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The Intricacies of Bioprinting Vascular Structures

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ABSTRACT: The field of tissue engineering and regenerative medicine has seen great advances in the last two decades. The overall implementation of these synthesized structures is still, however, greatly limited. One of the major contributors to this stagnation is the lack of functional vasculature within these structures. Given the role that vasculature plays in the distribution of oxygen and nutrients within the body, *in vitro* organs must also have this network of blood vessels to function properly. Herein, we review state-of-the-art bioprinting techniques and propose the best viable method amongst them for the creation of vascular structures. The three methods we analyzed are extrusion-based, jetting-based, and stereolithography. Information on specific bionics and the overall process for each method is provided, sourced from the primary and secondary scientific literature. While our findings indicated that extrusion-based bioprinting is the most popular bioprinting method used for printing vasculature, accounting for a majority of the papers reviewed it was determined stereolithography bioprinting holds the greatest potential in the creation of functional in vitro vasculature. This decision was made given its high resolution, affordable cost, and high cell viability percentage.

KEYWORDS: Biomedical Engineering; Cell and Tissue Engineering; Vasculature; Bioprinting; Bioink.

Introduction

Within the past decade, the field of regenerative medicine has gained recognition as a legitimate option for the regeneration of functional tissue and organs. One of the major contributors to this improved viability is the development of three-dimensional (3D) bioprinting. 3D bioprinting, like 3D printing, is an additive manufacturing technique. A solution containing cells and support material commonly referred to as "bio-ink" is deposited onto a support stage or liquid medium. It is then incubated to form a fully functioning tissue via crosslinking. Crosslinking refers to a bond that links a multitude of polymers. These bonds can be either covalent or ionic bonds. Bioprinting methodologies can be classified into one of two methods: Scaffold or scaffold-free. Scaffold bioprinting involves the printing of cells with support material to support the growth and proliferation of the cells.[1] The scaffolds mimic the extracellular matrix (ECM) as *in vivo*; the ECM provides structural support and can promote regeneration. An added benefit of scaffold bioprinting is the ability to support a higher cell density while also creating more complex geometries, unimpaired by gravity. Alternatively, scaffold-free bioprinting does not include any support material that is printed with the cells. Rather, the cells are first clumped together to form aggregates or clusters. From there, they secrete ECM for structural stability and can then be printed in pellets.[1] With scaffold-free bioprinting, cells are given more room for interaction resulting in the development of a 3D structure that more closely facilitates the high levels of cell communication we see in native tissue.[2] Bioinks, in this process, are limited to just tissue strands and tissue spheroids. Finally, an important aspect of bioprinting is the resolution of the structure. The print resolution refers to "the lowest measurable unit of printed material in the x and y dimensions" (Lee).[3] The lower the unit, the higher the resolution.

While bioprinting has had moderate success in the printing of simple tissue structures like skin, bladders, and ears, the creation of complex large-scale organs such as hearts and kidneys is yet to be seen *in vitro*. As of now, engineered organs are only a fraction of the size of their *in vivo* counterparts. This can be attributed to the lack of movement concerning nutrients within large-scale engineered organs. Nutrients enter tissue through a process called diffusion, where a substance moves from an area of high concentration to an area of low concentration. The rate at which this process occurs is known as the diffusion rate. Although important, the diffusion distance plays an equal, if not larger, role in the usefulness of nutrients. The larger an object is, the larger the diffusion distance. Ultimately, resources for cells could be used up before even reaching their destination or crossing a barrier, no matter how efficient the diffusion rate is. If a structure is too large to where it cannot receive nutrients due to a large diffusion distance the cells will die, hence rendering the organ nonfunctional. Instead of this slow and inefficient process, the use of vasculature will transport nutrients to where they are required in a much timelier manner. For example, instead of relying on sugar to diffuse through a multitude of tissues to go from the stomach to the muscles, vasculature facilitates the delivery of nutrients and oxygen throughout the tissue, preventing mass necrosis or cell death throughout the structure. Ultimately, this is the main challenge that is preventing the implementation of synthesized organs. In this review, we examine the structure of vasculature, the composition, and properties of bioinks, and novel bioprinting methods, assessing which bio-

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printing method has the best chance of achieving the mass generation of a functional vasculature.

Discussion

The vascular system:

The vascular system remains one of the important organ systems within our bodies. The main function of the system is the transportation of nutrients such as sugar, oxygen, blood, and hormones towards and away from different tissues and organs using different types of blood vessels. An example of this is the transport of insulin through the bloodstream. In tissue engineering and bioprinting, a functional vascular system is of the utmost importance concerning the survival of the synthesized tissue.

Blood Vessels Composition and Function:

There are three types of blood vessels: arteries, veins, and capillaries. The job of these vessels involves the transportation of oxygenated and deoxygenated blood to and from tissues. There are two systems of blood vessels: the pulmonary vessels and the systemic vessels. The pulmonary vessels transport blood from the right ventricle of the heart to the lungs and back to the left atrium. In contrast, the systemic vessels transport blood from the left ventricle to all the tissue in the organism, returning the blood to the right atrium.

Arteries Composition and Function:

Arteries are the blood vessels responsible for carrying blood away from the heart and the function is divided into pulmonary and systemic arteries. Pulmonary arteries transport deoxygenated blood from the right ventricle to the lungs. There, the blood is oxygenated by the lungs. Contrary to the pulmonary arteries, the systemic arteries transport oxygenated blood from the left ventricle to all tissue within the body. The further the blood is transported from the heart, the smaller the arteries become, branching out like a tree. The smallest arteries are known as arterioles.

Arteries are composed of three layers of tissue. These layers are the tunica adventitia, also known as the tunica externa, the tunica media, and the tunica intima as shown in Figure 1. The tunica adventitia is the outermost and thickest layer of the artery. It is composed of collagen and elastic tissues and the vasa vasorum. The vasa vasorum is a network of tiny blood vessels that can supply blood to larger blood vessels. They are the vessels of vessels. The main function of the tunica adventitia is to prevent the vessel from overextending and bursting. The middle layer of the artery, the tunica media, is composed of smooth muscle cells, elastic tissues, and collagen. In addition to providing support for the artery, the tunica media is also responsible for changing the vessel's diameter, increasing, or decreasing the blood flow and blood pressure within the vessels. The tunica intima is the innermost layer of the artery and is composed solely of endothelial cells. Endothelial cells are a specific type of cell that forms a boundary between a vessel and tissue and are responsible for regulating the distribution of substances that enter and exit tissue. Additionally, certain signals released by endothelial cells can induce the growth of vessels.[4]

