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Review

Vascularization in 3D printed tissues: emerging technologies to overcome longstanding obstacles

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Abstract: This review paper endeavors to provide insights into the emergence of 3D bioprinting as an alternative to longstanding tissue fabrication techniques primarily through an overview of recent advances in bioprinting vascularized tissues. Bioprinting has promise in resolving many issues that persist within tissue engineering including: insufficient perfusion of nutrients to tissue constructs, high rates of cell necrosis, and lack of cell proliferation and proper differentiation. These issues stem from a lack of proper angiogenesis, a primary challenge that remains to be overcome in tissue engineering. This review will discuss emerging 3D bioprinting techniques (such as inkjet printing, extrusion printing, and stereolithography, among others) that have been specially adapted to enhance and improve the vascularization process. Compatible bioinks are also discussed as they are vital to the 3D bioprinting process by allowing for the building of matrices that encourage vasculature to develop, survive, and prosper under physiological flow rates. Currently, these 3D bioprinting techniques have succeeded in increasing the long-term viability of thick tissues, generated luminal structures needed for vascularization, and allowed for differentiation factors to reach cells deep within thick constructs (~1 cm). While great progress has been made, 3D bioprinting continues to have deficits in high-resolution printing, viability at prolonged time scales and larger thicknesses required for organ transplantation, and the mechanical stability needed for long-term organ functioning. Nonetheless, the recent developments in the vascularization of tissues through bioprinting techniques are paving the way for lab-grown tissues and organs, which could have uses

in transplants, *in vitro* drug testing, and enhancing the current knowledge of organ function.

Keywords: Vascularization; tissue engineering; 3D bioprinting; scaffolds

Abbreviations: ADSC: Adipose-Derived Stem Cell; CHO: Chinese Hamster Ovary; DLP-SLA: Digital Light Processing-Based Stereolithography; DMD: Digital Micromirror Device; ECM: Extracellular Matrix; FGF: Fibroblast Growth Factor; FRESH: Freeform Reversible Embedding of Suspended Hydrogels; GelMA: Gelatin Methacryloyl; hADSC: Human Adipose-Derived Stem Cells; hiPSC: Human Induced Pluripotent Stem Cell; HMVEC: Human Microvascular Endothelial Cell; HNDF: Human Neonatal Dermal Fibroblasts; HUVEC: Human Umbilical Vein Endothelial Cell; IAMF: In-Air Microfluidic; iPSC: Induced Pluripotent Stem Cell; ITOP: Integrated Tissue-Organ Printer; MME: Molten Material Extrusion; MSC: Mesenchymal Stem Cell; PCL: Polycaprolactone; PCL-PEG-PCL: Polycaprolactone-Poly(Ethylene Glycol)-Polycaprolactone; PDGF: Platelet-Derived Growth Factor; PEG: Poly(Ethylene Glycol); PLGA: Poly(Lactic-co-Glycolic Acid); Plu-MP: Pluronic Monocarboxylate; SL: Stereolithography; TGF: Transforming Growth Factor; VE-Cadherin: Vascular Endothelial Cadherin; VEGF: Vascular Endothelial Growth Factor; 3DEAL: 3D Electrophoresis-Assisted Lithography

1. Introduction

Despite advances in medical technology, one of the leading causes of death in hospital intensive care units is loss of function of tissue or organs. In a multicenter cohort study of 96 intensive care units, at the time of death, 84% of patients had one or more organ failures and 89% required support of at least one organ [1]. A promising solution to the lack of sufficient organs and the potential for immunorejection is the creation of artificial organs. However, despite current advances in the field, artificial organs lack proper biocompatibility and functionality to warrant use. In order for tissue to successfully survive and function, it has to be less than 100–200 μm thick or it must have vasculature [2,3]. Oxygen, nutrients, and waste must be able to be transported to and from the tissue to ensure viability and prevent the development of necrotic tissue [4–7]. In search of a viable solution, scientists have turned to tissue engineering, which is a field that attempts to create functional organs *in vitro* and regenerate damaged tissues and organs without risk of immune rejection [8,9]. Due to the limited availability of donor tissues and organs for transplantation, the demand for engineered tissues has rapidly increased [10]. Despite the potential of tissue engineering, applications have been limited to mostly skin and cartilage replacements because of the issues that persist with vascularization [11].

