much lower. The venous system returns the blood to the heart through the superior and inferior vena cava that feed into the right atrium.
1.3 Tissue Engineered Heart Valves

Until the middle part of the 20th century, the prognosis for patients with problematic or non-functioning aortic valves was often terminal. At that time, the therapies for problematic valves took a huge leap with the development of the first replacement valves. In 1951, the descending aortic ball valve was described by Charles Hufnagel [25]. This was one of the first descriptions of a mechanical replacement valve, and was followed by further development of caged ball valves [26,27] (Figure 1-5), tilting disc valves [28], and artificial valves with leaflets [29,30]. These prosthetic valves, although functional, were not viable in that they did not use living cells, and thus could not grow with the patient. For viable aortic valve replacement solutions, pulmonary autografts [7] and cryopreserved allografts [31] were developed. More recently, tissue engineering has been used as a therapy involving viable cells.

In an ideal tissue engineering system, the tissue engineered construct will integrate with the native tissue around it, and will then grow with that tissue. Additionally, an engineered tissue, being a natural part of the patient would allow the patient to have a normal lifestyle. Because tissue engineering therapies allow for this return to a normal lifestyle for patients, with lower possibility of a requirement for future surgery, valve replacement is a good candidate for tissue engineering.

Tissue engineering is an emerging therapy in which synthetic or natural materials are coupled with living cells to form an artificial tissue (Figure 1-5). The engineered tissue can be implanted to replace problematic native tissue, with the idea that the engineered tissue will integrate with existing native tissue and grow with it, requiring no further therapeutic action [15]. In the optimal case, cells would be harvested from the patient and used to seed the scaffold, so that the engineered tissue would be immunologically equivalent to the patient’s own tissue. Tissue engineering has already been successfully implemented in burn therapies [20], and is receiving research attention in many major tissues such as liver [21], bone [22], bladder [23], and cartilage [24].
When tissue engineering emerged as a treatment option and started receiving large amounts of research attention, fibrous polymer scaffolds were seeded with living cells [32,33]. Since this work, many approaches for producing tissue engineered heart valves (TEHV) have been pursued. Scaffold research has involved decellularized valves [34,35], non-woven fibers [36,37], laser microfabricated polymers [38], and even scaffolds formed by the cells themselves [39,40]. Cells have been both seeded on scaffolds \textit{in vitro} [41] and captured \textit{in vivo} [42]. Stem cells have also been used in the production of TEHV, producing their own extracellular matrix in an approach that could be considered as regenerative medicine [43].

Aortic valve tissue engineering involves the seeding of living aortic valve interstitial cells onto polymer scaffolds. This process, at the basic level, is well
characterized [44,45], but new variations are being developed and tested constantly. Our tissue engineered heart valves use a polymer called poly(glycerol sebacate), which is a mixture of glycerol and sebacic acid, both of which are found naturally in the body [46]. Because of this, the polymer does not release toxic substances as it degrades. The polymer is micromolded into sheets approximately 300 microns thick with a diamond shaped mesh (Figure 1-7) that serve as the scaffolds. Aortic valve interstitial cells are then seeded on the scaffold and allowed to proliferate and form a collagenous tissue network.

![Diamond-shaped PGS tissue engineering scaffold shown at 300x magnification.](image)

The final goal of this work is to produce a tissue-engineered heart valve construct that can be implanted in a patient, and will be able to integrate with the patient’s natural tissue and grow, literally as their own heart valve. In this scenario, there would be no need for any lifestyle change or repeat surgery. The patient’s new tissue engineered heart
valve would become their own heart valve, and they would be able to live a normal lifestyle [47].

1.4 Mock Circulatory Loops

The analysis of fluid dynamics in the cardiovascular system is an important step towards a more complete understanding of the cardiovascular system, possibly leading to improvements in knowledge and treatment of cardiovascular problems. Mock circulatory loops (MCLs), with features designed to mimic the flow, pressure, and resistance changes present in the cardiovascular system are an important part of this analysis [48].

Using this system, measurements can be taken, often using optical flow visualization equipment, to determine the flow of fluid around structures present in the circulatory system, such as heart valves and arterial bifurcations. Also, flow and pressure meters can be used to determine the dynamics of the system, making MCLs useful for testing cardiac assist devices. An MCL system can be used to test the fluid flow around artificial valves as a step towards determining their usefulness in therapeutic applications. These loops are commonly used to test cardiac assist devices such as left ventricular assist devices [49], artificial hearts [50,51], and artificial valves [52], and have recently been applied to tissue engineering [53].

A mock circulatory loop uses a system of tubes, chambers, valves, and resistance equipment to simulate the flow of blood through the body [51,54]. The fluid in the loop is pumped using a pulsatile pump to simulate the heart pumping. The components of the loop are set up in such a way that the liquid pressures within the loop match those in the
body in both systole and diastole. Loops vary in complexity, depending on what part of the circulatory system is the focus of the study. Details of several key components that are common to nearly all mock circulatory loops follow.

The first is the pumping chamber, representing the left ventricle of the heart. There are a few variations of pumping chambers, but in both, the chamber is made of acrylic and lined with a silicon bag that contains the fluid (Figure 1-8). The silicon bag can be squeezed using air or a plate attached to a reciprocating motor. The plate pushing on or air squeezing the silicon bag is meant to simulate the contraction of the heart. Either form of pump can be run at a variety of speeds to simulate different heart rates. The inlet and outlet to the chamber each have valves placed in them one allowing for directed flow. Depending on the focus of the study, this chamber may be designed to provide an unobstructed view of the inlet or outlet valve.

Figure 1-8: An example of a ventricular assist device often used as a pump in a mock circulatory loop.
The next common loop component is one or more compliance chambers. These chambers mimic the small compliance movement of the aorta, atria, or blood vessels (depending on placement) as blood is pumped into them. These chambers can be large metal constructs that use metal rods or springs to adjust compliance (Figure 1-9), or chambers that use a pressurized air filled sac to do the same. Compliance is defined as the ratio of the change in volume to the change in pressure of the system (i.e. compliance = $\Delta V/\Delta P$). For an arterial compliance chamber to function properly, the compliance must be approximately $1 \times 10^{-8}$ m$^5$/N [54].

![Figure 1-9: A standard compliance chamber. The cylinder has an inlet and an outlet for fluid, and the compliance is controlled by the position of the metal bar.](image)

The third common mock circulatory loop component is a resistance element. This mimics the primary resistance in the vascular system, the arterioles. This feature is most often some form of screw clamp squeezing a particular location on the tubing in the loop (Figure 1-10). The clamp can be tightened or loosened to allow for different levels of resistance.
The fourth common component in a mock circulatory loop is a venous reserve vessel (Figure 1-11). This vessel represents the storage of blood in the venous return system. This component, in most systems, holds the largest amount of fluid in comparison to the other components.

