RICE UNIVERSITY

Computational and experimental models of vascular transport in engineered tissues

by

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Abstract

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As tissue engineering advances from developing simple two-dimensional (2D) constructs towards the development of thick three-dimensional (3D) tissues on the scale of human organs, the transport of oxygen and nutrients to cells via functional vasculature becomes a paramount engineering challenge. Our field lacks methodologies to fabricate the requisite architecture, while quantitative workflows to predict and evaluate the effectiveness of a given design are also lacking. We and others are adapting 3D printing technologies to generate complex and bioinspired vascular geometries that can support the transport needs of large 3D tissues. We applied computational tools and linked them to experimental analyses of convective and diffusive transport provided by three-dimensional vascular networks. Human vasculature is multiscale with fractal complexity; to begin to approach this complexity we designed and studied mimics of specific aspects of vascular anatomy such as branching blood vessel networks and intravascular bicuspid valves. Our perfusable vessels supported arterial pressures, so we further investigated the feasibility of surgically connecting our constructs directly to host vasculature in small and large animal studies. The objective of this work is to close the loop between computational and experimental models involving blood flow and mass transport in vascular networks, allowing scientists to more effectively design and fabricate vascularized tissues. This work provides a quantitative roadmap for the design of vascular networks and the evaluation of their function within 3D tissue constructs.
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<td>Two Dimensional</td>
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<td>Ambulatory Venous Pressure</td>
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<td>Matrix Metalloproteinase</td>
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<td>Human Mesenchymal Stem Cell</td>
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<td>milliQ</td>
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<td>Molecular Weight</td>
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<td>Multicellular Aggregate</td>
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<td>Multi Photon Polymerization</td>
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<td>Paraformaldehyde</td>
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<td>Particle Image Velocimetry</td>
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<td>Phosphate Buffered Saline</td>
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Overview of Thesis

The field of tissue engineering has generated significant progress toward the development of vascular structures in vitro, partially due to advances in fabrication techniques, such as 3D printing. Such advances in fabrication technologies have opened the possibility for fabricating nearly arbitrary vascular structures in vitro, while increased accessibility and performance of computational fluid dynamics (CFD) modeling software allows for countless possibilities to investigate different vascular topologies in silico. These studies are often done in isolation but would be boosted as a combined effort. Here, we seek to 1) establish parity between computational and experimental models of convective and diffusive transport and 2) use computational models to more efficiently screen and improve which vascular topologies should be fabricated in vitro.

Chapter 1 provides an overview of the importance of vascular structures in the body, their potential in the field of tissue engineering, and current methods for fabricating vascular networks in vitro with a focus on 3D printing. We further explore the application of computational modeling in the design and evaluation of vascular networks.

In Chapter 2 we begin by experimentally characterizing the printability and physical properties of different biocompatible hydrogel formulations based on poly(ethylene glycol) (PEG). Next, we establish parity between our computational models for fluid flow and diffusion for 3D printed vessel networks. Further, we expand the application of computational fluid dynamics by screening different vascular architectures for their efficiency of nutrient delivery, and we conclude this chapter by implementing Doppler ultrasound to map 3D fluid flow profiles through 3D channel networks. These studies provide a computational and experimental framework to design and evaluate efficient vascular topologies for engineered tissue constructs approaching the scale of whole human organs.

In Chapter 3 we apply our computational and experimental paradigm toward bicuspid valves, which are dynamic intravascular structures found in human veins and lymphatic vessels. We demonstrated a structural family of bicuspid valve designs that can be fabricated with our stereolithography system. We developed time-resolved CFD models with dynamic fluid structure interactions (FSI) to computationally probe the structure-function relationship of intravascular architecture on valve performance. Experimentally, a combination of high time-resolution particle image velocimetry (PIV) and valve pressure response in an in vitro flow loop support our computational studies and demonstrate valve architectures which may
have utility in engineered tissues and could have therapeutic potential for diseases such as chronic venous insufficiency (CVI).

We designed our biocompatible 3D printed vascular networks to support mammalian arterial blood pressures, so in Chapter 4 we describe initial feasibility studies of a transplant model based on surgical anastomosis of printed vascular networks with native arteries and veins in small and large animal studies. Direct surgical integration of tissue constructs with host vasculature is a nascent area, but remains an essential target for the eventual therapeutic delivery of metabolically active tissues and engineered organs. Our preliminary data suggest good material performance and an ability to withstand arterial pressures in vivo, but that more work on hemocompatibility is warranted.

We conclude in Chapter 5 with a summary of this thesis and an extrapolation of future studies, which are more broadly applicable in the design of vascularized tissues for tissue engineering applications. We further elaborate the potential of testing advanced imaging techniques and 3D bioprinting technologies.
Chapter 1

Introduction

Portions of this chapter have been previously published in Paulsen & Miller, 2015.

1.1 Vascularization is a key challenge in tissue engineering

In the developing embryo, the cardiovascular system is the first functioning organ system\(^2\). During the first few stages of development, an embryo is entirely supported by the diffusion of oxygen and nutrients from surrounding fluids. However, for a mouse embryo, after day 8 the embryo has surpassed the diffusion limit of oxygen and needs a secondary method to transport oxygen and nutrients to rapidly dividing cells\(^3\). Following a similar principle, in most adult human tissues, metabolically active cells are typically found within 100-200 µm from a capillary that can deliver oxygen and nutrients to, while removing waste products from, the micro-environmental cellular niche\(^4\). Engineered tissues must therefore provide similar levels of oxygen and nutrient exchange in order to maintain cell viability. As engineered tissues progress from relatively simple, flat structures to more complex solid organs (Figure 1.1), it becomes essential that engineered tissues incorporate sufficient vascularization and fluid flow to prevent hypoxia and, ultimately, necrosis at the core of the tissue.

Figure 1.1 Tissue engineering aims to produce increasingly complex tissues. As tissue engineering progresses towards developing complex solid organs, such as the kidney, the incorporation of vascular networks into the tissue becomes a necessity. Without functioning vasculature, solid organs with physiologic cell densities will quickly develop a necrotic core. From Atala, Kasper & Mikos 2012\(^5\).
However, native vascular architectures often have very complex structures that range in size from over 1 cm for the largest arteries in the human body down to less than 10 µm in diameter for the smallest capillaries: a 3000× range in scale. In addition to static vessels, veins and lymphatic vessels often contain valves, which are dynamic intravascular structures (Figure 1.2). These valves are essential for maintaining the unidirectional flow of blood in low pressure vessels where blood must move against gravity to flow back towards the heart.

![Figure 1.2: Diagram of vascular structures.](image)

Vasculature is made up of hollow vessels forming complex channels ranging in size from ~ 8 µm to >1 cm in diameter. Vascular networks include both static and dynamic elements, where common dynamic elements include the valves of low pressure vessels, such as veins and lymphatic vessels.

Vascular architectures are also highly tissue specific to meet the specific metabolic needs or unique functional requirements of a given tissue. For instance, Figure 1.3 shows capillaries from three different mouse tissues: the cerebral cortex, the heart, and a glomerulus from the kidneys. In the cerebral cortex, neurons undergo rapid changes in metabolic rate and require matching oxygen delivery. To meet this need, the vessels are highly tortuous with many branches to make oxygen readily available (Figure 1.3, left). Capillaries from the heart are aligned with cardiomyocytes to promote rapid diffusion of ions and nutrients while allowing for the bulk contraction of the tissue (Figure 1.3, middle), and capillaries of the glomerulus are tightly wound to aid in filtration (Figure 1.3, right). The differences between the capillary structures in Figure 1.3 illustrates the importance of vascular morphology both for optimizing between flow resistance and diffusion, while enhancing tissue specific functions. Furthermore, this discussion of vascular complexity focuses only on the geometric complexity.
of vessels rather than the biological complexity, where vessels consist of many different types of extracellular matrix (ECM) and cell types beyond just endothelial cells. However, for initial investigation in incorporating blood vessels into engineered tissues, we will take a reductionist approach, primarily focusing on the transport properties of vessels and the role of endothelial cells in the development of capillary networks.

![Image](image_url)

**Figure 1.3: Demonstrating tissue-specific structure of capillaries from murine tissues.** (a) Capillary bed from a mouse cerebral cortex contains tortuous, highly branched vessels to enhance oxygen delivery. (b) Capillaries in the heart tissue are aligned with underlying cardiomyocytes to allow for the rapid diffusion of ions and nutrients while allowing for the bulk contraction of the tissue. (c) Capillaries in a glomerulus within the kidney are tightly wound to aid in filtration. From Udan et al. 2013.

Given the complexities present in native vascular networks, we currently do not have the available technology to directly recreate native vascular structures in vitro, and we estimate it is unlikely 3D printing will achieve capillary-scale resolution within organ-sized volumes in the next 2-5 years. Therefore, this poses a fundamental question about how researchers should develop vascular structures that are sufficient for their intended engineering purpose, whether that be maintaining interstitial cell viability within an engineered tissue, developing a gradient of growth factors to influence cell behavior, or encouraging the formation of capillary networks by endothelial cells. To address this challenge, researchers must have the capacity to fabricate a wide variety of vascular structures, while computational modeling and high-throughput screening techniques will accelerate this progress. In this thesis, we seek to define, model, fabricate, and characterize important features of native vasculature whose architecture could support the proliferation and homeostasis of 3D volumetric tissues.
1.2 Current methods for developing perfusable tissue constructs

Though thin sheets\textsuperscript{10,11} or small clusters of cells\textsuperscript{12,13}, such as early-stage embryos\textsuperscript{3}, can survive on diffusive transport of oxygen and nutrients alone, researchers have investigated a multitude of approaches to improve mass transport into metabolically active engineered tissues whose size spans beyond the diffusion limit of oxygen. Many groups have overcome this transport dilemma by culturing tissues in bioreactors to continuously perfuse porous scaffolds with media\textsuperscript{14–16}. However, as cells deposit extracellular matrix (ECM) material during macro-porous flow, the cells tend to fill pores and restrict flow\textsuperscript{17}, and perfused scaffolds generally lack the necessary anastomosis sites to survive outside of the bioreactor, potentially limiting options for \textit{in vivo} implantation and anastomosis with host vessels. Furthermore, contact between blood and most non-endothelialized surfaces results in rapid clot formation\textsuperscript{18,19}, which can clog existing pores and prevent future blood flow through the structure\textsuperscript{20}. To maintain open channels for fluid flow prevention of clotting, researchers have focused on different methods to incorporate vascular networks into engineered tissues. Common approaches, as described in more detail below, include the guided development of capillary networks, microfluidic patterning techniques, the decellularization and recellularization of natural tissues, and additive manufacturing techniques or 3D printing.

\textbf{Directed growth of endothelial cells:} It has been well demonstrated that endothelial cells can form capillaries, a process known as vasculogenesis\textsuperscript{21}, particularly in the presence of mural cells and the proper combination of growth factors or extracellular matrix components\textsuperscript{14,22–24,21}. By incorporating angiogenic factors, such as vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), platelet derived growth factor (PDGF), or heparin, into engineered scaffolds implanted \textit{in vivo}, many groups have demonstrated that they can direct sprouting of existing vascular networks from the host into the implanted material\textsuperscript{25–29}. Another approach incorporates endothelial cells or endothelial progenitor cells (with or without mural cells) into engineered tissues and allowing these cells to self-organize into tubules and capillary beds\textsuperscript{14,21,30–33}. Baranski, et al. demonstrated the geometric patterning of endothelial cell cords to enhance tissue vascularization in addition to providing researchers with some control over vascular geometry\textsuperscript{34}. Though these methods have significantly improved our understanding of vasculogenesis (the de novo formation capillaries)\textsuperscript{35} and angiogenesis (the formation of new capillaries from existing branches)\textsuperscript{35} within the field of tissue engineering, directed growth of
endothelial cells does not result in immediately perfusable networks in the time scale necessary for cell survival in densely populated, metabolically active tissues.

**Microfluidic patterning of vascular networks:** Microfluidic approaches are another common method for incorporating vascular networks into engineered tissues, often with the purpose of studying the behavior of endothelial cells under various flow or signaling conditions. Microfluidic approaches commonly implement a combination of endothelial cell tubulogenesis and angiogenesis from pre-formed channels. Multiple groups have demonstrated the anastomosis between endothelialized microfluidic channels and capillary networks formed via vasculogenesis, a function necessary for scaling this vascularization technique to engineered tissues containing physiologically relevant cell densities. Milica Radisic and colleagues have also demonstrated the implantation of multi-layer microfluidic networks in vivo. These networks increased cell viability within the construct and remained patent for up to one week following surgical anastomosis with femoral vessels in the rat hind limb. Though microfluidic vascular networks have significantly increased our knowledge of vascular biology, these fabrication approaches are often difficult to scale up to the size of full organs, which can limit their ultimate applicability for the development of replacement tissues and organs.

**Decellularization and recellularization of native tissues:** An additional approach for improving transport to engineered tissues involves the decellularization and recellularization of existing organs or tissues. This method uses a detergent to remove cells from a tissue while leaving most of the native ECM intact, thereby preserving the underlying tissue structure, tissue mechanics, and vasculature. This technique takes advantage of the complex tissue and vascular geometry developed by the body; but, during the process of decellularizing and recellularizing tissues there is a significant level of uncertainty with regards to which ECM proteins maintain functionality or where new cells will adhere. Initial experiments implanting recellularized organs in vivo have demonstrated significant challenges with both leaking of vasculature and clotting within the first few hours following implantation. Additionally, high variation between samples restricts progress to low throughput trial and error and limits fundamental insights attained from these experiments. Despite advantages of this approach with respect to the recapitulation of native tissue
architectures, we desire higher levels of control over vascular geometry and cellular environment to better understand key principles involved in the development of vascular networks in vitro.

**3D printing of vascular networks:** Though the previously described approaches for developing vascularized tissues have improved mass transport through engineered tissues, most of these techniques lack the capacity to generate vascularized tissues with complex, hierarchical vascular structure, while also providing control over the cellular environment and vascular geometry. 3D printing provides significant promise to the field of tissue engineering because 3D printers provide researchers with control over nearly every X, Y, and Z coordinate within the print volume\(^52\). Using 3D printing, researchers can develop complex, branching vascular architectures while maintaining control over the position of interstitial cells and the surrounding ECM materials.

3D printing involves the layer by layer production of a new geometry. The most common methods used in tissue engineering or biological applications include extrusion-based methods, inkjet printing, laser sintering, and photolithography (Figure 1.4)\(^52\). Extrusion printing is the most common 3D bioprinting approach\(^53\)–\(^55\), and compatible with a wide variety of print materials relative to laser sintering and photolithography\(^56\),\(^57\). A modification of traditional extrusion-based printing that is highly relevant for generating vascular structures includes the co-axial extrusion of open channels\(^58\),\(^59\). Another modification on tadditional extrusion printing that is commonly used for the development of vascular networks is sacrificial templating. In this approach, the intended vascular channels are printed using temporary material that can be melted or dissolved away under physiological conditions, resulting in hollow lumens\(^48\),\(^60\),\(^61\). One example from our lab involves the extrusion of a carbohydrate-glass networks that can be cast in the material of choice (fibrin, PEG, alginate, agar) either with or without cells. The carbohydrate-glass networks can then be dissolved using PBS (phosphate buffered saline), and the resulting channels are seeded with endothelial cells\(^48\). Using this method, the interstitial regions are generally cast rather than printed, which allows users to utilize a wider variety of materials and cell types, because materials and cells are not limited to those compatible with 3D printing. Another modification of extrusion-based printing involves extrusion printing into a supportive bath, as pioneered by Adam Feinberg and
Figure 1.4: Schematic of common 3D printing techniques for developing vascular networks. A) Extrusion-based printing typically involves the extrusion of a liquid or semi-liquid substrate that cures upon cooling or other methods (light, calcium crosslinking, etc.) on the build platform. B) Inkjet printing also starts with a liquid substrate that is expelled from the cartridge dropwise before curing in the desired location. C) Selective laser sintering begins with a powder bed where individual layers are sintered together using heat from a laser. Powder from previous layers acts as a support to added layers within the build volume. D) Stereolithography uses either a laser or projected light source to cure photo-sensitive liquid materials layer by layer. Modified from Albritton & Miller 2017.63,64

Though inkjet printing has been used for generating vascular networks within engineered tissues,65 this approach is not as commonly used for fabricating vascular networks. Additionally, selective laser sintering is used less commonly for the direct patterning of vascular networks, due to the harsh heating from the laser. However, preliminary success has been shown using laser sintered networks as sacrificial templates,66 which could offer great methods for fabricating vascular networks if the dissolution of the template could be conducted in physiological conditions.

Another approach using additive printing methods involves crosslinking of photopolymerizable materials through stereolithography or multiphoton polymerization (MPP) techniques (Figure 1.4). Both techniques use a laser to induce photopolymerization, but stereolithography is generally used for larger scale applications with a resolution between
5-50µm, while MPP can achieve resolutions below 1 µm, but is often limited in total print volume\textsuperscript{56,67}. Recently, Kristi Anseth and colleagues have applied both MPP and stereolitography to fabricate channel structures using four different biocompatible α, ω-dihydroxy-polytetrahydrofuran-diacrylates (PTHF-DA). Using stereolitography, the team produced straight channels with diameters of 2 mm, while the MPP approach resulted in bifurcating channels with a diameter of 18 µm, wall thickness of 4 µm, and a height of 160 µm\textsuperscript{56}. Furthermore, Xing et al. used MPP to fabricate hydrogel lattices using 700 kDa PEGDA with features as small as 200 nm in diameter\textsuperscript{68}. Applying additional steps to the traditional stereolitography process, colleagues of Kristi Anseth have also fabricated biocompatible hydrogels using laser stereolitography, with added chemistries to allow patterning of additional functionalities into hydrogels, such as hydrogel stiffness\textsuperscript{69}. Though MPP outperforms most other printing techniques with respect to resolution, it is often limited in the scope of total print volume, which limits its application towards the development of tissues and organs on the centimeter scale.

The fabrication technique used most in this thesis is projection-based stereolitography (pSLA). pSLA printing involves layer-by-layer crosslinking of photo-curable polymers via serial projection of sequential photomasks\textsuperscript{70}. Early work with pSLA using biocompatible PEG-based hydrogels and a visible-light photo-initiator was demonstrated in Lin et al.\textsuperscript{71}, where researchers fabricated solid and porous structures containing human adipose-derived stem cells using pSLA. Shaochen Chen and colleagues at UC San Diego have long been pioneers in the field of projection stereolitography, fabricating complex fractal patterns using low MW PEGDA hydrogels group\textsuperscript{72-74}. pSLA has also been used by the Niels Larson and colleagues, who used low molecular weight (MW) PEGDA (700 kDa) to generate perfusable channel structures\textsuperscript{75}. However, the low MW PEGDA used for this printing was toxic to cells, and the group was unable to incorporate cells within the bulk of their hydrogels. Given these challenges, this work has focused on implementing pSLA using a new, non-toxic photoinitiator to generate complex channel structures with high resolution and high cytocompatibility. Furthermore, the setup-costs associated with pSLA are very low, approximately $2,000 per printer, with the main components consisting of a commercial DLP (digital light processing) projector, a stepper motor and axis for the z-stage, a RAMBO board, and custom 3D printed housing components (Figure 1.5). The current system in our lab, developed by Bagrat
Grigoryan and Anderson Ta, has an XY build size of 64x40 mm with an XYZ resolution of 50 µm and a light source at 405 nm, as determined by the optics of the commercial projector. We have previously demonstrated the capacity to fabricate complex channel networks in high MW PEGDA hydrogels with high fidelity (Figure 1.6). Ultimately, this fabrication technique gives us the capacity to fabricate a wide array of complex vascular topologies using a biocompatible material as necessary to complete the aims of this thesis.

Figure 1.5: Schematic of pSLA setup and fabrication workflow. (A) Schematic of pSLA 3D printer built in our lab. (B) Schematic demonstrating the fabrication process starting from a 3D model to a completed object. (C-D) Example photomasks that would be projected onto photo-curable resin to fabricate the rook model layer-by-layer. (Photo courtesy of Bagrat Grigoryan).

Figure 1.6: Example PEG-based hydrogels containing multiple channel networks fabricated with pSLA. A) Hydrogel containing a single straight channel (blue) surrounded by a helical secondary channel (red). B) Example of hydrogel containing two channels based on a 1° (blue) and 2° (red) Hilbert curve. (Photo courtesy of Bagrat Grigoryan).
1.3 Using computational modeling to systemically evaluate vascular architectures

Since 3D printing can generate a wide variety of vascular structures, but not yet matching the complexity of native vasculature, optimizing vascular geometry using in vitro experiments alone would be very time intensive and resource consuming. For these reasons computational models are commonly used to calculate variables such as flow rates, oxygen diffusion, and shear forces within a vascular network. This information can then be used to improve vascular structures prior to fabrication in vitro. Additionally, computational models can be used to calculate key factors of the cellular environment, such as shear stresses on cells within the vascular wall or oxygen concentrations, that have significant effects on cell behavior, but are difficult to monitor in vitro. Modeling approaches span a range of techniques with varying complexity. The majority of situations in tissue engineering can be modeled using CFD approaches using equations for laminar fluid flow, porous fluid flow, chemical reactions, and diffusion, such as the Navier-Stokes equations, Darcy’s Law, Michaelis-Menten Mechanics, and Fick’s Law (Figure 1.7).

