3D BIOPRINTING OF VASCULARIZED HUMAN TISSUES

A dissertation presented

by

DAVID BARRY KOLESKY

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3D Bioprinting of Vascularized Human Tissues

ABSTRACT

The ability to manufacture human tissues that replicate the spatial, mechano-chemical, and temporal aspects of biological tissues would enable myriad applications, including drug screening, disease modeling, and tissue repair and regeneration. However, given the complexity of human tissues, this is a daunting challenge. Current biofabrication methods are unable to fully recapitulate the form and function of human tissues, which are composed of multiple cell types, extracellular matrices, and pervasive vasculature.

My Ph.D. thesis focuses on advancing the capabilities of human tissue fabrication. Specifically, we demonstrate a multimaterial bioprinting method capable of producing 1D, 2D, and 3D vascularized tissue constructs by co-printing ECM, cell-laden, and fugitive inks. After these heterogeneous tissue constructs are printed and infilled with ECM, they are cooled to 4°C to remove the fugitive ink leaving behind a pervasive network that is subsequently lined with endothelial cells. These constructs are ~ 1 mm in thickness and can be sustained for up to 14 days via rocking-based flow through their vasculature. We then created a new extracellular matrix that enabled the fabrication of tissues that exceed 1 cm in thickness that are perfused on a microfluidic chip for long time periods (> 6 weeks). To demonstrate functionality, growth factors are perfused via the vasculature to differentiate stem cells toward an osteogenic lineage in situ. Finally,
we created renal proximal tubules (a sub-unit of kidney tissue) by this approach. Specifically, we constructed 3D tubules circumscribed by renal proximal tubule epithelial cells (PTECs). The PTECs form confluent, leak-tight epithelial monolayers that exhibit primary cilia and express Na\textsuperscript{+}/K\textsuperscript{+} ATPase, Aquaporin 1, and K-cadherin. The combination of 3D geometry and on-chip perfusable nature gives rise to enhanced, polarized PTEC phenotypes that develop an enhanced brush border, basement membrane protein deposition, basolateral interdigitations, enhanced cell height, megalin expression, and albumin uptake relative to 2D controls.

In summary, this multimaterial 3D bioprinting platform enables production of engineered human tissue constructs in which multiple cell types and vasculature are programmably placed within extracellular matrices. These 3D tissues may find potential applications in drug screening, disease models, and ultimately, tissue engineering and regenerative medicine.
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CHAPTER 1
INTRODUCTION

The ability to manufacture functional human tissue would be beneficial for numerous applications in drug screening \(^1\), disease modeling \(^2\), and, ultimately, tissue and organ repair \(^3\). However, human tissues are inherently complex and often arranged into composite architectures composed of multiple cell types, extracellular matrix, and vasculature \(^4\). For example, the kidney contains 20 specialized cell-types, has complex extracellular matrix comprised of proteins and glycosaminoglycans \(^5\), and arranged into approximately 1 million tubular microarchitectures known as nephrons, which itself consists of substructures, including the glomerulus, proximal tubule, loop of henle, and the collecting duct \(^6\). Moreover, the kidney, like all tissues, is replete with a complex vasculature through which blood is delivered. The vasculature is a hierarchical network of blood vessels that pervade all tissues in the body. The primary role of the vasculature is to sustain surrounding tissue by rapidly delivering oxygen and removing metabolic byproducts in response to changes in the local cellular microenvironment \(^7\). Notably, every cell in the body is within a few hundred microns from the nearest blood vessel ensuring constant access to oxygen and other nutrients as needed \(^8\).

Current strategies for fabricating acellular scaffolds \(^9\),\(^10\) are incapable of creating the complex, heterogeneous composite architectures that contain different cell types supported by a pervasive vascular network. New biofabrication methods are needed to better replicate the essential spatial, mechano-chemical, and temporal aspects of human tissue. Emerging methods, such as laser induced forward transfer (LIFT) \(^11\), inkjet printing \(^12\), and extrusion bioprinting \(^13\) enable printing myriad cell-types and
biomaterials into intricate 2D and 3D architectures, but lack the ability to embed perfusable vasculature, limiting the resulting tissue thicknesses < 1mm. Recently, researchers created vascular channels by printing a sacrificial sugar ink at elevated temperature (> 100°C). After coating the features with a biocompatible polymer, they cast a cell-laden hydrogel around the structure to form a 3D tissue construct. The sacrificial sugar filaments are then removed to yield an open network of interconnected channels, i.e., vasculature. Because this ink requires printing at elevated temperatures, they cannot co- or pre-print cell-laden inks. An ideal bioprinting method would allow one to simultaneously print multiple cell types, ECM, and embedded vasculature to create engineered tissue microenvironments.

My Ph.D. dissertation focuses on developing a multimaterial bioprinting method for fabricating functional vascularized human tissues. Several criteria must be met to achieve this overarching goal. First, this method must enable concurrent printing of the key tissue components (cells, ECM, and vasculature). Second, these engineered tissues must be perfusable, so they can be produced at thicknesses beyond > 1 mm, while possessing long-term cell viability. Third, this method must be capable of printing both tissue-specific cells as well as stem cell inks. Finally, the resulting tissues should function similar to their in vivo counterparts.

1.1 DISSERTATION SCOPE

The first aim of this dissertation is to fabricate three-dimensional human tissues composed of multiple printed cell types, extracellular matrix, and a pervasive vasculature. The second aim of this work is to enhance the physiological relevance of these printed tissues through controlled perfusion demonstrating a route to enhanced thickness, and
long-term stability. Finally, the third aim is to create an organ-specific tissue that recapitulates the native structure and function required to facilitate drug toxicity screening \textit{in vitro}.

The principal outcome of this thesis is the development of a multimaterial 3D bioprinting method and a suite of biological inks required for printing vascularized human tissues. As an initial demonstration, we concurrently print, at room temperature, gelatin-methacrylate inks that are either acellular (acting as ECM) or replete with multiple types of fibroblast-laden cell inks, and Pluronic F-127 as a fugitive ink. Upon cooling the printed constructs to 4 °C, the fugitive ink liquefies and can be removed leaving behind open luminal architectures, which are lined with human umbilical vein endothelial cells to form a confluent endothelium within the embedded vascular network. Next, we developed a new ECM that facilitates improved cell-ECM interactions as well as printing behavior. Specifically, a gelatin-fibrin network is used as both an ink and matrix system. Additionally, we printed custom- perfusion interface chips that enable the the long-term perfusion ( > 45 days) and enhanced tissue thickness ( ≥ 1 cm). For this demonstration, we printed human mesenchymal stem cells into 3D architectures and differentiated them towards the osteogenic lineage by delivering factors through the perfusible vascular network. Finally, using the same materials and methods, we developed a 3D renal proximal tubule, which is also printed within our perfusion chips. By printing Pluronic-F127 fugitive ink into a convoluted pattern, and encapsulating with the gelatin-fibrin matrix we are able to create a convoluted tubular architecture that, upon removal of the fugitive ink, can be controllably perfused, and sampled over time. The open lumen of the tubules are lined with human proximal tubule cells, which
circumscribe the inner surface to form a confluent epithelium. Compared to 2D models, these renal proximal tubules exhibit enhanced cell height, microvilli length and density, and megalin-mediated albumin uptake. Upon exposing this 3D renal proximal tubule to a known nephrotoxin, cyclosporine-A, we observed dosage-dependent increase in its diffusional permeability and physical damage to the epithelium, with increased with increasing drug concentration.

1.2 DISSERTATION ORGANIZATION

In Chapter 2, the relevant literature is reviewed with an emphasis on tissue composition, biofabrication methods (LIFT, inkjet, and extrusion printing), and emerging applications in drug screening and tissue engineering. In Chapter 3, the creation of heterogeneous, vascularized tissues via multimaterial bioprinting is discussed, where ink rheology, printing behavior, and cell viability over time is investigated. In Chapter 4, methods to print thick perfusable tissue are described and their long-term behavior is characterized. In Chapter 5, bioprinting and characterization of a perfusable perfusable, 3D renal proximal tubule model, including cellular phenotype, protein expression, and drug response are discussed. Finally, the conclusions of this dissertation are provided in Chapter 6.
CHAPTER 2
LITERATURE REVIEW

2.1 MOTIVATION AND SCOPE

The ability to manufacture functional human tissue would be beneficial for numerous applications in drug screening\(^1\), disease modeling\(^2\), and, ultimately, tissue and organ repair\(^3\). In this chapter we will review prior research in this field with the goal of describing key aspects of fabricating human tissues. First, we will describe the composition of human tissues, with an emphasis on bone and kidney tissue. Next, we will describe the essential components available for recreating human tissues, namely the extracellular matrices (ECM), cell sources, strategies to vascularize engineered tissues. Finally, we will introduce current biofabrication strategies, as well as key applications in enabling applications in drug screening and tissue engineering.

2.2 HUMAN TISSUES: VASCULARIZED COMPOSITE ARCHITECTURES

2.2.1 ECM, cells, and vasculature

Human tissues are inherently complex multicellular composites composed of extracellular matrix, cells, and a pervasive vasculature\(^3,15\) (Figure 2.1). There are hundreds of specialized cell types within human tissues, which can be divided into four main types: epithelial, muscle, nervous, and connective. Physical properties of these tissues vary greatly; for example, their elastic modulus spans several orders of magnitude ranging from 0.1 – 1 kPa for soft tissues, like the brain, to 10-20 GPa for hard tissues like mature bone\(^16\). Soft and stiff tissues often form heterogeneous that are several hundred microns wide, e.g., tendon, bone-muscle junctions, or the spinal cord\(^17\). During
development, these complex architectures arise naturally amongst a supportive circulatory system of blood vessels that pervades all tissues (Figure 2.2a). In tissues, cells depend on vasculature to constantly deliver nutrients and remove metabolic byproducts on demand, notably, every cell within vascularized tissue is ~300 μm to the nearest blood vessel in vivo.8

At a basic level, tissue is composed of a dynamic ECM is that supports surrounding cells and vasculature (Figure 2.2b). There are two distinct ECM domains: (1) the basement membrane, which consists of a thin, dense matrix layer that supports a

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**Figure 2.1 Characteristics of human tissues.** (a) Four types of human tissues: epithelial, muscle, nervous, and connective tissue. (b) Elastic modulus of human tissues that span three orders of magnitude.16
confluent layer of parenchymal surface epithelial or endothelial cells and (2) an underlying ECM-rich stroma containing fibroblasts and immune cells \(^{18,19}\). The apical surface of the epithelium or endothelial lining provides barrier properties against the external environment, and in some cases provides organ-specific functions, such as metabolism, filtration, and reabsorption (e.g., liver, kidney, and gut). These cells are anchored to the underlying basement membrane via a series of integrin and matrix adhesion molecules expressed on the basal side of the cells. The stroma dynamically interacts with the surface cells through secretory soluble molecules or chemokines, while physically supporting them via the matrix-rich ECM. Many of the ECM components exist in both domains; however, the exact compositions and architectures vary greatly between different human tissues \(^{20}\).

The three primary components of the ECM are (1) fibrous networks, such as collagen (I, II, III, V, XI), elastin, and fibronectin, (2) adhesive glycoproteins, such as laminins and tenascin, and (3) glycosaminoglycans (GAGs), carbohydrate polymers that maintain hydration and bind growth factors and chemokines \(^{21}\) (Figure 2.1). GAGs are bound to the ECM through a protein domain forming a proteoglycan along the fibrous super-structures (e.g., heparin sulfate, chondroitin sulfate, and keratin sulfate). Notably, hyaluronic acid does not form proteoglycans, but instead fills the extracellular space imparting a resistance to compression by providing a counteracting osmotic pressure via the absorption of significant amounts of water \(^{22}\). In each tissue, the ECM is arranged into tissue-specific architectures to achieve specialized functions, and mechanical properties, as well as to modulate the function of cells through gene expression levels, migration (durotaxis), and lineage-specified differentiation \(^{16}\). In the next section, we will describe
the interdependence between architecture, composition, and functionality in two organs of interest, bone and kidney. Both are compositionally heterogeneous, vascularized, and essential for organismal homeostasis, yet they vary dramatically in their structure, composition, and function.

Figure 2.2 Vascular networks and ECM in human tissues. (a) Image of the large vessels in the porcine heart. (b) The dynamic ECM microenvironment containing the parenchyma, stroma, and endothelium.
2.2.2 Bone

Bones are rigid, multifunctional organs that form the structural endoskeleton of vertebrates, store and release minerals, and are the origin of hematopoiesis. Bone organs have three main tissue-subunits, namely, cancellous and cortical tissue, and bone marrow. Together, these structures form a tough, lightweight, adaptive, self-healing, and multifunctional composite. Generally, bone is composed of cells embedded into a highly specialized and architected extracellular matrix, which is primarily composed of two major phases: collagen I fibrils and hydroxyapatite nanocrystals distributed along the collagen fibrils. These two phases are responsible for about 95% of the dry weight of bone. Cancellous (spongy) bone tissue consists of an irregularly shaped mineralized organic matrix, which forms a 3D interconnecting organized network (Figure 2.3). The second type of bone tissue is cortical (compact) bone, which is principally composed of shafts (diaphyses) of mature long bones (Figure 2.3) and composed of densely packed subunits called osteons. Osteons consist of a central Haversian canal containing a blood vessel surrounded by lamellae of mineralized organic matrix.

Ossification refers to the specific cellular synthesis of organic matrix by cells called osteoblasts, whereas mineralization or calcification refers to only the deposition of solid calcium phosphate crystals into this organic matrix, which is primarily comprised of collagen I. Generally, mineralization quickly follows the deposition of collagen fibrils, where the calcium and phosphate precursors are derived from the specific extracellular fluids that permeate the bone tissues. After osteoblasts have completely surrounded themselves with synthesized mineral, they change their cellular functionality and become...
osteocytes, or mature, bone-lining cells (Figure 2.3). Meanwhile, a third cell type, the osteoclast, is responsible for reabsorption (bone turnover). Over time, undifferentiated mesenchymal stem cells are recruited from the bone marrow into the void formed by the osteoclasts, differentiate into osteoblasts, and the cycle repeats throughout the lifetime of the individual. It is estimated that a young adult human replaces the total bone mass every five to six years, but absolute rates vary across bones. However, if the rate of reabsorption ever outpaces the rate of formation, the mass of bones begins to decrease, resulting in osteoporosis and an increased risk of bone fracture.

The open pores between the stiff, boney trabeculae (struts) are filled with blood vessels and soft, fatty marrow, which is composed mainly of blood forming hematopoietic stem cells (HSC), fat, and undifferentiated mesenchymal stem cells (MSCs). Figure 2.3 shows the complete bone marrow “stem cell niche”, which refers to the unique microenvironment of these regenerative cells in the bone marrow. Table 2.1 lists main cellular components of bone marrow, their location, and functional surface markers (if known). This niche is a functional unit that is responsible for endocrine, autocrine, and paracrine signaling and serves the needs of the whole organism by sustaining the stem cell population. HSC and MSCs from the bone marrow are mobilized and exported through the vasculature in response to injury, acute inflammation, biochemical stress, or during normal bone tissue repair. Changes in this delicate microenvironment can happen in response to invading cancer cells that invade and hijack the function of this niche and can be detrimental to organismal-level homeostasis.
Figure 2.3 Structural and compositional heterogeneity of bone and marrow.
(a) The macro- and microstructural architectures of cortical and trabecular bone. Each is formed of a hierarchical assembly of mineralized collagen fibers, composed of collagen protein molecules (tropcollagen), which is formed by amino acid chains and hydroxyapatite linked to form fibril arrays. (b) The bone marrow niche contains a variety of cells from both the mesenchymal and hematopoetic lineages. The presence of bone cells, microvasculature, and sinusoids creates a unique stem cell environment. Skeletal remodeling and whole body homeostasis is highly influenced by this region {Reagan:2015ca}. 
2.2.3 Kidney

Kidneys purify toxic and metabolic waste products from the blood. Each kidney contains a million functional units called nephrons, which are composed of a glomerulus and a hairpin-shaped convoluted tubule that drains the filtrate into the renal pelvis (Figure 2.4). The glomeruli are adjacent to the Bowman’s capsule, which is lined with parietal epithelial cells and contains the mesangium, which interfaces with many, densely packed capillaries to filter the blood. The glomerular filtration barrier consists of endothelial cells, the glomerular basement membrane, and podocytes (Figure 2.4) All molecules below the size of albumin (~65 kDa) pass through the glomerular filter and enter the convoluted tubule. The tubule consists of several substructures, including the proximal convoluted tubule, loop of Henle, distal convoluted tubule, and the collecting duct. To concentrate the filtrate, a countercurrent system forms a high osmotic gradient in the renal medulla. Further, the kidney contains a special intrarenal immune system, which is composed of various cell types (e.g., dendritic cells, macrophages, and fibroblasts), which is largely contained in the interstitium (the space between tubules).
The proximal tubule epithelium is responsible for reabsorbing water, small proteins, amino acids, carbohydrates and electrolytes, thereby regulating blood plasma osmolality, extracellular volume, blood pressure, and pH. Overall, about 85% of the fluid is resorbed in this region of the nephron. The renal convoluted tubule possesses a dense apical brush border with extensive basolateral plasma membrane infoldings (Figure 2.4e). High magnification shows microvilli contain numerous micropits, which may act to increase the effective cell surface area, improving sampling of the local environment as well as reabsorption efficiency.
The ability to take up substances from the surrounding environment provides proximal tubule cells with vital nutrients and also enables the selective transport of...
substances from one compartment to another. Essential to organismal homeostasis, these cells are able to regulate levels of various substances using an extensive apical endocytotic apparatus, leading to the reabsorption of low-molecular weight plasma proteins (e.g., albumin, hormones, vitamin-binding proteins and cytokines) and many drugs (e.g., gentamicin and amikacin). This process begins with the specific binding of ligands to a receptor on the apical surface, followed by the receptor-ligand complex being internalized by invagination of the plasma membrane, transport of the ligand, and, finally, receptor recycling to the cell surface (Figure 2.5). This receptor-mediated endocytosis primarily involves two apically located multiligand-binding receptors, megalin, and cubilin (Figure 2.5). Specifically, megalin is a 600 kDa transmembrane glycoprotein that functions as a low-selectivity, high-capacity scavenger receptor and cubilin is a 460 kDa peripherally attached glycoprotein that interacts and functions with megalin in a dual-receptor complex, which aids in enhanced specificity receptor binding. Specifically, this complex is essential for reabsorption of albumin, which is synthesized in the liver and is the most abundant plasma protein serving many diverse and important functions in vivo. Controversially, it has been argued that the specificity of the megalin/cubilin-mediated tubular uptake of albumin is low, and that they are serving as low-affinity, high-capacity scavenging receptors. Importantly, this implies that total albumin uptake is heavily dependent on proximal tubule length (total number of PT cells), flow rate (time in which albumin is colocalized with the megalin/cubilin complex), and concentration of albumin in the ultrafiltrate. Further, the per-cell albumin uptake efficiency strongly depends on the presented surface area, and thus, microvilli length and density. Interestingly, prior studies reveal the fraction of reabsorbed albumin compared
to the filtered load is smaller in mice and rats, which have relatively short PTs compared to larger species with longer proximal tubules \(^{34}\). These observations support the hypothesis that geometry of the proximal tubule affects functionality.

**Figure 2.5 Megalin, cubulin in the kidney.** (a) Megalin (right) is a 600kDa transmembrane protein that contains four cysteine-rich clusters of low-density lipoprotein-receptors that constitute the ligand binding domains. Cubilin (left) is ~460 kDa transmembrane protein it contains 27 CUB domains, which facilitates the molecular binding interaction with several proteins \(^{32}\). (b) Megalin and cubulin can act independently or as a complex to facilitate receptor-mediate binding and resorption in the proximal tubule. Following uptake ligands are internalized, packaged into vesicles, and ultimately, recycled to the apical surface \(^{33}\).
2.3 ENGINEERED TISSUE CONSTRUCTS

To recreate the complexity of human tissues, such as those described in the previous section, engineers have attempted to incorporate the key elements of tissues (ECM, cells, and vasculature) in tissue constructs in vitro. In this section we will describe the components that comprise engineered tissues and comment on their benefits and limitations, to date. First, we will describe the various materials that have been used to recreate the ECM. Next, we will describe the various cells that are available for imparting function into tissue constructs, and finally, the essential aspects of vasculature that need to be recreated to enable tissue fabrication.

2.3.1 Biological, Synthetic, and Hybrid Extracellular Matrices

In vivo, cells grow within complex networks of protein fibers and polysaccharides called ECM. The ECM is an intricate 3D matrix that guides cell adhesion, proliferation, differentiation, morphology, and gene expression. The ECM directs cell function through local variations in structural organization and chemical composition across tissue types. Important structure and chemical considerations occur at multiple length scales (e.g., from fibrillar microstructures to chemical functional groups), thus, advances in both biofabrication and synthetic chemistry have led to novel materials ECM for 3D tissue culture. Currently, materials used for 3D culture in vitro include biological, synthetic, and hybrid ECM materials (Figure 2.6) with each material possessing inherent advantages and disadvantages.
Natural biomaterials used in 3D tissue culture include both network-forming proteins (e.g., collagen, gelatin, fibrin, and matrigel) and polysaccharides (e.g., hyaluronic acid (HA), alginate, agarose, chitosan, heparin, chondroitin sulfate, and dextran) \(^{39}\) (Figure 2.6a). While many of these materials are derived directly from the native ECM and thus have the advantage of inherent biocompatibility and/or bioactivity, the customizability of chemical, structural, and rheological properties of these materials is...
limited. Conversely, hydrogels created from synthetic building blocks do not natively promote cell adhesion, cell migration, or degradability, but can be controllably functionalized with moieties such as cell adhesive (RGD) or MMP-cleavable peptides along the polymer backbone \(^{42,43}\). Synthetic materials of interest may include poly(acrylic acid) (PAA), poly(ethylene glycol) (PEG), poly(lactic) and poly(lactic-co-glycolic) acids (PLA, PLGA), and poly(caprolactone) (PCL) \(^{42}\). Alternatively, hybrid materials have been developed to mimic natural ECM \(^{42}\) (Figure 2.6b), such as supramolecular peptide amphiphiles that recreate the dynamic and reversible self-assembly processes that occurs in natural systems \(^{44}\). These structures have been used to mimic natural ECM microstructures, induce differentiation of stem cells, and bind or mimic functional proteins, such as vascular endothelial growth factor (VEGF) \(^{45,46,41}\).

Overall, both synthetic or semi-synthetic matrices allow for the precise design of artificial matrices with customized molecular structure \(^{47}\), which may offer potential for better understanding of cell behavior relative to complex native ECM. Moreover, these materials have the added benefit of being able to program their rheological properties through chemical modifications \(^{41}\). However, these materials are often produced in small quantities, which, to date, has limited their transition from small-scale assays to large-scale tissue manufacturing applications. In the next section, we will discuss several off-the-shelf biomaterials that are suitable for large-scale tissue manufacturing including collagen, gelatin, and fibrin, which are used in this thesis.