Figure 1-10: An example of a resistance plate for a mock circulatory loop. The plate squeezes the tube that runs in on the left and out on the right, generating resistance to flow.

Figure 1-11: An example of a venous reserve vessel. The large tube on the left is the inlet, and the
To properly set up the loop, the pressures must be tuned to the average natural pressures found within a person. For the arterial side of the loop, assuming a healthy adult is being modeled, those pressures are 120 mmHg systolic and 80 mmHg diastolic (traditionally expressed as 120/80). Many factors in the loop have effects on these pressures, but, generally speaking, the resistance of the resistance element determines the average pressure (desired in this case to be ~100 mmHg), while the compliance determines the amplitude of the pressure waveform. Achieving a proper waveform requires both of these factors to be adjusted together, as well as adjustments to the amount of fluid in the loop and the positions of the loop elements.

1.5 Mock Circulatory Loop Fluids

Currently, the fluid used in MCLs is often either a complex aqueous solution of glycerin, xanthan gum, and sodium iodide mixed such that its fluid properties (kinematic viscosity and elasticity) match those of blood and its refractive index matches that of the model material [55], or a simpler 40% glycerin solution in water [56] that matches the kinematic viscosity of blood. The matching of blood properties allows for conclusions to be drawn regarding the natural flow of blood over anatomical structures using the artificial fluid instead of blood, which would present problems in a loop environment such as clotting and separation. The refractive index matching allows little to no alteration in the light path from refraction when using flow imaging techniques such as particle image velocimetry [57].
However, these mixtures cannot be used with native tissue or cultured living cells because of their incompatibility with living tissue. The xanthan gum and sodium iodide in the more complex mixture are not cell-safe, and the simple glycerin-water mixture lacks the proper compounds to support cell growth and proliferation. A cell-safe fluid with the properties of blood would be useful in TEHV applications. It could be both used to compare fluid flow over native valves to that over TEHV and to condition a TEHV construct.

1.6 Proposed Work

The objective of this work is to develop and test blood analogs that are useful in a cardiac flow loop and are not toxic to living cells or tissue. Along with the biocompatibility goal, the fluid should have properties similar to blood (viscosity, osmolality, and elasticity). The developed blood analogues will be used for two applications relating to the production and study of tissue engineered heart valves.

1.6.1 TEHV/MCL Application 1: Fluid Flow Comparison

Flow visualization is a common method used in conjunction with MCLs. Particle image velocimetry (PIV) uses a laser reflecting off particles in a flowing fluid to visualize in detail the flow field, including any eddies or flow disturbances. This is useful for determining the exact flow patterns, which can be used to better design a feature for greater efficiency. For viewing how a moveable feature, such as a heart valve, reacts to
fluid flow, a high speed camera can be used. The high speed camera can record the movements of the feature at a very small time scale, allowing for differences between designs to be picked up more readily under fluid flow.

A large part of constructing tissue-engineered heart valves is ensuring that the construct performs as similarly as possible to the native heart valve in a physiological situation. Native aortic valve leaflets can be placed in the loop and the flow profiles over them could be compared to that over tissue-engineered constructs. The data from this sort of experiment could supplement mechanical property data acquired previously in an effort to produce a tissue-engineered aortic valve construct that behaves as similarly as possible to native aortic valve tissue. Currently, mechanical property data is useful in determining the structure and composition of a scaffold used for aortic valve tissue engineering. Fluid flow data would be useful in determining the proper size and shape of the construct.

1.6.2 TEHV/MCL Application 2: Bioreactor Construct Conditioning

An important part of tissue engineering is construct conditioning [58]. This is a process by which tissue-engineered constructs are subjected to conditions and forces similar to those they will experience in vivo in an effort to increase tissue formation and properly structure tissue formed on the construct. Construct conditioning has been shown to improve cell proliferation and tissue formation on engineered tissues. It has also been shown to improve the alignment of formed tissue such that it is closer to that of the native tissue.
For a heart valve, the physiological forces are stretch (as valve closes against pressure), flex (as valve opens to allow flow), and fluid shear (as fluid flows over valve) [59]. Systems built for the purpose of construct conditioning are called bioreactors, and come in many different forms. A common form of bioreactor for aortic valve constructs is one that cyclically stretches the construct, simulating the cyclic stretch that the valve will undergo in vivo [60]. However, this only addresses the cyclic stretch of native heart valves, and not the flex or fluid flow. With a MCL used as a bioreactor, all three physiological forces that heart valves are subject to could be mimicked, allowing for better conditioning of the construct.
Chapter 2

MOCK CIRCULATORY LOOP DESIGN SPECIFICS

The loop assembled for this work is similar in terms of parts and functionality to that developed by Lukic et al. [61]. In this loop design, an adult-size Pierce-Donachy pneumatic pump without valves was used as an aortic compliance chamber and a venous reservoir (Terumo Capiox 400 mL venous reservoir, Tokyo, Japan) was used as venous compliance (Figure 2-1).

To provide adequate volume for the desired amount of aortic compliance, a 4-liter glass filter flask was attached to the air side of the Pierce-Donachy pneumatic pump (Figure 2-2). This chamber was connected to a pressure source (Barnant Company Vacuum Pressure Station, Barrington, IL), which maintained constant pressure in the...
aortic compliance chamber using an outlet-tube regulator. Two screw clamps were positioned on the tube connecting the aortic and venous compliance chambers to act as systematic resistance.

Average arterial pressure in the loop was maintained at approximately 95 mmHg with systolic and diastolic pressures of approximately 120 and 70 mmHg respectively (120/70). A trace of pressure versus time in the loop can be seen in Figure 2-3. This pressure trace is not optimal, as the systolic and diastolic phases of the trace aren’t constant pressure. The systolic peak and diastolic pressure are in the acceptable range.

Figure 2-2: The atrial compliance setup. The air line from the compliance chamber (C in Figure 2-1) runs into the flask at the point indicated. The pump holds pressure within the flask while the clamp allows excess pressure to escape.
Pressure was measured using Argon Medical Devices (Plano, TX) disposable pressure transducers connected to the loop in the following three locations. One on the arterial side before the aortic compliance chamber, one between the aortic and venous compliance chambers placed after the systematic resistance clamps, and one on the outlet tube from the venous compliance chamber (Figure 2-4). Pressures in the loop were tuned by changing the pressure in the aortic compliance chamber and the set point on the systematic resistance clamps.