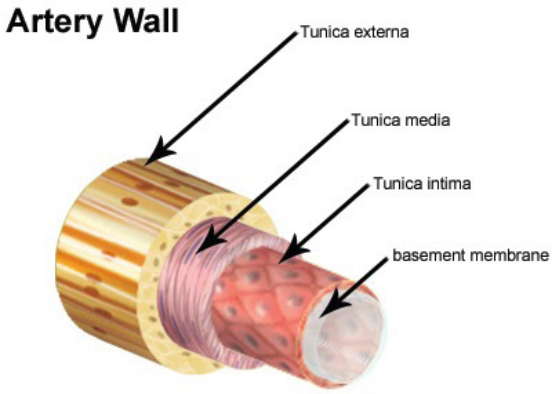

Figure 1: The layers of tissue in an artery are shown. Image referenced from National Cancer Institute SEER Training.[5]

Veins Composition and Function:

In contrast to the function of arteries, veins are responsible for carrying blood back from the tissues toward the heart. Most of the veins within the body carry deoxygenated blood back to the heart after the exchange of nutrients has occurred in tissues. However, there are exceptions to this function such as pulmonary veins. Both veins carry oxygenated blood back to the heart. Veins are composed of the same three layers of tissue as arteries: The tunica externa, tunica media, and tunica intima. However, there is less smooth muscle and tissue and thus the walls of veins are thinner compared to arteries.[5] Some unique structural features that veins have specifically are valves to prevent backflow. During transportation, blood is transported to the right ventricle to be pumped to the lungs to be oxygenated again. With pulmonary veins, oxygenated blood is returned from the lungs to the left atrium and is ejected into the left ventricle.

Capillaries Composition and Function:

The third type of blood vessel within the vascular system is a capillary. While veins and arteries are responsible for the movement of blood throughout the body, capillaries are responsible for the transfer of oxygen, nutrients, and waste between tissue and blood. This transfer occurs through passive diffusion, where a substance travels down a concentration gradient through a membrane. Additionally, this process can also occur through pinocytosis, where vesicles (membrane-bound sacs) take in nutrients and merge with the cellular membrane. Unlike veins and arteries, capillaries are composed of only two layers of tissues. There is an inner layer composed of endothelial cells and an outer layer composed of epithelial cells.

There are three primary classifications of capillaries: continuous, fenestrated, and sinusoidal. Continuous capillaries are, as the name suggests, continuous and uninterrupted, allowing only small molecules to pass. Fenestrated capillaries have small pores that allow the passage of slightly large molecules. These capillaries are found in areas that frequently experience exchanging of blood such as the small intestine and kidneys. Sinusoidal capillaries are the most disrupted capillary type as the large pores within the capillary allow for the passage of large molecules and even cells. Sinusoidal capillaries are found in tissues such as the liver, spleen, and bone marrow.

Growth of Blood Vessels:

The growth of new blood vessels is vital to the function and health of an organism. Subsequently, the independent growth of vasculature after fabrication will give a synthesized organ much higher viability. Two mechanisms form blood vessels: vasculogenesis and angiogenesis.

Vasculogenesis is the process of creating blood vessels in the embryo. It is the first stage in the development of the circulatory system and is often followed by angiogenesis. One key aspect that separates vasculogenesis from angiogenesis is that vasculogenesis occurs where there are no pre-existing blood vessels. Vasculogenesis occurs through the differentiation of endothelial precursor cells known as angioblasts and through that differentiation, a de novo formation of a vascular network is created.[6]

Unlike vasculogenesis, angiogenesis is the formation of new blood vessels from pre-existing vessels. The initiation of angiogenesis is controlled by several growth factors such as vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF), and tumor necrosis factor (TNF-α).[7] A growth factor is a protein that can instruct a specific cellular response. VEGF is responsible for promoting the growth of new proteins and forms a part of the mechanism that restores the blood supply when there is compromised blood circulation. There are two types of angiogenesis: Sprouting angiogenesis and intussusceptive angiogenesis.

Sprouting angiogenesis (SA) is characterized by the growth of endothelial cells and this type of angiogenesis can add blood vessels to areas that did not have blood vessels previously. It is initiated by the detection of hypoxia or a lack of oxygen. In these areas parenchymal cells (functional cells of organs, e.g., neurons) secrete VEGF. Subsequently, tip cells travel toward the growth factor, leading to the growth of new capillary sprouts. Concurrently, endothelial cells become stalk cells and build the blood vessels behind the tip cells.[8] In simpler terms, SA can be thought of as the creation of a deviation from the main blood vessel such as an exit lane from the main highway. As shown in Figure 2, a new deviation from the main vessel is being created as the tip cell leads the development of the new vessel.

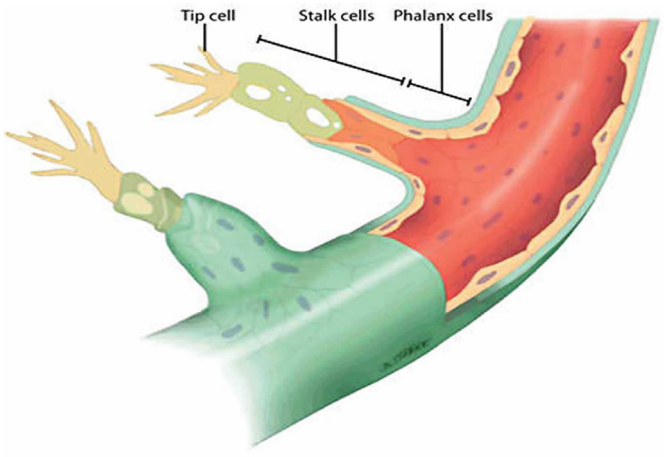

Figure 2: The sprouting angiogenesis process is shown. Image referenced from Spiegelaere *et al.*[9]

The discovery of intussusceptive angiogenesis occurred in the 1980s and is a novel and relatively unexplored phenomenon. Intussusceptive angiogenesis (IA) can be thought of as the splitting of a pre-existing blood vessel into two smaller copies, somewhat similar to DNA replication. A defining characteristic of IA is the formation of intraluminal tissue pillars, formed when endothelial walls of the vessel migrate towards each other, similar to cytokinesis in mitosis and meiosis. As shown in Figure 3 section C, after the initial pillar is created, pericytes and myofibroblasts (vascular mural cells in the basement membrane),[10] inject an extracellular matrix into the pillar and finally, two new vessels are formed.[9] IA can only occur in a network formed through vasculogenic or SA. Typically, the initial network is formed through SA and is further developed by IA.

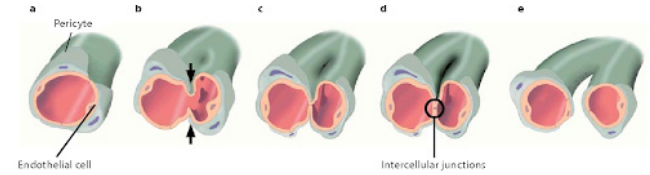

Figure 3: The Process of intussusceptive angiogenesis is shown. Image referenced from Spiegelaere *et al.*[9]

Extrusion-based bioprinting:

Given the uses of vasculature within an organism, it has been made evident that the need for functional blood vessels is dire. Luckily, bioprinting is slowly removing this roadblock, opening the door for functional, life-size, and implantable *in vitro* organs.

