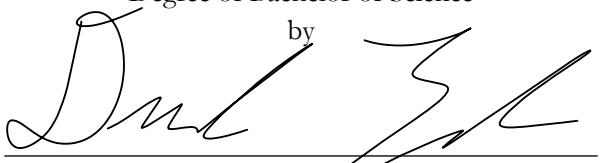
Project Number: GRG-1801

DESIGN OF A PERFUSABLE STRUCTURE FOR 3D BIOPRINTING UTILIZING DECELLULARIZED PLANT VASCULATURE

A Major Qualifying Project Report:

Submitted to the Faculty
of the
WORCESTER POLYTECHNIC INSTITUTE

In partial fulfillment of the requirements for the
Degree of Bachelor of Science
by



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Date: April 25, 2018

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ABSTRACT

Lack of a viable vascular network impedes 3D bioprinting from creating functional tissues. Utilizing the native vasculature of decellularized spinach leaves as a scaffold for cell seeding provides the potential to form a viable vascular network. This network coupled with a gel matrix has the potential to provide the support and nutrients necessary for printing functioning tissues. Our data shows cell viability within a seeded scaffold after 36 hours and demonstrates cellular escape from the spinach leaf scaffold. When combined, the data shows that cells have the ability to adhere and escape from the scaffold, into a gel matrix, mimicking the initial steps for vascular growth.

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AUTHORSHIP

All authors contributed equally to all aspects of the project and this report.

ACKNOWLEDGMENTS

We would like to thank Worcester Polytechnic Institute for the knowledge and guidance to complete this project. A special thanks to Glenn Gaudette, Josh Gershlak, Emily Robbins for all of their support through this experience. We would also like to extend our gratitude to DEKA Research and Development for this wonderful opportunity. Especially Dean Kamen, Andrew Capulli, Stu Jacobson, Richard Gautney, Derek Kane, Tom Bollebach, and Mike Tilley. And a final thanks to our peers, family, and friends.

I. INTRODUCTION

Every year, over 116 thousand people are in dire need of a transplant and every ten minutes, someone is added to a transplant waiting list. Roughly 50% of the patients in need of a transplant are under the age of 49, as presented in Figure 1. This need, coupled with the average lifespan in the United States of approximately 79 years, stresses the need for long term solutions to organ replacements. However, with only 12 thousand organ donors, many of the needed transplants cannot be performed. Many transplants involve major organs such as the kidney, liver, heart, and lungs, and even with adequate organ donor supply, there are many criteria that need to be met to ensure a donor organ will be compatible with the recipient. These criteria include, but are not limited to, blood type, organ type and size, and patient physiology. These and other factors greatly reduce the number of successful transplants [1].

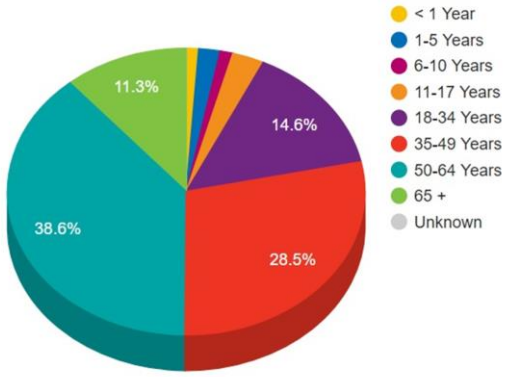

Figure 1: Ages of patients receiving transplants last year [1]

Although organs can sometimes be retrieved within the short time following the death of a patient, there is a short time frame for organ viability. This time frame ranges from 24-36 hours in kidneys to 4-6 hours in heart and lung tissue. This adds yet another variable, complicating the organ transplant process and lessening the possibility of a successful transplant [1].

Tissue engineering aims to alleviate this problem by developing functioning in vitro tissues via three-dimensional (3D) bioprinting. Currently, the designated cell types for the intended tissue or organ are being printed into supportive gel matrices. The gel acts as a supportive environment with physical characteristics similar to those found within the human body. However, one of the major obstacles with this process is vascularization and the diffusion of nutrients. While the gel provides the proper structural support, cells and nutrients cannot be adequately delivered through the matrix, resulting in cell death. Vascularization is critical because it provides an oxygen-rich environment for the diffusion of nutrients, similar to that found naturally in human tissue. Without proper diffusion of nutrients through tissue engineering matrices, the newly seeded cells do not survive long enough to differentiate into functioning tissues [2] [3]. Therefore, there is a need for a functioning vascular network to support cell growth, survival, and development within the contexts of 3D bioprinting of new tissues and organs.

The project aims to establish a network to mimic vasculature in the body and improve oxygen and nutrient diffusion to the cells being printed. The system will utilize existing vascular networks found naturally in other tissues as a structural base. Tissue engineers have tried to address this issue in the past using decellularization. Through this, the cells that make up a tissue, defining its biological properties, are washed away, leaving behind only the structural components of the tissue. Traditionally, this process has been performed on organs to allow for the possibility to seed new cells and provide non-specific donor organs [4]. Although this application would widen the donor pool, it still relies on the availability of organ donors for tissues.

An alternative example of such a system is using the existing vasculature of decellularized plant materials. Two potential plant scaffolds for this application include decellularized spinach leaves and decellularized parsley stems. Spinach leaves have a main stem and a small, intricate system of vasculature that is similar to that of human veins, while parsley stems provide a simple, more robust, one-dimensional scaffold. In order to utilize these materials, the plants need to be decellularized, removing the cells that biologically define the plant, leaving only those necessary for structure. With only the extracellular, or structural, components remaining, the plant structure now serves as a

possible scaffold for seeding human cells [5]. In the body, new vasculature is formed by a process known as angiogenesis. During this process, new vessels sprout from the original vessel to create a more intricate network [6]. By utilizing the scaffold of the plant material and angiogenic signaling pathways, this project aims to establish a larger, viable network.

Feasibility studies were performed to determine which potential scaffold would be more suitable for inducing these angiogenic pathways. The first step necessary for sprouting new blood vessels is the adequate adhesion of human cells to the chosen plant scaffold. Because of the large surface to volume ratio of the parsley stems, the data showed inadequate adhesion of the human cells to the plant stem. This, along with the published success of seeding human cells into decellularized spinach leaves led the team to ultimately move forward with decellularized spinach leaves as the scaffold for inducing angiogenesis. Adequate adhesion of human cells to the spinach leaves, in addition to proper cellular escape from the leaf vasculature into the gel matrix, would promote the growth of new blood vessels and eventually lead to the development of a vascular network within the gel matrix used for 3D printing. This would allow for the diffusion of oxygen to the cells being printed into the gel matrix, eventually promoting the growth of new tissues and reducing the overall need for organ transplants.

II. LITERATURE REVIEW

People get sick or injured every day, leaving organs and tissues in high demand without a reliable means to meet that need. According to the United Network for Organ Sharing (UNOS), someone is added to the national transplant waiting list every ten minutes, and an average of 20 people die each day waiting for transplants [1]. Tissue engineering and regenerative medicine have begun various initiatives to engineer tissues and organs that would be both biocompatible and functional in the body, thus reducing the pressing and unattainable need for organ transplants [2].

A. Regenerative Medicine

The field of regenerative medicine covers a broad range of topics and is looked at as a highly interdisciplinary field of study encompassing past surgeries, hospital procedures, and the overall advancement of biological scaffolds [3]. Its primary focus is aimed at replacing or regenerating human cells, tissues, or organs to fully functioning use, through *in vivo* or *ex vivo* methods [7]. This is accomplished through the integration of human cell therapies, gene-based methods, biomaterials, and molecular medicines [3].

Stem cells offer a large potential for the field of regenerative medicine. Stem cells are undifferentiated cells with the potential to differentiate or specialize [8]. Adult stem cells are present in different tissues in the body and tend to differentiate into organ-specific cells; however, there is less potential for adult stem cells to mature because they are further along the "differentiation" path. In the clinical setting, these cells have been isolated and implanted back into patients as a method for tissue repair [9]. The ability to isolate cells has been coupled with biomaterials in an attempt to restore organ function [3].

In 1988, fetal and adult rat and mouse hepatocytes, pancreatic islet, and small intestine cells were isolated and seeded onto a synthetic scaffold. The scaffold contained an intertwined, branching network of polymers which allowed for vascularization and diffusion to aid in cell survival. Isolated clinical trials have demonstrated successful implantation of these scaffolds into mice, indicating a potential for the use of engineered vasculature in biomaterials [7]. Biomaterials are multifaceted in their contribution towards regeneration. For example, the implanted biomaterial can induce a cellular response allowing the patient's cells to interact with the scaffold without first seeding cells *ex vivo*. Additionally, biomaterials can be used as a vehicle for the transport of cells and medication when implanted [9]. In one study, seven patients with myelomeningocele, a disorder resulting in poor bladder compliance, received artificial bladder scaffolds to repair the damage. The scaffolds were created from either collagen, or a mix of collagen and polyglycolic acid. Biopsies were taken from individual patients and their cells were cultured, seeded onto artificial scaffolds, and re-implanted into the patients. Postoperatively, bladder function was improved with an average follow-up time of 46 months, resulting in reduced bladder pressure and increased liquid capacity [10].

When selecting biomaterials for regenerative medicine, it is important to consider features such as biocompatibility, biodegradability, defined shape/size, the ability to perfuse oxygen and nutrients, and the ability to remove waste. Additionally, each level of degraded polymer should be nontoxic, and a defined breakdown time should be established [11]. Two crucial aspects of biomaterials that incur the biggest problems are vascularization and the ability to maintain stress and strain. Other challenges to biomaterial use involve maximizing cell attachment, proliferation and differentiation on said material, reducing long term karyotype changes, and overcoming the immune response of the body [9]. In addition to biomaterials, the use of donated organs has also been presented as a potential scaffold for seeding cells and providing an organ replacement.

Donated human organs provide a good structural scaffold after being decellularized. Once the organs are decellularized, cells can be re-seeded. These organs provide biochemical and mechanical guidance for cells to adhere, proliferate, and differentiate [4]. Prior to implantation, the organs need to be primed to adapt to the microenvironment of the body. Alexis Carrel, the father of modern cardiovascular and transplant surgery, provided the basis of regenerative medicine, collaborating with Charles Lindbergh to create a perfusion pump, allowing organs to survive outside of the body. This was a big step towards the bioreactors and perfusion systems used today[7].

There are several advantages to using harvested organs as replacements. As previously mentioned, the organ structure can stimulate cell behavior, attachment, replication, and differentiation. There is very little immune response because the extracellular matrix, which provides structural support for cells and mechanical cues for cell differentiation, is similar amongst a species [4] [12]. The decellularized organs are apt to structurally change and adapt to in vivo conditions. The vasculature is maintained in these organs, combatting a big problem faced with biomaterial constructs [4]. However, the production of such organs requires a vast amount of time and resources and the application is still far from commercialization.

B. Bioprinting

Despite the potential of decellularization, the ability to use donor organs as a scaffold for a patient's cells still relies on the availability of organs. Another option that has been recently explored is the possibility to grow, or more specifically, print biological organs for patient transplants. In the past, scientists have achieved three-dimensional (3D) fabrication through methods such as electrospinning, freeze-drying, and particle leaching; however, these methods create a large bulk product lacking the necessary precision for the development of micro-scale structures. Seeded scaffolds present the opportunity for more precise cell placement, but these structures take considerable amounts of time and money to construct [13]**.** In recent years, there has been interest in the application of additive manufacturing for tissue engineering. Use of such technology would allow for precise cell placement, as well as faster processing, providing a reliable source for engineered organs and tissue [14].

1. Classes of Bioprinters

There are three different classes of bioprinters currently available; these classes, depicted in Figure 2, include inkjet (a), extrusion-based (b), and light-assisted (c,d) [14]. Each printer has different considerations for the application of printing cells. Inkjet and extrusion-based printers have been explored for the biofabrication of complex heterogeneous structures. Both printers build structures by layer and have the potential to disperse different cell types utilizing multiple nozzle attachments. The main difference between the two types is their printing surface: inkjet printers print on top of a multiaxial surface, while extrusion-based printers print inside a hydrogel [13] [15]. Light-assisted bioprinting functions a little differently; it includes two major subclasses of printers: DLP (digital light processing)-based printers and laser-based printers. DLP-based printers utilize a digital micrometer to project UV light into a pattern, while laser-based printers transfer cells from one slide to another through a medium via evaporative gas pressure [13] [15].