Figure 2-3: Arterial pressure versus time acquired from the mock circulatory loop setup. The maximum systolic pressure is approximately 120 mmHg and the average diastolic pressure is approximately 70 mmHg.
The aortic and venous compliance chambers are mounted on a vertical Plexiglas board and connected to the inlet (on the venous side) and the outlet (on the aortic side) of an acrylic chamber containing an inlet valve and a place for a native aortic valve or TEHV to be placed. The sample aortic valve is contained within an acrylic L-shaped tube, allowing a clear view of the valve along its axis (Figure 2-5). This allows for high-speed photography of the valve as it opens and closes. The acrylic chamber contains a blood sac that is pressed cyclically by a Harvard Apparatus Pulsatile Blood Pump. The speed of the blood pump is controllable to allow for heart rates from 30 to 100 beats per minute (figure 2-6).
Figure 2-5: Two views of the valve chamber and elbow. (A) The shape of the elbow with the chamber on the left and an arrow indicating flow direction. (B) The view of the valve (circled) from the end of the elbow for data collection. A caged ball valve is currently installed for pressure testing.

Figure 2-6: The pump setup (without fluid). When full, the plate (circled) presses into the diaphragm, forcing fluid out of the chamber.
Chapter 3

FLUID REQUIREMENTS

The main requirements for both fluids are that they mimic the fluid properties of blood and support living tissue and cells. To properly represent the fluid flow paths that blood would take over the valves, the fluid must have mechanical properties similar to those of blood. To support living cells and tissue, the fluid must have a number of specific properties.

The mechanical property focus is viscosity, specifically, the kinematic viscosity. This is calculated by dividing the dynamic (or absolute) viscosity by the density of the fluid. The kinematic viscosity of blood varies from about 3.5 to 4.5 mm\(^2\)/s, depending on hematocrit. The goal is to achieve a kinematic viscosity that is within this range.

Support of living cells and tissue requires precise tuning of several properties. First, obviously, the fluid cannot contain toxic substances that would kill the cells quickly. For this, material safety data sheets (MSDS) were consulted. Within the MSDS, the National Fire Protection Association 704 (NFPA 704, commonly known as the fire diamond) was checked for the health risk number. For possible fluid components, only liquids with a health number of 0 (poses no health hazard) or 1 (exposure poses minor health hazard) were considered.

Simply not killing cells, however, is not enough. The fluids must be able to support cellular growth and division. For this, something to feed the cell is required. The best source of cellular food is glucose, as it is used often in cell culture media [62]. Thus,
high glucose content was required for the fluid. Along with glucose, culture media contains many molecules to aid in cellular growth and repair such as amino acids, vitamins, and inorganic salts.

The biological properties of blood were also considered important in fluid formulations. The biological properties of blood include pH and osmolality. The native pH of blood is found within a very narrow range, from approximately 7.35 to 7.45 [18]. The osmolality of blood is important for osmotic balance with the cells. If the solute concentration in the fluid is too high, water will be pulled out of the cells by osmotic pressure and the cells will shrivel. If the solute concentration in the fluid is too low, water will be pulled into the cells by osmotic pressure and the cells may burst. Native blood has an osmolality of approximately 300 mOsmol/L, and, for the fluid, a goal was set to be within 5% of this value. These properties were not specifically tested in the development of the fluid, but were assumed to be within acceptable ranges for the cells in fluids that passed cell toxicity tests. During cell toxicity test, cells were imaged to assess osmotic content of fluid, as higher osmolality fluids lead to shrunken cells and lower osmolality fluids lead to bursting cells due to water movement.

For the flow imaging fluid, optical properties are also important. To ensure compatibility with laser imaging, the fluid must be clear and colorless. A slight yellow color is allowable, as many previous loop fluids are this color due to the sodium iodide present in them. Any red color, however, is not allowable, as this would absorb light wavelengths used for laser imaging. An additional requirement is the refractive index of the fluid. When light moves between media of different refractive index, it is refracted and the light path changes. The acrylic used in construction of the loop has a refractive
index of approximately 1.48, so the fluid must have a refractive index as close to this as possible. If there is some difference between the refractive index of the fluid and the acrylic loop, calculations can be done to correct for the changing light path, but, to simplify the imaging protocol, refractive index was limited to between 1.45 and 1.50. To achieve this high refractive index, stronger chemicals were required, which reduced the biological compatibility of the fluid.

The timescale of use is different for the two fluids. The flow imaging fluid will need to be used for up to eight hours while the video or laser imaging takes place. Because of the shorter time requirements of this fluid, long-term biocompatibility and cell support are not as important. This makes up for the above-mentioned challenges presented in the biocompatibility of high refractive index fluids. The bioreactor fluid, however, will need to be in use for weeks as the cells divide and produce the tissue surface of the TEHV. Most of the bioreactor studies we conduct using other bioreactors run for two to four weeks [58], so bioreactor fluids were tested with cells for four weeks. Without the high refractive index requirements, it should be easier to develop a fluid that can support cell growth over this longer period.

Finally, we wanted fluids that were relatively simple to produce in any quantity, whether it was small quantities for our testing purposes or large quantities for running in the loop. The goal was to minimize the number of components in both fluids, not only to keep production simple, but to avoid unforeseen consequences that could come out with more complex mixtures.
Chapter 4

FLUID DEVELOPMENT

Developing these fluids involved first picking a liquid as a starting point, and then adding other liquids to tune the properties of the mixture to match the desired properties. The starting point liquid would have to be cell-safe, and provide the necessary compounds for cell growth and division, while the additives would have to alter the mechanical and/or optical properties of the mixture without compromising the cell-sustaining properties of the original fluid.

4.1 Initial Fluid Selection

As a starting point, we decided to use culture media. For most cell culture in our lab, including that of heart valve cells, we use a modified version of medium 199, developed by Joseph Morgan in the 1950s [63]. Medium 199 was originally developed for nutritional studies of chick embryo fibroblasts, but is used in wide variety of cell culture applications, mainly in the culture of non-transformed mammalian cells. Gibco medium 199 has added Earle’s salts (a salt-balancing mixture), L-glutamine (an amino acid that supports cell growth), and 2.2 g/L sodium bicarbonate (a buffer used to stabilize pH). In addition to these factory additives, we add fetal bovine serum (FBS, 10% by volume) and Gibco antibiotic-antimycotic (5% by volume) to produce the media we use for our cell culture work. This media, however, could not be used for this MCL fluid, as
it contains phenol red, a pH indicator that colors the media red under neutral pH conditions. This red color would not be permissible in a fluid for the applications we needed, as it would absorb the laser used for imaging. Fortunately, medium 199 is available with the same factory additives listed above and without the phenol red. Our usual media mix, the 10% FBS and 5% antibiotic-antimicotic mixture mentioned above, using the medium 199 without phenol red was produced using the same sterile filtration techniques that are used for regular culture media. This became the basis for both fluids, as it already contains the sugars, amino acids, vitamins, and inorganic salts required to sustain the valve cells.

4.2 Potential Additives

The next step was finding additives that would alter the mechanical or optical properties of the fluid while maintaining the cell support of the culture media. For this, we consulted the MSDS of a number of chemicals, looking for chemicals with a low health hazard rating. We also looked at other chemicals we used in our lab as media additives, knowing that these chemicals were cell safe, but not knowing their effect on refractive index or viscosity.