One of the most common printing methods is extrusion-based bioprinting due to its low production cost and wide range of printable biomaterials. The printable viscosities of biomaterials that can be extruded range from 30 millipascal seconds (mPa/s) to more than 6 * 107 mPa/s.[11] This covers a range of materials that have the viscosity of motor oil up to window putty including, honey, lard, and ketchup. In extrusion-based bioprinting, a continuous filament of a cell-laden ink is extruded out of a nozzle by a pneumatic system or a screw. Layers of bioink are serially built on top of one another to form a three-dimensional figure following a CAD model. After the primary structure is formed via extrusion, ultraviolet (UV) light is used to induce crosslinking between polymers, forming a durable and viable structure (Figure 4).

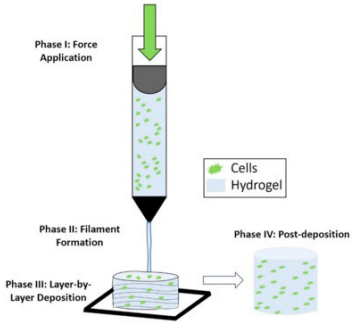

Figure 4: The various phases of Extrusion-Based Bioprinting (EBB) are shown. Phase 1: The bio-ink consists of various cells and a hydrogel medium for stability and force is applied to force the bio-ink out. Phase 2: The bio-ink is extruded in a continuous filament. Phase 3: The bio-ink is deposited in

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layers, forming a three-dimensional structure. Phase 4: Post-extrusion modifications are shown, which include the crosslinking of hydrogels. This fuses the layers, ensuring mechanical durability. Image referenced from Ramesh *et al.*[12]

Extrusion Printing Methods:

There are three categories of methods by which bioink can be extruded out of the nozzle. These methods are pneumatic, screw-based, and piston-based (Figure 5). In pneumatic bioprinting, the bio-ink is forced out of the nozzle using pressurized air. Contrary to a pneumatic system a screw-driven printer uses a rotating screw, driven by a motor, to extrude the bio-ink.[13] The benefits of using a pneumatic system include its simplicity and lack of maintenance needed, yet accuracy may differ based on the viscosity of the bio-ink. Screw-driven methods provide more direct control over the placement of the bio-ink. However, screw-driven extrusion results in a higher percentage of cell damage in comparison to pneumatic extrusion bioprinting.[13] This cell damage can be attributed to the shear stress that cells experience when being extruded as the depth of the screw threads and geometry of the screw may deform the cells themselves. Ning *et al.*[13] found that screws with a smaller pitch distance and higher thread depth are more likely to cause cell damage. The use of a piston in extrusion-based bioprinting is very similar to pneumatic-based bioprinting where a piston is used instead of pressurized air.

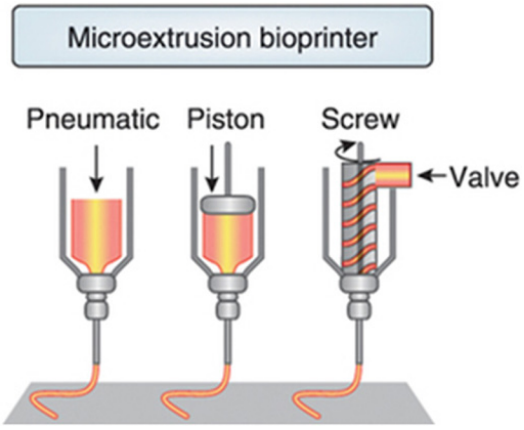

Figure 5: This image has been referenced from Wilson *et al.*[14] The three different methods of extrusion-based bioprinting are shown.

Bio inks:

The variety of bioinks that can be utilized in extrusion-based bioprinting is immense. Several biomaterials are compatible with the printing method including hydrogels[14-18], spheroids[19-20], micro-carriers, and the extracellular matrix.[21] Due to the flexibility of nozzle diameters, bioinks of many viscosities can be extruded.

Hydrogels:

A hydrogel is a three-dimensional substance that is composed of hydrophilic polymers and can retain large amounts of water while maintaining its shape. Hydrogels are often used as bioinks as they mimic the extracellular matrix and are biocompatible while also having high printability.[22]

There are three categories of hydrogels: natural, synthetic, and hybrid. Natural hydrogels can be classified as hydrogels whose polymers originate from organic material like collagen, and other components of the ECM.[23] As a result, an environment that enhances cellular proliferation is acquired, but at the cost of durability as natural hydrogels have a weak structure and the distortion of one layer could subsequently destroy the printed tissue.[24] Compared to natural hydrogels, synthetic hydrogels are created using synthetic polymers such as polyamides. Since synthetic hydrogels are, as the word suggests, "synthetic" they lack the big interactive capabilities of natural hydrogels.[25] However, this disadvantage is somewhat offset by their mechanical strength and durability. As the name suggests hybrid hydrogels are a combination of both synthetic and natural hydrogels. They are composed of proteins, peptides, and can even contain nano/microstructures, and are interconnected, undergoing chemical modifications.[26] Thus, they retain the bioactivity seen in natural hydrogels as well as some of the mechanical properties visible in synthetic hydrogels. Some common properties that all hydrogels share are swelling in which they can take in water like a sponge while retaining their physical orientation.

Alginate is an example of a natural hydrogel. It is a hydrophilic polymer that originates from the cell wall of seaweed and brown algae. It is composed of d-mannuronic acid and a-L-guluronic acid.[27] The carboxylic chains within the structure allow for ionic crosslinking that is often induced by a CaCl2.[18] In 3D bioprinting, alginate remains one of the most widespread biomaterials due to its biocompatibility and ability to form porous structures.

Gelatin is an example of a hybrid hydrogel that is the result of structural degradation of collagen and the process of gelation occurs when polymer chains either undergo physical or chemical crosslinking. Thermal gelation is one method of gelation that forms a gel by heating a solution, inducing cross-linking. The chemical modification of gelation with methacrylic anhydride forms a photocrosslinkable natural bioink and the Gelatin methacryloyl (GelMA).[17] Hence it can be classified as a hybrid hydrogel. GelMA can be categorized into two types, one that is produced from acid treatment (Type 1) and the other produced from alkali treatment (Type 2). In contrast to pure gelatin, which is soluble at body temperature, GelMA can maintain its structural form at body temperature.[17] This makes GelMA a much more viable bioink as the 3D structure formed will not be degraded by the heat of the human body when implanted in vivo. Additionally, GelMA is shown to have unparalleled scaffold strength, enabling it to support cell viability for 14 days.[17] A study conducted by Lee *et al.*[17] found that both Type A and Type B constructs had high cell viability when the cell concentration was at 20%, indicating that GelMA is a viable bioink for extrusion-based bioprinting.