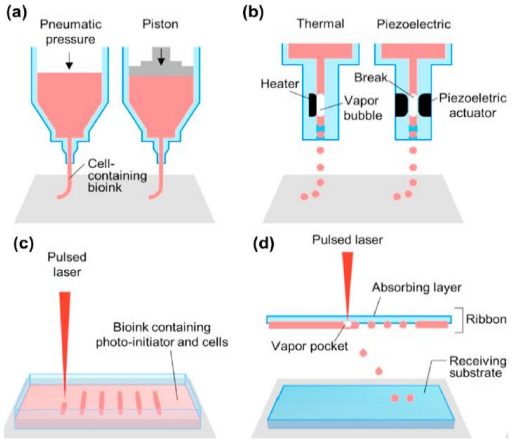

Figure 2: Available 3D printing techniques [14]

Two overarching obstacles with bioprinting cells and organs are the time for natural cell growth and the development of a vascular network [13][15]. Without a reasonable growth time, substituting engineered organs for a transplant is not a major improvement; without a viable vascular network, cells cannot gain the oxygen and nutrients needed for growth and survival [16].

C. Materials

Biomaterials have been explored as a potential medium for the delivery of cells or regeneration of afflicted organs and tissues. As the field of regenerative medicine advances, a growing array of polymers and cell attractive materials have been explored for use in both 3D printing and scaffolds. Polymers may be formulated to provide a stable matrix for printing live cells for tissue production. Cell adhesive materials and chemoattractants can be used to prompt cell movement in order to sustain cell growth and vascular activity within these polymers. After obtaining cell viability and movement within a polymer matrix, vasculogenesis can begin. The sections below detail several materials that can be used in combination to contribute to the development of this vascular network.

1. Matrix Materials for Cell Printing

Hydrogels are a potential medium to provide a support system for cell viability and movement for the development of artificial organs and tissues. Hydrogels are unique because of their mechanical properties. Their properties allow for cell growth and expansion without mechanical stress buildup. The strength of the materials is typically dependent on gel formation - either via natural gelation or crosslinking. [17] Rheological data suggest that hydrogels can be formulated at varying concentrations depending on the intended cellular applications [17]. The flexible mechanical properties allow diffusion of nutrients, small molecules, and proteins, which is necessary for cell survival within a matrix [18]. The flow curve, measuring shear rate versus shear stress, is a hysteresis loop [19]. Several polymers detailed in Table 1 have been researched and/or used to promote cell viability in regenerative medicine. Alginate is a natural anionic polymer extracted from brown seaweed and has been found in many commercial biomedical applications. Due to anionic nature, alginate must be cross-linked with cations (typically Ca2+) in order to form structurally sound hydrogels [19]. Alginate is a hydrophilic material with variable mechanical properties. Naturally occurring alginate has a molecular weight that ranges from 32,000 to 400,000g/mol [19]. While increasing the molecular weight of alginate improves the polymer's mechanical properties, high molecular weight alginate becomes extremely viscous, which is not an ideal quality for a cell-printing matrix. Alginate is a highly desired material for biomedical applications because it is structurally similar to the extracellular matrix cells are familiar with, along with its biocompatible tendencies. Alginate has been used for cell encapsulation experiments for tissue engineering research, and its biocompatible properties have shown to reduce the body's immune response to the foreign body [19]. Various peptides such as RGD can be added as side chains to the alginate backbone to further increase biocompatible properties and induce cell adhesive behavior. Alginate has been in a variety of applications including wound healing, tissue engineering, and drug delivery. Although alginate is extremely biocompatible and is capable of easy gelation, the polymer is inherent nondegradable in mammals,

Table 1: Properties of Cell Matrix Materials

Material	Mechanical Properties	Cell Adhesion	Natural vs. Synthetic
Alginate [20] [19]	Viscosity increases as pH decreases; physical properties increase with MW; structure resembles ECM	Yes, with the help of proteins (RGD)	Natural, can be synthetically modified with surface proteins
Fibrin Gel [21]	Very high elasticity, high stability, relatively low protein content, stiffening occurs in response to shear/ tension/ compression	Yes	Natural
Gelatin [22]	Properties resemble ECM; crosslinking; structure resembles collagen	Yes	Natural, derived from collagen hydrolysis
Hyaluronic Acid [18]	viscous; poor mechanical properties	Yes	Natural, in connective, epithelial & neural tissue

thus, limiting its productivity in mammalian tissue engineering [20].

Fibrin is a protein polymer that exhibits both viscous and elastic material properties. Despite its relatively low protein content, fibrin has a high stability in addition to a very high level of elasticity [21]. In addition to its role in thrombosis, fibrin assists in determining the functionality of cellular processes such as adhesion, migration, proliferation, differentiation, angiogenesis, inflammation, etc. [21]. Fibrin exhibits strong cell adhesive properties as a result of its role in blood clot formation and functionalizing cell processes within the body. Thus, theoretically, fibrin in the form of a gel would allow for cell adhesion upon seeding, as well as cell movement and nutrient diffusion.

Gelatin is a natural protein obtained from the hydrolysis of collagen molecules. To enhance the properties of gelatin, cross-linking with calcium phosphates allows for increased mechanical stability. The natural mechanical properties of gelatin resemble those of the extracellular matrix and collagen fibers, thus contributing to an ideal cell printing environment. Recent studies have also shown that combining gelatin with polyaniline or other carbon-based substrates can induce conductive properties within the gelatin matrix for specialized cardiac and nerve tissue engineering [22]. Gelatin is biocompatible and has been shown to induce minimal immune responses inside the body. With cross-linking, gelatin can achieve bioactive properties as described above. Proteins such as RGD can be bound to the gelatin molecules to promote cell adhesion in tissue engineering applications. Due to its wide range of properties, gelatin has been used in a variety of biomedical applications including tissue engineering, 3D cell scaffolds, and electrospun nanofibers and nanoparticles [22]. While the success of gelatin is owed in large part to the natural properties of collagen, gelatin itself exhibits better solubility and antigenicity than collagen, leading to a promising environment for 3D cell printing.

Hyaluronic acid (HA) is a glycosaminoglycan copolymer naturally found in connective tissues such as synovial fluid at body joints and natural scaffolding within cartilage. As a result of its natural applications within the body, hyaluronic acid is naturally viscous and has very poor mechanical properties. Natural hyaluronic acid also has a high rate of degradation, limiting its function during in vivo testing. Studies have shown that crosslinking hyaluronic acid and modifying the carboxylic acid and/or alcohol groups of the HA backbone can improve the mechanical limitations of the material [23]. HA has shown improved cell adhesion by incorporating RGD as a binding peptide to the molecule's chemical sequence. Because hyaluronic acid is found naturally throughout the body, it has been known to significantly contribute to cell proliferation and migration. With the proper chemical crosslinking and protein additives, hyaluronic acid can be a promising material for cell adhesion and growth.

2. Cell Adhesion

In addition to the matrix material in which the cells are seeded, the other vital materials are those that induce cell adhesion. These materials may be chemically combined with a matrix material to promote cell adhesion within the matrix, or they may also be printed within the matrix to promote cell adhesion followed by cell proliferation along the new material. Several materials commonly used for cell adhesion are listed in Table 2 and further detailed.

Fibronectin is a glycoprotein that occurs in two different forms: soluble and insoluble. Soluble fibronectin is found naturally in the blood and body fluids, while insoluble fibronectin is naturally found in various connective tissues and is associated with basement membranes of tissue layers [23]. Because of its varying forms and functions, fibronectin has a variety of mechanical properties that are promising for cell adhesion applications in regenerative medicine. As detailed in Table 2, fibronectin has a molecular weight around 440kDa and a coefficient of friction around 1.7 [23]. Fibronectin yields extremely promising cell adhesion properties, and it has been proven successful in cell adhesion, growth, migration, and differentiation, along with wound healing and embryonic development applications. In the body's natural environment, fibronectin binds to integrin proteins along cell membranes, and it works to bind the components of the extracellular matrix (including collagen and fibrin). If kept in its soluble form, fibronectin would potentially combine with a matrix material to promote cell adhesion to the matrix; if kept in its insoluble form, fibronectin would provide a sub-matrix for cell adhesion, growth, and proliferation within the printing matrix.

In addition to its gel form, fibrin can also be formulated into single-strand fibers. The proper extrusion of specified concentrations of fibrinogen and thrombin result in the formation of these fibers. Due to the viscoelasticity of fibrin, its fibers can be considered "self-repairing"; the fibers grow stiffer in response to shear stress, tension, and/or compression [21]. Fibrin exhibits strong cell adhesive properties because of its role in blood clot formation and functionalizing cell processes within the body [21]. Fibrin fibers have the potential to be printed within a matrix material to provide a cell adhesive material to promote cell growth and proliferation along a sub-matrix.

Collagen is a protein that is widely distributed throughout all connective tissue in the body and thus is considered the primary structural protein of the extracellular matrix. There are 29 types of collagen, but most biomedical applications highlight Types I, II, III, V, and XI. Each type is found in different part of the body, and these five types are known to form collagen fibers [25]. Because of its role in the human body, collagen is extremely biocompatible and exhibits promising cell adhesive properties. In addition, collagen can be used to increase adhesion of various proteins, such as VEGF, for use in cellular applications. Type I collagen is successful in tissue engineering applications [25]. Utilizing collagen's natural tendency to form fibers may be useful for printing a cell adhesive, collagen sub-matrix within a matrix material.

3. Chemoattractants

The use of various matrix materials for cell growth and cell adhesive materials allow for sufficient cellular seeding on or within biomaterials. In addition to these materials, chemoattractants may be used to promote the sprouting of vessels from biomaterials in tissue engineering applications. Vessel sprouting is essential for creating a vascularized network within matrices. Chemoattractants, or growth factors, can be useful in guiding cellular growth and movement. This can help to move cells within a matrix by attracting them in specified directions. Table 3 details several growth factors that can be used in promoting angiogenesis in various tissue engineering applications.

D. Vasculature

As discussed previously, when producing alternative tissues and organs, the largest issue is providing nutrients and oxygen to the cells and tissues. That is, the lack of a stable vascular network hinders the growth of healthy tissues. Viable vasculature must be within 200μm or less from all cells, act as a selective barrier, and minimize pressure of blood flow [32].

Table 2: Properties of Cell Adhesion Materials

Material	Mechanical Properties	Cell Adhesion	Natural vs. Synthetic
Fibronectin [23]	MW ~440kDa, coefficient of friction ~1.7	Yes (cell adhesion, growth, migration, differentiation, wound healing, embryonic development)	Natural
Fibrin [21]	Very high elasticity, high stability, relatively low protein content, stiffening occurs in response to shear/ tension/ compression	Yes	Natural
RGD Peptide [24]	Principal integrin-binding component in ECM proteins	Yes	Natural or Synthetic
Collagen [25]	High tensile strength	Yes	Natural

One method to provide nutrients to engineered organs is achieved using a bioreactor to allow for vessel growth and development, but this often takes days or weeks, and many times the vasculature still will not reach the center of the organ. Bioprinting vascular networks was another option explored to help provide nutrients and oxygen to cells in a timely manner; however, the resolution of commercially available bioprinters is not small enough to create a dense enough microvascular network [33] [34] [35].

Studies have also explored scaffolds and channels built to act as vasculature, but these were often too dense and impermeable, or would shift after implantation due to vascular remodeling [5].

Methods to stimulate angiogenesis and induce vascularization in tissue engineered organs through natural pathways could allow for the development of smaller, more stable vasculature. By utilizing angiogenic pathways to grow a vascular network, this could potentially create a network that cells could be seeded or printed into, acting as a predeveloped, nutrient rich cellular scaffold [33] [34] [35].

