EVALUATION OF BLOOD VESSEL MIMIC 
SCAFFOLD BIOCOMPATIBILITY

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ABSTRACT

Evaluation of Blood Vessel Mimic Scaffold Biocompatibility

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The Tissue Engineering Research Lab at California Polytechnic State University, San Luis Obispo focuses on creating tissue-engineered blood vessel mimics (BVMs) for use in preclinical testing of vascular devices. These BVMs are composed of electrospun scaffolds made of an assortment of polymers that are seeded with different cell types. This integration of polymers with cells leads to the need for biocompatibility testing of the polymer scaffolds. Many of the lab’s newest scaffolds have not been fully characterized for biologic interactions. Therefore, the first aim of this thesis developed methods for in vitro cytotoxicity testing of polymers used in the fabrication of BVMs. This included cytotoxicity testing using direct contact and elution-based methods, along with fluorescent staining to visualize the scaffold effects on cells.

The second aim of this thesis implemented the newly developed cytotoxicity protocols to evaluate the biocompatibility of existing polymers, ePTFE and PLGA, used in the tissue engineering lab. The results demonstrated that ePTFE and PLGA were noncytotoxic to cells. The third aim of this thesis evaluated the biocompatibility of novel polymers used to fabricate BVMs: PLGA with salt, PLLA, and PCL. Elution-based methods concluded that PLGA with salt, PLLA, and PCL were noncytotoxic to cells; however, the direct contact method illustrated PLGA with salt and PCL were mildly cytotoxic at 24 and 48 hours. Potential causes of this variability include the addition of salt to PLGA, dissolving PCL in dichloromethane, inadequate sample sizing, and the inherent differences between the test methods. Overall, this thesis developed and implemented methods to evaluate the biocompatibility of polymer scaffolds used in the BVM model, and found that ePTFE, PLGA, and PLLA scaffold materials were biocompatible and could be implemented in future BVM setups without concerns. Meanwhile, PLGA with salt and PCL’s toxicity was mild enough to urge future cytotoxicity testing on PLGA with salt and PCL before further use in the lab.
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CHAPTER 1. INTRODUCTION

1.1 Overview

The Tissue Engineering Research Lab at California Polytechnic State University, San Luis Obispo is run by Dr. Kristen Cardinal, who focuses the lab’s innovative work on creating tissue-engineered models for use in preclinical testing. With cardiovascular disease being the leading cause of death and disability worldwide [1], the field continuously works to meet the critical need for reliable and improved treatments. Hence, the creation of tissue-engineered blood vessel mimics (BVMs) intended to serve as preclinical models for evaluating intravascular stents and other vascular devices. BVMs are composed of electrospun polymer scaffolds seeded with human vascular cell types and cultivated in a flow-based bioreactor system to mimic the vasculature’s physiological environment in vitro. The scaffolds can be composed of an assortment of polymers, all of which will contact different cell types, leading to the need for biocompatible scaffold materials. Many of the lab’s newest scaffold materials have not been fully characterized for biocompatibility. Therefore, the overall goal of this master’s thesis was to evaluate biocompatibility of existing and novel BVM scaffolds materials. This Introduction will serve to provide background and foundation in the areas relevant to this work—including basic blood vessel anatomy, pathology, and treatments for cardiovascular disease, the components and fabrication of blood vessel mimics for device testing, a deeper look at BVM scaffolds, the electrospinning process, and biocompatibility testing.

1.2 Native Blood Vessel Anatomy

To establish a comparison between the blood vessel mimics and the anatomy of the native vasculature, researchers must first understand the native blood vessel anatomy. A blood vessel is made up of three layers: the tunica intima, the tunica media, and the tunica adventitia (Fig. 1). The adventitia, the outermost layer of the vessel, provides structural support and shape to the vessel. The tunica media, the middle layer of the vessel, regulates the vessel's internal diameter and is
composed of elastic and muscular tissue. The tunica intima, the innermost layer of the vessel, provides housing for the flow of blood. The vessel's size and location determine the amount of muscle and collagen fibrils present within each vessel layer [2].

![Tunica adventitia, tunica media, and tunica intima](image)

**Figure 1. Blood Vessel Anatomy.** The vessel wall comprises three layers: tunica adventitia, tunica media, and tunica intima [3].

The tunica media, or vascular wall, comprises a broad layer of connective tissue and numerous layers of smooth muscle cells (SMCs). In contrast, the tunica adventitia consists of loosely organized adventitial fibroblasts. The endothelium lines the lumen, the hollow passageway through which blood flows, and consists of a dense layer of endothelial cells (ECs) [2]. An abundance of endothelial cells is always present, no matter the blood vessels' size or function (Fig. 2). Each layer and cell type provides a unique function. ECs and SMCs are critical cell types due to their role in vessel strength preservation and mechanical property management. More specifically, SMCs have secretory capabilities that help maintain elasticity and radial compliance of the vessel. In contrast, the EC layer provides selective permeability, a thrombo-resistant barrier, facilitation of laminar blood flow through the lumen, vessel tone, platelet activation, cell adhesion, and much more [2].
Figure 2. Cross-section of a Small Artery. The endothelial cells are the fundamental component of the blood vessel, although seemingly scarce [4].

1.3 Cardiovascular Disease Pathology and Treatments

As stated in Section 1.1, cardiovascular disease is the leading cause of death and disability worldwide. An estimated 17.9 million people die of related cardiac disorders each year, including heart and vascular diseases [5]. These diseases can be caused by several factors, the most common being atherosclerosis, plaque buildup, irregular heartbeats, congenital heart defects, and cardiomyopathies [6]. Atherosclerosis (Fig. 3) is an inflammatory disease that involves the accumulation of lipids within the arterial wall. It leads to the hardening and narrowing of coronary arteries, which eventually restricts blood flow to the heart [7]. In the United States, 1.4 million patients per year are subjected to operations requiring arterial prostheses, and about 100,000 patients require vascular bypass of arteries to treat atherosclerotic disease [8]. These alarming statistics justify the need for improved treatments of cardiovascular disease.
Figure 3. Stages of Atherosclerosis. Normal artery compared to a mild atherosclerotic artery and a severe atherosclerotic artery [9].

Current techniques used to treat cardiovascular disease include lifestyle changes, medications, bypass surgery, and intravascular stents. These treatments have shown success, but also come with limitations [10]. The most commonly used grafts for bypass surgeries are autologous arteries and veins, or those obtained from the same individual. Because of either previous surgery, burns, trauma, disease, etc., these options are unavailable to 10% of cardiovascular disease patients [11]. Along with this, purely synthetic polymer constructs have led to failures due to the early formation of thrombosis [2]. These limitations have led to the development of tissue-engineered blood vessels [12].

Intravascular devices are also a common option for treating cardiovascular disease. Mechanical devices such as bare-metal stents, drug-eluting stents [13], bioresorbable vascular scaffolds [14], amongst others are types of intravascular devices that increase the efficacy of angioplasty while decreasing the possibility of restenosis, or reduction in vessel lumen diameter after angioplasty. Limitations of these intravascular devices include, but are not limited to, access site bleeding, stent under-expansion, stent thrombosis, and incomplete revascularization [14].
Therefore, novel treatments for cardiovascular disease continue to be researched and developed to overcome these challenges. With the development of novel treatments comes the need for new methods and models to help establish safety and efficacy.

1.4 Blood Vessel Mimics for Device Testing

Extensive preclinical testing must be done to characterize medical device safety before being introduced into animal models or human clinical testing. The traditional progression of medical device testing before being approved for market involves extensive in vitro/benchtop testing, followed by in vivo/animal testing, and lastly clinical testing in humans (Fig. 4). Preclinical studies are an essential first step in demonstrating the safety of a medical device and, if done correctly, can provide considerable data and insight into the future and effectiveness of a device or material being tested. Using tissue-engineered models as a part of preclinical testing can provide a valuable understanding of devices or materials before introducing them into animals and humans. One such example of a tissue-engineered model is a blood vessel mimic, shown in the Figure 4 below, which provides a scalable, biologic vascular model that serves as an intermediate testing environment.

![Figure 4: Typical Progression of Device Testing](image)

**Figure 4. Typical Progression of Device Testing.** New devices typically undergo initial in vitro testing on materials or components, followed by testing in animal models, and eventually clinical trials. Blood vessel mimics have been developed as an intermediate testing environment to provide information about a device in a more complex environment before being used in animal models [15].
1.5 Blood Vessel Mimic Components and Fabrication

Blood vessel mimics provide a valuable, vascular testing model and it is important to understand their components. The fundamental components of an in vitro tissue-engineered blood vessel mimic (BVM) include three traditional building blocks: cells, scaffolds, and bioreactors. For BVMs to best mimic the native vasculature, they must provide, at the least, a simple blood vessel structure. Therefore, the BVM models are comprised of vascular cell types, an electrospun polymer scaffold, and a perfusion system bioreactor (Fig. 5).

![Figure 5. The bioreactor system: peristaltic pump connected to a media reservoir and chamber containing the vessel of interest [16].](image)

The components of the BVMs used in Kristen Cardinal’s lab consist of a polymer scaffold with a cellular lining made of human endothelial cells (ECs) and sometimes smooth muscle cells (SMCs), because these are the cell types found within the native blood vessel. Human Umbilical Vein Endothelial Cells (HUVECs) are most commonly used in the BVM lab after being cultured in medium, passed, and maintained at 37 °C and 5% CO₂ for expansion.

While cells are being cultured for use in the BVMs, scaffolds are simultaneously prepared. In general, the role of a scaffold is to mimic the extracellular matrix (ECM) of a native blood vessel.
A scaffold is the biomaterial that contributes to the structure and mechanical properties of the BVM, as well as to the formation of new tissue in vivo. Therefore, the scaffold material should be functionally non-thrombogenic, non-immunogenic, compatible with high blood flow rates, and have similar viscoelasticity to native vessels [2]. Along with this, the scaffolds used in tissue engineering applications must be easily sterilizable and able to maintain their sterility. Scaffolds can be composed of several different types of biomaterials or polymers depending on the application and desired use. A closer look at BVM scaffolds will be provided in Sec 1.6.

Once the desired cells are properly expanded and scaffolds are prepared, a bioreactor system becomes necessary. A bioreactor is defined as a device capable of creating the proper environment for a biologic product [17]. The bioreactor’s primary role is to provide housing and a controlled environment for a scaffold and cell construct. This involves controlling temperature, pH, and gas, transporting nutrients, and simulating blood flow. There are several types of bioreactors found in the tissue engineering field: including spinner flasks, rotating wall bioreactors, and flow-based bioreactors. In the BVM lab, a flow-based bioreactor is assembled and utilized for blood vessel cultivation. The bioreactor is connected to a peristaltic pump and media reservoir that allow for continuous transluminal or transmural flow of media throughout the system and scaffold.

With all the components selected and prepared, BVMs are fabricated by combining the polymeric scaffold with previously cultured cells and cultivating the construct in the assembled bioreactor (Fig. 6). First, the fabricated scaffold is cut to the appropriate size and sutured onto fittings for later conditioning. Once the scaffold fits the desired measurements, it undergoes the process of conditioning within the bioreactor before adding and cultivating cells. Conditioning involves the scaffold being flushed and coated with “conditioning media” that contains proteins necessary for cell adhesion and growth. The polymeric scaffolds are then sodded, or injected and covered, with ECs and/or SMCs and cultivated to create a complete BVM. The resulting scaffold and cell construct is incubated in the bioreactor system for a specified timepoint while connected
to a peristaltic pump and media reservoir. Once the timepoint is completed, the cultivated construct is then harvested from the system and chemically fixed to preserve its current state for imaging and analysis.

Figure 6. The sequential steps in preparing the cells, scaffold, and bioreactor for the creation of a tissue-engineered blood vessel [15].

1.6 A Deeper Look at BVM Scaffolds

A key component of a BVM is the scaffold, which provides the vessel's structural and mechanical integrity. To fabricate BVMs, researchers must choose an appropriate biomaterial for the scaffold. These biomaterials are determined based on specific criteria, for example, whether they are natural or synthetic and degradable or non-degradable. In Dr. Cardinal’s Tissue Engineering Lab, the standard polymers used have been expanded Polytetrafluoroethylene (ePTFE) and Poly(lactic-co-glycolic acid) (PLGA). Newer polymers and polymer combinations that the lab
has more recently explored include PLGA with salt, Poly-L-lactic Acid (PLLA), and Polycaprolactone (PCL).

Polytetrafluoroethylene (PTFE), also known as Teflon, is a synthetic polymer made via tetrafluoroethylene polymerization (Fig. 7). This polymer can be expanded to create ePTFE, which has a microporous structure that enhances the polymer by providing a high strength-to-weight ratio, considerable biocompatibility, high thermal resistance, and high porosity [18]. This polymer is well-established with notable biocompatibility [19]. ePTFE has been used in many medical devices, including vascular grafts, stents, cardiovascular and soft tissue patches, facial implants, surgical sutures, and endovascular prostheses [20]. While this polymer seems to hold many desirable characteristics for tissue engineering, it is not degradable, and it is not something that can be fabricated or customized internally at Cal Poly. Along with this, the expanded polymer’s hydrophobic surface provides an unfavorable non-adhesion property, making it challenging to coat its surface with any enhancing technologies (i.e. coatings that increase cell adhesion and/or growth) [21]. Therefore, although ePTFE was used for many years as the lab’s BVM scaffold, it has been recently replaced by several other degradable and more tailorable polymers.

Figure 7. Polytetrafluoroethylene. Made from the monomer tetrafluoroethylene by free radical vinyl polymerization [22].

The most common, recently used scaffold in the BVM lab is Poly(lactic-co-glycolic acid) (PLGA). PLGA consists of two monomers, lactic acid and glycolic acid, and has the molecular structure displayed in Figure 8 below. PLGA is used in many biomedical applications including as
scaffolds, hydrogels, and injectable microspheres in bone tissue engineering [23], as nanoparticles in drug delivery systems [24], and in vascular tissue engineered devices [25]. This is a widely used polymer due to its low level of toxicity, biocompatibility, tailorable biodegradation rate, prevalence in devices approved for clinical use in humans, and its potential to be surface-modified to enhance cell interaction, adhesion, and growth [23]. One disadvantage of PLGA is its suboptimal mechanical properties when used in load-bearing applications. This is why PLGA is commonly combined with other materials to enhance its mechanical properties [23]. Additionally, PLGA has shown undesirable stiffness within the BVM lab, especially when compared to ePTFE [26]. Although there are limitations of using PLGA, it was ultimately selected as a polymer for BVM fabrication for the reasons stated previously, along with the fact that it dissolves in many common solvents, including tetrahydrofuran, acetone, ethyl acetate, and chlorinated solvents [27]. This solubility factor makes it beneficial for the lab’s BVM, as electrospinning requires the polymer to be dissolved in various solvents [28]. Another benefit of PLGA is its porous matrix, which provides a cell-favorable structure that promotes cell adhesion, migration, and growth. Overall, PLGA was deemed an appropriate polymer for electrospinning BVM scaffolds due to its ability to be electrospun into fibrous, porous constructs, its ability to provide critical cellular responses under shear stress, its ability to be ethyl alcohol (EtOH) gas sterilized, and its low cost [27].

Figure 8. Poly(lactic-co-glycolic acid). The molecular structure of PLGA with x and y indicating the number of times each unit repeats [28].
While PLGA possesses desirable polymer properties and characteristics for scaffold fabrication, the addition of sodium chloride (NaCl) or other salts into the polymer solution may be beneficial. The addition of salt results in a more viscous electrospinning solution, which creates scaffolds with uniformly distributed pores from the interior region to the surface of the scaffold. While PLGA plus salt scaffolds can maintain their structural integrity, the salt addition also seems to weaken the mechanical properties of scaffolds [29]. More specifically, experiments have shown how scaffolds constructed of PLGA without salt result in constructs with denser morphology, larger pores, and substantial leaching, while scaffolds constructed of PLGA with salt result in constructs with smaller pores and thus less leaching [30]. Pore size and interconnection between pores are essential components in scaffold fabrication when thinking about eventual cell sodding, adhesion, and growth; therefore, the addition of salt to PLGA was desirable for the BVM lab. This will be further introduced in Section 1.7.1.

An even more recent polymer that has been implemented is PLLA. PLLA is the L-isomer of polylactic acid (PLA) (Fig. 9), a thermoplastic polymer common in biodegradable medical device applications. PLLA is a known biodegradable, biocompatible, and biologically inert polymer [31]. PLLA’s notable degradability occurs from the natural degradation of the polymer in situ through the mechanism of hydrolysis. The disadvantages of PLLA include low toughness (due to brittleness), slow degradation rate (due to crystalline structure and molecular weight), hydrophobicity (due to a static water contact angle of about 80°), and the lack of reactive side chain groups (due to chemical inertness). These disadvantages can lead to plastic deformation at high-stress levels, an extensive lifetime of devices in vivo, low cell affinity, inflammatory response upon direct contact to biological fluids, and challenging surface functionalization and bulk modification [32]. Although PLLA has several undesirable properties, the polymer’s material properties, such as composition or molecular weight, can be tailored to potentially better suit the BVM. This ability to be tailored makes it an ideal candidate in applications such as bone fixation screws, bioreabsorbable
suture threads, and stent coatings [32]. When PLLA is fabricated, the polymer can provide highly porous and fibrous scaffolds. The resulting structure from electrospinning PLLA closely mimics the natural extracellular matrix and leads to improved cell adhesion and growth throughout the scaffold [33]. Although PLLA comes with limitations, the polymer has many desirable characteristics that make it valuable to the BVM lab.

![Figure 9. Poly-L-lactic Acid (PLLA).](image)

**Figure 9. Poly-L-lactic Acid (PLLA).** The molecular structure of PLLA with n indicating the number of times each unit repeats [34].

Another more recent polymer that has been introduced to the BVM lab is PCL. PCL is a synthetic polyester (Fig. 10) that degrades by hydrolysis under physiological conditions and is used in many medical applications, such as in implantable biomaterials and in capsules for controlled release and targeted drug delivery [35]. This synthetic polyester is biodegradable, biocompatible, nontoxic, has desirable mechanical properties (i.e., high strength), and is readily available. Like PLLA, PCL has a slow degradation rate and high permeability [35]. Disadvantages of PCL in tissue engineering include its hydrophobic surface, which can decrease cell retention, along with the fact that solvents used in fabrication of this polymer can be toxic to cells [36]. Although there are several disadvantages to PCL, it is known to be a material with exceptional electrospinning properties. The polymer's nanofibrous structures when spun provide a large surface area, small diameter of pores, and a high porosity that make it ideal for the fabrication of an electrospun polymer [37]. Therefore, it is a favorable polymer for use in BVM scaffold fabrication.
Overall, a variety of polymer scaffolds have been explored and implemented for BVMs. A summary of these polymers is provided in Table 1. As mentioned previously, fabrication plays a key role in polymer properties. As described in Section 1.5, Cal Poly’s Tissue Engineering Lab uses an electrospinning process to fabricate structured microfiber or nanofiber scaffolds. This process contributes to the polymer structure and properties, and requires the use of a solvent, which impacts biocompatibility. Specifics about the electrospinning process will be covered in the next section.
Table 1. Summary table of polymers that have been used as BVM scaffolds.

<table>
<thead>
<tr>
<th>Polymers</th>
<th>Example Uses in Research Lab</th>
<th>Example Uses in the Medical Field</th>
<th>Advantages</th>
<th>Disadvantages</th>
</tr>
</thead>
<tbody>
<tr>
<td>ePTFE</td>
<td>Tissue-engineered vascular grafts as <em>in vitro</em> BVMs [38], Assessment of BVMs with optical coherence tomography [39], Development of an in vitro tissue engineered BVM [40]</td>
<td>Vascular grafts, stents, cardiovascular and soft tissue patches, facial implants, surgical sutures, and endovascular prostheses [20]</td>
<td>Microporous structure, high strength-to-weight ratio, biocompatibility, high thermal resistance, high porosity [18]</td>
<td>Nondegradable, cannot be fabricated or customized internally at Cal Poly, hydrophobic surface [21]</td>
</tr>
<tr>
<td>PLGA</td>
<td>Preparation and characterization of electrospun PLGA scaffolds [27], Electrospinning polymer scaffolds for tissue engineered BVMs [41], Custom tissue engineered aneurysm models [42], Tissue-engineered aneurysm models[43]</td>
<td>Porous or fibrous scaffolds, hydrogels, and injectable microspheres in bone tissue engineering [23], nanoparticles in drug delivery systems [24], vascular tissue engineering devices [25]</td>
<td>Minimal toxicity, biocompatibility, tailored biodegradation rate, ability to modify surface properties to provide better cell interaction [23], ability to provide critical cellular responses under pulsatile flow, ability to be EtO gas sterilized in house, low cost [27]</td>
<td>Less than ideal mechanical properties when used in load-bearing applications [23], undesirable stiffness [26]</td>
</tr>
<tr>
<td>PCL</td>
<td>Evaluation of new polymers for electrospinning tissue engineered BVMs [44]</td>
<td>Implantable biomaterials, providing a capsule for controlled release, targeted drug delivery [35]</td>
<td>Biodegradable, biocompatible, nontoxic, strength, ease of availability, slow degradation rate, high permeability [35]</td>
<td>Hydrophobic surface, low cell retention, solvents that can be toxic to cells [36]</td>
</tr>
</tbody>
</table>
1.7 Electrospinning

Electrospinning is a widely used scaffold fabrication technique in the tissue engineering field. Researchers use electrospinning for many different applications, including tissue engineering and drug delivery [45]. Except for ePTFE, which has been obtained from commercial vendors, all other BVM scaffolds in Dr. Cardinal’s Tissue Engineering Lab have been created in-house via electrospinning. The electrospinning process utilizes a DC voltage to spin fine, small diameter fibers from a chosen polymer solution onto an area of the researcher’s choice to create the desired scaffold shape. An electrospinning apparatus, shown generically below in Figure 11, consists of three components: a high voltage power supply, a spinneret, and a grounded collector (in the lab’s case, a rotating mandrel or a flat plate). Once the polymer of choice is completely dissolved within a specific solvent and placed into a syringe, an electric charge is applied to the polymer solution surface. The charged solution is then forced out of the syringe tip and rapidly whipped around within the gap between the syringe tip and the collector, thus evaporating the solvent and leaving fibers of the polymer on the collecting mandrel [46]. The resulting scaffold is then removed from the collecting mandrel and placed into a desiccator for moisture removal.

![Electrospinning setup diagram](image)

Figure 11. Electrospinning set up. Schematic diagram of the horizontal setup of an electrospinning apparatus [45].
The key variables and parameters in the electrospinning process are the polymer type, solvent type, collector/mandrel size, gap distance between the spinneret and the collector, syringe pump flow rate, and applied voltages. An advantage of the electrospinning process is the ability to create different fiber morphology and fiber diameters from the adjustment of these parameters. In addition, the process is a very simple, versatile, and cost-effective way to create non-woven fibers with high surface area to volume ratio, controllable porosity, and adequate mechanical properties. Along with this, electrospinning creates scaffolds with fiber diameters that mimic the native tissue environment, which promote cell-matrix and cell-cell interactions, and thus encourage cell growth, as seen in Figure 12 below [46], [47].

![Figure 12. Comparison of Electrospun Scaffolds to that of a Native Airway ECM.](image)

Scanning electron microscope (SEM) images of a decellularized basement membrane (A), a cross-section of a decellularized airway bronchiole, (C), and a histological section of an airway smooth muscle bundle stained with hematoxylin and eosin (H&E), scale bar 40 um (E) compared with nanofiber (B) biphasic (D) and aligned (F) PET electrospun scaffolds [47].
1.7.1 Electrospinning of More Tailored Scaffolds

Along with using the electrospinning process to fabricate the BVM lab’s standard tissue-engineered blood vessels, the lab has also used it to create more specialized approaches and scaffolds. One of these approaches includes the addition of benzyl triethylammonium chloride (BTEAC) salt into the polymer solution during the electrospinning fabrication process (known in the lab as “salt spins”). The goal of this approach is to create nanofibrous scaffolds with smaller pore sizes that allow for better endothelial cell adhesion without the use of smooth muscle cells. This provides a sometimes preferred single-sodded model (one cell type instead of two) that allows for a more straightforward BVM setup and analysis. Adding BTEAC salt into the scaffold requires performing the standard electrospinning technique using a 75:25 PLGA to chloroform ratio with a small addition of BTEAC salt. Based on PLGA’s mass, the addition of 3 percent by weight of BTEAC salt addition was found to be optimal for creating the smallest electrospun fibers. Adding more than 3 percent by weight led to insignificant changes in fiber diameter. Overall, the addition of BTEAC salt to PLGA scaffolds provides an effective and reproducible method of creating nanofiber scaffolds [41].

Along with the specialized electrospinning approach mentioned above, researchers in the lab have also developed custom aneurysm-shaped molds to create aneurysm BVM scaffolds. To create these aneurysm geometries, molds with a negative impression of pre-dimensioned CAD models (Fig. 13) were machined, a stainless-steel mandrel was placed inside the empty molds, and, finally, melted, water-soluble wax was injected around the mandrels to form casts. The aneurysm scaffolds were characterized by their overall dimensions and microstructural components and were later used for cell deposition and vessel cultivation. Results from aneurysm BVM studies demonstrated feasibility in creating custom aneurysm in vitro models due to appropriate fiber morphology within the various aneurysm shapes, consistent cell deposition, and successful cultivation of the aneurysm BVMs [43].
Figure 13. Design of Electrospun Aneurysm Scaffolds. Saccular, fusiform, and blister geometries (a-c) [43].

While the electrospinning fabrication process provides adequate polymer structures and properties in both the standard and more specialized approaches used by tissue engineering lab researchers, it does require the use of a solvent, and may include additional aspects such as salt additives or wax molds, which can have significant impacts on biocompatibility.

1.8 Definition and Fundamentals of Biocompatibility

Understanding and evaluating the biocompatibility of a scaffold is paramount, regardless of which polymer is chosen for the scaffold, how specific electrospinning parameters are set, and whether any additional complexities are added. Biocompatibility can be defined as the ability of a material to perform with an appropriate host response in a specific situation [48]. The phrase ‘appropriate host response’ refers to the ability of a material to be harmless to the host tissue and/or to not fail due to biological mechanisms of the body [49]. To limit any detrimental reactions or life-threatening complications, host tissue responses must be well characterized and evaluated before the material is used clinically.

The understanding of biocompatibility has changed over time and, therefore, more detailed descriptions of it continue to emerge as the medical field grows. With regard to the traditional medical device field, biocompatibility is described as summarized above, with a focus on the basic interaction of a material with cells. With regard to the tissue engineering field, biocompatibility is described as the ability of three-dimensional artificial tissue to be accepted by host defense
mechanisms upon implantation, while still maintaining functional capacity [50]. In short, biocompatibility involves how an organism responds to contact with a foreign material and how that material is affected by the organism. An organism includes either a human or animal and/or a local tissue or tissue distant from the site of contact [51]. Organism responses include things like healing, developing a tumor, blood clots, or even death. A foreign material can be fully implanted into an organism or can be something that simply contacts a mucous membrane, the skin, etc. Additionally, contact timepoints for a foreign material can be brief (< 24 hours, < 30 days) or lengthy (> 30 days). Overall, many factors contribute to the biocompatibility of a material and, therefore, researchers must carefully select appropriate biomaterial(s) to be used when designing a contact device.