PluronicF127 is an example of a synthetic hydrogel as it is composed of amphiphilic copolymers that contain ethylene oxide and polypropylene oxide.[28] While it is a synthetic hydrogel it has some natural hydrogel properties such as biocompatibility and biodegradability. A unique characteristic of PluronicF127 is thermosensitivity which can cause a sol-gel phase transition. This phase can be defined as the change from a liquid to a gel. Due to this phase PluronicF127 can encapsulate cells better and be more adhesive.[28] PluronicF127 has been applied in the

creation of vasculature as Suntornnond *et al*.[15] used a combination of PluronicF127 and GelMA for a bioink.

Spheroids:

Recently the use of tissue spheroids as potential bioink has gained more popularity as the benefit of using tissue spheroids is that instead of having cells attempt to proliferate through a hydrogel, the starting number of cells can be relatively high which will then cause ECM to be deposited, eventually forming a 3D structure.[29]

Tissue spheroids refer to three-dimensional cell aggregates that are in a spherical orientation. Cell aggregates refer to cells that have clustered together. To date, the primary usage of these spheroids has been in cancer research and the discovery of new drugs as the testing of novel drugs can be done *in vitro*.

Several methods are applicable in the printing of tissue spheroids. As always, however, certain restrictions and considerations must be taken care of before printing. The two most important requirements concerning tissue spheroids are that during the extrusion process, the pressure and shear stress placed on the spheroids should not cause major DNA or structural damage and the ability of fusion is not compromised.[19] The process of fusion is shown in Figure 6 and one can see that the fusion of the spheroids is integral to the development of a smooth and interconnected structure.

To use tissue spheroids as a bio-ink, one must first generate the spheroids themselves. Additionally, the spheroids themselves must be uniform as their use in organ bioprinting is largely dependent on their scalability. In 2011, a novel method of tissue bio fabrication was developed by Mehesz *et al*.[30] Some previous methods that were used were centrifugation, gravity-reliant hanging drops, and cultivation within a flask. However, all these methods have size and shape parameters outside of the experiment's control and thus their scalability is unreliable. Mehesez *et al*. utilized wells that were filled with non-adhesive hydrogels. From there, suspended cells would form spheroids, with their weight dragging them towards the rounded bottom, forming a spherical shape. To dispense the suspended cells a pipette system was used. Tissue spheroids were seen just 48 hours after being placed in the micro recessions.

In the past decade, success has been found in the printing of tissue spheroids in 2019 Aguilar *et al*.[20] and was used to print mesenchymal stem cells using a Regenova printer. The target diameter for the printer was 500 μm. To generate the spheroids, Aguilar et al. used centrifugation and gravity and cultured each group for up to 28 days. After printing the viability of all cells present excluding a cell group known as trypan blue was higher than 85%. Additionally, it was seen that the spheroids cultured in an osteogenic medium expressed a higher level of osteogenic genes and the DNA content of all cells increased each day. These findings give light to the fact that the use of tissue spheroids as bioink can increase proliferation and cellular interactions, and generate a viable 3D structure that truly mimics an *in vivo* organ.

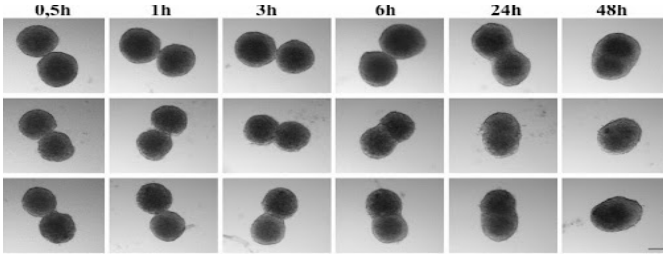

Figure 6: The process of fusion is shown by three samples of tissue spheroids over 48 hours. The image is referenced from Kosheleva *et al*.[31]

Micro-carriers:

Micro-carriers (MC) are support matrices that help cells grow in bioreactors. They are usually spherical with diameters of 150-200 μm.[32] Their development in bioprinting has stemmed from certain limitations that occur within the use of hydrogels in bioprinting. One problem that may occur with hydrogels is that no matter the *in vivo* cellular environment, in the bio-ink, the cell is placed in a hydrophilic, or water-loving environment and is constrained to a spherical shape.[33] A benefit of using MCs is that they can be embedded in hydrogels and can increase the mechanical strength of the hydrogel in the process.[33]

Decellularized Extracellular matrix components:

While biomaterials such as hydrogels and micro-carriers do provide the cells with a safe and durable environment, a major flaw that they possess is that they cannot accurately replicate the behavior and function of the extracellular matrix (ECM). This can be attributed to the fact that many hydrogels are composed of a singular component of the ECM such as collagen, instead of the ECM in its entirety. The purpose of the ECM is to regulate the cell shape as well as initiate many intracellular reactions and is a vital component in cell communication. Additionally, the ECM contains many growth factors which are key to the development of functional tissue. In terms of bioprinting, an environment that has a resemblance to the ECM is integral to fabrication as after the primary structure is printed, proliferation and differentiation are needed to mimic an *in vivo* organ or tissue structure. A simple solution to this problem is the use of decellularized ECM as a bio-ink. For clarification, decellularized ECM (dECM) simply refers to ECM that has been isolated from its cellular environment. To create a dECM bioink, first, the ECM is removed from cells, and then solubilized, and finally, the pH is adjusted to prevent cell damage due to the acidic pH of the ECM.[21] A study conducted by Pati *et al*. found that the use of dECM bioink resulted in the formation of ECM within the 3D printed structure, high cell viability, and no stress-induced apoptosis during extrusion.[21]

Disadvantages:

While extrusion-based bioprinting remains one of, if not the most, accessible bioprinting methodologies it has several key flaws that have spurred on the development of drop-based bioprinting and stereolithography. One particular disadvantage that occurs in extrusion-based bioprinting is the tendency for cells to be damaged due to shear stress, impacting the functionality and viability of the cells (as low as 40% viability).[34] This occurs during the extrusion of bio-ink through a nozzle of a small diameter and can also occur when the bioink is being

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extruded by a screw-driven process.[13] In terms of nozzle diameter, a nozzle with a smaller diameter will result in greater accuracy but it comes at the cost of cell viability as the smaller diameter results in greater stress which will deform the cells due to the lack of space. For clarification, shear stress is when a force acts parallel to the area of a cross-section, unlike normal force which acts perpendicular to the area. Examples of shear stress include chewing food, applying the brake in a car, or running. While the issue of shear stress does play an impact on cell viability the scope of the issue has been reduced due to the introduction of shear-thinning bioinks such as alginate. Shear-thinning refers to how the viscosity of the gel decreases with an increase in stress, mitigating the amount of stress that affects the ink. This can result in higher cell viability as the amount of deformation within the bio-ink will decrease.