Tissue engineering often involves the development of cell scaffolding. Cell scaffolding refers to creating structures from suitable biomaterials or biomimetic materials that allow for cell attachment and tissue development [9,12,13]. These scaffolds mimic the function of a native tissue's extracellular matrix (ECM) [13]. Scaffolds can be synthetic, natural, or a hybrid of the two. Natural scaffolds such as collagen and gelatin are highly compatible and biodegradable [14], but, they are difficult to reproduce and standardize and have poor mechanical properties compared to synthetic polymers [12,15]. Synthetic scaffolds can be easily reproduced and standardized, but typically lack

the necessary precision and biocompatibility. Hybrid structures provide reinforcement to weaker, natural scaffolds, but the tensile strengths and compatibility of these scaffolds are still not comparable to native vessels [13,16]. Although scaffolds are engineered to promote vascularization, they often contain imprecisions such as partially connected or contorted pathways that impede the nutrient supply and reduce viability [7]. Additionally, scaffolds need to naturally have or be engineered to have appropriate surface conditions for cell adhesion and proliferation, limited toxicity to prevent uncontrolled cell death, adequate porosity to encourage the transport of nutrients and waste as well as cell motion, and mechanical strength matching the targeted tissue [17,18]. Sacrificial materials can be employed in tissue engineering and involves molding cells around a temporary or sacrificial component that can either be physically removed or chemically dissolved to leave the biomaterial. While this can result in higher precision in network architecture, this removal process can be non-trivial as the cells risk both chemical damage and harm from residual materials [19].

Another method of modeling the ECM is decellularization, which takes advantage of the naturally occurring ECMs and vascularization in tissues. This process uses physical or chemical agents to remove the cellular material from tissue, leaving only the structural matrix [16]. Unfortunately, decellularization is a compromise between maintaining the ECM (preserving mechanical properties) and removing cellular material (eliminating immune reactions) [11,16]. Other techniques, such as rotating and perfusion bioreactors, are capable of providing the growing scaffold tissue with nutrients, waste removal, and mechanical stimulation all of which aid in promoting vascularization but fail to fully address the issues surrounding vascularization [20]. The nutrient flow from bioreactors, for instance, occurs along the path of least resistance, resulting in problems of heterogeneity in tissue development [2].

The majority of these methods, while applicable, are still not viable for practical usage due to issues regarding precision, form, and the generation of vasculature. Three-dimensional (3D) bioprinting is an emerging field that could one day resolve many of the issues that persist within tissue engineering. As discussed earlier, tissue engineering has been plagued with difficulties including: designing and maintaining microenvironments, recreating the ECM, and precisely controlling the deposition of cells to encourage differentiation and proliferation [21]. These processes are all vitally important to angiogenesis. 3D bioprinting involves the precise layering of cells, scaffolds, and bioactive molecules, which means it has potential to be one of the most viable approaches due to its precision and reproducibility. Although the bioprinting technology is still under development, it has the capacity to overcome the longstanding problems of toxicity, lack of precision, and other weaknesses that more established methods face [19].

This review will cover types of bioinks employed in 3D bioprinting and a variety of 3D bioprinting methods that have been developed in recent years to overcome the prevalent challenges in the vascularization of tissue constructs. The goal of this review is to provide the reader with a deeper understanding of the pros and cons associated with the techniques previous researchers have applied to tissue engineering. A brief overview of the process of vascularization will be given in order for the readership to better understand the process and why researchers have found it so difficult to reproduce *in vitro*. The cells commonly used in bioinks will be examined to emphasize their roles and shortcomings in the vascularization process. This will be followed by a discussion of the current 3D bioprinting techniques and how well they are able to recapitulate the process of vascularization *in vitro*. While vascularization in tissue engineering is a broad and rapidly developing

field, we hope this review paper will serve to incorporate and synthesize some of the most recent and significant work that has been done using an assortment of 3D bioprinting techniques.