1.8.1 Biocompatibility Background

Before a medical device or biomaterial come in contact with the body—whether directly or indirectly—researchers must perform extensive biocompatibility testing on the material and/or device. The material characteristic of biocompatibility distinguishes a biomaterial from other material types due to its ability to perform its intended function within the human body without unintentionally harming the body or material.

One harmful effect that may occur if a material or device is not biocompatible is the potential of triggering the foreign body response. Because the surfaces of most engineered materials and devices do not hold biological surface markers, the body recognizes them as foreign. Proteins from the blood react to this foreign article by immediately adsorbing to the surface of it, ultimately initiating the body’s immune response that attempts to eliminate the material or device. The body’s foreign body immune response typically involves a cascade of five events: 1) material/device implant, 2) protein adsorption, 3) cellular recognition, 4) inflammation, and 5) foreign body response/resolution. Each of these biological steps can involve short-term or long-term effects to the body. Another harmful effect that may occur is cytotoxicity, or any toxic agent or process that
kinds living cells [52]. Therefore, a critical component of designing a biomaterial is to ensure that these undesirable responses will not occur.

Although the polymers introduced above—PLGA, PLLA, and PCL—have displayed acceptable biocompatibility in the field [23][31][35], the electrospinning process requires these polymers to be dissolved in a solvent for later fabrication of the scaffold. Therefore, the solvents, along with the polymers, all must be tested for their biocompatibility, as the scaffold (solvent and polymer) come in direct contact with cells during BVM setups, as described in Section 1.5.

1.8.2 Various Biocompatibility Tests

To assess a material’s biocompatibility, various tests can be performed. As part of the overall risk management process, there are many factors to consider when choosing which test procedures to implement, for example, nature, degree, duration, frequency, and conditions of exposure to humans [48]. Biocompatibility tests include cytotoxicity, sensitization, hemocompatibility, pyrogenicity, implantation, genotoxicity, carcinogenicity, reproductive and developmental toxicity, and degradation assessments. Each medical device is different and therefore requires unique forms of biological evaluation. Biocompatibility testing of polymers used in BVM fabrication requires testing the effect of the polymer on cells, as this is the application in which they are used. Although a variety of biocompatibility tests involve contact with cells, a key test that all materials are subject to is cytotoxicity.

1.8.2.1 Cytotoxicity Testing

Cytotoxicity is an important test for all medical devices that come in contact with the body. Cytotoxicity is an in vitro test performed to evaluate whether a medical device causes cell death via leaching of toxic substances or via direct contact. Primary cytotoxicity experiments include cell viability testing using dyes that stain for live and dead cells in culture. Cells are then differentiated based on the ratio of both living to dead cells and assessed further [52]. For novel materials, two primary methods for cytotoxicity testing are recommended including direct contact and elution
procedures. Direct contact involves placing a material directly onto the cells and evaluating cell reaction to the material (i.e., whether cells live or die). Direct contact provides a preliminary insight into how cells react to certain material types and is an initial qualitative and quantitative assessment of cytotoxicity. Elution involves leaching all possible toxic substances out of a material and into a solution that is then used for culturing cells. This technique provides a more thorough qualitative and quantitative analysis of a material’s effect on cells, as it utilizes cell viability assays to screen for direct cytotoxic effects of specific compounds [48]. To qualitatively determine cytotoxic effects, the cells are examined microscopically and assessed for changes in, for example, general morphology, detachment, cell lysis, and membrane integrity. To quantitatively determine cytotoxic effects, the ratio of live to dead cells and the number of cells are assessed by objective means. These methods are utilized to determine the biological response of mammalian cells \textit{in vitro} using the appropriate biological parameters [53].

1.9 Biocompatibility Concerns of BVMs

With the materials and solvents required for BVM scaffold fabrication in mind, there are several concerns with the biocompatibility of these scaffolds. The biocompatibility of polymers that can be fabricated and customized internally at Cal Poly—PLGA, PLLA, and PCL—are all well-researched and well-understood, but their combination with corresponding solvents (Table 2) necessary for scaffold fabrication may impact their effect on cells.

<table>
<thead>
<tr>
<th>Polymer</th>
<th>Solvent</th>
</tr>
</thead>
<tbody>
<tr>
<td>PLGA</td>
<td>Chloroform</td>
</tr>
<tr>
<td>PLGA with salt</td>
<td>Chloroform</td>
</tr>
<tr>
<td>PLLA</td>
<td>Chloroform</td>
</tr>
<tr>
<td>PCL</td>
<td>Dichloromethane (DCM)</td>
</tr>
</tbody>
</table>

Table 2. The polymers used in BVM fabrication along with their corresponding solvents utilized during the electrospinning process.
As Table 2 displays, PLGA, PLGA with salt, and PLLA are dissolved in chloroform. Chloroform, or CHCl₃, is a colorless, volatile, liquid derivative of trichloromethane that was formerly used as an inhaled anesthetic during surgery. This compound is currently used as a solvent or a substance that helps other substances dissolve [54]. Chloroform has been studied for its effect on animals and humans and has demonstrated undesirable toxic effects. Due to its past use as an anesthetic, chloroform in high concentrations can result in narcosis, anesthesia, and in the most severe effects of acute exposure, depression of the central nervous system (CNS), and cardiac sensitization [55]. When studied for its effects on cells, chloroform has also shown concerning cytotoxic effects in vitro [56]. However, chloroform is also a common electrospinning solvent, and successful desiccation and rinsing may negate the cytotoxic effects. For example, an in vitro cytotoxicity study demonstrated the use of chloroform as a solvent in electrospun nanofibers had no cytotoxicity and was deemed biocompatible [57].

PCL is dissolved in dichloromethane (DCM), or CH₂Cl₂. Dichloromethane is a colorless, flammable liquid that exhibits high volatility and stability, but is widely used as an organic solvent. DCM’s applications include paint removal, cleaning agents, aerosol products, and insecticides. Although it has many applications, the solvent’s ability to vaporize quickly and remain in the air makes it a toxic and potentially fatal chemical to humans. Similar to chloroform, this solvent has presented with inhalation, neurotoxicity, carcinogenicity, and skin irritation dangers [58]. The dangerous toxicological effects found with chloroform and dichloromethane raise a concern about their role in the biocompatibility of the polymer solution used in BVM scaffold fabrication, although similar to chloroform, successful use in electrospun scaffolds has been documented. For example, electrospun PLGA fibrous scaffolds were dissolved in a mixture solvent containing DCM and were deemed biocompatible [59].

In addition to the solvents themselves, the addition of benzyl triethylammonium chloride (BTEAC) to the PLGA solution raises a biocompatibility concern as well. BTEAC is an organic
compound added to PLGA to decrease fiber diameter, resulting in thinner nanofibers and decreased pore size. Although thought to be beneficial, the addition of BTEAC into the BVM scaffold solution adds another component that may impact biocompatibility of the scaffolds. While BTEAC presents a concern, acute toxicity tests done with BTEAC on small planktonic crustaceans showed toxicity levels far below what would be required to induce immobilization. Therefore, BTEAC may result in only minimal toxicity to cells (i.e. not enough to induce cell death) [60]. The various biocompatibility concerns summarized above demonstrate the need for biocompatibility testing of the solvents and additives of the BVM polymers.

1.10 Summary and Aims of the Thesis

BVMs continue to be optimized to improve their consistency and utility as preclinical testing models. As new modifications are made, it is important that biocompatibility testing is done to evaluate the improved models. BVM scaffolds can be composed of an assortment of polymers that will contact different cell types, leading to the need for biocompatibility testing of the polymers prior to their implementation as BVM scaffolds. Many of the lab’s newest scaffolds have not been fully characterized for biologic interactions, and future scaffold development will benefit from early-stage biocompatibility testing. The overall goal of this master’s thesis was to evaluate the biocompatibility of existing and novel BVM scaffolds, with a specific focus on cytotoxicity. To accomplish this goal, three aims were pursued: 1) Develop the proper and necessary protocols for cytotoxicity testing, including both direct contact and elution methods, 2) Assess the cytotoxicity of standard polymers—ePTFE and PLGA—used in the tissue engineering lab, and 3) Assess the cytotoxicity of recent polymer additions to the lab—PLLA, PCL, and PLGA with salt.

1.10.1 Aim 1: Protocol Development for Cytotoxicity Testing

The first aim of this thesis was to develop the necessary protocols for in vitro cytotoxicity testing of polymers used in the fabrication of BVMs. This included direct contact and elution cytotoxicity methods, along with a live/dead assay. The direct contact procedure involved placing
each electrospun polymer directly onto cells and evaluating the viability and morphology of the cells at various timepoints. This technique helped to gather preliminary qualitative data on the cytotoxicity of the different polymer types. The elution procedure involved investigating the cytotoxicity of the scaffolds through exposure of cells to scaffold elution products. Cell viability was quantified through a calcein AM and ethidium homdimer-1 (EthD-1) staining assay. These protocols were developed and optimized in Aim 1.

1.10.2 Aim 2: Biocompatibility of Existing BVM Scaffold Polymers: ePTFE and PLGA

The second aim of this thesis was to implement the newly developed cytotoxicity protocols to evaluate the biocompatibility of existing polymers, ePTFE and PLGA, used in the tissue engineering lab. This was done through implementing the direct contact, elution, and cell viability assays. Results from this aim served as a baseline of acceptable outcomes, since these two polymers have been used successfully as BVMs for many years.

1.10.3 Aim 3: Biocompatibility of Novel BVM Scaffold Polymers: PLGA with salt, PLLA, and PCL

The third aim of this thesis was to evaluate the biocompatibility of three novel electrospun polymers used for BVMs: PLGA with salt, PLLA, and PCL. This was done by implementing the protocols developed in Aim 1, including direct contact, elution, and cell viability assays.
CHAPTER 2. AIM 1: PROTOCOL DEVELOPMENT FOR CYTOTOXICITY TESTING

2.1 Introduction

The first aim of this thesis was to develop cytotoxicity protocols for evaluating the biocompatibility of electrospun polymers used for BVMs. To accomplish this goal, numerous studies were performed, each of which implemented both direct contact and elution-based methods. A variety of polymer types were implemented in these studies due to the frequent troubleshooting of the electrospinner. Issues with the electrospinner necessitated the use of whichever polymer could be spun effectively at the time. Trial #1 involved a preliminary assessment of cytotoxicity protocols for PLGA, PLGA with salt, and PLLA. Trial #2 involved performing the cytotoxicity protocols on ePTFE with a calcein AM and ethidium homodimer-1 cell viability assay. Calcein AM identifies live cells by their intracellular esterase activity and dyes the cells green. Ethidium homodimer-1 identifies dead cells by their lack of plasma membrane integrity and dyes the cells red [61]. Trial #3 involved performing cytotoxicity testing on ePTFE again but included other sterilization methods to ensure soaking the scaffold in EtOH and rinsing with DPBS (the sterilization method used in Trials #1 and #2) was an effective form of sterilization. Trial #4 involved cytotoxicity testing on PLGA, PLGA with salt, PLLA, and PCL. Trial #5 involved an elution test on the experiment’s controls. Trial #6 utilized positive and negative controls while implementing various timepoints and temperatures to the elution procedure for optimization of the elution protocol. Trial #7 involved a stain screen with several different stain concentrations and incubation times to optimize the staining protocol. Trial #8 involved the positive and negative controls with different extraction ratios for further optimization of the elution protocol.
2.2 Methods and Results

2.2.1 Cell Culture Procedure

To begin all experiments, mouse fibroblast (3T3) media was prepared following the lab’s standard operating procedure (SOP) 3010 rev A. Mouse fibroblast (3T3) cells were stored in vials in a liquid nitrogen dewar. A vial of 3T3s was thawed into 1 T75 and later passed into 1 T225 cell culture flask according to SOP 3020 rev C and SOP 3021 rev C. Cells were then incubated at 37 degrees Celsius (°C) and 5% Carbon Dioxide (CO₂).

2.2.2 Trial #1

This study involved a preliminary assessment of cytotoxicity protocols—developed from previous work done in the lab—on PLGA, PLGA with salt, and PLLA to ensure efficacy of the protocols. The direct contact procedure was implemented first, followed by the elution procedure to gather further qualitative and quantitative data.

2.2.2.1 Cell Seeding Procedure

Direct Contact Cell Seeding

Passage 28 3T3 cells at ~90% confluence in 1 T225 cell culture flask were harvested and resuspended in 24 mL of 3T3 media, referred to as the “cell solution.” Two mL of 3T3 media was placed into each well of a 6-well plate (Fig. 14) along with 0.5 mL of the cell solution, resulting in a final cell seeding density of ~187,500 cells per well or ~19,700 cells/cm² [62][63]. The 3T3 cells were counted according to SOP 3040 rev A. The 6-well plate was then placed in the incubator at 37 °C and 5% CO₂ for 24 hours.
Elution Cell Seeding

One 35 mm micro-dish (Fig. 15A) and two 8-well micro-slides (Fig. 15B) were used for cell seeding [65]. Passage 28 3T3 cells at ~90% confluence in 1 T225 cell culture flask were harvested and resuspended in 20 mL of 3T3 media. Two mL of 3T3 media and 0.5 mL of the cell solution was placed into the micro-dish, resulting in a final cell seeding density of ~225,000 cells per well or ~75,000 cells/cm². Additionally, 0.25 mL of 3T3 media and 0.05 mL of the cell solution was placed into each well of the micro-slides, resulting in a cell seeding density of ~22,500 cells per well or ~22,500 cells/cm². The seeding density was higher in these elution set-ups than in the direct contact experiment because the optimal cell seeding density was yet to be determined. The micro-dish and micro-slides were then placed in the incubator at 37 °C and 5% CO₂ for 24 hours.

Figure 15. Cell culture plates. A) Micro-dish 35 mm, high glass bottom, B) micro-slide 8-well [66][65].
2.2.2.2 Direct Contact Procedure

The scaffold sample used for this trial study was a thin, PLGA trial spin. The scaffold was cut into 4 pieces and sterilized in sterile 70% Ethanol (EtOH) for 20 minutes. The EtOH was aspirated out and replaced with Dulbecco’s Phosphate Buffer Solution (DPBS) for 2 minutes to rid the samples of any residual EtOH. After 2 minutes, the DPBS was aspirated out and forceps were used to transfer the sterile samples into their corresponding wells. The sterile samples were placed directly on top of the cells to incubate for various lengths of time before evaluation. Two of the 6 wells were left without polymer (thus containing only 3T3 cells in 3T3 media) to serve as a negative control. A negative control involves the same experimental procedures except that the treatment is changed to something that is predicted to have no result [67]. Well plates were observed at 10X magnification using a white-light fluorescence microscope at 12-, 24-, and 48-hour timepoints and qualitatively evaluated according to a scale from 0-3. Representative white-light images and their corresponding toxicity levels on the 0-3 scale are outlined below (Table 3).
Table 3. Representative white-light images on a scale from 0-3 of toxicity.

<table>
<thead>
<tr>
<th>Cytotoxicity Scale</th>
<th>Interpretation</th>
<th>Representative White-Light Images</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>Noncytotoxic</td>
<td><img src="image1" alt="Image" /></td>
</tr>
<tr>
<td>1</td>
<td>Mildly cytotoxic</td>
<td><img src="image2" alt="Image" /></td>
</tr>
<tr>
<td>2</td>
<td>Moderately cytotoxic</td>
<td><img src="image3" alt="Image" /></td>
</tr>
<tr>
<td>3</td>
<td>Severely cytotoxic</td>
<td><img src="image4" alt="Image" /></td>
</tr>
</tbody>
</table>

2.2.2.3 Elution Procedure

To begin the elution procedure, 600 mL of water was warmed to 50 °C in a 1000 mL beaker on a hot plate with a stir bar. Samples of PLGA with salt and PLLA were used for this experiment (Fig. 16). The PLGA with salt sample was cut into three sections, while the PLLA sample was left intact, as it was too thin and fragile to section off.
The polymer samples were sterilized in conicals of sterile 70% EtOH for 20 minutes and then subjected to DPBS for 2 minutes to rinse any residual EtOH from the polymers. The samples were then transferred to conicals of 10 mL 3T3 media and placed into the beaker of water at 50 °C for 72 hours (Fig. 17). A conical of only 3T3 media (no polymer) was included to serve as a negative control. After 72 hours, the samples were aseptically removed from the conicals and 0.3 mL of media from each conical was placed into the wells of the micro-slides previously seeded with cells. The micro-dish was left with only 3T3 cells and warmed 3T3 media from the fridge for later use as a control during staining. The micro-dish and micro-slides were then placed and left in the incubator for 24 and 48 hours.
2.2.2.4 Staining and Imaging Procedure

After 24 hours of incubation with the eluted media, the micro-dish and micro-slides from the elution procedure were stained and imaged. One of the micro-slides was stained with Bisbenzimide (BBI), a nuclear stain, and imaged with the widefield fluorescence microscope at 10X magnification following the lab’s SOP 5030 rev B. The other micro-slide was stained with Trypan Blue to stain any dead cells (Appendix A).

A positive control is a group in an experiment that is not exposed to the experimental treatment, but receives a treatment with a known result to ensure efficacy of the stain [68]. In this case, the micro-dish was subjected to 100% EtOH for 20 minutes to serve as a positive control. It was expected that all cells would die after being subjected to EtOH and therefore demonstrate the efficacy of the Trypan Blue stain in accounting for dead cells.
2.2.2.5 Trial #1 Results

Direct Contact Results

White-light images from the Trial #1 direct contact procedure can be found in Appendix B. The results of Trial #1 are summarized in Table 4. In summary, PLGA did not show cytotoxic effects at 24 hours and mild cytotoxic effects were observed at 48 hours.

Table 4. Results of Trial #1 direct contact procedure with PLGA. Numbers separated by commas represent different fields of view.

<table>
<thead>
<tr>
<th>Timepoint</th>
<th>Sample</th>
<th>Cytotoxicity Scale</th>
<th>Interpretation</th>
</tr>
</thead>
<tbody>
<tr>
<td>12-hour</td>
<td>PLGA</td>
<td>0, 0, 0, 0</td>
<td>Noncytotoxic</td>
</tr>
<tr>
<td></td>
<td>3T3 media (neg. control)</td>
<td>0, 0, 0, 0</td>
<td>Noncytotoxic</td>
</tr>
<tr>
<td>24-hour</td>
<td>PLGA</td>
<td>0, 0, 0, 0</td>
<td>Noncytotoxic</td>
</tr>
<tr>
<td></td>
<td>3T3 media (neg. control)</td>
<td>0, 0, 0, 0</td>
<td>Noncytotoxic</td>
</tr>
<tr>
<td>48-hour</td>
<td>PLGA</td>
<td>1, 1, 1, 0</td>
<td>Mildly cytotoxic</td>
</tr>
<tr>
<td></td>
<td>3T3 media (neg. control)</td>
<td>0, 0, 0, 0</td>
<td>Noncytotoxic</td>
</tr>
</tbody>
</table>

Elution Results

The results of the elution procedure (Appendix B) revealed an unsuccessful staining and imaging protocol. It became evident that a stain able to differentiate between live and dead cells would be more useful; therefore, BBI was ineffective for this application. No results were taken from the micro-dish (positive control) because all the cells had died and detached from the bottom of the well after subjection to 100% EtOH for 20 minutes. No results were gathered from the Trypan Blue stained micro-slide because no cells were found when counting cells with the hemocytometer (SOP 3040 rev A).
2.2.2.6 Trial #1 Issues Noted

Direct Contact Issues Noted

Issues presented by the direct contact procedure in Trial #1 included sterile forceps not being long enough to transfer the polymer from the conical of DPBS to the wells; therefore, the DPBS had to be aspirated from the conical and the polymer had to be brought up high enough to be transferred with shorter sterile forceps. Additionally, there was no replacement of media 24 hours after the cells were seeded and before the polymers were placed into the wells. Furthermore, due to a high volume of media in the wells, the polymer samples were not sitting directly on top of the cells.

Elution Issues Noted

Issues presented by the elution procedure in Trial #1 included over-confluency in the micro-dishes and under-confluency in the micro-slides at 24 hours. Along with this, 600 mL of water in the beaker was too high, causing the conicals of media to be briefly submerged during the elution. After 24 hours of the elution, the 600 mL of water from the beaker had evaporated. When removing the PLGA with salt sample from the conical of media, the polymer broke apart in the media because it was electrospun months before use and became brittle (Fig. 18). Several issues were also seen with the staining and imaging protocol. Specifically, BBI and Trypan Blue were unsuitable stains for this specific application. Additionally, the use of 100% EtOH for 20 minutes was an ineffective positive control due to observed cell detachment from the well plate.
2.2.2.7 Trial #1 Next Steps to Try

Direct Contact Next Steps to Try

Due to the direct contact issues described above, the next direct contact experiment involved longer sterile forceps for transfer of the polymer samples, a replacement of media 24 hours after cell seeding before polymer placement, and less volume of media in the wells. Less volume of media would hopefully allow the polymer samples to rest on top of the cells instead of floating above.

Elution Next Steps to Try

In response to the elution issues described above, 500 mL of water was placed in the beaker so the conicals would not submerge in the water. Tinfoil was also placed on top of the beaker during the elution to reduce the evaporation of water. To ensure the water was kept at 50 °C and 500 mL, the water was evaluated every day throughout the 3-day timepoint. Polymers were freshly spun before use to overcome the brittleness observed with PLGA. Cell seeding densities were adjusted accordingly to seed at ~60-70% confluence. Only 6-well plates were utilized, rather than 8-well plates. Calcein AM and EthD-1 were implemented instead of BBI and Trypan Blue. The positive
control was created with the use of sterile 70% EtOH for 30-45 seconds instead of 100% EtOH for 20 minutes.

2.2.3 Trial #2

Trial #2 involved performing adjusted cytotoxicity protocols on ePTFE with the calcein AM and EthD-1 cell viability assay. ePTFE was selected for this trial because it was a well-established biocompatible scaffold material and, for this reason, proved useful in the development of protocols for cytotoxicity testing. The direct contact procedure was implemented first, followed by the elution procedure to gather further qualitative and quantitative data.

2.2.3.1 Cell Seeding Procedure

Direct Contact Cell Seeding

Passage 34 3T3 cells at ~90% confluence in 1 T225 cell culture flask were harvested and resuspended in 24 mL of 3T3 media. Next, 1.5 mL of 3T3 media and 0.5 mL of the cell solution was placed into each well of a 6-well plate, resulting in a final cell seeding density of ~187,500 cells per well or ~19,700 cells/cm² [62]. The 6-well plate was then placed in the incubator at 37 °C and 5% CO₂ for 24 hours.

Elution Cell Seeding

Passage 34 3T3 cells at ~90% confluence in 1 T225 cell culture flask were harvested and resuspended in 24 mL of 3T3 media. Next, 1.5 mL of 3T3 media was placed into all wells of two 6-well plates and two micro-dishes. One mL of the cell solution was placed into one 6-well plate and one micro-dish (24-hour timepoints), resulting in a cell seeding density of ~375,000 cells per well or ~39,500 cells/cm². Additionally, 0.5 mL of cell solution was placed into the other 6-well plate and micro-dish (48-hour timepoints), resulting in a cell seeding density of ~187,500 cells per well or ~19,700 cells/cm². Half of the cell seeding density was placed into the 48-hour timepoint well plate because it would be incubating for 24 hours longer than the 24-hour timepoint well plate.
The 6-well plates and micro-dishes were then placed in the incubator at 37 °C and 5% CO₂ for 24 hours.

### 2.2.3.2 Direct Contact Procedure

ePTFE scaffolds were sterilized in sterile 70% EtOH for 20 minutes and rinsed with DPBS for 1 minute. One ePTFE scaffold was left in EtOH unrinsed to serve as a positive control. The ePTFE samples were transferred into their corresponding wells (Fig. 19). Two wells were left without samples (only 3T3 cells with 3T3 media) to serve as a negative control. The well plate was then placed in the incubator until being qualitatively evaluated under the white-light fluorescence microscope at 12-, 24-, and 48-hour timepoints.

![Image of 6-well plate with ePTFE scaffolds](image)

**Figure 19.** Trial #2 direct contact procedure with ePTFE scaffolds.

### 2.2.3.3 Elution Procedure

Three ePTFE scaffolds were placed in sterile 70% EtOH for 20 minutes and rinsed with DPBS for 1 minute before being transferred to 10 mL conicals of 3T3 media (Fig. 20A). One conical was left without a scaffold to serve as a negative control. The conicals were placed in water at 50 °C for 72 hours (Fig. 20B). The scaffolds were then removed from the media and 2.5 mL of the eluted media was placed into previously prepared wells of 3T3 cells. The well plates were then placed in the incubator for 24 and 48 hours.
Figure 20. **Trial #2 elution procedure.** A) Three ePTFE scaffolds placed into 10 mL of 3T3 media, B) the conicals placed into water at 50 °C for 72 hours.

### 2.2.3.4 Staining and Imaging Procedure

After 24 hours of incubation with the eluted media, the well plates were evaluated and imaged under the white-light microscope. Media was aspirated out of the well plates and the micro-dish was subjected to sterile 70% EtOH for 30-45 seconds to serve as a positive control for the staining protocol. All wells were rinsed with DPBS, and the live/dead stock stain solution was created with 5 mL DPBS + 4 uL EthD-1 + 3 uL calcein AM. Next, 0.5 mL of the stain solution was placed into all wells for 20 minutes before being aspirated out carefully as to not aspirate any cells. The wells were imaged using the widefield fluorescence microscope. The staining and imaging procedure was repeated for the 48-hour timepoint wells with a replacement of the eluted media at 24 hours. Due to unclear images and low fluorescence of the stain at 24 hours, a 10 mL DPBS + 12 uL EthD-1 + 10 uL calcein AM stock stain solution was used at 48 hours and 1.5 mL of the stain was placed onto the wells for 30 minutes.
2.2.3.5 Trial #2 Results

Direct Contact Results

White-light images from the Trial #2 direct contact procedure can be found in Appendix C. The results from Trial #2 are summarized in Table 5. ePTFE demonstrated noncytotoxic effects on 3T3 cells at each timepoint. Additionally, the ePTFE left to soak in EtOH and unrinsed served as an effective positive control because it demonstrated cell death.

Table 5. Results of Trial #2 direct contact procedure with ePTFE. Numbers separated by commas represent different fields of view.