Applications

FRESH Bioprinting:

A common problem that occurs with many bioinks in extrusion-based bioprinting is that they are soft. Unlike plastics and metals used for 3D printing, biomaterials such as collagen, alginate, decellularized ECM, and many others do not have the durability to maintain their geometry after being extruded. This phenomenon often occurs when these materials are extruded onto a flat support stage, such as a petri dish. However, researchers at Carnegie Mellon University, directed by Professor Adam Feinberg have developed a bioprinting method that involves the "freeform reversible embedding of suspended hydrogels", otherwise known as FRESH The purpose of FRESH is to embed the printed hydrogels within a secondary hydrogel material composed of gelatin microparticles. However, one key innovation within the hydrogel bath is that the material acts as a high viscous material when exhibiting low shear stress but acts as a low viscous material when exhibiting high shear stress.[35] In layman's terms, the extrusion needle will encounter little resistance when penetrating the hydrogel and when exiting, yet when the bioink is extruded it will keep its current orientation as the secondary hydrogel will exhibit a high viscosity. After the bioink achieves crosslinking, forming a viable 3D structure, the support bath can then be melted in a non-destructive manner by raising the temperature. Hinton *et al.*[35] were able to successfully print a viable femur and heart; structures that would otherwise be impossible to print given the durability of these soft hydrogels. Thus, using FRESH the orientation of bio-inks will no longer be constrained by the weight or durability while still in gel form.

Creating Vasculature:

As of today, there has been a multitude of studies that have successfully developed perfusable vasculature. Suntornnond *et al.*[15] were able to use a composition of Pluronic F127 and GelMA to create a hydrogel and used human umbilical vein endothelial cells (HUVEC) to create a vascular structure. After printing, the cells were cultured for 7 days and achieved a maximum number of 600,000 cells, staying alive until day 10.

To create their vasculature conduits Zhang *et al.*[36] used human umbilical vein smooth muscle cells (HUVSMCs) and alginate as the bio-ink. A coaxial extrusion system was used to create the conduits. The initial viability of the cells was 73±2%

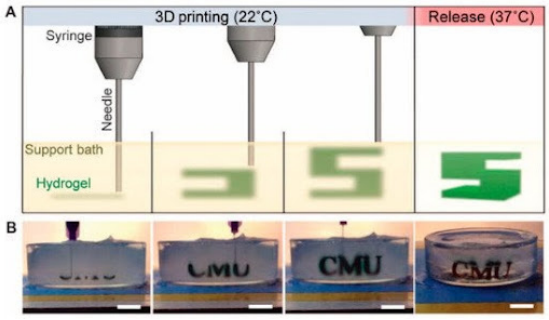

Figure 7: A visual representation of the FRESH technique can be seen. (A). A needle containing a hydrogel-based ink extrudes said ink into a support bath at a temperature of 22 degrees Celsius. Layer by layer the figure is built. After the figure has been oriented and cross-linked, the temperature is raised to 37 degrees Celsius, melting the support material, leaving only the finished structure. (B). An example is shown with the letters "CMU" being printed. This image was used from Hinton *et al.*[35]

and on day 7 the cells had maximum viability of 84±1%. Additionally, the conduits also showed high perfusion with 405±11 µl per hour. Finally, extracellular matrix development was seen on the printed cells, indicating that the cells are communicating and becoming more like their *in vivo* counterparts.

Like Zhang *et al.* Tabriz *et al.*[18] also used alginate as the support material for the bio-ink with Human glioma U87-MG cells being the cell culture. An interesting procedure conducted by Tabriz and Co. was that they partially crosslinked their alginate with CaCl2 and after extrusion, the cell-laden alginate was again crosslinked. Finally, after the second crosslinking BaCl2 was used to perform one final crosslinking. After printing the cells had a viability of 92.9 ±+0.9% and over 11 days the cell viability was always above 82%.

Another study that was able to create vascular structures was Jia *et al.*[37], which used a blend bioink composed of GelMa, alginate, and 4 arm polyethylene glycol-tetra-acrylate (PEG-TA). Like Tabriz *et al.* they partially crosslinked the bioink to form stable constructs. The printing system used multilayered coaxial nozzles with HUVECs and human mesenchymal stem cells (MSCs) being the cell culture. After printing the bio-ink, the constructs were exposed to UV light to induce covalent crosslinking. It was found that the cell viability for UV exposure times of 20 seconds and 30 seconds was above 80% after 7 days of culture.

Extrusion-based bioprinting has great potential in the field of tissue engineering and regenerative medicine. This can be attributed to the versatility of bio-inks as well as the low cost of synthesis. Additionally, there is a great variety in printers as the website Aniwaa listed 11 bioprinters.[38] Out of those 11, 6 printers could perform extrusion-based bioprinting, all for affordable prices. The highest-priced was the BioScaffold Printer BS3.2, priced at $150,000. In stark contrast the lowest-priced bioprinter, Dr. INVIVO 4D was priced at $20,000. Products of extrusion-based bioprinting can be used in tissue transplants as well as seeing the effects of certain drugs on cells with zero risks to a patient's life. This could result in increased development of experimental drugs as well as personalized organ transplants, eliminating the need for donor lists. These studies do indicate that the development of functional vasculature is plausible using extrusion-based bioprinting.

Droplet-Based Bioprinting:

Droplet-based bioprinting is an additive manufacturing technique where a cell-laden bioink is extruded out of a nozzle in the form of droplets. There are three overarching methods of droplet-based bioprinting, each with its sub-methods. These three methods are inkjet bioprinting, acoustic droplet ejection, and micro-valve bioprinting. Inkjet bioprinting has several sub-methods but the two most common methods are drop-on-demand bioprinting and electrohydrodynamic bioprinting. Finally, drop-on-demand has three sub-methods that each involve different actuators, specifically thermal and piezoelectric actuators, and electrostatic forces. The general process behind droplet-based bioprinting is very similar to extrusion-based bioprinting where droplets are deposited onto a support stage. From there the droplets spread, fusing. Finally, a crosslinking agent is applied to the layer hardening it. The crosslinking agents can vary, consisting of a UV light, a reservoir, mist, or other droplets as shown in Figure 8.