Initially, tests were done using the fluids and additives available in our lab, as they were used for cell culture so we knew that they would be safe. These additives include fetal bovine serum (FBS), antibiotic-antimicotic agent, and L-ascorbic acid 2-phosphate (AA2P). These fluid additives were tested for refractive index and viscosity.
Mixtures were then tested, and an effort was made to understand not only the effect of individual additives on the mixture, but the effect of the interactions between additives. It was found, however, that anything made from cell culture fluids would be too water-like in its properties (refractive index close to 1.33, viscosity close to 1 cP), as all of the fluids used for cell culture are water based.

Chemicals that are often used in blood analogues were also considered. These are glycerin, xanthan gum, and sodium iodide. In most blood analogues, glycerin and xanthan gum are used to increase the viscosity of the fluid, while sodium iodide is used to increase the refractive index. However, both xanthan gum and sodium iodide have an NFPA 704 health rating of 2 (intense exposure poses risk of injury), and were therefore deemed not cell-safe. While glycerol does have a health rating of 1 (exposure poses minimal risk), is not able to raise either refractive index or viscosity high enough to be useful on its own.

More additives were needed. In consulting Materials Safety Data Sheets of many chemicals, as well as work previously done on this project, the field of fluids was narrowed down to two. These fluids met criteria in additives we felt we needed, those being high refractive index, high viscosity, and cell safety.

One possible additive was mineral oil. Mineral oil is colorless and odorless, and is an organic mixture of alkanes. Mineral oil is used in certain cell culture applications, mainly involving embryos and oocytes for in vitro fertilization and other related procedures, so is cell-safe. The mineral oils we tested were found to have a refractive index in close to 1.47, which is comfortably within the desired range. However, the
challenges in properly mixing the oil with our water-based cell culture media would make this fluid unusable as an additive.

Another possible additive was dimethyl sulfoxide (DMSO). This fluid is a polar aprotic solvent, but is less toxic than other members of this class. DMSO is frequently used as a solvent in chemical reactions such as nucleophilic substitutions, and is also used extensively in biochemistry and cell biology. It has a variety of biological uses, including use as an inhibitor of secondary structures in DNA primers in polymerase chain reaction [64], as a cryoprotectant in cell freezing for storage [65], and as an agent to induce differentiation in certain stem cell populations [66,67]. DMSO is also used in the medical field in topical-application pharmaceuticals, as it increases absorption of some compounds though the skin and other tissues. For our purposes, DMSO has a health hazard rating of 1 (exposure poses minor health hazard), and a high refractive index. In small quantities, we believed that DMSO would provide a large increase in refractive index, and would still be non-toxic to the cells. Because of its high refractive index and clear and colorless appearance, DMSO was used as a part of the imaging fluid.

Another possible additive was 3,4-dimethoxybenzyl alcohol (DMBA). This fluid is a high-viscosity alcohol used in a variety of chemical processes. Like DMSO, DMBA has a health hazard rating of 1 (exposure poses minor health hazard). That, coupled with the fluid’s high viscosity and refractive index, was what made this a potential additive, considering it might be a cell-safe way to increase the viscosity of our fluids.
Therefore, DMSO and DMBA could be used in conjunction with our culture media to produce a fluid that was relatively simple to make, and would meet our requirements for both the imaging and bioreactor fluids.

4.3 Additive Effects

As mentioned above, additive effects on both viscosity and refractive index were important in the development of these blood analogues. As is apparent in table 4-1, DMBA has a higher viscosity than blood, and a high refractive index, while DMSO has a slightly lower viscosity than blood and a high refractive index. The culture media has properties very similar to water in terms of refractive index and viscosity (low refractive index and lower viscosity than blood). At a basic level, we found that we could increase both refractive index and viscosity of our mixtures by adding more DMBA and DMSO.

Table 4-1: Numerical information on the considered fluids.

<table>
<thead>
<tr>
<th>Fluid</th>
<th>Refractive Index</th>
<th>Health Rating</th>
<th>Density (g/cm³)</th>
<th>Dynamic Viscosity (cP)</th>
<th>Kinematic Viscosity (mm²/s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Culture Media</td>
<td>1.331</td>
<td>0</td>
<td>0.979</td>
<td>0.88</td>
<td>0.89</td>
</tr>
<tr>
<td>DMSO</td>
<td>1.473</td>
<td>1</td>
<td>1.096</td>
<td>2.20</td>
<td>2.01</td>
</tr>
<tr>
<td>DMBA</td>
<td>1.549</td>
<td>1</td>
<td>1.106</td>
<td>7.76</td>
<td>7.02</td>
</tr>
</tbody>
</table>
It has been shown in previous studies involving refractive index [68] that the change in refractive index and viscosity is not linear with respect to the percent composition of a certain fluid in a mixture. Adding DMBA to media alone would not produce a fluid with a proper viscosity and refractive index, and thus would not be useful for the imaging fluid. However, since the only concern in producing a testable bioreactor fluid was viscosity, it was found that a mixture of DMBA and media would work for a bioreactor fluid. However, to tune both the viscosity and the refractive index for the imaging fluid, a binary mixture is not enough. The three fluids we decided to use for the imaging blood analogue created a system of fluids that could raise or lower the properties we were looking at based on the proportions of each of the individual additives.

For viscosity, media and DMSO both have lower viscosity than blood. Thus, if the viscosity is too high, mixtures with higher media and DMSO content were considered. DMBA has a higher viscosity than blood, and therefore was used to raise viscosity.

For refractive index, DMBA and DMSO both have higher refractive indices than was required for laser imaging, while media has a much lower refractive index. Thus, DMBA and DMSO proportions were increased to raise the refractive index, and media proportion was increased to lower it.

<p>| | | | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Glycerin</td>
<td>1.475</td>
<td>1</td>
<td>1.261</td>
<td>14.12</td>
</tr>
<tr>
<td>Xanthan Gum</td>
<td>NA</td>
<td>2</td>
<td>NA</td>
<td>NA</td>
</tr>
</tbody>
</table>
The final concern, cell safety, was a little harder to directly define by numbers than the other two properties, mainly because so many factors, including pH, molecular composition, and osmolality can have an effect. The metric used to evaluate additives, the NFPA 704 health rating, is defined more for human exposure than individual cellular exposure, so it was possible that this metric would be somewhat meaningless. Both of the chosen additives, DMSO and DMBA, have a health rating of 1 (exposure poses minimal risk), so it was assumed that they both presented approximately the same level of danger to the living cells. However, it was also assumed that both of them would be less cell-friendly than the culture media. Therefore, efforts were made to minimize the proportion of both blood analogues that was made up of these two fluids.