2. Vascularization of tissue constructs

2.1. Process of vascularization and its role in organ and tissue function

Blood vessels are formed *in vivo* by two mechanisms, namely vasculogenesis and angiogenesis, which share the same purpose of creating new blood vessels but differ in the process by which the new vessels are constructed [22]. While the former is largely restricted to embryogenesis, the latter takes place not only during embryonic growth and development, but also during the adult lifespan. Angiogenesis refers to the process of creating new blood vessels from existing ones [23]. The process has been described to take place by means of two mechanisms: intussusception and sprouting [22–24]. The former occurs when interstitial cellular columns get inserted into the lumen of already existing vessels, whose growth results in the remodeling of the local vascular network. Sprouting consists of a vessel detaching from its environment through the dissolution of its basement membrane and the ECM surrounding it [25].

The need to include vascularization in any implantable tissue originates in its essential role for tissue growth and functionality. Therefore, enabling the presence of a biomimetic vascular network within the implantable construct is a key factor for the successful implantation of a biofabricated tissue/organ, as long as anastomosis with the host's vasculature is performed [7]. Since the dimensions of constructs presented in research papers are usually relatively low compared to those of real organs [26], the readers are advised to look at the advancements presented in this review in light of not only functionality and performance, but method scalability as well. More details about the limitations and potential improvements will be discussed in Section 4.

One challenging aspect of creating vascular tissues or vascularized organs resides in the complexity of the blood vessel structure and its hierarchical network. Blood vessels display a concentric arrangement of three layers, each with different properties and functions, connected by additional layers of ECM [27]. Their composition and biological make-up have been studied at length [23–26,28–31] to reveal their architecture which is described as follows. The innermost layer, the intima, is made of endothelial cells whose cohesion is supported by intercellular adhesion molecules, such as vascular endothelial cadherin (VE-Cadherin). This is followed by a basement membrane which contains both laminin and type IV collagen as well as an elastic membrane. The second layer consists mainly of smooth muscle cells, dispersed in a matrix of type I and type III Collagen, in a spiral formation along the vessel axis. The second layer also has an elastic membrane that connects it to the next layer. The outermost layer, adventitia, consists of fibroblasts in a collagen matrix and usually contains nerves. Moreover, from a functional point of view, vessel integration with its environment is realized by means of adhesion molecules such as integrins, without which vessel stability would be compromised [32]. The vasculature of organs, also known as microvasculature, follows a similar organization into arterioles and venules, where the layers are present but register lower thicknesses [33]. Capillaries also develop that consist of only an endothelial layer and a basement membrane, to allow for rapid diffusion of nutrients to cells [29]. This level of complexity has proved difficult to replicate in many tissue engineering processes and is further complicated by the cell types required for the formation of vasculature.

2.2. Bioinks-cell types for obtaining vascularized tissues

3D bioprinting stands out as a powerful fabrication method due to its technological advantages over earlier presented techniques. An essential part of 3D bioprinting is developing and refining bioinks. Bioinks are mixtures of biocompatible materials and viable cells that are deposited into desired configurations by means of the chosen bioprinter [34]. They are required to possess certain physicochemical properties, such as printability that is determined by the viscosity, density, surface tension, crosslinking properties, stiffness, and degradation profiles that correspond with the application they are intended for [35]. The most important property, perhaps, is biocompatibility to ensure that the viability of cells, their cell-cell interaction, proliferation and organization, and where necessary, differentiation are preserved or readily enabled in the 3D construct [36].