<table>
<thead>
<tr>
<th>Timepoint</th>
<th>Sample</th>
<th>Cytotoxicity Scale</th>
<th>Interpretation</th>
</tr>
</thead>
<tbody>
<tr>
<td>12-hour</td>
<td>ePTFE</td>
<td>0, 0</td>
<td>Noncytotoxic</td>
</tr>
<tr>
<td></td>
<td>ePTFE in EtOH (pos. control)</td>
<td>3, 3</td>
<td>Severely cytotoxic</td>
</tr>
<tr>
<td></td>
<td>3T3 media (neg. control)</td>
<td>0, 0</td>
<td>Noncytotoxic</td>
</tr>
<tr>
<td>24-hour</td>
<td>ePTFE</td>
<td>0, 0</td>
<td>Noncytotoxic</td>
</tr>
<tr>
<td></td>
<td>ePTFE in EtOH (pos. control)</td>
<td>3, 3</td>
<td>Severely cytotoxic</td>
</tr>
<tr>
<td></td>
<td>3T3 media (neg. control)</td>
<td>0, 0</td>
<td>Noncytotoxic</td>
</tr>
<tr>
<td>48-hour</td>
<td>ePTFE</td>
<td>0, 0</td>
<td>Noncytotoxic</td>
</tr>
<tr>
<td></td>
<td>ePTFE in EtOH (pos. control)</td>
<td>3, 3</td>
<td>Severely cytotoxic</td>
</tr>
<tr>
<td></td>
<td>3T3 media (neg. control)</td>
<td>0, 0</td>
<td>Noncytotoxic</td>
</tr>
</tbody>
</table>

Elution Results

White-light images from the Trial #2 elution procedure can be found in Appendix C. Images taken on the widefield fluorescence microscope are displayed in Figures 21 and 22. Over-confluency was observed in all wells. Along with this, the images demonstrated that ePTFE scaffolds caused severe cell death, as cells were detaching from the surface of the flask and there were large areas where no cells were found. The negative and positive controls—warmed 3T3 media and samples subjected to EtOH—were ineffective in providing a basis for comparison.
between cells cultured with the ePTFE eluted media and cells cultured with the controls. The inefficacy of the controls was due to the positive control not fluorescing dead cells and the negative controls detecting a high number of dead cells.

Failed Positive Control – EtOH subjection for 30-45 seconds

Failed Negative Control – 3T3 media

Failed Negative Control – 3T3 media warmed in 50 °C water for 72 hours

ePTFE

Calcein AM

Ethidium Homodimer-1

Dead cell
Figure 21. Trial #2 widefield fluorescence images of elution procedure at 24-hour timepoint.
10X magnification, 6% power, 500 ms exposure time.

- Failed Positive Control – EtOH subjection for 30-45 seconds
- Failed Negative Control – 3T3 media
Failed Negative Control – 3T3 media warmed in 50 °C water for 72 hours

Figure 22. Trial #2 widefield fluorescence images of elution procedure at 48-hour timepoint.
10X magnification, 6% power, 500 ms exposure time.
2.2.3.6 Trial #2 Issues Noted

Direct Contact Issues Noted

A notable issue from the Trial #2 direct contact procedure was that the positive control well containing the unrinshed ePTFE scaffold subjected to EtOH still contained confluent, live cells. Additionally, the negative control of only 3T3 media suggested that cell death was due to over-confluency rather than cytotoxic factors, which could result in false conclusions.

Elution Issues Noted

Over-confluency in all wells was also noted as an issue with the Trial #2 elution procedure. Severe cell death of cells immersed in the ePTFE eluted media was observed and determined by cell detachment from the flask surface as well as large areas where no cells were found. However, it was not possible to attribute the cell death to ePTFE, due to the failure of the negative controls. Observed cell death could have been attributed to the media not completely cooling from 50 °C to 37 °C before being placed onto the cells. The 24-hour widefield fluorescence images displayed an unsuccessful staining and imaging protocol due to unclear images and low florescence of cells. Adjustments were made to the protocol for the 48-hour timepoints: 10 mL DPBS + 12 uL EthD-1 + 10 uL calcein AM stock stain solution, 1.5 mL of stain placed into each well, and a 30-minute incubation time. While images looked clearer, the positive control was still unsuccessful in demonstrating cell death. In addition, the 48-hour timepoint images displayed a significant number of dead cells in warmed 3T3 media from the fridge, which was unexpected.

2.2.3.7 Trial #2 Next Steps to Try

Direct Contact Next Steps to Try

Due to the issues described above, the next direct contact procedure implemented a longer soak of the polymer in EtOH before placing it onto cells for the positive control, as well as lower cell seeding densities.
Due to the issues described above, the scaffolds were rinsed more thoroughly in DPBS after being sterilized in EtOH. In response to the previously unsuccessful positive control, the micro-dish was subjected to 2 mL of 100% methanol for 20 minutes instead of the previously used 100% EtOH for 20 minutes and sterile 70% EtOH for 30-45 seconds. This decision was based on knowledge gained from classes taken during protocol development. The cell seeding density was decreased to mitigate the observed over-confluency and cell death seen in warmed 3T3 media from the fridge. Regarding the staining and imaging protocol, to get clearer images, the power was increased, and the exposure time was decreased on the widefield fluorescence microscope. Additionally, to ensure the efficacy of scaffold sterilization, another round of ePTFE cytotoxicity testing involving other forms of sterilization methods was performed.

2.2.4 Trial #3

Trial #3 involved performing cytotoxicity testing on ePTFE again but included other sterilization methods to ensure efficacy of the sterilization method used in Trials #1 and #2. This trial included only an elution procedure. A direct contact procedure was not performed because this trial was focused on optimizing the elution procedure and sterilization parameters.

2.2.4.1 Cell Seeding Procedure

Passage 28 3T3 cells at ~90% confluence in 1 T225 cell culture flask were harvested and resuspended in 24 mL of 3T3 media. Next, 1.5 mL of 3T3 media was placed into four 6-well plates and two micro-dishes. Additionally, 0.5 mL of the cell solution was placed into two of the 6-well plates and one micro-dish (24-hour timepoints), resulting in a cell seeding density of ~187,500 cells per well or ~19,700 cells/cm². Next, 0.25 mL of cell solution was placed into the other 6-well plates and micro-dish (48-hour timepoints), resulting in a cell seeding density of ~93,750 cells per well.
or \(~9,900 \text{ cells/cm}^2\). The 6-well plates and micro-dishes were then placed in the incubator for 24 hours at 37 °C and 5% CO₂.

### 2.2.4.2 Elution Procedure

Three ePTFE scaffolds were subjected to different forms of sterilization. ePTFE scaffold #1 was sterilized by being placed onto fittings, soaked in a conical of sterile 70% EtOH for 20 minutes, and aseptically flushed with DPBS three times transmurally and transluminally. Sterile gloves and forceps were utilized to ensure a sterile environment. The fittings were cut off and the scaffold was placed into a 10 mL conical of 3T3 media. ePTFE scaffold #2 was sterilized via autoclaving and placed into a 10 mL conical of 3T3 media. ePTFE scaffold #3 was sterilized using the method from Trial #1 and #2 – soaked in a conical of sterile 70% EtOH for 20 minutes and then thoroughly rinsed in DPBS for 1 minute. This scaffold was placed into another 10 mL conical of 3T3 media. A conical of 10 mL 3T3 media (no scaffold) was included to serve as a negative control. All conicals were placed into the water bath at 50 °C for 72 hours. After 72 hours, the scaffolds were removed, and 1.5 mL of the eluted media was added to the cells. The well plates were then placed into the incubator for 24 and 48 hours.

### 2.2.4.3 Staining and Imaging Procedure

After 24 hours of incubation with the eluted media, the well plates were evaluated and imaged under the white-light microscope. The media was aspirated out of the well plates and the micro-dish was subjected to methanol for 20 minutes to serve as a positive control for the staining protocol. All wells were rinsed with DPBS, and the live/dead stock stain solution was made of 10 mL DPBS + 12 uL EthD-1 + 10 uL calcein AM. Next, 1.5 mL of the stain solution was placed into each well for 30 minutes and aspirated out carefully as to not aspirate any cells. The wells were then imaged using the widefield fluorescence microscope. This staining and imaging procedure was repeated for the 48-hour timepoint wells with a replacement of the eluted media at 24 hours.
2.2.4.4 Trial #3 Results

White-light images from the Trial #3 elution procedure illustrated noncytotoxic effects at 24-hours and mild cytotoxic effects at 48-hours with each sterilization method and the negative control of 3T3 media warmed to 50 °C for 72 hours. The other negative control (warmed 3T3 media from the fridge) was effective in demonstrating noncytotoxic effects, while the positive control (methanol subjection) was effective in demonstrating severe cytotoxic effects. Images can be found in Appendix D and results are summarized in Table 6.

Table 6. Results of Trial #3 elution procedure with ePTFE including various sterilization methods. Numbers separated by commas represent different fields of view.
Results from the widefield fluorescence images (Fig. 23, 24) illustrated that methanol for 20 minutes acted as an effective positive control for demonstration of cell death. Over-confluency was still observed in all wells. The fluorescence stain was much brighter than it had been in previous trials due to increased proficiency with the widefield fluorescence microscope and the increase in power from 6% power to 12% power. ePTFE was no longer demonstrating cell death and each sterilization method was effective and comparable. This elution procedure reinforced that each sterilization technique demonstrated low cell death; therefore, it was concluded that soaking the scaffold in EtOH and thoroughly rinsing with DPBS was an effective form of sterilization.

<table>
<thead>
<tr>
<th>Calcein AM</th>
<th>Ethidium Homodimer-1</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Effective Positive Control</strong> – methanol subjection for 20 minutes</td>
<td></td>
</tr>
<tr>
<td><img src="image1" alt="Calcein AM" /></td>
<td><img src="image2" alt="Ethidium Homodimer-1" /></td>
</tr>
<tr>
<td><strong>Effective Negative Control</strong> – 3T3 media</td>
<td></td>
</tr>
<tr>
<td><img src="image3" alt="Calcein AM" /></td>
<td><img src="image4" alt="Ethidium Homodimer-1" /></td>
</tr>
<tr>
<td><strong>Effective Negative Control</strong> – 3T3 media warmed in 50 °C water for 72 hours</td>
<td></td>
</tr>
<tr>
<td><img src="image5" alt="Calcein AM" /></td>
<td><img src="image6" alt="Ethidium Homodimer-1" /></td>
</tr>
</tbody>
</table>
ePTFE scaffold soaked in EtOH and flushed with DPBS

ePTFE scaffold autoclaved

ePTFE soaked in EtOH and thoroughly rinsed with DPBS

**Figure 23. Trial #3 widefield fluorescence images of 24-hour timepoint elution procedure.**
10X magnification, 12% power, 500 ms exposure time.

Calcein AM

Ethidium Homodimer-1

Effective Positive Control – methanol subjection for 20 minutes
Effective Negative Control – 3T3 media warmed in 50 °C water for 72 hours

ePTFE scaffold soaked in EtOH and flushed with DPBS

ePTFE scaffold autoclaved
2.2.4.5 Trial #3 Issues Noted

Over-confluency was still a notable issue from Trial #3. Future studies did not utilize the flushing with DPBS or autoclave sterilization techniques because polymers were spun on a flat sheet collector (mandrel collector was out for maintenance). Another issue was that no successfully established, positive control had been utilized to ensure cytotoxic substances were effectively being leached out of samples at an extraction condition of 50 °C for 72 hours.

2.2.4.6 Trial #3 Next Steps to Try

Due to the issues described above, the cell seeding density was decreased further. The sterilization technique of soaking the polymer in EtOH and thoroughly rinsing in DPBS was confirmed as an acceptable sterilization method. A longer rinsing time was implemented to ensure all residual EtOH was removed from the polymer. A sample of sterile, powder-free latex glove was used in the following elution procedures to serve as a positive control for the leaching of cytotoxic substances during the elution procedure.

2.2.5 Trial #4

All previous study takeaways were implemented in Trial #4, which involved cytotoxicity testing on PLGA (solvent: chloroform), PLGA with salt (solvent: chloroform), PLLA (solvent:
chloroform), and PCL (solvent: DCM). These polymer types were used to gather preliminary biocompatibility insight on these scaffold materials. Sterile, powder-free latex gloves were used as a positive control, as it has been shown that they leach out toxins from the polymer sample and kill cells that are cultured with the eluted media [69]. This trial included only an elution procedure. No direct contact procedure was performed because the elution procedure was still in need of optimization.

### 2.2.5.1 Cell Seeding Procedure

Passage 28 3T3 cells at ~90% confluence in 1 T225 cell culture flask were harvested and resuspended in 24 mL of 3T3 media. Two mL of 3T3 media was placed into each well of six 6-well plates and two micro-dishes. Next, 0.3 mL of the cell solution was placed into three of the 6-well plates and one micro-dish (24-hour timepoints), resulting in a cell seeding density of ~112,500 cells per well or ~11800 cells/cm². Additionally, 0.15 mL of cell solution was placed into the other 6-well plates and micro-dish (48-hour timepoints), resulting in a cell seeding density of ~56,250 total cells per well or ~6000 cells/cm². The 6-well plates and micro-dishes were then placed in the incubator for 24 hours at 37 °C and 5% CO₂.

### 2.2.5.2 Elution Procedure

To begin the elution procedure for Trial #4, each electrospun polymer and a powder-free latex glove sample were sterilized in a conical of sterile 70% EtOH for 20 minutes and then rinsed thoroughly with DPBS for 1 minute. The samples were transferred to separate conicals containing 15 mL of 3T3 media. In addition, 15 mL of 3T3 media (negative control) and 15 mL of 3T3 media with the sample of sterile, powder-free latex glove (positive control) (Fig. 25A) were included in the trial. All conicals were placed in water at 50 °C for 72 hours (Fig. 25B).
Figure 25. Trial #4 elution preparation. A) Sterile, powder-free latex glove (positive control), B) conicals placed in water at 50 °C for 72 hours.

After 72 hours, the samples were aseptically removed from the conicals (Fig. 26A) and the eluted media (Fig. 26B) was placed into the fridge to cool. Once cooled, the conicals were warmed in the water bath to 37 °C and 2 mL of the eluted media was placed into corresponding wells of the 6-well plates. The well plates were placed in the incubator for 24 and 48 hours.

Figure 26. Trial #4 samples after elution. A) Samples of polymer removed from the conicals after being placed in water at 50 °C for 72 hours, B) the resulting eluted media before being placed onto the cells.
2.2.5.3 Staining and Imaging Procedure

After 24 hours of incubation with the eluted media, the well plates were evaluated and imaged under the white-light microscope. The media was aspirated out of the well plates and the micro-dish was subjected to methanol for 20 minutes to serve as a positive control for the staining protocol. All wells were rinsed with DPBS, and the live/dead stock stain solution was made with 30 mL DPBS + 36 uL EthD-1 + 30 uL calcein AM. Additionally, 1.5 mL of the stain solution was placed into each well for 30 minutes and aspirated out carefully as to not aspirate the cells. The wells were then imaged using the widefield fluorescence microscope. This staining and imaging procedure was repeated for the 48-hour timepoint wells with a replacement of the eluted media at 24 hours.

2.2.5.4 Trial #4 Results

White-light images from Trial #4 at 24 hours (Appendix E) displayed variability between each polymer. Specifically, PLLA, PLGA, and PLGA with salt illustrated moderate cytotoxic effects while PCL scaffold and flat sheet samples illustrated mild cytotoxicity. These results are summarized in Table 7. The negative controls – 3T3 media and 3T3 media that was warmed to 50 °C for 72 hours – were successful in demonstrating limited cell death. The positive control—powder-free latex glove eluted media—was successful in invoking cell death. At 48 hours, white-light images (Appendix E) displayed insufficient cell confluence as well as dead cells with all images other than the negative control of warmed 3T3 media from the fridge. Additionally, while the negative control of 3T3 media displayed limited cell death, the negative control of 3T3 media warmed to 50 °C for 72 hours displayed significant cell death. The positive control of powder-free latex glove eluted media also presented the expected cell death.
Table 7. Results of Trial #4 elution procedure. Numbers separated by commas represent different fields of view.

<table>
<thead>
<tr>
<th>Timepoint</th>
<th>Sample</th>
<th>Cytotoxicity Scale</th>
<th>Interpretation</th>
</tr>
</thead>
<tbody>
<tr>
<td>24-hour</td>
<td>PLLA</td>
<td>2, 1</td>
<td>Moderately cytotoxic</td>
</tr>
<tr>
<td></td>
<td>PLGA</td>
<td>2, 2</td>
<td>Moderately cytotoxic</td>
</tr>
<tr>
<td></td>
<td>PLGA with salt</td>
<td>2, 1</td>
<td>Moderately cytotoxic</td>
</tr>
<tr>
<td></td>
<td>PCL scaffold</td>
<td>1, 1</td>
<td>Mildly cytotoxic</td>
</tr>
<tr>
<td></td>
<td>PCL flat sheet 210310</td>
<td>1, 1</td>
<td>Mildly cytotoxic</td>
</tr>
<tr>
<td></td>
<td>PCL flat sheet 210215</td>
<td>1, 1</td>
<td>Mildly cytotoxic</td>
</tr>
<tr>
<td></td>
<td>3T3 media warmed to 50 °C for 72 hours (neg. control)</td>
<td>0, 0</td>
<td>Noncytotoxic</td>
</tr>
<tr>
<td></td>
<td>3T3 media (neg. control)</td>
<td>0, 0</td>
<td>Noncytotoxic</td>
</tr>
<tr>
<td></td>
<td>Latex glove (pos. control)</td>
<td>3, 3</td>
<td>Severely cytotoxic</td>
</tr>
<tr>
<td></td>
<td>Methanol (pos. control)</td>
<td>3, 3</td>
<td>Severely cytotoxic</td>
</tr>
<tr>
<td>48-hour</td>
<td>PLLA</td>
<td>0, 2</td>
<td>Mildly cytotoxic</td>
</tr>
<tr>
<td></td>
<td>PLGA</td>
<td>0, 3</td>
<td>Moderately cytotoxic</td>
</tr>
<tr>
<td></td>
<td>PLGA with salt</td>
<td>2, 3</td>
<td>Severely cytotoxic</td>
</tr>
<tr>
<td></td>
<td>PCL scaffold</td>
<td>3, 3</td>
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<td>PCL flat sheet 210310</td>
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</tr>
<tr>
<td></td>
<td>PCL flat sheet 210215</td>
<td>3, 3</td>
<td>Severely cytotoxic</td>
</tr>
<tr>
<td></td>
<td>3T3 media warmed to 50 °C for 72 hours (neg. control)</td>
<td>3, 3</td>
<td>Severely cytotoxic</td>
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<tr>
<td></td>
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<tr>
<td></td>
<td>Methanol (pos. control)</td>
<td>3, 3</td>
<td>Severely cytotoxic</td>
</tr>
</tbody>
</table>

Widefield fluorescence images from Trial #4 at 24 hours (Fig. 27) demonstrated limited cell death for all polymer types. The controls—subjection to methanol for 20 minutes, warmed 3T3 media from the fridge, 3T3 media warmed to 50 °C for 72 hours, and powder-free latex glove eluted media—seemed to have worked effectively at 24 hours. In contrast, at 48 hours (Fig. 28), much...
more cell death was observed across wells. The positive control of powder-free latex glove eluted media displayed no cells because all cells had died, detached from the well plate, and were aspirated during the staining and imaging protocol. The negative control of 3T3 media warmed to 50 °C for 72 hours was ineffective because it displayed almost no cells. This result was contradictory to prior results seen with the negative control at these same parameters. This result suggested inconsistency with the elution procedure’s extraction condition of 50 °C for 72 hours.

<table>
<thead>
<tr>
<th>Calcein AM</th>
<th>Ethidium Homodimer-1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Effective Positive Control – methanol subjection for 20 minutes</td>
<td></td>
</tr>
<tr>
<td>Effective Negative Control – 3T3 media</td>
<td></td>
</tr>
<tr>
<td>Effective Negative Control – 3T3 media warmed to 50 °C for 72 hours</td>
<td></td>
</tr>
</tbody>
</table>
Effective Positive Control – powder-free latex glove eluted media

PLL

PLGA

PLGA with salt
Figure 27. Trial #4 widefield fluorescence images of 24-hour timepoint elution procedure. 10X magnification, 50% power, 1 ss exposure time.
Effective Negative Control – 3T3 media

Failed Negative Control – 3T3 media warmed to 50 °C for 72 hours

Effective Positive Control – powder-free latex glove eluted media

PLLA
2.2.5.5 Trial #4 Issues Noted

An issue during Trial #4 included using different cell seeding densities for the 24- and 48-hour timepoints (i.e., half of the cell seeding density was used for the 48-hour timepoint because it would be incubated for 24 hours longer). Additionally, when removing the PLGA and PLGA with salt samples from their conicals after the elution, the polymers broke down and sunk to the bottom, leaving polymer debris (Fig. 29). This, unfortunately, caused debris to be placed onto the cells with the media. Another issue noted was the discoloration of the PLGA conical compared to other conicals (yellow-ish rather than pink) when removed from the 50 °C water. This suggested the eluted media had become rather acidic after the elution procedure. A critical issue found was the inconsistency of the 50 °C for 72 hours extraction condition. In past trials, 3T3 media warmed to 50 °C for 72 hours illustrated confluent, live cells; however, this trial’s negative control demonstrated significant cell death within 48 hours.
Figure 29. Trial #4 PLGA and PLGA with salt conicals after being removed from the 50 °C water for 72 hours. The polymer broke down in the conical when being removed. The PLGA conical is also discolored, compared to the other polymer conicals.

2.2.5.6 Trial #4 Next Steps to Try

Due to the previously described issues, the same cell seeding density was to be implemented for the 24- and 48-hour studies, instead of seeding half into the 48-hour studies. Additionally, an experiment with only the elution controls, what eventually became Trial #5, was to be performed to ensure that these controls were working properly.

2.2.6 Trial #5

Trial #5 involved an elution test with only the negative and positive controls to ensure efficacy of the controls and elution extraction conditions. The negative controls included 3T3 cells cultured with warmed 3T3 media from the fridge as well as 3T3 media that had been warmed to 50 °C for 72 hours. The positive control included 3T3 cells cultured with media that was eluted by a powder-free latex glove warmed to 50 °C for 72 hours. This study included only an elution procedure. No direct contact was performed because the trial was focused on the elution procedure and controls.
2.2.6.1 Cell Seeding Procedure

Passage 28 3T3 cells at ~90% confluence in 1 T225 cell culture flask were harvested and resuspended in 24 mL of 3T3 media. Two mL of 3T3 media was placed into each well of two 6-well plates. Next, 0.3 mL of the cell solution was then placed into the 6-well plates, resulting in a cell seeding density of ~112,500 total cells per well or ~11,800 cells/cm². The 6-well plates were then placed in the incubator for 24 hours.

2.2.6.2 Elution Procedure

To begin the Trial #5 procedure, two conicals were prepared, each with 10 mL of 3T3 media. The negative control included 3T3 media with no sample. The positive control included a sterile, powder-free latex glove sample in the media. The conicals were warmed to 50 °C for 72 hours. After 72 hours, 3T3 cells were cultured with warmed 3T3 media from the fridge, the 3T3 media warmed to 50 °C for 72 hours, and the sterile, powder-free latex glove eluted media warmed to 50 °C for 72 hours. The well plates were then incubated for 24 and 48 hours.

2.2.6.3 Staining and Imaging Procedure

After 24 hours of incubation with the eluted media, the well plates were evaluated and imaged under the white-light microscope. The media was aspirated out of the well plates and rinsed with DPBS for 1 minute. Next, 1.5 mL of a live/dead stock stain solution consisting of 30 mL of DPBS + 36 uL EthD-1 + 30 uL calcein AM was placed into each well for 30 minutes and aspirated out carefully as to not aspirate any of the cells. The wells were imaged immediately using the widefield fluorescence microscope. This staining and imaging procedure was repeated for the 48-hour timepoint wells.

2.2.6.4 Trial #5 Results

Trial #5 results illustrated confluent, live cells cultured with warmed 3T3 media from the fridge; however, large gaps between cells were observed with the cells cultured in 3T3 media.
warmed to 50 °C for 72 hours and the latex glove eluted media. White-light images at 24 and 48
hours (Appendix F) displayed and confirmed significant cell death and detachment from the well
plate when cultured with 3T3 media that had been warmed to 50 °C for 72 hours. These results are
summarized in Table 8. This result further indicated and reinforced that the elution extraction
conditions of 50 °C for 72 hours was suboptimal.

Table 8. Results of Trial #5 elution procedure. Numbers separated by commas represent
different fields of view.

<table>
<thead>
<tr>
<th>Timepoint</th>
<th>Controls</th>
<th>Cytotoxicity Scale</th>
<th>Interpretation</th>
</tr>
</thead>
<tbody>
<tr>
<td>24-hour</td>
<td>3T3 media</td>
<td>0, 0</td>
<td>Noncytotoxic</td>
</tr>
<tr>
<td></td>
<td>Latex glove</td>
<td>2, 2</td>
<td>Moderately cytotoxic</td>
</tr>
<tr>
<td></td>
<td>3T3 media warmed to 50 °C for 72 hours</td>
<td>2, 1</td>
<td>Moderately cytotoxic</td>
</tr>
<tr>
<td>48-hour</td>
<td>3T3 media</td>
<td>0, 0</td>
<td>Noncytotoxic</td>
</tr>
<tr>
<td></td>
<td>Latex glove</td>
<td>3, 2</td>
<td>Severely cytotoxic</td>
</tr>
<tr>
<td></td>
<td>3T3 media warmed to 50 °C for 72 hours</td>
<td>2, 2</td>
<td>Moderately cytotoxic</td>
</tr>
</tbody>
</table>

The negative control of 3T3 cells cultured in warmed 3T3 media from the fridge displayed
consistent cell morphology and ~90% confluence (Fig. 30, 31). Meanwhile, the negative control of
cells cultured in 3T3 media that had been warmed to 50 °C for 72 hours displayed inconsistent cell
morphology and ~60% confluence, which was unexpected. The positive control of cells cultured
with media previously subjected to sterile, powder-free latex glove eluted media demonstrated
significant cell death and detachment from the well plate, which was expected.
Calcein AM

Effective Negative Control – warmed 3T3 media

Ethidium Homodimer-1

Effective Negative Control – warmed 3T3 media

Failed Negative Control – 3T3 media at 50 °C for 72 hours

Failed Negative Control – 3T3 media at 50 °C for 72 hours
Effective Positive Control – Powder-free latex glove eluted media at 50 °C for 72 hours

Effective Positive Control – powder-free latex glove eluted media at 50 °C for 72 hours

**Figure 30.** Trial #5 widefield fluorescence images of 24-hour timepoint elution procedure.  
10X magnification, 12% power, 500 ms exposure time.

Calcein AM

Ethidium Homodimer-1

Effective Negative Control – 3T3 media

Effective Negative Control – 3T3 media
Failed Negative Control – 3T3 media at 50 °C for 72 hours

Failed Negative Control – 3T3 media at 50 °C for 72 hours

Effective Positive Control – Powder-free latex glove eluted media at 50 °C for 72 hours

Effective Positive Control – Powder-free latex glove eluted media at 50 °C for 72 hours

Figure 31. Trial #5 widefield fluorescence images of 48-hour timepoint elution procedure.