**Collagen.** Collagen is the major component of ECM and the most abundant fibrous protein in mammals \(^{21}\). To date, over 28 types of collagen have been identified in
vertebrates. The majority of collagen molecules form a triple-stranded helix that subsequently assembles into supramolecular fibrils and networks, while the macro-scale architecture depends on the type of collagen and location in vivo (Figure 2.7). The complex structure of fibrillar collagen type 1 is presented in different morphologies in different tissues. Capturing the intrinsic properties of collagen is of particular interest for 3D tissue culture, as it makes up much of the interstitial tissue space and the basement membrane, and can undergo gelation in vitro. However, the ability to create intricate 3D shapes and structurally complex tissues has been relatively limited due to the lack of mechanical robustness of the native isotropic gels. Much work has been done to improve its mechanical properties via crosslinking or blending with other biomaterials. However, lack of structural integrity of pure collagen has limited its applications to simple bulk casting and molding applications.

Figure 2.7 Collagen structure, fibril assembly, and microstructure. (a) Collagens contain three polypeptide chains extended in conformation, with each chain containing Glycine-X-Y repeat triplet in which the glycl is every third position and X and Y typically occupied by porline and 4-hydroxyproline (b) High resolution image of single collagen fibril and schematic of assembly. Fibril-forming collagens occur as the principal source of tensile strength in animal tissues and are assembled via binding sites along the triple helices that pack into highly periodic (67-nm repeats) (c) Transmission electron microscopy image of collagen fibrils in a developing tendon. EC, extracellular fibril; Fp, fibril cluster; IC, intracellular fibril. Adapted from.
**Gelatin.** Gelatin is a common polypeptide mixture of partially, and irreversibly, hydrolyzed collagen\(^{38}\) that exhibits thermally reversible gelation. The resulting material undergoes gelation when collagen triple helices partially reform by cooling below ~ 30°C\(^{57}\), and liquefies upon heating. While this thermal reversibility is useful for dispersing cells in the low viscosity state, these gels lack thermal stability at biological temperatures (37°C) and require chemical crosslinking for use as tissue culture (Figure 2.8). To overcome this limitation, several crosslinking strategies have been introduced, including the use of fixatives\(^{58,59}\) (e.g., glutaraldehyde, formaldehyde, and genepin) and photocrosslinking methods like UV-irradiation, which requires chemically modifying gelatin to add methacrylate or free thiol groups and the use of a photoinitiator\(^{60,61}\) (Figure 2.8). These methods, while effective, can have unwanted effects on cells through due to both photoinitiator toxicity and UV-exposure. Enzymatic crosslinking via transglutaminase\(^{62}\) or tyrosinase offers a mild alternative for rendering the gels thermally stable yet proteolytically degradable\(^{63}\). Specifically, transglutaminase is a Ca\(^{2+}\) enzyme that catalyzes protein crosslinking via the formation an isopeptide bond between glutamine and lysine residues (Figure 2.8). Moreover, gelatin exhibits rheological properties amenable to many biofabrication approaches and has been widely used in casting, electrospinning, inkjet printing, and extrusion bioprinting\(^{36,64}\).
**Figure 2.8 Gelatin crosslinking mechanisms.** (a) Gelatin is hydrolyzed collagen and exhibits a thermally reversible gelation as the remaining collagen helices stabilize \((T < 30^\circ C)\) or dissociate \((T > 30^\circ C)\). (b-c) Various methods to crosslink and thermally stabilize gelatin. (b) Gelatin can be chemically modified using methacrylic anhydride, to covalently attach a UV sensitive methacrylate group to (gelMA). Upon addition of UV-sensitive initiator (Irgacure 2959) and exposure to UV light, the gel crosslinks. (c) Enzymatic crosslinking can be achieved using transglutaminase (TG), which irreversibly couples terminal glutamine groups.

**Figure 2.9 Fibrin formation from fibrinogen.** (a) Schematic of fibrin assembly. Thrombin cleaves fibrinogen molecules which dissociate into fibrin monomer and fibropeptides. The fibrin monomer then becomes unstable and rapidly aggregates into fibrin protofibrils that, ultimately, assemble into fibrin networks. (b) High resolution TEM images of these steps of showing monomer creation, fibril aggregation, and network assembly. (c) SEM of a typical fibrin network microstructure after assembly. Adapted from.

Adapted from 67.
**Fibrin.** Fibrin is a naturally occurring protein-based material, which has been used as an injectable scaffold for tissue engineering and as a model for studying cell behavior in 3D culture. Fibrin is formed through a rapid, thrombin-initiated aggregation of insoluble chains of fibrinogen polypeptides into a fibrillar 3D network (Figure 2.9)\(^{65-67}\). The variables effecting network assembly have been studied extensively, especially the effects of pH, Ca\(^{2+}\), and thrombin concentration on microstructural changes in the final network\(^{68}\).

One main advantage of using fibrin for tissue engineering are its biochemical properties, such as cell adhesive binding domains and natural degradability via fibrinolysis. These attributes alleviate the need to perform additional chemistry to introduce such moieties. Moreover, fibrin is known to specifically bind many important proteins and polysaccharides, such as fibronectin, hyaluronic acid, von Willebrand factor (vWF), and VEGF. There are two RGD sites per fibrinogen molecule through which cell-surface integrins can naturally interact\(^{67}\). The mechanical properties as well as the degradation time are also tunable based on clot kinetics and addition of aprotinin, respectively\(^{69}\). Faster network assembly leads to a denser network, and to stiffer gels. Aprotinin is a proteolytic enzyme that inhibits fibrinolysis leading to slower degradation rates. Over the last 15 years, fibrin scaffolds have been used for *in vitro* and *in vivo* applications to generate new cartilage tissues\(^{70}\), pro-angiogenic scaffolds\(^{71}\), and in cardiovascular tissue engineering\(^{72}\).

To summarize, biomaterials must be carefully selected to mimic the native ECM’s structure, properties, processing, and performance, for tissue engineering applications.
Figure 2.10 summarizes the various chemical and physical entities within the ECM that directly affect the constitutive cells within the engineered tissues (e.g., degradation, adhesion, bioactivity, transport, and mechanical properties). In principle, most of these features can be engineered into ECM-mimics; however, cell source often plays an equally important role in achieving tissue-specific functionality, and thus, must be carefully considered.

**Figure 2.10 A summary of the various chemical cell-ECM interactions.** (A) Bioactivities of hydrogels can be achieved through protein engraftment. Degradation sites may be either hydrolytic (B) or proetolytic (F). functional groups are readily displayed along backbone of macromolecular networks. Stronger and more stable networks can be present via covalent crosslinking (G). Many secondary interactions should be taken into account when studying cell-ECM interactions, including hydrophobic interactions between the polymer chains (C), hydrogen bonding between polymer and cell-surface proteins (D) and ionic interactions between the polymer and cell membranes (E). Adapted from Ref73
2.3.2 Primary Cells, Immortalized Cell-lines, and Stem Cells

An essential step in any tissue manufacturing process is to select the appropriate cell types from which to build the tissue of interest. There are over 300 unique cell types in the body, many of which are not capable of being utilized to reconstruct human tissue due to issues with scarcity, isolation, or loss of function in vitro. Broadly, three major classes of cells exist for this application: primary cells, cell-lines, and stem cells. This section will discuss each class as well as their advantages and disadvantages.

*Primary cells.* Primary cells are extracted directly from a parent tissue source and have the key advantage of identical karyotype and chromosome number to the parent source. Primary cell culture has been used as a means of studying organ-specific cells in vitro. In fact, some basic *in vivo* behaviors can be recapitulated during cell culture making hypothesis regarding the parent tissue more simple to test than invasive *in vivo* investigations. For example, osteoblasts, isolated from bone, deposit collagen and calcium phosphate, cardiomyocytes extracted from the heart spontaneously beat in cell culture, hippocampal neurons controllably synapse, and renal proximal tubule cells transport water across their membranes using specialized aquaporins. To date, primary cells have been isolated and stored for every major organ and tissue (e.g., brain, heart, lungs, kidneys, eyes, muscle, bone and blood vessels). However, their main disadvantage is that these cells generally have a limited lifetime in cell culture. Primary cells only divide a limited number of times before they reach senescence—the genetically determined limit of their mortality beyond which they exhibit decreased proliferation and viability, which is known as the Hayflick limit. Gradually, after each cell division,
most of a cell’s telomeres shorten consistently after replication and once the telomeres reach a critical length, then cell proliferation ceases \(^{80,81}\) (Figure 2.11). The inability to grow cells indefinitely leads to more expensive cell culture, batch-to-batch variability (as the source tissue is always different), and, ultimately, unstable cell functionality during culture \(^{81}\). Theses issues have motivated the development of immortalized cell lines.

**Figure 2.11 Schematic representation of the telomere shortening phenomena.** After every cell division the telomere ends of the chromosomes shorten, and once a critical size is reached cells can no longer divide. In stem cells, the enzyme telomerase adds DNA stretches to the telomeres \(^{82}\).

**Immortalized cell lines.** Immortalized cell lines have been used extensively in cell culture as a means of producing continuously proliferating cells for monitoring both basic cell function and performing tissue-specific functional assays. There are dozens of immortalized cell lines ranging from endothelial, epithelial, connective tissue-derived, and cancerous cells. With the ability to culture immortalized cells continuously, these cells have clear advantages in decreased variability, ease of use, and stable function over time. However, with the exception of cancer cells, which are naturally immortalized \(^{83}\).
(e.g., HeLa cells), these cells must be manipulated genetically to induce immortality—this should always be taken into account when performing biological analysis and drawing conclusions suggestive of the natural \textit{in vivo} environment \textsuperscript{84}. While the proliferative potential of immortalized cells make them appealing for therapeutic applications in tissue engineering as a scalable cell source, their unlimited proliferation can lead to uncontrolled growth and tumor formation \textsuperscript{84} \textit{in vivo}. Despite the clear advantages in long-term cell culture, the use of immortalized cells in tissue engineering will require novel advances in safety-focus strategies such as inducible telomere protection or selective expression of human telomerase reverse transcriptase (h-TERT) \textsuperscript{84}. By contrast, stem cells possess the unique ability to naturally proliferate extensively, but coupled with the ability to be directly differentiated towards multiple lineages \textsuperscript{85}.

\textit{Stem Cells}. Stem cells are essential to the development, maintenance, and regeneration of tissues within the human body. They exist as undifferentiated cells that exhibit both self-renewal and multi-lineage differentiation potential. In humans, they are broadly classified as either embryonic or adult stem cells, but, more recently, researchers have developed induced pluripotent stems (iPSCs) by delivering a series of transcription factors to coax terminally differentiated cells into a pluripotent, embryonic-like state \textsuperscript{86,87}. Embryonic stem cells (ESCs) are isolated from the inner cell mass of a blastocyst \textsuperscript{88}—these cells uniquely have pluripotent differentiation potential, meaning that they can differentiate into each of the three germ layers (e.g., endoderm, ectoderm, and mesoderm), which consist of over 200 different cell types found in the body \textsuperscript{89}. ESCs possess impressive potential as a universal cell source; however, it is currently controversial to isolate these
cells from humans, as they must be removed from a discarded embryo and, more importantly, the unlimited growth potential of these cells can lead to uncontrolled growth and differentiation, which gives rise to teratoma formation upon implantation \(^{88}\). These main disadvantages have limited ESC adoption beyond developmental biology studies. Like ESCs, iPSCs have the dramatic ability to differentiate into cells from the three germ layers, but they possess a key advantage of being patient-derived, which makes them suitable for studying patient-specific genetic diseases or being an autologous cell source for transplantation \(^{90}\) (Figure 2.12). Since they are derived from a mature fibroblast cell, these cells lack the controversy of being sourced from human embryos, but akin to ESCs, they can lead to uncontrolled proliferation and nonspecific differentiation, which has limited their translation potential to date \(^{86}\).
Alternatively, adult stem cells are isolated from various mature tissues in the body such as adipose, bone marrow, and blood. Instead of being pluripotent, these cells are generally characterized as multipotent – meaning that they have several lineages to which they can differentiate, instead of cells in all three germ layers. These cells do proliferate in culture, but not indefinitely. For example, human bone-marrow derived stem cells (hMSCs) are of specific interest, as they can differentiate into a number of lineages including adipose, bone, muscle, tendon, and cartilage (Figure 2.13). Beyond their multi-potentency, these cells are far more predictable than pluripotent stem cells, hence, have already been used as an autologous cell source for many therapies, including bone marrow transplantation and other directly injectable cell therapies.
Moving forward, next generation cell-based therapies will likely include localized deliveries of cell-laden biomaterials as a delivery vehicle. Notably, each of these key cell sources have been incorporated into tissue constructs by different biofabrication approaches. However, to date, current tissue engineered constructs have been limited to thin geometries due to the lack of a pervasive vasculature to sustain cells deep with the tissue. It is difficult to overcome this limitation without ability to embed a pervasive vasculature within the engineered tissues that keeps the therapeutic stem cells alive and thriving.
2.3.3 Vasculature

The vascular system is a conduit for blood to transport nutrients, oxygen, carbon dioxide, hormones, and blood cells to and from the various tissues in the human body. Vascular networks are essential for nourishing tissue, fighting disease, controlling local and organismal temperature, and maintaining homeostasis. They are intricate branched networks that pervade all tissues in the body and their diameters range from 5 \( \mu \text{m} \) (\( \mu \)-capillary) to 25 mm (aorta). Mature, large vessels (> 200\( \mu \text{m} \)) possess a trilayer architecture consisting of a tunica intima, media, and adventitia (Figure 2.14a). The intima consists of a layer of endothelium lining the inner layer of vessel, the media is primarily composed of a smooth muscle layer, and the adventitia contains a surrounding layer of fibroblasts and matrix. For large vessels, the composite architecture provides both mechanical support and the ability to rapidly expand and contract to accommodate changes in blood flow in response to local metabolic needs of the tissues which they pervade (Figure 2.14) \(^98\). However, in some vessels, namely the microvascular networks, the outer layers are absent and they consist solely of a single layer of endothelium, which allows rapid oxygen exchange due to the increased permeability without the stabilizing outer layers \(^100\).
Figure 2.14 Architecture of mature blood vessels and vascular networks. (a) Mature blood vessels possess a composite, tri-layer architecture composed of the tunica intima (endothelium), media (elastic smooth muscle layer), and adventitia (stiff fibroblast-laden connective tissues) (b) MRI and 3D reconstruction of a heart vasculature. Adapted from open source images: University of Washington http://www.lab.anhb.uwa.edu.au/mb140/corepages/vascular/Images/VesWall.jpg. and Cornell University http://www.biotech.cornell.edu/heart-vasculature-3d-ct-dataset
While all blood vessels serve as a conduit for carrying oxygen and removing metabolic by-products from tissues within the body, the smaller microvascular networks are the vessels responsible for exchange. Lacking the outer layers of tunica, media, and adventitia, they are far more permeable than larger vessels and given their small dimension, prone to rapid exchange. Additionally, blood vessels are responsible for proper water balance in tissues through selective permeability, shield surrounding parenchyma from stress, and route chemical signals throughout the body. The endothelial monolayer is responsible for much of this selectivity and regulation, as well providing anti-thrombogenic resistance to the luminal surface. Hence, the presence of a confluent, uniform endothelium is essential for proper vascular function.

In recreating vascularized tissue, the primary objective is to deliver nutrients and remove waste in engineered constructs that are too thick to rely solely on diffusion (<1mm). In Figure 2.15 sliced tissue is placed in various oxygen rich environments and monitored over time. The cells in the fresh sliced tissue showed high viability throughout the thickness, whereas the other tissues showed cell viability decreased at increasing depths from the surface, in which the absolute distance was proportionate to the distance to the oxygen source. This experiment highlights the essential need for vasculature in delivering oxygen into thick tissues rather than solely relying on diffusion from the outer surface. The ability to create 3D vascularized tissue has been a major challenge over the past two decades, hindering the biofabrication of physiologically relevant tissues.
Figure 2.15 The effect of oxygen concentration on tissue viability. Tissue slices were cut under sterile conditions with 1.5 mm thickness image taken after 8 h in different oxygen conditions. Changes in cell morphology were apparent in the sharp difference between the superficial compact layer of normal cells and the central area of obviously abnormal cells. Adapted from: 102.
2.4 TISSUE FABRICATION METHODS

Traditional tissue fabrication methods have been limited to the creation of homogenous cell-laden tissue constructs due to the inability to prescribe precise locations of different cells or materials \textit{a priori}. Ideally, one can assemble all key tissue elements described in the previous section (e.g, ECM, cells, and vasculature), concurrently and with high precision. To move beyond simple, homogeneous biofabrication strategies like freeze-casting \textsuperscript{104}, molding \textsuperscript{105}, and cell-sheets \textsuperscript{106}, biofabrication methods aim to recreate structure and function of human tissues. Several methods have recently emerged including electrospinning \textsuperscript{107}, soft-lithography \textsuperscript{108}, multi-photon polymerization \textsuperscript{109}, and bioprinting \textsuperscript{110}. In this section, three bioprinting methods: (1) laser induced forward transfer printing (LIFT), (2) inkjet printing, and (3) extrusion printing (Figure 2.16) will be described in detail.

![Figure 2.16](image)

**Figure 2.16 Selected biofabrication approaches capable of delivering cells in various methods.** Laser-induced forward transfer, thermal and piezoelectric inkjet, and pneumatic, piston, and screw extrusion. Adapted from \textsuperscript{111} \textsuperscript{36}. 


2.4.1 Laser induced forward transfer printing (LIFT)

LIFT is a biofabrication process that uses coated glass slide with a laser energy absorbing layer and cell-laden bioink \(^{112}\) called a donor substrate. A laser pulses focused light onto the absorbing layer causing it to locally evaporate, which subsequently generates a high gas pressure ejecting the bio-ink towards the collector slide (Figures 2.16-2.17). Using this method, precise deposition of relatively thin, cell-laden structures can be achieved while retaining high cell viability \(^{11,112}\) (Figure 2.16). This approach does not rely on deposing inks through fine nozzles, thus bio-inks with a range of viscosities can be deposited (from 1-300 mPa s\(^{-1}\) \(^{113}\). Material is locally ejected from the donor substrate precisely where the laser is focused, which places strict requirements on having a uniform distribution of cells within the gel coating, as aggregates can lead to unwanted heterogeneities. This process is well suited for creating thin tissue structures, such as skin and corneal tissue. However, the generation of larger clinically relevant 3D constructs is time-consuming, which has thus far limited the widespread adoption of this technology \(^{114}\). Moreover, this method is only capable of printing cells and ECM, without the ability to embed vascular networks, it is not capable of constructing truly 3D human tissues \(^{112}\).
During inkjet printing, small volumes (1-100 picoliters) of bioink are ejected through a small orifice (10-50 µm) onto an underlying substrate. The two most common approaches for droplet generation are thermal and piezoelectric inkjet printing (Figure 2.15). In thermal inkjet, a micro-heater is used to locally vaporize small volumes of fluids to generate a pulse of pressure that ejects liquid from the print head. In piezoelectric-based inkjet printing, a direct mechanical pulse is applied to the fluid in the nozzle by the piezoelectric actuator, and a shock wave ejects the bioink through the nozzle.
Inkjet printing has numerous advantages such as high speeds (1-10,000 droplets second\(^{-1}\)), low cost, and compatibility with many cell-types and materials\(^{110,118}\). Although droplets are can be as small as 10 µm, the final feature sizes are generally greater than 50 µm due to the tendency for low viscosity inks to undergo wetting and spreading once in contact with the underlying substrate\(^{119}\). Additionally, building thick, three-dimensional structures requires multiple passes due to the printed features possessing low aspect ratios (< 0.1). The physics of drop formation have been characterized extensively\(^{119}\). Notably, the key parameters governed by groupings of physical constants into several dimensionless numbers, namely, the Reynolds (Re), Weber (We), and Ohnesorge (Oh) numbers\(^{119}\):

\[
Re = \frac{\nu \rho a}{\eta}
\]

\[
We = \frac{\nu^2 \rho a}{\gamma}
\]

\[
Oh = \frac{\sqrt{We \rho a}}{Re} = \frac{\eta}{\sqrt{\gamma \rho a}}
\]

where \(\rho\), \(\eta\), and \(\gamma\) are the density, dynamic viscosity, and surface tension of the fluid, respectively, \(\nu\) is the velocity, and \(a\) is a characteristic length. The processing map delineates the appropriate regions for reliable jetting, as shown in Figure 2.18. If surface tension is too high or the droplet size is too large, then satellite droplets form due to Plateau-Rayleigh instabilities. If the viscosity of the ink is too high, droplet formation will not occur. If jetting velocity is too high, then splashing may occur upon droplets hitting the underlying substrate. \(Re\) relates the material’s inertial and viscous forces, with typical
values in the range of 0.5 - 10 for reliable jetting. \( We \) relates the inertial and surface tension forces and can have a much broader range, but reliable jetting is heavily dependent on \( Oh \), which relates \( Re \) and \( We \). Importantly, only a narrow range of \( Z \) values (\( Oh^{-1} \)) between 1 and 10 yield reliable jetting.

Figure 2.18 Operable inkjet printing fluid regimes. The narrow range of \( Z = Oh^{-1} \) that is suitable for stable, reliable jetting is plotted along the Reynolds and Weber numbers. Also, the delineating \( Z \) numbers that correspond to satellite droplets, splashing, and fluids too viscous to print are shown.\textsuperscript{119}
To date, this method been used to print thin, avascular samples, such as skin\textsuperscript{120} and cartilage\textsuperscript{121} (Figure 2.19), respectively. However, this approach also has a few drawbacks. First, large stresses generated during droplet formation affect cells, resulting in transient pores in the cell membrane\textsuperscript{115}. In addition, as \( \rho \) and \( \gamma \) of most biomaterials biomaterials are similar, only bio-inks that possess a narrow range of narrow range viscosities (\( \sim 3.5 - 12 \text{ mPa s}^{-1} \)) can be jetted. Droplets undergo significant wetting and spreading leading to poor resolution (\( > 50 \mu m \) features), low cell concentrations (making physiological tissues difficult to achieve), and the high risk of nozzle clogging\textsuperscript{110}. Moreover, creating vasculature using this approach is very challenging, as low aspect ratio droplets make building tubular architectures cumbersome. Subsequently, only few, simple examples of inkjet printed vasculature exist in literature\textsuperscript{115}. 