![Image of vascular transport](image)

**Figure 1.7**: Equations outlining vascular transport of oxygen and nutrients to interstitial cells. Oxygen is transported to cells through convective and diffusive transport all while being consumed by interstitial cells and filtered through endothelial cells. (Modified from: Miller 2014)\(^{52}\)

Such computational modeling techniques are employed for a variety of applications within developmental biology and cancer research. For instance, Welter and Rieger modeled interstitial pressure in developing tumors, which has been shown to play a role in the delivery
of chemotherapy and other cancer treatments. As another example, Spencer et al. used a unique computing method based on the Lattice Boltzmann Method (LBM) to predict events on multiple scales in perfusion bioreactors. Since the LBM relies on kinetic theory and allow for multi-scale computation, using LBM allowed the group to predict the diffusion of oxygen, nutrients, and the transport of cells through the porous scaffolds. Using predictive models, researchers could identify uneven flow patterns or hypoxic regions within a scaffold and correct these problems during the design process. Truslow et al. used computational fluidic dynamics models to model oxygen delivery to a cell laden hydrogel through hexagonally packed channels. However, many research groups work exclusively with computational or experimental models and there is often not enough overlap to close the loop between in silico and in vitro systems.

Though computational models have been shown to be effective in predicting flow rates, diffusion levels, and other key tissue parameters, very few groups have been able to use computational models to improve vascular structures they fabricate. In order for computational models to aid in developing vascular structures for 3D printed tissues, researchers must first close the loop between computational and experimental tissue models. Researchers can use these verified models to optimize their printed tissue structures prior to the printing process and use the models to better understand the biochemical and mechanical environment of cells within a tissue (Figure 1.7). This enhanced understanding and increased printing efficiency will improve the quality and throughput of vascularized tissue models within the field of tissue engineering.

In summary, vascular geometry and flow rates play a crucial role in maintaining cell viability and tissue specific functions in vivo. The advancement of 3D printing techniques allows researchers to generate complex, hierarchical structures in vitro, providing a tool for the production of tissues for regenerative medicine applications and high throughput disease models. Furthermore, these advancements in fabrication will be advanced by the use of computational modeling techniques and in vivo studies.
Chapter 2

Design and validation of 3D printed vascular networks for efficient nutrient delivery

Here, we explore the utility of 3D printed perfusable PEG-based hydrogels along with computational models for convective and diffusive transport. We performed extensive characterization of these hydrogels and established the reliability of computational models. Further, we introduce the screening of vascular geometries based on results of computational modeling.

*Portions of this chapter have been previously published in Paulsen & Miller 2015.*

2.1 Acknowledgements

I thank my collaborators and co-workers for their help on this aim. Specifically, Amanda Randles and John Gounley of Duke University developed the Lattice-Boltzman calculations used in this chapter, while Jessica Rosenkrantz and Jesse Louis-Rosenberg of Nervous System developed the complex branching architectures described in this aim. Thank you to Shail Mehta from Rice University for his assistance with mechanical testing. Thank you to Richard Bouchard and James Long for reconstructing the Doppler ultrasound data. Ian Kinstlinger provided incredible insight for this chapter, and his research has been a crucial continuation of this work. Finally, this work was in part supported by the John S. Dunn Collaborative Research Award.

2.2 Introduction

In Chapter 1 we discussed the importance of vascular networks in delivering oxygen and nutrients within engineered tissues and the advantages of using 3D printing to fabricate
these vascular networks. In this chapter, we look at the design of different vascular networks, implementing CFD modeling to assess the nutrient delivery of different vascular networks and introduce preliminary screening techniques to improve the design of vascular architectures within engineered tissues.

2.2.1 Using PEGDA for 3D fabrication of vascular networks

Throughout this work we have used poly(ethylene glycol) (PEG) as our base material for fabricating hydrogels containing micro-channel networks. PEG is a synthetic hydrogel that is a hydrophilic, biocompatible, FDA-approved material with highly tunable mechanical and diffusive properties depending on polymer chain length and concentration\(^{80-82}\). PEG also contains no natural biologically active sites\(^{83}\), which allows a high degree of control over cell-matrix interactions due to ease of functionalization\(^{84-91}\). Additionally, PEG is compatible with a variety of chemical crosslinking reactions, including radically initiated step or chain-growth reactions\(^{88,92}\). In previous work, we have demonstrated that radical chain-growth polymerization of PEG-diacrylate (PEGDA), using lithium phenyl-2,4,6-trimethylbenzoylphosphinate (LAP) as a photoinitiator and tartrazine (Sigma-Aldrich) as a photoabsorber results in the formation of consistent gel structures (Figure 1.5). LAP has been shown previously to be cyto compatible and a more effective photoinitiator than Irgacure I2959 at visible light wavelength of 405 nm\(^{93}\), which corresponds well to the peak absorbance of our photoabsorber, tartrazine, at 425 nm\(^{94}\). Furthermore, though our pSLA printing technology is compatible with more advanced crosslinking chemistries, such as thiol-ene step growth reactions\(^{92}\), chain growth reactions using PEGDA, as outlined in Figure 2.1 and Figure 2.2, are cost effective and provide sufficient control over crosslinking to generate high fidelity hydrogels with optics-limited features resolution of around 250 µm.

Though the experiments for this thesis have been conducted using PEGDA containing no added bioactive molecules, in future studies we can incorporate cell adhesive and MMP-degradable (matrix metalloproteinase) ligands into our PEG-based hydrogels, as has been previously demonstrated in our lab\(^{88}\), allowing cells to interact with our 3D printed hydrogel networks. Though the addition of cell adhesive or MMP-degradable ligands does not perfectly recreate all of the native bioactivity of natural extracellular matrices (ECM), these specific modifications to PEG chemistry give users specific control over the interactions of cells with
the surrounding matrix, which is useful for probing cell-matrix and cell-cell interactions within engineered systems.

![Figure 2.1: Cleavage of LAP molecule in the presence of light to form free radicals.](image1)

![Figure 2.2: Chain-based radical polymerization of PEGDA to form a hydrogel network.](image2)

### 2.2.2 Using computational modeling to compute convective and diffusive transport

As mentioned previously, CFD models offer valuable information for researchers designing vascular networks about fluid flow profiles and oxygen or nutrient delivery. These models are generally based off of Navier-Stokes equations (Equation 2.2), which work well for single phase fluid flows where the continuum assumption applies\(^\text{96}\). However, as models become more complex, reaching smaller size scales where the continuum assumption does not readily apply, or in situations requiring complex boundary conditions or time dependent calculations, the highly parallelizable Lattice Boltzman Method (LBM) offers significant advantages\(^\text{78,96,97}\). Unlike traditional CFD models which require a triangular mesh, LBM is derived from the Boltzmann equation of kinetic theory and implements a regular array of lattice points within the desired fluid geometry on which the traditional Boltzmann equation can be solved to calculate the movement of synthetic particles\(^\text{96,97}\). LBM solves fluid flow within discrete packed voxel arrays, instead of attempting to interpolate rough mesh topologies in order to correctly solve Navier-Stokes. Therefore, when moving from simpler test geometries to the complex branching networks developed by our collaborators from Nervous System, we move from traditional CFD models to the LBM. However, even when using the LBM, predicting oxygen transport in complex 3D geometries is a computationally intensive task and
is not always practical for screening the oxygen transport in large numbers of potential vascular networks. For this reason, we developed a simplified method to compare the capacity of different vascular networks to transport oxygen into surrounding tissues and to maintain cell viability.

2.2.3 Ultrasound imaging for 3D measuring 3D fluid flow profiles

Lastly, while computational models can easily calculate fluid flow profiles in 3D, making in vitro measurements of fluid flow profiles in 3D is much more difficult. Most light-based techniques can be used to map fluid flow profiles in 2D, but have limited capacities to measure flow profiles through thick geometries in 3D. Therefore, we have turned to ultrasound-based Doppler imaging to map fluid flow profiles through our 3D vascular networks. Ultrasound imaging has long been a valuable tool for medical diagnostics and for biomedical engineering due to its non-invasive nature, centimeter-scale penetration depth, and rapid image acquisition rates. Ultrasound scans an entire XZ-plane, while the imaging plane must still be raster-scanned across a sample to build up a full XYZ volume. Additionally, ultrasound imaging has become more widely used in the field of tissue engineering to assess flow through vascular networks and assess integration of tissue engineered constructs. As ultrasound expands to include more and more quantitative techniques, including measuring of elastic modulus and 3D fluid flow mapping through Doppler imaging, this imaging modality will become increasingly valuable for non-invasive monitoring of vascular tissue development.

2.3 Objectives

In this work we sought to establish parity between our computational and experimental models for convective and diffusive transport through 3D printed vascular structures. Our approach started with characterization of physical properties of our PEGDA hydrogels then moved on to develop parallel computational and experimental models for fluid flow and small molecule diffusion through channel networks fabricated using pSLA. Our goals were to:

1) Characterize properties of different PEGDA formulations, including required curing time, swelling ratio, and mechanical properties.

2) Establish parity between computational and experimental models for convective and diffusive transport.
3) Develop methods to assess efficiency of oxygen transport of vascular architectures.
4) Map fluid flow profiles through 3D printed channel networks in 3D using Doppler ultrasound.

Though fabrication of simple vascular networks has previously been done using different 3D printing techniques, and though various groups have used computational modeling to assess vascular transport or even to improve the oxygen delivery of theoretical channel networks, few groups have combined both experimental and computational models for the development of complex vascular networks in vitro. By utilizing medium-throughput screening in silico coupled with lower-throughput screening in vitro, we propose to develop an integrated workflow through which biomaterial scaffolds containing living cells and patterned vascular networks can be designed and fabricated.

2.4 Materials and Methods

2.4.1 Fabricating PEGDA hydrogels using pSLA fabrication

The majority of structures used in this chapter were generated using software such as SolidWorks or Blender. However, more complex geometries (as noted later in the chapter) were generated using an algorithm inspired by leaf venation, which was first developed by Runions, et al. and implemented through a collaboration with Jessica Rosenkrantz and Jesse Louis-Rosenberg of Nervous System (Figure 2.3). After the desired structure was generated and converted to a stereolithography (STL) format, the 3D model was sliced using the software Creation Workshop, which also controls the z-stage and projector of the pSLA system. Hydrogels were fabricated using pre-polymer solution containing 34 mM LAP, 2.65 mM tartrazine, and 10% w/w 6 kDa PEGDA, unless otherwise specified. Gels were fabricated using a layer height of 50 µm, where layer exposure times were adapted for each PEGDA formulation as listed in Figure 2.8B.
Figure 2.3: Schematic describing the multiple stages of the Nervous System leaf venation algorithm. A) The user first designates root from which sprouting will originate. Then, hormone sources are distributed in a desired area (or volume) using a randomized dart throwing algorithm. B) The influence of each hormone source on the root is calculated and the vein grows in the average direction of the hormone sources. C) As the vein grows, source neighborhoods are identified, which influence the growth of individual branches. D) Vein thickness of the final network is modified during post-processing. Final networks depend on a wide variety of specified parameters controlling hormone influence and vein branching parameters. Modified from Nervous System108.

2.4.2 Material characterization

To assess the range of PEGDA formulations compatible with pSLA printing, we mixed print solutions containing varying ratios of 6 kDa to 35kDa PEGDA ranging in concentration from 5 to 50% w/w (Figure 2.9). These print solutions were then used to print a small 3×3×3 mm cube gels containing a single horizontal channel 1 mm in diameter (Figure 2.4). This gel was allowed to swell to equilibrium and the fidelity of the channel was assessed along with the capacity of the gel to withstand basic manipulation, such as transferring between wells of a multiwall plate. Printed gels were graded on a scale from “poor gel stability”, where no clear channel remained after equilibrium swelling, “Inconsistent Printing”, where a channel was clearly present but had ill-defined edges or the gel was too soft to handle, or “Good Printing”, where gels had clean edges and self-supporting structure (Figure 2.4).
Finally, the dimensional and mass swelling ratios of the gels were calculated for each formulation (Figure 2.9). The dimensional swelling ratio was defined as the change in dimensions at the time of printing relative to the dimensions at equilibrium swelling. This parameter was calculated by measuring the diameter of a cylindrical gel immediately after printing relative to the diameter of the same gel after reaching equilibrium swelling. The mass swelling ratio is defined as the gel mass after reaching equilibrium swelling divided by the dried mass. After printing, cylindrical gels were allowed to reach equilibrium swelling over a period of at least 24 hours before being weighed. The gels were then allowed to air dry over 2 days before being lyophilized overnight and weighed again.

2.4.3 Mechanical testing of PEGDA materials

Uniaxial tensile testing was conducted using a Bose Enduratec ELF 3200 system, similar to the procedure described previously in Stephens, et al.\textsuperscript{109}. First, test strips for all “printable” PEGDA formulations were 3D printed at scaled dimensions so that the final gels would have approximate dimensions of $4 \times 25 \times 1$ mm at equilibrium swelling. Dimensions of the swollen hydrogels were recorded prior to testing. Ends of the hydrogel were glued between two pieces of bibulous paper, which were in turn secured within the clamps of tensile testing system. The tension tests were conducted at a rate of 0.5 mm/s to sample failure. The tensile modulus for samples was calculated using a custom MATLAB script, which calculated the slope of a stress strain curve between 0.05 and 0.1 engineering strain. Ultimate strain was assessed by finding the strain value associated with the maximum stress, which occurred just prior to sample failure.
2.4.4 Experimental models of convective transport using particle image velocimetry

Flow rates through 3D printed channel networks were measured using PIVlab\textsuperscript{110}, an open-source PIV package for MATLAB. PIV works by mapping the displacement of groups of tracer particles between frames (Δt) then computing the local fluid flow velocity\textsuperscript{110}. For these experiments we used 10 µm red fluorescent beads (Magsphere Inc) as our tracer particles at a 1:100 dilution in milliQ water. The bead suspension was flowed into the channel at a rate of 100 µL/min and images were captured at a rate of 40 frames per second with a pixel size of 10.6 µm/pix and a total image size of 2048×2048 pixels. Prior to calculating flow velocity, a static mask was applied to the images, which were then pre-processed using a high-pass filter of size 7 pixels, using intensity capping, and a Wiener2 denoise filter with a window size of 4. After preprocessing, fluid flow velocities were analyzed using direct cross correlation with in integration area of 20 pixels and a step size of 10 pixels. Results were filtered by a manually applied bounding box based on velocity, and final results were calculated by averaging velocities over 500 frames.

2.4.5 Experimental models of diffusive transport

Diffusion through PEG gels was approximated as described previously by Hasan, et al. and Cuchiara, et al.\textsuperscript{36,111}. Briefly, fluorescently labeled molecules of different molecular weights (Rhodamine 0.125 mg/mL (688 Da; PolySciences), FITC-dextran 0.25 mg/mL (4 kDa; Sigma Aldrich), and FITC-dextran 0.25 mg/mL (10 kDa; Sigma Aldrich)) were flowed through channels within 3D printed PEGDA hydrogels (10% w/w 6 kDa) and the fluorescence intensity surrounding the channel was imaged every 2 minutes over a period of 80 minutes using epifluorescence microscopy. Images were then analyzed using a custom MATLAB script to compare diffusion rates of different fluorescent dyes into the bulk of the gel\textsuperscript{36,111,112}.

2.4.6 Computational models of convective transport

CFD models for convective transport were developed in COMSOL Multiphysics 5.3a using the Single Phase Laminar Flow module (Reynold’s number <1, Equation 2.1), which derives velocity fields using Navier-Stokes equations (Equation 2.2)\textsuperscript{104}. The Flow Through Porous Media add-on, based on the Brinkman Equations (Equation 2.3), was used to model convective transport into the surrounding PEG hydrogel. Brinkman Equations were used
instead of Darcy’s law because Brinkman’s equations allow for more efficient coupling between the free fluid flow and porous media flow\textsuperscript{113–115}.

\[
Re = \frac{\text{Inertial Forces}}{\text{Viscous Forces}} = \frac{\rho v L}{\mu} = \frac{v L}{\nu}
\]

**Equation 2.1: Equation for Reynolds number to assess flow regime for pipe flow.** $v =$ flow velocity, $\nu =$ kinematic viscosity of the fluid, $L =$ characteristic length (channel diameter)\textsuperscript{76}.

\[
\rho (u \cdot \nabla) u = \nabla \cdot \left[ p + \frac{\rho}{\mu} (\nabla u + (\nabla u)^T) - \frac{2}{3} \mu (\nabla \cdot u) I \right] + F
\]

\[
\nabla \cdot (\rho u) = 0
\]

**Equation 2.2: Navier-Stokes equations for laminar fluid.** $\rho =$ density of the fluid, $u =$ flow velocity, $p =$ pressure, $\mu =$ fluid viscosity, and $F =$ additional external forces such as gravity.

\[
\frac{\partial}{\partial t} \left[ \epsilon_p^u \right] + \nabla \cdot \left( \frac{\rho}{\epsilon_p} \right) = -\nabla p + \nabla \cdot \left[ \frac{1}{\epsilon_p} \left[ \mu (\nabla u + (\nabla u)^T) - \frac{2}{3} \mu (\nabla \cdot u) I \right] - \left( k^{-1} + \frac{Q_{br}}{\epsilon_p^2} \right) u + F \right]
\]

**Equation 2.3: Governing equations for flow through porous media based on Brinkman Equations.** $\rho =$ density of the fluid, $\epsilon_p =$ scaffold porosity, $u =$ flow velocity, $p =$ pressure, $\mu =$ fluid kinematic viscosity, $I =$ identity matrix, $k =$ scaffold specific permeability, $Q_{br} =$ volumetric flow rate, and $F =$ sum of external forces such as gravity\textsuperscript{113}.

To ensure that computations are run as efficiently as possible, we began by conducting a mesh analysis to assess the mesh density necessary to achieve stable results. We first constructed a 3D channel at 250 $\mu$m in diameter to represent our smallest potential feature size, then applied a pressure differential of 5 kPa (~ 40 mmHg) and solved the model at increasingly finer meshes. After plotting the flow profile across the channel relative to the number of mesh nodes, we determined that measurements for maximum velocity magnitude level out at an element volume ratio around 0.007, corresponding with a mesh level of “Fine” in COMSOL (Figure 2.5). This study will ensure that results from following studies are both accurate and efficient with regards to computing time.
To model convective transport through our 3D channel networks, the 3D geometry used to print the gels were scaled up according to the gel swelling ratio for the target PEGDA formulation and imported into COMSOL 5.3a. The inlet and outlet of the channels were identified, and the inlet flow rate was set to the equivalent of 100 µL/min to match the inlet flow rate of the bead tracking experiments. Physical values including fluid viscosity and hydraulic conductivity (specific conductivity) of the PEGDA hydrogel were found in the literature (Table 2.1).

<table>
<thead>
<tr>
<th>Variable</th>
<th>Description</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>U</td>
<td>Normal inflow velocity</td>
<td>$1 \times 10^{-5}$ to 0.1 m/s</td>
</tr>
<tr>
<td>$\rho$</td>
<td>Density of fluid</td>
<td>1000 kg/m$^3$</td>
</tr>
<tr>
<td>$\mu$</td>
<td>Dynamic viscosity of fluid</td>
<td>0.001 Pa*s (1 cP)$^{76}$</td>
</tr>
<tr>
<td>$\kappa$</td>
<td>Specific conductivity of PEG hydrogel</td>
<td>$0.75 \times 10^{-15}$ m$^2$/Pa*s$^{116}$</td>
</tr>
<tr>
<td>$\epsilon_p$</td>
<td>Porosity of PEG hydrogel</td>
<td>100%</td>
</tr>
</tbody>
</table>

Table 2.1: Physical parameters to calculate convective transport in PEGDA hydrogels.

2.4.7 Computational models of diffusive transport and reactive species

Diffusive transport was modeled in 2D using the Transport of Diluted Species interface, where the primary driving force is diffusion by Fick’s Law ($\text{Equation 2.4}$), and any consumption of molecules by cells ($R_i$) is assumed to be uniform through the interstitial area.
\[ \nabla \cdot (-D_i \nabla c_i) + \mathbf{u} \cdot \nabla c_i = R_i \]
\[ N_i = -D_i \nabla c_i + \mathbf{u}c_i \]

**Equation 2.4: Governing equations for Transport of a Diluted Species Module.** \( D_i \) = diffusivity of the species, \( c_i \) = concentration of the species, \( R_i \) = species reaction rate, \( N_i \) = flux of the species.

Another mesh analysis was conducted for diffusive transport using a similar procedure to section 2.4.6. We established a simple 2D test geometry and computed the concentration of a sample molecule at steady state (including a reaction term) at increasingly finer meshes. Concentration measurements level out at an element area ratio around 0.468, corresponding with a mesh level of “Finer” (Figure 2.6).

![Figure 2.6: Mesh analysis for 2D convection-diffusion models](image)

**Figure 2.6: Mesh analysis for 2D convection-diffusion models.** Mesh density of Finer (~50,000 elements) appears sufficient for consistent solutions.