1. Angiogenesis and Endothelial Activity in Vascular Growth

Angiogenesis is defined as the formation of new blood vessels from existing networks; this process can be spontaneous or induced by pathways within the cell [6]. Angiogenesis contains 4 key steps: the degradation of the ECM, migration of cells from the original vessel towards a nonvascularized region, the proliferation of cells, and the maturation of those cells [36]. For this to occur, endothelial cells, which line blood vessel walls, must squeeze out through the degraded ECM, and escape from the original vessel. There are two main ways in which this happens: angiogenic sprouting, which sprouts new vessels from existing vasculature, and intussusceptive angiogenesis, which inserts a pillar into existing capillaries, and branches them internally [37]. One important consideration for the information provided about angiogenesis is that the details of sprouting mechanisms are well understood while intussusceptive mechanisms are not. The formation of a new vessel sprout occurs in response to invasion of endothelial cells at a distinct site in the surrounding tissue or matrix. This process of choosing a distinct sprouting site repeats as the new sprout branches and forms a network with other surrounding sprouts [28]. The branching occurs by elongation of the sprouts, which is prompted by the proliferation and migration of endothelial cells. The repeated sprouting and elongation through cell growth and proliferation results in branched vasculature [38]. The newly created networks provide structure for oxygen and nutrient diffusion through the desired tissue vasculature.

Table 3: Key Chemoattractants in Angiogenic Signaling

Material	Natural or Synthetic	Function
FGF-2 [26]	Natural	Maturation of fibroblast vessels
PDGF [27]	Natural	Fibroblast growth, vessel wall maturation
VEGF [28]	Natural	Endothelial cell growth, migration
ANG – 1 [29]	Natural	Stabilizes vessels during linear growth
Nell – 1 [30]	Natural	Enhance pericyte proliferative, osteogenic, and angiogenic capabilities
CCN2 [31]	Natural	Pro-angiogenic functions during development, would, repair, cancer. Direct regulator of endothelial cell activities

2. Chemoattractants and Angiogenesis

As discussed previously, chemoattractants can be leveraged as a means to promote vessel sprouting and endothelial cell migration. These activation pathways naturally exist in the cell as the body secretes factors to signal for vessel growth into a new region. The main pathways involved with angiogenesis are signaled by proteins such as vascular endothelial growth factors (VEGF), fibroblast growth factor (FGF-2), and platelet-derived growth factors (PDGF), as outlined in Table 3 [39] , the most prominent being VEGF. Mechanisms for sprouting are well known; Figure 3 depicts the varying stages of vessel sprouting in response to VEGF and endothelial cell activity.

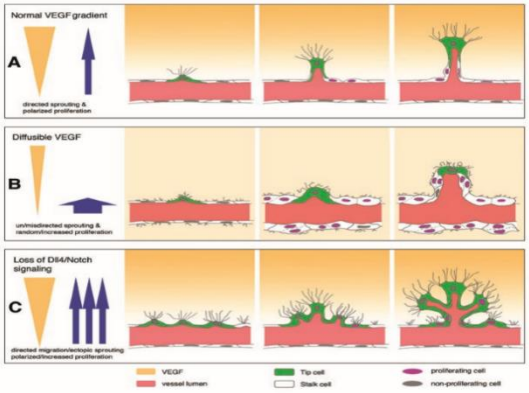

Figure 3: Stages of Vessel Sprouting in Response to VEGF and Endothelial Activity [40]

These pathways can be targeted to help induce angiogenesis; for example, VEGF can be used as a guide for remodeling. When these are added to the hydrogel, the vascularization will follow the path of the factor [34]. Several experiments have shown the influential role VEGF plays in cellular movement and angiogenesis. For cellular movement, scientists have seeded cells in a transwell insert, and coated the bottom of the plate with VEGF and other ligands. By doing this, they can measure the number of cells that pass through the transwell membrane and compare those results to uncoated plates to see increased rates of cell migration. Similarly, to measure angiogenic sprouting, cells were seeded on beads and placed in fibrin gel which was supplemented with VEGF and several other ligands. The added VEGF resulted in increased sprout length and number of sprouts [41].

Studies exploring the combination of various signal pathways have shown promising results [34]. The combination of different factors may help to increase the rate a vessel reaches maturation [33]. A study, represented in Figure 4, targeted pathways with VEGF and angiopoietin 1 (ANG-1) to increase the growth and maturity of the vascular structure at an increased rate. Over time, the number of cells attached to each vessel tip increased, but the cells that incorporated both growth factors had significantly more than the other groups, suggesting that this may speed up development [34].

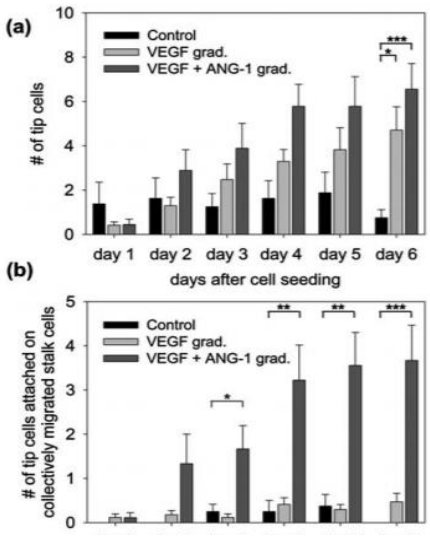

Figure 4: Effect of Targeted VEGF and ANG-1 Pathways on Growth and Maturity [34]

Similarly, platelet-derived growth factors (PDGF) andANG-1 can be used in concert to achieve similar results to those seen when using VEGF alone. In a study performed on the PDGF signaling pathway, platelet-derived microparticles were added to an existing vasculature. The addition of these factors greatly induced angiogenesis and was comparable to traditional VEGF factors [33]. Figures 5-2, 5-3, 5-4, and 5-5 demonstrate the growth of the vasculature over time. Figure 5-6 demonstrates growth using VEGF factors, and Figure 5-1 demonstrates growth without inducing angiogenesis. The results of this study suggest that various pathways can be explored to promote the growth and angiogenic response of endothelial cells.

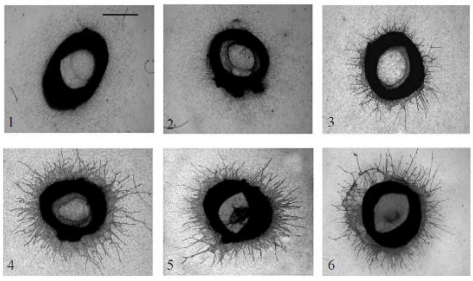

Figure 5: Growth of vasculature over time with PDGF pathway [33]

3. Creating Leaky Vasculature in Vessels

As discussed previously, cells need to be able to escape and migrate to allow for new vessel formation. Angiogenic sprouting requires increased porosity in the walls of preexisting vasculature; under the normal mechanisms of VEGF growth factors, porosity is first increased, by breaking down the ECM, then angiogenesis can occur. Without the natural breakdown of the ECM, the vasculature needs to be made more porous to ensure escape is possible [42]. A couple of methods to increase porosity in cell walls by degrading different concentrations of the present cellulose have been explored in previous studies. These methods include the use of different alkali treatments and cellulase digestion [43].

One study compared an alkali, or basic treatment, to treatments with acid and hot water solutions. In this study, leaves of the species *Populus* (trees in the Salicaceae family) were tested; the porosity of each leaf was measured following each treatment. The study found that treatment with hot water and dilute acid decreased porosity with increased temperatures over time, while treatment with basic solutions increased porosity with increased temperatures over time [43]. In a similar study, the effects of different alkali solutions on porosity were tested. The study analyzed four different dilute acids: K_2CO_3, Na_2CO_3, KOH, and NaOH. In this study cellulose was boiled in 0.1M of each solution, and it was found that the bases created varying ranges of porosity. The porosity each base, from the most porous to the least, is as follows: K_2CO_3 > Na_2CO_3 > KOH > NaOH [44].

Another option to break down the cell wall is to use cellulase as a catalyst for digestion. Cellulase cuts the polysaccharide chain of sugars in cellulose (one of the main components in plant cell walls) into individual monosaccharides, breaking down the structure of the plant's cell wall. Cellulose is organized into long chains that make up microfibrils, depicted in Figure 6. The hydrophobic regions indicated by the arrows are thought to be the point for cellulase attack. This however, is done at random, and due to variations in different regions of the wall, is not always the most efficient way to evenly break down cellulose. These enzymes contain glucanases, which help to cleave the bonds on the cellulase chain and allow for hydrolysis to occur [45].

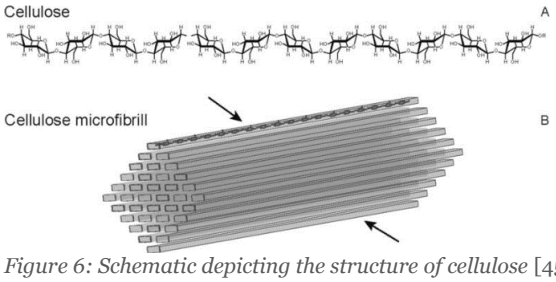

Figure 6: Schematic depicting the structure of cellulose [45]

Many studies have been done to improve the digestion of cellulose. Pretreatment to the biomass may make the cellulose more accessible to the enzymes and allow for a more even breakdown of the cell wall. In one study, porosity was increased utilizing cellulase, a digestive enzyme, to digest portions of the cellulose in the cell wall. In this study, performed on corn stalk cell walls, the plants were pretreated with 2% H_2SO_4 and then digested with cellulase enzymes. The study found that the pretreatment increased cellulase activity, therefore increasing porosity. Additionally, with increased temperatures, the enzyme activity increased, creating relatively more porous cell walls [46]. Other studies have explored other pretreatments such as alkaline chemicals, and organosolvents that break down the other components of the cell wall, but acidic treatments are the only ones to reduce conversion time from the untreated stem and thought to increase accessibility of cellulose [47].

4. Leaf Anatomy: Commonalities between plant & animal kingdoms

As discussed above, the current method of using decellularized organs has proven successful only in isolated instances. One of the main problems with this method, however, lies in the availability of the necessary organs to decellularize and use in patients. The current demand for organs gravely outnumbers the supply available for medical use. It is nearly impossible to account for more than 100,000 patients in need of transplanted organs or related structures [1]. The field of regenerative medicine is moving quickly in the direction of developing tissue engineered systems to provide a more reliable solution for obtaining viable organs. These systems would soften the urgent timeline necessary for harvesting and transplanting organs between patients. Utilizing viable materials that are more readily available can help to overcome this major challenge in the field of regenerative medicine.

Engineers have recently discovered that the complex structure of leaves can be broken down into various base, structural elements and systems comparable to those found in human cells and tissue layers. While animal and plant kingdoms differ in basic cellular properties and functions, the vascular networks found in the tissues of each are very similar.

Figure 7 depicts the similarities in vasculature between animal and plant tissue layers [5]. Figure 7 shows the obvious macroscopic structural differences between a decellularized human organ and a decellularized leaf. However, the microscopic images depict the similarities between human and plant vasculature on the tissue level. This is due in part to the correlation between plant vasculature patterns and Murray's Law, the accepted physiological rule describing the branching network structures found in the human cardiovascular system [48].

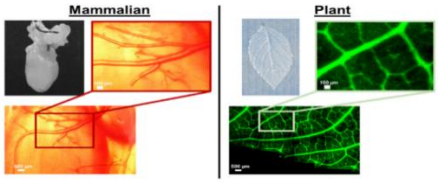

Figure 7: The Similarities in Vasculature Between Animal and Plant Tissue Layers [48]

Other similarities include varied mechanical properties common within the animal and plant kingdoms that allow for a variety of functions. For example, the polysaccharides such as cellulose that make up plant cell walls have high biocompatibility and strong wound healing capabilities [49]. Engineers believe that utilizing related vasculature and structures found in plant tissue can provide tissue engineers with viable living scaffolds that are available in much higher quantities and at much lower costs compared to transplanted human organ [5].