Extrusion-based bioprinting is filamentary-based method in which concentrated bio-inks are deposited through fine nozzles (~100 µm diameter) (Figure 2.16). These filaments are patterned in layer-by-layer sequence to create three-dimensional tissues. Filaments are extruded with aspect ratios of near unity, which reduces the number of layers required to build a three-dimensional object compared to LIFT or inkjet. During printing, pneumatic, piston, or screw driven systems (Figure 2.16) can be used to drive material.
deposition. Mechanically driven systems (piston or screw) tend to provide better control over flow rates relative to pneumatic systems. Importantly, compared to LIFT and inkjet, extrusion based-bioprinting allows for a broader range of materials and viscosities to be printed. To date, inks spanning six orders of magnitude (\(\sim 10^1 \text{ mPa s}^{-1} \cdot 10^7 \text{ mPa s}^{-1}\)) have been successfully printed by this technique, including alginate, gelatin, chitosan, hyaluronic acid, Pluronic F-127, PEG, and methylcellulose.

A common strategy for maintaining print fidelity upon deposition is to design viscoelastic inks that possess both shear-thinning behavior and a well-defined yield stress \((\tau_y)\). Due to the shear thinning behavior, the ink viscosity decreases as the local shear rate within the nozzle increases, which allows it to rapidly flow through the nozzle. However, upon deposition the shear rate goes to zero and ink rapidly solidifies \(^{122,123}\) (Figure 2.20). Inks that possess a yield stress require finite amounts of stress \((\tau > \tau_y)\) to induce flow, enabling rapid stops and starts in response to applied stress. In the as-printed state, the inks exhibit a substantial plateau modulus, enabling self-supporting filaments printed into 3D structures with high fidelity \(^{122}\).
Specifically, cell-based inks, including cell-laden hydrogels and cell spheroids\textsuperscript{125} have been successfully printed with high viability using a variety of ECM-based inks as carriers\textsuperscript{36}. The high resolution of extrusion printing has enabled the creation of complex multimaterial parts at physiologically relevant sizes that mimic tissue anatomy, such as aortic valves\textsuperscript{126}, ovine meniscus\textsuperscript{127}, and miniaturized human distal femur\textsuperscript{128} (Figure 2.21). Lacking a perfusable, interconnected microvasculature, these cell-laden tissues are sustained via passive diffusion of oxygen and nutrients from the surrounding environment, which restrict their thickness to $\leq 1$ mm.

\textbf{Figure 2.20 Examples of viscoelastic materials.} (a) Flow curves of alginate at 2,3,5 wt\%. (b) Stress amplitude sweep of a prototypical yield stress material, microcrystalline wax at two different compositions\textsuperscript{123}. The material is subjected to increasing stress while in the linear elastic regime, revealing the plateau modulus (point of constant $G'$) and the yield stress (stress at which $G'$ equals 90\% of plateau modulus).\textsuperscript{124}
Figure 2.21 Extrusion-based bioprinting examples. (A) A model of aortic valve reconstructed from a μ-CT images (red indicates leaflet and green indicates valve root) and an anatomically similar structure after printing (B). (C-D) Ovine meniscus μCT-scan and subsequent print. (E-F) A miniaturized distal femur from a human knee (Rhino software) and print of a heterogeneous bone (yellow) and cartilage interface (green).

Figure 2.22 Examples of cell spheroid printing and assembly. (a) Cell spheroids are extruded through a nozzle onto an underlying substrate. (b) Example of printed spheroids after fabrication and post fusion (typically with 24 - 48 h). (c) As cells must remain in their printed position prior to cellular fusion, strategies have been adopted to immobilized spheroids into agarose scaffolds. (d) Example of large 2.5 mm blood vessel printed using spheroids and agarose molds. Adapted from.
As a final example, cell spheroids that consist of dense aggregates of cells (~ 200 - 750 µm diameter), bound together by their own deposited ECM, have been printed into various tissues architectures (Figure 2.22)\textsuperscript{129}. This method enables the patterning of cells at near native tissue-level cell densities (10\textsuperscript{8} - 10\textsuperscript{9} cells mL\textsuperscript{-1}), which should enhance tissue-level functionality. This method has been used to create large diameter blood vessels (0.9 - 2.5 mm outer diameter); however, all cells remain 1 mm from the surface. By printing layers of cell spheroids, it might be possible to create functional organs at physiological scales \textsuperscript{130-132}. However, to date, these printed tissues lack pervasive microvasculature vasculature both within and adjacent to individual spheroids, limiting tissues to thin, avascular architectures.

To date, extrusion-based printing of cell-laden tissue constructs has been primarily limited to solely ECM and cells. Due to the lack of supporting microvasculature, these tissues are restricted to thin layers that are less physiologically relevant \textsuperscript{133}. Their lack of vasculature also limits tissue-level functionality, since this is a crucial component of the cellular microenvironment \textit{in vivo}.

Recent advances have harnessed printing to create vascularized tissue constructs \textsuperscript{14,134,135}. Notably, fused deposition modeling a molten sugar solution was first 3D printed into a rigid carbohydrate glass template, forming a sacrificial scaffold that is dissolved after casting with a cell-laden hydrogel (Figure 2.23)\textsuperscript{14}. After the sugar template dissolves, the open channels were lined with endothelial cells. These vascular networks were then perfused using an oscillatory rocking motion that moved cell media through the open channels, sustaining the surrounding cell-laden tissue. The hepatocytes in the perfused system demonstrated both higher cell viability and enhanced functionality (e.g.,
albumin secretion). However, the sugar scaffold must be heated to over 100°C to facilitate fused deposition modeling, which makes co-printing concurrently with cell-laden hydrogels quite difficult as the high temperature nozzle would damage cells upon contact. While elegant, this hybrid printing/molding method is currently limited to the construction of simple tissue architectures composed of homogeneous cell-laden matrices, whereas natural tissues are structurally complex and composed of multiple cell types.

In summary, several biofabrication methods have been introduced to date. Yet, no method offers the ability to simultaneously pattern ECM, multiple cell types, and embedded vasculature concurrently with high precision. Existing engineered tissues are limited in their size, complexity, and functionality, relative to native human tissues of interest.
Figure 2.23 Vascularized tissues via fused deposition modeling of carbohydrate glass. (a) Schematic of the process of using carbohydrate glass as sacrificial scaffold. (b) Images of this sequence include printing of carbohydrate scaffold, casting cell-laden ECM, and removal of scaffold. (c) Image of homogeneous, vascularized tissue construct containing endothelialized channels and a surrounding fibroblast interstitium. (d) Comparison of functionality in both acellular (left) and vascularized (right) tissue, showing enhanced cell activity in vascularized tissue. Adapted from 14.
2.5 APPLICATIONS FOR ENGINEERED HUMAN TISSUES

There is a tremendous need for engineered human tissues that recapitulate function in both *in vitro* and *in vivo* environments. Both near-term applications in pre-clinical drug screening and long-term applications in tissue and organ replacement would greatly benefit from this capability.

2.5.1 Drug screening

Currently, *in vitro* applications for engineered human tissues are within reach. High drug failure rates and rising costs are leading to serious concerns for drug developers, regulators, and patients. Overall, drug candidates have a likelihood of approval by the Food and Drug Administration (FDA) of just 10%\textsuperscript{136}, which suggests an opportunity to increase efficiency through various means including more predictive disease models and earlier toxicology evaluations. It is estimated that this inefficiency in drug development has lead to over $300 Billion in spending on drugs that ultimately fail to reach to be approved by the (FDA)\textsuperscript{136,137}.

Given the dramatic improvements in high throughput drug discovery screening, the increasing number of lead drug candidates should result in an increase in approved drugs per billion US$ on R&D\textsuperscript{137}. Unfortunately, the number of molecules entering clinical trials has remained largely unchanged over the past 50 years, even while the costs to bring new drugs to the market has escalated. Facetiously, this empirical trend has been named Eroom’s Law (in reference to the famed Moore’s law), stating that the number of new drugs approved by the FDA per billion dollars of R&D expenditure has roughly halved every 9 years over the last 60 years (Figure 2.24)\textsuperscript{137}.
Ultimately, drug-screening platforms need to recreate human-level physiology to properly predict clinical drug success with accurate preclinical data. Realistic, functional human tissue models could supplement existing animal models, which often do not fully recapitulate the drug toxicity in humans. Success in this area could ultimately reduce the number of drugs that fail during clinical trials, lower the development costs and associated risks of bringing drugs to market. Ultimately, inferences made using these advanced platforms should help to advance tissue-level biology, troubleshoot failed drugs in the pipeline, and inform the creation of next-generation therapeutics.

Figure 2.24 Eroom’s law in pharmaceutical R&D. (a) The number of new drugs approved by the FDA per billion dollars of expenditure has roughly halved every 9 years over the last 60 years. (b) The pipeline for drug approval has seen improvements in the number of leads drugs, but the number of drugs that reach trials remains constant and the number of drugs approved continues to deline according to Eroom’s law\textsuperscript{137}.
Organs-on-chips. Recent pioneering work in organs-on-chips\textsuperscript{139,140}, has shown that recapitulating aspects of organotypic chemo-mechanical microenvironments in 2D culture can preserve or restore native tissue functionality as demonstrated in several key embodiments (e.g., lung\textsuperscript{140}, liver\textsuperscript{141}, heart\textsuperscript{142}, gut\textsuperscript{143}, and renal proximal tubule\textsuperscript{144}) (Figure 2.25). Although promising, this microfluidics-based approach lacks several key elements for recreating human tissue environments, notably three dimensionality and highly simplified ECM mimics (surface coatings), which, may limit the physiological relevance\textsuperscript{133} of these devices. Importantly, these devices are strictly designed for \textit{in vitro} applications, and, hence, are not suitable for implantation or large-scale tissue reconstruction.

\textbf{Figure 2.25} The renal proximal tubule on a chip is a representative microfluidic organ-on-chip. (A) The microfluidic device consists of a flat apical channel and a bottom reservoir separated by a porous ECM-coated porous membrane upon which primary human proximal tubule cells (PTEC) are cultured. This system allows for fluid sampling and the testing of compounds. (B) The device is assembled by plasma bonding of the two PDMS layers to the central porous membrane and then seeding the PTECs through the device inlet onto the porous ECM-coated membrane.\textsuperscript{138,139}
Spheroids. Cell spheroids offer an alternative to 2D microfluidics-based approaches for drug screening platforms \(^{145}\). Inherently, these models consist of aggregates of cells that are bound together using their own secreted ECM, which improves their clinical and biological relevance compared to 2D culture. Spheroids can be composed of either terminally differentiated cells or stem cells, which can serve as either mature \(^{145}\) or developing tissues \(^{6}\), respectively. In these models, the goal is often to recreate miniaturized organs or organoids to elucidate organ-level function or toxicity in the liver \(^{146}\), kidney \(^{6}\), gut \(^{147}\), and brain \(^{148}\).
Figure 2.26 Summary of spheroid formation, differentiation, and printing. (a) Spheroids are created by assembling loose mobile cells suspensions into spherical shapes. Generally, cells are aggregated using low-adhesion plates or conical wells and grown in suspension culture to improve nutrient exchange compared to static culture. (b) iPSC spheroids differentiated towards specific organ lineages are called organoids. This example of a kidney organoid demonstrates the exquisite microstructural complexity organoids are capable of achieving by mimicking developmental biology. Kidney organoids contain developing nephrons, stroma and vasculature with progressive maturation over time.
2.5.2 Tissue and organ replacements

Clinical need exists for tissue manufacturing approaches that can repair or regenerate functional human tissues or organs. In the U.S, over 100,000 people are currently awaiting a replacement organ, and hundreds of thousands more undergo replacement and repair surgeries to tissues annually (Figure 2.27). Moreover, the mismatch between supply and demand is growing, as the average age of the population increases.

![Plot of the number of donors, transplants and recipients on the waiting list over time.](image)

**Figure 2.27 Plot of the number of donors, transplants and recipients on the waiting list over time.** Over 100,000 people are currently waiting for organs, yet only around 20,000 are performed every year. Adapted from the organ donor website

Regardless of the complexity of the target tissue in need of repair, tissue engineering strategies generally involve the application of combinations of biomaterials, cells, and biologically active factors designed to effect tissue formation upon implantation. Broadly, strategies are either based on the assembly of tissue *in vitro* or the induction of tissue regeneration *in vivo*. To date, flat, tubular, or hollow structures
have been recreated, but solid organs such as the liver and kidney remain highly allusive (Figure 2.28) \(^4\). Meeting this grand challenge will take a concerted effort by many researchers over the next several decades.

**Figure 2.28 Four structural levels of complex tissues and organs.** Human tissues can be categorized into increasing levels of increased engineering complexity. Examples include flat structures include the skin or cornea, tubular structures such as the trachea, hollow tissues such as the bladder and solid organs such as the kidney \(^4\).

### 2.6 CONCLUSIONS

In summary, as engineers move towards the creation of arbitrarily complex 3D architectures that contain more of the chemical, mechanical, and biological complexity of living tissues, native tissue function will become increasingly observable in *in vitro* and *in vivo* applications. However, continued advances are required in multiple areas, including materials selection, cell sources, biofabrication, and developmental biology, to achieve this overarching goal.
CHAPTER 3

3D BIOPRINTING OF VASCULARIZED, HETEROGENEOUS TISSUE CONSTRUCTS

This chapter has been adapted from the publication:


3.1 INTRODUCTION

The ability to create 3D vascularized tissues on demand would enable scientific and technological advances in tissue engineering, drug screening, and organ repair. To produce 3D engineered tissue constructs that mimic natural tissues and, ultimately, organs, several key components—cells, extracellular matrix (ECM), and vasculature—need to be patterned in precise geometries. Each of these components plays a vital role in imparting, supporting, or sustaining the biomimetic function of the engineered tissue construct, respectively. Perhaps the most important of these components is the vasculature; without proximity to a perfused microvasculature that provides efficient nutrient, growth/signaling factor, and waste transport, most cells within bulk tissue constructs will not remain viable. In fact, 3D engineered tissue constructs quickly develop necrotic regions without perfusable vasculature within a few hundred microns of each cell. Unfortunately, the inability to create vasculature networks within engineered tissue constructs has hindered progress in the field of tissue engineering for decades.
One emerging strategy for creating engineered tissue constructs is 3D printing\textsuperscript{110}. To date, this technique has been primarily used to create acellular 3D scaffolds and molds\textsuperscript{9,10,13,154} which must be seeded with cells post-fabrication. Building tissue constructs by directly depositing cells or cell aggregates, known as bioprinting, has also been reported\textsuperscript{125,130,155}. However, neither approach is currently capable of embedding vasculature, which severely constrains the overall dimensions of tissue constructs produced by these strategies. More recently, researchers have introduced vascular channels by printing sacrificial carbohydrate glass filaments at temperatures above 100°C, which are then encapsulated within cell-laden hydrogels via a molding process\textsuperscript{14}. While elegant, this hybrid printing/molding technique is relegated to constructing simple tissue architectures composed of homogeneous cell-laden matrices. Yet, natural tissues are structurally complex and heterogeneous, i.e., composed of multiple cell types.

In this chapter, we will report a method of 3D printing vascularized tissue constructs composed of multiple cell-types that are patterned adjacent to a pervasive vascular network. Specifically, three types of inks are developed: (1) extracellular matrix (ECM) ink composed of gelatin methacrylate (GelMA), (2) cell-laden inks composed of different types of fibroblast cells and ECM, and (3) a fugitive ink composed of a triblock copolymer PEO-PPO-PEO (pluronic F-127). These inks are printed into 1D, 2D, and 3D vascularized tissue constructs, which are lined with endothelium upon removal of the fugitive ink. The initial and long-term viability of printed cells is assessed.
3.2 EXPERIMENTAL METHODS

3.2.1 Ink Formulations

We created several inks for 3D printing of engineered tissue constructs. The first two inks are composed of pure or cell-laden GelMA solutions. We synthesized GelMA following a modified procedure, reported previously \(^{14,60}\). First, a 10 w/v\% gelatin solution is prepared by dissolving gelatin (Type A, 300 bloom from porcine skin, Sigma) in Dulbecco’s phosphate buffered saline (DPBS) warmed to 60°C for 2 h under vigorous stirring. The solution temperature is maintained between 60°C and 70°C during gelatin dissolution, after which it is lowered to 50°C. To produce GelMA with 50% degree of methacrylation \(^{60,156,157}\), 0.14 mL of methacrylic anhydride (Sigma) for each gram of gelatin in solution is then added drop-wise to the gelatin solution. The gelatin solution is covered to prevent evaporation, methacrylation reaction is allowed to proceed for 4 h at 50°C under vigorous stirring. The methacrylation reaction is then quenched via dilution of the reaction solution with DPBS warmed to 40°C to yield a GelMA concentration of 4.5 w/v\%. To remove excess methacrylic acid and methacrylic anhydride, GelMA product is precipitated overnight by the addition of ice-cold acetone to the reacted solution at a 1:4 ratio of GelMA to acetone. Acetone is then decanted from the precipitated GelMA, and the precipitate is dried under flowing air for 30 min before being dissolved in 10 w/v\% in DPBS warmed to 40 °C. This warm GelMA solution is vacuum filtered through a 0.2\µm filter (Corning Bottle-Top Vacuum Filtration System), transferred to 12-14 kDa MWCO dialysis tubing, and dialyzed against DI water for 3 days (the dialysis media is changed twice daily) to remove any remaining methacrylic acid and salts from DPBS. Lastly, the GelMA is frozen overnight at -80°C, lyophilized
for four days, and stored at -20 °C.

Pure GelMA inks are prepared by first dissolving GelMA powder in warm 1:1 DMEM:EGM-2 cell culture media at 15 wt/v%. Irgacure 2959 (BASF) is added to the solution at 0.3wt% as a photoinitiator, the solution is briefly vortex mixed, and stored at 37°C until fully dissolved. Once dissolved, the solution is centrifuged to remove air bubbles. Unused GelMa solution is stored in dark conditions at 2 - 8 °C to prevent unintentional crosslinking via ambient light.

Cell-laden GelMA inks are created by first removing 10 T1/2 or HNDFs from culture flasks via standard trypsinization technique. The cells are then dispersed in 15 wt% GelMA/media solutions at 2 x 10⁶ cells mL⁻¹. The cell-laden ink is pipetted up and down to mix thoroughly. Each GelMA-based ink is then loaded into a syringe at 37°C and allowed to cool to room temperature (~ 22°C) for 15 min prior to use.

The third ink is composed of 40 wt% Pluronic F127 (Sigma) in deionized, ultrafiltrated (DIUF) water. The ink is homogenized using a Thinky mixer until powder is fully dissolved, and subsequently stored at 4°C. Prior to use, the ink is loaded in a syringe (EFD Inc., East Providence, RI) at 4°C and centrifuged to remove any air bubbles. This ink is used to print 1D, 2D, and 3D vascular networks.

A final ink is composed of a two-part silicone elastomer (SE 1700, DOW Chemical) with a 10:1 base to catalyst (by weight) that is homogenized using a mixer (AE-310, Thinky Corp, Japan) and subsequently loaded into a syringe (EFD Inc., East Providence, RI) at room temperature and centrifuged to remove any air bubbles. This ink is dyed with different fluorophores (Risk Reactor Inc., Santa Ana, CA) to demonstrate
multimaterial printing. This ink is also used to print a border region composed of high-aspect ratio walls around each tissue construct.

3.2.2 Rheological Characterization

The rheological properties of each ink are measured using a controlled stress rheometer (DHR-3, TA Instruments, New Castle, DE) with a 40mm diameter, 2° cone and plate geometry. The shear storage ($G'$) and loss ($G''$) moduli are measured at a frequency of 1 Hz and an oscillatory strain ($\varepsilon$) of 0.01. Temperature sweeps are performed using a peltier plate over the range from -5°C to 40°C. Samples are equilibrated for 5 min before testing and for 1 min at each subsequent temperature to minimize thermal gradients throughout the sample.

3.2.3 Cell Culture and Maintenance

C3H/10T1/2, Clone 8 cells (ATCC® CCL-226TM) and green fluorescent protein-expressing human neonatal dermal fibroblast cells (GFP-HNDFs, Angio-Proteomie) are maintained in Dulbecco’s Modified Eagle Medium containing high glucose and sodium pyruvate (DMEM, GlutaMAXTM, Gibco) and supplemented with 10% fetal bovine serum (FBS, Gemini Bio-Products). Primary human umbilical vein endothelial cells (HUVECs, Lonza) and red fluorescent protein-expressing HUVECs (RFP-HUVECs, Angio-Proteomie) are maintained in EMG-2 media (complete EGTM-2 BulletKitTM, Lonza). All cell cultures are passaged per the respective vendor’s instructions. HUVECs, GFP-HNDFs, and RFP-HUVECs are not used beyond the ninth passage.

3.2.4 Glass Slide Treatment

The engineered tissue constructs are printed onto glass slides that are pre-treated to
promote bonding of GelMA-based inks. Glass slides are first cleaned via sonication in a series of solvents (isopropyl alcohol, ethanol, and deionized water) and subsequently air-dried. The slides are then soaked in a 5% 3-(Trimethoxysilyl)propyl methacrylate (Sigma) in toluene solution at 60 °C overnight, rinsed with isopropanol, and air-dried prior to their use as substrates.

### 3.2.5 Multi-material 3D Bioprinting

All fabricated 3D structures are printed using a custom-built 3D printer with an overall build volume of 725mm x 650mm x 150mm (ABG 10000, Aerotech Inc., Pittsburgh, PA) equipped with four independent, z-axis controlled ink reservoirs. Inks are housed in separate syringe barrels, and nozzles of varying size are attached via luer-loks. Several types of nozzles are used, including borosilicate (30 µm in diameter produced using a P-2000 micropipette puller, Sutter Instrument Co., Novato, CA), stainless steel (100 µm or 410 µm in diameter), and tapered plastic (200 µm in diameter) nozzles (EFD Inc., East Providence, RI). Each ink is extrusion printed through the nozzle orifice under an applied air pressure (800 Ultra dispensing system, EFD Inc., East Providence, RI) at speeds of 1-10 mm s⁻¹ and pressures ranging from 20-60 psi. Before printing, the nozzles are aligned using orthogonally mounted optical micrometers (LS-7600 series, Keyence, Japan) to determine their respective X-Y offsets. The cell-laden inks are printed for a maximum of 2 h after mounting to minimize cell damage due to lack of oxygen.