To incorporate diffusive transport into the model, gel geometries were imported into COMSOL as described previously in section 2.4.6. When applicable, fluid flow calculations were coupled with the transport of a diluted species. Values for diffusivity of the target species and cell consumption rates of oxygen were found in the literature (Table 2.2). However, because low oxygen concentrations result in cell death, the oxygen consumption rate was modeled as a step function where the consumption rate (\( R_{O_2} \)) transitions to 0 at a set critical oxygen concentration (\( C_{min} \)) with a transition zone of \( 1 \times 10^{-8} \) mol/cm\(^3\).
<table>
<thead>
<tr>
<th>Variable</th>
<th>Description</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>( C'_{\text{Rho}} )</td>
<td>Concentration of rhodamine at inlet</td>
<td>( 1.88 \times 10^{-4} \text{ M} )</td>
</tr>
<tr>
<td>( C'_{\text{FITC}} )</td>
<td>Concentration of 10kDa FITC dextran at inlet</td>
<td>( 2.5 \times 10^{-5} \text{ M} )</td>
</tr>
<tr>
<td>( D_{\text{Rho}} )</td>
<td>Diffusion coefficient for rhodamine</td>
<td>( 2.67 \times 10^{-6} \text{ cm}^2/\text{s} ) \textsuperscript{117,118}</td>
</tr>
<tr>
<td>( D_{\text{FITC}} )</td>
<td>Diffusion coefficient 10kDa FITC dextran</td>
<td>( 1 \times 10^{-7} \text{ cm}^2/\text{s} ) \textsuperscript{81,119}</td>
</tr>
<tr>
<td>( C'_{\text{O}_2} )</td>
<td>Concentration of oxygen at inlet</td>
<td>( 1.78 \times 10^{-4} \text{ M} ) \textsuperscript{120}</td>
</tr>
<tr>
<td>( D_{\text{O}_2} )</td>
<td>Diffusion coefficient for oxygen</td>
<td>( 3 \times 10^{-5} \text{ cm}^2/\text{s} ) \textsuperscript{79}</td>
</tr>
<tr>
<td>( R_{\text{O}_2} )</td>
<td>Oxygen consumption rate per cell</td>
<td>( -2.5 \times 10^{-18} \text{ mol/s/cell} ) \textsuperscript{121}</td>
</tr>
<tr>
<td>( C_{\text{cell}} )</td>
<td>Cell concentration</td>
<td>( 1-100 \times 10^6 \text{ cells/mL} )</td>
</tr>
<tr>
<td>( c_{\text{min}}(\text{O}_2) )</td>
<td>Critical oxygen concentration</td>
<td>( 4.56 \times 10^{-8} \text{ mol/cm}^3 ) \textsuperscript{79}</td>
</tr>
</tbody>
</table>

Table 2.2: Physical parameters used to model the diffusive transport of oxygen and other species. Values in the top half of the table are used in time-dependent models of the diffusion of fluorescent molecules, while the bottom half of the table details parameters used to model oxygen transport in steady state models for assessing vascular efficiency.

### 2.4.8 Fluid dynamics modeling using Lattice Boltzmann method

The lattice Boltzmann method, implemented by collaborators Amanda Randles and John Gounley, was used to compute fluid flow profiles through complex channel networks developed in collaboration with Nervous System. Calculations were performed using a massively parallel computational hemodynamics application, HARVEY, as described previously in Randles, et al 2014\textsuperscript{97}. Initial results using LBM were first validated and compared to CFD results through similar ladder geometries with the same inflow rate (Figure 0.1). First, the desired geometries were converted from a triangular mesh to a Cartesian grid needed for LBM. Steady-state flow profiles were then obtained by applying the LBM to the system with a set pressure differential at the inlet and outlet of each geometry. By setting a specified pressure differential these models take into consideration variations in the fluidic resistance of each individual geometry. These results were then analyzed via a custom workflow to predict vascular efficiency.

### 2.4.9 Assessing vascular efficiency

Assuming that oxygen is the limiting factor for cell viability\textsuperscript{79}, we developed a screening technique for assessing how efficiently different vascular architectures can deliver nutrients to cells. An ideal vascular architecture would minimize total vascular volume (thereby maximizing interstitial space where cells execute tissue-specific functions), while
maintaining high levels of cell viability. Therefore, we developed a fitness function we call “vascular efficiency” that is directly correlated with the square of the total viable volume within a hydrogel model and is inversely correlated with the product of the non-viable and vascular volumes (Equation 2.5).

\[
\text{Vascular Efficiency} = \frac{\text{Viable Volume}^2}{(\text{Non} - \text{Viable Volume})(\text{Vascular Volume})}
\]

Equation 2.5: Vascular efficiency fitness function.

To calculate the vascular efficiency of a given vascular network, simulation results from LBM models were discretized into mm thick slices, as shown in Figure 2.7. Then, using a custom MATLAB script, for each slice in the x-direction, the simulated fluid velocity was extracted and used to calculate a “viable” radius from the center of each channel. This radius is defined as the region around each channel in which enough oxygen can diffuse to support cell viability, as calculated using COMSOL Multiphysics simulations described in section 2.4.7. Figure 2.7D depicts the calculation of the viable radius per channel. The output of the model was a measurement of “vascular efficiency.” To account for variations in resistance of different channel networks, all models were solved using the same pressure differential between the inlet and outlet, as described in section 2.4.8.
2.4.10 3D flow reconstruction using ultrasound imaging

To measure fluid flow profiles in 3D, deionized (DI) water containing 25% glycerol and 3 µm polystyrene beads (Magsphere Inc) was infused into the desired channel network at a constant velocity. The polystyrene beads provided ultrasound backscatter while glycerol prevents the beads from settling out of suspension during imaging. During perfusion ultrasonic B-mode and color Doppler images were taken with the Vevo 2100-LAZR photoacoustic-ultrasonic imaging platform at 24 MHz. Six partially correlated color Doppler acquisitions were obtained at transducer orientations of 0°, 45°, and 90° and transmit steering angles of +/-15°. The acquisitions were then retrospectively co-registered based on B-mode data of the open channel. Matched COMSOL-based numerical modeling and optical flow tracking were implemented for comparison and validation, respectively.
2.5 Results

2.5.1 Material characterization and printability screen

We demonstrated that a wide variety of PEGDA formulations containing both 6 kDa and 35 kDa chains are compatible with our pSLA printing technique, where formulations were considered “printable” if they supported the formation of a stable gel with a clearly defined edges and an open channel (Figure 2.4, Figure 2.8). We noticed a trend in exposure time necessary to sufficiently crosslink the desired formulation, where formulations containing higher overall concentration of PEGDA, regardless of MW ratio, required less exposure time to form a solid gel with clearly visible edges (Figure 2.8). The mass swelling ratio of different PEGDA formulations depended most strongly on the ratio of 6:35 kDa chains, where lower relative concentrations of 6 kDa chains resulted in higher swelling ratios. Additionally, a lower total concentration of PEGDA resulted in a higher swelling ratio. One challenge that occurs from using a variety of PEGDA formulations is the difference in swelling ratios, which affects equilibrium swelling and the final size of the gel. Therefore, we also measured changes in the dimensional swelling ratio to provide a scaling factor for generating hydrogels with a target feature size after equilibrium swelling. This quantitative analysis of swelling ratio is implemented in Chapters 3 and 4 when developing valve structures and vascular shunts with target dimensions that proved crucial in proper function of the hydrogels.

![Figure 2.8: Exploration of PEGDA formulations compatible with pSLA. A) Heat map highlighting PEGDA compatible with pSLA fabrication. B) Per layer exposure times used for crosslinking different formulations, where the thick black line distinguished between consistently printable and less consistently printable formulations.](image)
Changes in overall PEGDA concentration and MW ratio also affected the mechanical properties of the hydrogels. Tensile modulus for the printable formulations ranged from approximately 0.5 kPa (40% w/w 35 kDa) to over 400 kPa (50% w/w 6kDa) (Figure 2.9), where we were limited on the lower end of the stiffness regime due to a material’s ability to support itself during the printing process and were limited by PEGDA solubility on the upper end. Meanwhile, the stiffness of native human tissues range from less than 100 Pa for very soft tissues such as neural or blood tissues to over 1 GPa for bone tissue\(^{122}\). Therefore, though our pSLA system is compatible with a wide range of PEGDA, we cannot fully recapitulate the range of elastic moduli that occur in native tissues using PEGDA alone. However, this challenge could likely be addressed by exploring additional MW PEGDA formulations or alternative photo-crosslinkable materials altogether.

Additionally, we demonstrated that it is possible to achieve similar stiffness with very different ultimate strains by using different PEGDA formulations. For example, while the 10% w/w of 6 kDa has a similar tensile modulus to the 40% 1:3 6:35 kDa formulation their average ultimate strains are 38 ± 2% and 60 ± 12%, respectively. These results suggest that adjusting the overall concentration of PEGDA along with the ratio of different molecular weights offers the potential for researchers to control more than just the stiffness of the resulting hydrogel. For the majority of future experiments, we chose to continue using the 10% w/w 6 kDa formulation because it is highly soluble, it is easy to print, and the resulting gels are durable. However, for future experiments requiring larger, more resilient structures we will use the 40% w/w formulation with a 1:1 ratio of 6:35 kDa chains (Chapters 3 & 4). This formulation is stiffer than the 10% 6 kDa formulation at ~70 kPa, but also has a higher ultimate strain at ~50%, which should make the structures more resilient to rupture under applied pressure.
Figure 2.9: Characterizing properties of different PEGDA formulations for 3D printed hydrogels. A) Dimensional swelling ratio for different PEGDA formulations. B) Swelling ratio for different PEGDA formulations. C) Tensile modulus of printable PEGDA formulations. D) Ultimate strain of printable PEGDA formulations. NT refers to groups not tested due to poor printability of samples. N=3 for all data.

2.5.2 Comparing computational and experimental models of convective transport

After characterizing the physical properties of different PEGDA formulations, we fabricated simple channel networks from 10% w/w 6 kDa PEGDA for initial flow analysis. We began with a simple “ladder” channel structure containing a single inlet and outlet with multiple branches in between. With an inflow rate of 100 µL/min, PIV measurements revealed maximum flow rates of 1.6 mm/s in the top and bottom channels of the ladder (Figure 2.10). Computational models of fluid flow through the model channel geometry followed similar
patterns to the PIV data, but with slightly lower flow rates. This slight discrepancy in flow rates is likely due to the slight over-curing effect that occurs while printing overhanging regions (Figure 2.11), resulting in channel geometries that are slightly more narrow than intended. However, results from both experimental and computational models demonstrate parabolic flow profiles and show the same trend with respect to average flow rate through each channel, where the central channels have lower average flow rates than the first or last channel (Figure 2.10). Though PIV measurements show a decent level of variation and there is a slight under-prediction in flow rates form our COMSOL model, there is still a high degree of correlation between the computational and experimental models for convective transport within channel networks, instilling confidence in future applications of computational models of convective transport.

Figure 2.10: Comparison between experimental and computational measurements of fluid flow through branching channel networks. A) Channel geometry used for printing ladder networks and developing computational models of fluid flow. Inflow rates were set at 100 µL/min, and maximum flow rates were measured across channel mid-points as shown in red. B) PIV calculations for velocity magnitude through channel networks. (N=5, representative image). Scale bar = 1 mm. C) COMSOL model calculating velocity magnitude through geometry shown in (A) after scaling the model to account for hydrogel swelling. Scale bar = 1 mm. D) Average maximum velocity through channels 1-4 as shown in (A) where N=5 for PIV measurements. Velocity data shows good correlation between computational and experimental models of fluid flow.
Figure 2.11: Demonstration of over-curing effects within channels fabricated using pSLA at 50 µm layer height. A) Hydrogel geometry used in B, where the print direction was parallel to the axis of the central channel. B) PEGDA hydrogel containing fluorescent FITC-dextran, where central channel (dashed white line) shows no over-curing effects. C) Same hydrogel geometry as shown in A, but the print direction is rotated 90°, perpendicular to the axis of the central channel. D) Hydrogel containing FITC-dextran as shown in C, where the central channel shows minor over-curing at the bottom of the channel (white asterisk). All scale bars = 1 mm. (Photo Courtesy of Dan Sazer)

2.5.3 Comparing computational and experimental models of diffusive transport

After comparing convective transport in experimental and computational models, we measured diffusive transport from our channel structures into the surrounding hydrogel over time. Using epifluorescence microscopy we monitored the accumulation of fluorescent molecules in the bulk of a 10% w/w 6 kDa PEGDA hydrogel over time by measuring the changing intensity at a fixed distance of 200 µm from the edge of the printed channel (Figure 2.12). Experimental results show that rhodamine (MW 688 Da) diffused rapidly from the channel into the interstitial space within the first 15 minutes, while minimal diffusion was observed from 3 or 10 kDa FITC-dextran over an 80 minute time span (Figure 2.12). For computational models of diffusive transport we used coefficients of diffusion from the literature (Table 2.2), while starting concentrations of our species as established in experimental models. Computational models display similar trends to experimental models,
where low MW rhodamine accumulated rapidly in the interstitial space over the first 15 – 20 min, then appeared to saturate shortly afterwards. Meanwhile, 10 kDa FITC-dextran diffused only very slowly out of the main channel, showing hardly any accumulation in the interstitial space after a period of 60 minutes. These results indicate a strong correlation between the computational and experimental models for diffusive transport within PEG-based hydrogels. Though diffusion experiments were only conducted using one formulation of PEGDA, we predict that PEGDA formulations using lower MW or higher concentrations of PEGDA would have lower diffusion rates due to decreased hydrogel mesh size. Lin et al. previously calculated the mesh size of 10% w/w 6 kDa PEGDA hydrogels using the Peppas-Merrill model with an additional step to calculate mesh size. They determined that the average mesh size is of 10% w/w 6 kDa PEGDA hydrogels is 7.75 (± 0.5) nm, while 10% w/w 20 kDa PEGDA hydrogels had a calculated mesh size of 13.09 (± 0.8) nm. Hagen et al. additionally showed that average mesh size of PEGDA hydrogels decreased with increasing polymer volume fractions, supporting this hypothesis.

![Figure 2.12](image)

**Figure 2.12: Measuring macroscale diffusion of fluorescent dyes in PEGDA hydrogels.** A) Experimental setup for measuring diffusion of fluorescent dyes out of a 3D printed channel. B) Normalized fluorescence intensity at 200 μm from the channel wall over time. N=2. C) Computational results for rhodamine and 10 kDa FITC-dextran concentration over time.

### 2.5.4 Calculating vascular efficiency

After establishing a level of reliability for our computational models, we expanded our application to predict cell viability within hydrogels under flow perfusion. To explore how well different channel architectures deliver oxygen to surrounding interstitial cells, we developed a workflow to calculate the “vascular efficiency” of different channel networks based on the total viable volume (as calculated by oxygen concentration) relative to the total...
vascular volume. We first explored the role cell concentration and flow rate of oxygenated media affect oxygen concentrations within our engineered PEGDA hydrogels. Though per cell oxygen consumption rates vary largely depending on cell type, size, and growth phase, ranging from \(>1\) amol \(O_2/\text{cell/s}\) to greater than 50 amol/cell/s\(^{121}\), here we assumed an average consumption rate of 2.5 amol/cell/s\(^{121}\). As shown in Figure 2.13, in samples with sufficiently low oxygen consumption rates (as occurs with low cell densities or cells with low metabolic rates), the applied inflow rate can deliver enough oxygen to maintain cell viability throughout the gels, here assumed to be 0.045 mM oxygen\(^{79}\), even at relatively low flow rates. However, at higher cell concentrations (or higher oxygen consumption rates), a significant portion of the scaffold drops below this critical oxygen concentration, resulting in a “viable radius” surrounding the central channel. Furthermore, at low flow rates but high oxygen consumption, an axial gradient also develops along the central channel, which determines the “decay constant” within the vascular efficiency screening (Figure 2.13). However, at higher flow rates, the viable radius remains relatively constant along the length of the gel and the diameter of this viable radius is also dependent on the inflow rate of the channel.

![Image](image.png)

**Figure 2.13: Computed oxygen concentration in 2D hydrogel models.** Oxygen concentration in PEGDA hydrogels containing simulated cell populations of 10 - 100 \(\times\)10\(^6\) cells/mL and an inflow of oxygenated media at \(1 \times 10^{-4}\) to 0.01 m/s through the central channel. Though gels with cell populations of 10 million cells/mL (A-B) did not consume oxygen rapidly enough to reach the critical oxygen concentration commonly associated with cell death\(^{79}\), the oxygen concentration in hydrogels with cell concentrations of 100 million cells/mL (C-D) did drop below this threshold. Scale bar = 2 mm.

As an example of the entire workflow, we started with a branched architecture generated by Nervous System, where fluid flow rates were computed using the LBM-based
HARVEY solver as established by Amanda Randles and colleagues. Again, LBM was used over traditional CFD approaches because of improved handling of complex boundaries and the highly parallelizable nature of LBM. Adding a slight crimp in one channel allowed us to demonstrate that a change in geometry will affect the fluid flow rate, which in turn affects the total vascular efficiency of this topology (Figure 2.14). As fluid flow in the crimped channel decreases, the flow rate falls below the level necessary for a constant viable radius, and therefore the viable radius linearly decreases across the length of the channel (Figure 2.14), as shown in (Figure 2.13). Though computational models can calculate oxygen transport in 3D, these models are computationally intensive, which restricts their application in the screening of vascular architectures. Therefore, this algorithm provides an alternative approach to screen different vascular architectures for rapid oxygen delivery to surrounding cells.

![Figure 2.14: Example calculation of vascular efficiency within two similar vascular architectures. A) Volumetric rendering of a vascular architecture, highlighting slice planes for D and E. B, C) 3D rendering of two different geometries based on the model given in A. Opaque channels in center of geometries highlight differences between the two models. D, E) 2D slices through geometries in B and C at 7 and 9 mm. Images show vascular area (white), viable cellular area (red), and remaining gel area (black). Dashed yellow circles highlight differences in viable area within the central channels. We calculate these modifications to the vascular topology results in a 15% increase of vascular efficiency relative to the original geometry B.](image)
In addition to these example geometries, we can calculate the fluid flow profiles through a wide range of vascular geometries as generated by Nervous System. By changing the parameters entered into the topological design algorithm, we can generate vascular geometries with a range of branching angles, channel densities, and channel tapering profiles. The LBM successfully solved fluid flow profiles through all generated vascular geometries and the results further support the importance of screening algorithms to determine how efficiently these different structures can deliver oxygen and nutrients to surrounding tissues (Figure 2.15).

![Diagram](image)

**Figure 2.15: Example fluid flow profiles through complex vascular geometries as solved using the LBM.** Example geometries with different branching parameters and channel densities demonstrate how the vascular structure plays a significant role in the resulting fluid flow profiles and the distribution of fluid flow throughout the different branches of the channel network, where red indicates high fluid flow rates and blue indicates regions of low fluid flow rates.

### 2.5.5 3D flow reconstruction using ultrasound imaging

We have previously demonstrated the capacity to measure fluid flow velocity in 2D, but the complex, branching networks described in this chapter have 3D flow profiles that cannot be sufficiently approximated in 2D. Therefore, we turned to Doppler ultrasound to measure 3D...
flow profiles our engineered channel networks. As demonstrated in Figure 2.16, we were successfully able to reconstruct 3D flow profiles using ultrasonic Doppler imaging. Furthermore, despite local irregularities in fluid flow, computational models generally aligned well with measured flow rates, though computed flow was up to 20% slower, likely due to the presence of some over-curing effect that likely reduced the size of the channels.

![Figure 2.16: Using ultrasound Doppler imaging to reconstruct 3D flow profiles through hydrogel channel networks.](image)

A) Schematic of the serpentine vasculature phantom. B) COMSOL model of expected flow patterns of 25% glycerol solution in the serpentine phantom. C) Cross-section of serpentine geometry in color Doppler (24 MHz) showing flow of suspended polystyrene beads into and out of the page. D) Reconstructed fluid flow profiles in 3D, where the red and green boxes are expanded in panels E and F, respectively. E) Flow profile inconsistencies were noted in regions with physical channel irregularities. F) Radially symmetric and parabolic flow profiles through the center of the channel. Color Doppler images acquired with collaborators Dr. Richard Bouchard and James Long.

### 2.6 Conclusions

Work in this chapter represents the first steps towards the closed-loop design of vascular structures in engineered tissues. We first explored the range of PEGDA formulations compatible with our pSLA fabrication technique, focusing on mixtures of 6 kDa and 35 kDa PEGDA, ranging in concentration from 5 to 50% w/w. Mass swelling ratio of the hydrogels depended on the PEG formulation, ranging from $5.6 \times (50\% \text{ w/w } 6\text{kDa})$ to $51.9 \times (20\% \text{ w/w } 35\text{kDa})$, while mechanical properties similarly depended on the PEG Formulation, ranging from $0.63 (20\% \text{ w/w } 35\text{kDa})$ to $403.8 \text{ kPa} (50\% \text{ w/w } 6\text{kDa})$. Next, to establish parity between our computational and experimental models, we fabricated simple microchannel networks then
used PIV to measure fluid flow rates through hydrogel channel networks and compared these results with computed flow rates through the same structures. Results showed only minor differences between these two models, signaling strong overlap between measured and computed flow profiles. After validating our computational models, we used these models to develop a custom algorithm for screening different vascular architectures for efficient delivery of oxygen to engineered tissues. Finally, we demonstrated the capacity to measure fluid flow profiles in 3D printed channel networks using ultrasonic Doppler imaging.