The overall anatomy of a leaf is comparable to that of human tissue. Figure 8 depicts the cross-sectional anatomy of leaf tissue. Like human tissue, plant tissue is comprised of various functional layers. The upper epidermis provides protection, the mesophyll layers allow for transport within the tissue and house the leaf vasculature, and the lower epidermis provides a second layer of protection [50]. Found within the

epidermal layers of the leaf tissue are stomatal pores acting as openings into the mesophyll layers. These pores allow for oxygen diffusion through the inner vascular networks. Like capillary action found in human veins and arteries, the inner veins and vasculature in leaves allow for the movement of oxygen and other biomaterials throughout the entire tissue [48]. The vascular networks in plants provide the necessary movement of nutrients throughout each tissue layer, potentially providing a viable living structure for regenerative medicine applications to be used in human patients.

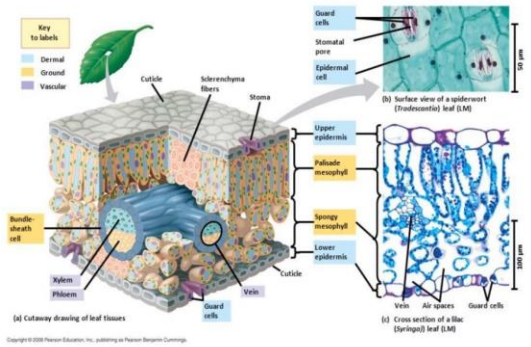

Figure 8: Cross-Sectional Anatomy of Leaf Tissue [50]

III. PROJECT STRATEGY

The following section outlines the overall approach for the project. This includes expectations for the project, objectives, and requirements. This provides a guideline to inform the final design and ensure that the design is best meeting the client's needs and specifications.

A. Client Statement

Well characterized and reproducible macro- and microscale vasculature are needed for the development and assembly of engineered tissues. Macroscale vasculature must be perfusable and allow for manual and/or surgical anastomosis to non-biological hardware and native vasculature respectively. As a result, the anastomosis-capable end(s) of the macroscale vasculature should be no smaller than ~26G needle and be able to be sutured or otherwise attached to hardware/native vasculature. The scaffold or any other means of producing the microvasculature should be biocompatible and allow for both endothelial cell seeding and barrier formation within its lumen. Microscale vasculature can be entirely comprised of self-assembled, stabilized endothelial cell tubules. Microscale vasculature should be on the order of single digit to tens of microns in diameter. Microscale vasculature would ideally connect engineered or otherwise produced macroscale vasculature to make possible or at least enhance the transport of large molecules including from macroscale vasculatures spaced at least 200µm apart.

Microscale vasculature can arise from vasculogenesis and/or angiogenesis. Size distribution, diffusive properties, and bio-integration (cell seeding, barrier formation [macro-], and vessel density formation [micro-] should be known and controlled for. All materials must be biocompatible and cell viability of greater than or equal to 80% is acceptable.

B. Design Requirements: Technical

Presented next is an evaluation of critical design objectives and constraints. These have been grouped into three major design attributes for consideration: biological, mechanical, and industrial attributes. Objectives, presented in Table 4, are defined as statements of the client's needs or wants.

Table 4: Objectives

Biological Objectives
● Successfully utilize the VEGF signaling pathway ● Minimize time it takes for cellular movement from scaffold ● Maximize the percentage of HUVECs that escape from scaffold ● Provide a hydrogel matrix that maintains cell viability
Mechanical Objectives
● Allow for diffusion of oxygen and nutrients within the gel ● Provide a scaffold with enough porosity to allow for cellular escape ● Reduce internal pressure on cells and scaffold ● Reduce shear stress/cell deformation within the gel ● Provide a hydrogel matrix that allows for cellular movement
Industrial Objectives
● Allow for production of scaffold-gel construct in 4-6 weeks ● Allow for large scale production of decellularized material ● Allow for production of sterile gel in mass quantity ● Translatable for large scale incubation for cell growth ● Opportunity to automate cell loading process

Next, the constraints for the project are presented. Constraints are defined as critical design components needed for design function; without these the design would fail. These attributes are highlighted in Table 5.

Table 5: Constraints

Biological Constraints
• Must allow for diffusion of oxygen and nutrients to the cells in the construct • Materials used must be proven biocompatible or similar to known biomaterials • Must allow for cellular adherence to scaffold
Mechanical Constraints
• Oxygen and nutrients must be able to diffuse within the gel
Industrial Constraints
• No one step in the process should take longer than 8 hours, not including incubation times • Must have consistent protocols, to ensure identical results

Design functions are outlined Table 6. These functions are defined as important or relating to the operation of the design.

Table 6: Functions

Biological Functions
• Allow for cellular movement within the gel, including endothelial cell escape • Cells must adhere to and escape from the scaffold
Mechanical Functions
• Gel must provide support and structure for the cells • Gel must allow for cellular movement • Stem must act as a scaffold for cell seeding
Industrial Functions
• Allow for a scalable and reproducible process

Finally, the specification for the design are presented. Specifications are defined as statements relating to operational limits, ranges, etc. These attributes are outlined in Table 7.

Table 7: Specifications

Biological Specifications
• Seed HUVECs and fibroblasts into stem scaffold at a ratio of 1:3 and a density of at least 100,000 [26] • 80% of cells must remain viable for 48 hours after seeding • Cells must escape the scaffold within 7 days [51]
Mechanical Specifications
• Vessel walls must maintain integrity under pressure of at least 150mmHg (calculated max pressure within a 10μm vessel using average blood pressure information provided by the American Heart Association) [52] • Cells must allow for 30-50% cellular escape from the scaffold
Industrial Specifications
• Production of structure must take between 4-6 weeks

C. Design Requirements: Standards

The following table outlines industry standards that would need to be adhered to and considered in the design of this project in accordance with ISO guidelines.

Table 8: Key industry standards for the final design

Important Industry Standards
• Sterility (ISO 11737-2:2009, sterilization of medical devices) [53] • Endotoxin and pyrogens (ISO 10993-11: Systemic Effects) [54] • Blood transfusion (ISO/TC 76 - Transfusion, infusion and injection, and blood processing equipment for medical and pharmaceutical use) [55] • Biocompatibility (ISO 10993-1 Biological Evaluation and Biocompatibility Testing of Medical Devices) [56] • Human Cell Applications (ISO 13022:2012, Medical Products Containing Viable Human Cells - Applications of Risk Management and Requirements for Processing Practices) [57]

D. Revised Client Statement

Design a perfusable scaffold for endothelial growth and attachment to support cellular escape and migration from the original scaffold. The construct will incorporate a hydrogel matrix that allows for cell viability and proliferation after migration. The construct should allow the beginning stages of angiogenesis to occur and provide the foundation for further expansion of the matrix into a functioning vascular network.

E. Project Approach

To begin the project, the group first became familiar with the mechanisms and characteristics of angiogenesis and 3D printing. This allowed for the proper knowledge to adequately integrate the two. Research was conducted regarding previous studies involving angiogenic behavior of endothelial cells. Other experiments of interest included scaffold use for regenerative applications as well as different materials used for cell adhesion and migration. After initiating communication with the project sponsor, DEKA Research and Development Corporation, the team outlined expectations for the project, and specified short-term and long-term goals. The team determined that the overarching problem DEKA is facing with printing viable organs and tissues was the lack of established vasculature within the printing matrix. To overcome this, the team decided to focus the project on the initial steps of angiogenesis, mainly cellular adhesion to an existing vessel or structure, cellular escape from this vessel, and migration into the printing matrix. This would be accomplished by inducing cell movement from an existing scaffold through a gel.

The group brainstormed different possible solutions for the problem, considering previous research in the designs. After narrowing down possible solutions, the group drafted preliminary designs for the project. These preliminary designs addressed certain criteria such as objectives, constraints, functions, and specifications. For testing purposes, the team chose to work with human mesenchymal stem cells (hMSCs) to model cellular adhesion based on their ability to be loaded with quantum dots. These quantum dot loaded cells would be useful in imaging the cells within the scaffold and verifying the design. Success with these cell types would verify the intended cellular behavior and lead to ultimately using human umbilical vein endothelial cells (HUVECs) in the final design because of their role in vessel formation within the body.

Additionally, recent discoveries in utilizing decellularized plant material as scaffolds for cell seeding motivated the team to use such materials, specifically parsley stems and spinach leaves, as a main scaffold for the project. Different gels were also investigated, and the decision to use fibrin was based on its mechanical properties as well as its role in wound healing and high affinity for cell viability and movement [21] [26] [41] [51]. This would be used to create a scaffold-gel construct to allow for the escape and migration of HUVECs from the main plant stem.

As mentioned, the scaffold-gel construct needed to exhibit certain characteristics to achieve the desired functions. These characteristics included: adequate cell adhesion, leaky vasculature allowing cellular escape, and cell viability and movement within the chosen gel. Cellular adhesion was monitored through fluorescent imaging of the quantum dot loaded cells seeded within the scaffold. Porosity was quantified by extruding microspheres 10μm in size, similar to the size of HUVECs, through the plant stems and counting the microspheres that escaped using a hemocytometer. Cell viability was tested by seeding fibrin gels with human fibroblasts and performing live/dead assays after 48 hours to determine cell survival. The plant scaffold would be seeded with hMSCs and fluorescently imaged to ensure adequate adhesion of the human cells to the decellularized plant material. The seeded cells would then be monitored for migration into the gel from the plant scaffold, and fluorescent images would be taken of the gel to determine cellular escape from the original scaffold and migration through the gel.

IV. DESIGN PROCESS

The following section discusses the overall design process. This presents an in-depth analysis of the client's needs as well as multiple conceptual and alternative designs that meet those needs. Additionally, preliminary studies and findings are presented, and the design selection is discussed.

A. Needs Analysis

To evaluate the importance of various design criteria, a pairwise analysis has been performed. This outlines major categories for the design. It has been discussed in detail with the client to determine which features are most important moving forward with a final design. From this analysis, it was determined that the most important criteria to be met were the diffusion of nutrients, induction of angiogenesis, and the induction of vasculogenesis/connectibility of

vessels. The entire copy of the pairwise analysis can be viewed in Appendix A.

B. Conceptual Designs, Modeling, Feasibility

Based on the design criteria, different designs were developed and explored through prototypes and feasibility studies. The design was narrowed down to one optimal design for the project based on the results of these prototypes and studies.

1. Concept Map

The concept map in Figure 9 depicts the initial thought process and demonstrates how each component fits together to ultimately result in vasculogenesis.

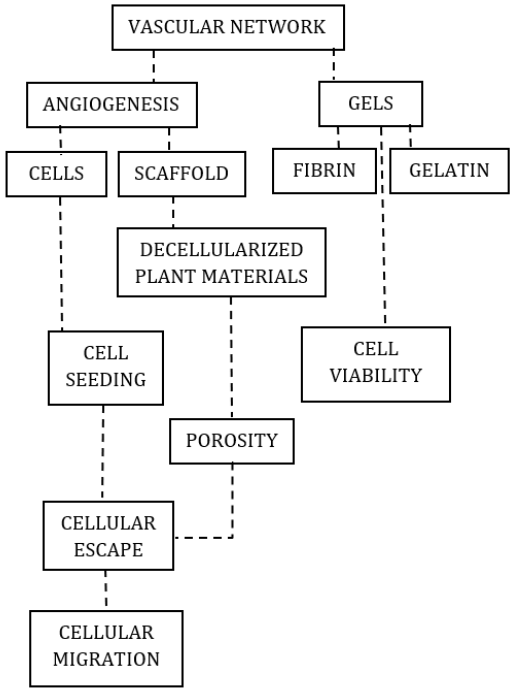

Figure 9: Concept map for vasculogenesis

2. Conceptual Designs

Here, the main concept of the vascular network is presented in Figure 10. In this design, two decellularized spinach leaves are placed within a gel matrix and the stems of both leaves are perfused with human endothelial cells (HUVECs). This would be completed using a bioreactor to allow constant perfusion. Ideally, the endothelial cells would then escape into the gel through the natural vascular scaffold provided by the veins in the leaf. The endothelial cells would then begin to exhibit angiogenic markers and there would be visible sprouting and cellular movement through the gel. The movement of the cells through the gel matrix would eventually result in the growth of new blood vessels and the formation of a full vascular network (labeled as the vascular network in Figure 10).