Engineered tissue constructs are produced by sequentially co-printing multiple inks. First, a border is printed by depositing the PDMS ink through a 200 µm tapered nozzle. A thin layer of the GelMA matrix is then deposited into bordered region at 37°C. This layer is then cooled to 15°C to induce rapid gelation. Next, the fugitive Pluronic F-127
and cell-based GelMA inks are printed directly onto GelMA-coated, bare glass, or treated surfaces. After printing, the patterned vascular and cell-laden features are encapsulated by depositing a pure GelMA ink heated to 37°C. The entire structure is illuminated with UV light source (Omnicure EXFO, 5mW cm$^{-2}$ for 60 s) to crosslink the GelMA species within the bulk matrix as well as cell-laden, patterned features. The entire structure is then cooled to 4°C to liquefy the printed Pluronic F127 features. Two empty syringes outfitted with 200 mm diameter stainless steel nozzles are then inserted through the printed construct into the liquefied regions. A modest vacuum is applied to remove the Pluronic F127 ink from those regions, leaving behind open, interconnected microchannels that yield the desired embedded vascular networks.

3.2.6 Endothelialization of Vascular Networks

The embedded vascular networks are flushed with ~500 µL EGM-2 cell media and then injected with ~5-10 µl of HUVEC suspensions ( > 1x10$^7$ cell mL$^{-1}$). The structures are inverted and incubated at 37°C for 30 min. After 30 min the entire structure is flipped and again incubated at 37°C for 30 min to ensure that the top and bottom of the channels are exposed to HUVECs. The structures are then incubated at 37°C for 5 h, after which they are placed on a rocker oscillating at ~1 Hz frequency within the incubator. After 24 h, non-adherent cells are flushed out of the network with fresh EGM-2 media and the construct is placed back on the rocker at ~1 Hz frequency. For improved cell adhesion, the channels may be coated with fibronectin (BD Technologies) solution (10 mg mL$^{-1}$) for 30 min prior to introducing the HUVEC suspension.
3.2.7 Cell Viability Assay

Cell viability is determined by printing a cell-laden GelMA ink composed of $2 \times 10^6$ cells mL$^{-1}$ 10 T1/2 fibroblasts and HNDFs. Four printed structures are fabricated for each time point. The samples are prepared by staining with calcein-AM (“live”, 1µL mL$^{-1}$, Invitrogen) and ethidium homodimer (“dead”, 4 µL mL$^{-1}$, Invitrogen) for 20 min. Control samples are produced by casting cell-laden GelMA films (200-300 µm thick) composed of the same cell type, density, and GelMA composition, and exposed to the same curing process as the printed samples. Live dead data are acquired using confocal microscopy for each time point ($t = 0,1,3,5,7$ days, $n = 10$), and average viability and standard deviations are determined for each sample. A student's t-test is used to compare the viability of printed versus control cell populations. Differences with p values less than 0.05 are denoted with asterisks.

3.2.8 Imaging and Image Analysis

Photographs and videos of the 3D printed, engineered tissue constructs are captured using a DSLR camera (Canon EOS 5D Mark II, Canon U.S.A Inc.). To aid in visualizing the embedded vascular networks, an aqueous-based fluorescent red dye (Risk Reactor) is directly injected into the network using a syringe. Microscopy is performed using a Keyence zoom microscope (VHX-2000, Keyence, Japan), an inverted fluorescence microscope (Axiovert 40 CFL, Zeiss), an upright confocal microscope (LSM710, Zeiss), and an upright fluorescent microscope (Axiozoom V16, Zeiss). Composite microscopy images are generated using ImageJ by combining bright field and fluorescent channels. 3D projections and Z-stacks are generated using manual and automated processes in Imaris (Imaris 7.6.4, Bitplane Scientific Software) and ImageJ software. For cell
counting, a semi-automated counting algorithm in Imaris is used to produce counting statistics. For extended microscopy experiments, samples are first fixed using 4% paraformaldehyde solution (Electron microscopy Sciences), stained with DAPI nuclear stain (Life Technologies) and hydrated using DPBS with 0.05% Tween-20 (Sigma).

### 3.3 RESULTS AND DISCUSSION

#### 3.3.1 Heterogeneous Tissue Fabrication

We created a new 3D bioprinting method for fabricating engineered tissue constructs replete with vasculature, multiple types of cells, and ECM. Creating these intricate, heterogeneous structures requires the ability to precisely co-print multiple materials in 3D (Figure 3.1). Hence, we first implemented a custom-designed, large-area 3D bioprinter with four independently controlled printheads (Figure 3.2).

![Figure 3.1 Schematic views of our 3D bioprinting concept.](image)

Figure 3.1 Schematic views of our 3D bioprinting concept. (left) ECM, cells, and vasculature are co-printed to yield engineering tissue constructs composed of heterogeneous subunits (right).
As a simple demonstration of our multimaterial printing capability we first printed four PDMS inks, each of which is dyed with a different fluorophore, in a predefined sequence to produce a heterogeneous 3D architecture. Notably, undyed PDMS ink is used to print high-aspect ratio borders around the farbricated engineered tissue constructs (Figure 3.3-3.4).

**Figure 3.2 Multimaterial 3D printer.** (a) Schematic view and (b) image of our multimaterial 3D printing apparatus which is composed of an X-Y airbearing gantry on a granite slab equipped with four independently controlled, printheads that move along the Z-axis. [Total build area is 750 mm x 650 mm]
Building on earlier work printing 3D micro-fluidic devices\textsuperscript{156}, and self-healing materials\textsuperscript{157} with embedded vasculature, we designed fugitive and cell-laden hydrogel inks to meet several important criteria. First, the inks must be compatible with one another during printing under ambient conditions. Second, the patterned cells and surrounding ECM must not be damaged during printing or the fugitive ink removal procedure. Notably, sacrificial inks, such as sugar and wax, are not suitable; as they either require elevated printing temperatures\textsuperscript{14} or harsh solvents for removal\textsuperscript{156}, and/or they lack biocompatibility. Third, the resulting vasculature must be perfusable. Finally, both printed cells and those introduced via perfusion must remain viable.

Figure 3.3 Multimaterial 3D printing of heterogeneous architectures. (a) Multimaterial 3D printing using four, independently addressable printheads. (b) Fluorescence image of 4-layer lattice printed by sequentially depositing four PDMS inks each dyed with a different fluorophore. (c) Image sequence acquired during the 4-layer printing process.
3.3.2 Ink and Matrix Rheology

To fabricate embedded vasculature, we developed an aqueous fugitive ink composed of Pluronic F-127 that can be easily printed and removed under mild conditions. We selected this triblock copolymer, in part because of its demonstrated effectiveness in printing synthetic microvascular networks \textsuperscript{3,157}. More importantly, it is biologically inert to multiple cell types over the short time periods needed to complete the fabrication process. Pluronic F-127 is composed of a hydrophobic poly(propylene oxide) (PPO) segment and two hydrophilic poly(ethylene oxide) (PEO) segments arranged in a PEO-PPO-PEO configuration (Figure 3.4a). This material undergoes thermally reversible gelation above a critical micelle concentration (CMC, \(\sim 21\) wt\%) and temperature (which decreases from approximately \(10^\circ\text{C}\) to \(4^\circ\text{C}\) as the PEO-PPO-PEO concentration increases above the CMC) \textsuperscript{157}. When both of these critical parameters are exceeded, micelles form as the hydrophilic PEO segments self-assemble into corona that are well solvated by water, while the hydrophobic PPO segments tightly associate within the micelle cores \textsuperscript{136,158,159}. However, below the gelation temperature, the hydrophobic PPO units are hydrated, such that individual PEO-PPO-PEO species become soluble in water giving rise to a gel-to-fluid transition for systems whose concentration exceeds the CMC. We exploit this behavior to produce a highly concentrated ink (40 wt\% Pluronic F127) that exhibits a strong shear-thinning response when the applied shear stress exceeds the shear yield stress \((\tau_y)\) (e.g., during printing), as well as a plateau shear elastic modulus \((G')\) that exceeds the shear loss modulus \((G'')\) when the applied shear stress is below \(\tau_y\) (e.g., after printing). We find that the ink elasticity is \(\sim 2 \times 10^4\) Pa at \(22^\circ\text{C}\). Below \(\sim 4^\circ\text{C}\), the ink liquefies and its elasticity decreases by several orders of magnitude, thereby facilitating
To create the ECM, we synthesized gelatin methacrylate (GelMA) for use as a bulk matrix and cell carrier. This material is selected due to its low-cost and abundant base material, ease of modification, and biocompatibility. GelMA is denatured collagen that is modified with photopolymerizable methacrylate (MA) groups, allowing the matrix to be covalently cross-linked by UV light after printing. Physical gelation arises from the assembly of intermolecular triple helices that possess a structure similar to collagen (Figure 3.4b). By varying the concentration, degree of methacrylation, and temperature, the shear yield stress and elastic modulus of aqueous GelMA systems can be systematically tuned. We specifically produce a concentrated ECM-like ink by dissolving 15wt/v% GelMA in cell culture media. Above approximately 25°C, the ink is a low viscosity fluid with a $G'$ value below $10^{-1}$ Pa. Upon cooling below 25°C, the ink undergoes gelation yielding a clear, viscoelastic matrix material. The ink elasticity

Figure 3.4 Schematic views of thermally reversible gelation and the corresponding shear elastic ($G'$) and loss moduli ($G''$) measured as a function of temperature. (a) Pluronic F127 fugitive, (b) pure GelMA, and (c) 10T1/2 fibroblast cell-laden GelMA inks.
increases with decreasing temperature, with $G'$ values of $\sim 10^3$ Pa and $2 \times 10^4$ Pa observed at 22°C and 4°C, which correspond to typical conditions for printing and fugitive ink removal, respectively.

We used the same aqueous GelMA system to create cell-laden inks for 3D bioprinting. Prior studies have shown that cells adhere, remodel, and migrate through GelMA due to the presence of collagen-binding motifs and matrix metal-proteinase sensitive groups $^{14,60,163}$. We find that the incorporation of a moderate concentration ($2 \times 10^6$ cells mL$^{-1}$) of fibroblast cells into the 15 wt/v% GelMA ink (Figure 3.4b-c) does not significantly alter the temperature at which gelation ensues or the ink elasticity over the temperature range of interest, i.e., 2°C to 40°C. Hence, both the pure and cell-laden GelMA inks can be printed and further processed, as needed, in the same manner.

The differences in thermally reversible gelation observed for the fugitive Pluronic F127, pure GelMA, and cell-laden GelMA inks give rise to three distinct processing windows. Between approximately 4°C and 22°C, each ink is stiff and exhibits a solid-like response, where $G' > G''$. At $T \geq 22°C$, the Pluronic F127 fugitive ink is stiff and solid-like ($G' > G''$), while the pure and cell-laden GelMA inks are liquids that flow readily. Below $\sim 4°C$, the Pluronic F127 fugitive ink is a liquid that flows readily, while the pure and cell-laden GelMA inks are stiff and solid-like ($G' > G''$).
3.3.3 Printing 1D, 2D, 3D Vascular Networks

We take advantage of this complimentary behavior to print representative 1D, 2D, and 3D vascular networks, which are subsequently embedded in a pure GelMA matrix, i.e., acellular ECM. The schematic views and corresponding images of each printed vascular network design are shown in Figure 3.5-3.6. After introducing and photocrosslinking the GelMA matrix, the fugitive ink is removed by cooling the printed constructs below 4°C yielding open channels. The process for creating a 3D vascularized

Figure 3.5 1D, 2D, and 3D vasculature. Schematic illustrations, optical images, and fluorescent images of 1D (Column 1:a-c), 2D (Column 2:d-f), and 3D (Column 3:g-i) embedded vascular networks that are printed, evacuated, and perfused with a water-soluble fluorescent dye. Bottom row: (j) Optical image of representative microchannel within a 2D vascular network perfused with a HUVEC suspension and (k) confocal image of live HUVEC cells lining the microchannel walls.
channel is shown in more detail in Figure 3.6. After this process, representative vascular networks are injected with a fluorescent red dye to aid visualization. Within each construct, the diameter of printed filaments can be altered on demand by modifying the printing pressure, speed, or nozzle height. For example, we printed 1D microchannel arrays with diameters increasing from 45 µm to 500 µm using a single 30 µm nozzle and increasing the printing pressure and nozzle height in a stepwise fashion between each printed feature (Figure 3.5 a–c). Cross-sectional images of these channels, shown in Figure 3.6 in the Supporting Information, reveal that their final diameters range from ca. 100 µm–1 mm. Since the GelMA ink has higher water content than the fugitive ink, the printed vascular features swell as water diffuses into fugitive Pluronic F127 ink from the surrounding matrix. Indeed, we find that their channel diameters nearly double in size, with a swelling ratio that is independent of initial feature size for this ink combination.

Figure 3.6 Cross-sectional views and swelling of printed channels. (a) Printed channel diameters range from 100 µm – 1 mm as observed with optical microscopy. (b) The channels diameters are measured immediately after printing and evacuation to characterize the swelling of the channels, which is ~ 2x original diameters for all channels tested.
The 2D vascular network design mimics the hierarchical, bifurcating motifs found in biological systems; large channels bifurcate to form smaller channels that maximize efficient blood flow, nutrient transport, and waste removal while minimizing the metabolic cost\textsuperscript{3,8}. These 2D hierarchical vascular networks are printed using 30 μm nozzle (Fig. 3.5d-f). The as-printed, largest channels (~650 μm in diameter) provide a single inlet and outlet for perfusion, while the smallest channels (~150 μm in diameter) reduce the characteristic diffusion distance between adjacent conduits. Finally, we printed the 3D microvascular network design shown in Fig. 3.5g-I, Fig. 3.7, which consists of a 3D periodic array of uniform microchannels. Because the embedded
microchannels are interconnected in all three dimensions, the fugitive ink can be removed from the surrounding GelMA matrix quickly and with high fidelity.

Engineered tissue constructs must be able to support the attachment and proliferation of endothelial cells, which line vascular walls providing a barrier to fluid diffusion, while simultaneously facilitating homeostatic functions and helping establish vascular niches specific to various tissues. To promote endothelialization, we injected representative 2D hierarchical bifurcating networks with HUVEC suspension followed by gentle rocking (Fig. 3.5j). After 48 h, we find that these cells retained greater than 95% viability and assembled into a nearly confluent layer, as determined by live/dead staining coupled with confocal imaging within a representative, bifurcated microchannel (Fig. 3.5k).

Figure 3.8 Various demonstrations of printed vascular network versatility. (a-b) Images of a printed 2D bifurcating vascular network before and after injecting animal blood. (c) Optical and (d) confocal images of endothelialized vascular channels embedded within a fibrin gel. The samples are prepared by staining with calcein-AM (“live”, 1 µL mL\(^{-1}\), Invitrogen) and ethidium homodimer (“dead”, 4 µL mL\(^{-1}\), Invitrogen) for 20 min.
In a separate experiment, we directly injected animal blood into the inlet of the 2D vascular network and observed that it rapidly flowed through the entire network to outlet (Figure 3.8a). To further demonstrate the versatility of our approach, we printed and encapsulated a 1D vascular network within another biologically relevant matrix, i.e., fibrin gel, and showed that HUVECs attach and proliferate in those channels as well (Figure 3.8). These initial demonstrations, while simple, illustrate the potential to create perfusable vasculature of nearly arbitrary design with dimensions akin to those found in natural tissues. While it is possible to print finer capillaries, those would ideally be generated via directed capillary growth (e.g., angiogenesis)\textsuperscript{167,168}, which could be explored in the future.

3.3.4 Printing Vascularized, Heterogeneous Tissue Constructs

We fabricated the multilayer tissue construct shown in Figure 3.9 by co-printing two inks: the fugitive Pluronic F127 ink and a cell-laden GelMA ink that contains green fluorescent protein-expressing HNDFs at a concentration of \(2 \times 10^6\) cells mL\(^{-1}\) through 200 \(\mu\)m nozzles in a predefined sequential process. We then deposited pure GelMA ink at 37°C to fully encapsulate the printed features, followed by photo-based cross-linking of the GelMA matrix. Next, the fugitive ink is liquefied and removed from the 3D construct and the evacuated channels are endothelialized, as described earlier. Using confocal microscopy, we observe the 3D tissue construct, both the green fluorescent protein expressing HNDFs in GelMA and the red-HUVECs that line the 3D vascular network.
To demonstrate patterning of multiple cell types, we printed an engineered tissue construct composed of semi-woven features printed in and out of plane (Figure 3.10). This 4-layer construct is produced in a layer-wise build sequence by co-printing four inks through 200 \( \mu \)m nozzles: PDMS, fugitive Pluronic F127 and two different cell-laden GelMA inks, followed by deposition of pure GelMA ink at 37°C to fully encapsulate the printed features, and, finally, UV photocrosslinking of the GelMA matrix. The cell-laden GelMA inks contained either GFP-HNDFs or non-fluorescent 10T1/2s at concentrations of \( 2 \times 10^6 \) cells mL\(^{-1}\). These cell types are used solely for demonstration purposes; in practice, human and animal cells would not be combined in engineered tissue constructs produced by our approach. Fig. 3.10c shows an image of the 3D structure directly after printing. After fabrication, the fugitive ink is liquefied and removed from the 3D construct. The evacuation procedure, which is identical to that used for all printed constructs described, involves placing empty syringe tips into the inlet and outlet microchannels and then suctioning out the entire vascular network under a modest Figure 3.9 Printing of heterogeneous, vascularized tissue lattice. (a) Schematic view and (b) fluorescence image of an engineered tissue construct, in which red and green filaments correspond to channels lined with RFP HUVECs and GFP HNDF-laden GelMA ink respectively. (c) Fluorescent image of engineered tissue construct after two days of culture. Cross-section view indicates endothelial cells lining lumen of microvascular networks.
vacuum (Fig 3.10f). The removal process is rapid and yields a high fidelity, interpenetrating vasculature, which is then endothelialized and perfused with cell culture media. Using microscopy, we identified the locations of the three cell types that are independently stained: GFP HNDF (green), DAPI 10T1/2 (blue), and RFP HUVEC (red) cells. The semi-woven nature of this engineered tissue construct is clearly identifiable in the composite fluorescence microscopy image taken after 2 days of culture shown in Figure 3.10g.
Figure 3.10 Multicellular, vascularized tissue constructs and viability (a,b) Schematic views of the top-down and side views of a heterogeneous engineered tissue construct, in which blue, red, and green filaments correspond to printed 10T1/2 fibroblast-laden GelMA, fugitive, and GFP HNDF-laden GelMA, inks, respectively. The gray shaded region corresponds to pure GelMA matrix that encapsulates the 3D printed construct. [Note: The red filaments are evacuated to create open microchannels, which are endothelialized with RFP HUVECs.] (c) Bright field microscopy image of the 3D printed construct, which is overlayed with the green fluorescent channel. (d) Image showing the spanning and out-of-plane nature of the 3D printed construct. (f) Image acquired during fugitive ink evacuation. (g) Composite image (top view) of the 3D printed construct acquired using three fluorescent channels: 10T1/2 fibroblasts (blue), HNDFs (green), HUVECs (red). (h) Cell-viability assay results of printed 10T ½ fibroblast-laden and HNDF-laden GelMA features compared to a control sample (200-300 mm thick) of identical composition. Asterisks indicate differences with p<0.05 obtained from student’s t-test.
3.3.5 Printed Cell Viability

Additionally, we investigated the viability of both the printed 10T1/2 fibroblast and HNDF cells in GelMA over the course of 1 week. At Day 0, the cell viability of the 10T/12 was 61%, and 70% for the HNDFs; however, it increased to 82% and 81% respectively, after 7 days (Figure 3.10h). While we find that the initial cell viability is lower compared to the control, the printed cells proliferate over time leading to similar levels of viability after 1 week in culture (Figure 3.11, Figure 3.10h).

This data suggests that our printing approach is non-destructive to both primary human fibroblasts and an immortalized mouse fibroblast line. The decreased initial viability could arise from the shear or extensional stress experienced by the cells during the printing process. Others have reported that the applied pressure, nozzle diameter, cell type, and environmental conditions influence cell viability after printing. Another important parameter is the total build time required to fabricate the
desired engineered tissue construct. We anticipate that there is a maximum time over which the cell-laden inks can be stored in the ink reservoir prior to being harmed. By implementing multinozzle printheads \(^9,10,13,154,172\), which we reported previously for high-throughput, multimaterial printing, the characteristic build times would be vastly reduced over single nozzle printing. For example, it would require 3 days to print an engineered tissue construct with a volume of \(\sim 1000 \text{ cm}^3\), comparable to a typical adult human liver \(^{125,130,155,173}\), using a single (200 mm) nozzle at typical printing speeds; however, this same volume could be printed in one hour using a 64-multinozzle array. The scalable nature of our approach is also relevant for applications such as drug screening, in which multiple small constructs could be printed in parallel within well plates (Figure 3.12).
3.4 CONCLUSIONS

In summary, we report a new approach for creating vascularized, heterogeneous tissue constructs on demand based on multimaterial 3D bioprinting. This highly scalable platform allows one to produce engineered tissue constructs in which vasculature and multiple cell types are programmably placed within extracellular matrices. Nevertheless, several deficiencies remain, including the use of a UV-curable ECM mimic, the lack of controlled perfusion, and limited functionality. In the next two chapters, we overcame these limitations and demonstrate large-scale, functional tissue fabrication.
CHAPTER 4

3D BIOPRINTING OF THICK VASCULARIZED TISSUES ON PERFUSABLE CHIPS

This chapter has been adapted from the publication:


4.1 INTRODUCTION

The ability to manufacture human tissues that replicate the essential spatial, mechano-chemical, and temporal aspects of biological tissues would enable myriad applications, including 3D cell culture, drug screening, disease modeling, and tissue repair and regeneration. Over the past decade, bioprinting has evolved from patterning cellular inks to vascularized tissues. However, due to shortcomings in biological stability, materials processing, and active perfusion, these engineered constructs have been limited to thin tissues (i.e., 1 mm) that are sustained over short time periods (i.e., 14 days). To improve their physiological relevance, we printed 3D architectures of cell-laden, vascularized tissues that exceed 1 cm in thickness and are perfused on chip for long time periods (> 6 weeks). As an important demonstration of this platform, we 3D printed parenchyma, stroma, and endothelium into a single thick tissue composed of custom extracellular matrix, human mesenchymal stem cells (hMSCs), human neonatal dermal fibroblasts (hNDFs) and embedded vasculature lined with human umbilical vein endothelial cells (HUVECs). To demonstrate vascular functionality and tissue longevity, we perfused these tissues with
growth factors to differentiate hMSCs toward an osteogenic lineage in situ. This longitudinal study of emergent biological phenomena in complex microenvironments represents a foundational step in human tissue generation.