10X magnification, 12% power, 500 ms exposure time.
2.2.6.5 Trial #5 Issues Noted

A notable issue from Trial #5 was the unsuccessful elution method of warming the extraction subjects to 50 °C for 72 hours. This timepoint and temperature was ineffective in safely leaching all toxic substances from the samples without harming the 3T3 elution medium.

2.2.6.6 Trial #5 Next Steps to Try

Due to the issue noted above, new extraction conditions of the elution procedure were to be tested to determine the optimal temperature and time combination. The optimal combination should leach all toxic substances from the extraction samples without negatively impacting the culture medium in which the samples are eluted.

2.2.7 Trial #6

This trial tested new temperature and timepoints for the elution procedure—50 °C for 24 hours, 50 °C for 72 hours, 37 °C for 24 hours, and 37 °C for 72 hours—to determine the optimal elution method. These extraction conditions were determined based on recommendations by the FDA document ISO10993 - Biological Evaluation of Medical Devices [53]. The negative controls included 3T3 cells cultured with warmed 3T3 media from the fridge and 3T3 media that had been warmed to either 50 or 37 °C for 24 or 72 hours, although it was noted that based on prior trials the 3T3 media at 50 °C may or may not actually serve as an effective negative control. The positive control included 3T3 cells cultured with media eluted with a sterile, powder-free latex glove warmed to either 50 or 37 °C for 24 or 72 hours. An extraction ratio of 6 cm²/mL for samples thinner than 0.5 mm was implemented to ensure use of proper, consistent, and successful extraction ratio for toxins to leach from the sample [70]. This study included only an elution procedure. No direct contact procedure was performed because the trial was focused on the elution procedure.
2.2.7.1 Cell Seeding Procedure

Passage 31 3T3 cells at ~90% confluence in 1 T225 cell culture flask were harvested and resuspended in 24 mL of 3T3 media. Two mL of 3T3 media was placed into each well of eight 6-well plates and two micro-dishes. Next, 0.3 mL of the cell solution was placed into each well of the 6-well plates and micro-dishes, resulting in a cell seeding density of ~112,500 cells per well or ~11,800 cells/cm². The 6-well plates and micro-dishes were then placed in the incubator for 24 hours.

2.2.7.2 Elution Procedure

As introduced above, different elution temperatures and timepoints were implemented in this trial as outlined in Table 9. Additionally, an extraction ratio of 6 cm²/mL for samples thinner than 0.5 mm was implemented to ensure consistency amongst samples and to ensure use of the proper extraction ratio for toxins to leach from the sample successfully.

Table 9. Trial #6 elution controls at each different extraction condition.

<table>
<thead>
<tr>
<th>Control</th>
<th>Temperature (°C)</th>
<th>Time (hours)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3T3 Media</td>
<td>37</td>
<td>24</td>
</tr>
<tr>
<td></td>
<td></td>
<td>48</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>24</td>
</tr>
<tr>
<td></td>
<td></td>
<td>48</td>
</tr>
<tr>
<td>5.4 cm² latex sample</td>
<td>37</td>
<td>24</td>
</tr>
<tr>
<td></td>
<td></td>
<td>48</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>24</td>
</tr>
<tr>
<td></td>
<td></td>
<td>48</td>
</tr>
<tr>
<td>60 cm² latex sample</td>
<td>37</td>
<td>24</td>
</tr>
<tr>
<td></td>
<td></td>
<td>48</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>24</td>
</tr>
<tr>
<td></td>
<td></td>
<td>48</td>
</tr>
</tbody>
</table>
Conicals of 10 mL 3T3 media were prepared for each extraction condition listed in Table 9, with either no sample (only 3T3 media), a 5.4 cm² sample of a sterile, powder-free latex glove (size of the lab’s standard BVM scaffolds), or a 60 cm² sample of a sterile, powder-free latex glove (recommended extraction ratio) (Fig. 32A). Each of these conicals were then subjected to the temperature and timepoint combinations described above. The conicals warmed to 50 °C were placed in a water bath at 50 °C while the conicals warmed to 37 °C were placed in the incubator. After the 24- and 72-hour elution procedures, the samples were removed from the conicals (Fig. 32B) and 3T3 cells were cultured with the eluted media for 24 and 48 hours.

**Figure 32. Trial #6 elution procedure.** A) Latex samples, B) samples removed from the conicals before media was placed onto cells.

2.2.7.3 Staining and Imaging Procedure

After 24 hours of incubation with the eluted media, the well plates were evaluated and imaged under the white-light microscope. The media was aspirated out of the well plates and the micro-dish was subjected to methanol for 20 minutes to serve as a positive control for the staining protocol. All wells were rinsed with DPBS, and the live/dead stock stain solution was made with 30 mL DPBS + 36 uL EthD-1 + 30 uL calcein AM. Next, 1.5 mL of the stain was placed into all
wells for 30 minutes and aspirated out carefully as to not aspirate the cells. Wells were then immediately imaged after using the widefield fluorescence microscope. This staining and imaging procedure was repeated for the 48-hour well plates.

2.2.7.4 Trial #6 Results

Results from Trial #6 illustrated cell detachment from the surface of the flask and large gaps between cells with 3T3 media and latex glove eluted media subjected to 50 °C for 24 and 72 hours and 37 °C for 72 hours. Meanwhile, cells cultured with 3T3 media and 5.4 cm² latex glove sample eluted media warmed to 37 °C for 24 hours illustrated confluent, live cells while the 60 cm² latex glove sample eluted media illustrated severe cell death. White-light images from Trial #6 are found in Appendix G and results are summarized in Table 10. The optimal extraction condition was 37 °C for 24 hours due to its resulting noncytotoxic 3T3 media and severely cytotoxic 60 cm² latex-eluted media. Alternately, the 5.4 cm² latex sample (standard size of the lab’s BVMs) was noncytotoxic at 24 and 48 hours. This observed noncytotoxic effect may be due to the sample being too small to sufficiently leach out enough toxins to cause cell death.
Table 10. Results of Trial #6 elution procedure. Numbers separated by commas represent different fields of view.

<table>
<thead>
<tr>
<th>Timepoint</th>
<th>Extraction Condition</th>
<th>Control</th>
<th>Cytotoxicity Scale</th>
<th>Interpretation</th>
</tr>
</thead>
<tbody>
<tr>
<td>24 hours</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>50 °C, 24 hours</td>
<td>3T3 media</td>
<td>1, 1</td>
<td>Mildly cytotoxic</td>
<td></td>
</tr>
<tr>
<td></td>
<td>5.4 cm² Latex</td>
<td>1, 1</td>
<td>Mildly cytotoxic</td>
<td></td>
</tr>
<tr>
<td></td>
<td>60 cm² Latex</td>
<td>3, 3</td>
<td>Severely cytotoxic</td>
<td></td>
</tr>
<tr>
<td>50 °C, 72 hours</td>
<td>3T3 media</td>
<td>1, 2</td>
<td>Moderately cytotoxic</td>
<td></td>
</tr>
<tr>
<td></td>
<td>5.4 cm² Latex</td>
<td>2, 2</td>
<td>Moderately cytotoxic</td>
<td></td>
</tr>
<tr>
<td></td>
<td>60 cm² Latex</td>
<td>3, 3</td>
<td>Severely cytotoxic</td>
<td></td>
</tr>
<tr>
<td>37 °C, 24 hours</td>
<td>3T3 media</td>
<td>0, 0</td>
<td>Noncytotoxic</td>
<td></td>
</tr>
<tr>
<td></td>
<td>5.4 cm² Latex</td>
<td>0, 0</td>
<td>Noncytotoxic</td>
<td></td>
</tr>
<tr>
<td></td>
<td>60 cm² Latex</td>
<td>3, 3</td>
<td>Severely cytotoxic</td>
<td></td>
</tr>
<tr>
<td>37 °C, 72 hours</td>
<td>3T3 media</td>
<td>0, 3</td>
<td>Moderately cytotoxic</td>
<td></td>
</tr>
<tr>
<td></td>
<td>5.4 cm² Latex</td>
<td>2, 2</td>
<td>Moderately cytotoxic</td>
<td></td>
</tr>
<tr>
<td></td>
<td>60 cm² Latex</td>
<td>3, 3</td>
<td>Severely cytotoxic</td>
<td></td>
</tr>
<tr>
<td>48 hours</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>50 °C, 24 hours</td>
<td>3T3 media</td>
<td>2, 2</td>
<td>Moderately cytotoxic</td>
<td></td>
</tr>
<tr>
<td></td>
<td>5.4 cm² Latex</td>
<td>2, 2</td>
<td>Moderately cytotoxic</td>
<td></td>
</tr>
<tr>
<td></td>
<td>60 cm² Latex</td>
<td>3, 3</td>
<td>Severely cytotoxic</td>
<td></td>
</tr>
<tr>
<td>50 °C, 72 hours</td>
<td>3T3 media</td>
<td>2, 2</td>
<td>Moderately cytotoxic</td>
<td></td>
</tr>
<tr>
<td></td>
<td>5.4 cm² Latex</td>
<td>3, 2</td>
<td>Severely cytotoxic</td>
<td></td>
</tr>
<tr>
<td></td>
<td>60 cm² Latex</td>
<td>3, 3</td>
<td>Severely cytotoxic</td>
<td></td>
</tr>
<tr>
<td>37 °C, 24 hours</td>
<td>3T3 media</td>
<td>0, 0</td>
<td>Noncytotoxic</td>
<td></td>
</tr>
<tr>
<td></td>
<td>5.4 cm² Latex</td>
<td>0, 0</td>
<td>Noncytotoxic</td>
<td></td>
</tr>
<tr>
<td></td>
<td>60 cm² Latex</td>
<td>3, 3</td>
<td>Severely cytotoxic</td>
<td></td>
</tr>
<tr>
<td>37 °C, 72 hours</td>
<td>3T3 media</td>
<td>0, 2</td>
<td>Mildly cytotoxic</td>
<td></td>
</tr>
<tr>
<td></td>
<td>5.4 cm² Latex</td>
<td>1, 2</td>
<td>Moderately cytotoxic</td>
<td></td>
</tr>
<tr>
<td></td>
<td>60 cm² Latex</td>
<td>3, 3</td>
<td>Severely cytotoxic</td>
<td></td>
</tr>
</tbody>
</table>
2.2.7.5 Trial #6 Issues Noted

The most notable issue seen with Trial #6 was an ineffective use of the stain. After applying the stain to the wells and incubating for 30 minutes, all 3T3 cells detached from the bottom of the well plates (Fig. 33), resulting in well plates that were unable to be imaged under the widefield fluorescence microscope due to unidentifiable cells.

![Dead cells](image)

**Figure 33. Trial #6 unsuccessful staining protocol.** The far left and far right wells of the well plate display 3T3 cells that have completely detached from the bottom of the well plate (identified by the arrow) after being subjected to stain for 30 minutes.

2.2.7.6 Trial #6 Next Steps to Try

Due to the issues noted above, a new staining protocol was developed to ensure cells no longer detached from the bottom of the well plate after being subjected to the stain.

2.2.8 Trial #7

This trial involved a stain screen with several different concentrations of the stain along with incubation times. It was evident from Trial #6 that the staining protocol was lifting the cells from the bottom of the flask and therefore fluorescence images were unobtainable. New stain was purchased because this issue was not noted in prior trials and the previously used stain may have
expired. The goal of this trial was to optimize the staining protocol and ensure there was no detachment of cells from the bottom of the flask.

**2.2.8.1 Cell Seeding Procedure**

Passage 32 3T3 cells at ~90% confluence in 1 T225 cell culture flask were harvested and resuspended in 24 mL of 3T3 media. Two mL of 3T3 media was placed into each well of two 6-well plates. Next, 0.3 mL of the cell solution was placed into each well of the 6-well plates, resulting in a cell seeding density of ~112,500 cells per well or ~11,800 cells/cm². The 6-well plates were then placed in the incubator for 24 hours.

**2.2.8.2 Staining and Imaging Procedure**

After 24 hours of incubation, the media was aspirated out of the well plates and all wells were rinsed with DPBS. Three different live/dead stock stain solutions were made with the newly purchased stain (Table 11). Next, 1.5 mL of each different stain concentration was placed into corresponding wells. One well plate was incubated for 5 – 10 minutes and the other well plate for 20 – 25 minutes. After the incubation time, the stain was aspirated and wells were imaged immediately using the widefield fluorescence microscope to determine which combination of incubation time and concentration of stain resulted in the most effective staining protocol (i.e., clear and intense fluorescence images with no cell detachment from the well plate).

<table>
<thead>
<tr>
<th>Stain Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 mL DPBS + 12 uL EthD-1 + 10 uL calcein AM</td>
</tr>
<tr>
<td>10 mL DPBS + 20 uL EthD-1 + 10 uL calcein AM</td>
</tr>
<tr>
<td>10 mL DPBS + 12 uL EthD-1 + 3 uL calcein AM</td>
</tr>
</tbody>
</table>
2.2.8.3 Trial #7 Results

Widefield fluorescence images from the Trial #7 stain screen (Fig. 34) illustrated clear and intense images with each different stock stain concentration. Since the stock stain solution of 10 mL DPBS + 12 uL EthD-1 + 3 uL calcein AM had the lowest concentration of stain in the solution and was still able to produce a bright and distinguishable image for both stains, this stock stain concentration was deemed optimal. However, after 20 – 25 minutes of incubating the stain, cells began to lift off the surface of the flask. This was not observed with the 5 – 10-minute incubation time. Therefore, it was determined that 5 – 10 minutes of incubating the stain was the optimal incubation time.

<table>
<thead>
<tr>
<th>Incubation Time / Concentration</th>
<th>Calcein AM</th>
<th>Ethidium Homodimer-1</th>
</tr>
</thead>
<tbody>
<tr>
<td>5 – 10 minutes incubation time</td>
<td><img src="image1.png" alt="Calcein AM Image" /></td>
<td><img src="image2.png" alt="Ethidium Homodimer-1 Image" /></td>
</tr>
<tr>
<td>10 mL DPBS + 12 uL EthD-1 + 10 uL calcein AM</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Incubation Time / Concentration</th>
<th>Calcein AM</th>
<th>Ethidium Homodimer-1</th>
</tr>
</thead>
<tbody>
<tr>
<td>5 – 10 minutes incubation time</td>
<td><img src="image3.png" alt="Calcein AM Image" /></td>
<td><img src="image4.png" alt="Ethidium Homodimer-1 Image" /></td>
</tr>
<tr>
<td>10 mL DPBS + 20 uL EthD-1 + 5 uL calcein AM</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Figure 34. Trial #7 widefield fluorescence images of stain screen.
10X magnification, 3% power, 500 ms exposure time.

2.2.8.4 Trial #7 Issues Noted

No issues were noted with Trial #7.
2.2.8.5 Trial #7 Next Steps to Try

The Trial #7 stain screen determined that incubation of the stain for 5 – 10 minutes with a stock stain concentration of 10 mL DPBS + 12 uL EthD-1 + 3 uL calcein AM was to be implemented in future staining and imaging procedures. As compared with prior trials where 10 mL DPBS + 12 uL EthD-1 + 10 uL calcein AM of the stain solution was incubated on the cells for 30 minutes. ThermoFisher Scientific customer support suggested that the stain used in past trials may have expired after a year of use and that excessive use of calcein AM (containing DMSO) could be contributing to the observed cell detachment from the surface of the flask.

2.2.9 Trial #8

The optimal extraction condition of 37 °C for 24 hours was implemented in this trial. Various extraction ratios were tested by implementing different sample sizes of sterile, powder-free latex glove. The goal of this trial was to determine if there was a threshold of sample size where cell death became evident. This trial included only an elution procedure.

2.2.9.1 Cell Seeding Procedure

Passage 32 3T3 cells at ~90% confluence in 1 T225 cell culture flask were harvested and resuspended in 24 mL of 3T3 media. Two mL of 3T3 media was placed into each well of four 6-well plates and two micro-dishes. Additionally, 0.3 mL of the cell solution was placed into each well of the 6-well plates and micro-dishes, resulting in a cell seeding density of ~112,500 cells per well or ~11,800 cells/cm². The 6-well plates and micro-dishes were then placed in the incubator for 24 hours.

2.2.9.2 Elution Procedure

To begin Trial #8, six conicals were prepared with 10 mL of 3T3 media. The negative control included 3T3 media with no sample. The positive controls included media eluted with samples of a sterile, powder-free latex glove in various sizes: 6, 15, 30, 45, and 60 cm². The
prepared conicals were then warmed in the incubator to 37 °C for 24 hours. After 24 hours, the samples were removed from the conicals, leaving only the eluted media. 3T3 cells were cultured with warmed 3T3 media from the fridge, the 3T3 media that had been warmed to 37 °C for 24 hours, and the sterile, powder-free latex glove eluted media warmed to 37 °C for 24 hours. The well plates were then incubated for 24 and 48 hours.

2.2.9.3 Staining and Imaging Procedure

After 24 hours of incubation with the eluted media, the well plates were evaluated and imaged under the white-light microscope. The media was aspirated out of the well plates and the micro-dish was subjected to methanol for 20 minutes to serve as a positive control for the staining protocol. All wells were rinsed with DPBS, and the live/dead stock stain solution was made with 20 mL DPBS + 24 uL EthD-1 + 6 uL calcein AM. Next, 1.5 mL of the stain solution was placed into each well for 5-10 minutes and aspirated out carefully as to not aspirate the cells. The wells were then imaged using the widefield fluorescence microscope. This staining and imaging procedure was repeated for the 48-hour timepoint wells with a replacement of the eluted media at 24 hours.

2.2.9.4 Trial #8 Results

Results from Trial #8 illustrated no cytotoxic effects from the negative control of 3T3 media warmed to 37 °C for 24 hours. This reinforced an extraction condition that was not detrimental to the cells. The media eluted with a 6 and 15 cm² sterile, powder-free latex sample also demonstrated no cytotoxic effects. The 30 cm² sample eluted media showed mild cytotoxic effects, but it was not until the 45 cm² sample eluted media that severe cytotoxic effects were seen. These results suggested that the threshold of where cell death becomes evident happens with an extraction ratio of ~45 cm² sample size in 10 mL of media, at least for latex gloves. White-light images are found in Appendix H and Figure 35. Results are summarized in Table 12.
Table 12. Results of Trial #8 elution procedure. Numbers separated by commas represent different fields of view.

<table>
<thead>
<tr>
<th>Timepoint</th>
<th>Controls – all warmed to 37 °C for 24 hours</th>
<th>Cytotoxicity Scale</th>
<th>Interpretation</th>
</tr>
</thead>
<tbody>
<tr>
<td>24-hour</td>
<td>3T3 media</td>
<td>0, 0</td>
<td>Noncytotoxic</td>
</tr>
<tr>
<td></td>
<td>6 cm² latex</td>
<td>0, 0</td>
<td>Noncytotoxic</td>
</tr>
<tr>
<td></td>
<td>15 cm² latex</td>
<td>0, 0</td>
<td>Noncytotoxic</td>
</tr>
<tr>
<td></td>
<td>30 cm² latex</td>
<td>1, 1</td>
<td>Mildly cytotoxic</td>
</tr>
<tr>
<td></td>
<td>45 cm² latex</td>
<td>3, 3</td>
<td>Severely cytotoxic</td>
</tr>
<tr>
<td></td>
<td>60 cm² latex</td>
<td>3, 3</td>
<td>Severely cytotoxic</td>
</tr>
<tr>
<td>48-hour</td>
<td>3T3 media</td>
<td>0, 0</td>
<td>Noncytotoxic</td>
</tr>
<tr>
<td></td>
<td>6 cm² latex</td>
<td>0, 0</td>
<td>Noncytotoxic</td>
</tr>
<tr>
<td></td>
<td>15 cm² latex</td>
<td>0, 0</td>
<td>Noncytotoxic</td>
</tr>
<tr>
<td></td>
<td>30 cm² latex</td>
<td>1, 1</td>
<td>Mildly cytotoxic</td>
</tr>
<tr>
<td></td>
<td>45 cm² latex</td>
<td>3, 3</td>
<td>Severely cytotoxic</td>
</tr>
<tr>
<td></td>
<td>60 cm² latex</td>
<td>3, 3</td>
<td>Severely cytotoxic</td>
</tr>
</tbody>
</table>

Effective Negative Control – warmed 3T3 media from the fridge

Effective Negative Control – 3T3 media warmed to 37 °C for 24 hours
Figure 35. Trial #8 white-light images of 24- and 48- timepoint elution procedure, 10X.
Widefield fluorescence images showed no cell presence with the 45 and 60 cm² latex sample eluted media. This occurred because most of the cells died, lifted off the bottom of the flask, and were then aspirated during the staining process. These results are seen in Figures 36 and 37.

**Calcein AM**

- Effective Negative Control – 3T3 media
- Effective Negative Control – 3T3 media at 37 °C for 24 hours
- Failed Positive Control – 6 cm² latex glove eluted media at 37 °C for 24 hours
- Failed Positive Control – 15 cm² latex glove eluted media at 37 °C for 24 hours

**Ethidium Homodimer-1**

- 
- 
- 
-
Partially Failed Positive Control – 30 cm² latex glove eluted media at 37 °C for 24 hours

Effective Positive Control – 45 cm² latex glove eluted media at 37 °C for 24 hours

Effective Positive Control – 60 cm² latex glove eluted media at 37 °C for 24 hours

Figure 36. Trial #8 widefield fluorescence images of 24-hour timepoint elution procedure. 10X magnification, 50% power, 500 ms exposure time.

Calcein AM

Ethidium Homodimer-1

Effective Negative Control – 3T3 media
Effective Negative Control – 3T3 media at 37 °C for 24 hours

Failed Positive Control – 6 cm$^2$ latex glove eluted media at 37 °C for 24 hours

Failed Positive Control – 15 cm$^2$ latex glove eluted media at 37 °C for 24 hours

Partially Failed Positive Control – 30 cm$^2$ latex glove eluted media at 37 °C for 24 hours
Effective Positive Control – 45 cm² latex glove eluted media at 37 °C for 24 hours

Effective Positive Control – 60 cm² latex glove eluted media at 37 °C for 24 hours

Figure 37. Trial #8 widefield fluorescence images of 48-hour timepoint elution procedure. 10X magnification, 50% power, 500 ms exposure time.

2.2.9.5 Trial #8 Issues Noted

No issues were noted with Trial #8.

2.2.9.6 Trial #8 Next Steps to Try

Since no issues were found with Trial #8, the next step was to utilize the final, optimized protocols to test the cytotoxicity of polymers used in the BVM lab—ePTFE, PLGA, PLGA with salt, PLLA, and PCL.

2.3 Discussion and Conclusion

In developing the necessary protocols for in vitro cytotoxicity testing of polymers used in the fabrication of BVMs, it became evident after each trial run that some components of the protocol would be kept, and some needed changing. A summary of each trial is outlined in Table 13.
### Table 13. Summary of trials.

<table>
<thead>
<tr>
<th>Trial #</th>
<th>What was done</th>
<th>What was kept</th>
<th>What needed to be changed</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trial #1</td>
<td>Cytotoxicity protocols on PLGA, PLGA with salt, and PLLA – direct contact and elution procedures.</td>
<td>- 6-well plates and micro-dishes&lt;br&gt;- Elution extraction conditions&lt;br&gt;- Sterilization method</td>
<td>- Staining protocol / positive control&lt;br&gt;- Media replacement at 24 hours&lt;br&gt;- Cell seeding density&lt;br&gt;- Volume of water in elution beaker / tinfoil&lt;br&gt;- Newly spun polymers</td>
</tr>
<tr>
<td>Trial #2</td>
<td>Cytotoxicity protocols on ePTFE with cell viability assay of calcein AM and ethidium homodimer-1 – direct contact and elution procedures.</td>
<td>- Staining protocol&lt;br&gt;- Direct contact procedure</td>
<td>- Cell seeding density&lt;br&gt;- Stain positive control&lt;br&gt;- Sterilization method&lt;br&gt;- Imaging protocol</td>
</tr>
<tr>
<td>Trial #3</td>
<td>Cytotoxicity protocols on ePTFE including other sterilization methods – elution procedure.</td>
<td>- EtOH soak and DPBS rinse sterilization&lt;br&gt;- Imaging protocol</td>
<td>- Cell seeding density&lt;br&gt;- Elution positive control</td>
</tr>
<tr>
<td>Trial #4</td>
<td>Cytotoxicity protocols on PLGA, PLGA with salt, PLLA, and PCL – elution procedure.</td>
<td>- Elution positive control&lt;br&gt;- Cell seeding density&lt;br&gt;- Sterilization method</td>
<td>- Elution extraction conditions</td>
</tr>
<tr>
<td>Trial #5</td>
<td>Cytotoxicity protocols on controls – elution procedure.</td>
<td>- Positive and negative controls</td>
<td>- Elution extraction conditions</td>
</tr>
<tr>
<td>Trial #6</td>
<td>Cytotoxicity protocols on controls with different extraction conditions – elution procedure.</td>
<td>- New elution extraction conditions</td>
<td>- Staining protocol</td>
</tr>
<tr>
<td>Trial #7</td>
<td>Stain screen.</td>
<td>- Staining protocol</td>
<td>N/A</td>
</tr>
<tr>
<td>Trial #8</td>
<td>Cytotoxicity protocols on controls with different extraction ratios – elution procedure.</td>
<td>- Elution extraction ratios</td>
<td>N/A</td>
</tr>
</tbody>
</table>
Overall, the trial iterations described above introduced several challenges that necessitated addressing. The first limitation included troubleshooting of the staining and imaging protocol. It was quickly determined that BBI and Trypan Blue would not be the proper stains for this specific application and, therefore, calcein AM and ethidium homodimer-1 became the desirable assay. Although this live/dead assay effectively differentiated between live and dead cells, the staining protocol took several trials to optimize. Along with this, the proper cell seeding density became a challenge early on as over-confluency of the cells was regularly observed. This was a problem because over-confluency can lead to cell death, which could lead to false conclusions regarding scaffold material cytotoxicity.

Another challenge included the controls. First, determining the appropriate positive and negative controls for the direct contact and elution procedure proved challenging. The elution controls displayed notable cell death with the extraction condition of 50 °C for 72 hours. This high temperature for a long timepoint may have compromised components of the cell culture medium and therefore caused detrimental effects to the cultured cells. The 3T3 culture media is made of many different amino acids and vitamins, along with fetal bovine serum (FBS), penicillin-streptomycin, and fungizone. Cell culture media is usually stored in the fridge (2 – 8 °C) or the water bath/incubator (37 °C). Subjecting this media to the high temperature of 50 °C for 72 hours may have led to the denaturing of proteins within the media. Studies have shown that temperature shifts are one of the main causes of precipitation (of proteins or other media components) in cell culture. Precipitates can be harmful to cell viability due to their potential alteration of media composition by removing nutrients and other desirable components [71]. Additionally, heat treatment of FBS at 56 °C for longer than 30 minutes has proven to adversely affect cell health and the heating of serum products can result in the formation of precipitates [72]. This trial finding was what led to the incorporation of ISO 10993 cytotoxicity testing recommendations that alleviated the issues found with the controls and elution-based methods.
Another challenge came from the variability of the scaffold material samples. Throughout these trial iterations, the electrospinner was unable to produce consistent polymer scaffolds via mandrel spins. Fortunately, it became evident that the electrospinner was able to produce more consistent flat sheet sample spins. Therefore, it was decided that the cytotoxicity testing would be done on a combination of flat sheet samples and mandrel spins (i.e., whatever the electrospinner could produce at that time). This was the reason for the seemingly random selection of polymer/material types throughout the eight trials.