4.4 Property Determination

Viscosity for the fluids was determined using a Vilastic 3 viscoelasticity analyzer (Vilastic Scientific, Austin, TX). This machine measures the viscoelastic behavior of the fluids, and we used a laboratory balance (Mettler-Toledo, Columbus, OH) to measure density of the fluids we produced, so we could determine the kinematic viscosity (Figure 4-1). The machine can measure liquid viscosities at a variety of temperatures, which is adjustable using a Haake Thermo temperature control unit (Thermo Fisher Scientific, Waltham, MA).
Refractive indices of liquids were measured using a Bausch & Lomb refractometer (Figure 4-2). Individual fluids and possible blood analogues were both tested using this equipment.

Figure 4-1: The viscometer setup. On the left is the temperature control, on the right is the viscometer itself. Both are controlled by the computer.

Figure 4-2: The refractometer setup.
4.5 Cell Toxicity Testing

For cell toxicity testing, porcine aortic valve interstitial cells were used. Pig hearts were acquired from Penn State University’s Meat Lab and transported back to the lab. Using aseptic techniques in a cell culture hood, the aortic valve leaflets were cut out of the heart and rinsed in phosphate buffered saline (PBS). Both the top and bottom surface of the leaflets were then rubbed roughly with sterile surgical sponges to remove any remnant red blood cells and the outer layer of endothelial cells on the valves. The valves are then placed in TPP bioreactor tubes with specially formulated medium 199 for cell isolation containing 10% type I collagenase [69]. If cells were being isolated from multiple hearts, sets of valve leaflets are segregated into different tubes by the heart from which they came.

After approximately 24 hours of incubation at 37º C in the isolation media, the tubes are spun in a centrifuge for 10 minutes at approximately 900 rpm (120 g), which pellets the cells at the bottom of the tube. The isolation media is then removed and replaced with our standard culture media (medium 199, 10% FBS, 10% antibiotic-antimicotic), and the pellet is resuspended and transferred to a T25 cell culture flask. Cells from different hearts are kept separate, and culture flasks are labeled with the date, passage number (0 after isolation) and the heart from which they came.

Cells are observed using an Olympus CKX41 inverted microscope (Olympus Corporation, Tokyo, Japan) to confirm cell type by morphology [69] and passaged once or twice before cell toxicity testing so that some cells could be left over for future tests. For the testing, cells in a single flask are treated with trypsin and allowed to detach. The
suspended cells are moved into new culture media in 8 or 12 wells of a Falcon 24-well culture plate (Figure 4-3). The 24-well plates are filled with cells from either two or three hearts, with cells from different hearts separated in different wells (either 8 wells per heart for 3 hearts or 12 wells per heart for 2 hearts).

![Image of a 24-well plate]

Figure 4-3: A 24-well plate ready for cell toxicity testing. Cells in this plate were isolated from the hearts of 3 different animals (labeled H1, H2, H3). For the test, the top row will be control (medium 199, 10% FBS, 5% antibiotic-antimicotic) and each lower row will be filled with one testing fluid. For each fluid and the control, there are 6 wells, 2 from each heart.

For the toxicity testing, cells are first imaged using light microscopy after confluence has been achieved in the wells and while still in standard culture media. The
media is then changed such that there is an entire row of wells with each test fluid in it, and an entire row with standard culture media to serve as a control.

For the imaging fluid, the cells were imaged twice over the course of 8 hours (at t=4 hrs and t=8 hrs) and compared to controls to determine their health and growth in the fluid. For the bioreactor fluid, cells were imaged every two days over the course of 4 weeks, with media/fluid changes twice a week.

After the toxicity tests, cells were trypsinised and removed from the plate and transferred either to another plate or to a T25 tissue culture flask and placed in our standard culture media. The cells were observed the next day to make sure that they were still growing and proliferating.

Tables 4-2 to 4-3 show results of a selection of tested fluids. Figures 4-4 and 4-5 display the dynamic viscosity and elasticity curves for the bioreactor and imaging fluids. Both dynamic viscosity and elasticity are plotted versus shear rate for these graphs.
Table 4-2: A sample of tested bioreactor fluids, in order of media volume percent (BR 1-BR 3). BR F represents the final mix. Fewer bioreactor fluids were tested, as the single vital property was easier to tune in a binary mixture. An asterisk next to a property indicates that it falls outside the acceptable range. Fluids with a blank in the last column were not toxicity tested due to poor properties. (goal property: kinematic viscosity between 3.5 and 4.5)

<table>
<thead>
<tr>
<th>Ref #</th>
<th>Media (vol %)</th>
<th>DMBA (vol %)</th>
<th>Dynamic Visc (cP)</th>
<th>Kinematic Visc (mm²/s)</th>
<th>Passed Toxicity Test?</th>
</tr>
</thead>
<tbody>
<tr>
<td>BR 1</td>
<td>50</td>
<td>50</td>
<td>7.80</td>
<td>6.00*</td>
<td></td>
</tr>
<tr>
<td>BR 2</td>
<td>60</td>
<td>40</td>
<td>5.24</td>
<td>4.03</td>
<td></td>
</tr>
<tr>
<td>BR 3</td>
<td>75</td>
<td>25</td>
<td>3.20</td>
<td>2.46*</td>
<td></td>
</tr>
<tr>
<td>BR F</td>
<td>64</td>
<td>36</td>
<td>5.08</td>
<td>3.91</td>
<td>Yes</td>
</tr>
</tbody>
</table>
Table 4-3: A sample of tested imaging fluids, in order of media volume percent (IM 1-IM 12). IM F represents the final mix. An asterisk next to a property indicates that it falls outside the acceptable range. Fluids with a blank in the last column were not toxicity tested due to poor properties. (goal properties: refractive index above 1.45, kinematic viscosity between 3.5 and 4.5)