To advance the fields of tissue and, ultimately, organ engineering, one must be able to recapitulate biological context (organized cells, vasculature, and extracellular matrix) while simultaneously enabling control over spatial (3D), chemical (growth factors) and mechanical (perfusion) cues over prolonged time periods \(^{133}\). Towards this objective, three competing methods have recently emerged. Organ decellularization \(^{183}\) offers the primary advantage of harvesting organ-specific scaffolds with intact vasculature at scale. However, prior to achieving desired tissue functionality, these scaffolds must be repopulated with cells. Achieving a heterogeneous distribution of cells in the correct spatial locations for parenchyma, stroma, and endothelium of a decellularized organ remains challenging. Alternately, naïve populations of human induced-pluripotent stem cells have been self-assembled into cell aggregates and differentiated to create brain \(^{148}\), liver \(^{184}\), and kidney \(^{185}\) organoids. While this approach leads to complex and heterogeneous tissues, organoid growth is limited by a lack of vasculature, which gives rise to necrotic regions when their size exceeds roughly 1 mm in diameter \(^{148}\). By contrast, 3D multimaterial bioprinting enables the programmed assembly of cell-laden, vascularized living tissues. To date, only thin tissues (~1 mm thick) have been created due to a lack of stable, intravascular perfusion over long time periods \(^{4,111,133}\).

In this chapter we expand our multimaterial bioprinting platform to include stem cell-laden inks, new extracellular matrix materials and directed perfusion. This new
approach enables the creation of stable, thick, and functional tissues without the use of UV-photocrosslinkers and toxic initiators. Moreover, cell viabilities close to 100% are achieved upon printing. By incorporating these tissues directly into 3D printed perfusion chips we demonstrate unprecedented control over tissue composition, architecture, and microenvironment.

4.2 EXPERIMENTAL METHODS

4.2.1 Solution Preparation

Matrix and ink precursor solutions are prepared before printing tissue engineered constructs. A 15wt/v% gelatin solution (Type A, 300 bloom from porcine skin, Sigma) is produced by warming in DPBS (1X Dulbecco’s phosphate buffered saline without calcium and magnesium) to 70°C (unless otherwise noted) and then adding gelatin powder to the solution while vigorously stirring. The gelatin is allowed to fully dissolve by stirring for 12 h at 70°C (unless otherwise noted), and the pH is then adjusted to 7.5 using 1M NaOH. The warm gelatin solution is sterile filtered and stored at 4°C in aliquots for later usage (< 3 months). Fibrinogen solution (50 mg mL\(^{-1}\)) is produced by dissolving lyophilized bovine blood plasma protein (Millipore) at 37°C in sterile DPBS without calcium and magnesium. The solution is held at 37°C (undisturbed) for 45 minutes to allow complete dissolution. The transglutaminase (TG) solution (60 mg mL\(^{-1}\)) is prepared at by dissolving lyophilized powder (Moo Glue) in DPBS without calcium and magnesium and gently mixing for 20 sec. The solution is then placed at 37°C for 20 minutes and sterile filtered before using. A 250 mM CaCl\(_2\) stock solution is prepared by dissolving CaCl\(_2\) powder (Corning) in DPBS without calcium and magnesium. To prepare stock solution of thrombin, lyophilized thrombin (Sigma Aldrich) is reconstituted
at 500 U mL$^{-1}$ using sterile DPBS, aliquotted and stored at -20°C. The thrombin aliquots are thawed immediately prior to use.

### 4.2.2 Matrix Formulations

To create gelatin-fibrin interpenetrated polymer networks (IPNs), solutions of fibrinogen, gelatin, calcium and TG are mixed together at various concentrations at 37°C. A typical final concentration is 10 mg mL$^{-1}$ fibrinogen, 7.5 wt% gelatin, 2.5 mM CaCl$_2$ and 0.2 wt% TG. For printing the large-scale tissues, we use 1 wt% TG to account for diffusion and dilution into printed cell filaments. The amount of time these components are allowed to sit before mixing with thrombin determines matrix optical clarity. This wait step, termed TG preincubation time, is typically 15-20 minutes. After TG preincubation time, the solution is rapidly mixed with thrombin at a ratio of 500:1 resulting in a final concentration of 1 U mL$^{-1}$. After mixing, the matrix must be cast within 30 sec, as irreversible, enzymatically driven polymerization of fibrinogen into fibrin gel occurs rapidly.

### 4.2.3 Ink Formulations

Several inks are created for 3D bioprinting of thick vascularized tissues. The first ink, which is used to create customized perfusion chips, is composed of a two-part silicone elastomer (SE 1700, DOW Chemical) with a 10:1 base to catalyst (by weight) that is homogenized using a mixer (2000 speed, AE-310, Thinky Corp, Japan). The silicone ink is printed within two hours of mixing with catalyst. The second ink, which is a fugitive ink used to print the vasculature, is composed of 38 wt% Pluronic F127 (Sigma) and 100 U mL$^{-1}$ thrombin in deionized, ultrafiltrated (DIUF) water. To prepare this ink, 40% stock Pluronic F127 is homogenized using a Thinky mixer until the powder
is fully dissolved, and subsequently stored at 4°C. Prior to use, a 2000 U mL⁻¹ thrombin solution is added the fugitive (Pluronic) ink at a ratio of 1:200, and homogenized using a Thinky mixer. This ink is then loaded in a syringe (EFD Inc., East Providence, RI) at 4°C and centrifuged to remove any air bubbles. Before printing, this ink is brought to room temperature.

The third ink, a cell-laden printable ink, is composed of 7.5 wt/vol% gelatin and 10 mg mL⁻¹ fibrinogen, unless otherwise noted. Notably, ink stiffness is tuned by varying the gelatin process temperature (70°C-95°C). This ink is prepared similarly to the IPN matrix, however, no TG or thrombin is used in this formulation. Crosslinking of printed ink is achieved through diffusion of thrombin and TG from the surrounding matrix after casting. To uniformly disperse cells into the ink, the fibrinogen-gelatin blend is maintained in a liquid state at 37°C and then cell suspensions with concentrations greater than 2 x 10⁶ cells mL⁻¹ are mixed via gentle pipetting. After the ink is thoroughly mixed, the ink is held at 4°C for 15 min to drive thermal gelation of the gelatin phase. Next, the ink is removed from the refrigerator and allowed to equilibrate to room temperature for at least 15 min, mounted to the 3D bioprinter and used immediately for up to 2 h.

4.2.4 Fibrinogen-fluorophore conjugation

To visualize the fibrin network in printed filaments and the casting matrix, fibrinogen was conjugated to two fluorophores. Specifically, 1g of bovine fibrinogen was dissolved in 100 ml of 50 mM borate buffer, pH 8.5 (Thermo Scientific) to form a 10 mg mL⁻¹ solution. To this solution, N-hydroxysuccinimide (NHS), conjugated with either fluorescein or rhodamine was added at a molar ratio of 10:1 dye:fibrinogen. After reacting for 2 h at room temperature, the labeled fibrinogen was separated from
unconjugated dye by dialysis using 10 kDa MWCO dialysis tubing in a 2 L bath against PBS for 3 days, changing the PBS in the bath twice daily. After dialysis was complete, the fluorescently conjugated fibrinogen was frozen at -80°C, lyophilized, and stored at -20°C before use.

4.2.4 Rheological Characterization

The ink rheology is measured using a controlled stress rheometer (DHR-3, TA Instruments, New Castle, DE) with a 40 mm diameter, 2° cone and plate geometry. The shear storage ($G'$) and loss ($G''$) moduli are measured at a frequency of 1 Hz and an oscillatory strain ($\gamma$) of 0.01. Temperature sweeps are performed using a Peltier plate over the range from -5°C to 40°C. Samples are equilibrated for 5 min before testing and for 1 min at each subsequent temperature to minimize thermal gradients throughout the sample. Time sweeps are conducted by rapidly placing a premixed solution onto the temperature-controlled Peltier plate held at 37°C or 22°C, unless otherwise noted. It is important to minimize bubble generation during mixing, because the solution rapidly gels upon casting.

4.2.5 Cell Culture and Maintenance

Human bone-marrow derived mesenchymal stem cells (hMSCs) (Rooster Bio) are cultured in Booster Media (Rooster Bio) and are not used beyond 2 passages. Green fluorescent protein-expressing human neonatal dermal fibroblast cells (GFP-HNDFs, Angio-Proteomie) are cultured in Dulbecco’s modified Eagle medium containing high glucose and sodium pyruvate (DMEM) (GlutaMAX™, Gibco) and supplemented with 10% fetal bovine serum (FBS) (Gemini Bio-Products). Primary red fluorescent protein expressing human umbilical vein endothelial cells (RFP-HUVECs) (AngioProteomie) are
cultured in EGM-2 media (complete EGM\textsuperscript{TM}-2 BulletKit\textsuperscript{TM}, Lonza). All cell cultures are passaged per the respective vendor’s instructions. GFP-HNDFs and RFP HUVECs are not used beyond the fifteenth and ninth passages, respectively.

4.2.7 3D perfusion chip fabrication

All vascularized tissues are created on a custom-designed multimaterial 3D bioprinter equipped with four independently addressable printheads mounted onto a 3-axis, motion-controlled gantry with build volume 725 mm x 650 mm x 125 mm (AGB 10000, Aerotech Inc., Pittsburgh, PA USA). Each ink is housed inside a separate syringe barrel to which nozzles of varying size (i.e., 100 µm - 410 µm diameter) are attached via a leur-lock (EFD Inc., East Providence, RI, USA). Inks are extruded through each deposition nozzle by applying air pressure (800 Ultra dispensing system, EFD Inc., East Providence, RI, USA), ranging from 10-140 psi, corresponding to print speeds from 1 mm s\textsuperscript{-1} to 5 cm s\textsuperscript{-1}. Prior to printing, the relative X-Y offsets of the four printheads are determined using orthogonally mounted optical micrometers (LS-7600 series, Keyence, Japan).

To manufacture the customized perfusion chips, the silicone ink is loaded into a 10 mL syringe, centrifuged to remove air bubbles, and deposited through a tapered 410 µm nozzle. The gasket design is created using custom MATLAB software and the structures are printed onto 50 mm x 75 mm glass slides. After printing, the chips are cured at 80°C in an oven for > 1 h and stored at room temperature.

To produce thick vascularized tissues, multiple inks are sequentially co-printed within the customized perfusion chips. To form a base layer, a thin film of gelatin-fibrin matrix, containing 0.1%wt TG, is cast onto the base of the perfusion chip and allowed to
dry. Next, the fugitive Pluronic F127 and cell-laden inks are printed onto the surface. The fugitive (Pluronic F127) and cell-laden inks are printed using 200 µm straight and tapered nozzles, respectively. After printing, stainless metal tubes are fed through the guide channels of the perfusion chip and pushed into physical contact with printed vertical pillars composed of the fugitive ink, which are positioned at the inlets and outlets. Prior to encapsulation, TG is added to the molten 37°C gelatin-fibrin matrix solution and preincubated for 2-20 min depending on the desired matrix transparency. To form a cell-laden matrix, the molten 37°C gelatin-fibrin matrix is first mixed with HNDF-GFP cells and then mixed with thrombin. Next, this matrix is immediately cast within the perfusion chip to fully encapsulate the printed tissue, where it undergoes rapid gelation due to thrombin activity. The tissues are then stored at 37°C for 1 h to allow fibrin polymerization to terminate and TG to crosslink the network. The entire structure is then cooled to 4°C to liquefy the printed fugitive ink, which is then flushed through the device using cold cell media, leaving behind open conduits that serve as the desired vascular network embedded within the printed tissue and surrounding cell-laden matrix.

The 3D perfusion chips are loaded onto a machined stainless steel base, and a thick acrylic lid is placed on top. The lid and base are clamped together by four screws, forming a seal around the silicone 3D printed gasket top. Next, sterile two-stop peristaltic tubing (PharMed BPT) is filled with media and connected to the outlet of a sterile filter that is attached to a 10 mL syringe (EFD Nordson), which serves as a media reservoir. Media that has been equilibrating for > 6 h in an incubator at 37°C, 5% CO₂ is added to the media reservoir, and by means of gravity, is allowed to flow through the filter and peristaltic tubing, until all the air is displaced, before connecting the peristaltic tubing to
the inlet of each perfusion chip. To complete the perfusion circuit, silicone tubing is connected between the outlet of the perfusion chip and an inlet at the top of the media reservoir. Hose pinch-off clamps are added at the inlet and outlet of the perfusion chip to prevent uncontrolled flow when disconnected from the peristaltic pump, which can damage the endothelium or introduce air bubbles to the vasculature. The media reservoir is allowed to equilibrate with atmospheric pressure at all times by means of a sterile filter connecting the incubator environment with the reservoir. This process is detailed in Figure 4.1.
Figure 4.1 Schematic illustration of the construction and perfusion of a thick vascularized tissue.
4.2.6 Endothelialization of Vascular Networks

With the peristaltic tubing removed from the chip outlet, 50-500 µL HUVEC suspensions (1 x 10^7 cells mL^-1) are injected via pipette to fill the vascular network. Next, the silicone tubing is replaced on the chip outlet and both the outlet and inlet pinch-clamp is sealed. The perfusion chip is incubated at 37°C to facilitate cell adhesion to the channels under zero-flow conditions. After 30 min, the chip is flipped 180° to facilitate cell adhesion to the other side of the channel, and achieve circumferential seeding of cells in the channel. Finally, the cells are further incubated for between 5 h and overnight at 37°C before commencing active perfusion.

4.2.7 Active Perfusion

After endothelial cell seeding, the peristaltic tubing is affixed to a 24-channel peristaltic pump (Ismatec), after which the inlet and outlet hose clamps are unclipped to prepare for perfusion. The peristaltic pump is then started at a desired perfusion rate. For single vascular channels, the perfusion rate was set at 13 µL min^-1, while for the thick, large-scale tissues, it was set at 27 µL min^-1.

4.2.8 Cell Viability Assay

Cell viability is determined post-printing by printing inks with 2 x 10^6 cells mL^-1 for each condition. For printed cell viability, the printed filaments are deposited onto a glass substrate and then stained using calcein-AM (“live”, 1 µL mL^-1, Invitrogen) and ethidium homodimer (“dead”, 4 µL mL^-1, Invitrogen) for 20 minutes prior to imaging via confocal microscopy (n = 3 unique samples, imaged n = 10 times). Live and dead cell
counts are obtained using the 3D objects counter plugin in ImageJ software. The results are averaged and standard deviations determined for each sample.

4.2.9 Imaging and Analysis

Macroscopic photographs and videos of printing and assembly of tissues are collected using a DSLR camera (Canon EOS, 5D Mark II, Canon Inc., USA). Fluorescent dyes are used to improve visualization of Pluronic F127 (Red, Risk Reactor) and gelatin-fibrin ink (Fluorescein, Sigma Aldrich). Various microscopes are used to visualize printed tissue structures including a Keyence Zoom (VHX-2000, Keyence, Japan), an inverted fluorescence (Axiovert 40 CFL, Zeiss), and an upright confocal microscope (LSM710, Zeiss). ImageJ was used to generate composite microscopy images by combining fluorescent channels. 3D rendering and visualization of confocal stacks is performed in Imaris 7.6.4, Bitplane Scientific Software and ImageJ software. Cell counting is performed using semi-automated native algorithms in Imaris and ImageJ counting and tracking algorithms.

4.2.10 Immunostaining

Immuno-staining and confocal microscopy are used to assess the 3D vascularized tissues. Printed tissues are first washed with phosphate buffered saline (PBS) via perfusion for several minutes. Next, 10% buffered formalin is perfused through the 3D tissue for 10 to 15 min. The tissue is removed from the perfusion chip and bathed in 10% buffered formalin. Time of fixation with formalin varied with tissue construct thickness; approximately 2 h of fixation is required for a 1 cm thick tissue. The 3D tissues are then washed in PBS for several hours and blocked overnight using 1 wt% bovine serum albumin (BSA) in PBS. Primary antibodies to the cell protein or biomarker of interest are
incubated with the constructs for 2 days at the dilutions listed in Supporting Table S1 in a solution of 0.5 wt% BSA and 0.125 wt% TritonX. Removal of unbound primary antibodies is accomplished using a wash step against a solution of PBS or 0.5 wt% BSA and 0.125 wt% TritonX in PBS for 1 day. Secondary antibodies are incubated with the constructs for 1 day at the dilutions listed in Supporting Table S1 in a solution of 0.5 wt% BSA and 0.125 wt% TritonX in PBS. Samples are counter-stained with NucBlue or ActinGreen (Life Technologies) for 2 h and then washed for 1 day in PBS prior to imaging. Confocal microscopy is performed using an upright Zeiss LSM 710 with water immersion objectives ranging from 10X to 40X employing spectral lasers at 405, 488, 514, 561, and 633 nm wavelengths. Image reconstructions of z-stacks are performed in ImageJ using the z-project function with the maximum pixel intensity setting. Three dimensional image reconstructions are performed using Imaris software.

Supporting Table 1: Summary of staining protocols and reagents

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<th>Antibody or stain</th>
<th>Source</th>
<th>Catalog #</th>
<th>Host Species &amp; Reactivity</th>
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<td>ab24590</td>
<td>Mouse anti-human</td>
<td>1:200</td>
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</tr>
</tbody>
</table>

4.2.11 hMSC Staining

Fast Blue (Sigma Aldrich) and alizarin red (SigmaFast, Sigma Aldrich) are used to visualize AP activity and calcium deposition. One tablet of Fast Blue is dissolved in 10
mL of DI water. This solution is stored in the dark and used within 2 h. Cells are washed using 0.05% Tween 20 in DPBS without calcium and magnesium and fixed as described above. The samples are then covered with Fast Blue solution and incubated in the dark for 5-10 min and washed using PBS-Tween buffer. To assess mineralization, 2% alizarin red solution is dissolved in DI water, mixed vigorously, filtered, and used within 24 hours. Samples are equilibrated in DI water and incubated with Alizarin Red solution for 2-5 minutes, then the staining solution is removed, and samples are washed 3X in DI water or until background dye is unobservable. Representative slices of both avascular and vascularized thick tissues are digested using 2 wt% Collagenase I in PBS without Ca\textsuperscript{2+}, Mg\textsuperscript{2+} at 37\degree C for > 24 h. The resulting solutions are filtered using a 0.2 \( \mu \)m sterile filter and rinsed with DI water. SEM/EDS is used to carry out elemental analysis on harvested mineral particulates.

**4.2.12 FITC-Dextran Permeability Testing**

To assess barrier function of the printed vasculature, diffusional permeability is quantified by perfusing culture media in the vascular channel, while alive, containing 25 \( \mu \)g mL\(^{-1}\) FITC-conjugated 70 kDa dextran (FITC-Dex, Sigma product 46945) at a rate of 20 \( \mu \)L min\(^{-1}\) for 3 min and 1 \( \mu \)L min\(^{-1}\) thereafter for ~33 min. The diffusion pattern of FITC-Dex is detected using a wide-field fluorescent microscope (Zeiss Axiovert 40 CFL). Fluorescence images are captured before perfusion and every 3 to 5 min after for 33 min. Diffusional permeability of FITC-Dex is calculated by quantifying changes of fluorescence intensity over time using the following equation;

\[
P_d = \frac{1}{I_1-I_b} \left( \frac{I_2-I_1}{t} \right) d
\]

where \( P_d \) is the diffusional permeability coefficient, \( I_1 \) is the average intensity at an initial
time point, \( I_2 \) is an average intensity after some time (t, \( \sim 30 \) min), \( I_b \) is background intensity (image taken before perfusion of FITC-Dex), and \( d \) is diameter of the channel. The permeability measurement was performed on two types of channel structures: (1) perfused channel with cell lining, and (2) perfused channel without cell lining (empty channel). For each type, the diffusional permeability is calculated from the measurement of three independent samples (\( n = 3 \)).

### 4.3 RESULTS AND DISCUSSION

Central to the fabrication of thick living tissues is the design of biological, sacrificial, and elastomeric inks for multimaterial 3D bioprinting. To satisfy the concomitant requirements of processability, heterogeneous integration, biocompatibility, and long-term stability, we first developed new printable cell-laden ink and castable extracellular matrix (ECM) comprising a blend of gelatin and fibrinogen. Specifically, these materials form a tough interpenetrating polymer network (IPN) composed of gelatin-fibrin cross-linked by a dual-enzyme, thrombin and TG, strategy (Figure 4.2 - 4.3).
Figure 4.2 3D vascularized tissue fabrication. (a) Schematic illustrating the tissue manufacturing process. (i) Sacrificial ink comprising pluronic and thrombin, and cell-laden inks comprising gelatin, fibrinogen, and cells are printed within a perfusion chip. (ii) Matrix material comprising gelatin, fibrinogen, cells, thrombin, and transglutaminase is cast over the printed inks. After casting, thrombin induces fibrinogen cleavage and rapid polymerization into fibrin in both the cast matrix, and via diffusion, in the printed cell ink. Similarly, transglutaminase diffuses from the molten casting matrix and slowly crosslinks the gelatin and fibrin. (iii) Upon cooling, the sacrificial ink liquefies and is evacuated, leaving behind a patent vascular network, which is (iv) endothelialized and perfused via an external pump. (b) Photographs of interpenetrated sacrificial (red) and cell inks (green) as printed on chip. [Scale bar = 2 mm] (c) Top-down bright field image of sacrificial and cell inks.[Scale bar = 50 μm]. (d) Photograph of a completed construct housed within a perfusion chamber (d) and corresponding cross sections (e-f). [Scale bars = 5 mm]