2.7 Future Work

Future work for this chapter involves correlating in silico vascular efficiency results with in vitro models containing cells and, in return, to use our in vitro results to improve computational modeling of nutrient transport in vascularized tissues.

2.7.1 In vitro analysis of vascular efficiency

Ian Kinstlinger has advanced our vascular efficiency analysis by developing a workflow to assess vascular efficiency in vitro. Using vascular architectures developed with Nervous System, Ian has seeded hepG2 cells within an agarose gel around a complex channel network and cultured these gels under perfusion over a period of days. Following perfusion, Ian has stained live sections of the hydrogel with MTT, a tetrazolium salt that is reduced by mitochondria of living cells to form a purple dye to map metabolic activity of the cells throughout the entire volume of the perfused hydrogel (Figure 2.17). These results demonstrate decreased cell viability over time, where the cells that remain viable on Day 3 are generally close to the perfused channel networks. Additionally, the purple staining around some channels appears stronger than others, which could suggest higher flow rates through specific branches of the vascular network. Though more work is necessary to make any conclusions, these preliminary in vitro results suggest that vascular efficiency assessments could be used to improve the design of perfused channel networks for better delivery of oxygen and nutrients in engineered tissues.
Figure 2.17: Example images for in vitro vascular efficiency analysis conducted by Ian Kinstlinger. A) Schematic of vascular network generated by Nervous System (blue) embedded in an agarose hydrogel (yellow). B) Top view of vascular geometry shown in A where open channels have been perfused with a blue dye. C) Slice of a gel seeded with hepG2 cells (taken from region within dashed lines in A and B), stained with MTT immediately after seeding. D) Similar slice of a hydrogel stained with MTT after 3 days of perfusion culture. Scale bar = 5mm.

2.7.2 Incorporation of vascular efficiency analysis into LBM models

The vascular efficiency analysis algorithm was designed to be simple enough that collaborators could incorporate efficiency analysis directly into HARVEY without the need to incorporate computationally expensive diffusive transport models. Because LBM easily handles complex geometries, it is possible that additional work with Amanda Randles and colleagues could incorporate efficiency parameters and adapt models in real time as they are solving, resulting in improved vascular architectures for oxygen and nutrient delivery.
Chapter 3

**Design and characterization 3D printed hydrogels containing functional bicuspid valves**

**3.1 Acknowledgements**

We thank Dr. Grande-Allen for her helpful suggestions regarding the structure and biology of venous valves. We thank Pete Galie of Rowan University and his students Nesrine Bouhrira and Brandon DeOre for their work in gathering the µPIV measurements and help with initiating fluid-structure interaction models. Thanks to Richard Figliola, Laura Chopp, and Kyle Yeomans at Clemson University for their work testing valve structures in a flow loop mimicking the venous muscle pump. Additionally, thank you to Charlene Sixuan Pan for her help developing the Python script used to incorporate stiffness patterning into our 3D printed hydrogels and for her work incorporating scattering agents into our print solution. Thanks to Palvasha Deme, and Nick Calafat and Charlene Pan for their assistance in 3D printing test valve gels. And finally, thanks to the NIH 3D print exchange for their 3D models of heart valves used in initial designs of bicuspid valves in this chapter (http://3dprint.nih.gov/discover/3dpx-000452; Model 3DPX-000452).

**3.2 Introduction**

In Chapter 2 we characterized different PEGDA formulations compatible with our pSLA fabrication technique and established computational models for fluid flow through 3D printed vascular structures. In this chapter, we apply these data to design, fabricate, and test dynamic vascular valves, which are essential structures within human venous and lymphatic networks. However, to effectively fabricate functioning bicuspid valves, further investigation into the structure-function relationship of engineered bicuspid valves is necessary. Therefore,
using the principle of closed-loop vascular design laid out in Chapters 1 and 2, we use computational and experimental models to improve the design of a functional bicuspid valve fabricated using our custom pSLA system.

3.2.1 Role of valves in vessels in vivo

Venous valves are essential structures in directing blood flow within the body. Venous and lymphatic valves are generally bicuspid (Figure 3.1)\textsuperscript{127,128}, containing two leaflets, and range from 20 µm to over 10 mm in diameter\textsuperscript{8,129}. Though monocuspid or tricuspid venous valves have been occasionally reported in vivo, Ghassan Kassab and colleagues have demonstrated that bicuspid valves have lower mechanical cost (lower solid shear stresses with higher wall shear stresses) than either mono- or tricuspid valves. This lower mechanical cost indicated that bicuspid valves were less likely to mechanically fail than mono- or tricuspid valves, and the group hypothesized that this increased resilience resulted in the predominance of bicuspid valves within the human venous system\textsuperscript{127}. Valves allow skeletal muscles to aid in the pumping process in low pressure vessels, such as veins of the legs and lymphatic vessels, where blood and lymph must fight against gravity to move back towards the heart\textsuperscript{7,8}. An example of this venous-muscle pumping mechanism in the legs and feet is outlined in Figure 3.2. During rest, blood from the superficial veins flows into the deep veins of the legs. When muscles of the calf contract, they compress the deep veins and valves force blood to flow towards the heart. Similar mechanisms apply during flexion and compression of the foot. This system of pumping works effectively, but requires a system of competent valves within the venous network.
Figure 3.1: Structure of the saphenous valve in the open and closed positions. A) Human saphenous vein valve in open position. Scale bar = 1 mm. B) Valve in closed position. C) Flow profile through valve in the open state, highlighting vortex formation within valve sinus (arrows) and development of low flow regions (boxes). Modified from Nam et al 2012.

Figure 3.2: Schematic of venous muscle pump action in the foot and calf. In the relaxed state, blood moves from the superficial veins (SV) to the deep veins (DV) within the legs. Then, during walking (active state), muscles (M) of the calf and foot contract, compressing the deep veins and causing blood to evacuate the vessels. Valves within these vessels ensure that blood flows in one direction, back towards the heart. From Stranden 2011.

When venous valves fail, pressure builds up within the veins and surrounding tissues (Figure 3.3), resulting in a condition termed chronic venous insufficiency (CVI). An
estimated 2.5 million people suffer from CVI in the US, with symptoms ranging in severity from the formation of varicose veins and edema, to ulceration, and chronic wound formation in approximately 20% of cases\textsuperscript{132}. CVI costs between 150 million – 1 billion dollars per year\textsuperscript{133} and results in the loss of nearly 2 million work days/year\textsuperscript{132}. Valve functionality is commonly measured by the pressure differential across a valve. The ambulatory venous pressure (AVP) refers to lowest mean pressure across the valve during activity, which is typically around 30 mmHg in healthy subjects, while the recovery time (RT) is the time it takes the vein pressure to return to resting pressures after activity is ceased, typically 20-30s\textsuperscript{131}. Insufficiency within venous valves inhibits the effectiveness of the venous-muscle pump system, causing AVP to rise and beginning the progression of CVI (\textbf{Figure 3.3})\textsuperscript{131}. Currently, CVI is typically addressed with mild treatments such as compression stockings; however, physicians have used more invasive treatments such as valvuloplasty\textsuperscript{134} or bioprosthetic valves\textsuperscript{135} to address severe cases of CVI. Ultimately, the causes of CVI and stages of progression remain unknown, supporting increased research into the structure-function relationship within venous valves.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure3.3.png}
\caption{\textbf{Figure 3.3: Schematic highlighting changes in venous pressure during activity}. During activity, contraction of muscles in the lower limbs forces blood out of the deep veins, while valves force blood to flow back towards the heart. During this process, the ambulatory venous pressure (AVP) typically decreases to around 30 mmHg in healthy veins. However, venous insufficiency decreases the effectiveness of the muscle pump and increases AVP. Additionally, the recovery time (RT) during which pressure in the veins returns to resting levels, is usually around 20-30s in healthy patients. Insufficient or leaky valves typically have a much faster RT than healthy valves. \textbf{From Stranden 2011}\textsuperscript{131}.}
\end{figure}
3.2.2 Current design and fabrication of valves

When designing dynamic vascular elements, the structure-function relationship becomes even more critical for the successful design of functional structures. Furthermore, because native valve function is undoubtedly related to the microstructure of the extracellular matrix, using our currently available palette of biomaterials, we may actually need to fabricate a slightly different structure to achieve similar function. For tissue engineering applications, various groups have engineered functional heart valves, or less commonly venous valves, using a wide variety of fabrication techniques, such as the recellularization of decellularized valves\textsuperscript{136,137}, molding cells in a sacrificial material\textsuperscript{138}, or 3D printing\textsuperscript{139}. However, because we have the capacity to fabricate a wide array of valve structures using our pSLA fabrication technique, we were interested in exploring the closed-loop design of functional bicuspid valves. Though researchers have demonstrated the use of computational models to explore the structure-function relationships within venous valves\textsuperscript{140,141}, we could not find instances where researchers used computational models to improve the design of their valve and fabricate structures based on the insight of their computational models. Here, we apply the exploration of materials and CFD models conducted in Chapter 2 to implement the closed-loop design of multi-scale bicuspid valves. Using this framework, we have developed highly adaptable \textit{in vitro} models for bicuspid valves to better understand the key structure-function relationships within tissue engineered bicuspid valves.

Later in this chapter we explore the potential to incorporate mechanical heterogeneity into our valve structures. Previous groups have demonstrated non-linear, anisotropic mechanical properties in heart valves\textsuperscript{142} as well as venous valves\textsuperscript{143}, where venous valves typically have higher circumferential than radial elastic moduli and mechanical properties of the valve leaflets depend on their location within a vein (proximal vs. distal)\textsuperscript{143}. Researchers have previously developed methods for incorporating mechanical heterogeneity into monolithic hydrogels by manipulating gel formulation\textsuperscript{144,145}, exposure time, gel post-processing\textsuperscript{146}, or through cell-directed matrix re-organization\textsuperscript{136}. However, our goal was to adjust mechanical properties we were interested in developing a method using only crosslinking time rather than changing materials or incorporating additional post-processing steps.
3.2.3 Computational modeling of valves

Computational modeling of fluid flow and stresses within biological valves presents many challenges compared with the modeling of static structures. First, computational models involving fluid-structure interactions present challenges due to the complex nature of modeling fluid flow through continuously changing structures. Additionally, models focusing on fluid flow or stress distribution within a closed valve must resolve the multiple contact points within closed leaflets. Finally, native valves typically display complex, non-linear mechanical characteristics requiring more advanced mathematical formulations. Previous groups have addressed these challenges using a variety of computational techniques to model fluid flow and stresses within native and engineered valves.

Early work by Charles Peskin and colleagues focused on developing improved methods for modeling fluid-structure interactions within complex fluids for better understanding of fluid flow through native and artificial heart valves. Buxton and Clarke used the LBM to model fluid flow through a valve, incorporating a lattice spring model to incorporate fluid structure interactions within the model. They demonstrated that valve response and total opening area time depends on the pressure differential across the valve. Additionally, their model highlighted the presence of recirculation zones within the valve sinus. Similarly, Hart et al. used computational models to calculate stresses experienced by aortic valves under flow, while Weinberg et al. developed multiscale models to analyze the progression of calcific aortic stenosis in aging heart valves. These model are unique in that they incorporate structural changes in valves over a patient’s lifetime, and, due to the multiscale nature of these simulations, they incorporate changes on the structural and tissue level scale within aortic heart valves. However, despite these impressive advances in modeling techniques, there remains a challenge in validation techniques for these models. Though many groups have made significant progress developing computational or experimental models of valves, we have not come across any work applying computational models of valves towards the closed-loop design of functional bicuspid valves in vitro.

3.3 Objectives

The objective of this chapter is to apply the material characterization and computational models developed in the second chapter of this thesis and apply these data to dynamic valve
structures. We additionally began to explore the potential for incorporating heterogeneous mechanical properties into our 3D fabrication technique. Our goals were as follows:

1) Demonstrate feasibility for fabricating functional mesoscale bicuspid valves using pSLA of biocompatible hydrogels.
2) Develop fluid structure interactions models of valves using COMSOL Multiphysics, then screen the function of different valve geometries.
3) Fabricate selected valve structures and assess fluid flow profiles through valves.
4) Assess valve function compared to prosthetic valves using an in vitro flow loop.
5) Demonstrate feasibility to incorporate 3D heterogeneity into monolithic hydrogels using pSLA.

3.4 Materials and Methods

3.4.1 Valve design and fabrication

We first generated a 3D model for a bicuspid valve based on the geometry the 3D human aortic valve model available from the NIH 3D Print exchange (Model ID 3DPX-000452). We fabricated our models using our pSLA system described previously, in Chapter 2. Valve geometries were later adapted based on investigations using a computational fluid dynamics model. Additionally, to prevent any over-curing defects from interfering with leaflet closure in small valves, we fabricated valves so the axis of the channel was parallel with the projection. For these small valves the concentration of tartrazine in the print solution was increased to 3.45 mM to help prevent over-curing of the gels.

3.4.2 Developing a preliminary FSI model of venous valves:

Computational models were developed in COMSOL Multiphysics 5.3a using the fluid-structure interactions (FSI) physics module. Models were developed in 2D using a time-dependent study, where valve geometry was constructed within COMSOL to match a cross-section at the center of a given valve geometry. Using this computational model, we iteratively altered valve geometry, focusing on three parameters: leaflet length (LL), leaflet gap width (LG), and sinus depth (SD) (Figure 3.5). The gel geometry was built around a channel 2 mm in diameter and 200 µm thick leaflets. LL ranged from 1000 to 2000 µm in 250 µm increments, LG ranged from 400 to 800 µm in 100 µm increments, and SD ranged from 0 to 850 µm in 200 µm increments. To prevent contact between the leaflets (where contact would
significantly increase complexity of solving), our workaround involved introducing a transient spring force (20 N/m) applied to the inner most tip of the leaflets if the leaflets came within 100 µm of each other. After screening 125 leaflet geometries using a Young’s modulus of 25 kPa (corresponding to a PEGDA formula of 10% w/w 6kDa), select geometries were screened using a Young’s modulus of 75 kPa (corresponding to 40% w/w using a 1:1 ratio of 6:35 kDa PEGDA), as would be used to fabricate large gels for flow studies. Initial valve gels were fabricated using the 10% w/w formulation because this formulation demonstrated consistent printability and favorable mechanical properties in small scale gels. However, with the increased mechanical demands of larger scale gels in the in vitro flow loop, we needed a more robust PEGDA formulation and turned to the stiffer, 40% w/w formulation. A parameter list for the COMSOL models can be found in Table 3.1, where the dynamic viscosity of the fluid and the Poisson’s ratio for PEGDA where taken from the literature. Leaflet location was analyzed using two COMSOL domain probes, tracking the inner most locations of each leaflet, while the fluidic resistance was calculated by integrating the flow profile at the valve outlet divided by the total pressure differential (10 Pa) as described in Equation 3.1.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Description</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>ρ</td>
<td>Density of fluid</td>
<td>1000 kg/m³</td>
</tr>
<tr>
<td>μ</td>
<td>Dynamic viscosity of fluid</td>
<td>0.001 Pa·s (1 cP)</td>
</tr>
<tr>
<td>E</td>
<td>Young’s modulus</td>
<td>25 &amp; 75 kPa</td>
</tr>
<tr>
<td>ν</td>
<td>Poisson’s ratio</td>
<td>0.49</td>
</tr>
<tr>
<td>P_in</td>
<td>Time dependent pressure at inlet</td>
<td>10<em>abs(cos(2</em>pi*t)) Pa</td>
</tr>
<tr>
<td>P_out</td>
<td>Time dependent pressure at outlet</td>
<td>5<em>abs(cos(2</em>pi*t)) Pa</td>
</tr>
</tbody>
</table>

Table 3.1: Physical parameters used in FSI model of valve movement.

3.4.3 Mapping flow profiles through valve:

Micro-particle image velocimetry (µPIV), with collaborator Pete Galie and colleagues, was used to quantify flow fields through the microscale valve geometries (Figure 3.9). Polystyrene beads labeled with Nile Red (Peak excitation: 640 nm; mean diameter 1.95 µm; Spherotech Inc, Lake Forest, IL) were suspended in PBS at a dilution of 1:500 and were illuminated with a dual pulse Nd:YAG laser (125 mJ/pulse, 15 Hz, 532 nm; Dantec Dynamics; Skovlunde, Denmark). The pulse width of the laser was set to 50 µs with a total testing period of one second. Particles were visualized 4× objective on a Nikon Ti-E inverted microscope, where the focal plane was set to the mid-plane of the valve channel. Images were captured
using a high-speed, high-resolution double-frame CCD camera (FlowSense EO, Dantec Dynamics; Skovlunde, Denmark), while images were analyzed using Dynamic Studio software (Dantec Dynamics, Skovlunde, Denmark) using an adaptive PIV algorithm, as described previously in Theunissen, Scarano and Riethmüller\textsuperscript{156}.

**3.4.4 Testing functionality of valves:**

To conduct more in-depth characterization of our valves, we worked with collaborator Richard Figliola and colleagues, using a lumped parameter model modified from Snyder and Rideout\textsuperscript{157} to generate a fluidic circuit mimicking the pressure occurring in the deep veins within the legs. The final model is shown in Figure 3.4\textsuperscript{157} and was developed by R. Gorman and L. Chopp and improved upon by K. Yeomans\textsuperscript{158,159}.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure34.png}
\caption{Lumped parameter model mimicking venous circulation of the legs. From Laura Chopp\textsuperscript{160}.}
\end{figure}

Resistance elements in the model are categorized as either resistors due to valves or tubes. Resistance (R) in the flow loop is controlled by a combination of needle and ball valves and the resistance presented by the tubing itself and can be determined by using pressure transducers and a flow probe. Resistance in the flow loop in a fluid circuit is expressed by Equation 3.1. Compliance (C) in a fluid circuit is given by Equation 3.2. Compliance elements in the flow loop consist of air chambers and a calf muscle pump compliance chamber. In the case of the muscle pump the transmural pressure is specified in an elastic tube, because the varying external pressure applied by the calf muscle upon the deep veins powers the pumping action. For static compliance elements, we used air chambers as compliance elements, where the compliance of the air chambers used in the flow loop can be calculated using Equation 3.3.
\[ R = \frac{\Delta P}{Q} \]

**Equation 3.1: Resistance in flow loop.** Where \( \Delta P \) presents the pressure drop across the resistor and \( Q \) is the flowrate through the resistor\(^{161} \).

\[ C = \frac{\Delta V}{\Delta P} \]

**Equation 3.2: Compliance in a fluid circuit.** Where \( \Delta V \) is the change in volume and \( \Delta P \) is the change in pressure\(^{162} \).

\[ C = \frac{V}{nP} \]

**Equation 3.3: Compliance within air chambers within flow loop.** Where \( V \) is the volume of trapped air, \( P \) is the absolute pressure, and \( n \) is the process factor\(^{162} \).

### 3.4.5 Incorporating 3D heterogeneity into monolithic valve structures:

To incorporate stiffness heterogeneity into our 3D printed structures, we developed a custom Python script to rearrange the ASCII G-code generated by the slicer and host controller, Creation Workshop. Briefly, this script allows the user to generate a 3D model containing defined regions with different exposure times, as indicated by their position in the z-direction. After slicing the stacked 3D model, the Python script then re-shuffles the generated G-code resulting in different lengths of curing for different regions of the hydrogel. The script results in automated printing including mechanical heterogeneity of the hydrogels.

### 3.4.6 Ultrasound imaging of hydrogels containing differentially patterned regions

With collaborator Richard Bouchard and colleagues, we used ultrasound imaging to demonstrate changes in hydrogel properties due to differential light exposure. Ultrasound imaging was performed using a maximum intensity projection, where C-scans were acquired with a Verasonics128 ultrasound platform using an L11-4V linear array with a nominal 7 MHz center frequency; 3D data were acquired with an automated stage translating in the elevation dimension.

### 3.4.7 Statistical Analysis

Data are reported as mean ± standard deviation and are compared using an unpaired, two-tailed Student’s t-test unless otherwise specified.
3.5 Results and Discussion

3.5.1 Valve fabrication and design analysis

We have demonstrated the capacity to 3D print functional bicuspid valve structures using our pSLA printing technique. These 3D printed bicuspid valves function as fluidic diodes, opening under forward flow and closing in the presence of retrograde flow. Due to the transparency of our hydrogels, the leaflet performance was readily observable under trans-illuminated microscopy with manually-actuated flow via syringe (Figure 3.5). However, further exploration of a small range of valve architectures revealed that not all valves closed properly under manual retrograde flow (Figure 3.5). Three-dimensional printing could in theory fabricate nearly arbitrary geometries; to focus structural permutations worth exploring, we identified three geometric parameters of valves whose roles in valve structure function relationships we sought to investigate further: Leaflet Gap (LG), Leaflet Length (LL) and Sinus Depth (SD) (Figure 3.5).