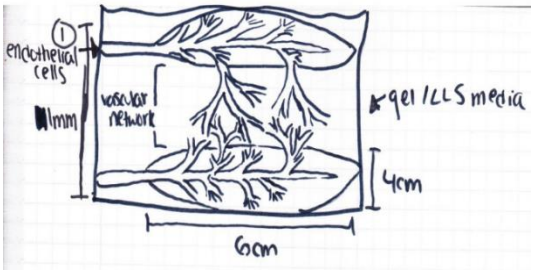

Figure 10: Conceptual Design 1, Vascular Network

Figure 11 depicts a second conceptual design through which two decellularized parsley stems would be laid side by side within a gel matrix. The inside of two decellularized parsley stems will be seeded with HUVECs after being treated with fibronectin, a cellular adhesive protein that would help to increase the likelihood of the cells sticking to the plant stem wall. The outside of each parsley stem will be coated in growth factors that would act to promote cellular escape of the HUVECs through the parsley stem wall and into the gel matrix. Once the cells would escape through the parsley stem walls, they would migrate through the gel matrix and begin forming new blood vessels; this would eventually lead to the formation of a full vascular network within the gel.

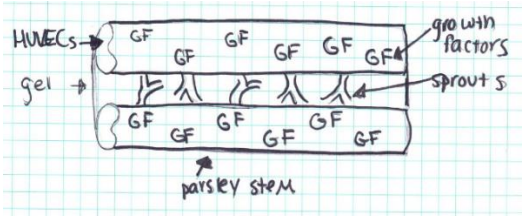

Figure 11: Conceptual Design 2, Coated Growth Factors

The next conceptual design, depicted in Figure 12, includes again the use of growth factors to stimulate angiogenesis. Similarly, this design consists of two decellularized parsley stems positioned side by side within a gel matrix. In this design, the growth factors would be extruded in

the gel between the two stems. Again, the inside of both stems would be seeded with HUVECs. The extrusion of growth factors within the gel matrix would in theory encourage endothelial escape from the plant stem, followed by the sprouting of new blood vessels within the gel matrix in the direction of the growth factors. This would ultimately lead to the continued growth of these blood vessels and the creation of a vascular network (not depicted in Figure 12) between the stems.

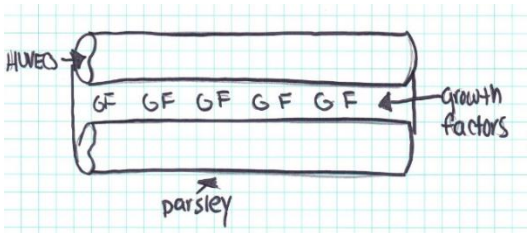

Figure 12: Conceptual Design 3, Extruded Growth Factors

The final conceptual design depicts two decellularized parsley stems placed within a gel matrix. Each will be seeded internally with HUVECs and externally with human fibroblasts. Figure 13 shows how the fibroblasts on the outside of the stems will act as natural chemoattractants via their natural secretion of growth factors. This would promote cellular escape of the HUVECs through the parsley stem wall and induce sprouting of vessel walls from the parsley stem into the gel. This would ideally lead to the continued growth of new blood vessels that would develop into a full vascular network within the gel matrix.

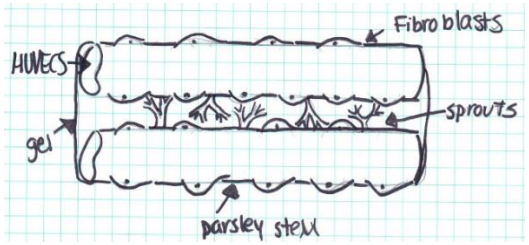

Figure 13: Conceptual Design 4, Fibroblast Coating

3. Feasibility Studies

The following studies aim to determine the feasibility of different experimental designs. This section summarizes some of the initial tests conducted to ensure the success of the project. The tests looked at key features such as biocompatibility, decellularization, cellular adhesion, porosity treatments, and gel characterization.

i. Biocompatibility

Throughout the duration of this project, there was a concurrent study conducted to analyze the biocompatibility of decellularized plant scaffolds. This study implanted a decellularized spinach leaf subcutaneously in rat stomachs and kept the implant within the rat for up to 4 weeks. The sections of the rat were dissected and processed using histology techniques. The final images, included in the preliminary data, model the immune response within the rats.

ii. Decellularization of Parsley Stems

Since this project analyzed the potential for utilizing parsley stems as a scaffold, the ability to decellularize parsley was analyzed. The parsley was submerged in 1% SDS and left on the rotator for 48 hours. Then, the stems were transferred to a 0.1% Triton-X-100 in a 10% sodium chlorite bleach for another 48 hours. Finally, the stems were then rinsed in deionized water (diH_20). The decellularization of these stems was assessed by appearance compared to the spinach leaves

iii. Cell Adhesion

To ensure the cells would adhere to the decellularized parsley, a feasibility study was conducted, mimicking the methods used in the paper Gershlak, et al.

A dried, decellularized parsley stem was rehydrated in sterile PBS, then left under UV light for 20-30 minutes. A 10µg/mL solution of fibronectin was created in sterile PBS. A syringe was used to draw up air, 100µL of the 10% solution, and then air. The first bubble of air was pushed through the stem to rid the stem of excess PBS. The solution was pushed through until just visible as a bubble formed on the opposite end. The stem was then placed in a new petri dish and incubated in 37°C and 5% CO_2 for 30-45 minutes. One end of the stem was then tightly sutured, and 500,000 fibroblasts, suspended in 100µL of media, were inserted into the stem with the syringe. The open end of the stem was then tightly sutured and placed in a petri dish. The stem was left to sit for 1-2 hours. Then, 15mL of media was added to the petri dish. The dish was left inside an incubator at 37°C and 5% CO_2 for 76 hours. After 76 hours the stem containing cells was fixed in 4% paraformaldehyde.

iv. Porosity Treatments

Different treatments were explored to improve the porosity of the parsley stems and allow for improved diffusion out of the scaffold. Three different base treatments (NaOH, KOH, and Na2CO3) were explored. Observations about the stem integrity and availability to diffuse were made. Additionally, treatments of 2.5% cellulase were tested. The cellulase was allowed to digest for 20 minutes and 40 minutes to observe the effect upon the stems and diffusion of the cells.

5. Gel Characterization

Finally, different gels were explored for support in cell migration. After conducting research about existing gels, the design was narrowed down to two main options: fibrin or gelatin. Both gels were made, allowed to gelate, and were sterilized. The study assessed ease of gelation and filtering, time to gelate, temperature for gelation, and overall characteristics of the gels.

6. Histology

Sections of the stem coated with HUVECs were taken along the axial and transverse faces. Sections were taken at 10µm and stained with an H&E stain to look for the presence of cell nuclei and cytoplasm within the decellularized parsley stem.

4. Preliminary Data

i. Biocompatibility

The biocompatibility of the scaffolds was tested by implanting the decellularized leaves subcutaneously on the stomach of rats. The results of the 4-week test are presented in Figure 14.

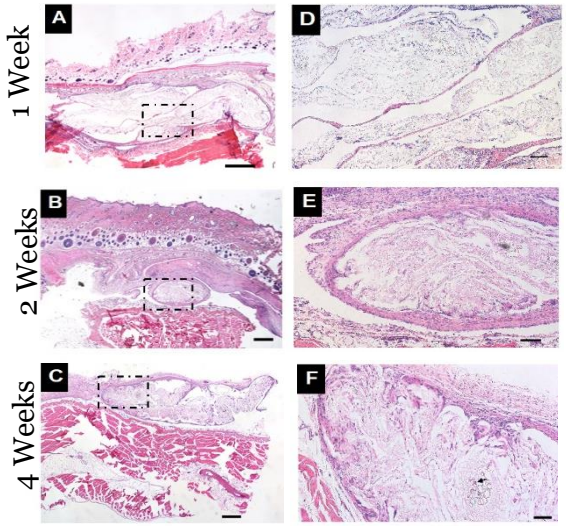

Figure 14: Results from biocompatibility testing on decellularized spinach leaves

Figures 14-D, 14-E, and 14-F serve as a magnified image of the box indicated in Figures 14-A, 14-B, and 14-C. At one week, the scaffold is visible within the rat. By 2 weeks the body has encapsulated and begun to wall off the material, indicating an immune response; however, by week 4, the scaffold has started to become integrated into the subcutaneous layer. This suggests that the material is in fact biocompatible and would serve as a viable scaffold for cellular adhesion and eventual implantation into the body.

ii. Decellularization of Parsley

From the preliminary testing, we have obtained data relating to the decellularization of parsley stems. Figure 15 is a 2in parsley stem after being treated with SDS and 0.1% Triton X-100 in a 10% sodium chlorite bleach. This protocol was obtained from Gershlak et al. and yielded a decellularized parsley stem containing no traces of pigment nor plant cells after being treated with the preceding chemicals, as seen in the Figure 15. This served as a positive indicator that parsley could be decellularized as in the study done by Gershlak et al [5].

Figure 15: Decellularized parsley stem, approximately 2in in length

iii. Cell Adhesion

Cellular adherence was tested in two different plant models: spinach and parsley. The Gershlak, et al. paper demonstrated the ability to seed human umbilical endothelial cells (HUVECs) into the main stem of the decellularized spinach leaf. Based on that research, the following experiment aimed to test cellular adhesion to the decellularized parsley stem and compare the results to the established cellular adhesion of decellularized spinach leaves. The following tests seeded fibroblasts into a parsley stem to confirm the viability of the scaffold for cellular adhesion. The stem was hydrated in sterile PBS and sterilized using UV sterilization, and pre-coated with fibronectin to increase cellular adhesion to the plant material. The parsley stem was seeded with 500,000 fibroblasts and left to incubate for three days. Figure 16 presents the histology samples, stained with an H&E stain.

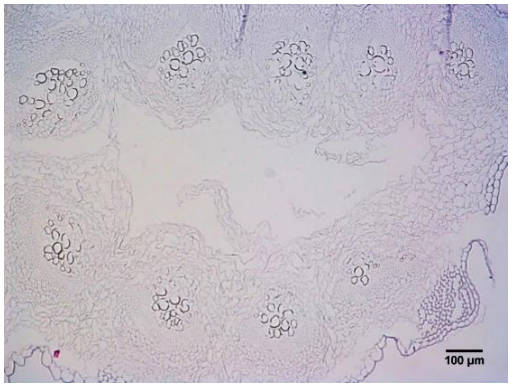

Figure 16: Fibroblasts seeded into a 2in, decellularized parsley stem (800,000 cells) for 48 hours

Figure 16 demonstrates the cellular adhesion for the potential parsley scaffold. As seen through the histology sample, the parsley stem appeared to have little to no seeding after three days and remained consistent with past seeding attempts. The lack of cells lining the inside of the parsley stem indicates that this scaffold would allow for inadequate cellular adhesion.

iv. Porosity Treatments

Porosity of the plant stems was analyzed to ensure diffusion from the scaffold was possible. This was modeled with parsley stems and the stems were treated with a 2.5% cellulase solution over different time points. The major initial conclusions are outlined in Table 9.

Table 9: Observations from different treatment considerations

Treatment	Observations
NaOH	Stem appears stiffer and thinner
KOH	Stem appears more degraded
$NaCO_2$	Stem appears very degraded and much softer
Cellulase 20 minutes	Stem appears intact
Cellulase 40 minutes	Stem appears intact
Cellulase 12 hours	Stem is majorly degraded
Cellulase 18 hours	Stem is nearly entirely degraded and cannot hold original shape
Cellulase 24 hours	Stem is entirely degraded and unusable

From these conclusions, cellulase treatments under an hour were interpreted to be the most promising solution to increasing porosity and diffusion. This data would be useful for future experiments in establishing the appropriate porosity for cellular escape through the chosen plant scaffold.

Additionally, the stems with the most promising initial structure post treatment (cellulase treatments under an hour) were perfused with 200,000 microspheres, 10µm in size. These microspheres were collected and counted using a hemocytometer and the percentage of microspheres escaped was calculated. These results are also presented in Table 10.