The cell-laden inks must simultaneously facilitate printing of self-supporting filamentary features under ambient conditions and subsequent infilling of the printed tissue architectures by casting without dissolving or distorting the patterned cell-laden and fugitive (vasculature) inks (Figure 4.2a). The thermally reversible gelation of the gelatin-fibrinogen network enables its use in both printing and casting, where gel and fluid states are required, respectively (Figure 4.3). Thrombin is used to rapidly polymerize fibrinogen 65, while TG is a slow-acting Ca$^{2+}$-dependent enzymatic crosslinker that
imparts mechanical and thermal stability to the gel in culture \textsuperscript{188}, enabling long term perfusion. Notably, the printed cell-laden ink does not contain either enzyme to prevent polymerization during printing. The fugitive ink also contains a high concentration of thrombin (100 U mL\textsuperscript{-1}). Hence, when it is surrounded by the ECM during the casting process, soluble fibrinogen is rapidly transformed to insoluble fibrin, templating fibrin around the lumen and facilitating the desired, long-term perfusion of cell media. Whereas the castable matrix contains both thrombin and TG, which diffuse into adjacent printed filaments forming a continuous, IPN, in which the native fibrillar structure of fibrin is preserved (Figure 4.4). Importantly, our approach allows arbitrarily thick tissues to be fabricated, since the matrix does not require UV irradiation \textsuperscript{189} (which has a low penetration depth in tissue \textsuperscript{190}) to cure, and it can be extended to other biomaterials, including fibrin and hyaluronic acid (Figure 4.5).
Figure 4.3 Structure and rheology of gelatin-fibrin matrix. (a) A schematic showing the three-step gelation of gelatin-fibrin. First, upon adding thrombin, the fibrinogen rapidly polymerizes into a fibrin gel. Second, gelatin undergoes a liquid to gel transition as the temperature decreases. Third, after an extended period of time (> 1h), the gelatin is crosslinked to the fibrin IPN by action of transglutaminase. (b) A fluorescent image of rhodamine labeled fibrinogen within a gelatin-fibrin IPN demonstrates that a dense fibrillar network of fibrin is formed upon addition of thrombin.[Scale bar = 50µm] (c) At 37°C, fibrinogen increases the solution viscosity of gelatin upon addition, yet only marginally alters the shear plateau modulus (d) of the resulting gel at room temperature. (d) Viscoelastic behavior of gelatin fibrin inks is highlighted by several orders of magnitude of shear elastic modulus. At room temperature (22°C) and below, the modulus of printable gelatin-fibrinogen inks is between $1 \times 10^4$ – $1 \times 10^5$ Pa. When the temperature is increased above 30°C the solution is rheologically suitable for cell dispersion and casting – the solution exists in the viscous state with a shear elastic modulus approaching zero. (e) The dynamic shear elastic modulus of fibrin-gelatin rapidly increases with time upon addition of thrombin, indicating fibrin network formation, while the plateau elastic modulus increases with increasing fibrin content. (f) Within a fibrin-gelatin composite, the fibrin phase of the IPN imparts more stiffness to the resulting gel.
Figure 4.4 Fibrin structure within the printed ink and matrix. (a) Printed gelatin + fibrin-fluorescein filaments are encapsulated in a gelatin + fibrin-rhodamine matrix containing thrombin and transglutaminase. [Scale bar = 250 µm] After gelation occurs, the fibrin in the (b) matrix, and (c) printed filaments polymerizes into a continuous network. [Scale bar = 50 µm] (d) Increasing the TG preincubation time before adding thrombin results in increasingly transparent matrices, and correspondingly, finer pore and filament sizes within the fibrin network. The TG concentration used in these gels is 0.2 %wt. [Scale bar = 50 µm]
The gelatin-fibrin matrix supports multiple cell types of interest to both 2D and 3D culture conditions, including human umbilical vein endothelial cells (HUVECs), human neonatal dermal fibroblasts (HNDFs), and human bone marrow-derived mesenchymal stem cells (hMSCs) (Figure 4.6 - 4.8). We find that endothelial cells express vascular endothelial-cadherin (VE-Cad) (Fig. 4.6a), and HNDFs (Fig. 4.6b) and hMSCs (Fig 3.6c) proliferate and spread on this matrix surface and in bulk. Moreover, the printed cell viability can be as high as 95% depending on how the gelatin is preprocessed. (Figure 4.7) At higher temperatures, the average molecular weight of

**Figure 4.5 Printed cells and vasculature in alternative ECMs.** (a) A co-printed pattern of gelatin-fibrinogen (fluorescent green) and fugitive ink (clear) prior to casting. Image of evacuated channels surrounded by different matrices: (b) pure-fibrin gel, (c) methacrylated hyaluronic acid, and (d) fibrin-matrigel lined with RFP-HUVECs and perfused.
gelatin is reduced (69 kDa at 70°C processing to 32 kDa at 95°C processing) resulting in softer gels with lower viscosity, shear yield stress, and shear elastic modulus (Figure 4.7 - 4.9). Cell-laden inks can be printed with ease and accommodate cell densities ranging from $0.1 \times 10^6$ cells mL$^{-1}$ to $10 \times 10^6$ cells mL$^{-1}$ (Figure 4.8, Figure 4.9). Upon printing, hMSCs within this soft gelatin-fibrinogen matrix continue to spread, proliferate, and contract into dense, cellular architectures that align along the printing direction (Figure 4.9), likely arising due to cellular confinement $^{191}$ and contraction via the Poisson-effect $^{192}$.

Figure 4.6 Gelating-Fibrin IPN Supports cell growth and proliferation. (a) HUVECs growing on top of the matrix in 2D (b) HNDFs growing inside the matrix in 3D, and (c) hMSCs growing on top of the matrix in 2D. [Scale bar = 50 µm]
Figure 4.7 Processing temperature effects on gelatin printability and cell viability. (a) Gelatin processing temperature affects the plateau modulus and cell viability after printing. Higher temperatures lead to lower modulus and higher HNDF viability post-printing. (b-c) Images of printed hMSC-laden ink prepared using 95°C processed gelatin (b) as printed and (c) after 7 days in the 3D printed filament where actin (green) and nuclei (blue) are stained. [Scale bar = 1 mm]

<table>
<thead>
<tr>
<th>Matrix material tested with HUVECs spread on top in 2D</th>
<th>Area per cell (1000-2000 sq µm)</th>
<th>Area per cell (2000-3500 sq µm)</th>
<th>Area per cell (3500-5500 sq µm)</th>
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<tr>
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<td></td>
<td>X</td>
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<tr>
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<td>X</td>
<td></td>
<td></td>
</tr>
<tr>
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<tr>
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Figure 4.8 HUVEC spreading on various matrix materials. (a) A table showing the adhesion behavior and relative cell spreading on various matrix formulations. Fluorescent micrographs of RFP-HUVECs show examples of (b) low, medium, and high levels of cell spreading on gels. Fibrinogen is gelled at pH 7 to generate an opaque fibrin gel, and at pH 7.5 to generate a transparent or “clear” fibrin gel. pH is adjusted using 1M NaOH. [Scale bar = 50 µm]
Figure 4.9 Gelatin processing temperature improves cell viability and printability. Dissolving gelatin at higher temperatures for 12 h results in gelatin-fibrinogen inks that exhibit lower (a) plateau shear elastic modulus and (b) shear yield stress. Printed cell-laden filaments are (c) less uniform when gelatin is solubilized at 70°C compared to (d) those produced by gelatin solubilized at 95°C. Correspondingly, live-dead assays demonstrate that printed fibroblast viability is (e) significantly lower in stiffer, 70°C solubilized gelatin than in (f) 95°C solubilized gelatin. hMSC-cell laden inks of varying cell densities: (g) 0.1M mL$^{-1}$, (h) 1M mL$^{-1}$, and (i) 10M mL$^{-1}$ imaged immediately after printing, in which a live/dead assay is carried out using calcein (live) and ethidium homodimer (dead). [Scale bars = 250µm]
To construct thick living tissues within perfusion chips, we co-printed cell-laden, fugitive, and silicone inks (Figure 4.2b-f). First, the silicone ink is printed on a glass substrate and cured to create customized perfusion chips (Figure 4.1). Next, the cell-laden and fugitive inks are printed on chip, and then encapsulated in the castable ECM matrix (Figure 4.1, Figure 4.2). The fugitive ink, which defines the vascular network, is composed of a triblock copolymer (i.e., polyethylene oxide (PEO)-polypropylene oxide (PPO)-PEO). This ink can be removed from the fabricated tissue upon cooling to roughly 4°C, where it undergoes a gel-to-fluid transition\textsuperscript{157,182}. This process yields a pervasive network of open channels, which are then lined with HUVECs. The vasculature is then perfused over long time periods using an external pump that generates a smooth flow over a wide range of flow rates\textsuperscript{193}.

To demonstrate the formation of stable vasculature, we printed a simple tissue construct composed of two parallel channels embedded within a fibroblast cell-laden matrix (Figure 4.10). The channels are lined with HUVECs and subsequently perfused with endothelial growth media to form a confluent monolayer that lines each blood vessel (Fig. 4.10a). Importantly, after six weeks of active perfusion, these endothelial cells maintain endothelial phenotype and remain confluent, characterized by expression of CD31, von-Willebrand Factor (vWF), and vascular endothelial cadherin (VECad) (Fig. 4.10b-c). The cross-sectional view of a representative vessel reveals lumen formation (Fig. 4.10d). Confirming the barrier function of the endothelium, we measured a five-fold reduction in the diffusional permeability compared to unlined (bare) channels (Figure 4.10e, Figure 4.11). Stromal HNDFs residing within the surrounding matrix exhibit cell spreading and proliferative phenotypes localized to regions within ~1 mm of
the vasculature (Figure 4.10f, Figure 4.12); cells further away from these regions become quiescent likely due to an insufficient nutrient supply. As cell density increases, their viability rapidly decreases at distances beyond 1 mm from the embedded blood vessels (e.g., only 5% of the cells remain viable at 7 mm). Clearly, the perfusable vasculature is critical to support living tissues thicker than 1 mm over long time periods.

Figure 4.10 3D vascularized tissues remain stable during long-term perfusion. (a) Schematic depicting a single HUVEC-lined vascular channel supporting a fibroblast cell-laden matrix and housed within a perfusion chip. (b-c) Confocal microscopy image of the vascular network after 42 days, CD-31 (red), vWF(blue), and VE-Cadherin (magenta). [Scale bars = 100 µm]. (d) Long-term perfusion of HUVEC-lined (red) vascular network supporting HNDF-laden (green) matrix shown by top-down (left) and cross-sectional confocal microscopy at 45 days (right). [Scale bar = 100 µm]. (e) Quantification of barrier properties imparted by endothelial lining of channels, demonstrated by reduced diffusional permeability of FITC-dextran. (f) GFP-HNDF distribution within the 3D matrix shown by fluorescent intensity as a function of distance from vasculature.
Figure 4.11 Barrier properties of embedded vasculature. (a) FITC-labeled Dextran (70 kDa) is perfused through channels with, and without a lining of HUVECs. The fluorescence signal is recorded every 5 minutes up to 30 minutes to measure the degree of diffusion into the gel. (b) Calculated diffusional permeability with and without the cell lining. (c) FITC-dextran fluorescence signatures with cell lining demonstrate the slow spreading of the fluorescence signal with time. [Scale bar = 500 µm]
To explore emergent phenomena in complex microenvironments, we created a heterogeneous tissue architecture (>1 cm thick and 10 cm³ in volume) by printing a hMSC ink into a 3D lattice geometry along with an interpenetrating, branched vascular network lined with HUVECs, and infilled with an HNDF-laden extracellular matrix.

Figure 4.12 Fibroblast cell proliferation and viability within vascularized tissues. (a) A GFP-expressing fibroblast laden gelatin-fibrin hydrogel (50K cell mL⁻¹) is perfused through four embedded channels lined with RFP-expressing HUVECs. The cell density, as measured by GFP levels, is highest near the channels, and decreases in the central region far from the channels. [Scale bar = 500 µm] Viability, proliferation, and morphology of fibroblast-laden tissues (b-d) (500K cell mL⁻¹) are assessed during perfusion over a two-week period using calcein (live) and ethidium homodimer (dead). At Day 0 (b) cells are uniformly distributed and highly viable. At Day 14 (c) cells within 1 mm of vascular channel retain high viability forming a dense, interconnected network, while cells beyond this region exhibit a density-dependent viability and morphology. (d) Cell viability as a function of distance from the vascular channel for day 0 and day 14. [Scale bar = 500 µm]
uniformly distributed within the interstitial space (Figure 4.13a). The fibroblast-laden matrix forms a connective tissue that both supports and binds the printed stem cell-laden and vascular features. In this example, fibroblasts serve as a model extracellular support cell that homogeneously surrounds the heterogeneously patterned cells and vascular networks. However, those cells could easily be replaced with myriad support cells (including immune cells, or pericytes) or tissue specific cells (hepatocytes, neurons, or islets) for future embodiments. The embedded vascular network is designed with a single inlet and outlet that provides an interface between the printed tissue and the perfusion chip. This network is symmetrically branched to ensure uniform perfusion throughout the tissue, including deep within its core. In addition to providing transport of nutrients, oxygen, and waste materials, the perfused vasculature is used to deliver specific differentiation factors to the tissue in a more uniform manner than bulk delivery methods, in which cells at the core of the tissue are starved of factors. This versatile platform (Figure 4.13) is used to precisely control growth and differentiation of the printed hMSCs. Moreover, both the printed cellular architecture and embedded vascular network are visible macroscopically with this thick tissue (Fig. 4.13b-c).
Figure 4.13 Osteogenic differentiation of thick vascularized tissue. (a) Schematic depicting the geometry of the printed heterogeneous tissue within the customized perfusion chip, wherein the branched vascular architecture pervades hMSCs that are printed into a lattice architecture, and HNDFs are cast to fill the interstitial space. (b) Photographs of a printed tissue construct within and removed from the customized perfusion chip, from the side. (c) Confocal microscopy image through a cross-section of 1 cm thick vascularized osteogenic tissue construct after 30 days of active perfusion and in situ differentiation. [Scale bar = 1.5 mm] (d) Osteocalcin intensity across the thick tissue sample inside the red lines shown in (c). (e) High resolution image showing osteocalcin (purple) localized within hMSCs and they appear to take on symmetric osteoblast-like morphologies. [Scale bar = 100 µm] After 30 days, (f-g) thick tissue constructs are stained for collagen-I (yellow), which appears localized near hMSCs. [Scale bars = 200 µm] (h) Alizarin red is used to stain CaP deposition and fast blue is used to stain alkaline phosphatase, indicating tissue maturation and differentiation over time. [Scale bar = 200 µm]
To develop a dense osteogenic tissue, we transvascularly delivered growth media to the tissue during an initial proliferation phase (6 days) followed by an osteogenic differentiation cocktail that is perfused for several weeks. Our optimized cocktail is composed of BMP-2, ascorbic acid, and glycerophosphate, to promote mineral deposition and alkaline phosphatase (AP) expression (Figure 4.14)\textsuperscript{194}. To assess tissue maturation, changes in cell function and matrix composition are observed over time. In good agreement with prior studies \textsuperscript{191}, we find that AP expression in hMSCs occurs within 3 days, while mineral deposition does not become noticeable until 14 days, which coincides with visible collagen-I deposition by hMSCs (Figure 4.15)\textsuperscript{191}. Figure 4.13c shows an avascular tissue with comparable hMSC density, in which positive alizarin stains are only observed near its surface (within a few hundred microns). By contrast, the thick vascularized tissue stains positive in hMSC regions deep within its core after 30 days of osteogenic differentiation via perfusion. We characterized the mineral deposits, which consist of particulates \textasciitilde20-200 nm in size, using SEM/EDS analysis. Calcium and phosphorous peaks are only observed for vascularized tissues, not the avascular control (Figure 4.16). The phenotype of hMSCs varies across the printed features: compacted, and associated with high mineralization, while those in the periphery are more elongated, encapsulating the printed filaments, and exhibit less mineralization. We observe that subpopulations of HNDFs and hMSCs migrate from their initial patterned geometry towards the vascular networks and wrap circumferentially around the channel (Figure 4.13d). After 30 days, the printed hMSCs express osteocalcin within the tissue, and osteocalcin expression is proportional to distance from the nearest vessel (Figure 4.13e). Further, we
find that collagen deposition is localized within printed filaments and around the circumference of the vasculature (Figure 4.13f-h and Figure 4.15).

**Figure 4.14. Optimization of the osteogenic cocktail for differentiating hMSCs to osteocytes.** (a) hMSC’s are cultured in a polystyrene well in the presence of different media conditions. At day 14, the cells are stained with fast blue and alizarin red to visualize osteocytes and deposited minerals, respectively. [Scale bar = 2 mm] (b) A graph depicting average absorbance at 500 nm at 9 different locations within each well. 500nm absorbance is a measure of the amount of alizarin red in the wells. Base media = Rooster Media. Growth media = Rooster media + GTX media booster. Osteo supplement = 10 mM beta-glycerophosphate and 50 µg mL⁻¹ L-ascorbic acid. BMP-2 concentration = 100 ng mL⁻¹
Figure 4.15. Maturation of thick vascularized tissues differentiated toward an osteogenic lineage in situ. Thick vascularized tissue is analyzed at various time points to visualize maturation. (a) Collagen-I (pink) deposition is not present in acellular scaffolds and is instead secreted by the cells in the tissue, after 3 days there is very little collagen in regions near hMSCs and HNDFs, however, by day 30, printed hMSCs have produced significant collagen in both filaments and circumferentially around the vascular channels. [Scale bars = 100 µm] (b) The delivery of an osteogenic cocktail through the vascular network has lead to first the osteogenic lineage commitment of hMSCs and then the deposition of mineral over the course of 30 days. This is evidenced by appearance of alkaline phosphatase expression, which is observable via fast blue, and subsequent CaP mineralization – observable via alizarin red stain. [Scale bars = 100 µm]
In summary, thick, vascularized human tissues with programmable cellular heterogeneity that are capable of long-term (> 30 days) perfusion on chip have been fabricated by multimaterial bioprinting. The ability to recapitulate physiologically relevant, 3D tissue...
microenvironments enables the exploration of emergent biological phenomena, as demonstrated by our observations of the *in-situ* development of hMSCs within tissues containing a pervasive, perfusable, endothelialized vascular network. Our 3D tissue manufacturing platform opens new avenues for fabricating and investigating human tissues for both ex vivo and in vivo applications.

Not only is this approach suitable for creating vascular networks, but other tubular architectures, such as the lymphatics, intestine, bile ducts, and nephrons can be integrated within printed human tissue constructs, which opens up new avenues for creating physiologically relevant *in vitro* models, and, ultimately, tissue repair and regeneration.
CHAPTER 5

FUNCTIONAL 3D RENAL PROXIMAL TUBULES VIA BIOPRINTING

This chapter has been adapted from the publication:

5.1 INTRODUCTION

Engineering human tissues, and ultimately organs, that recapitulate native function for use in drug screening, disease modeling, and regenerative medicine is a grand challenge. The kidney is arguably the most important organ of interest, as more than 100,000 people are waiting for kidney transplantation in the U.S. alone. Moreover, incidence rates of chronic and acute kidney injury are spiking due to increased use of prescription drugs, further accelerating the demand for replacement organs. Notably, up to 25% of acute renal failure observed in the clinic is drug induced. However, the prediction of nephrotoxicity in preclinical studies is difficult. Renal toxicity accounts for only 2% of failures in preclinical drug testing, yet it is responsible for nearly 20% of failures in Phase III clinical trials. There is a critical need for improved kidney tissue models that can both predict human drug toxicity in longitudinal preclinical testing and serve as a modular building block for engineering human nephrons and, ultimately, kidneys.

While renal injury can occur in many locations, including the renal vascular network, glomerulus, tubulointerstitium, and collecting ducts, the convoluted proximal
tubule (PT) is the site most frequently damaged. The PT is responsible for 65-80% of nutrient absorption and transport from the renal filtrate to the blood. Circulating drugs and their metabolites often accumulate in the PT at high concentrations in both intra- and intercellular spaces. Hence, an ideal in vitro PT model would recapitulate in vivo functional transporter activity specific to nutrients and drugs of interest, enabling mechanistic studies of their effects. Unfortunately, proximal tubule cells grown in traditional 2D cell culture lack significant transporter expression and cell polarity, hindering accurate predictions of in vivo nephrotoxicity. In vitro models that restore the in vivo phenotype and function of proximal tubule cells could lead to more predictive nephrotoxicity models.

Towards this objective, several kidney PT models have been developed. In 2D, proximal tubule cells have been cultured on biomimetic basement membrane coatings or on hollow fibers, improving their proliferation and ability to self-organize and maintain a differentiated state. In 3D microenvironments, proximal tubule cells have been shown to assemble into 3D structures within thin gels and maintain a differentiated state. More recently, researchers have begun to elucidate the cues required to drive stem cells towards renal lineages enabling the self-assembly of 3D kidney organoids. While their cellular heterogeneity is exceptional, the organoid-laden gels are currently limited to millimeter size scales and cannot be perfused due to a lack of addressable tubules and vasculature. In fact, to date, perfusion has only been achieved on planar proximal tubules in kidney-on-a-chip devices. Notably, the shear stress in that environment significantly enhances the differentiated state of the proximal tubule cells as well as their response to nephrotoxic drugs. However, these existing models lack many
characteristics of a truly biomimetic PT model including 3D convolution, open luminal architecture, perfusion at physiological shear stresses, and longevity.²⁰³ ²¹⁰

In this chapter, we describe a method for fabricating 3D PT constructs composed of perfusable open lumens in a programmable, convoluted architecture that can support cellular heterogeneity. Specifically, we construct a 3D open lumen architecture circumscribed by proximal tubule epithelial cells (PTECs) embedded within the same extracellular matrix on tissue chip that can be perfused to mimic physiological shear stresses. PTECs form a confluent, leak-tight epithelial monolayer that exhibits primary cilia and expresses Na⁺/K⁺ ATPase, Aquaporin 1 (AQP1), and K cadherin. The unique combination of their 3D geometry and perfusable nature allows for controlled shear stress conditions, which give rise to a more differentiated, polarized PTEC phenotype that develops an enhanced brush border, basement membrane protein deposition, basolateral interdigitations, enhanced cell height, megalin expression, and albumin uptake relative to 2D controls. Modes of PT damage due to the nephrotoxin cyclosporine A are analyzed by direct imaging of actin reorganization in the PTEC monolayer and quantified by diffusional permeability measurements of the PTEC barrier. To our knowledge, this is the first demonstration of 3D bioprinted convoluted proximal tubules with an addressable open lumen that can be maintained longitudinally over 60 days.
5.2 EXPERIMENTAL METHODS

5.2.1 Extracellular Matrix Preparation and Rheology

The extracellular matrix (ECM) is composed of a network of gelatin and fibrin. To prepare the ECM components for casting, first a 15 wt/v% gelatin solution (Type A, 300 bloom from porcine skin, Sigma) is produced by adding gelatin powder to a warm solution (70°C) of DPBS (1X Dulbecco’s phosphate buffered saline without calcium and magnesium). The gelatin is allowed to fully dissolve by stirring for 12 h at 70°C, and the pH is then adjusted to 7.5 using 1 M NaOH. The solution is sterile filtered and stored at 4°C in aliquots for later usage (< 3 months). A fibrinogen solution (50 mg/mL) is produced by dissolving lyophilized bovine blood plasma protein (Millipore) at 37°C in sterile DPBS without calcium and magnesium. The solution is held at 37°C without agitation for at least 45 min to allow complete dissolution. The transglutaminase (TG) solution (60 mg/mL) is prepared by dissolving lyophilized powder (Moo Gloo) in DPBS without calcium and magnesium and gently mixing or vortexing for 20 sec. The solution is then placed at 37°C for 20 min and sterile filtered before using. A CaCl$_2$ stock solution (250 mM) is prepared by dissolving CaCl$_2$ powder in DPBS without calcium and magnesium (Corning). To prepare stock solution of thrombin, lyophilized thrombin (Sigma Aldrich) is reconstituted at 500 U mL$^{-1}$ using sterile DPBS, aliquotted and stored at -20°C. Thrombin aliquots are thawed immediately prior to use.

A controlled stress rheometer (DHR-3, TA Instruments, New Castle, DE) with a 40 mm diameter, 2° cone and plate geometry is used for ink rheology measurements. The shear storage ($G'$) and loss ($G''$) moduli are measured at a frequency of 1 Hz and an oscillatory strain ($\gamma$) of 0.01. Time sweeps are conducted by rapidly placing a premixed
ECM solution (with thrombin, note ECM formulation is given in the bioprinting section below) onto the Peltier plate held at 37°C. It is important to minimize bubble generation during mixing, because the solution rapidly gels upon casting.