![Figure 3.5: Feasibility of design and fabrication of dynamic fluidic valves using pSLA. A) Schematic of bicuspid valve demonstrating two leaflets followed by an enlarged sinus region, highlighting structural elements explored in this paper. LL = leaflet length, LG = leaflet gap, SD = sinus depth. B) Initial valve design in the open position under forward flow, and the same valve in the closed position under retrograde flow. C) Modified valve design in the open under forward flow, but still partially open under retrograde flow.]

3.5.2 Fluid-structure interactions models for valve structures

We next used computational models to investigate the role of valve geometry and matrix stiffness on the functionality of our printed valves, where example models are shown in Figure 3.6. When assessing how specific features affected valve function (Leaflet Length, Leaflet Gap, Sinus Depth), we noticed that the width between valve leaflets had the most
noticeable effect on the movement of valve leaflets, where a starting gap greater than ~25% of the original channel diameter (greater than 500 µm for a 2000 µm channel) prohibited the leaflets from fully closing under initial retrograde flow (Figure 3.7). However, decreasing the initial gap distance between leaflets resulted in an increasing fluidic resistance of the valve. These opposing leaflet design considerations may provide a tractable means to optimize a given starting topology. The length of the valve leaflets also had a noticeable difference on the capacity of the valve to close, where leaflets shorter than 1750 µm (87.5% the width of the channel) also failed to fully close. However, leaflet length had an inverse correlation with fluidic resistance of the valve, where longer leaflets resulted in lower levels of fluidic resistance (Figure 3.7).

![Figure 3.6: Example solutions from FSI models for valves under pulsatile flow.](image)

**Figure 3.6: Example solutions from FSI models for valves under pulsatile flow.** COMSOL model of bicuspid valves under forward and retrograde where dotted blue lines indicate starting position of valve leaflets. Both valve geometries have leaflet lengths of 2000 µm and leaflet gap distances of 500 µm. However, the valve geometry in A does not have a sinus while the valve in B has a sinus depth of 650 µm. White asterisks in B (right) highlight additional fluid flow moving behind the valve leaflets, as compared to flow in valves without a sinus. Scale bar = 500 µm.

The next valve element we investigated is the sinus region. The valve sinus has been suggested to aid in valve leaflet closure and the prevention blood stagnation and clot formation between valve leaflets and the vessel wall\(^{163,164}\). However, an enlarged sinus is often not incorporated into bioprosthetic valves due to the added fabrication challenge\(^{165}\). To investigate the role of the sinus region in valve function, we tested valve structures with sinus depths ranging from 0 to 850 µm, and with respect to leaflet movement, the sinus depth appeared to
have only a very subtle influence (Figure 3.7). Though we did not observe significant influence of the valve sinus in regulating leaflet movement or leaflet strain, we wanted to investigate the role of sinus depth in regulating stagnant volumes within the valve, because stagnant blood is prone to clotting\textsuperscript{18,166}, and the sinus regions of valves have been suggested as potential initiators of deep vein thrombosis\textsuperscript{132,167}. From our computational models, there appears to be only a subtle difference between the flow patterns of valves containing sinuses of different depths (Figure 3.6). Valves containing a larger sinus region (deeper than 250 µm) appear to have more fluid flowing back behind the leaflets under retrograde flow, as shown by the yellow arrows and white asterisks in Figure 3.6, which may aid in the closure of the valves \textit{in vitro}. Given these results, we decided to further investigate the role of the valve sinus \textit{in vitro} using µPIV measurements to map fluid flow profiles through the valve and using an \textit{in vitro} flow loop to assess valve functionality in the presence or absence of a sinus region.

![Figure 3.7: Results from FSI models exploring structure-function relationships in bicuspid valves. A) Plot of leaflet movement over time for 125 different valve geometries. B) Minimum distance between leaflets during valve closure. (t=0.77 s) C) Fluidic resistance of valves during fully open state (t=0.51 s).](image)
Additionally, for studies using an *in vitro* flow loop, ideal valves would withstand pressure differentials upwards of 80 mmHg as occur *in vivo*. Therefore, we also ran our computational models for the selected geometries using a Young’s modulus of 75 kPa, corresponding to a PEGDA formulation of 40% w/w 1:1 6:35 kDa. Results from these models suggest that a smaller gap distance between the leaflets is necessary for efficient closure of the valves (Figure 3.8). An additional screen of leaflet gap from 500 to 300 µm revealed that a 300 µm gap should be sufficient for valve closure using this stiffer PEGDA formulation. These results confirm our previous hypothesis that mechanical properties can have a noticeable influence on valve function and will need to be considered for future applications involving different PEGDA formulations or the incorporation of anisotropic properties into valve design.

**Figure 3.8** Results from FSI computational models exploring the influence of hydrogel stiffness on valve function. A) Previous valve geometry (LG = 500 µm; LL = 2000 µm; SD = 650 or 0 µm) but with increased material stiffness at 75 kPa. B) Modified valve geometries with LG reduced to 300 µm. C) Plot of leaflet movement over time demonstrating that increased materials stiffness interferes with effective valve closure at the given pressures. However, decreasing the initial gap distance between leaflets restores the valve’s capacity to close under retrograde flow.
3.5.3 Experimental measurements of fluid flow patterns through valves

To map flow profiles through our two different valve structures (with or without a sinus) *in vitro*, we used a µPIV system to experimentally track the movement of suspended fluorescent particles through the different valve structures in both the open and closed states. Valves for µPIV testing were designed and fabricated according to dimensional parameters given in Table 3.2, where final measured dimensions correlated well with the expected dimensions.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Desired Dimension (mm)</th>
<th>Measured Dimension (mm) (±Standard Deviation)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Channel width</td>
<td>2</td>
<td>1.97 (± 0.06)</td>
</tr>
<tr>
<td>Leaflet thickness</td>
<td>0.2</td>
<td>0.18 (±0.04)</td>
</tr>
<tr>
<td>Leaflet length (LL)</td>
<td>2</td>
<td>2.02 (± 0.09)</td>
</tr>
<tr>
<td>Leaflet gap (LG)</td>
<td>0.5</td>
<td>0.52 (± 0.07)</td>
</tr>
<tr>
<td>Sinus depth (SD)</td>
<td>0.65</td>
<td>0.58 (± 0.06)</td>
</tr>
</tbody>
</table>

Table 3.2: Dimensions for small valves used in µPIV testing. Values given as mean (± standard deviation). N = 37 for all values except valve sinus, where N = 20.

Fluid flow profiles demonstrate that both valves with and without a sinus allowed forward flow while preventing retrograde flow (Figure 3.9). Under forward flow both valves had small vortices forming just past the leaflets, but flow appeared to be relatively stagnant near the junction between the leaflet and the wall of the channel. This is a different observation than found in a previous design where we saw notable vortices formation in the sinus region in valves with shorter leaflets positioned at angles that were more interrupting to flow (Figure 3.10). However, under retrograde flow, both valve geometries had noticeable vortices forming on either side of the leaflets. Vortices in valves without enlarged sinus regions had chaotic, disorganized flow, while flow in valves with enlarged sinus regions resulted in the formation of four organized vortices with counter-current rotation (Figure 3.9), as has been described in native valves\(^\text{168,169}\). Despite the reduced vortices in the sinus region of this updated geometry under forward flow, we believe that the geometries described in Figure 3.9, are superior to previously used geometries (Figure 3.10) due to their reduced fluidic resistance (as shown by FSI models) and their increased resilience, handling pressures up to 40 mmHg while previous design (Figure 3.10) only managed to withstand 20 mmHg within an *in vitro* flow loop.
Figure 3.9: Results from µPIV testing for selected valve geometries (LL = 2mm, LG = 0.5 mm, and SD = 0.65 or 0 mm). A) Image of valve geometry containing a sinus. Scale bar = 500 µm. B) PIV-computed flow profile through valve geometry (with sinus) under forward flow at 10 mL/min, where dashed white lines highlight the edges of valve geometry. Representative image from N = 2. C) Fluid flow profile through valve with sinus under retrograde flow at 4 mL/min. N = 1. D) Image of valve geometry without an enlarged sinus region. Scale bar = 500 µm. E) Forward flow through valve without enlarged sinus at 7 mL/min. Representative images from N = 2. F) Retrograde flow through valve without an enlarged sinus region, representative image from N = 5.

Figure 3.10: Previous valve geometry with shorter leaflets and a smaller leaflet gap distance. A) Valve geometry in the open position. B) Valve geometry in the closed position. C) PIV image data demonstrating formation of vortices in the valve sinus region, even during the open phase. Scale bar = 500 µm.
### 3.5.4 Gel functional parameters

Some of the most essential venous valves are those within the deep veins of the leg, which typically have diameters up to 1 cm\(^1\)\(^2\). Therefore, we were interested in testing the functionality of valves with diameters of 1 cm fabricated using the 40% 1:1 6:35 kDa (Young’s modulus of 75kPa and ultimate strain of 50%) under physiologically relevant pressure differentials. Valves fabricated at this scale likewise matched the desired dimensions, where dimensions were measured using phase contrast imaging after the gels had reached equilibrium swelling (Table 3.3).

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Desired Dimension (mm)</th>
<th>Measured Dimension (mm) (± Standard Dev)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Channel width</td>
<td>10</td>
<td>9.96 (± 0.17)</td>
</tr>
<tr>
<td>Leaflet thickness</td>
<td>1</td>
<td>1.01 (± 0.05)</td>
</tr>
<tr>
<td>Leaflet length (LL)</td>
<td>10</td>
<td>10.03 (± 0.18)</td>
</tr>
<tr>
<td>Leaflet gap (LG)</td>
<td>1.5</td>
<td>1.31 (± 0.29)</td>
</tr>
<tr>
<td>Sinus depth (SD)</td>
<td>3.25</td>
<td>3.09 (± 0.13)</td>
</tr>
</tbody>
</table>

**Table 3.3: Dimensions for large valves used in flow loop testing.** Values given as mean (± standard deviation). \(N = 29\) for all values except valve sinus, where \(N = 15\).

Though native valves must regularly function in pressures upwards of 80 mmHg\(^3\), pressures this high caused tearing and prolapse in the leaflets of our fabricated valves. However, we demonstrated that valves could consistently handle pressures of 40 mmHg, half the pressure regularly experienced \textit{in vivo}. When placed on a flow loop, designed and implemented by collaborator Richard Figliola and colleagues, that mimics the pumping action of the muscles within the lower leg\(^4\)\(^5\) both valves geometries resulted in pressure curves similar to those of native valves, albeit at lower pressures (Figure 3.11). Further analysis indicates subtle differences between the two valve geometries, where valves with a sinus had a significantly lower average AVP than valves without a sinus at 15.22 (± 4.44) and 18.72 (± 3.65) mmHg, respectively. Additionally, valves with a sinus had a longer recovery time than valves without a sinus (7.37 and 6.36 s), though results were not significant. These results indicate that the sinus region influences valve function and may help in preventing reflux (Table 3.4).

Though we had varying success with the robustness of printed valve structures, successful valves were able to open and close over 3600 times at pressures of 40 mmHg and a cycling frequency of 1 Hz. We also noticed that valves containing a sinus region appeared to
be more robust, withstanding a significantly higher number of cycles to failure than valves without a sinus, which suggests that the valve sinus could play a role in stress distribution and handling within the valves (Figure 3.11D). In summary, our 3D printed bicuspid valves were able to mimic the function of native valves at pressures up to 40 mmHg. Additionally, data from flow loop testing suggests that the valve sinus does play a notable role in valve function with respect to preventing reflux and in managing stresses on the valve leaflets and preventing valve failure (Figure 3.11). Though these critical roles of the valve sinus were not indicated by computational models, computational modeling did allow us to effectively analyze a wide variety of valve structures and select the most promising geometries for further testing in vitro.

In these studies we investigated the influence of three different geometric parameters on valve function, but using our CFD models, we could just as easily modify any given valve geometric parameter or mechanical property and investigate its effect on valve function. We can then apply these results to measure functional parameters of select valve geometries in vitro. This process demonstrates the versatility of our design approach, using medium-throughput screening of valve structures in silico before continuing lower-throughput testing in vitro. As an additional note, we noticed some discrepancies between computational and experimental valve models, which likely result from using a 2D CFD model predict functionality in a 3D valve. However, using 2D CFD models significantly increased our screening throughput for these time-dependent models as compared to 3D models, which often take hours rather than seconds to solve for a single geometry. Ultimately, we believe that the added breadth of 2D models outweighs the subtle inconsistencies between in silico and in vitro results. Indeed, for future studies we could also first apply our 2D FSI models to select valve geometries for further testing in 3D FSI models.
Figure 3.11: Ankle venous pressure response for selected valve geometries. A) Image of a valve geometry containing a sinus, scaled up to have a channel diameter of 10 mm. Scale bar = 2 mm B) Image of the same valve geometry without a sinus. Scale bar = 2 mm. C) Valve ankle pressure response using the fluidic lumped parameter model, where the maximum pressure was 40 mmHg. D) Plot showing measurements for cycles to failure in valves with (N = 24) and without (N = 12) a sinus. Bars indicate median with upper and lower quartiles. * indicates significant difference as determined by Student’s two-tailed t-test, p<0.05.

<table>
<thead>
<tr>
<th>Functional Parameter</th>
<th>Valve with Sinus (N=18)</th>
<th>Valve without Sinus (N=10)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AVP (mmHg)</td>
<td>15.22 (± 4.44)*</td>
<td>18.72 (± 3.65)*</td>
</tr>
<tr>
<td>Time to AVP (s)</td>
<td>7.94 (± 1.26)</td>
<td>7.20 (± 1.03)</td>
</tr>
<tr>
<td>RT (s)</td>
<td>7.37 (± 3.66)</td>
<td>6.36 (± 3.95)</td>
</tr>
</tbody>
</table>

Table 3.4: Comparison of functional parameters for valves with or without a sinus. Summary of valve functional parameters, where data is displayed as mean (± standard deviation). * indicates significant difference as determined by Student’s two-tailed t-test, p<0.05.

3.5.5 Incorporating mechanical heterogeneity into 3D printing PEG hydrogels

Native valve function is highly dependent on the mechanical properties of the valve leaflets, which are viscoelastic and anisotropic. To test our capacity to incorporate mechanical heterogeneity into our hydrogel structures, we started by generating simple strips for mechanical testing to assess whether curing time would affect the mechanical properties of our PEG hydrogels. Results indicated that for 10% w/w 6 kDa PEGDA hydrogels increasing the exposure time from 8.5 seconds per layer to 20 seconds per layer increased the tensile modulus significantly from approximately 80 kPa to above 100 kPa (Figure 3.12). Additionally, we demonstrated that patterning our increased exposure times resulted in
anisotropic mechanical properties within the gels. Hydrogel strips with additional exposure time and vertical patterns had significantly higher tensile modulus than gels with horizontal stripes, but non-significantly different stiffness than hydrogels exposed for the full 20 seconds.

Figure 3.12: Demonstration that curing time affects mechanical properties of 3D printed PEGDA hydrogels. A) Schematic of printing for hydrogel strips. B) Young’s Modulus of printed hydrogels fabricated using different, or patterned, exposure times. * indicates p<0.05 and ** indicates p<0.01 for multi-way ANOVA with Tukey-HSD post-hoc test.

After starting with a simple 2D pattern, we moved onto demonstration of 3D manipulation of mechanical properties. Using our pSLA setup and our custom Python script we incorporated 3D stiffness patterning into our hydrogels. In Figure 3.13 we show a stiffer volumetric star pattern fully encased in an otherwise monolithic PEGDA hydrogel. Additionally, we have demonstrated that this stiffness patterning can be visualized using ultrasound imaging, suggesting this approach could be utilized as reproducible anisotropic tissue phantoms for ultrasound elastography imaging, which uses shear waves to map the mechanical properties of specimens in 3D and has found increasing uses in cancer and tissue engineering research.99,170,171
Figure 3.13: 3D stiffness patterning using pSLA in monolithic PEGDA hydrogels. A) Front view photograph of a gel printed using pSLA where the bulk of the gel is printed with an 8.5s exposure, while the volume of the star was printed with an additional 20s exposure (28.5 s exposure total). B) Top view of the gel shown in A, demonstrating 3D patterning capabilities. C) Ultrasound image of gel shown in A demonstrating that patterning has an effect on gel properties. Scale bar = 2.5 mm.

3.6 Conclusions

In this chapter we progressed from the development of static vascular networks to the development of dynamic intravascular elements, focusing on bicuspid valves. Valves are essential structures within the body and demonstrate the complexity of native vascular architectures. Moving from the fabrication of static channel networks to dynamic vascular structures is a non-trivial task, in which the structure-function relationship in engineered tissues becomes even more critical in achieving the desired function.

We have demonstrated the use of computational modeling in the closed-loop design of dynamic bicuspid valves the function effectively as fluidic diodes. After screening 125 valve geometries in silico we identified key geometric parameters that influence valve closure and fluidic resistance. We demonstrated the formation of mirror-image vortices in valve sinus regions and that our fabricated valves demonstrate functionality similar to native valves, albeit at lower pressures. Additionally, though we noticed some discrepancies between our in silico and in vitro results, likely due to using 2D computational models to predict functionality of 3D structures, we believe the added screening throughput of 2D computational models outweighed these minor discrepancies. Though this work provides a more comprehensive
understanding of structure-function relationships within venous valves, it also demonstrates the necessity for the use of computational and experimental models in concert when developing complex vascular structures.

3.7 Future work

Though we began this work using relatively simple materials with isotropic mechanical properties, we have developed a framework that can be applied for future work using more advanced materials. As we continue our work exploring stiffness patterning of 3D printed hydrogels, we can expand this work to incorporate anisotropy in our printed valves. As the field of 3D printing advances including the expansion into more complex, printable materials incorporating cells, which can reorient, degrade, and deposit their own ECM, computational modeling can incorporate more complexities resulting in more physiologically relevant structures for tissue engineering and regenerative medicine applications.

3.7.1 Patterning anisotropy into valve leaflets

For future extensions of this work, our capacity to incorporate heterogeneity into our fabricated structures could allow the incorporation of heterogeneity within individual leaflets akin to native valve leaflet anisotropy. In addition to the research describing anisotropic properties of valves\textsuperscript{142,143}, Buxton and Clarke describe that the sinus region within the valve is less stiff than other regions of the valves\textsuperscript{151}. Therefore, this approach could allow detailed investigation of the effect of different stiffness patterns, such as those shown in Figure 3.14, on fluid flow and valve function.

![Figure 3.14: Schematic of anisotropic stiffness patterning in valve leaflets.](image)

To generate anisotropic behavior in the leaflets we can vary exposure time to alter mechanical properties of the PEGDA hydrogel. Here, we highlight three different patterning options for the valve leaflets, where darker regions indicate areas with increased exposure time and stiffness.
3.7.2 Use printing to develop models of diseased or insufficient valves

Various research groups have investigated the many causes of venous insufficiency, including poor attachment of valve leaflets to the wall of the valve, leaving a gap\textsuperscript{172}, or that varicose veins may result from infiltration of inflammatory cells and an increase in fibrous connective tissue\textsuperscript{173}. Using 3D printing could allow for the selective and sequential modification of valve structure, while the stiffness patterning could allow us to locally manipulate valve stiffness, mimicking disease progression \textit{in vivo}.
Chapter 4

Development and in vivo surgical anastomosis of 3D printed vascular networks

Portions of this work have been previously published in Sooppan et al. 2016.

4.1 Acknowledgements

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4.2 Introduction

As mentioned in Chapter 1, adequate graft vascularization and efficient integration with host circulation are paramount in translating large-scale engineered tissues into clinically relevant therapies\textsuperscript{174}. Engineering a thick and complex scaffold that can sustain a metabolically active cell population requires a highly organized, built-in vascular network. Although tremendous effort has been placed on studying new 3D scaffolds among other novel engineering techniques to enhance cell survival and integration, less effort has been placed on a direct transplant surgery model for graft implantation\textsuperscript{175}. Currently, most techniques for implanting pre-vascularized tissue constructs rely on the ingrowth of host vessels and wrapping and tapping anastomosis for perfusion of the engineered vasculature\textsuperscript{176–179}. This method of anastomosis has been shown to take days to weeks to perfuse the implanted microvascular network and appears to be highly dependent on the geometry of the microvascular network\textsuperscript{34,179,180}. However, for scaffolds larger than a few millimeters this time frame may be too slow to ensure the viability of cells within metabolically active tissues\textsuperscript{48}.

4.2.1 Challenges with small diameter vascular structures

Though there has been noted success in the development of large diameter vascular grafts from materials such as expanded polytetrafluoroethylene (ePTFE), polyethylene terephthalate (Dacron), and polyurethane, these materials do not function well for small diameter vascular grafts (<6 mm) due to challenges with clotting at reduced flow rates of smaller vessels and due to challenges with compliance mismatch between the vessel wall and the stiff graft material\textsuperscript{181}. Clotting has been a major challenge in the development of vascular replacements, where clotting is typically triggered by three factors commonly referred to as Virchow’s Triad: blood stasis, endothelial injury, and altered coagulability\textsuperscript{182}. For example, part of the success in developing large diameter vascular grafts has partially resulted from the fact that high blood flow rates through these large diameter vessels minimizes clotting; however, as blood flow rates decrease into smaller vessels, the opposite occurs. Additionally, these vascular grafts only focus on bypassing occluded or damaged vessels, and rarely include branches for connections with capillaries or smaller vessel networks that would nourish surrounding tissues. Therefore, to address the challenge of an implantation strategy to rapidly perfuse pre-vascularized networks, we demonstrated surgical strategies where perfusion
through 3D-printed vascular networks can be instantiated during surgery. Following implantation, we used a modified hind limb ischemia model to assess the patency and flow of gels containing 3D-printed vascular networks following in vivo implantation.