The final working protocol for cytotoxicity testing included direct contact and elution-based methods. The direct contact procedure involved initial cell seeding, followed by sterilization of the scaffold materials and the implementation of a proper positive control (samples left in EtOH and unrinised) and negative control (warmed 3T3 media from the fridge). Once sterilized, 3T3 cells were directly exposed to the test and control articles and microscopically evaluated at 12-, 24-, and 48-hour timepoints. The elution procedure involved initial cell seeding, followed by sterilization of the scaffold materials and implementation of positive controls (powder-free latex glove and methanol subjection) and negative controls (3T3 media subjected to extraction conditions and warmed 3T3 media from the fridge). Extracts were obtained by placing the test and control materials in conicals of 3T3 media at 37 °C for 24 hours. The 3T3 cells were then cultured with each fluid extract for 24- and 48-hour timepoints and microscopically examined for visible signs of toxicity in response to the test and control materials. This final working protocol was implemented in Chapters 3 and 4 to evaluate the biocompatibility of existing and novel BVM scaffold polymers: ePTFE, PLGA, PLGA with salt, PLLA, and PCL.
CHAPTER 3. AIM 2: BIOCOMPATIBILITY OF EXISTING BVM SCAFFOLD POLYMERS: EPTFE AND PLGA

3.1 Introduction

The second aim of this thesis was to utilize the cytotoxicity protocols developed in Aim 1 to evaluate the biocompatibility of currently used polymers in the BVM lab: ePTFE and PLGA. This study was important because it served as an additional confirmation of functional protocols—since ePTFE and PLGA have been historically relevant scaffold materials in the fabrication of BVMs. While ePTFE and PLGA are standard polymers used in BVM fabrication, they have also been extensively studied for their biocompatibility in the field. Both polymers are well-established with notable biocompatibility and low toxicity to cells. For example, in a post-market approval (PMA) for Gore’s ePTFE Viabahn Endoprosthesis Implant, cytotoxicity elution tests demonstrated noncytotoxic results from the device material on cells [73]. Additionally, in a different study, cytotoxicity assays done on pure PLGA composite scaffolds were noncytotoxic to cells [74]. To accomplish this aim, several studies involving direct contact and elution-based methods were performed.

3.2 Methods and Results

3.2.1 Cell Seeding Procedure

Direct Contact Cell Seeding

Cells were seeded according to the Cell Seeding SOP in Appendix K. Passage 28 3T3 cells at ~90% confluence in 1 T225 cell culture flask were harvested and resuspended in 24 mL of 3T3 media. Two mL of 3T3 media was placed into each well of two 6-well plates. Additionally, 0.3 mL of the cell solution was placed into each well, resulting in a final cell seeding density of ~112,500 cells per well or ~11,800 cells/cm². The 6-well plates were then placed in the incubator for 24 hours at 37 °C and 5% CO₂.
Elution Cell Seeding

Cells were seeded according to the Cell Seeding SOP in Appendix K. Passage 33 3T3 cells at ~90% confluence in 1 T225 cell culture flask were harvested and resuspended in 24 mL of 3T3 media. Two mL of 3T3 media was placed into each well of four 6-well plates and two micro-dishes. Additionally, 0.3 mL of the cell solution was placed into each well of the 6-well plates and micro-dishes, resulting in a cell seeding density of ~112,500 cells per well or ~11,800 cells/cm². The 6-well plates and micro-dishes were then placed in the incubator for 24 hours.

3.2.2 Direct Contact Procedure

To begin the direct contact procedure with ePTFE and PLGA, an ePTFE tubular scaffold (Fig. 38) and an electrospun, PLGA flat sheet sample (Fig. 39) were cut into sections (~6 cm²). The direct contact SOP in Appendix L was then followed. The samples were sterilized in sterile 70% EtOH for 20 minutes. After 20 minutes, the conicals were replaced with DPBS and shaken for 1 minute to rid the polymers of any residual EtOH. One sample of PLGA and ePTFE were each left in EtOH to serve as positive controls. Using sterile forceps, the samples were transferred into corresponding wells and well plates were placed in the incubator until imaging. The well plates were evaluated and imaged under the white-light microscope at 12-, 24-, and 48-hour timepoints.

Figure 38. ePTFE tubular scaffold sample.
Figure 39. Electrospun PLGA flat sheet sample. A) PLGA sample 15 wt.% PLGA/CHCL3 on flat sheet collector, B) the same PLGA sample removed from the collector.

3.2.3 Elution Procedure

To begin the elution procedure (Appendix M), six conicals were prepared, each with 10 mL of 3T3 media. The negative control was 3T3 media with no sample. The positive control was prepared by placing a 45 cm² sterile, powder-free latex glove sample in media. The polymer samples tested included an ePTFE tubular scaffold (~10 cm²), a PLGA tubular scaffold (~7 cm²), and a PLGA flat sheet sample (~45 cm²). Samples were placed in conicals that were warmed to 37°C in the incubator for 24 hours. After 24 hours, the samples were removed from the conicals, leaving only the eluted media. The 3T3 cells were cultured with warmed 3T3 media from the fridge (negative control), 3T3 media warmed to 37 °C for 24 hours (negative control), media eluted with a sample of sterile, powder-free latex glove and warmed to 37 °C for 24 hours (positive control), as well as the medias eluted with the ePTFE tubular scaffold, PLGA tubular scaffold, and PLGA flat sheet. The well plates containing the cultured cells were then incubated for 24 and 48 hours.
3.2.4 Staining and Imaging Procedure

After 24 hours of incubation with the eluted media, the well plates were evaluated and imaged under the white-light microscope. The media was aspirated out of the well plates and the micro-dish was subjected to methanol for 20 minutes to serve as a positive control for the staining protocol (Appendix N). All wells were rinsed with DPBS and 1.5 mL of a live/dead stock stain solution of 20 mL DPBS + 24 uL EthD-1 + 6 uL calcein AM was placed into each well for 5-10 minutes. After 5-10 minutes, the stain solution was aspirated out carefully as to not aspirate the cells. Each well was imaged using the widefield fluorescence microscope (Appendix O). This staining and imaging procedure was repeated for the 48-hour timepoint wells with a replacement of the eluted media at 24 hours.

3.2.5 Cell Counting Procedure

A cell counting procedure was implemented in Aim 2 to provide a quantitative representation of scaffold material biocompatibility. Images taken with the widefield fluorescence microscope were opened in ImageJ for proper cell counting (Appendix P). Each image was converted to 16-bit and the threshold was adjusted to ensure each nuclei was individually counted. Each particle was then analyzed in pixel² units. Live cells were counted based on the calcein AM stained images and dead cells were counted based on the ethidium homodimer-1 stained images. The results were tabulated as the number of live cells, the number of dead cells, and the ratio of live to dead cells for the positive controls, negative controls, ePTFE, and PLGA.

3.2.6 Results

Direct Contact Results

Results from this direct contact study illustrated that ePTFE and PLGA (solvent: chloroform) were noncytotoxic to 3T3 cells. White-light images from the ePTFE and PLGA direct contact procedure can be found in Appendix I. These results are summarized in Table 14.
Table 14. Results of ePTFE and PLGA direct contact procedure. Numbers separated by commas represent different fields of view.

<table>
<thead>
<tr>
<th>Timepoint</th>
<th>Sample</th>
<th>Cytotoxicity Scale</th>
<th>Interpretation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ePTFE</td>
<td>0, 0</td>
<td>Noncytotoxic</td>
</tr>
<tr>
<td>12-hour</td>
<td>ePTFE in EtOH (pos. control)</td>
<td>3, 3</td>
<td>Severely cytotoxic</td>
</tr>
<tr>
<td></td>
<td>PLGA</td>
<td>0, 0</td>
<td>Noncytotoxic</td>
</tr>
<tr>
<td></td>
<td>PLGA in EtOH (pos. control)</td>
<td>3, 3</td>
<td>Severely cytotoxic</td>
</tr>
<tr>
<td></td>
<td>3T3 media (neg. control)</td>
<td>0, 0</td>
<td>Noncytotoxic</td>
</tr>
<tr>
<td>24-hour</td>
<td>ePTFE</td>
<td>0, 0</td>
<td>Noncytotoxic</td>
</tr>
<tr>
<td></td>
<td>ePTFE in EtOH (pos. control)</td>
<td>3, 3</td>
<td>Severely cytotoxic</td>
</tr>
<tr>
<td></td>
<td>PLGA</td>
<td>0, 0</td>
<td>Noncytotoxic</td>
</tr>
<tr>
<td></td>
<td>PLGA in EtOH (pos. control)</td>
<td>3, 3</td>
<td>Severely cytotoxic</td>
</tr>
<tr>
<td></td>
<td>3T3 media (neg. control)</td>
<td>0, 0</td>
<td>Noncytotoxic</td>
</tr>
<tr>
<td>48-hour</td>
<td>ePTFE</td>
<td>0, 0</td>
<td>Noncytotoxic</td>
</tr>
<tr>
<td></td>
<td>ePTFE in EtOH (pos. control)</td>
<td>3, 3</td>
<td>Severely cytotoxic</td>
</tr>
<tr>
<td></td>
<td>PLGA</td>
<td>0, 0</td>
<td>Mildly cytotoxic</td>
</tr>
<tr>
<td></td>
<td>PLGA in EtOH (pos. control)</td>
<td>3, 3</td>
<td>Severely cytotoxic</td>
</tr>
<tr>
<td></td>
<td>3T3 media (neg. control)</td>
<td>0, 0</td>
<td>Noncytotoxic</td>
</tr>
</tbody>
</table>

Elution Results

Results from the elution procedure illustrated that ePTFE and PLGA were noncytotoxic at 24 hours. Negative controls displayed highly confluent, live cells and almost no dead cells. Positive controls displayed high cell death and cell detachment from the surface of the flask. At 48 hours, results illustrated that ePTFE and PLGA were noncytotoxic as well. At this timepoint, negative controls continued to display highly confluent, live cells and almost no dead cells. Positive controls displayed further cell detachment from the surface of the flask and even greater cell death. White-light images from the ePTFE and PLGA elution procedure can be found in Appendix I. These results are summarized in Table 15.
Table 15. Results of ePTFE and PLGA elution procedure. Numbers separated by commas represent different fields of view.

<table>
<thead>
<tr>
<th>Timepoint</th>
<th>Sample</th>
<th>Cytotoxicity Scale</th>
<th>Interpretation</th>
</tr>
</thead>
<tbody>
<tr>
<td>24-hour</td>
<td>3T3 media (neg. control)</td>
<td>0, 0</td>
<td>Noncytotoxic</td>
</tr>
<tr>
<td></td>
<td>3T3 media warmed to 37 °C for 24 hours (neg. control)</td>
<td>0, 0</td>
<td>Noncytotoxic</td>
</tr>
<tr>
<td></td>
<td>45 cm² latex (pos. control)</td>
<td>3, 3</td>
<td>Severely cytotoxic</td>
</tr>
<tr>
<td></td>
<td>Methanol (pos. control)</td>
<td>3, 3</td>
<td>Severely cytotoxic</td>
</tr>
<tr>
<td></td>
<td>ePTFE tubular scaffold</td>
<td>0, 0</td>
<td>Noncytotoxic</td>
</tr>
<tr>
<td></td>
<td>PLGA tubular scaffold</td>
<td>0, 0</td>
<td>Noncytotoxic</td>
</tr>
<tr>
<td></td>
<td>PLGA flat sheet</td>
<td>0, 0</td>
<td>Noncytotoxic</td>
</tr>
<tr>
<td>48-hour</td>
<td>3T3 media (neg. control)</td>
<td>0, 0</td>
<td>Noncytotoxic</td>
</tr>
<tr>
<td></td>
<td>3T3 media warmed to 37 °C for 24 hours (neg. control)</td>
<td>0, 0</td>
<td>Noncytotoxic</td>
</tr>
<tr>
<td></td>
<td>45 cm² latex (pos. control)</td>
<td>3, 3</td>
<td>Severely cytotoxic</td>
</tr>
<tr>
<td></td>
<td>Methanol (pos. control)</td>
<td>3, 3</td>
<td>Severely cytotoxic</td>
</tr>
<tr>
<td></td>
<td>ePTFE tubular scaffold</td>
<td>0, 0</td>
<td>Noncytotoxic</td>
</tr>
<tr>
<td></td>
<td>PLGA tubular scaffold</td>
<td>0, 0</td>
<td>Noncytotoxic</td>
</tr>
<tr>
<td></td>
<td>PLGA flat sheet</td>
<td>0, 0</td>
<td>Noncytotoxic</td>
</tr>
</tbody>
</table>

At 24 hours, the positive control of powder-free latex glove eluted media was effective in killing the cells. This was confirmed by the absence of cells in the positive control stained and imaged wells and was due to cell detachment and aspiration during the staining procedure. The other positive control of methanol for 20 minutes was effective in killing the cells and demonstrating the efficacy of the stain. This was confirmed by the intense fluorescence of ethidium homodimer-1 in the stained and imaged well. At 24 hours, the PLGA tubular scaffold, PLGA flat sheet, and ePTFE tubular scaffold eluted media illustrated a high confluency of live cells and no
dead cells (Fig. 40). Images at 48 hours illustrated similar results, but with an even greater confluency of live cells (Fig. 41).

Calcein AM

Ethidium Homodimer-1

Effective Negative Control – 3T3 media

Effective Negative Control – 3T3 media at 37°C for 24 hours

Effective Positive Control – 45 cm² latex glove eluted media at 37°C for 24 hours

Effective Positive Control – subjection to methanol for 20 minutes
Figure 40. PLGA and ePTFE widefield fluorescence images of 24-hour timepoint elution procedure. 10X magnification, 50% power, 500 ms exposure time.

PLGA tubular scaffold eluted media at 37°C for 24 hours

PLGA flat sheet eluted media at 37°C for 24 hours

ePTFE tubular scaffold eluted media at 37°C for 24 hours

Effective Negative Control – 3T3 media

Calcein AM

Ethidium Homodimer-1
Effective Negative Control – 3T3 media at 37°C for 24 hours

Effective Positive Control – 45 cm² latex glove eluted media at 37°C for 24 hours

Effective Positive Control – subjection to methanol for 20 minutes

PLGA tubular scaffold eluted media at 37°C for 24 hours
At 24 hours, the negative controls of warmed 3T3 media from the fridge and 3T3 media incubated at 37 °C for 24 hours resulted in high cell viability (923 live cells and 1175 live cells, respectively) and minimal cell death (1 dead cell and 0 dead cells, respectively). At 48 hours, these negative controls continued to illustrate high cell viability (1056 live cells and 1017 live cells, respectively) and minimal cell death (4 dead cells and 0 dead cells, respectively). At 24 and 48 hours, the powder-free latex glove eluted media resulted in no live or dead cells due to cell aspiration during the staining protocol. At 24 and 48 hours, the subjection of methanol for 20 minutes resulted in no live cells and high cell death (1290 dead cells at 24 hours and 1641 dead cells at 48 hours). At 24 hours, the PLGA tubular scaffold, PLGA flat sheet, and ePTFE tubular scaffold eluted media also resulted in high cell viability (1051 live cells, 966 live cells, and 945 live cells, respectively) and minimal cell death (0 dead cells, 1 dead cell, and 0 dead cells, respectively). At 48 hours, these polymers continued to illustrate high cell viability (1013 live cells,
1003 live cells, and 1125 live cells, respectively) and minimal cell death (3 dead cells, 1 dead cell, and 1 dead cell, respectively). These results are summarized in Table 16.

Table 16. Results of ePTFE and PLGA elution procedure cell counts.

<table>
<thead>
<tr>
<th>Timepoint</th>
<th>Sample</th>
<th># of Live Cells</th>
<th># of Dead Cells</th>
<th>Ratio of Live/Dead</th>
</tr>
</thead>
<tbody>
<tr>
<td>24-hour</td>
<td>3T3 media (neg. control)</td>
<td>923</td>
<td>1</td>
<td>923/1</td>
</tr>
<tr>
<td></td>
<td>3T3 media warmed to 37 °C for 24 hours (neg. control)</td>
<td>1175</td>
<td>0</td>
<td>1175/0</td>
</tr>
<tr>
<td></td>
<td>Latex (pos. control)</td>
<td>0</td>
<td>0</td>
<td>0/0</td>
</tr>
<tr>
<td></td>
<td>Methanol (pos. control)</td>
<td>0</td>
<td>1290</td>
<td>0/1290</td>
</tr>
<tr>
<td></td>
<td>PLGA tubular scaffold</td>
<td>1051</td>
<td>0</td>
<td>1051/0</td>
</tr>
<tr>
<td></td>
<td>PLGA flat sheet</td>
<td>966</td>
<td>1</td>
<td>966/1</td>
</tr>
<tr>
<td></td>
<td>ePTFE tubular scaffold</td>
<td>945</td>
<td>0</td>
<td>945/0</td>
</tr>
<tr>
<td>48-hour</td>
<td>3T3 media (neg. control)</td>
<td>1056</td>
<td>4</td>
<td>1056/4</td>
</tr>
<tr>
<td></td>
<td>3T3 media warmed to 37 °C for 24 hours (neg. control)</td>
<td>1017</td>
<td>0</td>
<td>1017/0</td>
</tr>
<tr>
<td></td>
<td>Latex (pos. control)</td>
<td>0</td>
<td>0</td>
<td>0/0</td>
</tr>
<tr>
<td></td>
<td>Methanol (pos. control)</td>
<td>0</td>
<td>1641</td>
<td>0/1641</td>
</tr>
<tr>
<td></td>
<td>PLGA tubular scaffold</td>
<td>1013</td>
<td>3</td>
<td>1013/3</td>
</tr>
<tr>
<td></td>
<td>PLGA flat sheet</td>
<td>1003</td>
<td>1</td>
<td>1003/1</td>
</tr>
<tr>
<td></td>
<td>ePTFE tubular scaffold</td>
<td>1125</td>
<td>1</td>
<td>1125/1</td>
</tr>
</tbody>
</table>

Results from this elution study confirm that ePTFE and PLGA (solvent: chloroform) were noncytotoxic to 3T3 cells.

3.3 Discussion and Conclusion

The second aim of this thesis involved cytotoxicity testing of standard BVM polymers used in the tissue engineering lab—ePTFE and PLGA—through direct contact and elution-based methods. The direct contact method involved placing the scaffold materials directly onto 3T3 cells.
and evaluating the cells at 12-, 24-, and 48-hour timepoints for signs of toxicity. Results from the direct contact procedure illustrated that ePTFE and PLGA were noncytotoxic at 12-, 24-, and 48-hour timepoints (Table 17). The elution method involved incubating ePTFE, PLGA, and control materials in 3T3 media at 37 °C for 24 hours and culturing 3T3 cells with the fluid extracts. The cells were then observed for signs of toxicity at 24- and 48-hour timepoints. Results from the elution procedure illustrated that ePTFE and PLGA were noncytotoxic to 3T3 cells (Table 17).

**Table 17. Results from cytotoxicity testing on ePTFE and PLGA.**

<table>
<thead>
<tr>
<th>Scaffold Material</th>
<th>Elution Procedure</th>
<th>Direct Contact Procedure</th>
</tr>
</thead>
<tbody>
<tr>
<td>ePTFE</td>
<td>Noncytotoxic</td>
<td>Noncytotoxic</td>
</tr>
<tr>
<td>PLGA (solvent: chloroform)</td>
<td>Noncytotoxic</td>
<td>Noncytotoxic</td>
</tr>
</tbody>
</table>

Challenges and limitations from this aim included the inability of the electrospinner to produce mandrel spins (aka “tubular scaffolds”) effectively and consistently. Thus, the cytotoxicity testing was done on a PLGA flat sheet sample instead of the lab’s standard scaffold spins. Ideally, to better model the standard BVM characteristics, cytotoxicity testing in the tissue engineering lab would be performed with higher quality and adequately-sized tubular scaffolds. This would ensure more representative biocompatibility results.

The results from this study were consistent with results from cytotoxicity testing done in previously published ePTFE and PLGA studies. As mentioned in Section 3.1, ePTFE and PLGA are well-established polymers with notable biocompatibility and low toxicity to cells. Therefore, this study provided a baseline for the comparison of new BVM materials, including PLGA with salt, PLLA, and PCL, which was Aim 3 and is described in Chapter 4.
CHAPTER 4. AIM 3: BIOCOMPATIBILITY OF NOVEL BVM SCAFFOLD POLYMERS: PLGA WITH SALT, PLLA, AND PCL

4.1 Introduction

The third aim of this thesis was to utilize the cytotoxicity protocols developed in Aim 1 to evaluate the biocompatibility of novel polymers in the BVM lab: PLGA with salt, PLLA, and PCL. This study was important because biocompatibility of the newer polymers had yet to be fully characterized. As described in Section 3.1, PLGA is well-established with well-known biocompatibility, but adding BTEAC salt into the scaffold could have significant impacts on biocompatibility. PLLA and PCL are also well-known biocompatible polymers. For example, cytotoxicity testing performed on an electrospun PLLA fiber membrane demonstrated noncytotoxic effects on cells [75]. Additionally, in a different study, cytotoxicity testing done on a PCL nanofibrous scaffold was noncytotoxic to cells [76]. However, because these polymers are dissolved in solvents—chloroform and dichloromethane—cytotoxicity testing was still necessary. To accomplish this aim, several studies involving direct contact and elution-based methods were performed.

4.2 Methods and Results

4.2.1 Cell Seeding Procedure

Direct Contact Cell Seeding

Cells were seeded according to the Cell Seeding SOP in Appendix K. Passage 28 3T3 cells at ~90% confluence in 1 T225 cell culture flask were harvested and resuspended in 24 mL of 3T3 media. Two mL of 3T3 media was placed into each well of three 6-well plates. Next, 0.3 mL of the cell solution was placed into each well, resulting in a final cell seeding density of ~112,500 cells per well or ~11,600 cells/cm². The 6-well plate was then placed in the incubator for 24 hours at 37°C and 5% CO₂.
Elution Cell Seeding

Cells were seeded according to the Cell Seeding SOP in Appendix K. Passage 34 3T3 cells at ~90% confluence in 1 T225 cell culture flask were harvested and resuspended in 24 mL of 3T3 media. Two mL of 3T3 media was placed into each well of six 6-well plates and two micro-dishes. Next, 0.3 mL of the cell solution was placed into each well of the 6-well plates and micro-dishes, resulting in a cell seeding density of ~112,500 cells per well or ~11,600 cells/cm². The 6-well plates and micro-dishes were then placed in the incubator for 24 hours.

4.2.2 Direct Contact Procedure

To begin the direct contact procedure, samples of PLGA with salt, PLLA, and PCL (Fig. 42-44) were cut into sections (~6 cm²). The direct contact SOP in Appendix L was then followed. The samples were sterilized in sterile 70% EtOH for 20 minutes. The conicals were replaced with DPBS and shaken for 1 minute to rid the polymers of any residual EtOH. A sample of each polymer was left in EtOH to serve as a positive control.

Figure 42. PCL samples. A) 12.5% PCL flat sheet spun on 210310, B) 12.5% PCL flat sheet spun on 210215, and C) PCL scaffold.
Figure 43. PLLA sample. A) PLLA sample on the flat sheet collector, B) the same PLLA sample removed from the collector.

Figure 44. PLGA with 1.5 wt.% BTEAC salt sample.

Using sterile forceps, the samples were transferred into the well plates, which were promptly placed into the incubator until imaging (Fig. 45). The well plates were evaluated and imaged under the white-light microscope at 12-, 24-, and 48-hour timepoints.

Figure 45. Sterilized polymer samples placed onto the cells in the direct contact procedure.
4.2.3 Elution Procedure

To begin the elution procedure (Appendix M), eight conicals were prepared, each with 10 mL of 3T3 media. The negative control consisted of warmed 3T3 media from the fridge with no sample. The positive control was prepared by placing a 45 cm² sterile, powder-free latex glove sample in media. The polymer samples tested, and their corresponding surface areas, are outlined in Table 18.

Table 18. Aim 3 elution test materials and corresponding surface areas.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Total Surface Area (cm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PLGA with salt tubular scaffold</td>
<td>~ 15</td>
</tr>
<tr>
<td>PLGA with salt flat sheet</td>
<td>~ 45</td>
</tr>
<tr>
<td>PLLA tubular scaffold (x2)</td>
<td>~ 25</td>
</tr>
<tr>
<td>PLLA flat sheet</td>
<td>~ 25</td>
</tr>
<tr>
<td>PCL tubular scaffold (x2)</td>
<td>~ 20</td>
</tr>
<tr>
<td>PCL flat sheet</td>
<td>~ 30</td>
</tr>
</tbody>
</table>

Samples were placed in conicals and warmed to 37 °C for 24 hours in the incubator. After 24 hours, the samples were removed, leaving only the eluted media left. The 3T3 cells were cultured with warmed 3T3 media from the fridge (negative control), 3T3 media warmed to 37 °C for 24 hours (negative control), media that was eluted with a sample of sterile, powder-free latex glove and warmed to 37 °C for 24 hours (positive control), as well as the medias eluted with the PLGA with salt, PLLA, and PCL samples. The well plates containing the cultured cells were then incubated for 24 and 48 hours.

4.2.4 Staining and Imaging Procedure

After 24 hours of incubation with the eluted media, the well plates were evaluated and imaged under the white-light microscope. Media was aspirated out of the well plates and the micro-dish was subjected to methanol for 20 minutes to serve as a positive control for the staining protocol.
(Appendix N). All wells were rinsed with DPBS and 1.5 mL of a live/dead stock stain solution of 30 mL of DPBS + 24 uL Ethd-1 + 6 uL calcein AM was placed into each well for 5-10 minutes. After 5-10 minutes, the stain solution was aspirated out of each well carefully as to not aspirate the cells. Each well was then imaged using the widefield fluorescence microscope (Appendix O). This staining and imaging procedure was repeated for the 48-hour timepoint wells with a replacement of the eluted media at 24 hours.

4.2.5 Cell Counting Procedure

Similar to Aim 2, a cell counting procedure was implemented in these studies to provide a quantitative representation of scaffold material biocompatibility. Images taken with the widefield fluorescence microscope were opened in ImageJ for proper cell counting (Appendix P). Each image was converted to 16-bit and the threshold was adjusted to ensure each nuclei was individually counted. Each particle was then analyzed in pixel² units. Live cells were counted based on the calcein AM stained images and dead cells were counted based on the ethidium homodimer-1 stained images. The results were tabulated as the number of live cells, the number of dead cells, and the ratio of live to dead cells for the positive controls, negative controls, PLLA, PLGA with salt, and PCL.