5.2.2 Ink Formulations

Two inks are required for 3D bioprinting of perfusible PT models. The first ink, which is used to create the perfusion chip gasket, is composed of a two-part silicone elastomer (SE 1700, DOW Chemical) with a 10:1 base to catalyst (by weight) that is homogenized using a centrifugal mixer for 2 min (2000 rpm, AE-310, Thinky Corp, Japan). The silicone ink is printed within 2 h of mixing with catalyst. This ink is loaded in a syringe (EFD Inc., East Providence, RI) and centrifuged to remove any air bubbles before printing at room temperature. The second ink, a fugitive ink used to print the tubule, is composed of 38 wt% Pluronic F127 (Sigma) and 100 U/mL thrombin in deionized, ultrafiltrated (DIUF) water. The fugitive ink is dyed pink through the addition of a Risk Reactor dye for visualization in. To prepare this ink, a 40 wt% Pluronic F127 solution in water is homogenized using a Thinky mixer until the powder is fully dissolved, and subsequently stored at 4°C. Prior to use, a 2000 U/mL thrombin solution is added to the fugitive (Pluronic) ink at a ratio of 1:20, and homogenized using a Thinky mixer. The fugitive ink is then loaded in a syringe (EFD Inc., East Providence, RI) at 4°C and centrifuged to remove any air bubbles. Before printing, this ink is equilibrated at room temperature for at least 15 min.

5.2.3 Bioprinting of 3D Perfusable Proximal Tubule Constructs

3D PT constructs are fabricated using a custom-designed, multimaterial 3D bioprinter equipped with four independently addressable printheads mounted onto a 3-
axis, motion-controlled gantry with build volume 725 mm x 650 mm x 125 mm (AGB 10000, Aerotech Inc., Pittsburgh, PA USA). Inks are housed in separate syringe barrels to which nozzles of varying size (i.e., 50 µm - 410 µm diameter) are attached via a luer-lock (EFD Inc., East Providence, RI, USA). Inks are extruded through deposition nozzles by applying air pressure (800 Ultra dispensing system, EFD Inc., East Providence, RI, USA), ranging from 10-90 psi, corresponding to print speeds between 1 mm/s and 5 cm/s.

To create the customized perfusion chip gasket, the silicone ink is deposited through a tapered 410 µm nozzle onto 50 mm x 75 mm glass slides. The gasket design is created using a custom MATLAB script which generates G-code for a final gasket structure. After printing, the perfusion chip gasket is cured at 80°C in an oven for > 1 hour and stored at room temperature prior to use.

Printing 3D PTs into the perfusion chip gasket requires a combination of casting the ECM and printing the fugitive ink (Figure 5.1). First, a base layer of ECM on the bottom of the perfusion chip gasket is formed. To create this ECM base layer, the ECM solutions described above are first mixed to obtain a typical final concentration of 10 mg/mL fibrinogen, 7.5 wt% gelatin, 2.5 mM CaCl₂ and 0.2 wt% TG. These components are held at 37°C for 15-20 min before use (this preincubation step with TG improves optical clarity of the final ECM [21]). After TG preincubation time, the solution is rapidly mixed with thrombin at a ratio of 500:1, resulting in a final thrombin concentration of 1 U/mL. Directly after rapid mixing via pipette, the matrix must be cast (typically within 30 sec) onto the base of the perfusion chip gasket. Within two minutes at 37°C, an irreversible, thrombin driven polymerization of fibrinogen into fibrin gel occurs. The
base layer is then allowed to dry slightly under nitrogen, such that it forms a flat surface on which to bioprint. Next, the fugitive Pluronic F127 ink (with 100 U mL$^{-1}$ thrombin) is printed in a convoluted path on the surface of the ECM using a tapered 200 µm nozzle. A custom Python script, employing MeCode, is used to convert custom convoluted geometries into tool paths in G-code for fugitive ink printing. Directly after fugitive ink printing, metal hollow perfusion pins interfaced through the silicone gasket are brought into contact with the fugitive ink. A top layer of ECM (composed and TG preincubated like the base layer) is then cast over the printed fugitive ink using a similar process to that described above. If cells, such as HNDFs, are incorporated in the ECM, they are mixed in directly after the TG preincubation time, prior to thrombin mixing and subsequent casting. After the top ECM layer is cast, the construct is held at 37°C for 1 h to allow fibrin polymerization to terminate and TG to crosslink the network. The construct is then cooled to 4°C for 15-20 min to liquefy the printed fugitive ink, which is flushed out of the device using cold cell media, leaving behind open conduits that serve as the desired tubular network embedded within the ECM with or without cells in the extratubular ECM space.
Figure 5.1 3D proximal tubule maturation process. (a) A photo of a mature (fully confluent) tubule, (b) PTEC loading at Day 0, scale bar = 500 µm, (c) higher magnification view of PTEC loading, scale bar = 300 µm, (d) PTECs adhering to the tubule at Day 1 after non-adherent cells are flushed away, scale bar = 200 µm, (e) low magnification view of PTECs growing into the tubule at Day 2, scale bar = 500 µm, (f) image at Day 4 where cells grow from colonies or clusters, scale bar = 100 µm, (g) image at Day 4 where cells are near confluence, scale bar = 100 µm, (h) image of a mature tubule at Day 38, scale bar = 500 µm, (i) higher magnification view of the confluent tubule at Day 38, scale bar = 100 µm, (j) image of the tubule, which approaches within 350 µm of itself due to its convoluted architecture, scale bar = 100 µm.
Each 3D PT construct is then placed onto a machined stainless steel base, and a thick acrylic lid is placed on top. The lid and base are clamped together by four screws, forming a seal around the printed silicone gasket. Next, sterile two-stop peristaltic tubing (PharMed BPT, 0.25 mm internal diameter) is filled with media and connected to the outlet of a sterile filter that is attached to a 10 ml syringe barrel (EFD Nordson), which serves as a media reservoir. PTEC media (designed for grown, so ATCC formulation plus 1% FBS, 1% aprotinin, and 1% Gibco antibiotic/antimycotic) that has been equilibrating for > 3 h in an incubator at 37°C, 5% CO₂ is added to the media reservoir, and tubing from the reservoir is connected to the outlet of the chip (metal hollow perfusion pin). To complete the perfusion circuit, silicone tubing from the reservoir is connected to the inlet metal perfusion pin on the chip. Hose pinch-off clamps are added at the inlet and outlet of the perfusion chip to prevent uncontrolled flow when disconnected from the peristaltic pump, which can damage the epithelium or introduce air bubbles into the system. The media reservoir is equilibrated with atmospheric conditions in the incubator at all times by means of a sterile filter on top of the media reservoir.

### 5.2.4 Cell Culture and Maintenance

Human immortalized PTECs (RPTEC/TERT1, ATCC CRL-4031) are cultured per ATCC’s instructions and are used for all PT model studies up to passage 20. For gene expression analysis, human primary RPTEC (Cell Science), immortalized PTECs (RPTEC-TERT1, Evercyte) and A498 (ATCC HTB-44) renal cancer cells are used and cultured per supplier’s instructions. Human neonatal dermal fibroblasts (HNDF), GFP expressing (Angio-Proteomie) are cultured per supplier’s instructions and used up to passage 15.
5.2.5 Gene Expression Analysis

Human primary RPTEC (Cell Science), immortalized RPTEC-TERT1 (Evercyte) and A498 (ATCC HTB-44) renal cancer cells are grown in 96-well plates according to supplier’s instructions and collected at Day 3 post-confluency by replacing culture medium with 100 ml per well of 1x RNA lysis mixture (QuantiGene® Sample Processing Kit, QS0101). Then 40 mL of lysate is mixed with an mRNA-capture magnetic bead set (Panomics QuantiGene® Plex Set 12631, catalog number 312631), incubated overnight, processed for branched DNA amplification, and analyzed according to the manufacturer’s instructions (Panomics QuantiGene® Plex Assay kit, QP1015). The PPIB probe is used as a housekeeping gene for normalization. Fluorescence Intensity (FI) data are presented as average and standard deviation of 3 biological replicates.

5.2.6 Epithelialization and Longitudinal Culture

Once each 3D PT construct is perfused for at least several hours with PTEC media in the incubator, it is ready for cell loading/seeding. PTECs (PTEC/TERT1, ATCC) are trypsinized from their culture dish and concentrated in media to ~2x10⁷ cells mL⁻¹. The cell suspension is then loaded into the perfusion chip through the outlet (Figure 5.1). The loaded construct is placed laterally in the incubator for several hours and then flipped 180° and incubated in the tubule with no flow overnight. The next day, non-adherent cells are flushed out of the tubule under flow by gravity. Perfusion of fresh media is then started and the remaining cells begin to cluster and then grow from those colonies (Fig. 5.1f) until they reach confluency at around 3 weeks post seeding. During the growth phase, PTECs are fed PTEC media prepared per ATCC guidelines plus 1% aprotinin (EMD Millipore, used to slow down the degradation of the ECM), 1% fetal
bovine serum (FBS), and 1% antibiotic-antimycotic. After maturation, FBS is removed, and PTECs packed into a tight epithelial monolayer. At Day 1 post-seeding, the PTECs were exposed to continuous, unidirectional flow at 1 µl min⁻¹, equating to shear stresses that varied between 0.01 and 0.5 dynes cm⁻² depending on the tubule cross section. Media was fed via a peristaltic pump in a closed loop circuit and changed every 2 days.

5.2.7 Albumin Uptake Study

Albumin uptake was assessed in three conditions: 2D with PTECs grown on tissue culture plastic, 2D with PTECs grown on ECM, and 3D bioprinted and perfused. For each case, PTECs are grown to confluency and allowed to mature in serum free media. Human serum albumin conjugated with FITC (HSA-FITC, Abcam ab8030) is suspended in PTEC media at 50 µg/mL. All samples are incubated with HSA-FITC in their media for 2 h (in the case of perfusion, it is perfused through the open lumen). After exposure, all samples are washed with 3x volume and then trypsinized with 10x trypsin to collect the individual cells. Cells are fixed and counterstained with primary and secondary antibodies for megalin (see Table S1 for the specific antibodies used). Cells from those samples, and naked cells, are analyzed by flow cytometry (BD LSR Fortessa) and data is collected from n = 10,000 cells per sample. To obtain images of HSA-FITC and megalin in PTECs, samples are fixed in place with formalin instead of being trypsinized after the wash step. Those samples are counterstained for megalin and imaged using confocal microscopy (Zeiss LSM710).
5.2.8 Cyclosporine-A Testing

The toxic effect of CysA on PTECs in 2D and 3D is explored. In 2D, cells are seeded in a 96-well format on tissue culture plastic and grown to confluency. They are fed media per ATCC’s guidelines. CysA (Sigma-Aldrich, SML1018) is suspended in their media at various concentrations and incubated with cells for 24 h. A viability assay using (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) in the presence of phenazine methosulfate (MTS) is run 24 h post exposure. This assay is completed many separate times: PTECs at early confluency, giving CysA the day they reached confluency, and late confluency, giving CysA at least 10 days after they reached confluency. In all cases the toxicity results are similar. For the 3D case, CysA is fed at various concentrations through the open lumen of mature tubules (i.e. reached confluency, past ~ 3 weeks, no serum included in the media for a minimum of 10 days). At 24 h post CysA exposure, a FITC-dextran leak test (described below) is
performed to assess and quantify perturbations to the barrier function of PTECs. Directly following, the PT is fixed using 10% buffered formalin for 1 h and counterstained for actin and DAPI (see Table S1 for specific stains).

### 5.2.9 FITC-Dextran Permeability Testing

To assess barrier function of the epithelium in 3D, diffusional permeability is quantified by perfusing PTEC media in the open lumen containing 25 µg/mL FITC-conjugated 70 kDa dextran (FITC-Dex, Sigma product 46945) at a rate of 15 µL/min for 3 min and 1 µL/min thereafter for ~30-45 min. The entire test is performed under live cell imaging with both the tubule and the surrounding ECM in the field of view (Figure 5.2). The diffusion pattern of FITC-Dex is detected using a wide-field fluorescent microscope (Zeiss Axiovert 40 CFL). Fluorescence images are captured before perfusion and every 3 to 5 min over a 30-45 min period. Diffusional permeability of FITC-Dex is calculated by quantifying changes in fluorescence intensity over time using the following equation:

\[
P_d = \frac{1}{I_1 - I_b} \left( \frac{I_2 - I_1}{t} \right) \frac{d}{4}
\]

\(P_d\) is the diffusional permeability coefficient, \(I_1\) is the average intensity at an initial time point, \(I_2\) is an average intensity at \(t \sim 30-45\) min, \(I_b\) is background intensity (image taken before perfusion of FITC-Dex), and \(d\) is the diameter of the channel.
Figure 5.2 Diffusional permeability measurements. FITC-labeled dextran (70 kDa) solution is perfused through the 3D PT lined with confluent PTECs and fluorescent images are captured at varying times: (a) $t = 0$ min, (b) $t = 45$ min, and (c, d) $t = 0$ min and 5 min, respectively, for control samples composed of a bare 3D PT (without PTECs), in which the FITC-labeled dextran diffuses much faster into the surrounding ECM, scale bars = 200 µm. (e) Measured diffusional permeability of 3D PT channels with and without proximal tubule epithelium.
5.2.10 Electron Microscopy

For transmission electron microscopy (TEM), PTECs in 2D or 3D architectures are fixed using 2.5% glutaraldehyde, 1.25% paraformaldehyde, and 0.03% picric acid in 0.1 M sodium cacodylate buffer (pH 7.4) for a minimum of several hours. Small samples (1 mm x 1 mm) are removed and washed in 0.1 M cacodylate buffer and bathed in 1% osmium tetroxide (OsO₄) (EMS) and 1.5% potassiumferrocyanide (KFeCN₆) (Sigma) for 1 h, washed in water 3x and incubated in 1% aqueous uranyl acetate (EMS) for 1 h followed by 2 washes in water and subsequent dehydration in varying grades of alcohol (10 min each; 50%, 70%, 90%, 2x10 min 100%). The samples are then put in propylene oxide (EMS) for 1 h and incubated overnight in a 1:1 mixture of propylene oxide and TAAB Epon (Marivac Canada Inc. St. Laurent, Canada). The following day the samples are embedded in TAAB Epon and polymerized at 60°C for 48 h. Ultrathin sections (about 60 nm) are cut on a Reichert Ultracut-S microtome, placed on copper grids stained with lead citrate and examined in a JEOL 1200EX Transmission electron microscope and images are recorded with an AMT 2k CCD camera. Image analysis is performed using ImageJ software.

For scanning electron microscopy (SEM), perfused PTECs in 3D are fixed using 10% buffered formalin for 1 h. The samples are thinly sliced (~1 mm thick) to expose cells circumscribing the open lumen. The fixative is washed away using PBSx2 and subsequent dehydration in varying grades of ethanol (20 min each; 30%, 50%, 70%, 90%, 3x20 min 100%). The samples are then placed in 50% ethanol and 50% hexamethyldisilazane (HMDS) for 30 min followed by 100% HMDS 3x30 min. All steps are performed in a closed and sealed glass container. After the final washing with HMDS,
the samples are removed and placed in an open container under N2 in the fume hood to dry. Dried samples are mounted to aluminum pin mounts using conductive carbon tape, sputter coated with gold, and imaged with a Tescan Vega SEM.

### 5.2.11 Immunostaining

Immunostaining followed by confocal microscopy is used to assess the cellular localization of proteins in 2D and 3D PTEC models. Prior to immunostaining, each construct is washed with PBS and then fixed for 20 min to 1 h using 10% buffered formalin. The fixative is removed using several washes in PBS for several hours and then blocked overnight using 1 wt% bovine serum albumin (BSA) in PBS. Primary antibodies to the cell protein or biomarker of interest are incubated with the constructs for 1 day at the dilutions listed in Table 5.1 in a solution of 0.5 wt% BSA and 0.125 wt% Triton X-100. Removal of unbound primary antibodies is accomplished using a wash step against a solution of PBS or 0.5 wt% BSA and 0.125 wt% Triton X-100 in PBS for 1 day. Secondary antibodies are incubated with the constructs for 1 day at the dilutions listed in Table 5.1 in a solution of 0.5 wt% BSA and 0.125 wt% Triton X-100 in PBS. Samples are counter-stained with NucBlue or ActinGreen for 2 h and then washed for 1 day in PBS prior to imaging.

### 5.2.12 Imaging and Analysis

Phase contrast microscopy is performed using an inverted Leica DM IL scope with objectives ranging from 1.25X to 40X. Confocal microscopy is performed using an upright Zeiss LSM 710 with water immersion objectives ranging from 5X to 40X employing spectral lasers at 405, 488, 514, 561, and 633 nm wavelengths. Image reconstructions of z-stacks are performed in ImageJ using the z-projection function with
the maximum pixel intensity setting. Any increases in brightness are performed uniformly across an entire z-projected image. Image analysis for quantification of diffusional permeability is performed using custom MATLAB scripts employing previously reported methods \(^{185}\). TEM image analysis is performed using ImageJ software to measure cell height \((n \geq 50)\), microvilli density \((n \geq 25)\), and microvilli length \((n \geq 200)\) over at least 3 independent samples for each condition.

5.2.13 Statistical Analysis

Data are expressed as means ± standard deviation. Statistical analysis is performed using MATLAB and statistical significance is determined at a value of \(p < 0.05\) as determined by a Student’s t-test without assuming equal variances. Different significance levels \((p\)-values\) are indicated with asterisks and specific \(p\)-values are provided in each figure legend.

5.3 RESULTS AND DISCUSSION

5.3.1 Proximal Tubule Fabrication

Our bioprinting method is used to construct a 3D convoluted proximal tubule segment of a nephron, as depicted in Fig. 5.3a \(^{182}\). First, as shown in Figs. 5.3b-c, a silicone gasket is printed on a glass slide that demarcates the outer border of the 3D tissue chip. A layer of ECM, which is composed of a gelatin-fibrin hydrogel \(^{211}\) is then evenly deposited within the gasket. Next, a fugitive ink, shown in pink, is printed onto the ECM layer. After printing, the fugitive ink is connected to hollow metal pins interfaced through the gasket walls and additional ECM is cast over the printed structure. The 3D tissue model is then housed within a perfusable chip, where it is cooled to 4°C to liquefy and subsequently remove the fugitive ink yielding an open convoluted tubular channel.
embedded within the ECM. Finally, cell media is perfused through the 3D convoluted tubular architecture on chip via an external peristaltic pump. Notably, our method can create 3D proximal tubule models in myriad configurations with precisely controlled size, curvature, and location. For instance, if multiple tubules are required to increase statistical relevance of an assay or provide basal-side access channels, they can be printed alongside one another (Figure 5.4) and can be perfused independently or through a single inlet.

The composition and rheological properties of the ECM and fugitive ink are specifically tailored for our method of fabrication. The ECM consists of fibrinogen, gelatin, and two enzymes (thrombin and transglutaminase)\textsuperscript{211}. The dual enzyme scheme enables rapid solidification of the ECM around printed features, through thrombin action on fibrinogen to make fibrin. The second enzyme, transglutaminase, provides a slower crosslinking of gelatin with fibrin, enabling a seamless integration of the upper and lower ECM layers during assembly (Figure 5.5a). Furthermore, the elastic modulus of the ECM (~3.5 kPa) mimics that of the cortex of a healthy kidney (~4 kPa)\textsuperscript{212} – matrix stiffness and composition is important for the retention of tissue-specific cell functionality\textsuperscript{195, 213}. The fugitive ink is composed of a triblock copolymer of polyethylene-polypropylene-polyethylene (Pluronic-F127), which forms a viscoelastic gel above a critical micelle concentration in water at room temperature. This ink exhibits a gel-to-fluid transition as the chip is brought from room temperature down to 4\textdegree C, enabling its removal from the ECM under those conditions\textsuperscript{211, 157}. The fugitive ink also contains a high concentration of thrombin (100 U mL\textsuperscript{-1}). Hence, when it is surrounded by the ECM during the casting
process, soluble fibrinogen is rapidly transformed to insoluble fibrin, templating fibrin around the lumen and facilitating the desired, long-term perfusion of cell media.