4.2.2 Surgical anastomosis between engineered and native vessels

Previous groups have demonstrated the direct anastomosis between engineered and native vascular networks, and a multitude of examples come from the field of tissue decellularization and recellularization. For example, Laura Niklason and colleagues have pioneered the field and have demonstrated preliminary success implanting engineered lungs in vivo for short time periods. Lungs were decellularized and seeded with rat lung epithelium and lung microvascular endothelium then cultured for a week in a bioreactor before being implanted in vivo for periods up to 2 hours. However, there were challenges in maintaining barrier function of small vessels and coverage with endothelial cells. Additionally, Uygun et al. demonstrated similar success implanting an engineered liver based on decellularization and recellularization technique, where the engineered tissue was implanted in vivo for up to 8 hours before significant clotting occurred.

As mentioned previously in section 1.3, Milica Radisic and colleagues have demonstrated the in vivo implantation of microfluidic channel networks in the femoral vessels of a rat, which remained patent for up to 1 week. The small scaffolds were fabricated by individual layer fabrication and sequential lamination. However, we believe that the automation of 3D printing will increase the opportunities for scaling up the overall size and production of vascularized tissues while providing more options for customization for vascular architectures, as demonstrated in Chapter 2. Another example from Jason Spector and colleagues involves 3D printing of a sacrificial Pluronic F127 loop which is embedded in a collagen gel before the pluronic loop is removed by aqueous dissolution, leaving an open channel. The Spector group demonstrated that using a polyglactin surgical mesh applied to the collagen gel, they could surgically anastomose the channels of their engineered vessel to the femoral artery and vein. These channels remained patent between 15 min to 24 hours in vivo. However, we believe that our pSLA system has more control over vessel geometry and we hypothesized that our constructs could yield equivalent or longer patency times.
4.3 Objectives

We moved from the development of vascular elements *in vitro* and *in silico* to the *in vivo* implantation and direct anastomosis of 3D printed micro-channel networks. The direct anastomosis of engineered vascular networks ensures that tissues are immediately perfused with blood and that cells within the tissues can rapidly receive oxygen and nutrients.

This chapter has been accomplished in three studies. In this first studies of this chapter, we focused on the development of a surgical technique to anastomose 3D printed micro-channels to native vessels. We first engineered and assessed the patency *in vivo* of polydimethylsiloxane (PDMS) constructs with built-in microchannel networks that mimic an integrated microvascular network. Although not hemocompatible, PDMS constructs enabled us to establish the initial surgical implantation model. In the second portion of this chapter, we adapted this surgical technique for the implantation of more hemocompatible PEG-based hydrogels to assess the hemocompatibility of our constructs in the same rat model. For the final stage of this chapter, we moved from a small-animal model to a larger porcine model. Our goals were to:

1) Develop a surgical technique to surgically anastomose 3D printed microchannel networks to native rat vasculature.

2) Design, test, and implant PEGDA hydrogels containing channel networks for surgical anastomosis in a rat model

3) Design, test, and implant PEGDA hydrogels containing channel networks for surgical anastomosis in a porcine model.

Despite mixed results, we believe this chapter serves as an important milestone toward the bioengineering and implantation of complex 3D tissues and potentially vital organs. The ability to develop an internal microvascular network within a hydrogel and surgically connect the microvasculature into the host vascular network is an essential step toward generating large vascularized grafts with cellularized interstitial tissue which can immediately integrate with host tissue.
4.4 Study 1: Develop a surgical technique to surgically anastomose 3D printed microchannel networks to native rat vasculature.

We started by developing a technique to surgically anastomose and monitor 3D printed channel networks in vivo.

4.4.1 Materials and Methods: Development of Surgical Techniques

4.4.1.1 Fabrication of PDMS Gel with micro-channels.

PDMS gels were generated as described previously in Miller et al, 2012. Briefly, sugar lattices of isomalt and dextran were printed using a ShopBot desktop D2418 router (ShopBot, Durham, NC) that we modified to hold an extrusion print head. Sugar lattices were printed using the geometry shown in Figure 4.1. The lattices consist of layered wells surrounding and supporting two primary vessels (approximately 1 mm in diameter) on top of 4 secondary vessels (approximately 300 μm in diameter). After the lattices cooled, the ends of the cross channels stretching between the primary channels and the well wall were removed using a fine tipped soldering iron. Additionally, one end of each of the main vessels was removed to generate a structure with a single inlet and outlet, as shown in Figure 4.1. The sugar-glass lattices were then cast in Sylgard 184 PDMS (Dow Corning, Midland, MI) by filling the printed well with 20:1 ratio of PDMS to Curing Agent, resulting in softer PDMS than optimally recommended by the manufacturer. The PDMS was then allowed to cure for two days at room temperature. Following curing, the sugar-glass was removed from the PDMS by placing the PDMS/sugar-glass constructs into a solution of 30% isopropanol in DI water for 2 days, replacing the isopropanol/water solution every 12 hours. To reduce the size of the final construct, the edges of the PDMS gels (not containing any channels) were removed using a razor blade.
**Figure 4.1: Description of sugar glass printing and initial flow testing.** A) Extrusion print head in the process of printing a sugar glass lattice. Print head can move x, y, or z planes. B) Final sugar lattice prior to casting. The lattice contains a network of filaments supported by a surrounding well. Red line denotes the outer edge of the well that will be filled with PDMS during casting. C) Schematic of printed sugar glass network. Drawing on the left denotes sugar filaments after printing, while the figure on the right shows filaments prior to casting. Unwanted filaments are removed using a fine tipped soldering iron prior to casting. D) Final PDMS gel with channel network. Excess PDMS has been removed using a razor blade. E) µCT reconstruction of internal channel network from a cast PDMS construct. F) Computational model of flow rates through cast channel geometry. Flow streamlines are color coded corresponding to flow rate. Flow rate at the inlet is equal to 0.12 mL/min. Computational models demonstrate continuity of flow and patency of channel networks. Scale bar = 4 mm.

**4.4.1.2 Microcomputed tomography scanning and computational flow analysis**

Microcomputed tomography (micro-CT) scans were performed on PDMS gels (n= 4) to obtain 3D models of the internal channel networks. The scans were conducted using a Bruker SkyScan 1272 at the Baylor College of Medicine Optical Imaging and Microscopy Core. The source voltage was set to 70 kV, with a source current of 142 mA, an exposure time of 2952 ms, and a rotation step of 0.5 degrees. The scans had a nominal resolution of 6 µm. The scans were then reconstructed using the NRecon software (Bruker, Kontich, Belgium), correcting for misalignment and ring artifacts. The reconstructed image stack was then filtered using a 3D Gaussian filter with a width of 5 pixels. Using the Mimics Innovation Suite software (Materialise, Plymouth, MN), pixel values were then inverted and thresholded to generate a clear surface for the internal channel network. Small unconnected objects smaller than 50 µm were removed. The image stack was then saved as a 3D model in a stereolithography (.stl) file. The mesh was adjusted to improve element quality and remove any holes or inverted elements.
(Figure 4.1). To estimate flow through the structure, the reconstructed channel geometries were imported into COMSOL Multi-physics 5.0. Using the single-phase laminar flow module, the inlet and outlet of the channels were defined under no slip conditions. The inlet flow was defined as 0.12 mL/min with no pressure drop between inlet and outlet. The resulting flow rates were plotted using streamlines where color corresponds with the flow rate (Figure 4.1).

4.4.1.3 Animal Care and Biosafety

Male Wistar rats weighing 400-450 g were obtained from Charles River Laboratories International Inc. (Wilmington, MA). All animals were provided with water and food *ad libitum* and maintained in a climate controlled environment accredited by the Institutional Animal Use and Care Committee of the University of Pennsylvania. All animal experiments were in compliance with the Guide for the Care and Use of Laboratory Animals, published by the US National Institute of Health (Eighth Edition, 2011).

4.4.1.4 PDMS Gel Implantation

Our experimental condition was to implant our PDMS-based microchannel networks in-line with the femoral artery and measure blood flow rates over time through the channels. To control for the experimental condition, experimental animals were divided into two groups based on the fate of their left common femoral artery, whether left intact or transected as a negative control. Ten male rats were evenly divided into a positive control and a negative control group. In the positive control group, the PDMS gel was implanted in the right common femoral artery and the left common femoral artery was left intact. In the negative control group, the PDMS gel was also implanted in the right common femoral artery, but the left common femoral artery was double ligated and transected proximally. Each rat underwent general anesthesia with a mixture of 4% isoflurane with 100% oxygen in an 2 liter induction chamber (VetEquip, Pleasantville, CA). Once sedated, the animals underwent endotracheal intubation with a 16-gauge (G) angiocatheter (Tycon Healthcare, Mansfield, MA) and were ventilated at a rate of 80 breaths per minute and tidal volume of 30 cc. The hind limbs of the animals were positioned with silk bandages. Bilateral inguinal areas of the animals were clipped and prepped in a sterile fashion. A 3 cm incision was made in the right infrainguinal region in both groups. Corresponding 3 cm incisions were made on the contralateral hind limb of the negative control.
group. No surgical operations were undertaken in the left hind limbs of the animals in the positive control group.

The entire procedure was performed under a Leica M60 modular stereo microscope (Leica Microsystems Inc. Buffalo Grove, IL). The incision was taken through the subcutaneous tissue until the femoral sheath was exposed (Figure 4.2A). The femoral artery was separated from the femoral vein and nerve (Figure 4.2B). PDMS gels were implanted in the right groins of all the animals in the experiment. Control of the proximal and distal femoral artery was obtained by placing a 5.0 silk suture (Ethicon. Somerville, NJ) around the vessel. The proximal and distal femoral arteries were cannulated in a stepwise fashion using two separate 26 G angiocatheters and secured in place by the silk sutures (Figure 4.2C - E). 100 units of heparin (Sagent Pharmaceuticals. Schaumburg, IL) were injected into the systemic circulation using the proximal catheter. The PDMS gels were flushed with 10 cc of heparinized saline _ex vivo_ to assess flow. Next, using a pair of fine hemostatic clamps, the proximal end of the femoral artery was temporarily clamped, the segment of artery between the two catheters was transected and the 26 G angiocatheters were trimmed to accommodate the length of the PDMS gel without any kinks along the system. The trimmed tip of the proximal angiocatheter was carefully mounted onto the proximal inlet of the PDMS gel micro-channel and the proximal hemostatic clamp was temporarily removed to assess pulsatile flow through the gel (Figure 4.2F). The distal angiocatheter was mounted onto the distal outlet of the PDMS gel and the hemostatic clamp was released (Figure 4.2G). Flow and pulsatility through the micro-channel of the gel were assessed visually and using Doppler imaging technology. In the negative control group, the left femoral artery was double ligated and transected proximally.
Figure 4.2: Implantation of PDMS gels containing channel networks in line with rat femoral artery. (A) Incision into the hind limb and exposure of the femoral bundle. (B) A indicates the femoral artery, v indicates the femoral vein, and n indicates the femoral nerve. (C) Cannulation of the femoral artery using an angiocatheter. (D) Angiocatheter is advanced into the arterial lumen and heparin is injected through the catheter. (E) The catheter is secured with a silk suture before transection. (F) The proximal catheter is connected with the inlet of the PDMS gel. (G) The distal catheter is connected with the outlet of the PDMS gel and hemostatic clamps are removed. (H) Blood is shown flowing through the femoral artery into the PDMS gel.

4.4.1.5 In vivo flow monitoring using laser Doppler imaging.

Following gel implantation and closure of the wound, blood flow through the gel micro-channels was assessed using a Moor Laser Doppler Imager LDI (Moor Instruments Inc. Wilmington DE) immediately, 1 hour, and 3 hours post-gel implantation. Moor LDI laser Doppler Imager software V.5.3 (Moor Instruments Inc. Wilmington DE) plots the relative flow rates as colored heat map (Figure 4.3). Prior to any imaging, each rat was injected with 10 cc of normal saline subcutaneously around the abdomen to increase intravascular volume and account for blood loss and insensible losses during the operations. The animals were kept ventilated under general anesthesia (2% isoflurane; 100% oxygen) for the duration of the Doppler imaging studies. In between flow measurements, the animals were kept on a warming platform and wrapped in a blanket to prevent hypothermia.

4.4.2 Results and Discussion – development of surgical techniques

4.4.2.1 Computational models of flow through microchannel networks

To validate patency and flow profiles of the channels before implantation, we used micro-CT scans of four different gels to assess the internal channel geometry and generate a 3D computational mesh. We then imported the geometry into COMSOL Multiphysics to assess flow patterns through the channels. Using an inlet velocity 0.12 mL/min and no slip conditions,
we determined that for all four gels, each channel received flow at the provided inlet velocity. A representative gel can be seen in Figure 4.1. Similar computational modeling will be extremely useful for optimizing vascular geometry to maximize cell viability in vascularized gels in future studies.

4.4.2.2 Patency post implantation

Ten gels were successfully implanted in vivo. Upon unclamping the proximal hemostatic clamp, anterograde bright red blood flow was observed filling the micro channels of the gel and pulsatile blood flow was observed out of the distal outlet of the gel (Figure 4.2). The distal angiocatheter was mounted on the gel outlet and pulsatile flow was observed through the distal femoral artery. The constructs withstood the physiologic pressure of the rat femoral system (80 - 100 mmHg). The patency and anterograde flow through the gel were assessed with a laser Doppler at four different time points. Figure 3A and B illustrates sample laser Doppler flow patterns in the positive and negative control groups, respectively. Flow through the gel, distal femoral artery, and the paw were similar in the surgery limb and the positive control. There was noticeably more flow through the gel, distal femoral artery, and the paw compared to the negative control limb immediately after, and at 1 and 3 hours post-implantation (Table 4.1). Though the implanted PDMS gels induce clotting within a few hours, as shown in Figure 4.3, patency for these gels is on a similar timescale to other tissue engineered constructs containing microvascular networks. Future in vivo studies using less thrombogenic materials and potentially lining channels with ECs should help to reduce clotting and improve patency of micro-channels.
Figure 4.3: Laser Doppler imaging of flow through implanted gel. The arrow in image A shows where the femoral artery was ligated. The negative control limb shows no blood flow to the hind limb after 1 hour, while the surgical limb shows blood flow (although at lower rates relative to the positive control) up to 3 hours post-surgery. The white arrows indicate the branching pattern of the inserted PDMS gel.

Table 4.1: p-value measured by t-test of unequal variance between Surgery limb and Positive Control. p-value measured by t-test of unequal variance between Surgery limb and Negative Control. Flux PU (Perfusion Unit) is a measure of the amount of blood flow.
In summary, we successfully developed a surgical methodology to connect 3D-printed gels containing microchannel networks with a rat’s native circulation for immediate perfusion of the construct. The vessels derived from 3D printing withstood the physiologic pressures of the rat femoral artery at 80–100 mmHg\textsuperscript{183}. Pulsatile flow through the micro-channels was confirmed upon implantation as well as up to 3 hours post-implantation. This time frame is similar to other tissue engineering methods incorporating micro-vessels, such as implanted organs engineered using tissue decellularization and recellularization techniques\textsuperscript{45,51,50}. The successful \textit{in vivo} implantation of our vascular construct is essential for using complex engineered tissues for regenerative medicine applications, particularly in tissues with a high metabolic activity. To follow up from this work, we moved from PDMS-based gels to more biocompatible PEGDA based hydrogels to further test our core hypothesis that 3D printed constructs could yield hemocompatible vascular grafts and pave the way for cellularized engineered tissues.
4.5 Study 2 – Design, test, and implant PEGDA hydrogels containing channel networks for surgical anastomosis in a rat model.

Leveraging the surgical technique developed in Study 1, we progressed to implanting channel networks fabricated using hemocompatible PEGDA hydrogels fabricated using our pSLA system. The new fabrication strategy and material selection improved patency time in the implanted gels, but the new material posed additional challenges in the surgical implantation process.

4.5.1 Materials and Methods

4.5.1.1 Fabrication and design of hydrogel networks for implantation in rat and pig models

Hydrogels were fabricated as described in Chapter 2. Implementing feedback from surgical collaborators, we modified the PEGDA formulation used for in vivo applications to make gels more durable, deciding on the 40% w/w 1:1 6:35kDa formulation. We incorporated the higher MW 35 kDa PEGDA, because gels consisting of 35 kDa PEGDA had higher ultimate strains than gels containing 6 kDa chains alone, as shown in Chapter 2. Furthermore, the higher overall concentration of PEGDA resulted in increased stiffness than the previously used 10% w/w 6 kDa PEGDA formulation.

All gels were printed using PBS containing 100 U/mL of heparin provided by veterinary staff to help prevent clotting\(^{182,185}\). Gels were allowed to reach equilibrium swelling overnight a PBS/heparin solution (100 U/mL). Then, to add a suture site to the hydrogels, a small piece of Vicryl mesh (Ethicon) was photocured to the bottom of each gel. The Vicryl mesh was adhered by placing a drop of PEGDA/photoinitiator solution (10% w/w 3.4 kDa PEGDA with 34 mM LAP, no tartrazine) between the mesh and the swollen hydrogel, then exposing the gel to the 405 nm light from our DLP projector for 30s at 16.5 mW/cm\(^2\). 3.4 kDa PEGDA was used to adhere the mesh due to its low swelling ratio to prevent puckering of the mesh as the added hydrogel reached equilibrium swelling.

4.5.1.2 Burst pressure and compliance testing of 3D printed hydrogels

To ensure that the gels could withstand arterial blood pressure, we tested the burst pressure of the hydrogel gel designs prior to implantation. Pressure was applied to the gels using a custom built pneumatic system which is capable of applying pressures up to 30 PSIG
(~1500 mmHg) (designed in our laboratory and available at the following URL: [https://github.com/MillerLabFTW/OpenSourcePneumaticSystem](https://github.com/MillerLabFTW/OpenSourcePneumaticSystem). First, printed hydrogels were allowed to swell to equilibrium prior to testing. The gels were then secured to a glass slide using cyanoacrylate glue and connected with 26 and 22 gauge catheters (as would be used in vivo), which were secured to the gel using cyanoacrylate glue. Tubing connected to the venous outlet of the gel was then clamped while the arterial end was connected to the pneumatic pressure system (Figure 4.4). Pressure was then ramped slowly from 0 to 15 PSI (775 mmHg), where the digital pressure gauge was used to record pressure at the point of channel rupture.

![Diagram](image)

**Figure 4.4: Burst pressure test setup.** A) Photograph of custom pneumatic system with key components labeled. The graphical user interface (GUI) was programmed in the integrated development environment (IDE) called Processing. B) Image of hydrogel setup during burst pressure testing.
4.5.1.3 PEGDA Gel Implantation

To implant PEGDA hydrogels it was necessary to modify the surgical procedure as described in Sooppan et al. 2016. First, rather than implanting the microchannel as a proximal/distal vascular shunt, the gels were implanted as an arteriovenous (AV) shunt, spanning the proximal femoral artery to femoral vein and bypassing the leg. Additionally, we used a small amount of cyanoacrylate glue between the catheters and the gel to secure the catheters in place and used 2 to 4 sutures to secure the Vicryl mesh within the surgical site. As mentioned previously, all surgical procedures were conducted according to protocols approved by the Institutional Animal Care and Use Committee of the University of Pennsylvania.

4.5.1.4 Monitoring flow through channels over time

Flow monitoring in rat models was conducted daily using Laser Doppler imaging as described previously in section 4.4.1.5.

4.5.2 Results and Discussions –Implanting PEGDA based scaffolds in rats

4.5.2.1 In vitro burst pressure measurements

Average burst pressure of the channels was 401.9 mmHg ± 11.9 mmHg (N=3), where the minimum burst pressure was 388.6 mmHg. All constructs burst along the inlet or outlet of the gel. Male Wistar rats typically have a systolic blood pressure around 80-120 mmHg, which suggests that our gel geometry had a burst pressure well above the natural ranges for the test subjects.

4.5.2.2 Implanting fabricated channels in rats and assessing gel patency over time

Transitioning from a PDMS scaffold to a PEG-based shunt posed additional challenges beyond material durability. First, because PDMS is hydrophobic it easily creates a water-tight seal with the polyurethane catheters and holds the gel securely in place. But, because PEGDA is highly hydrophilic, the hydrogels are much more prone to leaking. However, the higher precision of our pSLA fabrication allowed us to fabricate a taper to the inlet and outlet of the vascular channel to generate a pressure tight physical seal between the channel and each catheter (Figure 4.5). Next, we transitioned from ladder or straight channel geometries to an arteriovenous (AV) shunt model, which connects the femoral vein to the femoral artery, bypassing the leg (Figure 4.5). This change in geometry helped maintain the fluidic connection between the hydrogel and the catheters during leg movement. Additionally, we hypothesized
that the greater pressure differential between the artery and the vein (as compared to the proximal vs. distal artery) would aid in increasing blood flow rate and thereby help prevent clotting\textsuperscript{182,185}. Channel diameter played a noticeable effect on blood clotting, likely due to the reduction of blood flow rates in gels with narrower channel diameters. We tested gels containing channels ranging in size from (0.7 to 1.6 mm) and determined that a channel diameter of 1.6 mm was ideal for future studies (Figure 4.5). Finally, to prevent the gel from dislodging during movement of the leg, we added a Vicryl mesh to the gel that was sutured to secure the gel in place within the leg (Figure 4.5).