Table 10: Percentages of 10µm escaped with different treatment times of 2.5% cellulase

Treatment	n =	Average *
No Treatment	3	28.0 ± 7.07
20 Minutes Cellulase	1	27.8 ± 0**
40 Minutes Cellulase	2	35.7 ± 16.3

*Outliers were defined as stems that burst, or took greater than 24 hours to rehydrate leading to unreadable, or unrepresentative data

**Two of the three samples were considered outliers, standard deviation of zero is not representative of the true deviation

Examining the average percentage of microspheres escaped, it appears that the stems not receiving any treatment had the highest percentage of microspheres escaped with the lowest standard deviation. From these averages, it appears that the 40-minute cellulase treatment has the highest percentage of escaped microspheres; however, these stems also had the highest standard deviation. The 20-minute cellulase treatment did have the lowest standard deviation when the outliers would have been excluded, but that was due to a sample size of one, resulting from burst stems during the procedure.

v. Gel Characterization

2% Gelatin was created by heating the solution to 50°C until it became a viscous liquid, at which point it was filtered in the biosafety cabinet using a 0.8µm filter. It was allowed to gelate in the fridge overnight. Finally, a solution of equal volumes of fibrinogen and thrombin were incubated, to form a fibrin gel. Each of the components were filtered individually before mixing to make fibrin. Once the solution was removed from the incubator 30 minutes later, a gel was formed. An initial comparison of the two gels is presented in Table 11.

Table 11: Comparison of fibrin and gelatin for use in the final design

Fibrin	Gelatin
Gelation at 37°C	Gelation at -4-10°C
Known player in wound healing	Needs crosslinking to remain a gel in incubator
Biocompatible	Biocompatible

Based on the observations presented in Table 11, fibrin appears to be the more feasible gel with better potential for success. Fibrin is a known agent involved in wound healing and other cellular processes within the body and would thus be useful in working with our chosen cells to promote the growth of new blood vessels.

C. Alternative Designs

Prior to finalizing a concept and final design, we drafted several alternative designs. Figure 17 depicts a single spinach leaf suspended in a gel or liquid-like solid media. The endothelial cells are then perfused through a bioreactor. The intent of this design was to prompt vessel growth across the leaf and into the gel.

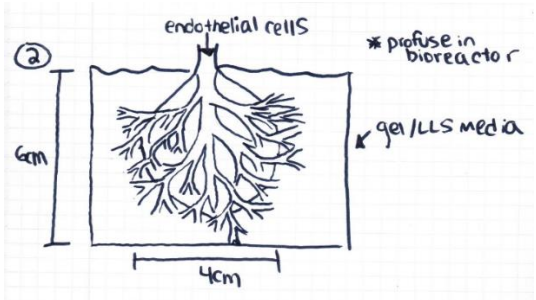

Figure 17: Alternate Design 1, Spinach Leaf Suspended in Gel

Figure 18 shows a side view of another alternative design. It shows two leaves sandwiching an area of gel between them. The premise of this concept was to print either columns (1) or fibers (2) of cell adhesive material from the surface of one leaf, through the gel, to the surface of the second leaf. This cell adhesive material would guide the endothelial cells through the gel and ultimately sprout vessels.

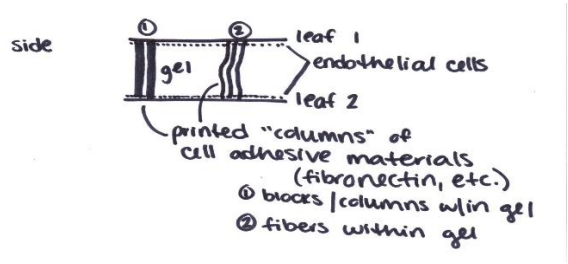

Figure 18: Alternative Design 2, Columns of Cell Adhesive Material

Figure 19 shows two spinach leaves positioned side by side submerged in a gel rather than stacked into a sandwich. To ease the stress of creating branched vessels in the Z direction, this design would allow for vessel sprouting in the same plane as the existing leaf vasculature.

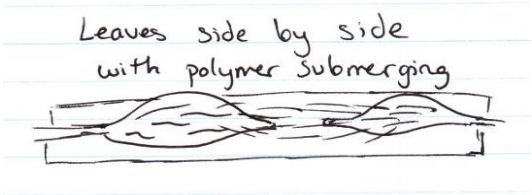

Figure 19: Alternative Design 3, Leaves Side by Side

This design, depicted in Figure 20 shows a single spinach leaf within a gel. As endothelial cells are perfused through the leaf stem, chemoattractants and/or growth factors would be printed in the Z direction within the gel. The endothelial cells would ideally "climb" the chemoattractants and sprout vessels in the Z direction within the gel.

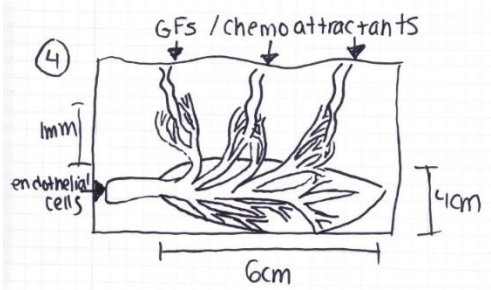

Figure 20: Alternative Design 4, Growth Factors in Gel to Guide Growth

A final alternative design, depicted in Figure 21, would be printing microfluidic channels as guides for endothelial cell migration within a gel. These channels would be constructed of a cell adhesive material (such as fibrin or collagen) to promote cell adhesion.

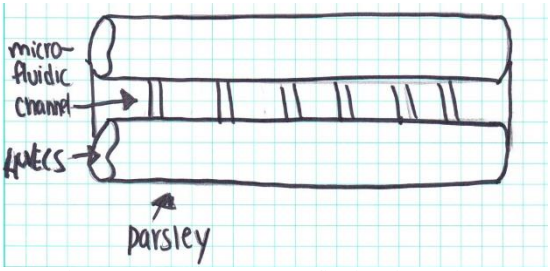

Figure 21: Alternative Design 5, Microfluidic Channels

D. Final Design Selection

Following these preliminary studies, the team came up with a final design, pictured in Figure 22. Decellularized spinach leaves, pictured in Figure 22-A, were chosen as the scaffold for this design based on the proven positive cellular adhesion to the spinach leaf stems. Figure 22-B shows the potential for modular designs using the decellularized spinach leaves for the final design. In a similar manner, the HUVECs would be seeded along the inside of the spinach leaf stems, and the cells would theoretically escape through the natural vascular of the spinach leaf veins. As pictured in Figure 22-C, this design would then be placed within a gel matrix. The HUVECs would escape into the gel matrix. If necessary, growth factors may be added to the gel matrix to encourage the growth of new blood vessels in specified directions. This would ultimately lead to the development of a full vascular network within the gel matrix. In terms of 3D bioprinting, this structure and vascular network would be within the gel matrix that the designated cell type is printed into. This would allow for the proper nutrient diffusion to the printed cells within the matrix. This process is depicted in Figure 22-C.

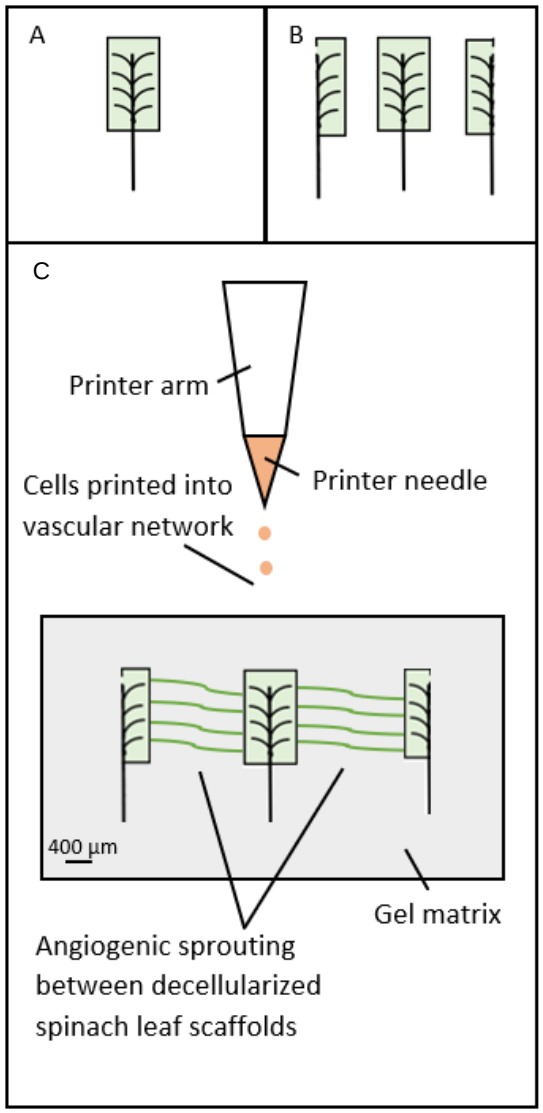

Figure 22: Schematic of final design construct

V. FINAL DESIGN VERIFICATION

The following section presents the raw data from tests for each individual component of the project design. The experiments aimed to ensure the functionality of the construct as a whole by evaluating cellular adhesion of the cells within the scaffold, escape from the scaffold, and migration through the gel. The compilation of these components demonstrates the proof of concept needed for the success of the final design.

As previously discussed in section IV, Design Process, a concurrent study confirmed the biocompatibility of the scaffold, so it was not explicitly examined in our results.

A. Cell Adherence

Cellular adherence of human cells to decellularized spinach leaves was tested with quantum-dot (Q-dot) loaded human mesenchymal stem cells (hMSCs). Cells were loaded with Q-dots 24 hours prior to seeding within decellularized leaves. 500,000 cells were seeded into a decellularized spinach leaf and cultured over 36 hours. These cells were then imaged on an inverted scope with fluorescents. Figure 23 presents cell seeding within the spinach leaf. The red fluorescents represent Q-dot loaded cells.

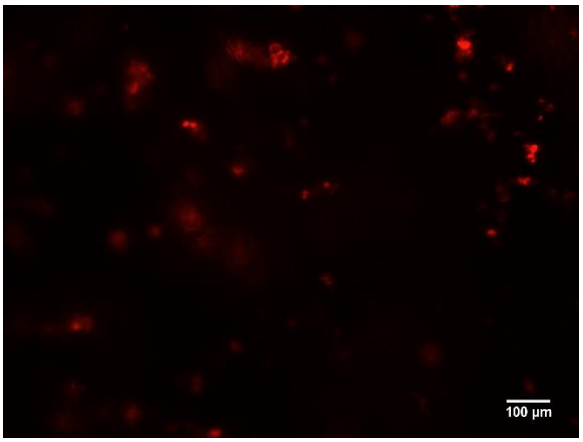

Figure 23: Q-dot loaded cells seeded within a decellularized leaf for 36 hours

The red fluorescents are dispersed throughout the inside of the leaf, indicating cellular adhesion to the leaf. The cells highlighted in the image display the distinct shape of Q-dot loaded hMSCs, being expressed only in the cytoplasm and not the nucleus. Thus, this indicates that the fluorescent signal is due to the cells rather than autofluorescence from the leaf.

B. Cellular Escape

To model the diffusion of cells from the scaffold, microspheres, 10µm in size, were loaded into a 5mL syringe. The microspheres were perfused through the stem using an electronic syringe extruder. 200,000 microspheres were resuspended in 5mL of diH_2O and extruded through the stem at a rate of 200µL/min. The average percentage of microspheres escaped from decellularized spinach leaves, n of 3, are outlined in Table 12.

Table 12: Percentages of microspheres escaped, 10µm in size, from the original vessel, n = 3

Average Percent Escaped	Standard Deviation
37.5	3.31

The average amount of microspheres escaped from a decellularized spinach leaf was 37.5% ± 3.31. This falls within the specification for 30-50% escape from the scaffold to allow for cellular escape and migration. This would indicate a vascular scaffold leaky enough to promote cellular escape, however not so leaky that all the cells seeded within the leaf would escape uncontrollably.