4.2.6 Results

Direct Contact Results

Results from this study illustrated no cytotoxic effects from PLLA, PLGA with salt, and PCL at 12 hours. At 24 hours, PLGA with salt and PCL began to display mild cytotoxic effects while PLLA displayed noncytotoxic effects. At 48 hours, PLGA with salt and PCL continued to exhibit mild cytotoxic effects. These mild cytotoxic effects included low confluence and limited cell growth seen in several areas of the well. The PLLA direct contact at 48 hours showed
necytotoxic effects. White-light images from the direct contact procedure can be found in Appendix J. These results are summarized in Table 19.

Table 19. Results of PLLA, PLGA with salt, and PCL direct contact procedure. Numbers separated by commas represent different fields of view.

<table>
<thead>
<tr>
<th>Timepoint</th>
<th>Polymer type</th>
<th>Cytotoxicity Scale</th>
<th>Interpretation</th>
</tr>
</thead>
<tbody>
<tr>
<td>12-hour</td>
<td>PLLA</td>
<td>0, 0</td>
<td>Noncytotoxic</td>
</tr>
<tr>
<td></td>
<td>PLLA in EtOH (pos. control)</td>
<td>3, 3</td>
<td>Severely cytotoxic</td>
</tr>
<tr>
<td></td>
<td>PLGA w/ salt</td>
<td>0, 0</td>
<td>Noncytotoxic</td>
</tr>
<tr>
<td></td>
<td>PLGA w/ salt in EtOH (pos. control)</td>
<td>3, 3</td>
<td>Severely cytotoxic</td>
</tr>
<tr>
<td></td>
<td>PCL tubular scaffold</td>
<td>0, 0</td>
<td>Noncytotoxic</td>
</tr>
<tr>
<td></td>
<td>PCL flat sheet 210310</td>
<td>0, 0</td>
<td>Noncytotoxic</td>
</tr>
<tr>
<td></td>
<td>PCL flat sheet 210215</td>
<td>0, 0</td>
<td>Noncytotoxic</td>
</tr>
<tr>
<td></td>
<td>PCL in EtOH (pos. control)</td>
<td>3, 3</td>
<td>Severely cytotoxic</td>
</tr>
<tr>
<td>24-hour</td>
<td>PLLA</td>
<td>0, 0</td>
<td>Noncytotoxic</td>
</tr>
<tr>
<td></td>
<td>PLLA in EtOH (pos. control)</td>
<td>3, 3</td>
<td>Severely cytotoxic</td>
</tr>
<tr>
<td></td>
<td>PLGA w/ salt</td>
<td>0, 2</td>
<td>Mildly cytotoxic</td>
</tr>
<tr>
<td></td>
<td>PLGA w/ salt in EtOH (pos. control)</td>
<td>3, 3</td>
<td>Severely cytotoxic</td>
</tr>
<tr>
<td></td>
<td>PCL tubular scaffold</td>
<td>2, 0</td>
<td>Mildly cytotoxic</td>
</tr>
<tr>
<td></td>
<td>PCL flat sheet 210310</td>
<td>0, 2</td>
<td>Mildly cytotoxic</td>
</tr>
<tr>
<td></td>
<td>PCL flat sheet 210215</td>
<td>0, 0</td>
<td>Noncytotoxic</td>
</tr>
<tr>
<td></td>
<td>PCL in EtOH (pos. control)</td>
<td>3, 3</td>
<td>Severely cytotoxic</td>
</tr>
<tr>
<td>48-hour</td>
<td>PLLA</td>
<td>0, 0</td>
<td>Noncytotoxic</td>
</tr>
<tr>
<td></td>
<td>PLLA in EtOH (pos. control)</td>
<td>3, 3</td>
<td>Severely cytotoxic</td>
</tr>
<tr>
<td></td>
<td>PLGA w/ salt</td>
<td>0, 2</td>
<td>Mildly cytotoxic</td>
</tr>
<tr>
<td></td>
<td>PLGA w/ salt in EtOH (pos. control)</td>
<td>3, 3</td>
<td>Severely cytotoxic</td>
</tr>
<tr>
<td></td>
<td>PCL tubular scaffold</td>
<td>0, 2</td>
<td>Mildly cytotoxic</td>
</tr>
<tr>
<td></td>
<td>PCL flat sheet 210310</td>
<td>1, 0</td>
<td>Mildly cytotoxic</td>
</tr>
<tr>
<td></td>
<td>PCL flat sheet 210215</td>
<td>0, 1</td>
<td>Mildly cytotoxic</td>
</tr>
<tr>
<td></td>
<td>PCL in EtOH (pos. control)</td>
<td>3, 3</td>
<td>Severely cytotoxic</td>
</tr>
</tbody>
</table>
Elution Results

Results from the elution procedure illustrated that PLGA with salt, PLLA, and PCL were noncytotoxic at 24 hours. Negative controls displayed highly confluent, live cells and almost no dead cells. Positive controls displayed cell detachment from the surface of the flask and high cell death. At 48 hours, results illustrated that PLGA with salt, PLLA, and PCL were noncytotoxic as well. At this timepoint, negative controls continued to display highly confluent, live cells and almost no dead cells. Positive controls displayed further cell detachment from the surface of the flask and even greater cell death. White-light images from the PLGA with salt, PLLA, and PCL elution procedure can be found in Appendix J. These results are summarized in Table 20.
Table 20. Results of PLLA, PLGA with salt, and PCL elution procedure. Numbers separated by commas represent different fields of view.

<table>
<thead>
<tr>
<th>Timepoint</th>
<th>Sample</th>
<th>Cytotoxicity Scale</th>
<th>Interpretation</th>
</tr>
</thead>
<tbody>
<tr>
<td>24-hour</td>
<td>3T3 media (neg. control)</td>
<td>0, 0</td>
<td>Noncytotoxic</td>
</tr>
<tr>
<td></td>
<td>3T3 media at 37 °C for 24 hours (neg. control)</td>
<td>0, 0</td>
<td>Noncytotoxic</td>
</tr>
<tr>
<td></td>
<td>Latex (pos. control)</td>
<td>3, 3</td>
<td>Severely cytotoxic</td>
</tr>
<tr>
<td></td>
<td>Methanol (pos. control)</td>
<td>3, 3</td>
<td>Severely cytotoxic</td>
</tr>
<tr>
<td></td>
<td>PLGA w/ salt tubular scaffold</td>
<td>0, 0</td>
<td>Noncytotoxic</td>
</tr>
<tr>
<td></td>
<td>PLGA w/ salt flat sheet</td>
<td>0, 0</td>
<td>Noncytotoxic</td>
</tr>
<tr>
<td></td>
<td>PLLA tubular scaffold</td>
<td>0, 0</td>
<td>Noncytotoxic</td>
</tr>
<tr>
<td></td>
<td>PLLA flat sheet</td>
<td>0, 0</td>
<td>Noncytotoxic</td>
</tr>
<tr>
<td></td>
<td>PCL tubular scaffold</td>
<td>0, 0</td>
<td>Noncytotoxic</td>
</tr>
<tr>
<td></td>
<td>PCL flat sheet</td>
<td>0, 0</td>
<td>Noncytotoxic</td>
</tr>
<tr>
<td>48-hour</td>
<td>3T3 media (neg. control)</td>
<td>0, 0</td>
<td>Noncytotoxic</td>
</tr>
<tr>
<td></td>
<td>3T3 media at 37 °C for 24 hours (neg. control)</td>
<td>0, 0</td>
<td>Noncytotoxic</td>
</tr>
<tr>
<td></td>
<td>Latex (pos. control)</td>
<td>3, 3</td>
<td>Severely cytotoxic</td>
</tr>
<tr>
<td></td>
<td>Methanol (pos. control)</td>
<td>3, 3</td>
<td>Severely cytotoxic</td>
</tr>
<tr>
<td></td>
<td>PLGA w/ salt tubular scaffold</td>
<td>0, 0</td>
<td>Noncytotoxic</td>
</tr>
<tr>
<td></td>
<td>PLGA w/ salt flat sheet</td>
<td>0, 0</td>
<td>Noncytotoxic</td>
</tr>
<tr>
<td></td>
<td>PLLA tubular scaffold</td>
<td>0, 0</td>
<td>Noncytotoxic</td>
</tr>
<tr>
<td></td>
<td>PLLA flat sheet</td>
<td>0, 0</td>
<td>Noncytotoxic</td>
</tr>
<tr>
<td></td>
<td>PCL tubular scaffold</td>
<td>0, 0</td>
<td>Noncytotoxic</td>
</tr>
<tr>
<td></td>
<td>PCL flat sheet</td>
<td>0, 0</td>
<td>Noncytotoxic</td>
</tr>
</tbody>
</table>

At 24 hours, the positive control of powder-free latex glove eluted media was effective in killing the cells. This was confirmed by the absence of cells in the positive control stained and imaged wells and was due to cell detachment and aspiration during the staining procedure. The other positive control of methanol for 20 minutes was effective in killing the cells and
demonstrating the efficacy of the stain. This was confirmed by the intense fluorescence of ethidium homodimer-1 in the stained and imaged well. At 24 hours, the PLGA with salt tubular scaffold and flat sheet, the PLLA tubular scaffold and flat sheet, and the PCL tubular scaffold and flat sheet eluted media illustrated a high confluency of live cells and almost no dead cells (Fig. 46). Images at 48 hours illustrated similar results, but with an even greater confluency of live cells (Fig. 47).
Effective Positive Control – methanol subjection for 20 minutes

PLLA tubular scaffold eluted media at 37 °C for 24 hours

PLLA flat sheet eluted media at 37 °C for 24 hours

PLGA with salt tubular scaffold eluted media at 37 °C for 24 hours
PLGA with salt flat sheet eluted media at 37 °C for 24 hours

PCL tubular scaffold eluted media at 37 °C for 24 hours

PCL flat sheet eluted media at 37 °C for 24 hours

Figure 46. PLLA, PLGA with salt, and PCL widefield fluorescence images of 24-hour timepoint elution procedure. 10X magnification, 50% power, 500 ms exposure time.

Calcein AM

Ethidium Homodimer-1

Effective Negative Control – 3T3 media
Effective Negative Control – 3T3 media at 37 °C for 24 hours

Effective Positive Control – 45 cm² latex glove eluted media at 37°C for 24 hours

Effective Positive Control – methanol subjection for 20 minutes

PLLA tubular scaffold eluted media at 37 °C for 24 hours
PLLA flat sheet eluted media at 37°C for 24 hours

PLGA with salt tubular scaffold eluted media at 37 °C for 24 hours

PLGA with salt flat sheet eluted media at 37 °C for 24 hours

PCL tubular scaffold eluted media at 37 °C for 24 hours
At 24 hours, the negative controls of warmed 3T3 media from the fridge and 3T3 media incubated at 37 °C for 24 hours resulted in high cell viability (1286 live cells and 1244 live cells, respectively) and minimal cell death (5 dead cells and 3 dead cells, respectively). At 48 hours, these negative controls continued to illustrate high cell viability (2020 live cells and 1635 live cells, respectively) and minimal cell death (0 dead cells and 2 dead cells, respectively). At 24 and 48 hours, the powder-free latex glove eluted media resulted in no live or dead cells due to cell aspiration during the staining protocol. At 24 and 48 hours, the subjection of methanol for 20 minutes resulted in no live cells and high cell death (1624 dead cells at 24 hours and 1635 dead cells at 48 hours). At 24 hours, the PLLA tubular scaffold and flat sheet, PLGA with salt tubular scaffold and flat sheet, and PCL tubular scaffold and flat sheet eluted media also resulted in high cell viability (1204 live cells, 1191 live cells, 982 live cells, 1293 live cells, 1174 live cells, and 1309 live cells, respectively) and minimal cell death (3 dead cells, 4 dead cells, 5 dead cells, 6 dead cells, 6 dead cells, and 11 dead cells, respectively). At 48 hours, these polymers continued to illustrate high cell viability (1740 live cells, 2287 live cells, 2287 live cells, 1264 live cells, 2142 live cells, and 1793 live cells, respectively) and minimal cell death (7 dead cells, 2 dead cells, 15 dead cells, 8 dead cells, 5 dead cells, 3 dead cells, respectively). These results are summarized in Table 21.
Table 21. Results of PLLA, PLGA with salt, and PCL elution procedure cell counts.

<table>
<thead>
<tr>
<th>Timepoint</th>
<th>Sample</th>
<th># of Live Cells</th>
<th># of Dead Cells</th>
<th>Ratio of Live/Dead</th>
</tr>
</thead>
<tbody>
<tr>
<td>24-hour</td>
<td>3T3 media (neg. control)</td>
<td>1286</td>
<td>5</td>
<td>1286/5</td>
</tr>
<tr>
<td></td>
<td>3T3 media warmed to 37 °C for 24 hours (neg. control)</td>
<td>1244</td>
<td>3</td>
<td>1244/3</td>
</tr>
<tr>
<td></td>
<td>Latex (pos. control)</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Methanol (pos. control)</td>
<td>0</td>
<td>1624</td>
<td>0/1624</td>
</tr>
<tr>
<td></td>
<td>PLLA scaffold</td>
<td>1204</td>
<td>3</td>
<td>1204/3</td>
</tr>
<tr>
<td></td>
<td>PLLA flat sheet</td>
<td>1191</td>
<td>4</td>
<td>1191/4</td>
</tr>
<tr>
<td></td>
<td>PLGA w/ salt scaffold</td>
<td>982</td>
<td>5</td>
<td>982/5</td>
</tr>
<tr>
<td></td>
<td>PLGA w/ salt flat sheet</td>
<td>1293</td>
<td>6</td>
<td>1293/6</td>
</tr>
<tr>
<td></td>
<td>PCL scaffold</td>
<td>1174</td>
<td>6</td>
<td>1174/6</td>
</tr>
<tr>
<td></td>
<td>PCL flat sheet</td>
<td>1309</td>
<td>11</td>
<td>1309/11</td>
</tr>
<tr>
<td>48-hour</td>
<td>3T3 media (neg. control)</td>
<td>2020</td>
<td>0</td>
<td>2020/0</td>
</tr>
<tr>
<td></td>
<td>3T3 media warmed to 37 °C for 24 hours (neg. control)</td>
<td>1635</td>
<td>2</td>
<td>1635/2</td>
</tr>
<tr>
<td></td>
<td>Latex (pos. control)</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Methanol (pos. control)</td>
<td>0</td>
<td>1635</td>
<td>0/1635</td>
</tr>
<tr>
<td></td>
<td>PLLA scaffold</td>
<td>1740</td>
<td>7</td>
<td>1740/7</td>
</tr>
<tr>
<td></td>
<td>PLLA flat sheet</td>
<td>2287</td>
<td>2</td>
<td>2287/2</td>
</tr>
<tr>
<td></td>
<td>PLGA w/ salt scaffold</td>
<td>2287</td>
<td>15</td>
<td>2287/15</td>
</tr>
<tr>
<td></td>
<td>PLGA w/ salt flat sheet</td>
<td>1264</td>
<td>8</td>
<td>1264/8</td>
</tr>
<tr>
<td></td>
<td>PCL scaffold</td>
<td>2142</td>
<td>5</td>
<td>2142/5</td>
</tr>
<tr>
<td></td>
<td>PCL flat sheet</td>
<td>1793</td>
<td>3</td>
<td>1793/3</td>
</tr>
</tbody>
</table>

Results from this elution study demonstrated that PLGA with salt (solvent: chloroform), PLLA (solvent: chloroform), and PCL (solvent: dichloromethane) were noncytotoxic to 3T3 cells.
4.3 Discussion and Conclusion

The third aim of this thesis involved cytotoxicity testing of novel BVM polymers used in the tissue engineering lab—PLGA with salt, PLLA, and PCL—through direct contact and elution-based methods. The direct contact method involved placing the scaffold materials directly onto 3T3 cells and evaluating the cells at 12-, 24-, and 48-hour timepoints for signs of toxicity. Results from the direct contact procedure illustrated that PLGA with salt, PLLA, and PCL were noncytotoxic at 12-hour timepoints. At 24- and 48-hour timepoints, PLGA with salt and PCL demonstrated mild cytotoxic effects (Table 22). The elution method involved incubating PLGA with salt, PLLA, PCL, and control materials in 3T3 media at 37 °C for 24 hours and culturing 3T3 cells with the fluid extracts. The cells were then observed for signs of toxicity at 24- and 48-hour timepoints. Results from the elution procedure illustrated that PLGA with salt, PLLA, and PCL were noncytotoxic to 3T3 cells (Table 22). The results from this elution study were consistent with results from cytotoxicity testing done in previously published PLGA, PLLA, and PCL studies. As mentioned in Section 4.1, PLGA, PLLA, and PCL are well-established polymers with notable biocompatibility and low toxicity to cells. However, cytotoxicity testing was still valuable because these polymers were dissolved in solvents (chloroform and dichloromethane), electrospun using protocols in our lab, and had yet to be characterized for their biocompatibility.

Table 22. Results from cytotoxicity testing on PLGA with salt, PLLA, and PCL.

<table>
<thead>
<tr>
<th>Scaffold Material</th>
<th>Direct Contact Procedure</th>
<th>Elution Procedure</th>
</tr>
</thead>
<tbody>
<tr>
<td>PLGA with salt (solvent: chloroform)</td>
<td>Mildly cytotoxic</td>
<td>Noncytotoxic</td>
</tr>
<tr>
<td>PLLA (solvent: chloroform)</td>
<td>Noncytotoxic</td>
<td>Noncytotoxic</td>
</tr>
<tr>
<td>PCL (solvent: dichloromethane)</td>
<td>Mildly cytotoxic</td>
<td>Noncytotoxic</td>
</tr>
</tbody>
</table>

While the elution procedure of PLGA with salt and PCL illustrated they were noncytotoxic to cells, the direct contact procedure showed mild cytotoxic effects from PLGA with salt and PCL.
Potential causes for the mild cytotoxic effects along with the observed variability between test methods are discussed below.

4.3.1 Discussion of Cytotoxic Variability

Potential causes of the mild cytotoxic effects observed with PLGA with salt and PCL in the direct contact procedure could be linked to the addition of benzyl triethylammonium chloride (BTEAC) salt to PLGA, or dichloromethane’s (DCM) use as a solvent for PCL. The addition of BTEAC salt to PLGA presents a potential cytotoxicity concern. The PLGA dissolved in chloroform did not result in cytotoxic effects from the elution or direct contact procedure, while PLGA with salt illustrated mild cytotoxic effects from the direct contact procedure. This reinforced the potential for cytotoxic effects of BTEAC salt. This salt has been shown in literature to cause serious irritation, along with acute toxicity [77]. While no studies were found specifically on the cytotoxicity of BTEAC salt on cells, future studies should be performed to determine if this addition of salt to PLGA does in fact present a biocompatibility concern.

Additionally, PCL was the only polymer dissolved in DCM, a solvent shown to have toxicological effects. In a study evaluating the cytotoxicity of a DCM extract on cells, it was concluded that the extract had significant cytotoxic effects on cells within a concentration range of 0.1 to 100 ug/ml [78]. DCM is an organic solvent known to be highly toxic and exposure can result in cough, pulmonary irritation, mucous membrane irritation, skin irritation, and corrosive burns [79]. Due to its inherent toxicity, using DCM as a solvent in the electrospinning of PCL could be a contributing factor to the mild cytotoxicity seen from PCL’s direct contact procedure.

Another potential cause of variability between the direct contact and elution results could include insufficient extraction ratios in the elution test method. For example, the scaffold and flat sheet samples of PCL were unable to reach the ~45 cm² in 10 mL of 3T3 media that was determined in Trial #8 to be the proper extraction ratio for powder-free latex gloves. Although this extraction ratio may not be compatible with each scaffold material type, it was able to properly leach toxins
from the latex gloves. Insufficient extraction ratios were due to the inability to spin large enough sample sizes. The PCL extraction ratio of ~30 cm² in 10 mL of media may not have been able to demonstrate signs of toxicity relative to the direct contact procedure that did present mild cytotoxic effects. The inability to reach this proper extraction ratio could have led to false conclusions with PCL from the elution procedure. For PLGA with salt, the flat sheet sample was able to reach the elution procedure’s sufficient extraction ratio of ~45 cm² in 10 mL of media and still demonstrated variability between the elution and direct contact procedure cytotoxicity results. Therefore, insufficient extraction ratios may not have been the primary cause of variability between the cytotoxicity results. Future elution methods should consider that not all polymer types sufficiently leach toxins with the same extraction ratio.

Both the addition of BTEAC salt to PLGA and the use of DCM as a solvent for PCL could be the potential causes of observed mild cytotoxic effects. Alternatively, the elution procedure’s potentially insufficient extraction ratio could be the cause of the differing cytotoxicity results observed between the elution and direct contact procedure.

4.3.2 Discussion of Test Method Variability

As implied in the discussion above, if the salt and DCM were definitively problematic, it would have been expected to see similar cytotoxicity results from the elution procedure. In addition to elution sample size, there are several other potential causes of the variability between the elution and direct contact test methods due to the methods themselves. First, the direct contact procedure has limitations due to physical interaction of the test sample with the cell layer. A study evaluating in vitro cytotoxicity of biomaterials illustrated that cell death and detachment was greatest in the direct contact tests when compared to the indirect contact tests of a cytotoxic control [80]. Additional studies have proven that the direct contact procedure is not recommended for very low or very high density material. Very low density material will tend to float in the cell growth media and very high density material will tend to sit on top of the cells, potentially damaging the cells.
when placed [81]. According to ISO 10993-5, extreme care must be taken to prevent any unnecessary movement of the scaffold materials when placed on the cell layer. For example, areas of dislodged cells could be due to the unnecessary movement of the test samples [53]. While extreme care was taken in all direct contact studies to ensure no unnecessary movement of the samples on top of the cells, it is possible that disturbance of the cells could have occurred during placement of the sample or during evaluation. For these reasons, in vitro testing using a test sample extract (i.e., the elution procedure) is more frequently used in cytotoxicity testing due to a mitigated potential risk of cellular trauma due to contact between the material and the cell layer [82].

Another potential cause for variability between the direct contact and elution-based cytotoxicity test methods have been identified in past studies. Under the assumption that no physical disruption of the cell layer occurs during placement of the sample, there is evidence that direct contact methods can better detect cytotoxic effects when compared to indirect contact methods. In a comparison between direct contact and elution-based methods done with polydioctylfluorene (PFO), it was determined that cytotoxicity tests of extracts in the elution-based methods were inadequate because PFO has been proven to cause blindness when in direct contact with human tissue [83]. Therefore, the direct contact method was more effective than the elution procedure. The UNE-ISO, the body legally responsible for the development of standards in Spain, suggests that testing procedures should reflect the application in which the test sample will be used [83]. In the case of our lab, our scaffold materials are coming in direct contact with cells and thus the direct contact method may be a more representative cytotoxicity testing procedure. This same study identified that the elution-based method was unable to detect cellular toxicity due to an insufficient amount of leachable toxic components from the samples. The direct contact method ensures that cells are exposed directly to the test subjects; therefore, providing more meaningful data regarding the potential toxicity of the test subject [83].

In summary, variability between the direct contact and elution-based test methods could be
due to the physical disruption of cells during the direct contact procedure or to the inherent nature of the direct contact procedure which is more sensitive to cytotoxic effects. Prior to implementing either cytotoxicity test method, it is important to consider the advantages and disadvantages of each method. Since each provides valuable data, future studies should continue to implement both direct contact and elution-based methods.

Challenges and limitations from this aim were consistent with studies performed in Aim 2, including the inability of the electrospinner to produce mandrel spins (aka “tubular scaffolds”) effectively and consistently. Thus, the cytotoxicity testing was done on a combination of flat sheet samples and partial mandrel spins. As mentioned in Aim 2, to better model the standard BVM characteristics, cytotoxicity testing in the tissue engineering lab would be ideally performed with higher quality and adequately-sized tubular scaffolds. This would ensure more representative biocompatibility results.
CHAPTER 5. DISCUSSION AND CONCLUSION

5.1 Summary and Aims of the Thesis

Tissue-engineered blood vessel mimics (BVMs) are useful preclinical testing models for vascular devices. Electrospun polymer scaffolds are the foundation of BVMs and, therefore, must be adequately tested for biocompatibility before being seeded with human vascular cell types. Because an assortment of polymer types are used in BVMs, general biocompatibility testing methods are necessary to ensure safe interaction between each polymer material and human vascular cells.

The overall goal of this thesis was to establish methods for evaluating the biocompatibility, specifically the cytotoxicity, of existing and novel polymers used in the fabrication of BVMs. Many of the newer polymers used in the tissue engineering lab have yet to be fully characterized for biocompatibility. Aim 1 of this thesis involved the development of both direct contact and elution-based protocols for cytotoxicity testing of polymers. In Aim 2, the protocols were used to assess the cytotoxicity of existing polymers used in the tissue engineering lab: ePTFE and PLGA. Finally, in Aim 3, the protocols were used to assess the cytotoxicity of recent polymer additions to the lab: PLLA, PCL, and PLGA with salt. Overall, the aims of this thesis successfully established methods that can be used to test and evaluate future scaffold materials used in the fabrication of BVM models.

5.2 Results and Discussion

5.2.1 Aim 1: Protocol Development

The development of protocols for in vitro cytotoxicity testing of polymers used in BVM fabrication included numerous trials. Throughout the trials, each iteration brought valuable contributions. It was shown that a live/dead stain consisting of calcein AM and ethidium homodimer-1 was a suitable assay for differentiating between live and dead cells in laboratory
experiments done with human vascular cell types. Additionally, a successful 3T3 cell seeding density for cytotoxicity testing was determined (i.e., one that did not cause over-confluency and therefore cell death).

The appropriate positive and negative controls for the direct contact and elution-based methods were also a significant contribution. As discussed in Section 2.3, the elution controls displayed inconsistencies and notable cell death with the extraction condition of 50 °C for 72 hours. The potential generation of precipitates within the cell culture media with a high temperature shift from 37 °C to 50 °C for a long timepoint may have caused detrimental effects to the cultured cells. Heat treatment of FBS within the cell culture media has been shown to adversely affect cell health and lead to these precipitates [72]. The undesirable cell death observed with the extraction condition of 50 °C for 72 hours led to an incorporation of ISO 10993 cytotoxicity recommendations, and inevitably brought worthwhile improvements (a new extraction condition of 37 °C for 24 hours and proper extraction ratios) to the elution-based methods.

Although there were a multitude of trials involved in the development of protocols for in vitro cytotoxicity testing, the final working protocol included direct contact and elution-based methods that could suitably evaluate the cytotoxicity of polymers used in BVM fabrication.