Figure 4.5: Schematic for \textit{in vivo} implantation of PEGDA channel networks in rat model. A) Drawing of final hydrogel geometry where dimensions are given following equilibrium swelling. The taper at the inlet and outlet of the gel helped prevent leaking around the inserted catheters and the crosshairs at the front and top edges of the gel aid in proper insertion of the catheters into the tapered channels. B) Image of gel following surgical anastomosis with the rat femoral vein and artery. The Vicryl mesh (which can be seen through the gel) has been sutured to the surrounding tissue outside the edge of the gel. Scale bar = 2mm.

After settling on a suitable design for our hydrogel-based channel networks, we implanted the AV shunts and monitored patency daily using laser-Doppler imaging and visual inspection of the hydrogels. After implanting a total of 23 gels \textit{in vivo}, the gels had an average patency time of just over 2 days, while some gels lasted as long as a week \textit{in vivo} (Figure 4.6). We had a total of 3 unsuccessful surgeries, where the construct was damaged during implantation or the rats had complications unrelated to the surgical procedure. These results demonstrate that our 3D printed channels could withstand the surgical implantation procedure and arterial pressures in a rat over a period of days, a notable improvement over the PDMS constructs from Study 1. This patency value is on par with decellularized organs implanted \textit{in vivo}\textsuperscript{51,50}, though these results still leave significant room for improvement. For future studies
we were interested in continuing our studies to larger animals to expand our surgical options, while still focusing on small diameter vessels below the 6 mm limit imposed on synthetic vascular grafts. We also wanted to use these additional studies to investigate the sources of clotting within our PEGDA hydrogels.

Figure 4.6: Results from hydrogels implanted *in vivo* in rat model. A) Image of a perfused gel immediately after implantation, along with the corresponding laser Doppler image. B) Image of the same gel after 6 days *in vivo*. Channel is still patent and blood is bright red and free of clots. Inset shows the corresponding laser Doppler image demonstrating blood flow through the channel. C) Example gel where a clot has formed 4 days after implantation. D) Histogram of the total number of days implanted *in vivo* for 23 gels, where the average patency time is 2.05 ± 1.9 days. Scale bar = 2 mm.
4.6 Study 3 - Design, test, and implant PEGDA hydrogels containing channel networks for surgical anastomosis in a porcine model

After results from *in vivo* implantation in rat models, we adapted our studies for larger porcine models carried out locally at Baylor College of Medicine.

4.6.1 Materials and Methods

4.6.1.1 Fabrication of hydrogel constructs for porcine model

Gels used for *in vivo* studies in a porcine model were similar to those used in previous studies, though the design was updated to accommodate differences in anatomy. Otherwise, the gels were fabricated as described previously using the 40% w/w 1:1 6:35 kDa PEGDA formulation with 100 U/mL heparin. To address challenges in securing Silastic tubing within the PEGDA hydrogels, hydrogels were housed in 3D printed plastic cases and tubing was secured to the plastic cases using cyanoacrylate glue. Plastic cases were fabricated with the Fortus 450 mc in the Rice Biomaterials Lab using the biocompatible polycarbonate filament (PC-ISO, stratasys) and were autoclaved prior to implantation (Figure 4.8).

4.6.1.2 Burst pressure testing

Burst pressure testing was conducted as described previously (Figure 4.4), though slight changes were made to accommodate new cannal geometry and implantation procedure. First, the catheters used to establish pneumatic connection with the gel were substituted with Silastic tubing and the gels were tested in the PC cases (as would be used *in vivo*).

4.6.1.3 Implantation in porcine model

All animal care and surgical procedures are performed according to Protocol AN-7216 as approved by the Institutional Animal Care and Use Committee (IACUC) of Baylor College of Medicine and secondarily approved by Rice University IACUC. Briefly, differences in construct assembly and the animal model resulted in a slightly different implantation procedure than used with rats. Constructs were implanted without totally severing the arteries and were implanted at either the leg or the neck locations (though eventually the neck was deemed preferable due to increased space). The surgical procedure, as shown in Figure 4.7, similarly starts with isolating the target vessels; however, the vessel is only partially transected and tubing is inserted into the vessel without the assistance of a needle.
Figure 4.7: Surgical diagram for inserting constructs into pig models. A) First, the target artery and vein are isolated and controlled using two silk suture loops. B) The sutures are pulled to restrict blood flow and a small incision is made partially through the vessel. C) The tubing (with the vascular construct attached) is inserted into the vessel and clamps are removed, allowing blood to flow through the implanted construct. Tubing is secured within the vessels using suture loops.

4.6.1.4 Flow imaging

During the final round of implantations, a GE Vivid 7 Ultrasound imaging system to measure flow using color Doppler trans-dermally following implantation.

4.6.1.5 Histology for in vivo samples

Two different methods were used for histological analysis the hydrogels: vibratome sectioning, which does not require embedding of the samples, and resin embedding using an ultramicrotome for sectioning. All samples were fixed in 4% paraformaldehyde overnight, then washed several times in PBS before sectioning or embedding.

Sectioning via vibratome: Sectioning was conducted using a Leica VT 1000S vibratome to generate 500 µm thick sections with the speed and frequency set to 3 and 9, respectively.
**JB4 resin embedding and sectioning:** Embedding using the JB4 resin (Polysciences) was done as described previously in Sullivan and Brown 2010188. Briefly, JB4 infiltration solution was prepared according to Sullivan and Brown. The gel samples were cut into smaller pieces before being placed in the infiltration solution until the samples sunk to the bottom of the tube. The infiltration solution was then changed, incubated at room temperature for a few hours, then replaced again for overnight incubation at room temperature. After infiltration the sections of gels were then placed in molds compatible with the Leica EM UC7 Ultramicrotome. The molds were then filled with the JB4 embedding solution and allowed to harden overnight under argon. Sectioning of resin-embedded samples was conducted using the Leica EM UC7 Ultramicrotome available through the Rice Shared Equipment Authority. Sections were cut at 6-8 µm thick using a glass knife.

**Staining of samples:** Hoechst stain was used to identify nuclear material in both JB4-embedded and vibratome-sectioned samples. Hoechst 6024 (Sigma) was added to sections at a concentration of 0.5 µg/mL and allowed to incubate for 20 min at room temperature before being rinsed several times using DI water.

Toluidine blue stain is a basic thiazine metachromatic dye commonly used as an alternative to H&E staining, where tissue and cell components will stain violet to red/yellow189. Toluidine blue was used only for JB4 embedded samples, which were stained according to a protocol optimized by Dr. Sadhna Dhingra at Baylor College of Medicine. Toluidine blue stain was mixed using 5 g sodium borate, 2.5 g toluidine blue (Sigma Aldrich) and 500 mL DI water. Slides containing resin-embedded sections were placed on a hot plate at 100-150 °C for at least 30 min prior to staining. The sections were then stained for 1-2 seconds before being rinsed with DI water several times. Slides were allowed to dry on hot plate for 5-10 min before being covered with a coverslip.

### 4.6.2 Results and Discussion

#### 4.6.2.1 Designing and implanting vascular conduits in porcine models

Design of the vascular conduit for the porcine model was similar to the rat model; however, the gel design had to be adapted for larger vessel diameter of the 5-10 kg piglets and the different anatomy of the surgical site. To accommodate larger vessel diameters, channel diameters were increased to accommodate 5 French Silastic tubing (0.76 mm ID, 1.65 mm OD;
Silastic), though inlet and outlet of the channels are still tapered to maintain a tight seal between the gel and the inserted tubing. Additionally, the implant site for piglets was changed from the leg to the neck (spanning the carotid artery and jugular vein), because the femoral bundle in piglets is located beneath a significant layer of muscle, unlike the superficial vascular bundle of rats. Finally, the PEGDA hydrogel was placed within a PC case to help secure the tubing in place within the gel following wound closure.

Figure 4.8: Schematic of PEGDA AV vascular shunt used for implantation in porcine model. A) Schematic of the modified hydrogel use for implantation in the neck of 5-10 kg piglets. B) Schematic of 3D printed PC case for hydrogel geometry in A. C) Image of a gel implanted as an AV shunt linking the carotid artery to the jugular vein. Scale bar = 5 mm.

Burst pressure for the updated gel design was 325.8 ± 29.3 mmHg (N = 5), with a minimum burst pressure of 305.6 mmHg. The gel geometry used for porcine models had a lower burst pressure than gels implanted in rats, likely due to the slimmer profile of these gels relative to the channel diameter. This reduced profile was implemented due to the restricted space within the implantation site, though the burst pressure of the constructs is still well above systemic blood pressure of piglets at ~ 120 mmHg. \(^{190}\)

Though preliminary in vivo studies in porcine models focused on refining the surgical procedure and design of the AV shunt, later studies have shown channels to remain patent for up to 4 hours in vivo (Figure 4.9). We determined that implantation in the neck is preferable to the leg, due to increased space and reduced movement of the construct following recovery from anesthesia. When implanted in vivo, the PC cases and cyanoacrylate glue prevented the
tubing from being dislodged from the hydrogels as the piglets moved about freely and the PEGDA hydrogels were capable of withstanding systolic pressures. Though only one gel was confirmed patent after four hours in vivo (where the piglet was recovered from anesthesia), a full list of results can be found in the Appendix in Table 0.3.

Figure 4.9: Assessment of PEGDA hydrogels implanted in a porcine model. A-B) Image of gel at time of implantation. Scale bar = 5 mm. C-D) Ultrasonic Doppler image of gel immediately after wound closure, displaying flow through both channels. E-F) Ultrasonic Doppler image 5 hours after implantation, where no signal was detected for Gel 1. G-H) Images of gel after explant and flushing with saline. Saline flush dislodges clots which form during animal sacrifice but preserve more stable clots seen in a few areas of (H). Scale bar = 2 mm.
4.6.2.2 Histology

Though we initially planned to use cryosectioning, as described in Ruan et al.\textsuperscript{191}, for histological analysis of implanted gels, the dense concentration of high MW PEGDA chains made this formulation incredibly difficult to section, despite the screening of different embedding and freezing techniques. Therefore, we turned to alternative approaches for sectioning our implanted hydrogels: vibratome sectioning at 500 µm and using an ultramicrotome to section samples embedded in JB4 resin\textsuperscript{188}. Initial results revealed the presence of nucleated cells within well-formed clots (**Figure 4.10, Figure 4.11**). Additionally, in thinner, resin embedded samples, toluidine blue highlighted the cells present at the edge of the gel. These histological techniques should also be used in future studies to assess protein adsorption, cell adhesion, and the timing of any clotting that occurs.

**Figure 4.10: 500 µm thick vibratome sections of gels implanted for 6hrs in vivo.** A) Reflected light color photo of a 500 µm thick vibratome section, where the red dotted line indicates the edges of the gel. Scale bar = 1 mm. B) Phase contrast/Hoechst overlay of Hoechst-stained channel, outlined in white in A. Scale bar = 250 µm. C) Zoomed in view of B showing individual nuclei. Scale bar = 100 µm.
Figure 4.11: Resin-based histology of gels implanted in porcine model. A) Phase contrast image of gel channel, where section is 6 µm thick. B) Zoomed image of the channel showing the presence of cells adhering to the channel wall. C) Hoechst stain of the gel demonstrated nucleated cells along the wall. D) Toluidine blue stain highlighting cell material in dark purple. Scale bars = 100 µm.

Ultimately, these results demonstrate that our pSLA fabrication approach is sufficient for producing vascular shunts that are compatible with surgical anastomosis in piglets, and though there was a high level of variation in this work, it suggests interesting hypotheses for future studies. First, preliminary histological data (Figure 4.11) suggest that surface roughness within the implanted channels could influence blood clotting due to the formation of static regions or potential damage to red blood cells or platelets. Additionally, though PEGDA is generally accepted as bio-inert, previous groups have demonstrated that blood proteins do indeed adsorb to PEG-based hydrogels\textsuperscript{192,193}. However, implementing changes in our PEGDA hydrogels, such as changes in formulation\textsuperscript{126}, curing percentage, or crosslinking chemistry, could potentially reduce the clotting response \textit{in vivo}. Ultimately, the flexibility of our pSLA fabrication system with respect to channel geometry and materials, allows us address any challenges with clotting or surgical implantation related to gel architecture or materials.

4.7 Conclusions

We began this chapter by developing a technique to surgically anastomose 3D printed channel networks with the femoral artery in a rat. We began with PDMS gels containing micro-
channel networks fabricated using lost-carbohydrate-glass casting due to the resilient mechanical properties of PDMS and the compatibility with autoclave-sterilization. Using our new surgical technique to implant these gels in-line with the femoral artery, we used a modified hind limb ischemia model to assess the perfusion and patency of the channels over time. As a proof-of-concept demonstration, we have established a workflow to rapidly perfuse the internal vasculature of 3D printed vascularized constructs implanted in vivo.

We then moved from our PDMS channel networks to more hemocompatible PEGDA-based networks fabricated using pSLA. Using this 3D printing technique, we designed channel networks capable of withstanding arterial pressures in rat and porcine models without leaking. Gels implanted in a rat model as AV shunts lasted an average of 2 days in vivo without clotting, while some samples lasted as long 1 week without clotting. Additionally, we expanded this work from a rat model to a porcine model, where we demonstrated preliminary success with channels patent for up to 4 hours in vivo. These results are on par with current research focusing on the in vivo implantation and anastomosis of decellularized/recellularized tissues. We also determined that JB4 resin-embedding and vibratome sectioning are feasible methods for sectioning dense, robust hydrogel networks that were incompatible with cryo- or paraffin embedding. For future work, the use of increasingly biocompatible materials to mimic the extracellular matrix of vascularized tissue will allow for prolonged patency of the channel lumens and better integration of the construct with the host tissue. Finally, these studies outline a method to overcome the challenge of oxygen and nutrient delivery when engineering complex and thick vascularized tissue by implementing surgical anastomosis for 3D printed hydrogel structures containing microchannel networks for rapid perfusion.

4.8 Future work

Though we describe a technique to implant and rapidly anastomose 3D printed channel networks and demonstrated preliminary patency (up to 6 days), more work remains with respect to prevention of clotting.
4.8.1 Improved gel design to reduce recirculation of blood and investigate influence of gel surface roughness on clotting.

As mentioned previously, blood stagnation is an important element of Virchow’s triad and contributes to unwanted blood clotting. Therefore, we began analysis into altering the channel design used in rat studies to reduce any stagnation regions that may occur in the gels along the sides of inserted catheters. Using COMSOL computational fluid dynamics models and a non-Newtonian model for the viscosity of blood flow, we screened different channel architectures along with different catheter placements to investigate this effect on stagnation zones within the channels. We demonstrated that our in silico models matched up well with results in our in vitro models, and for future in vivo experiments should further investigate the role of this new geometry in clot formation (Figure 4.12).
Figure 4.12: Using computational modeling to decrease stagnant regions and zones of recirculation within AV shunts. A) Bead tracking through original AV shunt geometry as used in rat studies, which demonstrates flow recirculation and dead zones on either side of the catheter. B) Table of parameters used for COMSOL models for blood flow through alternate channel networks. C-D) Computational model of blood flow through AV channel structures under different catheter placements. Models reveal that catheter placement can play a significant role in fluid flow and the total stagnant volume within the hydrogel networks, where arrows indicate zones of recirculation. E) Updated AV shunt geometry aimed at reducing the total dead volume within the shunt models. Furthermore, this geometry will minimize the variability in catheter placement during surgical anastomosis of the channel networks with native vessels.

As mentioned previously, Figure 4.11 suggests that surface roughness with hydrogel channels can affect clotting. To test this hypothesis constructs could be fabricated using smaller layer heights at 25 µm or rotate the printing orientation of the hydrogels, which would alter the location of any “stair stepping” effect within the channels. With this in mind, histological
analysis could be used to assess the formation of early clots within the channels relative to the geometry of the channel surface.

### 4.8.2 More advanced characterization of surface protein adhesion

Though PEG is typically considered to be bio-inert and resistant to protein adsorption, previous groups have demonstrated that proteins indeed adsorb to PEG hydrogels implanted \textit{in vivo}\textsuperscript{193}. Swartzlander, et al. used a proteomic analysis to demonstrate that the addition of cell adhesive ligands to PEG-based hydrogels can decrease the foreign body response \textit{in vivo}. In future work, similar techniques could be applied to study the adsorption rate of different proteins to PEG-based hydrogels \textit{in vitro}. The influence of different PEGDA formulations, different exposure times, and different surface treatments on protein adsorption could assist in preventing clotting \textit{in vivo}.

### 4.8.3 Adhesion of endothelial cells to 3D printed vessel grafts

Additionally, previous groups have shown that incorporating cell adhesive ligands or cells can reduce clot formation on engineered grafts\textsuperscript{193}. Therefore, we explored the potential to of incorporating gelatin-methacrylate (GelMa) into our scaffolds as a step towards pre-endothelializing our channels.

Unfortunately, the high concentration of PEGDA in our pre-polymer solution caused any added GelMa to precipitate out of solution. Therefore, we explored the potential to adhere GelMa to the surface of our PEGDA hydrogels after the gels had been printed and reached equilibrium swelling. After testing many methods of adhering the GelMa to our swollen hydrogels, we determined that the most effective method for adhering GelMa to the surface of swollen PEGDA hydrogels was interfacial polymerization\textsuperscript{195}. We first incubated swollen hydrogels in 34 mM LAP for a period of 5 hours. Then, any excess LAP solution was removed using a Kim wipe and 10\% w/w GelMa was added to the surface of the hydrogels and immediately but gently pipetted away. The gels were then exposed to light at 405 nm for 30 seconds from the bottom of the gel. Coated hydrogels were allowed to swell again in PBS for 24 hours before A549 epithelial cells were added to the gels at a concentration of 50k cells/cm\textsuperscript{2}. Cells were imaged 24 hours after printing, where cells adhered to the hydrogel surface and began to spread (\textbf{Figure 4.13}). These preliminary results suggest that such post-processing of
3D printed high concentration, high MW hydrogels could be an efficient method to incorporate cell-adhesive ligands onto the surface of our hydrogels for future studies.

Figure 4.13: Evidence of photocrosslinking of GelMa layer to PEGDA hydrogels after equilibrium swelling. Merged phase and fluorescent whole scan image of a 40% w/w PEGDA hydrogel printed with a hemi-cylindrical trough in the center of the gel seeded with A549 cells expressing H2B-mVenus/mCherry (Left). Right hand image shows a zoomed in image of the central trough, demonstrating noticeable cell spreading morphology 24 hours post-seeding. Scale bars = 1 mm (left) and 50 µm (right).
Chapter 5

Conclusion

5.1 Summary of Results and Implications

3D printing has the potential to vastly advance the field of tissue engineering, particularly with respect to the deployment of vascularized tissues in various investigations in vitro and in vivo, and has the potential to transform clinical medicine. The work presented here has demonstrated the closed loop design of vascular structures, whereby we established parity between computational and experimental models for convective and diffusive transport within 3D printed channel networks. In Chapter 1 we outlined the importance of vascular structures on maintaining cell viability and tissue function along with common techniques to engineer vascular networks in vitro.

In Chapter 2 we demonstrated the correlation between computational fluid dynamics models and in vitro bead tracking models. We have also demonstrated overlap between computational models of solute transport and in vitro diffusion models. We applied these models to screen different vascular architectures for increasing vascular efficiency, which compares the total viable cell volume within a vascularized tissue to the total vascular volume. After using computational models to screen static structures, we applied similar principles to new fluid structure interactions models when developing bicuspid valves in Chapter 3. We demonstrated that bicuspid valves fabricated using pSLA were capable of withstanding retrograde flow and that fluid flow profiles through our engineered valves related those observed in native valves. Additionally, valves fabricated from tougher PEGDA formulations and scaled up to 1 cm in diameter were able to withstand pressures up to 40 mmHg and were able to function in a similar manner to a native valve. Furthermore, this flow loop testing demonstrated that the valve sinus region influenced valve function despite having no noticeable difference in silico.
In Chapter 4 we developed a method to surgically anastomose 3D printed channel networks with host vascular networks, beginning with PDMS gels then moving on to hemocompatible PEGDA hydrogels fabricated using pSLA. Our PEGDA hydrogels using 40% w/w 1:1 6:35 kDa withstood arterial pressures in both rat and porcine models without any leaking. We demonstrated that our 3D printed vascular conduits remained patent for up to 1 week \textit{in vivo} in a rat model, and for up to 4 hours in a porcine model. Furthermore, using JB4-resin embedding we demonstrated the adhesion of nucleated cells to the walls of some gels, and further histological analysis could help identify a time course for clotting and uncover the source of clot formation.

Ultimately, knowledge gained from this research will improve the workflow used to design and fabricate vascular networks within engineered tissues and will help improve the quality of vascularized tissue models produced using 3D printing techniques.