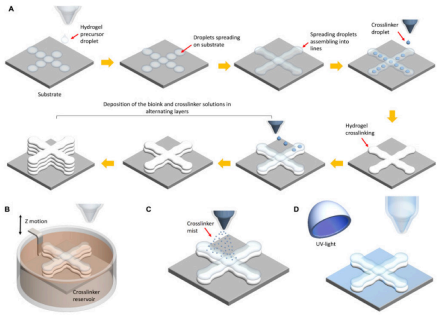

Figure 8: General droplet-based bioprinting process shown with varying crosslinking agents. (A) Bio ink droplets deposited and fused with crosslinking droplets. Multiple layers are deposited (B) Layer fused with a UV light. (C) Crosslinking mist is applied to layers. (D) Layers are placed in a crosslinking solution. Image referenced from Gudapati *et al.*[39]

Acoustic Droplet Ejection:

One major flaw that occurs within nozzle-based bioprinting methods is that the pressure of forcing bioink through a nozzle places mechanical stress on cells, causing cell death, thus reducing the overall viability of the present structure. One method that avoids these disadvantages is acoustic droplet ejection (ADE). In ADE the bioink is placed in a reservoir and acoustic radiation is used to apply force to the bio-ink, generating droplets. Then the droplet is attached to a build platform that is placed above the bioink reservoir. In ADE the size of the droplet is inversely proportional to the frequency of the signal. A higher signal will equate to a droplet with a smaller diameter and vice versa.[40] While this method does have the safety of the cells placed at the forefront it is still a relatively unexplored method, having only been used to create 2D layers and has rarely been used for 3D structures.[40]

Micro-valve bioprinting:

Micro-valve is a bioprinting method that uses an electromechanical valve to generate droplets. As seen in Figure 11, micro-valve bioprinting involves the use of pneumatic pressure, a solenoid coil, and a valve. Pneumatic pressure is applied to the bioink using a gas regulator. A voltage pulse generates a magnetic field in a solenoid coil that opens the nozzle and

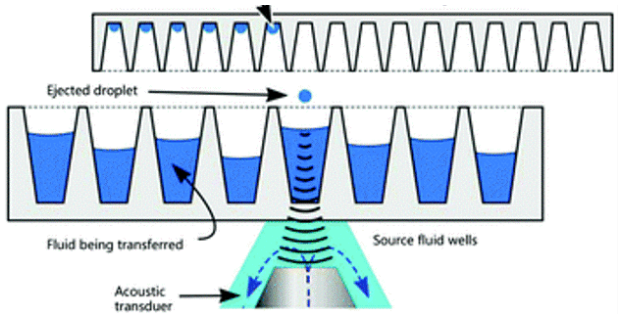

Figure 9: A schematic of the ADE printing process. The transducer (cylinder at the bottom) transmits an acoustic signal that travels upwards. The force generated from the sound generates a droplet that then travels upwards, sticking to the build platform shown at the top of the image. Image referenced from Guo *et al.*[41]

the bioink is then deposited.[41] Two unique factors controlling the deposition are the pressure generated by the gas regulator and the valve opening time.[41] Unlike other inkjet-based methods, microvalve bioprinters often contain multiple printheads, deposit bioinks in synchronization, and print close to 1000 droplets per second with 1-2 μm material thickness.41 Additionally, with a concentration of 1 mil cells/ml Ng and Yeong *et al.*[42] were able to achieve cell viability higher than 95%. However, this result should not be taken at face value since the purpose of the experiment was to determine the minimal cell concentration needed to achieve cell viability above 95%. Rather, in experiments not actively searching for high cell viability, it can be expected that the cell viability will be closer to 86%.[41]

Inkjet Bioprinting and its Sub-Methods:

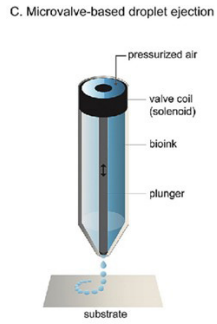

Figure 10: A schematic of a microvalve printhead is shown. Image referenced from Włodarczyk-Biegun *et al.*[43]

Inkjet bioprinting uses gravity, atmospheric pressure, and the fluid mechanics of the bio-ink such as the viscosity to eject droplets.[39]

Electrohydrodynamic Bioprinting:

Electrohydrodynamic (EHD) bioprinters function by using an electric field to pull the bio-ink through a nozzle and EHD is a subset of Inkjet Bioprinting. This benefits the cell viability as the bioink will not experience abnormally large pressure, preventing cell death. As a result of using electricity, the nozzle diameter of nozzles used in electrohydrodynamic printing has to be minuscule (≤ 100 μm) and the bioinks have to be highly concentrated.[39] The process of EHD is that exposure to an electric field causes mobile ions within a liquid to accumulate at the liquid surface. Eventually, a conical shape known as a

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Taylor cone is created and after the electric field exceeds its critical limit, the stress creates a droplet of bio-ink.[44]

Drop-on-Demand Bioprinting and its Sub-Methods:

Unlike electrohydrodynamic bioprinting, drop-on-demand bioprinting generates droplets of bioink by propelling the bio-ink through the nozzle. This is done by one of three mechanisms: piezoelectric actuators, thermal actuators, or electrostatic forces. For clarification, an actuator is any component that enables movement within a machine. Thermal actuators will simply convert thermal energy into motion. Piezoelectricity refers to the electricity that can accumulate within certain materials such as crystals, DNA, and proteins that are in response to mechanical stress. In simpler terms piezoelectricity refers to electricity that is the result of pressure and latent heat. Thus, piezoelectric actuators take the electric energy that can be generated from the bioink itself and can eject the bio-ink.

Piezoelectric Bioprinting:

Like EHD bioprinting, piezoelectric bioprinting utilizes electricity to generate droplets. Unlike EHD, piezoelectric actuators require crystals such as quartz to conduct electricity. To generate an electric current, the crystal is placed between two metal plates and once mechanical pressure is applied, a current can be forced out of the crystal. In addition to this method, electricity can also be applied to the crystal. This deforms the structure of the crystal releasing energy in the form of a sound wave.[45] In terms of generating a droplet, once the crystal is deformed, the fluid chamber is deformed as well,[39] resulting in a sudden change of volume. The result is that sound waves provide the needed pressure for the bio-ink to overcome the surface tension of the nozzle, delivering a droplet.[46]

Thermal Bioprinting:

Thermal bioprinting is somewhat like piezoelectric bioprinting as both processes require actuators. However, the overall process for thermal bioprinting is significantly more simple than piezoelectric bioprinting. In short, when voltage is applied, the thermal actuator heats the bio-ink. The heating of the bioink generates a bubble and once that bubble pops, pressure is generated which forces out a droplet of bio-ink. A particular concern of thermal inkjet bioprinting is that when the bioink is being heated up the heat will denature proteins and potentially kill cells.