<table>
<thead>
<tr>
<th>Ref #</th>
<th>Media (vol %)</th>
<th>DMSO (vol %)</th>
<th>DMBA (vol %)</th>
<th>Refr Index</th>
<th>Dynamic Visc (cP)</th>
<th>Kinematic Visc (mm²/s)</th>
<th>Passed Tox Test?</th>
</tr>
</thead>
<tbody>
<tr>
<td>IM 1</td>
<td>40</td>
<td>10</td>
<td>50</td>
<td>1.450</td>
<td>7.93</td>
<td>6.60*</td>
<td></td>
</tr>
<tr>
<td>IM 2</td>
<td>40</td>
<td>20</td>
<td>40</td>
<td>1.449*</td>
<td>4.33</td>
<td>4.12</td>
<td>No*</td>
</tr>
<tr>
<td>IM 3</td>
<td>42</td>
<td>10</td>
<td>48</td>
<td>1.460</td>
<td>7.56</td>
<td>5.82*</td>
<td></td>
</tr>
<tr>
<td>IM 4</td>
<td>43</td>
<td>14</td>
<td>43</td>
<td>1.448*</td>
<td>3.51</td>
<td>3.34</td>
<td>Yes</td>
</tr>
<tr>
<td>IM 5</td>
<td>45</td>
<td>5</td>
<td>50</td>
<td>1.454</td>
<td>8.98</td>
<td>7.48*</td>
<td></td>
</tr>
<tr>
<td>IM 6</td>
<td>45</td>
<td>8</td>
<td>47</td>
<td>1.454</td>
<td>7.99</td>
<td>6.15*</td>
<td></td>
</tr>
<tr>
<td>IM 7</td>
<td>45</td>
<td>9</td>
<td>46</td>
<td>1.453</td>
<td>7.64</td>
<td>5.87*</td>
<td>Yes</td>
</tr>
<tr>
<td>IM 8</td>
<td>45</td>
<td>10</td>
<td>45</td>
<td>1.446*</td>
<td>6.24</td>
<td>4.80*</td>
<td>Yes</td>
</tr>
<tr>
<td>IM 9</td>
<td>46</td>
<td>9</td>
<td>45</td>
<td>1.445*</td>
<td>6.39</td>
<td>4.91*</td>
<td></td>
</tr>
<tr>
<td>IM 10</td>
<td>46</td>
<td>12</td>
<td>42</td>
<td>1.448*</td>
<td>7.09</td>
<td>5.45*</td>
<td>Yes</td>
</tr>
<tr>
<td>IM 11</td>
<td>48</td>
<td>8</td>
<td>44</td>
<td>1.445*</td>
<td>6.70</td>
<td>5.16*</td>
<td></td>
</tr>
<tr>
<td>IM 12</td>
<td>50</td>
<td>12</td>
<td>38</td>
<td>1.449*</td>
<td>7.01</td>
<td>5.40*</td>
<td>Yes</td>
</tr>
<tr>
<td>IM F</td>
<td>40</td>
<td>18</td>
<td>42</td>
<td>1.451</td>
<td>4.46</td>
<td>4.25</td>
<td>Yes</td>
</tr>
</tbody>
</table>
Figure 4-4: (A) Dynamic viscosity versus shear rate and (B) elasticity versus shear rate curves for the bioreactor fluid referenced as BR F in Table 4-2.
Sample images from cell toxicity tests are presented (Figures 4-6 through 4-14).

Most cell toxicity tests included 6 wells in each group (1 control, 2-3 experimental).

Figure 4-5: (A) Dynamic viscosity versus shear rate and (B) elasticity versus shear rate curves for the imaging fluid referenced as IM F in Table 4-3.
Three timepoints were taken for the imaging fluid toxicity tests (0 hours, 4 hours, and 8 hours), while 16 timepoints were taken for the bioreactor fluid (0 days, and every other day to day 30). In all, 384 images were taken. In Figures 4-6 to 4-14, images of control wells are above and images of experimental wells are below. The health of cells was determined based on their morphology, taking into consideration several factors [71]. First, living cells are distinct from dead cells in that they are attached to the surface of the well. Second, attached cells display a spindle-shaped, elongated morphology. At confluence, elongated cells exhibit a swirling pattern, which is characteristic of fibroblasts. All images are labeled with the test and the time point.

Figure 4-6 shows images from a bioreactor fluid toxicity test. The top image is the control well containing standard culture media, and the bottom image is the experimental well containing the tested fluid (a 64-36 volume % mixture of culture media and DMBA, BR F in Table 4-2). The image is taken at time zero, so it just serves as an illustration of the similarities between the control and experimental wells at the beginning of the experiment. Both wells contain cells that are attached to the bottom of the well, and are elongated.

Figure 4-7 is two images from the same toxicity test, 8 days later. There are more cells in the field of view, as the cells have divided, but the morphology of cells in both the control and experimental wells is still indicative of healthy cells. Cells in both the control and experimental image are attached to the bottom of the well and elongated.

Figure 4-8 shows the same bioreactor fluid toxicity test, this time at the 15-day time point. The cells in the control well have reached full confluence (the cells
completely cover the field of view with no gaps), while the cells in the experimental well have not. This could be caused by the lower concentration of cell-necessary molecules in the bioreactor fluid because of the addition of the DMBA. However, cells in both images still display a health morphology, in that they are attached to the surface of the well and have an elongated, spindle-like morphology.

Figure 4-9 is images from the bioreactor fluid test on the 22nd day. The cells in both the control and experimental groups are still attached to the surface of the well and display an elongated morphology, indicating that the cells are healthy.

Figure 4-10 contains images from the bioreactor fluid test on the final day (day 30). Both wells contain attached cells which are elongated and spindle-shaped. This test was a success because the cells in the experimental well displayed a healthy morphology for the entire month that they were cultured in the bioreactor fluid.

Figure 4-11 contains images for a toxicity test of the imaging fluid (40% media, 18% DMSO, 42% DMBA, IM F in Table 4-3). These photos were taken at the start of the experiment (t = 0). The cells in these wells were allowed to grow to confluence prior to the experiment, unlike those used in the bioreactor fluid toxicity tests. Cells in both wells display similar healthy morphologies based on their attachment to the bottom of the well and elongated shape. Because of the confluence of the cells, a swirling pattern can be observed, further indicating cell health.

Figure 4-12 contains images for the same test as the previous figure, but at the 8 hour (ending) time point. The cells in both the control and experimental wells are attached to the surface and display an elongated, spindle-like morphology. They also
continue to display a swirling pattern indicating confluence of healthy cells. This test was a success because the cells displayed the same healthy morphology in the control and experimental wells for the duration of the test.

Figure 4-13 contains images for a failed toxicity test of the imaging fluid (40% media, 20% DMSO, 40% DMBA, IM 2 in Table 4-3). These images are from the initial time point (t = 0) and thus both contain healthy cells.