5.2.2 Aim 2 and Aim 3: Biocompatibility of Standard and Novel BVM Scaffold Materials

The protocol development trials refined a testing protocol that could properly evaluate the cytotoxicity of standard and novel BVM scaffold materials used in the tissue engineering lab. Results gathered from the final biocompatibility evaluation of standard and novel BVM scaffold materials showed that ePTFE, PLGA, and PLLA were nontoxic in elution and direct contact procedures while PLGA with salt and PCL were nontoxic in elution procedures and mildly cytotoxic in direct contact procedures. These outcomes are outlined in Table 23.
Table 23. Scaffold material with final cytotoxicity results.

<table>
<thead>
<tr>
<th>Scaffold Material</th>
<th>Elution Procedure</th>
<th>Direct Contact Procedure</th>
</tr>
</thead>
<tbody>
<tr>
<td>ePTFE</td>
<td>Noncytotoxic</td>
<td>Noncytotoxic</td>
</tr>
<tr>
<td>PLGA (solvent: chloroform)</td>
<td>Noncytotoxic</td>
<td>Noncytotoxic</td>
</tr>
<tr>
<td>PLGA with salt (solvent: chloroform)</td>
<td>Noncytotoxic</td>
<td>Mildly cytotoxic</td>
</tr>
<tr>
<td>PLLA (solvent: chloroform)</td>
<td>Noncytotoxic</td>
<td>Noncytotoxic</td>
</tr>
<tr>
<td>PCL (solvent: dichloromethane)</td>
<td>Noncytotoxic</td>
<td>Mildly cytotoxic</td>
</tr>
</tbody>
</table>

The scaffold materials ePTFE, PLGA, and PLLA consistently demonstrated noncytotoxic effects through elution-based and direct contact methods; therefore, these polymers can be deemed biocompatible and can be implemented in future lab BVM setups without concerns. Elution-based cytotoxicity testing of PLGA with salt and PCL illustrated noncytotoxic effects, while direct contact methods demonstrated mild cytotoxic effects at 24- and 48-hour timepoints (Table 23). Potential causes for these mild cytotoxic effects are summarized below and were discussed in further detail in Section 4.3. In summary, the addition of BTEAC salt to PLGA and the use of dichloromethane as a solvent for PCL could be the cause of cytotoxicity for these samples. Lastly, due to electrospinning troubleshooting and therefore inadequate sample sizing, an insufficient extraction ratio associated with the elution procedure could be the cause of differing cytotoxicity results observed between the two test methods. Future work should be performed to test these conjectures, which will be further discussed in Section 5.3.

However, if the salt and DCM were definitively problematic, it would have been expected to see similar cytotoxicity results from the elution procedure. Potential causes for the variability between test methods include (1) the direct contact procedure’s physical interaction of the test sample with the cell layer, and (2) the evidence supporting the direct contact procedure’s ability to better detect cytotoxicity, under the assumption that no physical disruption of the cell layer occurs during placement of the test sample [83]. As mentioned in Section 4.3, it is important to consider...
the advantages and disadvantages of each test method prior to implementation in cytotoxicity testing. Each method provides valuable and representative data; therefore, future studies should continue to implement both direct contact and elution-based methods.

5.3 Limitations and Future Steps

The most notable limitation of this thesis was the troubleshooting of the electrospinner. Throughout much of the thesis, the electrospinner was unable to produce consistent polymer scaffolds via mandrel spins (aka “tubular scaffolds). This was limiting because all cytotoxicity testing was originally intended for electrospun, tube-shaped scaffolds (i.e., standard mandrel spins used in typical BVM setups). Fortunately, as the year progressed, the electrospinner was able to produce consistent flat sheet sample spins. Therefore, this limitation was addressed by conducting cytotoxicity testing on flat sheet sample spins in combination with partial mandrel spins. However, this led to variability of polymer types and polymer configuration throughout the thesis.

Although the flat sheet samples were usable, they had their disadvantages. The flat sheet samples were very thin and fibrous which made it challenging to remove them from conicals, sterilize them in EtOH, and incubate them in media. The thin and fibrous nature of the polymer spins also made it difficult to obtain the scaffold surface area necessary to reach the desired extraction ratio in elution procedures. It was sometimes necessary to use multiple tubular scaffold samples, often more than was accessible, to reach a surface area large enough to properly leach toxic components from the sample. For example, due to electrospinner troubleshooting, several scaffold and flat sheet samples were unable to reach the elution procedure’s desired extraction ratio. To address this limitation, future elution procedures should utilize samples that produce an approximate extraction ratio of ~45 cm² in 10 mL of media. This ratio was defined in Trial #8 as an adequate ratio to properly leach toxins from powder-free latex gloves. Although the electrospinner limitation was addressed with the methods described above, it is recommended that future cytotoxicity testing be performed on higher quality and adequately-sized scaffold materials.
To obtain the elution procedure’s proper extraction ratio of ~45 cm$^2$ in 10 mL of media using a tubular scaffold, several samples would need to be spun to reach the proper extraction ratio.

Additional limitations associated with protocol development involved improper cell seeding densities during the first trials. This led to over-confluency of cells in the well plates and potentially false cytotoxicity conclusions. The positive control for the staining protocol was also a limitation during the first trials as 100% EtOH for 20 minutes and 70% EtOH for 30-45 seconds were causing cells to lift from the surface of the wells. Adjustment of the cell seeding protocol and implementation of methanol for 20 minutes alleviated the over-confluency and positive control limitations, respectively. Another limitation involved polymer brittleness which resulted in breakdown in the media during the elution procedure and debris when placing the eluted media on cells. The polymer brittleness limitation was remedied by using newly spun polymers. Another limitation involved the application of live/dead stain to cells for 30 minutes which caused the cells to lift off the surface of the well plates. The stain application troubleshooting was addressed by ordering new stain and applying a smaller concentration of stain for a shorter timepoint (5-10 minutes). Another notable limitation associated with protocol development involved the inconsistent outcomes observed with the elution procedure extraction condition of 50 °C for 72 hours. This led to the incorporation of ISO 10993 recommendations which included a new extraction condition of 37 °C for 24 hours and the introduction of improved extraction ratios. Once a final testing protocol was established, limitations emerged from the conflicting cytotoxicity results between the direct contact and elution-based methods.

Future work addressing the conflicting results between the test methods and more specifically the potential cytotoxic effects of DCM as a solvent for PCL should be conducted. This could involve dissolving PCL in chloroform—the solvent used for all other polymers—and evaluating cytotoxic effects thereafter. Chloroform can be a common solvent for PCL applications, but its use in lab was not pursued due to published protocols that utilized DCM [84],[85].
Additionally, studies have shown that acetone is a nonhazardous PCL solvent [86]. Other studies prefer ethyl acetate due to its relatively less toxic nature [87]. Therefore, future work could compare the feasibility and resulting biocompatibility of various PCL solvent types.

In addition to comparing PCL solvent types, future work could be performed to address the potential cytotoxic effects from the addition of BTEAC salt to PLGA. This could involve adding different amounts of BTEAC salt (without the PLGA polymer) directly onto 3T3 cells and evaluating the cytotoxicity results with a dose response curve. The hypothesis would be that with little to no salt there would be no cytotoxic response and when the dose of BTEAC salt is increased, cytotoxicity becomes apparent at a certain threshold. Additionally, various percentages of salt could be added into the scaffold solutions. For example, 1%, 2%, 5%, and 10% BTEAC salt could be added into the scaffold solutions and evaluated for a certain threshold where cytotoxic effects begin. If the BTEAC salt is determined to be cytotoxic at a certain threshold, the next steps would include determining if the cytotoxicity is permissible for application in the tissue engineering lab or the potential implementation of a rinsing step to mitigate the cytotoxic effects from the addition of salt to the scaffold solution.

Another limitation found with cytotoxicity protocols included the lack of quantification for the direct contact procedure. The lack of quantification made it difficult to distinguish nuances and make comparisons between each scaffold materials’ cytotoxic effects. Future work to better quantify the results from the direct contact procedure could include the use of a hemocytometer to count the number of cells found in the well plate or the further use of ImageJ capabilities. The phase contrast images taken from the white-light microscope could be evaluated in ImageJ for cell count or percent surface area covered by cells. Additionally, green fluorescent protein (GFP) 3T3 cells could be utilized for cytotoxicity testing to better quantify and compare the cytotoxicity of scaffold materials by means of cell count and cell coverage.

While ISO 10993 recommends established cell lines to be used for cytotoxicity testing, the
guidance does not recommend a specific cell type [53]. Standard cell types (i.e., mouse fibroblast cells) are commonly used in literature, but cytotoxicity has been shown to be cell-type dependent [82]. Therefore, future cytotoxicity testing should be performed using established cell lines other than 3T3 cells. In the tissue engineering lab, endothelial cells and smooth muscle cells are the primary cell types that come in direct contact with scaffold materials during BVM setups and therefore cytotoxicity tests could be performed to evaluate polymer interaction with these cell types.

Lastly, another iteration of the direct contact procedure with novel materials—PLGA with salt and PCL—should be conducted to confirm that the mild cytotoxicity observed was due to biocompatibility concerns of the scaffold materials and not an issue associated with the direct contact method (i.e., sample placement, physical disturbance of the cell layer). Agar diffusion techniques could also be implemented as another indirect contact test to provide redundancy and potentially offer more consistent results [53].

In summary, next steps of this project should include cytotoxicity testing using higher quality and adequately-sized scaffold materials and another iteration of the direct contact procedure with novel materials—PLGA with salt and PCL—to confirm observed results. Future work will be able to utilize the protocols developed in this thesis to prescreen any newly introduced polymers/materials for their biocompatibility before use in the tissue engineering lab.

5.4 Conclusion

In summary, Aim 1 successfully developed protocols for evaluating scaffold cytotoxicity with both direct contact and elution-based methods. Aim 2 utilized the protocols to perform cytotoxicity testing on standard, pre-existing polymers in the tissue engineering lab: ePTFE and PLGA. Aim 2 studies found that ePTFE and PLGA are nontoxic to mouse fibroblast cells. Therefore, ePTFE and PLGA are safe polymers to be used in scaffold fabrication for BVM models. Aim 3 utilized the protocols to perform cytotoxicity testing on the tissue engineering lab’s novel
polymers: PLLA, PCL, and PLGA with salt. Aim 3 studies found that PLLA, PCL, and PLGA with salt were noncytotoxic to mouse fibroblast cells in elution-based methods; however, the direct contact procedure illustrated that PLGA with salt and PCL were mildly cytotoxic at 24- and 48-hour timepoints. The toxicity was mild enough to urge future cytotoxicity testing on PLGA with salt and PCL before further use in the lab. Meanwhile, ePTFE, PLGA, and PLLA scaffold materials were deemed biocompatible and can be implemented in future BVM setups.
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PART THREE: CELL ViABILITY – TRYPA*N BLUE
1. Remove another petri dish of cells from the incubator – view cells under the microscope and estimate confluence.
2. On benchtop, aspirate off media and set aside. (This will be used to ensure dead cells are accounted for.)
3. Rinse with DPBS (non-sterile is fine)
4. Add 1 mL trypsin to the dish and wait for cells to detach.
5. Deactivate trypsin with 2 mL of set-aside media and mix cell solution by pipetting up and down.
6. Remove 150ul of cell solution and place in a microcentrifuge tube.
7. Carefully add 50ul of trypan blue to the microcentrifuge tube – cap tightly and flick to mix

PART FOUR: CELL ViABILITY – HEMOCYTMETER AND COUNTING
1. Remove 10 ul and inject solution into hemocytometer. (Note: this is 7.5ul cell soln and 2.5ul trypsin blue)
2. View under microscope and acquire an image of your view. Count cells in each of the five large squares, labeled 1-5, as indicated in the diagram below. Record the number of clear cells counted per square and the number of blue cells counted per square. Calculate total number of cells per square and averages.

3. Calculate the total number of cells in the petri dish (EQN 1) and the percent viability (EQN 2) as follows:

   EQN 1: Cells per mL = (cells in 5 large squares / 5) * dilution factor * 10^4 (volume factor)
   Cells per dish = Cells per mL x 3mL

   EQN 2: Percent viability = (Avg # clear cells per square) / (Avg total # of cells per square) *100

4. Discard petri dish, microcentrifuge tube, and hemocytometer in biohazard waste.
Appendix B: Trial #1

Appendix B-1: Trial #1 direct contact procedure with PLGA at the 12-hour timepoint, 10X

Well 1. PLGA
Well 2. PLGA
Well 3. Warmed 3T3 media from the fridge (neg. control)

Well 4. PLGA
Well 5. PLGA
Well 6. Warmed 3T3 media from the fridge (neg. control)

Appendix B-2: Trial #1 direct contact procedure with PLGA at the 24-hour timepoint, 10X

Well 1. PLGA
Well 2. PLGA
Well 3. Warmed 3T3 media from the fridge (neg. control)

Well 4. PLGA
Well 5. PLGA
Well 6. Warmed 3T3 media from the fridge (neg. control)
Appendix B-3: Trial #1 direct contact procedure with PLGA at 48-hour timepoint, 10X

Well 1. PLGA
Well 2. PLGA
Well 3. Warmed 3T3 media from the fridge (neg. control)

Well 4. PLGA
Well 5. PLGA
Well 6. Warmed 3T3 media from the fridge (neg. control)

Appendix B-4: Trial #1 elution procedure with PLLA at 24-hour timepoint, 10X

Well 1. Warmed 3T3 media from the fridge (neg. control)
Well 2. Warmed 3T3 media from the fridge (neg. control)
Well 3. 3T3 media subjected to 50 °C water for 72 hours (neg. control)
Well 4. 3T3 media subjected to 50 °C water for 72 hours (neg. control)
Well 5. PLLA eluted media
Well 6. PLLA eluted media
Well 7. PLLA eluted media
Appendix C: Trial #2

Appendix C-1: Trial #2 white-light images of ePTFE from direct contact procedure at 12-hour timepoint, 10X

Well 1. ePTFE scaffold
Well 3. ePTFE scaffold left in EtOH (pos. control)
Well 5. Warmed 3T3 media from the fridge (neg. control)

Well 2. ePTFE scaffold
Well 4. ePTFE scaffold left in EtOH (pos. control)
Well 6. Warmed 3T3 media from the fridge (neg. control)

Appendix C-2: Trial #2 white-light images of ePTFE from direct contact procedure at 24-hour timepoint, 10X

Well 1. ePTFE scaffold
Well 3. ePTFE scaffold left in EtOH (pos. control)
Well 5. Warmed 3T3 media from the fridge (neg. control)

Well 2. ePTFE scaffold
Well 4. ePTFE scaffold left in EtOH (pos. control)
Well 6. Warmed 3T3 media from the fridge (neg. control)
Appendix C-3: Trial #2 white-light images of ePTFE from direct contact procedure at 48-hour timepoint, 10X

Well 1. ePTFE scaffold
Well 3. ePTFE scaffold left in EtOH (pos. control)
Well 5. Warmed 3T3 media from the fridge (neg. control)

Well 2. ePTFE scaffold
Well 4. ePTFE scaffold left in EtOH (pos. control)
Well 6. Warmed 3T3 media from the fridge (neg. control)

Appendix C-4: Trial #2 white-light images of ePTFE from elution procedure at 24-hour timepoint, 10X

Well 1. Warmed 3T3 media from the fridge (neg. control)
Well 3. ePTFE scaffold #2 eluted media
Well 5. 3T3 media warmed to 50 °C for 72 hours (neg. control)
Micro-dish. Warmed 3T3 media from the fridge (later used as pos. control)

Well 2. ePTFE scaffold #1 eluted media
Well 4. ePTFE scaffold #3 eluted media
Well 6. Warmed 3T3 media from the fridge (neg. control)
Appendix C-5: Trial #2 white-light images of ePTFE from elution procedure at 48-hour timepoint, 10X

**Well 1.** Warmed 3T3 media from the fridge (neg. control)

**Well 3.** ePTFE scaffold #2 eluted media

**Well 5.** 3T3 media warmed to 50 °C for 72 hours (neg. control)

**Micro-dish.** Warmed 3T3 media from the fridge (later used as pos. control)

**Well 2.** ePTFE scaffold #1 eluted media

**Well 4.** ePTFE scaffold #3 eluted media

**Well 6.** Warmed 3T3 media from the fridge (neg. control)
Appendix D: Trial #3

Appendix D-1: Trial #3 white light images of ePTFE from elution procedure at 24-hour timepoint, 10X

Well 1. ePTFE scaffold soaked in EtOH and flushed with DPBS eluted media

Well 3. Autoclaved ePTFE scaffold eluted media

Well 5. ePTFE soaked in EtOH and thoroughly rinsed with DPBS eluted media

Well 2. ePTFE scaffold soaked in EtOH and flushed with DPBS eluted media

Well 4. Autoclaved ePTFE scaffold eluted media

Well 6. ePTFE soaked in EtOH and thoroughly rinsed with DPBS eluted media

Well 7. 3T3 media in 50 °C water for 72 hours (neg. control)

Well 9. Warmed 3T3 media from the fridge (neg. control)

Micro-dish. Warmed 3T3 media from the fridge (later used as pos. control)

Well 8. 3T3 media in 50 °C water for 72 hours (neg. control)

Well 10. Warmed 3T3 media from the fridge (neg. control)
Appendix D-2: Trial #3 white light images of ePTFE from elution procedure at 48-hour timepoint, 10X

Well 1. ePTFE scaffold soaked in EtOH and flushed with DPBS eluted media

Well 2. ePTFE scaffold soaked in EtOH and flushed with DPBS eluted media

Well 3. Autoclaved ePTFE scaffold eluted media

Well 4. Autoclaved ePTFE scaffold eluted media

Well 5. ePTFE soaked in EtOH and thoroughly rinsed with DPBS eluted media

Well 6. ePTFE soaked in EtOH and thoroughly rinsed with DPBS eluted media

Well 7. 3T3 media in 50 °C water for 72 hours (neg. control)

Well 8. 3T3 media in 50 °C water for 72 hours (neg. control)

Well 9. Warmed 3T3 media from the fridge (neg. control)

Well 10. Warmed 3T3 media from the fridge (neg. control)

Micro-dish. Warmed 3T3 media from the fridge (later used as pos. control)
Appendix E: Trial #4

Appendix E-1: Trial #4 white-light images of 24-hour timepoint elution procedure, 10X

Well 1. PLLA eluted media
Well 2. PLLA eluted media
Well 3. PLGA eluted media
Well 4. PLGA eluted media
Well 5. PLGA with salt eluted media
Well 6. PLGA with salt eluted media
Well 7. PCL spun on 210310 eluted media
Well 8. PCL spun on 210310 eluted media
Well 9. PCL spun on 210215 eluted media
Well 10. PCL spun on 210215 eluted media
Well 11. PCL scaffold eluted media
Well 12. PCL scaffold eluted media
Appendix E-2: Trial #4 white-light images of 48-hour timepoint elution procedure, 10X
Well 7. PCL spun on 210310 eluted media

Well 8. PCL spun on 210310 eluted media

Well 9. PCL spun on 210215 eluted media

Well 10. PCL spun on 210215 eluted media

Well 11. PCL scaffold eluted media

Well 12. PCL scaffold eluted media

Well 13. Warmed 3T3 media from the fridge (neg. control)

Well 14. Warmed 3T3 media from the fridge (neg. control)

Well 15. 3T3 media warmed to 50 °C for 72 hours (neg. control)

Well 16. 3T3 media warmed to 50 °C for 72 hours (neg. control)

Well 17. Powder-free latex glove eluted media (pos. control)

Well 18. Powder-free latex glove eluted media (pos. control)

Micro-dish. Warmed 3T3 media from the fridge (later used as pos. control)
Appendix F: Trial #5

Appendix F-1: Trial #5 white-light images of 24-hour timepoint elution procedure, 10X

Well 1. Warmed 3T3 media from the fridge (neg. control)

Well 2. Warmed 3T3 media from the fridge (neg. control)

Well 3. 3T3 media at 50 °C for 72 hours (neg. control)

Well 4. 3T3 media at 50 °C for 72 hours (neg. control)

Well 5. Powder-free latex glove eluted media at 50 °C for 72 hours (pos. control)

Well 6. Powder-free latex glove eluted media at 50 °C for 72 hours (pos. control)

Appendix F-2: Trial #5 white-light images of 48-hour timepoint elution procedure, 10X

Well 1. Warmed 3T3 media from the fridge (neg. control)

Well 2. Warmed 3T3 media from the fridge (neg. control)

Well 3. 3T3 media at 50 °C for 72 hours (neg. control)

Well 4. 3T3 media at 50 °C for 72 hours (neg. control)

Well 5. Powder-free latex glove eluted media at 50 °C for 72 hours (pos. control)

Well 6. Powder-free latex glove eluted media at 50 °C for 72 hours (pos. control)
Appendix G: Trial #6

Appendix G-1: Trial #6 white-light images of 24-hour timepoint elution procedure, 10X

50 °C for 24 hours:

Well 1. 3T3 media warmed to 50 °C for 24 hours
Well 2. 3T3 media warmed to 50 °C for 24 hours
Well 3. 5.4 cm² powder-free latex glove eluted media
Well 4. 5.4 cm² powder-free latex glove eluted media
Well 5. 60 cm² powder-free latex glove eluted media
Well 6. 60 cm² powder-free latex glove eluted media

50 °C for 72 hours:

Well 13. 3T3 media warmed to 50 °C for 72 hours
Well 14. 3T3 media warmed to 50 °C for 72 hours
Well 15. 5.4 cm² powder-free latex glove eluted media
Well 16. 5.4 cm² powder-free latex glove eluted media
Well 17. 60 cm² powder-free latex glove eluted media
Well 18. 60 cm² powder-free latex glove eluted media
Micro-dishes:

Well 25. Warmed 3T3 media from the fridge

Well 26. Warmed 3T3 media from the fridge

Appendix G-2: Trial #6 white-light images of 48-hour timepoint elution procedure, 10X

50 °C for 24 hours:

Well 1. 3T3 media warmed to 50 °C for 24 hours

Well 2. 3T3 media warmed to 50 °C for 24 hours

Well 3. 5 cm² powder-free latex glove eluted media

Well 4. 5 cm² powder-free latex glove eluted media

Well 5. 60 cm² powder-free latex glove eluted media

Well 6. 60 cm² powder-free latex glove eluted media
50 °C for 72 hours:

Well 7. 3T3 media warmed to 50 °C for 72 hours

Well 9. 5.4 cm$^2$ powder-free latex glove eluted media

Well 11. 60 cm$^2$ powder-free latex glove eluted media

Well 8. 3T3 media warmed to 50 °C for 72 hours

Well 10. 5.4 cm$^2$ powder-free latex glove eluted media

Well 12. 60 cm$^2$ powder-free latex glove eluted media

37 °C for 24 hours:

Well 13. 3T3 media warmed to 37 °C for 24 hours

Well 15. 5.4 cm$^2$ powder-free latex glove eluted media

Well 17. 60 cm$^2$ powder-free latex glove eluted media

Well 14. 3T3 media warmed to 37 °C for 24 hours

Well 16. 5.4 cm$^2$ powder-free latex glove eluted media

Well 18. 60 cm$^2$ powder-free latex glove eluted media
37 °C for 72 hours:

Well 19. 3T3 media warmed to 37 °C for 72 hours
Well 20. 3T3 media warmed to 37 °C for 72 hours
Well 21. 5.4 cm² powder-free latex glove eluted media
Well 22. 5.4 cm² powder-free latex glove eluted media
Well 23. 60 cm² powder-free latex glove eluted media
Well 24. 60 cm² powder-free latex glove eluted media

Micro-dishes:

Well 25. Warmed 3T3 media from the fridge
Well 26. Warmed 3T3 media from the fridge
Appendix H: Trial #8

Appendix H-1: Trial #8 white-light images of 24-hour timepoint elution procedure, 10X

**Well 1.** 3T3 media warmed to 37 °C for 24 hours

**Well 2.** 3T3 media warmed to 37 °C for 24 hours

**Well 3.** 6 cm² latex eluted media warmed to 37°C for 24 hours

**Well 4.** 6 cm² latex eluted media warmed to 37°C for 24 hours

**Well 5.** 15 cm² latex eluted media warmed to 37°C for 24 hours

**Well 6.** 15 cm² latex eluted media warmed to 37°C for 24 hours

**Well 7.** 30 cm² latex eluted media warmed to 37°C for 24 hours

**Well 8.** 30 cm² latex eluted media warmed to 37°C for 24 hours

**Well 9.** 45 cm² latex eluted media warmed to 37°C for 24 hours

**Well 10.** 45 cm² latex eluted media warmed to 37°C for 24 hours

**Well 11.** 60 cm² latex eluted media warmed to 37°C for 24 hours

**Well 12.** 60 cm² latex eluted media warmed to 37°C for 24 hours
Appendix H-2: Trial #8 white-light images of 48-hour timepoint elution procedure, 10X

**Well 1.** 3T3 media warmed to 37 °C for 24 hours

**Well 3.** 6 cm² latex eluted media warmed to 37°C for 24 hours

**Well 5.** 15 cm² latex eluted media warmed to 37°C for 24 hours

**Well 2.** 3T3 media warmed to 37 °C for 24 hours

**Well 4.** 6 cm² latex eluted media warmed to 37°C for 24 hours

**Well 6.** 15 cm² latex eluted media warmed to 37°C for 24 hours

**Well 7.** 30 cm² latex eluted media warmed to 37°C for 24 hours

**Well 9.** 45 cm² latex eluted media warmed to 37°C for 24 hours

**Well 11.** 60 cm² latex eluted media warmed to 37°C for 24 hours
Well 8. 30 cm² latex eluted media warmed to 37°C for 24 hours

Well 10. 45 cm² latex eluted media warmed to 37°C for 24 hours

Well 12. 60 cm² latex eluted media warmed to 37°C for 24 hours

Well 13. Warmed 3T3 media from the fridge

Well 14. Warmed 3T3 media from the fridge
Appendix I: Aim 3: Cytotoxicity Testing of ePTFE and PLGA

Appendix I-1: White-light images of 12-hour timepoint direct contact procedure, 10X

Well 1. Warmed 3T3 media from the fridge (neg. control)
Well 2. Warmed 3T3 media from the fridge (neg. control)
Well 3. PLGA
Well 4. ePTFE
Well 5. PLGA in EtOH (pos. control)
Well 6. ePTFE in EtOH (pos. control)

Appendix I-2: White-light images of 24-hour timepoint direct contact procedure, 10X

Well 1. Warmed 3T3 media from the fridge (neg. control)
Well 2. Warmed 3T3 media from the fridge (neg. control)
Well 3. PLGA
Well 4. ePTFE
Well 5. PLGA in EtOH (pos. control)
Well 6. ePTFE in EtOH (pos. control)
Appendix I-3: White-light images of 48-hour timepoint direct contact procedure, 10X