Electrostatic Bioprinting:

Electrostatic bioprinters, like piezoelectric bioprinters, generate droplets by increasing the volume of the fluid chamber. Electrostatic forces refer to forces that can pull or push without physical contact. Unlike thermal and piezoelectric printers, electrostatic printers can eject highly viscous ink.[47] This separates it from other inkjet methods, which can only print low viscosity materials, making it a highly preferred method, given its high-resolution results.

Bio inks:

Due to the nature by which the bioink is deposited in droplet-based bioprinting the viscosity range of bioinks remains quite limited as the use of high viscosity bioinks can result in the lack of droplets. The viscosity range for droplet-based bioinks is 3.5-12 mPa/s.[48] Thus, droplet-based bioprinting is limited to low viscosity bioinks. Within this range, hydrogels remain a popular choice for bioinks. Specific substances include alginate,[49-51] fibrin,[52] collagen,[49] and gelatin.[47] The reason that droplet-based bioprinting requires such low viscosity bioinks is that if the bioinks are in high viscosity there is the potential hazard of the nozzles becoming clogged.

Disadvantages:

Despite its great advantages such as method variety, non-contact nature, and accessibility, droplet-based bioprinting has flaws that prevent it from being a perfect bioprinting method. One of the more prominent flaws is the restriction that is placed on the bioink variety as droplet-based bioprinting has a limited selection of only low viscosity bioinks, else the nozzles will end up clogging up. One theoretical disadvantage present in droplet-based bioprinting and specifically thermal bioprinting is the notion that the high heat (up to 300 degrees Celsius) of the nozzle can denature cells, decreasing cell viability during the printing process.[34] However, Cui *et al*.[53] found this belief to be exaggerated. Using Chinese hamster ovary cells, the reported cell viability was 89% and the temperature of the bioink only increased between 4 and 10 degrees Celsius. Thus, while the limitation of the bioinks can be limiting in the application of droplet-based bioprinting there are little to no more disadvantages besides the bio-inks, making it a very usable method.

Applications:

Despite the limitations, droplet-based bioprinting has been at the forefront of vasculature fabrication. Both Cui and Boland *et al*.[52] as well Yao *et al*.[49] were able to successfully generate vasculature using droplet-based bioprinting. Cui and Boland used fibrin as the bioink with human microvascular endothelial cells as the cell culture. Using thermal inkjet bioprinting they were able to achieve cell viability of 90%. While pores did develop during the deposition of droplets, it was observed that these pores were repaired 2 hours after being deposited. Finally, the fabricated microvasculature remained stable and retained its integrity for 14 days. While also generating microvasculature, Yao et al. used both different cell cultures and bioink. Instead of using human microvascular endothelial cells and fibrin Yao and Co. use HUVECs and alginate microspheres coated in collagen. Additionally, instead of using thermal inkjet bioprinting, Yao and Co. used an electrohydrodynamic printer to dispense the droplets. The generated vasculature was then inserted into mice and the synthetic vascular channels were able to fuse with hose vessels, undergoing vasculogenesis. These two studies do indicate that droplet-based bioprinting is a viable method for generating functional vasculature structures. Finally, droplet-based bioprinting has proven itself to be an easily accessible method as according to Bishop *et al*.[34] droplet-based bioprinting has the lowest cost between itself, extrusion-based bioprinting, and stereolithography. Some printers that can perform droplet-based bioprinting are the BIO X printer and the 3DDiscovery printer, priced at $40,000 and $200,000.

Stereolithography:

Recently, stereolithography apparatus (SLA) has been acknowledged as a bioprinting technique. While extrusion and drop-based bioprinting rely on a nozzle to precisely dispense droplets of cell-laden bioink, SLA uses a light source to selectively illuminate sections of a liquid bioink. The exposure to

the light source induces cross-linking within the vat of bioink, creating a 3D structure.[54]

Process and Components:

When generating a structure using SLA there is a certain amount of variation that can occur when it comes to the structural design of the printer. The methodology used in SLA is that a concentrated light source will be reflected off a mirror into a certain section of photocurable resin, hardening it. However, the position of this light source and mirror can vary as they can either be placed below the resin tank or above (Figure 11).[55] When placed above the resin tank, the light will harden the very top layer of the resin. From there the build platform lowers the completed layers as a recoater blade applies a fresh coat of resin to be hardened. This continues until the structure is completed. In contrast, when the light is underneath the vat of resin, the build platform will immerse itself into the resin and from there the process occurs but in reverse as the build platform will go up as more layers are hardened. When it comes to the light source used for the laser the most common option is to use ultraviolet (UV) light to harden the bio-ink. However, the use of UV light will induce cell damage.[56] To counter this, Wang *et al.*[57] were able to use visible light as the laser and achieved 85% cell viability for at least 5 days.

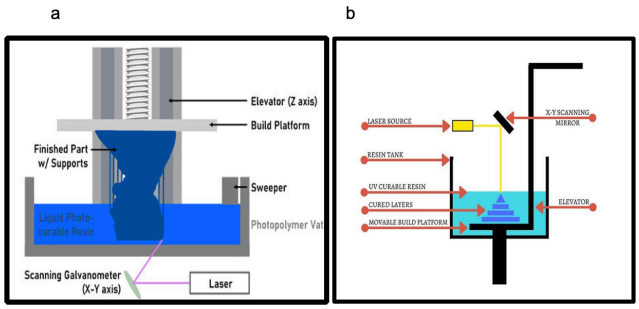

Figure 11: Two variations of stereolithographic printers are shown. (a) A printer with a laser beneath the vat. (b) A printer with a laser above the vat. Image (a) referenced from "All About Stereolithography 3D Printing".[58] Image (b) referenced from All3DP *et al.*[39]

Bio inks:

The bioinks used for SLA are generally derived from bioinks used for both extrusion and droplet-based bioprinting. However, to be applied in SLA these bioinks have to be modified with a functional group to allow for photo-crosslinking. A benefit of photopolymerization is that it can control the formation and structure of hydrogels spatially and temporally.[56] While UV light is the main method of initiating photopolymerization it has been found that when replaced with visible light, the hydrogel has higher cell compatibility as well as a more uniform structure due to increased penetration depth.[56]

To create a photo ink both Lam *et al.* and Thomas *et al.* combined GelMA and Hyaluronic acid, both of which can be used in extrusion-based bioprinting, and combined with Lithium phenyl-2,4,6-trimethylbenzoyl phosphinate (LAP), a photoinitiator, within phosphate-buffered saline.[60,61] By using a photoinitiator and extrusion-based bioinks, a photoink was synthesized. As shown by these two studies, a photoinitiator is vital to the creation of a photoink. The photoinitiators used most extensively in bioprinting can be classified as free radical photoinitiators. Free radical photoinitiators produce radicals (atom, molecule, or ion with an unpaired valence electron) when exposed to radiation (UV or visible light). Two subcategories of free radical photoinitiators are type 1 and type 2. Currently, the most common option for type 1 photoinitiators is LAP. In contrast, type 2 photoinitiators have a plethora more options such as ruthenium pyridine complex, EY, and camphorquinone.[56]