Figure 4-14 contains images from the same failed imaging fluid toxicity test as the previous figure, at the final time point (t = 8 hours). The control well still contains cells with a healthy morphology (attached to the bottom of the well and elongated). However, the experimental well contains many unhealthy and dead cells. Cells in this well appear more rounded because they have detached from the surface of the well. Some also appear out of focus because they are floating at different heights in the fluid. The cell death in the experimental group indicated failure in this cell toxicity test.
Figure 4-6: Successful toxicity test example for bioreactor fluid (BR F in Table 4-2). Pictures taken at t = 0, control above, experimental below. Cells have not yet grown to confluence in the dish. Scale bar = 200 μm.
Figure 4-7: Successful toxicity test example for bioreactor fluid (BR F in Table 4-2). Pictures taken at $t = 8$ days, control above, experimental below. Notice cells are elongated on the surface of the dish. Scale bar = 200 μm.
Figure 4-8: Successful toxicity test example for bioreactor fluid (BR F in Table 4-2). Pictures taken at $t = 15$ days, control above, experimental below. Notice cells are elongated on the surface of the dish. Scale bar = 200 μm.
Figure 4-9: Successful toxicity test example for bioreactor fluid (BR F in Table 4-2). Pictures taken at $t = 22$ days, control above, experimental below. Notice cells are elongated on the surface of the dish. Scale bar = 200 μm.
Figure 4-10: Successful toxicity test example for bioreactor fluid (BR F in Table 4-2). Pictures taken at $t = 30$ days, control above, experimental below. Notice cells are elongated on the surface of the dish. Scale bar = 200 $\mu$m.
Figure 4-11: Successful toxicity test example for imaging fluid (IM F in Table 4-3). Pictures taken at $t = 0$, control above, experimental below. Scale bar = 200 μm.
Figure 4-12: Successful toxicity test example for imaging fluid (IM F in Table 4-3). Pictures taken at t = 8 hours, control above, experimental below. Notice cells are confluent and elongated on the surface of the dish. Scale bar = 200 μm.
Figure 4-13: Failed toxicity test example for imaging fluid (IM 2 in Table 4-3). Pictures taken at $t = 0$, control above, experimental below. This initial time point shows the health of the cells at the beginning of the test. Scale bar = 200 μm.
Figure 4-14: Failed toxicity test example for imaging fluid (IM 2 in Table 4-3). Pictures taken at t = 8 hours, control above, experimental below. Notice cells in the experimental image are rounded and no longer attached to the plate. Scale bar = 200 μm.
Some additional cell toxicity testing was also done using the imaging fluid. Because of the addition of DMSO to the fluid, ensuring that the fluid was truly cell-safe was important. Two additional tests were done.

The first test was done using native porcine aortic valves. The valve leaflets were place in the loop shortly after being removed from the pig hearts. The loop was filled with imaging fluid (the IM F mix of 40% media, 18% DMSO, and 42% DMBA) and allowed to run for 8 hours, while additional valve leaflets were incubated in static culture in both cell culture media and imaging fluid for experimental controls. After this, all leaflets were placed in cell isolation media. The cell isolation protocol detailed above was performed to ensure viability of cells on aortic valves which were kept in pulsatile flow in the loop using the imaging fluid for 8 hours. Cells were successfully isolated from aortic valves kept in static culture in culture media, in static culture in imaging fluid, and in the loop in imaging fluid, showing that cells in aortic valve leaflets remain viable after 8 hours in the mock circulatory loop.

The second test involved the use of Trypan blue staining, which is taken up by cells with poor membrane integrity. Cells taking up Trypan blue stain are assumed to be unhealthy or dead. For this test, cultured porcine aortic valve cells were left for 8 hours in the imaging fluid (IM F mix from Table 4-3), with other cells left in culture media to serve as a control. The cells were then washed three times using phosphate-buffered saline, and were then cultured overnight in culture media. This was done to remove any imaging fluid from them, as the DMSO in the imaging fluid would disrupt the cell
membrane, which could lead to false positives in the Trypan blue staining. The next day, the cells were stained with Trypan blue and placed on a hemocytometer for counting. Based on the hemocytometer count, it was determined that an average of 18.2% of cells cultured in the bioreactor fluid absorbed Trypan blue, indicating that they were unhealthy. In the control group, 12.6% of cells were determined to have absorbed Trypan blue. According to the Trypan blue protocol, in a healthy log-phase culture, less than 5% of cells should absorb Trypan blue. Since the cells used in this experiment were beyond their log growth phase, this test was deemed a success, and the imaging fluid was re-validated as cell-safe.
Chapter 5

TEHV/MCL SYSTEM.

5.1 Increasing Fluid Volume

A challenge of translating fluid testing into useable fluids for the mock circulatory loop is the difference in the volumes we needed for testing and the volume required for the loop. For testing, 1 milliliter volumes of fluids were usually produced, as this was enough for viscosity and refractive index tests. There is a large difference between these produced volumes, and the methods used to make and mix them, and the volume of the mock circulatory loop, which is approximately 900 milliliters.

For the smaller volumes, high-accuracy measurements can be made using a pipette, which measures fluid volumes usually less than 1 milliliter. However, when the same proportions of liquids must be achieved on the order of hundreds of milliliters, precise measurements require different equipment. At this larger scale, weighing fluids with a known density using a laboratory balance is the best way to achieve very precise measurements.

The idea here is that care must be taken to ensure that the 900 milliliters of fluid for the loop is as similar as possible in terms of its properties (viscosity, refractive index, and cell safety) to the original 1 milliliter of fluid produced for testing. However, measurement and mixing is done for the larger volume, small portions of the fluid for use
in the loop must be tested for refractive index and viscosity to ensure that it preserves the properties of the original tested fluid.

5.2 Projected Studies

The TEHV/MCL system opens up a number of possibilities for future studies in tissue engineering of heart valves. The fluids that we have designed serve two purposes: imaging studies and bioreactor studies. Each of these studies has a different purpose in the development of tissue engineered heart valves, and both are equally necessary for the production of a working replacement valve for implantation in patients.

5.2.1 Imaging

There are two levels of imaging we expect to do with the TEHV/MCL system. The data acquired from both of these imaging levels will be compared between native heart valves and tissue engineered heart valves. With these comparisons, design of tissue engineered heart valves can be improved to enhance the fluid mechanics related to them.

The first level of imaging is using a high-speed video camera. This is useful in terms of the movement of the valves. From both the end of and above the valve chamber, a high speed camera can be used to view the opening and closing of the valve. Video can be taken for both the native and tissue engineered valves and then compared either qualitatively or quantitatively. Using qualitative methods, the movement of the valves can be compared frame by frame on video to improve the design of tissue engineered heart valves so that their movement is as similar as possible. Using quantitative methods,
models of valve movement can be produced and related to metrics measuring their movement. These metrics can then be used to compare the movement of tissue engineered heart valves to that of native valves.

Images of a porcine aortic valve in the loop acquired using the high speed camera (Kodak Motion Corder Analyzer, Series SR, Redlake MASD, San Diego, CA) are shown in Figure 5-1. The six images show one opening and closing of the valve, starting just before the valve opens and ending just after it closes. The images were taken over the course of approximately one second. For these images, the Harvard apparatus pump was set to 60 beats per minute and the camera was set to acquire images at 30 frames per second.
Figure 5-1: Six images, in order, of a porcine aortic valve working in the loop. The pump rate in the loop was set to 60 beats per minute, and these images were taken over the course of just under one second (27 frames at 30 frames per second). Valve opening is indicated in red.
The second level of imaging is the use of laser imaging methods, such as particle image velocimetry (PIV). This is useful in terms of the fluid dynamics around the valves. Using PIV, the fluid movement around the valves can be measured in detail, and the fluid flow profiles can be compared between the native heart valves and the tissue engineered heart valves. In these studies, features of the flow around tissue engineered heart valves will be examined and compared to those in native heart valves and literature sources detailing fluid flow around heart valves. In these studies, a primary flow feature that will be examined is the formation and maintenance of trapped vortices in the spaces behind the valves, or the aortic sinuses [70]. These vortices have been shown to play a role both as a feedback control for the position of the valve leaflets during the period of maximum velocity in the aorta and as a higher pressure-generating feature that provides most of the force to close the valves before the backflow occurs.