Figure 5.3 Kidney proximal tubule fabrication. (a) Schematic of a nephron highlighting the convoluted proximal tubule, reprinted with permission (b,c) corresponding schematics and images of different steps in the fabrication of 3D convoluted, perfusable proximal tubules, in which a fugitive ink is first printed on a gelatin-fibrinogen extracellular matrix, ECM (i), additional ECM is cast around the printed feature (ii), the fugitive ink is evacuated to create an open tubule (iii), and PTEC cells are seeded within the tubule and perfused for long time periods (iv); (d) a 3D reconstruction of the printed convoluted proximal tubule acquired by confocal microscopy, where actin is stained in red and nuclei are blue; the white dotted line denotes the location of the cross-sectional view shown below in which PTEC cells circumscribe the open lumens in 3D, scale bar = 500 µm, (e) higher magnification view of the region in (d) denoted by the white rectangle, scale bar = 200 µm, (f) higher magnification view of the tubule, scale bar = 30 µm.
Figure 5.4 Multiplexed 3D proximal tubules. (a) SEM image of 6 PTs printed adjacent to one another, scale bar = 500 µm. [Note: The image is acquired on a thin dried slice cut from the printed sample.], (b) High magnification image taken inside the larger 3D PT shown in the background, scale bar = 50 µm. As shown here, multiple PTs can be printed in parallel and lined with PTEC cells that grow to confluency.
Prior to introducing cells, we first perfuse the 3D tissue chip with cell media for several hours at 37°C to remove any residual fugitive ink and equilibrate the matrix at 37°C and 5% CO₂ in the incubator. We then introduce PTEC-TERT1 cells that consist of human proximal tubular cells immortalized through stable expression of the catalytic subunit of human telomerase reverse transcriptase (TERT) \(^{215}\). PTEC-TERT1 are reported to maintain morphological and functional properties of primary cells, including tight junctions and dome formation, expression of gamma-glutamyl transferase (GGT) activity, sodium-dependent phosphate uptake, and parathyroid hormone stimulation \(^{207}\). PTEC-TERT1 have demonstrated genomic stability up to 90 population doublings has been demonstrated \(^{215}\). To assess their utility, we carried out gene expression analysis on 33 key genes and compared PTEC-TERT1 against both primary renal PTEC and the renal cancer cell line A498 (Figure 5.5). The mRNA levels demonstrate that PTEC-TERT1 cells are transcriptionally close to primary renal PTEC. We chose these cells (hereby referred to as PTEC cells) based on this gene analysis combined with their replicative advantage.
Figure 5.5 Engineered extracellular matrix (ECM) and gene expression profiles for various PTEC lines. (a) Schematic representation of the ECM constituents and their gelation and cross-linking as a function of different stimuli, (b) relative mRNA levels of 33 selected genes related to renal epithelial function, transport, endocytosis, hormone response, injury response, and cell differentiation for three cell lines (primary renal PTEC, PTEC-TERT1, and the A498 cancer renal cell line). PTEC-TERT1 cells are transcriptionally similar to primary PTEC and different from the A498 renal cancer epithelial cell line.
To circumscribe the convoluted tubules with a confluent PTEC monolayer, PTEC cells are first trypsinized from a tissue culture plastic dish, concentrated, and perfused into the open lumen of the printed structure. The cells incubate in the tubule overnight with no flow to facilitate adherence to the ECM and are then flushed lightly at Day 1 to remove any non-adherent cells. A time sequence of their maturation process in the tubule is provided (Figure 5.1). Notably, PTECs grow to confluence within the tubule, circumscribing the open lumen in 3D over a period of approximately 3 weeks. For increasing levels of complexity, support cells, such as fibroblasts or immune cells, can be suspended in the ECM surrounding the tubule or printed into complex architectures. As shown in Fig. 5.6, fibroblasts can survive adjacent to the tubule in the extratubular space of the ECM. Tubule diameters ranging from 150 µm to 700 µm have been printed; however, most of the assays and quantitative measurements are carried out on PTs with diameters ranging from 400 µm to 550 µm under a flow rate of ~1 µL min⁻¹. Images of a mature PT at low (Fig. 5.3d) and higher magnifications (Figs. 5.3e-f) reveal that PTECs circumscribe the lumen and adopt a cuboidal morphology, as expected for their in vivo phenotype. These engineered 3D convoluted PTs are maintained longitudinally by feeding media in a closed loop system. Media is replaced every two days and the tubules remain viable for extended periods; the longest we tested was over two months (65 days).
5.3.2 3D Proximal Tubules Form Polarized Epithelium

After PTECs are seeded and grown to maturity in the tubule, a combination of light microscopy, scanning electron microscopy (SEM) and transmission electron microscopy (TEM) are used to characterize the printed and perfused 3D PT (Figure 5.7). Specifically, low (Figure 5.7a) and high (Figure 5.7b) magnification views in phase microscopy reveal that PTECs grow throughout the tubule packing together in a columnar fashion. TEM images of the tubule cross-section also show that PTECs assemble into a tightly packed, columnar renal tubular epithelium (Figure 5.7c-d). As shown schematically in Figure 5.7e, the epithelium should have a basement membrane formed on the basal side and a brush border of microvilli on the apical side facing the open lumen with cells in a columnar morphology. Using TEM images, we quantified the increase in cell height, owing to the columnar cell morphology within the 3D proximal tubule (Figure 5.7c) compared to the same cells grown for the same duration on 2D surfaces (i.e., a tissue
culture dish and on our ECM) in the absence of perfusion (Figure 5.7d). Importantly, the PTECs in our printed and perfused 3D PT constructs exhibit a two-fold increase in cell height relative to the planar controls (Figure 5.7e-f).
Figure 5.7. 3D proximal tubule morphology and molecular markers. (a) A phase contrast image of a mature 3D PT construct taken at 6 weeks, scale bar = 500 µm, (b) phase contrast image of the 3D PT construct at 6 weeks, scale bar = 250 µm, (c) TEM image of the PTECs within the tubule at 5 weeks, scale bar = 5 µm, (d) TEM image of the PTECs grown on a 2D dish coated with ECM, scale bar = 5 µm, (e) schematic view of the columnar epithelium seen in native tissue, in which PTECs pack together closely and exhibit a dense brush border on the apical side, tight junctions, and a solid basement membrane, reprinted with permission (35) (f) PTEC cell height as measured from TEM images of the 3D PT constructs as well as two 2D controls (i.e., in which the cells are either cultured on an ECM-coated or bare tissue culture dish), *p <0.001, **p<0.003, (g) SEM images at low (scale bar = 50 µm) and higher (scale bar = 20 µm) magnifications showing a confluent layer of PTECs within the 3D PT, white arrows highlight the presence of primary cilia at a density of one per cell, (h) 3D rendering of a partial tubule showing the apical side, which highlights the primary cilia (red), scale bar = 20 µm, (i) image of the PT highlighting the presence of Na/K ATPase in green, scale bar = 100 µm, (j) image of the 3D PT highlighting the presence of AQP1 in yellow, scale bar = 100 µm, (k) high magnification view of the image in (j) highlighting actin in red and showing AQP1 in yellow, scale bar = 20 µm.
SEM images of the apical side of the 3D PT (Figure 5.7g) reveal the formation of a confluent cell layer and the presence of primary cilia (one per cell, akin to that observed in vivo). The primary cilium is a sensory organelle that extends into the open lumen and responds to shear stress; it is important for the maintenance of the epithelial cell phenotype and is often lost once cells are isolated and cultured in 2D in the absence of shear stress. Primary cilia are also observed in our PT using immunofluorescence, by staining for acetylated tubulin (shown in red in the 3D rendering in Figure 5.7h and Figure 5.8). Furthermore, we confirmed the expression of the epithelial marker Na+/K+ ATPase (Figure 5.7h and Figure 5.7i), and its appropriate sub-cellular localization to the basolateral plasma membrane (Figure 5.8a), which is again akin to the in vivo PT epithelial phenotype. The proximal tubule-specific (versus distal tubule) water channel Aquaporin 1 (AQP1) is also predominant throughout our tubule (Figure 5.7j) and the AQP1 staining at higher magnification has a speckled pattern on the membrane surface (Figure 5.7k) as others have shown.
Figure 5.8 PTEC characterization within printed and perfused 3D proximal tubules. (A) 3D reconstruction of PTECs stained for Na\(^+/\)K\(^+\) ATPase (green) and acetylated tubulin (red) where basal-lateral expression of Na\(^+/\)K\(^+\) ATPase is apparent and two primary cilia are visible on the apical side, scale bar = 10 µm and (B) TEM image of primary cilia, scale bar = 1 µm.
Cell polarity is a fundamental feature of epithelial cells and is crucial to ensure vectorial transport by PTECs. Hence, we explored PTEC polarity by first characterizing the apical side of our 3D PT using TEM (Figure 5.9a). At the apical surface, microvilli are present and form a brush border that is significantly more pronounced than in 2D (compare Figure 5.9a and Figure 5.9c with Figure 5.9d). At the basal (Figure 5.9b) surface, basolateral interdigitations (BI) are prominent. These BI extend the surface area of the lateral and basal borders in vivo; cells in 2D (Figure 5.7d) are mainly flat and lack BI. Presence of circular invaginations in the lateral membrane, annotated with white arrows in Figure 5.9b, suggest that mechanisms of active transport are present at the lateral surface. Furthermore, there is a distinct difference between the morphology of the ECM and basement membrane (BM) proteins deposited by the PTECs. Further exploration of the BM protein composition revealed that in mature 3D PT constructs, PTECs deposited laminin and collagen IV (Figure 5.9c). Other important properties observed were the presence of tight junctions between neighboring cells in TEM (Figure 5.9d) and the presence of cell-cell junction proteins, such as K cadherin in Figure 5.9e which link cells in a characteristic cobblestone pattern. Lastly, properties of the brush border are quantified by TEM image analysis. We find that the microvilli length in the printed and perfused 3D PTs is double that observed for either 2D control (Figure 5.9f). Concurrently, microvilli density is also significantly higher for the printed and perfused 3D PT constructs (Figure 5.9g).
Figure 5.9 PTEC polarity in printed and perfused 3D proximal tubules. (a) TEM image of the brush border on the apical side of PTECs at 6 weeks, scale bar = 1 µm, (b) TEM image of the basal side of PTECs at 6 weeks highlighting the presence of the engineered extracellular matrix (ECM), basement membrane proteins secreted by the cells (BM), basolateral interdigitations (BI), and circular invaginations in the membrane marked with white arrows, scale bar = 1 µm, (c) PTECs at 6 weeks showing the basement membrane proteins the cells secreted, namely laminin (predominant protein in red) and collagen IV (green), scale bar = 10 µm, (d) tight junction (white arrow) between PTECs in the bioprinted tubule, scale bar = 500 nm, (e) the cell junction protein K Cadherin (magenta) stained in the PT, scale bar = 10 µm, (f) microvilli length and (g) microvilli density quantified through TEM images, p<0.001.
PTECs will form near leak tight barriers against the traffic of certain proteins, like high molecular weight dextran, when healthy and confluent. Barrier function is assessed by traditional methods. Specifically, FITC-labeled dextran (70 kDa) is perfused through the open lumen of mature PTs and the intensity of fluorescence is captured using a wide-field fluorescence scope with time. From the FITC intensity values, the diffusional permeability is calculated and compared against a 3D tubule without epithelial lining (Figure 5.2). The drastic reduction in the diffusional permeability coefficient (greater than an order of magnitude) indicates that the epithelial barrier in the printed and perfused 3D PT construct is nearly leak-tight and functional.

5.3.3 Albumin Uptake

Receptor-mediated endocytosis by PTEC cells is essential for body fluid homeostasis. Reabsorption of plasma proteins from the glomerular filtrate relies partially on the megalin-cubilin complex located in the brush border (29-31) and can be modeled in vitro by monitoring albumin uptake by PTECs. We tested the ability of PTECs, grown either on perfused 3D PT constructs or 2D controls, to uptake FITC-labeled human serum albumin (HSA). After exposure to FITC-HSA for 2 hours, PTECs were collected, stained for megalin expression, and analyzed by flow cytometry. The results for albumin uptake are provided in Figure 5.10A. Large populations of cells in the 2D controls exhibit fluorescence intensity similar to the non-fluorescent control, whereas cells lining the perfused 3D PT constructs exhibit a significant increase in the FITC-HSA intensity. Results for megalin, one of the transporters for albumin, show that its expression is also highest in the 3D PT (Figure 5.10b). Mean values for the fluorescence intensity of the populations analyzed by flow cytometry are listed in Table 5.2. Contrary to the 2D
controls, we find that enhanced megalin expression is strongly correlated with superior albumin functional uptake in the perfused 3D PTs, suggesting that both 3D architecture and perfusion improve epithelial function likely due to enhanced cell polarity and brush border (Fig. 5.9). Lastly, images of FITC-HSA (Fig. 5.10c), megalin (Fig. 5.10d), and the combination thereof (Fig. 5.10e) reveal an overlapping distribution of albumin and megalin in PTECs that line the 3D PT. Thus, our engineered 3D PT constructs exhibit superior albumin uptake function relative to either 2D control.

Table 5.2. Albumin uptake for PTEC cells. Mean values of the data shown in Figure 5.10 for each population of cells.

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<thead>
<tr>
<th>Mean Intensity</th>
<th>Albumin</th>
<th>Megalin</th>
</tr>
</thead>
<tbody>
<tr>
<td>2D on Plastic</td>
<td>201</td>
<td>571</td>
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<tr>
<td>2D on Printing Matrix</td>
<td>310</td>
<td>1127</td>
</tr>
<tr>
<td>3D Printed (Perfused)</td>
<td>1452</td>
<td>1670</td>
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Fig. 5.10 Albumin uptake in printed and perfused 3D proximal tubules. (A) Flow cytometry data comparing the fluorescence intensity of PTECs fed FITC-labeled human serum albumin for 2 h under several conditions, including 2D controls on bare (blue) and ECM-coated (green) plastic dishes and in 3D PTs perfused for 65 days (magenta). (B) Flow cytometry data comparing the fluorescence intensity of megalin for the same PTEC samples as shown in (A), (C) fluorescence image of the 3D PT constructs stained for FITC-labeled albumin (red), (D) megalin (blue), and (E) combined, scale bars = 20 µm.
5.3.4 Cyclosporine-A Testing

Cyclosporine A, a drug commonly given following transplant surgery to prevent rejection, is a known nephrotoxin that damages proximal tubule cells. To study its effect on the perfused 3D PT, we exposed them to various concentrations of Cyclosporine A (CysA) and monitored alterations of cell morphology and cytoskeleton organization by immunostaining of actin filaments. Bright field images of the tubules (Figure 5.11a-d) and corresponding 3D renderings of actin staining (Figure 5.11e-h and 5.11i-l) reveal dose-dependent manifestation of CysA-induced damages. Minor breaks in cell-cell junctions (Figure 5.12) and reorganization of actin (Figure 5.11j) are observed at 10 µM CysA, whereas discrete areas devoid of cells are readily evident at 50 µM CysA (Figures 5.11g and 5.11k) and exacerbates at 100 µM CysA (Figures 5.11d, 5.11h, and 5.11I). We also note that cell layers tighten and buckle at 50 and 100 µM CysA (Figs. 5.11g and 5.11k). Finally, we assessed CysA-induced disruption of the epithelial barrier function by quantifying the diffusional permeability of FITC-dextran (70 kDa) in treated tubules. As shown in Fig. 5.11m, exposure to 50 and 100 µM CysA increased the epithelial barrier permeability by almost 4 fold and 6 fold respectively. For comparison, cell viability of PTECs grown on 2D culture plastic dishes was decreased by 40 and 60% after treatment with 50 and 100 µM CysA (Fig. 5.11n). Overall, the results indicate that the 3D PT constructs can be used to qualitatively (immunostaining) and quantitatively (diffusional permeability measurements) assess nephrotoxicity.
Fig. 5.11 Drug toxicity testing. (a-d) Brightfield images, (e-h) 3D renderings, and (i-l) high magnification images of printed and perfused 3D PTs dosed with varying concentrations of Cyclosporine A for 24 h, where actin (green) and nuclei (blue) are stained, scale bars = 200 µm (a-h) and scale bars = 20 µm (i-l), respectively, (m) Diffusional permeability measurements taken after dosing with Cyclosporine A, *p < 0.003, **p < 0.02, (n) Cell viability measured for the 2D control (on bare dish) after dosing with Cyclosporine A (all populations shown are statistically significantly different with a p < 0.005).
5.3.5 Discussion

Recent advances in bioprinting have enabled the creation of pervasive and interconnected channels within 3D hydrogel matrices\textsuperscript{14,182}. We previously reported that such channels can be lined with endothelial cells and perfused to create embedded vascular networks\textsuperscript{182}. Building on that work, we now describe a pathway for fabricating perfusable, convoluted 3D proximal tubules via \textit{in vitro} epithelialization. To create a

![Figure 5.12. Observed damage for printed and perfused 3D proximal tubules dosed with 10 µM cyclosporine A.](image)

(a) brightfield image of a healthy proximal tubule at 4 weeks, scale bar = 100 µm, (b) brightfield image of a tubule after 24 h of cyclosporine A exposure, scale bar = 100 µm, (c) Live (green) and dead (red) staining of the tubule at 24 h after cyclosporine A exposure showing that < 5% of the total cells are dead, scale bar = 100 µm, (d) high magnification image showing the most dramatic, but quite uncommon, damage observed under these conditions, where actin (green) and nuclei (blue) are stained, scale bar = 20 µm.
more stable microenvironment for longitudinal study, we developed an extracellular matrix based on enzymatic crosslinking of fibrinogen and gelatin. This matrix promotes improved adhesion of PTECs (in comparison to our previous matrices), allowing the cells to form a confluent layer that exhibits the hallmarks of functional cells in vivo, including many important morphological features and functional markers, which can be sustained for > 60 days. Importantly, these engineered 3D PTs enable collection of hundreds of thousands of cells for analysis, much more than the 10,000 cells which are required for accurate sampling via flow cytometry. Such high cell populations are difficult to achieve in planar microfluidic-based devices.

We show that our 3D PT constructs can be used to elucidate mechanisms of drug-induced tubule damage, including weakening of cell-cell junctions, cell ejection from the monolayer, and cell death. In future studies, we plan to investigate the morphology and function of PTECs seeded within printed 3D tubules whose outer diameter (~60 µm is physiological) and curvature more closely mimics in vivo PTs to probe whether further improvements to the biomimicry of our model are possible. Moreover, we envision creating more complex constructs in which tubules are patterned alongside one another to facilitate basal side access or study crosstalk between PTECs in adjacent channels. Ultimately, we will co-print 3D PTs along with embedded vasculature with integrated podocytes and mesangial cells. Overall, our versatile fabrication method also allows one to incorporate multiple cells types in the extratubular space, thereby programming predefined levels of complexity required for studying cell-cell interactions.
5.4 CONCLUSIONS

In summary, we have demonstrated bioprinting, seeding, and characterization of perfusable, 3D functional renal proximal tubules embedded within an extracellular matrix on chip. Our 3D PT constructs promote the formation of a tissue-like epithelium with \textit{in vivo} phenotypic and functional properties relative to the same cells grown in 2D controls. Our bioprinting method opens new avenues for creating 3D tissues on chip that better recapitulate \textit{in vivo} microenvironments, which could enable advances in drug screening, mechanistic drug studies, disease models, and ultimately, regenerative medicine.
CHAPTER 6
CONCLUSIONS

My dissertation describes the fabrication and characterization of vascularized human tissues via multimaterial 3D bioprinting. Specifically, we used 3D bioprinting to manufacture functional vascularized tissues that recapitulate aspects of the structural and cellular heterogeneity found in human tissues. We developed a versatile method to actively perfuse printed vascularized tissues, which enables physiologically relevant size scales as well as organ-specific tissue models for drug testing. We reports a pathway for fabricating 3D tissues with embedded (tubular) channels that can either be confluent ly lined with human umbilical vein endothelial cells to yield vasculature or renal epithelial cells to create a proximal tubule model. The ability to fabricate lumenal tissue-specific architectures may open new avenues for investigating structure-function in complex human tissues, e.g., by co-patterning of epithelial and endothelial tissues in 3D. Moreover, this approach could be extended to other tubular architectures, such as lymph, or bile ducts.

The principal findings of my PhD research are summarized below:

1) 3D printing of vascularized, heterogeneous tissues

We developed a suite of mutually compatible ECM, cell-laden, and fugitive inks, engineered for multmaterial bioprinting of 1D, 2D, and 3D vascularized tissue constructs. These tissues are replete with multiple, printed cell types that demonstrate proliferative ability after printing, as well as a confluent endothelium.

2) Novel materials processing strategy for printing and casting
To satisfy the concomitant requirements of processability, heterogeneous integration, biocompatibility, and long-term stability, we developed a printable cell-laden ink and ECM comprising a blend of gelatin and fibrinogen. Specifically, these materials form a tough interpenetrating polymer network composed of gelatin-fibrin cross-linked by a dual-enzyme, thrombin and transglutaminase, strategy. Notably, tissues are created without the use of UV-photocrosslinkers or toxic initiators, in which printed cells exhibit ~95% viability after printing cell-laden inks through fine deposition nozzles (200 µm in diameter).

3) **Fabrication of thick vascularized tissues**

We produced stem cell-laden tissues with an embedded, perfusable vasculature network that exceed 1 cm in thickness and 1 cm³ in volume. As an important demonstration, we 3D printed parenchyma, stroma, and endothelium into a 3D tissue composed of custom extracellular matrix, human mesenchymal stem cells (hMSCs), human neonatal dermal fibroblasts (hNDFs) and embedded vasculature lined with human umbilical vein endothelial cells (HUVECs).

4) **3D printing of customized perfusion chips**

To enable perfusion of vascularized human tissues, we print these tissues within custom-designed and 3D printed perfusion chips that are interfaced with an external pump. Silicone walls act as a gasket between a stainless steel base and a thick acrylic lid is placed on top. These custom perfusion chips can be printed in nearly arbitrary form factors, in which multiple vascular channels or networks can be individually addressed and controllably perfused in a single chip.
5) **Printed vasculature demonstrate long-term stability**

Thick living tissues fabricated within customized perfusion chips possess the mechanical and chemical integrity to survive over six weeks of active perfusion. Importantly, the channels remain stably patent and the confluent endothelial maintains expression of key endothelial specific proteins (e.g., CD31, von-Willebrand Factor, and vascular endothelial cadherin).

6) **Perfusable vasculature is critical to support thicker than 1mm**

Stromal HNDFs residing within the surrounding matrix exhibit cell spreading and proliferative phenotypes localized to regions within ~1 mm of the vasculature cells further away from these regions become quiescent likely due to an insufficient nutrient supply. Clearly, the perfusable vasculature is critical to support living tissues thicker than 1mm over long time periods.

7) **Induced osteogenic differentiation of hMSCs via perfusable factors**

To demonstrate vascular functionality and tissue longevity, we perfused the thick vascularized tissues with BMP-2, glycerophosphate, and ascorbic acid to differentiate hMSCs toward an osteogenic lineage *in situ*. Prior to this experiment, we optimized the differentiation factors by comparing the expression of alkaline phosphatase and calcium phosphate deposition under various conditions. Enabled by our long-term perfusion capabilities, we studied the development of this tissue over 30 days and observed the deposition of collagen 1, which preceded calcium phosphate mineral deposition.
8) **Perfusable vasculature enhances mineral deposition of hMSCs**

In an avascular tissue with comparable hMSC density, mineral deposition is only observed within a few hundred microns of its outer surfaces. By contrast, the thick vascularized tissues stain positive in hMSC regions deep within its core after 30 days of osteogenic differentiation via perfusion. SEM/EDS confirms the elemental composition of mineral deposits to be calcium and phosphorous.

9) **Developed functional 3D renal proximal tubule model**

Leveraging our bioprinting platform, we printed a 3D open lumen architecture circumscribed by proximal tubule epithelial cells (PTECs) embedded within an extracellular matrix on a custom perfusion chip. The 3D model is perfused at physiologically relevant shear stresses over long durations (> 60 days). PTECs form a confluent, leak-tight epithelial monolayer that exhibits primary cilia and expresses Na\(^+/\)K\(^+\) ATPase, Aquaporin 1, and K cadherin. The unique combination of their 3D geometry and perfusable nature allows for controlled shear stress conditions, which give rise to a more differentiated, polarized PTEC phenotype that develops an enhanced brush border, basement membrane protein deposition, basolateral interdigitations, enhanced cell height, megalin expression, and albumin uptake relative to 2D controls.

10) **Proximal tubule model is suitable for testing nephrotoxicity**

We observe dosage-dependent nephrotoxicity in response to the known nephrotoxin, cyclosporine A (CysA). After exposure to 10 \(\mu\)M CysA, we observe minor breaks in cell-cell junctions and reorganization of actin, whereas discrete areas devoid of cells are readily evident at 50 \(\mu\)M CysA, which is further exacerbated at 100 \(\mu\)M. As a
consequence, exposure to 50 and 100 µM CysA dramatically increased the diffusional permeability of the epithelial barrier by nearly 4-fold and 6-fold respectively, relative to 3D tubules without CysA.
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