C. Cellular Migration

Cellular viability and migration throughout a fibrin gel is well understood and studied, as discussed previously [21] [26] [41] [51]. Due to time and resources, migration could not be studied in the following tests, however, the results in literature provide results to allow for a confident selection of a supportive gel matrix. Based on this knowledge, fibrin gel was selected as the ideal gel for the final construct. Despite this, the viability of the gel was confirmed with the specified fibrin concentration to ensure repeatability of the researched procedures. Fibroblasts were suspended in a 2.5mg/ml fibrin gel, allowed to solidify for 8hrs and cultured for 72hrs. Then a live dead assay was performed on the gels and analyzed under a fluorescent scope. In the following images, red represent dead cells while green represents live cells. The blue represents the nuclei of the cells. The results from these tests are presented in Figure 24.

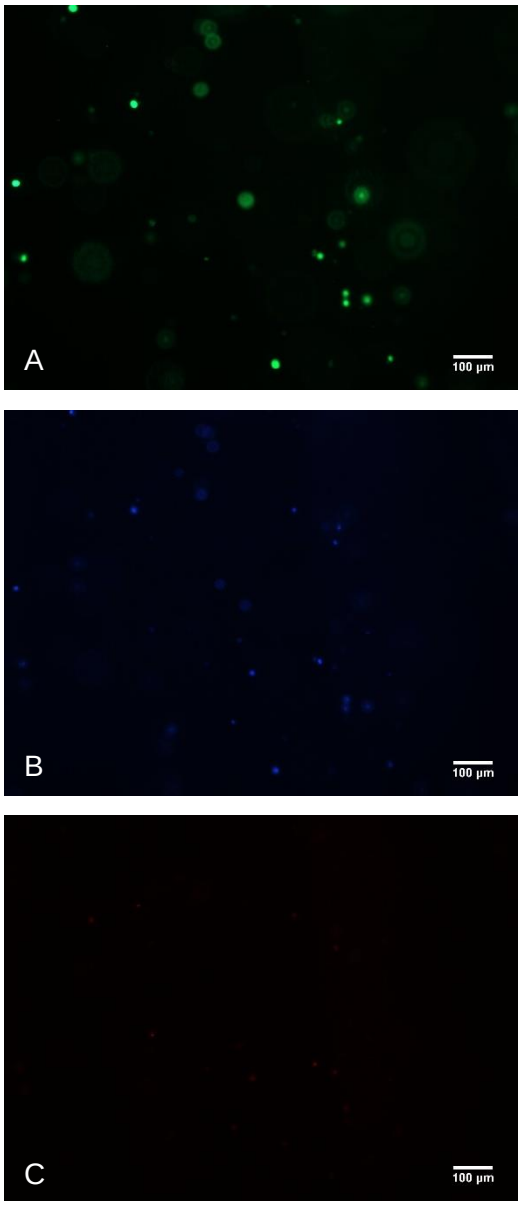

Figure 24: Live/dead assay of fibroblasts after 72 hours in fibrin gel. Approximately 500,000 cells seeded

Figure 24-A is an image of the live stain and Figure 24-B is an image of the nuclei stain. It is evident that many of the nuclei align with the live cells, indicating a plethora of live cells within the fibrin gel. Figure 24-C is an image of the dead stain. There are little cells that are present in the stain, confirming again that most of the cells, if not all of the cells, are viable after 72 hours within the fibrin gel.

VI. FINAL DESIGN AND VALIDATION

The following section discusses the overall procedures for ensuring the final design meets the listed specifications and standards. It also discusses the project's overall impact on society. The methodology breaks down the exact experimental means used to confirm the success of the project. The design was evaluated first by ensuring cellular adhesion was possible in the decellularized scaffolds. Then once adequate adhesion was confirmed, the ability for the scaffolds to allow for cellular escape was tested. Finally, the cell matrix was chosen based on literature about cell viability and migration within a fibrin gel. The compilation of these steps confirms the first steps of angiogenesis, cellular escape and migration, to eventually allow for the formation of blood vessels.

A. Methodology

The results of this project revealed adequate cell adhesion in spinach leaf scaffolds and sufficient cellular escape from the main veins. These components suggest that the initial stages of angiogenesis, cellular escape and migration, are feasible using decellularized spinach leaves as a cellular scaffold.

1. Decellularization of Parsley Stems

Spinach leaves were treated with 10% sodium dodecyl sulfate (SDS) for 24 hours, and then treated with 0.1% Triton-X-100 in a 10% sodium chlorite bleach in deionized water solution for 72 hours. After, they were rinsed with deionized water to remove the bleach.

2. Mammalian Cell Culture

Human mesenchymal stem cells (hMSCs) were cultured using a media containing IMDM, 2% Glutamax, 1% Penicillin Streptomycin, and 10% Fetal Bovine Serum.

3. Quantum Dot Loading

12.5µL of quantum dots were added to 10 mL of human mesenchymal stem cell media. Human mesenchymal stem cells were then exposed to

this media and allowed to incubate for 24 hours. Quantum dot loaded cells emit a specific fluorescent profile underneath the microscope, which can be described as a red fluorescent ring surrounding the nucleus of the cell.

4. Cell adherence to decellularized scaffolds

A lyophilized, decellularized spinach leaf was cut around the main vein to expose the vessels and reduce the overall surface area. Figure 25 demonstrates the cut for one spinach leaf. The leaf was then rehydrated with sterile PBS. The leaf was then left under UV light for 20-30 minutes. A 10µg/mL solution of fibronectin was created in sterile PBS. A syringe was used to draw up air, 100µL of the 10% solution, and then air. The first bubble of air was pushed through the main vein to rid the leaf of excess PBS. The solution was pushed through until the media was visible in the vein and the air was mostly removed from the syringe. The leaf was then placed in a new petri dish and left to dry at room temperature for 60 minutes. Then, 500,000 quantum dot loaded human mesenchymal stem cells, suspended in 200µL of media, were inserted into the leaf with the syringe. The leaf was then placed into a petri dish. The leaf was left to sit for 8 hours in a humid environment, then 15mL of media was added to the petri dish. The dish was left inside an incubator at 37°C and 5% CO_2 for 36 hours. After 36 hours the leaf containing cells was imaged with an inverted fluorescent scope.

Figure 25: Decellularized spinach leaf, preparation set up for cell seeding

5. Testing Cellular Escape with Microspheres

The syringe pump extruder was utilized to ensure a steady flow rate of microspheres into a rehydrated spinach leaf. The system was set up as presented in Figure 26. The leaf was rehydrated in PBS and 200,000 blue, dyed, microspheres, 10µm in size, were perfused through the leaf at a rate of 200µL/min and the escaped microspheres were collected in a petri dish. The microspheres were centrifuged for 5 minutes at 0.2rcf and then were counted using a hemocytometer to determine the percent escaped. Any microspheres not perfused through the leaf were counted separately and excluded from the original number of microspheres perfused.

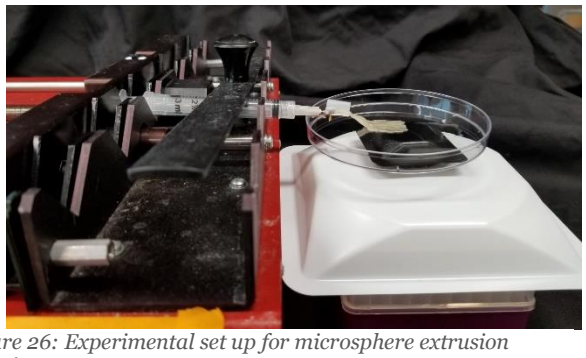

Figure 26: Experimental set up for microsphere extrusion experiments

6. Production of Fibrin

A 5mg/mL concentration of fibrinogen was prepared in a 20mM HEPES solution containing 0.9% NaCl. 0.5U/mL solution of thrombin was prepared in a 40mM solution of $CaCl_2$. The solutions were then filtered separately in a 0.22µm filter and combined at 1:1 ratio. The gel was incubated at 37°C and 5% CO_2 for 30-60 minutes.

7. Cell Viability within Gels

Fibrin was made as described in the section titled “Production of Fibrin”; however, the total volume when calculating concentration included the addition of 50µL of media for seeding the cells. A 1000µL gel was produced. The cells were mixed first with the fibrinogen, then the fibrinogen and thrombin were allowed to gel at 37°C for 30-60 minutes in a 24-well plate.

Afterwards, 1mL of media was added to the well. After 48 hours, a live/dead assay was performed on the gel to determine cell viability. Two solutions were made: solution 1 contained 1.0mL of Serum Free DMEM, 2.0µL of Ethidium Homodimer-1, and 0.5µL of Calcein AM, solution 2 contained the same concentrations with the addition of 0.5µL Hoescst Dye. Each solution was mixed within 1 hour of use. The media in each well was aspirated. Approximately 80µL of solution 1 was added to each well and left to incubate at 37°C for 15 minutes. Then approximately 80µL of solution 2 was added to each well and left to incubate at 37°C for 15 minutes. The dyes were then aspirated from the plate and the cells were washed with 1x PBS three times. The cells were then fixed in 4% paraformaldehyde for 10 minutes at room temperature and imaged under an inverted fluorescent microscope.

8. Microscopy

All cellular images were taken using fluorescent microscopy on an inverted microscope and all other images were taken on an upright scope without the use of the corresponding fluorescent wavelength.

B. Verification of Standards

The following is a list of the standards intended for this project to comply with and a brief description of how this project has met each standard.

1. Sterility (ISO 11737-2:2009, sterilization of medical devices)

In compliance with ISO 11737-2:2009, the International Standard on Sterility of Medical Devices, all cell culture practices, gel formation, scaffold seeding, and material handling was done in a sterile biosafety cabinet and/or sterile incubator. All tools used for these practices were autoclaved prior to use, and all materials coming into contact with cells were sterilized with rinses of ethanol and PBS [53].

2. Endotoxin and pyrogens (ISO 10993-11: Systemic Effects)

In compliance with ISO 10993-11, the International Standard on the Evaluation of Systemic Sterility, all components of the final construct have been evaluated for use within the body. The cells being used, human mesenchymal stem cells (hMSCs) and lymphoma fibroblasts, the gel being used, fibrin, and chemoattractants being used, VEGF/fibroblasts, are all sourced from and/or secreted naturally by the human body. Thus, utilizing these natural materials should avoid any systemic toxicity. The decellularized plant scaffolds being used for this project have undergone in vivo biocompatibility testing and have shown minimal inflammatory or toxicity response within the body [54].

3. Blood transfusion (ISO/TC 76 - Transfusion, infusion and injection, and blood processing equipment for medical and pharmaceutical use)

In compliance with ISO/TC 76, the International Standard for Blood Transfusion and Processing for medical or pharmaceutical use, all handling and testing of biological materials and materials intended for contact with human blood have been standardized, and quality testing for the industrial setting has been considered [55].

4. Biocompatibility (ISO 10993-1 Biological Evaluation and Biocompatibility Testing of Medical Devices)

In compliance with ISO 10993-1, the International Standard for Biological Evaluation and Biocompatibility Testing of Medical Devices, all components of the final construct have undergone biocompatibility testing or have been previously deemed biologically safe for use within the body. In vivo testing of the decellularized plant constructs being used have shown minimal signs of an inflammatory response in the surrounding cells and tissue layers. All other components are sourced from within the body and have been deemed biologically safe [56].

5. Human Cell Applications (ISO 13022:2012, Medical Products Containing Viable Human Cells - Applications of Risk Management and Requirements for Processing Practices)

In compliance with ISO 13022, the International Standard on Human Cell Applications and Risk Management, all cells being used in the final construct of this design have been handled sterilely - handled within a biosafety cabinet, cultured in a sterile incubator, frozen (if necessary) in sterile vials, loaded into scaffolds within a biosafety cabinet. The risks and benefits of using such materials have been evaluated, and the manufacturability of this construct utilizing human cells has been addressed throughout the project [57].