5.2.2 Bioreactor

The bioreactor studies for the TEHV/MCL system will be based on the bioreactor studies that are already undertaken in our lab using our cyclic stretch bioreactors. The initial goal is to determine a benchmark for the use of the system as a bioreactor. Previous studies have shown that the use of a bioreactor to condition tissue engineering constructs increases cell proliferation and structural formation on the construct (Engelmayr, 2008).
To test this, similar constructs are placed in the bioreactor and in a static culture state (in an ultra-low attachment plate) and are grown for 2 to 4 weeks. At the end of the culture period, constructs are removed from their respective culture setups and tests are performed. Some of the samples are fixed and stained using a nuclear stain (Vectashield with dapi) and an f-actin stain and imaged using confocal microscopy to determine the structure of the formed tissue. Samples are also imaged using an environmental scanning electron microscope (ESEM) for further determination of the structures formed by the cells on the construct. Samples are also quantified using a picogreen DNA assay (to determine cell proliferation) and a Biocolor Sircol collagen assay (to determine collagen production), both of which are normalized to the wet weight of the sample. The results of all of these tests are compared between the bioreactor samples and the static culture samples to determine the effect of the bioreactor on the tissue engineered construct.

The goal of the bioreactor is to alter the construct in a way that is conducive to its final use. In this case, with the aortic valve, the bioreactor should increase cell proliferation and collagen production, and also increase the alignment of cells and collagen fibers closer to their alignment in native valves. This would require using native valves as a benchmark and comparison point for the cellular and collagen alignment in the construct. In the cyclic stretch bioreactor, constructs were small strips, whereas in a TEHV/MCL system bioreactor, the constructs would need to be the size and shape of native aortic valve leaflets. This will provide a much more concrete comparison for the studies, as a full-sized native leaflet will be compared to full-sized tissue engineered leaflets, both statically cultured and cultured in the bioreactor.
Chapter 6

LIMITATIONS

Even with a well-produced fluid and a loop that properly mimics physiological conditions, there are still concerns regarding the introduction of living cells to a mock circulatory loop. Aside from the proper compounds in the fluid to allow for cell growth and division, temperature and oxygen content in the fluid must be above certain levels to allow for healthy cells. These concerns apply mainly to the use of the loop as a bioreactor, as this requires tissue engineered valve leaflets to be in the loop for long periods of time, on the order of weeks. For imaging applications, this is of less concern, as the native and tissue engineering valve leaflets will only be in the loop for the time required for imaging, which should be no more than 8 hours.

6.1 Temperature

For cell culture, a temperature of approximately 37° C is used, as this is human body temperature. Heating moving fluid in the loop to this temperature presents a challenge. There are cell culture incubators that are large enough to accommodate the entire loop, with all its auxiliary parts (the pump, the compliance volume), thereby solving this problem, but incubators of such size are rare.

A cheap, simple solution to this problem is a heating pad. Such pads are available at low cost in a variety of places, and are usually used to ease aching muscles or joints.
They often have variable setting for the level of heat. There is enough open tubing in the loop that a small heating pad could be wrapped around the tube in one or two locations on the loop, or wrapped around the venous compliance chamber. Unfortunately, with no temperature measurement or calibration available in the heating pad itself, temperature probes would have to be placed in the loop to measure the temperature of the fluid and ensure that it is constant throughout the loop. Using the temperature probes, the heating pads could be calibrated relatively easily, and then the temperature in the loop can be held constant at the required 37º C.

6.2 Gas Transfer

Another important requirement for cell culture is oxygen transfer to the cells. In normal cell culture, liquid media is added in the amount of 0.2 milliliters per square centimeter of culture area (a depth of 0.2 centimeters) to allow for oxygen dissolving in the media to reach the cells on the bottom surface of the container. Mock circulatory loops, which aren’t usually designed to allow for living tissue culture, don’t have a method for oxygenating the fluid. There are two possible solutions to this problem.

The first is using some sort of bubbling mechanism. A tube can be inserted into the venous reservoir and air could be pumped in to allow for gas transfer into the fluid. A sterile filter will need to be used to limit contamination from the air. In testing the entire system, this is something that must be investigated. Ensuring that cells are getting
enough oxygen during bioreactor studies when they are in the loop for weeks at a time is vital.

The second is using an oxygenator. Fluid oxygenators are being developed for clinical use in patients with certain deficiencies that limit the amount of oxygen they can carry in their blood. Something like this added to the system could be useful in increasing oxygen content of the loop fluids and improving the health of the cells.
Chapter 7

CONCLUSIONS

The purpose of this work was to provide new tools for the research of tissue engineered heart valves. The mock circulatory loop was seen as a powerful tool that has had much use in other areas of cardiac health, including the development of mechanical heart valves, cardiac assist devices, and even full artificial hearts. The adaptation of this tool for use in tissue engineered aortic valve research held major benefits for that research.

First, fluid flow around viable native aortic valves and viable tissue engineered aortic valves can be compared in conditions very similar to those found \textit{in vivo} using fluids with similar mechanical properties to those of blood. A direct comparison of fluid flow between native and tissue engineered aortic valves will provide necessary data to adjust the shape, size, and mechanical properties of the tissue engineered valves to the point where they are the best replacement possible for a native valve.

Second, the tissue engineered constructs can be conditioned in an environment closely matched to the \textit{in vivo} environment. This conditioning will expose properly shaped and sized tissue engineered constructs to the forces and stresses that they will experience when they are implanted, thus allowing for improved development of cells and tissue on the construct. Many forms of bioreactors exist in tissue engineering, and using a mock circulatory loop as a bioreactor will provide a tissue engineered construct with an environment more closely matched to the \textit{in vivo} than many of them.

The main challenge in this project was developing fluids for both of the above applications that retained the properties of blood analogues used in mock circulatory loops but allowed for the use of viable cells in these loops. Many of the common fluids used in blood
analogues are not cell safe, or do not provide the necessary nutrients for long-term cell culture. The development of new, cell safe blood analogues that can still be used in fluid flow imaging (a standard use of a mock circulatory loop) and can also be applied to long term bioreactor cell culture (a new use of a mock circulatory loop) has closed the gap between construct development and clinical implantation.
BIBLIOGRAPHY