### 5.2 Future Outlook

#### 5.2.1 Closed-loop development

While the use of computational modeling to design vascular structures is not novel \textit{per se}, this crucial implementation of parallel computational and experimental models is unique. For future work, we will continue the closed-loop design of efficient vascular networks for \textit{in vitro} and \textit{in vivo} applications (\textbf{Figure 5.1}). We also predict that this workflow will help engineers design vascular networks to meet the needs of specific tissues within the field of tissue engineering, as described in Chapter 1 (\textbf{Figure 1.2}). We also plan to continue working with the development of ultrasound phantoms, using our printing technique to develop more advanced phantoms with varying levels of backscatter and open channel networks for perfusion.
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Figure 5.1: Example model for closing the loop in the design of tissue engineering vasculature. Using an example from Miller et al. (2012), we demonstrate an opportunity where closing the loop between computational and experimental models could be used to improve vascular geometry prior to printing. Predictive computational models allow researchers to test the effects of multiple printing parameters, such as vessel geometry, material parameters, cell densities, on flow rates, oxygen diffusion, and cell viability in silico, though such models must first be verified using experimental models. Such high-throughput optimization offers significant promise for bioprinting applications (Figure from Paulsen & Miller 2015).

5.2.2 Developing advanced ultrasound imaging phantoms

Ultrasound is an important non-invasive imaging technique for medical applications and for the field of tissue engineering. However, as ultrasound imaging techniques improve to include more quantitative methods measuring materials stiffness, 3D flow patterns and oxygen tension, more complex phantoms consisting of soft materials mimicking native tissues are necessary to validate these new imaging and analysis methods. Using our pSLA technique, we have demonstrated the capacity to fabricate ultrasound phantoms using soft materials in a single step, without the need for material casting or back-filling. As mentioned previously, we have demonstrated the capacity to fabricate monolithic hydrogels containing both open channel networks and controlled stiffness regions. In addition to tissue engineering applications, this fabrication capability also has significant potential in the field of medical imaging in developing phantoms. We have demonstrated that we can use ultrasound imaging to
reconstruct 3D fluid flow profiles, but we can also use our stiffness patterning to generate variable levels of backscatter within our ultrasound phantoms.

Figure 5.2: Demonstrating increasing layer exposure time results in increased ultrasound backscatter. Example model of backscatter patterning based on exposure time (left), averaged scan using 40 MHz transducer (right). Scale bar = 5 mm.

Additionally, we have demonstrated that our pSLA fabrication approach is robust enough to handle the incorporation of common ultrasound and light scattering agents, 40 µm silica particles and intralipid\textsuperscript{196}. To prevent the silica particles from falling out of suspension during the printing process, 0.0833 % w/w of Xanthan gum (Sigma), a common food thickening agent, was added to the print solution. Unexpectedly, though the addition of silica particles required an increase in per-layer exposure time during printing, the addition of intralipid did not require increased exposure times, even at concentrations of 20% v/v (Figure 5.3).

Figure 5.3: Incorporation of ultrasound and light scattering agents into pSLA print solution. A) Schematic of gel. B) Including 40 µm silica particles and C) of intralipid into print solution, intralipid concentration in % v/v. Scale bar = 1 mm. Data acquired by Charlene Pan.
Combining this work with 3D channel networks, we can fabricate a tumor mimicking phantom containing both channels (representing vessels) and a hypoechoic region mimicking the tumor (Figure 5.4). The capacity to 3D print tissue-mimicking materials with varying levels of backscatter and open channel networks, can provide researchers the tools they need to fabricate ultrasound tissue phantoms for the validation of new imaging methods.\textsuperscript{171,170}

\textbf{Figure 5.4: Tumor phantom with hypoechoic tumor region and channel networks.} A) Model of phantom geometry where tumor-mimic has been cured for a total of 2.5 s per layer while the rest of the gel has been cured for 4.5 s per layer. B) B-mode reconstruction of geometry shown in A, where the lower exposure times within the central region result in less backscatter, as would occur in a tumor.
References


82. Parlato, Matthew; Reichert, Sarah; Barney, Neal; Murphy, W. Poly(ethylene glycol) Hydrogels with Adaptable Mechanical and Degradation Properties for Use in Biomedical Applications. *Macromol Biosci.* **14**, 687–698 (2014).


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Appendix

Results from additional vascular geometries using LBM

The lattice Boltzmann method has some key advantages over traditional CFD approaches, particularly when solving problems with complex geometries and boundary conditions. Therefore, to calculate fluid flow profiles through complex vascular structures, we turned to the LBM in collaboration with the Randles group from Duke University. Figure 0.1 shows some of the simpler channel designs used to verify results from initial LBM models.

**Figure 0.1: Models used to verify results from LBM.** A) Pressure differential across a 3D ladder structure. B) Fluid velocity magnitude within a single slice of the ladder model shown in A. C) Pressure differential across a torus knot structures.
Generating a lentiviral library generating cell lines that are easy to monitor using fluorescence microscopy

Expression of fluorescent proteins is a simple way to assess cell morphology, differentiate between cell types, and assess cell viability in real time using non-invasive fluorescence microscopy. For future experiments measuring cell viability and functionality in vitro, we have generated a lentiviral library for generating multiple cell lines with permanent expression of fluorescent proteins. Lentivirus was produced according to the Rice University Institutional Biosafety Committee oversight on Protocol #662023, and plasmids for lentiviral production were obtained Chris Chen and colleagues at Boston University, Addgene’s gene repository, and using GeneDesigner from DNA 2.0. **Table 0.1** lists all plasmids, their providers, and brief descriptions, with individual plasmid elements described in **Table 0.2**.

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<thead>
<tr>
<th>Gene</th>
<th>Supplier</th>
<th>Description</th>
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<tbody>
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<td>hEF1α-H2B-mVenus-IRES-mCherry PGK-Puro</td>
<td>DNA 2.0 (Custom Plasmid)</td>
<td>Signals for constitutive expression of nuclear mVenus, cytoplasmic mCherry, and Puromycin resistance</td>
</tr>
<tr>
<td>hEF1α-H2B-mOrange2-IRES-EGFP PGK-Puro</td>
<td>DNA 2.0 (Custom Plasmid)</td>
<td>Signals for constitutive expression of mOrange2 in the nucleus, EGFP in the cytoplasm and Puromycin resistance</td>
</tr>
<tr>
<td>hEF1α-H2B-mVenus-IRES-mPlum PGK-Puro</td>
<td>DNA 2.0 (Custom Plasmid)</td>
<td>Signals for constitutive expression of nuclear mVenus, cytoplasmic mPlum, and Puromycin resistance</td>
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<td>pHIV-Luc-ZsGreen</td>
<td>Addgene (39196)</td>
<td>Generates constitutive expression of luciferase and ZsGreen (496/506)</td>
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<td>pHAGE PGK-GFP-IRES-LUC</td>
<td>Addgene (46793)</td>
<td>Generates constitutive expression of luciferase and GFP</td>
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<td>HRE-Luciferase</td>
<td>Addgene (26713)</td>
<td>Codes for the expression of the HRE promotor (linked in hypoxia inducible pathways) for luciferase</td>
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<td>pUltra-Chili-Luc</td>
<td>Addgene (48688)</td>
<td>Codes for the constitutive expression of tdTomato and luciferase</td>
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<tr>
<td>pHIV-Luciferase</td>
<td>Addgene (21375)</td>
<td>Codes for the constitutive expression of luciferase</td>
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<td>piRFP670-N1</td>
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<td>Generates constitutive expression of iRFP (643/670)</td>
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<td>piRFP720-N1</td>
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<td>Generates constitutive expression of iRFP (702/720)</td>
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<td>Chen Lab</td>
<td>Envelope plasmid for second generation lentiviral systems</td>
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**Table 0.1**: List of plasmids used in our lab.
### Table 0.2: Table explaining individual elements within plasmids listed in Table 0.1.

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<th>Full Name</th>
<th>Description</th>
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<tr>
<td>hEF1α</td>
<td>Human Elongation factor A</td>
<td>Constitutive promoter involved in protein synthesis, promoting the transfer of aminoacylated mRNAs to a ribosome 197.</td>
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<tr>
<td>IRES</td>
<td>Internal Ribosome Entry Site</td>
<td>Constitutive promoter involved in protein synthesis, allows for initiation of translation in the middle of an mRNA sequence 198.</td>
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<td>PGK</td>
<td>Murine Phosphoglycerate Kinase-1</td>
<td>Constitutive promoter for long term expression, even in cell lines that are susceptible to promoter silencing (i.e. Embryonic stem cells). In vivo the promoter encodes for the enzyme 3-phosphoglycerate kinase, which is involved in glycolysis 199.</td>
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<td>pHIV</td>
<td>HIV Core Promoter</td>
<td>Produces high levels of constitutive expression 200.</td>
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<tr>
<td>HRE</td>
<td>Hypoxia Response Element</td>
<td>Promotor involved in altering gene expression under hypoxic stress 201.</td>
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<tr>
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<td>Human Ubiquitin C</td>
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<td><strong>Localization Sequences</strong></td>
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<td>H2B</td>
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<td>One of the 5 main proteins contributing to chromatin structure in eukaryotic cells. The protein has a low molecular weight is transported to the nucleus along with any other proteins linked to it 203.</td>
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<td><strong>Fluorescent proteins</strong></td>
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<tr>
<td>mVenus</td>
<td>Fluorescent protein with Ex/Em at 515/528 nm. mVenus has been optimized to create a monomeric form of the protein.</td>
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<td>mOrange2</td>
<td>Fluorescent protein with Ex/Em at 549/565 nm.</td>
<td></td>
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<tr>
<td>mPlum</td>
<td>Fluorescent protein with Ex/Em at 590/649 nm</td>
<td></td>
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<td>Fluorescent protein with Ex/Em at 488/507 nm</td>
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<td>ZsGreen</td>
<td>Fluorescent protein with Ex/Em at 493/505 nm</td>
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</tr>
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<td>mCherry</td>
<td>Fluorescent protein with Ex/Em at 587/610 nm</td>
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</tr>
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<td>tdTomato</td>
<td>Fluorescent protein with Ex/Em at 554/581 nm</td>
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<td>iRFP</td>
<td>Fluorescent protein with different potential Ex/Em of either 643/670 or 702/720 nm</td>
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<td><strong>Luminescent Proteins</strong></td>
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<td>Luc</td>
<td>Luciferase</td>
<td>Oxidative enzyme used in bioluminescence. Generates light by cleaving luciferin fuel 204.</td>
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<td><strong>Selection Molecule</strong></td>
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<tr>
<td>Puro</td>
<td>Puromycin</td>
<td>An aminonucleoside antibiotic that inhibits protein synthesis in both prokaryotic and eukaryotic cells. This gene is used to select for cells that have been successfully transduced with the construct 205.</td>
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</tbody>
</table>

After obtaining the plasmid in the form of a bacterial stab, the bacteria were grown and plasmids were then isolated using a Midi-Prep from Qiagen. The isolated DNA was then used to generate lentivirus using a second generation lentiviral system. Lentiviral vectors were used for cell transduction because they permanently incorporate genes into the cells’ genome and because lentiviral vectors are capable of holding relatively large genetic constructs (up to 18
To be incorporated into the virus, all plasmids must contain long terminal repeats flanking the desired components. To generate lentiviral vectors we followed the protocol outlined by Kutner et al. 2009 and Life Science’s Lipofectamine transfection protocol. Briefly, we transfected HEK 293T cells with three sets of different plasmids: the plasmid of choice, the packaging plasmid PSPAX2, and the envelope plasmid MD2.G. We then waited 3 days to collect the culture media containing the virus and used a PEG-it virus concentration kit from Systems Biosciences to concentrate the virus 20×.

Cell lines were transduced by adding concentrated virus to culture media and allowing transduction to occur over 3 days. Cell lines were then selected using puromycin (for cells expressing a puromycin selection cassette) or by fluorescence assisted cell sorting at the BCM Cytometry and Cell Sorting Core. Sorting was used in select cases instead of puromycin selection to prevent cells from losing their phenotype during the selection process. As an example, after transducing human mesenchymal stem cells (hMSCs) with two different concentrations of lentivirus containing the PGK-H2B-mCherry-CMV-EGFP-IRES-Puro construct, the virus was removed from the cells after three days, then the cells were sorted based on fluorescence intensity into High, Medium, or Low expression groups. Overall, cells receiving more virus appeared had higher levels of fluorescence, but did not appear to have an increased maximum expression level (Figure 0.2). Additionally, after sorting, a group of cells was analyzed from each sort group to test for purity (Figure 0.3).
Figure 0.2: FACS sorting data for hMSCs transduced to express PGK-H2B-mCherry-CMV-EGFP-IRES-Puro. Cells. Cells were given either a high, low, or no dose of lentivirus and were sorted into High, Medium, and Low expression groups.
Figure 0.3: Purity test corresponding with sorting data from Figure 0.2. Cells sorted into High, Medium, and Low groups were analyzed to determine the purity of each group.
Testing differentiation capacity of transduced cells to assess phenotype.

For use in future experiments, we have transduced multiple cell lines to express fluorescent proteins, including 344SQ mouse adenocarcinoma cells, C4-2 human prostate cancer cells, endothelial cells derived from iPSCs, HEK 293T cells, and hMSCs (kindly provided by Rooster Bio). The images in Figure 0.4 are presented as examples of two different cell lines transduced to express dual color plasmids. Cells expressing fluorescent proteins with overlapping spectra can be separated using multispectral imaging.

![hMSCs expressing PGK-H2B-mCherry CMV-EGFP-IRES-Puro](image1)

![C4-2s expressing hEF1α-H2B-mVenus-IRES-mPlum PGK-Puro](image2)

**Figure 0.4: Examples of fluorescently labeled cell lines expressing different fluorescent signatures**

To ensure that hMSCs maintained their capacity to differentiate following transduction, we tested their differentiation capacity after labeling. We cultured hMSCs expressing Medium or Low levels of PGK-H2B-mCherry-CMV-EGFP-IRES-Puro along with unlabeled cells. We then added adipogenic or osteogenic factors to the media and cultured the cells for 3 and 5 weeks, respectively. In addition to comparing the effects of expression level, we also compared differentiation between two different types of media: Rooster Bio’s Differentiation Basal Medium or with the lab’s standard growth medium (DMEM, 10% FBS, 1% P/S).
After three weeks we stained the adipogenic cultures with Oil Red O, which stains lipid droplets red, according to the protocol provided by ThermoScientific (Human Mesenchymal Stem Cell Protocol: Oil Red O Staining). Figure 0.5 shows representative images from each group, where the red stain indicated lipid droplets within cells. All groups cultured with adipogenic factors showed signs of adipogenic differentiation.

![Cell Line](Rooster + Adipo) ![Cell Line](Rooster) ![Cell Line](Standard + Adipo) ![Cell Line](Standard)

**Figure 0.5: Oil Red O stain Following 3 weeks in culture. Scale bar = 0.5 mm**

After five weeks in culture we stained the osteogenic cultures with alizarin red, which stains calcium deposits red, according to the Osteogenic Differentiation protocol provided by Life Technologies. Figure 0.6 shows an image of the cell culture plate after alizarin red staining, where black lines separate different media types, while yellow lines separate different cell lines. The staining was then quantified by solubilizing the alizarin red stain using acetic acid and measuring the absorbance at 420 nm, as described in Madurantakam, et al\(^{209}\) (Figure 0.7). Results show that the media type appeared to have a stronger effect on mineralization levels than transduction level.

Overall these results suggest that the transduction and sorting process did not alter the hMSC phenotype because cells could still undergo osteogenic and adipogenic differentiation.
Figure 0.6: Image of a cell culture plate following staining with alizarin red. Cells grown in DMEM media showed more noticeable staining than other groups, regardless of transduction.

Figure 0.7: Absorbance levels for solubilized alizarin red stain at 420 nm. Media type played a more significant role in staining level rather than transduction level.
### Summary of implantation procedures in porcine model

<table>
<thead>
<tr>
<th>Study Number</th>
<th>Date</th>
<th>Surgery Description</th>
<th>Results</th>
<th>Other Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.1</td>
<td>5/9/17</td>
<td>Non-survival, monitor under anesthesia</td>
<td>• Construct and tubing were connected using surgical mesh and cyanoacrylate glue.</td>
<td>Surgery was not successful</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Implant femoral AV shunt</td>
<td>• Connection between construct and tubing was not secure enough to withstand surgery</td>
<td></td>
</tr>
<tr>
<td>1.2</td>
<td>5/10/17</td>
<td>Non-survival, monitor under anesthesia</td>
<td>• Plastic case was introduced to secure tubing to construct</td>
<td>Surgery successful, but inconsistent signal from flow probe</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Implant femoral AV shunt</td>
<td>• Low signal quality from the flow probe, but flow was measured between 18-10 mL/min and confirmed upon removal of construct after ~2 hours</td>
<td></td>
</tr>
<tr>
<td>1.3</td>
<td>5/11/17</td>
<td>Non-survival, monitor under anesthesia</td>
<td>• Constructs (with PLA case) implanted in L and R leg.</td>
<td>Surgery successful, though gel dried slightly after surgery</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Implant femoral AV shunt (L) and AA (R)</td>
<td>• Flow was confirmed in Left leg after ~2 hours, but damage to vessels in R leg potentially halted flow on R side.</td>
<td></td>
</tr>
<tr>
<td>1.4</td>
<td>5/11/17</td>
<td>Non-survival, monitor under anesthesia</td>
<td>• Constructs implanted in L and R leg.</td>
<td>Surgery successful, though gel dried slightly after surgery</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Implant femoral AV shunt (L) and AA (R)</td>
<td>• Flow was confirmed through both constructs after ~2 hours, though gel in R leg had some visual clotting</td>
<td></td>
</tr>
<tr>
<td>2.1</td>
<td>9/5/17</td>
<td>Survival, recover after surgery and monitor for ~8 hours</td>
<td>• Smaller gel design fit better beneath skin without inhibiting implantation procedure</td>
<td>Implantations delayed 1 week due to Harvey</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Implant femoral AV shunt (L)</td>
<td>• Added bead at the end of tubing allowed us to reduce the total length of tubing inserted into the vessels</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>• Pig did not recover from anesthesia</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>• No apparent damage to the construct after removal ~20min after attempted recovery</td>
<td></td>
</tr>
</tbody>
</table>
| 2.2 | 9/7/17 | • Survival, recover after surgery and monitor for ~8 hours  
• Implant femoral AV shunt (L)  
• Surgery was successful, though signal from the flow probe was inconsistent.  
• After ~6 hours the construct was removed, but had folded back causing a kink in the tubing. Blood in the channel of the construct was completely clotted. | Implantations delayed 1 week due to Harvey |
| 3.1 | 11/29/17 | • Survival, recover after surgery and monitor for ~6 hours  
• Implant AV shunt (L) in neck  
• Fit between tubing and new case material was difficult  
• Eventually implanted a gel successfully without leaks, closed wound and recovered animal  
• After some initial bleeding, no apparent issues, despite low readings from flow probe  
• Construct was removed ~6 hours after implantation, fully clotted. | Pig was not ambulatory after recovering from anesthesia |
| 3.2 | 11/29/17 | • Survival, recover after surgery and monitor for ~6 hours  
• Implant AV shunt (L) in neck  
• Fit between tubing and new case material was difficult  
• Eventually implanted a gel successfully without leaks, closed wound and recovered animal  
• Construct dislodged shortly after recovery and animal was euthanized. |
| 4.1 | 1/18/18 | • Survival, recover after surgery and monitor for ~6 hours  
• Implant AV shunt (L) in neck  
• Hydrogel, case, and tubing were assembled prior to implantation and tubing was secured in place using cyanoacrylate glue.  
• No flow probe was used due to poor signal and potential to dislodge tubing. | While removing the construct there was some bleeding in the neck, but the construct appeared to be in place and remained patent |
| 4.2 | 1/18/18 | • Survival, recover after surgery and monitor for ~6 hours  
• Implant AV shunt (L) in neck  
• Hydrogel, case, and tubing were assembled prior to implantation and tubing was secured in place using cyanoacrylate glue.  
• No flow probe was used due to poor signal and potential to dislodge tubing. | After removing the construct there was a noticeable clot within the channel, but the clot did not appear adhered to gel channel and was easily flushed out of the channel using saline. |
| 4.3 | 1/22/18 | • Survival, recover after surgery and monitor for ~6 hours  
• Hydrogel, case, and tubing were assembled prior to implantation and tubing was secured in place using cyanoacrylate glue. | No Doppler signal was found after 5 hours, and the construct was clearly clotted upon removal. |
- Implant AV shunt (L) in neck
- Flow was measured using color Doppler ultrasound immediately after implantation and 5 hours later

| 4.4 | 1/22/18 | - Survival, recover after surgery and monitor for ~6 hours  
- Implant AV shunt (L) in neck  
- Hydrogel, case, and tubing were assembled prior to implantation and tubing was secured in place using cyanoacrylate glue.  
- Flow was measured using color Doppler ultrasound immediately after implantation and 5 hours later  
- Doppler signal was apparent after 5 hours, and the construct appeared patent upon removal. |

**Table 0.3: Summary of all surgeries performed on porcine model.**