Disadvantages:

While SLA does have many advantages such as great resolution and low printing time it does have some inherent disadvantages that prevent it from being a perfect bioprinting method. The biggest drawback that SLA faces is the fact that it must use a UV laser to harden the bio-ink, forming a 3D structure. The use of UV light damages the DNA of cells, decreasing their viability. However, this disadvantage is quickly losing its significance as visible light is becoming a more viable option for SLA as demonstrated by Wang *et al.*[57] Another disadvantage that comes with SLA is the extremely long post-processing time. This stage refers to the time when cells are cultured after the initial structure is formed. For example, Grigoryan *et al.* had to culture the hardened cells for up to 15 days.[54] Additionally, Thomas *et al.* cultured their cells for up to 28 days.[60] Thus it can be seen that the high resolution and accuracy seen in SLA can only be achieved after many days of post-processing. Finally, SLA is not the best method for creating multicellular structures.[56] Thus creating complex organs like a heart or liver using SLA is not the most efficient and viable method.

Applications:

While stereolithography does have its disadvantages, the results that can be achieved with SLA more than make up for the disadvantages. For one, SLA has a high resolution of 50 μm[56] with cell viability anywhere between 85% and 90%.[60] In terms of creating vascular structures, SLA has been able to produce viable structures as Thomas *et al.* were able to produce vascular channels using HUVEC and used a degradable photo ink to achieve maximum cell viability of 90% and the endothelial lined channels remained stable for all 28 days they were examined.[60] Stereolithography has no limitations on both bioink viscosity and cell density.[48] Finally, the cost for SLA is quite affordable as Bishop *et al.*[34] described it as "medium". Examples of stereolithography bioprinters include the NGB-R bioprinter and the BIO X printer, priced at $300,000 and $40,000. With all the benefits of stereolithography, there is no doubt that this method holds great potential.

Future Perspectives and Current Limitations:

Currently, bioprinting has unlimited potential and a variety of applications such as organ transplants, artificial meat, and drug testing. However, this subject is still very novel and has some challenges that need to be addressed. One of the challenges that currently exist, according to Zhang *et. al* is the creation of multilayered vascular networks.[63] Vascular structures such as veins and arteries have multiple layers of tissue such as the tunica adventitia and the tunica intima and given that each of these layers has its distinct functions yet also interacts with one another bioprinting must make further advan

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cements to where a vascular structure can be generated with distinct layers that can also interact with one another. This will further mimic how blood vessels operate within a living organism. One issue with bioprinting that is frequently brought up is the lack of materials within bioink. Researchers are often only limited to synthetic or natural polymers, each with its advantages and disadvantages. Yet, as previously stated, this issue can be solved with the use of hybrid hydrogels which contain the structural integrity of synthetic polymers and the increased cell communication of natural polymers. Finally, arguably the biggest challenge concerning vascular structures specifically in printing resolution. According to Bishop *et. al*, the diameter of a capillary can be as small as 3 μm whereas the highest printing resolution of a bioprinter is 20 μm.[34] What this means is that if a capillary has to be enlarged by nearly 6 times its diameter it means that the rest of the organ must also be enlarged to compensate, resulting in these engineered organs being unusable for human operation. Thus, the priority for bioprinting vascular structures is to increase the printing resolution, and one optimal way to do this could be by combining printing methods, using the high resolution of stereolithography with the speed of extrusion-based bioprinting could greatly increase efficiency while still delivering a viable product.

Table 1: A summary of the differences, advantages, and disadvantages of each of the three printing methods is shown.

Bioprinting Method	Extrusion Based	Droplet-Based	Stereolithography
Bio inks	Hydrogels, Spheroids, Microcarriers, Decellurized ECM	Hydrogels are composed of alginate, fibrin, collagen, and gelatin.	Hydrogels combined with photoinitiators. Ex. GelMA combined with Lithium phenyl-2,4,6-trimethylbenzoyl phosphinate.
Cell Viability	40- 86%[11]	70-90%[39]	85-90%[61]
Disadvantages	The use of a nozzle increases shear stress on cells, decreasing cell viability. This can be mitigated by using hydrogels with a lower viscosity as bioink.	The variety in bio-inks is limited, restricted only to low viscosity bioinks.	The use of ultraviolet light damages cell structure and the post-processing time is quite long, decreasing efficiency.
Maximum Cost of Printer (excluding cost of materials)	$150,000	$200,000	$300,000
Process of Printing	The bio-ink is continuously extruded through a nozzle in layers.	A nozzle generates droplets of bioink that are then placed onto a surface and the droplets then fuse forming a layer.	A light source is used to harden certain areas of a vat of photocurable bioink. After a layer is finished a recoater blade applies a fresh layer of ink to be hardened.
Cells Used	Human umbilical vein endothelial cells [15], human umbilical vein smooth muscle [36], human mesenchymal stem cells [37]	Human mesenchymal stem cells [40], human induced pluripotent stem cells [39], fibroblasts [51,64]	Fibroblasts [58], human umbilical cord endothelial cells [61], lung adenocarcinoma cells [55]
Dimensions	Outer Diameter: 1449 ± 27 μm [36]	Outer Diameter: 4-5 mm [39]	Outer Diameter: 360-720 μm [61]

Conclusion

Bioprinting vasculature remains one of the major challenges that researchers face today when creating synthetic organs. While the fabrication of blood vessels has been successful it has been seen that these synthesized vessels are unable to remain viable for any period longer than one month. This limits their use *in vivo* long-term as they cannot be relied upon to remain functional. Additionally, both veins and arteries are not composed of just one layer of cells. Therefore, another roadblock to the creation of implementable vasculature is the development of multicellular bioprinting, where multiple cell cultures can be placed in a bioink and grow both cohesively and independently, forming layers. This process is already in the works as both multi-material stereolithography and extrusion-based bioprinting have been developed and tested. To create these vasculatures, three bioprinting methods were discussed: Extrusion-based, droplet-based, and stereolithography. As shown in Table 1, while each method does have its drawbacks, all three methods have been able to generate vasculature. In terms of choosing a method for a study, there are multiple factors to consider such as resolution, bio-inks, and cell viability. For resolution and viability, stereolithography is by far the best method and has an affordable cost. Its lack of bio-ink viscosity and cell density only serves to increase its benefits. When on a low budget, droplet-based bioprinting will serve as the best method as out of the three methods it has the lowest cost and has high cell viability at the cost of limited bio-inks. While extrusion-based bioprinting is the most used method and its bioink variety does make it a serviceable method, the lower cell viability makes it much more of a risk to take on. Thus, through our findings, we have found that stereolithography shows the most potential in the creation of functional vasculature.

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