C. Economics

The final design construct for this project will significantly reduce the cost of organ/tissue replacement. Utilizing this construct would eliminate the need to use donor organs and undergo expensive surgical procedures, thus saving patients and insurance agencies money. The use of decellularized plant materials as scaffolds within this construct positively contributes to the agricultural industry and helps to maintain jobs for farmers. With these positive economic impacts, the overall revenue for the healthcare field, especially surgeons and transplant specialists, would decrease, and insurance coverage for this type of procedure must be evaluated.

D. Environmental Impact

While utilizing plants such as spinach and parsley would reduce the amount of fabricated materials used in tissue engineering, the increase in plant usage would negatively impact the environmental availability of these products. If/when this construct undergoes production within an industrial setting, the rate at which these plant materials will be used will drastically increase due to manufacturability needs production. While the availability of these materials for human consumption might decrease, the shift would be for use within the healthcare field, ultimately helping to save the lives of patients.

E. Society Influences

The main societal influence of this project is the use of spinach leaves as a medical application. The idea of such an application is highly shocking to most individuals and may not be well received for those in need of the technology. Currently, the use of spinach leaves for medical research has resulted in a lot of societal attention, with some speculation coming from people who may not understand the intended application or potential of this material. This construct would have a large positive societal influence as well. Individuals able to receive implants as a result of this technology would be able to regain normal lives, with less hospital stays.

F. Political Ramifications

Utilizing plants as tissue engineering scaffolds would allow for easier organ transplants in areas of the world where human donor organs are not always readily available. However, dependent upon the agriculture of the country, these types of produce might not be available in excess, or at all, to use for tissue engineering. This has the potential to result in biased applications of this construct to within areas with abundant economic and agricultural resources. As a result, this can be seen as a medical advancement intended for first-world nations.

G. Ethical Concerns

This product would increase the quality of life for those receiving this procedure by allowing them to live away from the hospital. Patient well-being would no longer be an inhibitory factor. Additionally, this alleviates ethical concerns for the use of animal products in certain transplants. The design of this product would be animal product free, providing an ideal solution for all lifestyles. Matching donor organs to patients would no longer pose as a problem for transplant patients, significantly reducing the amount of time the patient would need to live unhealthily prior to organ replacement.

H. Health and Safety Issues

This construct would allow for higher patient survival rates by reducing the wait time for donor organs. Reducing the amount of time patients are waiting for replacement organs likely would have a positive correlation to the average lifespan of patients needing organ transplants. With any implant, there is a risk of malfunction or infection, which could cause negative health effects for the patient. However, with current studies analyzing biocompatibility and inflammatory response in these tissues, the decellularized plant materials do not appear to have any negative response. This final construct has complied with relevant international standards pertaining to various elements including sterility, biocompatibility, and product manufacturing.

I. *Manufacturability*

The project is fairly reproducible and could be scaled up to a larger scale process. Many of the steps required (i.e. decellularization, incubation, seeding cells) can be easily scaled up. The only step to date that would cause potential issues in the process is the cannulation of the stems. This step has to be done by hand due to the delicate nature of the decellularized material. However, if an assembly line is established, this process can be easily optimized. Other important considerations for manufacturing this construct would be standardizing the necessary protocols, the time needed to create this construct, and the efforts needed to produce this construct on a large scale.

Table 13: Estimated cost of manufacturing

Item	Cost
Decellularization	$20.00
Construct Assembly	$2,400.00
Equipment	$580.00
Total Cost per Construct	$3,000.00

In regard to the pricing of the construct, the Table 13 is a breakdown of the cost to engineer one of these leaf constructs. Decellularization steps include purchasing spinach leaves, detergents and bleaches. The Construct Assembly price includes proteins, cells, and cell media. Equipment is representative of general laboratory equipment such as incubators, pipettes, pipette tips and cell culture dishes. The estimated total cost of this construct is $3,000. This estimate does not include the cost of 3D bioprinting into the construct, nor does it include other factors like the actual surgery once the organ is produced, quality control, or other additional upscale processes.

J. Sustainability

Production of our construct would reduce the negative industrial effects on the environment by eliminating the role of synthetic materials in tissue engineering. Utilizing plant structures and human cells/proteins would eliminate the need to manufacture materials in an industrial setting. While this process would emit less industrial fumes during production, automation of production would cause an increase in electricity usage due to incubation, automation of cell seeding processes, etc.

VII. DISCUSSION

Decellularized plant scaffolds were proven to be a viable option for seeding cells and the biocompatibility of the materials have been confirmed [5]. Since the scaffolds can be seeded and induce very little immune response, the option of using decellularized plants as scaffolds for promoting angiogenesis was explored. In the experiment conducted, cellular adhesion was tested in two different plant models (spinach and parsley). The two were compared and spinach was chosen as the superior scaffold. After adequate adhesion was obtained, cellular escape was modeled from the scaffold utilizing microspheres 10μm in size to model endothelial cells. These tests were performed on scaffolds and the microspheres were collected to measure the percent escape. Regarding cell movement within a gel, previous research has shown the ability of endothelial cells to move within fibrin gel matrices and supported the decision for the gel matrix. The compiled success of each of these components would allow for the initial stages of angiogenesis to occur. Angiogenesis begins with endothelial cell escape and migration to allow the new blood vessels to form. Therefore, ensuring adequate adhesion, escape, and migration would allow new vessels to form [6]. Once these new

vessels have formed, cells can be printed onto the construct to form tissues, and eventually functioning organs.

In comparing the two different plant materials, it is evident that there was greater adhesion in the spinach leaf. Despite previous successes in the cell seeding of parsley stems, our team was unable to achieve adequate cell adhesion. Histological samples of a parsley stem seeded with fibroblasts can be seen in Figure 16. This was due to the large surface to volume ratio of the stem, as well as the time constraint the team had to follow. To model cellular adhesion in spinach leaves, quantum dot loaded human mesenchymal stem cells were seeded and allowed to incubate for 36 hours. The results from this experiment can be seen in Figure 23. Cellular adhesion is evident considering quantum dot loaded human mesenchymal stem cells have a specific fluorescent profile characterized by a red color outlining the nucleus. Considering the cellular adhesion that was obtained when using decellularized spinach leaves as a scaffold, the team decided to continue with this plant material as a scaffold.

In the studies modeling cellular escape, the results in Table 12 show that 10 um microspheres had an average of 37.5% escape from the spinach leaf scaffold when perfused through. Although there is no published literature about the necessary percentage of cellular escape for angiogenesis, we believe 30-50% to be an appropriate amount, as it would allow for some escape and the formation of new vessels without a total loss of cells. The percent escape calculated from the leaf shows that the scaffold is porous enough to allow for cellular escape, but not so porous that seeded cells will not be able to adhere to the scaffold once perfused.

Experiments were then conducted to ensure that cells would be able to survive in a fibrin gel matrix. 1000μm fibrin gels were composed of 5mg/ml of fibrinogen and 0.5U/ml. They were then seeded with fibroblast cells. Using a live/dead assay, cell survival was evident after 48 hours within the gel. Due to time constraints and several other factors, cellular migration could not be tested. However, there is a large body of research that has proven endothelial cell movement is possible through a fibrin gel.

Although the results from this study show that cellular escape and migration from a decellularized spinach leaf scaffold into a gel should allow for angiogenic activity and consequently the ability to print cells into the newly formed vasculature, there are some limitations to the experiments and data collected. The stem and leaf were not seeded with the same density of cells, nor were they suspended in the same amount of media. This was to account for differences in main vein sizes; however, this could have skewed the results of the comparison. Additionally, although the cellular escape from the scaffold was measured, due to limited resources, no tests were completed to measure the pore size or porosity of the samples. Finally, the live/dead assays were only run for 48 hours with a 1000μm gel with fibroblasts. It is possible that a longer time and larger gel could impact the cells differently. Although, it is not suspected that this would be the case since fibrin is a key factor in the wound healing process and would interact the cells readily [61]. Most of the experiments were performed with fibroblasts or human mesenchymal stem cells to ensure that cells would be able to perform these functions, but confirming these experiments with HUVECs would be most beneficial since these cells line the blood vessels in the body.

VIII. CONCLUSIONS AND RECOMMENDATIONS

The lack of sustainable and mechanically intact vasculature hinders scientists from engineering functional organs for transplants. The lack of sustainable and mechanically intact vasculature hinders scientists from engineering functional organs for transplants. The scale on which scientists are having trouble diffusing oxygen and nutrients through a tissue is 200μm [32]. During wound healing, the body can regenerate vessels over larger distances while maintaining oxygen rich vasculature [21]. One potential solution to overcome this problem is to mimic angiogenic functions, and apply their sprouting mechanisms to in vitro tissues, with the ultimate goal of creating vasculature to supply nutrients to a functional organ.

This project is a proof of concept that cells can migrate from a scaffold and into a gel – mimicking the first stages of angiogenesis, and consequently, the printing of cells into this construct to create functioning tissue. These steps include cellular escape and migration from the original vessel. From the initial studies, it was

determined that the spinach leaf had better cellular adhesion than the parsley stem and was a more viable option for the final construct. These experiments coupled with previous research show the potential for cellular adhesion, escape, and migration from a scaffold and into a gel matrix. Mimicking the initial stages of angiogenesis would address the oxygen and nutrient diffusion problem engineers are facing when designing artificial organs. Plant scaffolds were treated with fibronectin and seeded with quantum dot loaded human mesenchymal stem cells showed adequate cellular adhesion throughout the inside of the scaffold and the scaffold also demonstrated adequate cellular escape through the walls.

Coupling our results with previous research, cellular escape from a scaffold and migration through a gel to form a complete vascular network is feasible with further time and resources. Additionally, this shows the potential for the leaf scaffold must integrate itself into its surrounding environment. This could be applied to different sects of regenerative medicine, particularly to the mass production of 3D bioprinted organs. This application could be expanded upon and coupled with a bioreactor, to potentially transform the scaffold into a functioning organ itself, while maintaining low cost and long-term durability. The next step would be to dictate cellular movement within the gel. Having the ability to direct cellular movement from an existing scaffold would be one step closer to solving the vascularization problem associated with 3D printed organs. This could be competed with the use of growth factors. Specifically, VEGF can be used to guide cells to the correct regions and allow for the formation of blood vessels that are no more than 200μm from the closest cell. The next step would be to culture the constructs for a longer time period to allow the cells to mature, and form established vasculature. These constructs would then need to be tested for mechanical durability and pressure tolerance within the vessels. Eventually, following the formation of a complete vascular network, different cells would be printed into the matrix to develop functioning tissues and organs. Following this, in vivo studies need to be conducted to test the mechanical viability of these decellularized scaffolds or the tissues formed within the vasculature.

This project shows the significance plant scaffolds can have on the field of regenerative medicine and tissue engineering. As seen in biocompatibility studies, cells of the host animal penetrated the plant scaffold and integrate themselves into its physiology. This biocompatibility allows plant scaffolds to be used for not only regenerative medicine, but to develop new functioning organs through 3D bioprinting. By creating a vascular network within a gel, scientists become one step closer to solving the oxygen and nutrient diffusion problem, allowing for the mass production of artificial organs.

IX. APPENDICES

A. Pairwise Analysis with the project sponsor

	Induce Angiogenesis	Induce Vasculogenesis	Connectibility	Minimize Cell Death	Print on/in Vasculature	Time for Vascular	Vasculature Durability	Diffusion of Nutrients	Scalable/Reproducible	Low Cost	**Sum**	**Rank**
Induce Angiogenesis		1	1	1	1	1	1	0	1	1	8	2
Induce Vasculogenesis	0		0.5	1	1	1	1	0	1	1	6.5	3
Connectibility	0	0.5		1	1	1	1	0	1	1	6.5	3
Minimize Cell Death	0	0	0		1	1	1	0	1	1	5	4
Print on/in Vasculature	0	0	0	0		0	0	0	0	0	0	9
Time for Vascular Growth	0	0	0	0	1		0	0	1	1	3	6
Vasculature Durability	0	0	0	0	1	1		0	1	1	4	5
Diffusion of Nutrients	1	1	1	1	1	1	1		0	1	8	1
Scalable/Reproducible	0	0	0	0	1	0	0	1		1	3	7
Low Cost	0	0	0	0	1	0	0	0	0		1	8

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