**Well 1.** Warmed 3T3 media from the fridge (neg. control)

**Well 2.** Warmed 3T3 media from the fridge (neg. control)

**Well 3.** PLGA

**Well 4.** PLGA in EtOH (pos. control)

**Well 4.** ePTFE

**Well 6.** ePTFE in EtOH (pos. control)

---

Appendix I-4: White-light images of 24-hour timepoint elution procedure, 10X

**Well 1.** Warmed 3T3 media from the fridge (neg. control)

**Well 2.** Warmed 3T3 media from the fridge (neg. control)

**Well 3.** 3T3 media warmed to 37 °C for 24 hours

**Well 4.** 3T3 media warmed to 37 °C for 24 hours

**Well 5.** 45 cm² latex eluted media warmed to 37 °C for 24 hours

**Well 6.** 45 cm² latex eluted media warmed to 37 °C for 24 hours
Appendix I-5: White-light images of 48-hour timepoint elution procedure, 10X

Well 1. Warmed 3T3 media from the fridge (neg. control)
Well 2. Warmed 3T3 media from the fridge (neg. control)
Well 3. 3T3 media warmed to 37 °C for 24 hours
Well 4. 3T3 media warmed to 37 °C for 24 hours
Well 5. 45 cm² latex eluted media warmed to 37 °C for 24 hours
Well 6. 45 cm² latex eluted media warmed to 37 °C for 24 hours

Well 7. PLGA scaffold eluted media
Well 8. PLGA scaffold eluted media
Well 9. PLGA flat sheet eluted media
Well 10. PLGA flat sheet eluted media
Well 11. ePTFE scaffold eluted media
Well 12. ePTFE scaffold eluted media
Well 7. PLGA scaffold eluted media

Well 8. PLGA scaffold eluted media

Well 9. PLGA flat sheet eluted media

Well 10. PLGA flat sheet eluted media

Well 11. ePTFE scaffold eluted media

Well 12. ePTFE scaffold eluted media
Appendix J: Aim 3: Cytotoxicity Testing of PLLA, PLGA with salt, and PCL

Appendix J-1: White-light images of 12-hour timepoint direct contact procedure, 10X

Well 1. PLLA
Well 3. PLLA in EtOH (pos. control)
Well 5. PLGA with salt

Well 2. PLLA
Well 4. PLGA with salt in EtOH (pos. control)
Well 6. PLGA with salt

Well 7. PCL flat sheet 210310
Well 9. PCL in EtOH (pos. control)
Well 11. PCL flat sheet 210215

Well 8. PCL flat sheet 210310
Well 10. PCL flat sheet 210215
Well 12. PCL scaffold
Well 13. PCL scaffold
Well 14. Warmed 3T3 media from the fridge (neg. control)
Well 15. Warmed 3T3 media from the fridge (neg. control)

Appendix J-2: White-light images of 24-hour timepoint direct contact procedure, 10X

Well 1. PLLA
Well 3. PLLA in EtOH (pos. control)
Well 5. PLGA with salt

Well 2. PLLA
Well 4. PLGA with salt in EtOH (pos. control)
Well 6. PLGA with salt

Well 7. PCL flat sheet 210310
Well 9. PCL in EtOH (pos. control)
Well 11. PCL flat sheet 210215
Appendix J-3: White-light images of 48-hour timepoint direct contact procedure, 10X
Well 7. PCL flat sheet 210310

Well 8. PCL flat sheet 210310

Well 9. PCL in EtOH (pos. control)

Well 10. PCL flat sheet 210215

Well 11. PCL flat sheet 210215

Well 12. PCL scaffold

Well 13. PCL scaffold

Well 14. Warmed 3T3 media from the fridge (neg. control)

Well 15. Warmed 3T3 media from the fridge (neg. control)
Appendix J-4: White-light images of 24-hour timepoint elution procedure, 10X

**Well 1.** Warmed 3T3 media from the fridge (neg. control)

**Well 3.** 3T3 media warmed to 37 °C for 24 hours (neg. control)

**Well 5.** Powder-free latex glove eluted media (pos. control)

**Well 2.** Warmed 3T3 media from the fridge (neg. control)

**Well 4.** 3T3 media warmed to 37 °C for 24 hours (neg. control)

**Well 6.** Powder-free latex glove eluted media (pos. control)

**Well 7.** PLGA w/ salt scaffold eluted media

**Well 9.** PLGA w/ salt flat sheet eluted media

**Well 11.** PLLA scaffold eluted media

**Well 8.** PLGA w/ salt scaffold eluted media

**Well 10.** PLGA w/ salt flat sheet eluted media

**Well 12.** PLLA scaffold eluted media
Well 13. PLLA flat sheet eluted media  
Well 15. PCL scaffold eluted media  
Well 17. PCL flat sheet eluted media

Well 14. PLLA flat sheet eluted media  
Well 16. PCL scaffold eluted media  
Well 18. PCL flat sheet eluted media

Micro-dish. Warmed 3T3 media from the fridge (later used as a pos. control)

Appendix J-5: White-light images of 48-hour timepoint elution procedure, 10X

Well 1. Warmed 3T3 media from the fridge (neg. control)  
Well 3. 3T3 media warmed to 37 °C for 24 hours (neg. control)  
Well 5. Powder-free latex glove eluted media (pos. control)
Well 2. Warmed 3T3 media from the fridge (neg. control)

Well 4. 3T3 media warmed to 37 °C for 24 hours (neg. control)

Well 6. Powder-free latex glove eluted media (pos. control)

Well 7. PLGA w/ salt scaffold eluted media

Well 9. PLGA w/ salt flat sheet eluted media

Well 11. PLLA scaffold eluted media

Well 8. PLGA w/ salt scaffold eluted media

Well 10. PLGA w/ salt flat sheet eluted media

Well 12. PLLA scaffold eluted media

Well 13. PLLA flat sheet eluted media

Well 15. PCL scaffold eluted media

Well 17. PCL flat sheet eluted media
Well 14. PLLA flat sheet eluted media

Well 16. PCL scaffold eluted media

Well 18. PCL flat sheet eluted media

Micro-dish. Warmed 3T3 media from the fridge (later used as a pos. control)
# Appendix K: Cytotoxicity Testing: Cell Seeding SOP

## Approx. Time:

1 hour

## Procedure:

### A. Preparation

1. Always wash hands thoroughly before beginning.
2. Assure lab safety by wearing gloves, shoes, and pants.
3. Place 3T3 media and 3T3 trypsin into a water bath to warm up.
4. “Open” the hood by raising the door to the marked line, turning on the light and pump, and thoroughly spraying with 70% EtOH.
5. Wipe microscope stage with 70% EtOH.
6. Check confluency of 3T3 cells.
   6.1. Should be 80-100% confluent before seeding.
7. Acquire the necessary number of packaged sterile 6-well plate(s) and bring into hood.
   7.1 If performing elution, bring sterile, glass bottom micro-dish into hood as well.* Ethanol treatment should not be used in the 6-well plate to prevent cross-contamination of EtOH into negative control and polymer samples*
   7.2. If performing elution, use double the number of 6-well plate(s) for 24- and 48-hour timepoints.

### B. Seeding

8. Using previously warmed 3T3 media, place 2 mL of media into each well of the 6-well plate(s) and micro-dish.
   1.1. Let these incubate while performing next steps.
9. *Follow steps B.1 - B.6 from SOP 3021 for passing 3T3 cells*
10. Pipette out all cell solution and place in a 50 mL conical.
    3.1. Spin down cells into a pellet using centrifuge at setting 4 for 4 minutes.
    3.2. Bring spun down cells into hood and aspirate out media, leaving cell pellet at the bottom.
    3.3. Resuspend in 24 mL of 3T3 media.
    3.4. Agitate the solution to ensure that all cells are evenly distributed.
11. Label the dishes with cell type (3T3), initials, date, and other relevant information.
12. Pipette 0.3 mL of the cell solution to each well of 6-well plates and micro-dish.
13. Carefully place the lids onto the 6-well plate(s) and micro-dish with aseptic technique and transfer to the incubator.
14. Allow cells to incubate overnight before performing direct contact and/or elution procedure.

## Abbreviations:

1. EtOH – ethanol

## Reminders:

1. Be sure to manually aspirate whenever the protocol says “Aspirate”
2. Always spray items with 70% EtOH before placing them in hood
3. Spray hands with 70% EtOH often, particularly after taking them out of the hood
4. Be organized in the hood

## Materials:

- Micro-dish
- 6-well plate(s)
### Appendix L: Cytotoxicity Testing: Direct Contact SOP

<table>
<thead>
<tr>
<th>Approx. Time:</th>
<th>Procedure:</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 hour</td>
<td>A. Preparation</td>
</tr>
<tr>
<td></td>
<td>1. Remove the 6-well plate(s) from the incubator.</td>
</tr>
<tr>
<td></td>
<td>2. Check and record cell confluency of each well under white-light microscope.</td>
</tr>
<tr>
<td></td>
<td>2.1 Well plate should be 60-70% confluent before procedure.</td>
</tr>
<tr>
<td></td>
<td>3. Take a <em>Phase Contrast</em> image of cell confluency under the white-light microscope.</td>
</tr>
<tr>
<td></td>
<td>4. Place the well plate(s) back into the incubator until needed for direct contact.</td>
</tr>
</tbody>
</table>

#### Abbreviations:
1. EtOH – ethanol
2. DPBS – Dulbecco’s phosphate buffered saline

#### Reminders:
1. Be sure to manually aspirate whenever the protocol says “Aspirate”
2. Always spray items with 70% EtOH before placing them in hood
3. Spray hands with 70% EtOH often, particularly after taking them out of the hood
4. Be organized in the hood

#### Materials:
- Scaffold samples
- 6 well plate(s)
- Sterile forceps
- Nonsterile forceps
- Sterile 70% EtOH
- DPBS

<table>
<thead>
<tr>
<th>Materials:</th>
<th>Procedure:</th>
</tr>
</thead>
<tbody>
<tr>
<td>Scaffold samples</td>
<td>B. Direct Contact</td>
</tr>
<tr>
<td>6 well plate(s)</td>
<td>1. Bring sterile and nonsterile forceps into the hood.</td>
</tr>
<tr>
<td>Sterile forceps</td>
<td>2. Bring (~ 6 cm² surface area) samples into hood on a labeled paper towel.</td>
</tr>
<tr>
<td>Nonsterile forceps</td>
<td>3. Place 12mL of 70% sterile EtOH into the proper amount of conicals.</td>
</tr>
<tr>
<td>Sterile 70% EtOH</td>
<td>3.1 One conical per sample.</td>
</tr>
<tr>
<td>DPBS</td>
<td>3.2 Invert conicals.</td>
</tr>
<tr>
<td></td>
<td>4. Use the nonsterile forceps to grab each sample and place into the corresponding conical of 70% sterile EtOH.</td>
</tr>
<tr>
<td></td>
<td>4.1 Label the conicals with the sample type.</td>
</tr>
<tr>
<td></td>
<td>4.2 Let these samples sit in EtOH for 20 minutes.</td>
</tr>
<tr>
<td></td>
<td>4.3 Invert all conicals at beginning and end of 20 minutes.</td>
</tr>
<tr>
<td></td>
<td>5. While waiting, replace the 2 mL of 3T3 media in 6-well plate(s).</td>
</tr>
<tr>
<td></td>
<td>6. After 20 minutes of sterilization, aspirate out the EtOH and replace with sterile DPBS.</td>
</tr>
<tr>
<td></td>
<td>6.1 Leave one of each sample subjected to EtOH (don’t rinse with DPBS) for use as positive control.</td>
</tr>
<tr>
<td></td>
<td>6.2 Thoroughly rinse the samples in DPBS for 1 minute by vigorously shaking the conical.</td>
</tr>
<tr>
<td></td>
<td>7. Bring the well plate(s) into the hood and uncover.</td>
</tr>
<tr>
<td></td>
<td>8. Use long sterile forceps to grab each sample out of DPBS.</td>
</tr>
<tr>
<td></td>
<td>9. Place the previously sterilized and rinsed samples into corresponding wells.</td>
</tr>
<tr>
<td></td>
<td>10. Cover the well plate(s) and label each well with sample type.</td>
</tr>
<tr>
<td></td>
<td>11. Place the well plate(s) back into the incubator.</td>
</tr>
</tbody>
</table>

#### C. White-Light Imaging

<table>
<thead>
<tr>
<th>Materials:</th>
<th>Procedure:</th>
</tr>
</thead>
<tbody>
<tr>
<td>Scaffold samples</td>
<td>1. At 12-, 24-, and 48-hours after placing the scaffold samples into the well plate(s), take <em>Phase Contrast</em> images of each well under the white-light microscope.</td>
</tr>
<tr>
<td>6 well plate(s)</td>
<td>1.1 Take three images per well (take note of where the image is in relation to the sample).</td>
</tr>
<tr>
<td>Sterile forceps</td>
<td>1.2 Save images and place into corresponding folder.</td>
</tr>
<tr>
<td>Nonsterile forceps</td>
<td>1.3 After 48 hours, remove the samples from the well plate and image underneath the sample.</td>
</tr>
<tr>
<td>Sterile 70% EtOH</td>
<td>2. Dispose of the well plate(s).</td>
</tr>
<tr>
<td>DPBS</td>
<td>3.1 Place samples into biohazard if still solid and aspirate out the cells and media before discarding into biohazard.</td>
</tr>
<tr>
<td><strong>Appendix M: Cytotoxicity Testing: Elution SOP</strong></td>
<td></td>
</tr>
<tr>
<td>---</td>
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</tr>
<tr>
<td><strong>Approx. Time:</strong></td>
<td><strong>Procedure:</strong></td>
</tr>
<tr>
<td>1 hour</td>
<td><strong>A. Preparation</strong></td>
</tr>
<tr>
<td></td>
<td>1. Bring nonsterile forceps, along with (~45 cm² surface area) samples into hood on a labeled paper towel.</td>
</tr>
<tr>
<td></td>
<td>2. Bring appropriate amount of conicals into hood and label with polymer type.</td>
</tr>
<tr>
<td></td>
<td>2.1 One conical per sample.</td>
</tr>
<tr>
<td></td>
<td>3. Place 12 mL of 70% sterile EtOH into each conical.</td>
</tr>
<tr>
<td></td>
<td>3.1 Invert conical.</td>
</tr>
<tr>
<td></td>
<td>4. Use the nonsterile forceps to grab each sample and place into the corresponding conical of 70% sterile EtOH.</td>
</tr>
<tr>
<td></td>
<td>4.1 If sample doesn’t easily fit into conical, cut into strips.</td>
</tr>
<tr>
<td></td>
<td>4.2 Let these samples sit in the EtOH for 20 minutes.</td>
</tr>
<tr>
<td></td>
<td>4.3 Invert all conicals at beginning and end of 20 minutes.</td>
</tr>
<tr>
<td></td>
<td>5. While waiting, prepare separate 15 mL conicals with 10 mL of 3T3 media and label with corresponding treatment and sample type.</td>
</tr>
<tr>
<td></td>
<td>5.1 One conical per sample, plus one conical for the incubated media control, plus one for latex glove control.</td>
</tr>
<tr>
<td></td>
<td>6. After 20 minutes of sterilization, aspirate out the EtOH and replace with DPBS.</td>
</tr>
<tr>
<td></td>
<td>6.1 Thoroughly rinse the samples in 10 mL DPBS for 1 minute by thoroughly shaking the conical.</td>
</tr>
<tr>
<td></td>
<td>7. Aspirate out DPBS and with long sterile forceps transfer the scaffolds from their conicals to corresponding conicals prepared with media.</td>
</tr>
<tr>
<td></td>
<td>8. Include 10mL of just media (no sample) as negative control.</td>
</tr>
<tr>
<td></td>
<td>9. Include a 45cm² sample of sterile, powder-free latex glove in 10 mL of media as positive control.</td>
</tr>
<tr>
<td></td>
<td>9.1 Use sterile scissors and sterile gloves to cut piece and place into media.</td>
</tr>
<tr>
<td></td>
<td>10. Place all labeled conicals in incubator at 37 °C for 24 hours.</td>
</tr>
<tr>
<td></td>
<td>11. 24 hours later... remove the scaffolds with sterile forceps.</td>
</tr>
<tr>
<td></td>
<td>11.1 Take pictures of scaffolds before discarding.</td>
</tr>
<tr>
<td></td>
<td>12. Place the eluted media into the fridge.</td>
</tr>
<tr>
<td><strong>Abbreviations:</strong></td>
<td><strong>B. Preparation for Placement onto Cells</strong></td>
</tr>
<tr>
<td>1. EtOH – ethanol</td>
<td>1. Remove the previously seeded well plate(s) and micro-dish from the incubator.</td>
</tr>
<tr>
<td>2. DPBS – Dulbecco’s phosphate buffered saline</td>
<td>2. Check and record cell confluency of each well under white-light microscope.</td>
</tr>
<tr>
<td></td>
<td>2.1. Wells should be 60-70% confluent before procedure.</td>
</tr>
<tr>
<td><strong>Reminders:</strong></td>
<td>3. Take a Phase Contrast image of cell confluency under the white-light microscope.</td>
</tr>
<tr>
<td>1. Be sure to manually aspirate whenever the protocol says “Aspirate”</td>
<td><strong>C. Cell Culture</strong></td>
</tr>
<tr>
<td>2. Always spray items with 70% EtOH before placing them in hood</td>
<td>1. Aspirate out old media from the 6-well plate(s) and micro-dish.</td>
</tr>
<tr>
<td>3. Spray hands with 70% EtOH often, particularly after taking them out of the hood</td>
<td>2. Place 1.5mL of eluted media into corresponding wells of 6-well plate(s) and label accordingly for 24- and 48-hour timepoints.</td>
</tr>
<tr>
<td>4. Be organized in the hood</td>
<td>3. Replace 3T3 media in micro-dish.</td>
</tr>
<tr>
<td><strong>Materials:</strong></td>
<td>3. Place well plate(s) and micro-dish back into incubator until staining and imaging.</td>
</tr>
<tr>
<td>6-well plate(s)</td>
<td></td>
</tr>
</tbody>
</table>
Appendix N: Staining SOP – Calcein AM & Ethidium Homodimer-1

<table>
<thead>
<tr>
<th>Approx. Time:</th>
<th>Procedure:</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 hour</td>
<td>A. Preparation</td>
</tr>
</tbody>
</table>

1. Remove the well plate(s) and micro-dish from the incubator.
2. Check and record cell confluency of each well under white-light microscope.
3. **Take 3 Phase Contrast images** of each well under the white-light microscope and take note of qualitative observations.
4. Place the well plate(s) and micro-dish back into the incubator until the stain solution is prepared.

<table>
<thead>
<tr>
<th>Abbreviations:</th>
<th>B. Calcein AM &amp; Ethidium Homodimer-1 Stain</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. EtOH – ethanol</td>
<td>5. Remove Calcein AM &amp; Ethidium Homodimer-1 from the freezer.</td>
</tr>
<tr>
<td>2. DPBS – Dulbecco’s phosphate buffered saline</td>
<td>5.1 Wrap in tinfoil to protect from light. Let thaw completely.</td>
</tr>
<tr>
<td></td>
<td>7. Aspirate media from micro-dish.</td>
</tr>
<tr>
<td></td>
<td>8. Pipette 2mL of Methanol into micro-dish and allow to sit for 20 minutes.</td>
</tr>
<tr>
<td></td>
<td>9. While this is sitting, make the Live/Dead assay stock solution in a 50mL conical.</td>
</tr>
<tr>
<td></td>
<td>11.1 Spray down sterile pipette tips and micropipette and bring into hood.</td>
</tr>
<tr>
<td></td>
<td>11.2 <strong>Stock Solution:</strong> 10 mL DPBS + 12 uL EthD-1 + 3 uL Calcein AM. <em>May need to adjust this depending on how many wells there are.</em></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Reminders:</th>
<th>10. Cover the stock solution in tinfoil to protect from light and bring 6-well plate(s) and micro-dish from incubator to center of hood.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Tilt the 6-well plate at an angle, using the pipette tip to “aspirate” into waste conical.</td>
<td>11. After 20 minutes, aspirate Methanol from micro-dish and rinse with DPBS.</td>
</tr>
<tr>
<td>2. Place any solution at edge of well, careful not to blast the cells.</td>
<td>12. Carefully aspirate media out of all other wells.</td>
</tr>
<tr>
<td>3. Always spray items with 70% EtOH before placing them in hood</td>
<td>13. Rinse each well with DPBS.</td>
</tr>
<tr>
<td>4. Spray hands with 70% EtOH often, particularly after taking them out of the hood</td>
<td>14. Carefully aspirate out DPBS from each well. Cover the 6-well plate(s) and micro-dish.</td>
</tr>
<tr>
<td>15. Uncap Live/Dead stock stain solution with aseptic technique.</td>
<td>16. Pipette 1.5mL of stock stain solution into each well and micro-dish.</td>
</tr>
<tr>
<td>16.1 Cover in tinfoil to protect from light.</td>
<td>16.1 Cover in tinfoil to protect from light.</td>
</tr>
<tr>
<td>17. Allow stain to sit on cells for 5-10 minutes.</td>
<td>17.</td>
</tr>
<tr>
<td>18.1 Immediately bring well plates to 330 for imaging.</td>
<td>18. After 5-10 minutes, aspirate out stain.</td>
</tr>
<tr>
<td>19. Image cells right away.</td>
<td>19.</td>
</tr>
<tr>
<td>21.1 Take 3 images per well.</td>
<td>21.2 On the microscope, unscrew all magnifications other than 4 and 10X.</td>
</tr>
<tr>
<td>21.3 Turret 2 for Calcein AM and turret 6 for EthD-1.</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Materials:</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Live/Dead stain</td>
<td></td>
</tr>
<tr>
<td>6-well plate(s)</td>
<td></td>
</tr>
<tr>
<td>Micro-dish</td>
<td></td>
</tr>
<tr>
<td>DPBS</td>
<td></td>
</tr>
<tr>
<td>Methanol</td>
<td></td>
</tr>
<tr>
<td>70% EtOH</td>
<td></td>
</tr>
</tbody>
</table>
# Appendix O: Imaging with Widefield Fluorescence Microscope SOP

## Approx. Time:

1 Hour

## Reminders:

1. Live/Dead is light sensitive. Always keep wrapped in foil and limit exposure to light.
2. Obtain permission from Dr. Cardinal and/or Dr. Lily Laiho before using the fluorescent microscope.
3. This is a VERY expensive piece of equipment—always use care during operation.
4. Lamp should be turned on 10-15 mins prior to taking images.
5. If sample is too wet, carefully blot edge of sample with a Kim wipe.
6. Promptly report all microscope issues to Dr. Laiho.

## Materials:

Samples

## Procedure:

### A. Microscope Operation

1. Log into notebook—fluorescence, initials, date, time, and lamp hours.
2. Turn microscope on using fluorescent settings.
   a. Turn turret to setting 2.
   b. Power on Olympus lamp (black switch on top of microscope).
   c. Turn on Olympus lamp (push “on/off” button and turn dial up).
   d. Turn on Optiscan wheels (green switch).
   e. Set filter wheel 1 to 10, and set filter wheel 2 to 10.
   f. Open shutter (by sliding to icon of open circle).
   g. Set Prior keypad to shutter S1.
   h. Dial objectives to desired magnification (typically 10x).
   i. Set thin bar to icon of camera (pull all the way out).
3. Sign into computer by clicking Kristen’s account (password can be obtained from Kristen).
4. Place samples on microscope stage.
5. Manually adjust microscope to clarify image.

### B. Capturing Images

1. Click QCapture Pro (on desktop).
2. Click camera icon at upper left corner of QCapture Pro window and preview.
3. “Snap “pictures”. Save pictures if desired (labeled with sample info, initials, and magnification).
4. Save files on a flash drive.
   4.1. Quit QCapture Pro. Log out of computer.
   4.2. Log out of notebook. Shut down microscope by switching off Olympus lamp, switching off Optiscan wheels, switching off camera (on top of microscope), closing shutter and setting thin bar to icon of eye.
**Appendix P: Cell Counting with ImageJ SOP**

<table>
<thead>
<tr>
<th>Approx. Time:</th>
<th>Procedure:</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 hour</td>
<td>A. Counting Cell Nuclei</td>
</tr>
<tr>
<td></td>
<td>1. Open the image in ImageJ.</td>
</tr>
<tr>
<td></td>
<td>2. Plugins ➔ Macros ➔ Record...</td>
</tr>
<tr>
<td></td>
<td>3. Image ➔ Type ➔ 16-bit</td>
</tr>
<tr>
<td></td>
<td>4. Edit ➔ Options ➔ Conversions to “scale when converting”</td>
</tr>
<tr>
<td></td>
<td>5. Image ➔ Adjust ➔ Threshold</td>
</tr>
<tr>
<td></td>
<td>5.1 Mess around with the threshold until looks clearer.</td>
</tr>
<tr>
<td></td>
<td>6. Adjust your threshold so that the nuclei will be counted individually.</td>
</tr>
<tr>
<td></td>
<td>6.1 Optimise this or else all counts will be skewed.</td>
</tr>
<tr>
<td></td>
<td>7. Process ➔ Binary ➔ Watershed</td>
</tr>
<tr>
<td></td>
<td>8. Analyze ➔ Analyze Particles (50-Infinity) in pixel² units</td>
</tr>
<tr>
<td></td>
<td>9. Change “show” to Bare Outlines</td>
</tr>
<tr>
<td></td>
<td>10. Make sure “summarise” and “display results” are checked.</td>
</tr>
<tr>
<td></td>
<td>11. In the summary pop up, the count will be the number of nuclei.</td>
</tr>
<tr>
<td></td>
<td>12. Cross reference the nuclei in the original image against the counted image to make sure non-nuclei are not being counted.</td>
</tr>
<tr>
<td></td>
<td>13. Hit “Create” on the Macro recorder.</td>
</tr>
<tr>
<td></td>
<td>The macro should read...</td>
</tr>
<tr>
<td></td>
<td>run(&quot;16-bit&quot;);</td>
</tr>
<tr>
<td></td>
<td>run(&quot;Conversions...&quot;, &quot;scale&quot;);</td>
</tr>
<tr>
<td></td>
<td>setAutoThreshold(&quot;Default dark&quot;);</td>
</tr>
<tr>
<td></td>
<td>//run(&quot;Threshold...&quot;);</td>
</tr>
<tr>
<td></td>
<td>setOption(&quot;BlackBackground&quot;, true);</td>
</tr>
<tr>
<td></td>
<td>run(&quot;Convert to Mask&quot;);</td>
</tr>
<tr>
<td></td>
<td>run(&quot;Watershed&quot;);</td>
</tr>
<tr>
<td></td>
<td>run(&quot;Analyze Particles...&quot;, &quot;size=50-Infinity show=[Bare Outlines] display summarize&quot;);</td>
</tr>
<tr>
<td></td>
<td>14. Use this Macro for all following images.</td>
</tr>
<tr>
<td></td>
<td>15. Open the image in ImageJ.</td>
</tr>
<tr>
<td></td>
<td>16. Control + R will perform the recorded Macro on each following image.</td>
</tr>
</tbody>
</table>

**Abbreviations:**

**Reminders:**

1. Optimise the threshold or else counts will be skewed.

**Materials:**

ImageJ software