Most bioinks aiming to suit applications such as organ biofabrication, take into account the need to obtain macro- and microvasculature and as such they contain endothelial cells. As previously discussed, endothelial cells comprise the innermost vascular layer. While animal cells such as bovine aortic endothelial cells (BAEC) [37,38], mouse fibroblasts [39], and Chinese hamster ovary (CHO) cells [40] are used as proof of concept, one human cell line commonly used is that of the human umbilical vein endothelial cells (HUVECs).

2.3. Endothelial cells

Multiple support materials have been used with HUVECs, including hydrogels [6,41,42], decellularized ECM [36], and cell platelets or constructs such as tissue strands [43]. Some notable examples include HUVECs embedded in alginate-gelatin methacryloyl (GelMA) scaffold, where the cells self-organized into a confluent layer surrounding the 3D-bioprinted microfibers after 15 days of *in vitro* maturing [44]. Moreover, HUVECs were employed in a layer-by-layer deposition method allowing for the formation of three concentric layers, which conveyed better mechanical properties and increased protection against potential thrombogenicity caused by disruption of an exposed endothelial layer [44]. However, this technique might be more suitable for obtaining vascular grafts and/or macro-vasculature, since the results indicated difficulty in obtaining vessels with diameters less than 6 mm, which is the upper limit of an aortic vessel. Figure 1 showcases another example of bioprinted constructs that use HUVECs [6].

Human microvascular endothelial cells (HMVECs) have also been utilized along with fibrin, as a bioink, to reproduce microvasculature. It has been shown that after 3 weeks, the cells were capable of self-aligning in a fibrin channel and had formed a confluent lining which possessed a tubular structure similar to human vasculature [45].

Although endothelial cells are able to create the lumen of the vessels, constructs that rely solely on the guided self-organization of these cells have been proven to lose cell viability in a relatively short amount of time [46]. In fact, early studies have shown that the blood vessel maturation process is strongly dependent on the interactions between smooth muscle cells and endothelial cells, as they control their respective quiescent phenotypes [47]. To this end, Lee et al. [48] used a fugitive-ink approach with endothelial cells alongside pericyte-like supporting cells and attained angiogenesis by sprouting, gaining increased permeability of the construct after 9 days (Figure 2). Jia et al. [49] used a HUVEC-mesenchymal stem cell (MSC) mixture in a blend of GelMA-alginate-poly(ethylene glycol)-tetraacrylate (PEGTA) and generated perfusable constructs, which showed improved

mechanical properties. Their constructs displayed markers of angiogenesis and smooth muscle cell differentiation indicating early-stage maturation of the vessel, similar to natural vasculature. However, after 21 days of culture, the compressive moduli of the construct sharply decreased, and the thin walls were found to be mechanically unstable [49].

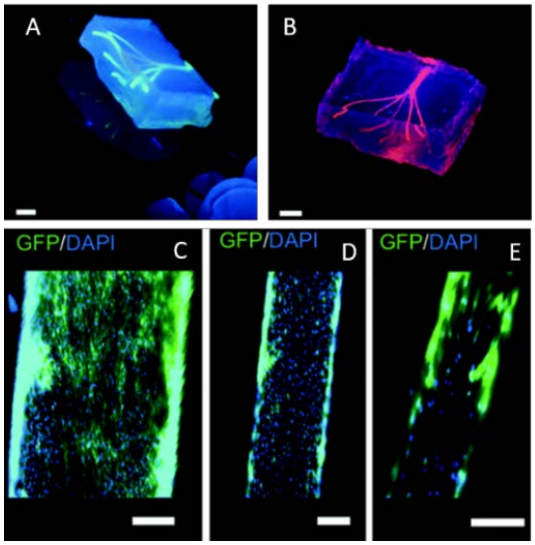

Figure 1. Top: 3D branching agarose templates embedded in (A) a GelMA hydrogel construct and (B) the resulting network after perfusion with fluorescent microbeads. Bottom: Proliferation of HUVECs in (C) 1000 μm, (D) 500 μm, (E) and 250 μm wide channels after 7 days of proliferation (scale bars =250 μm). Adapted figures from Bertassoni et al. [6]. Reprinted with permission.

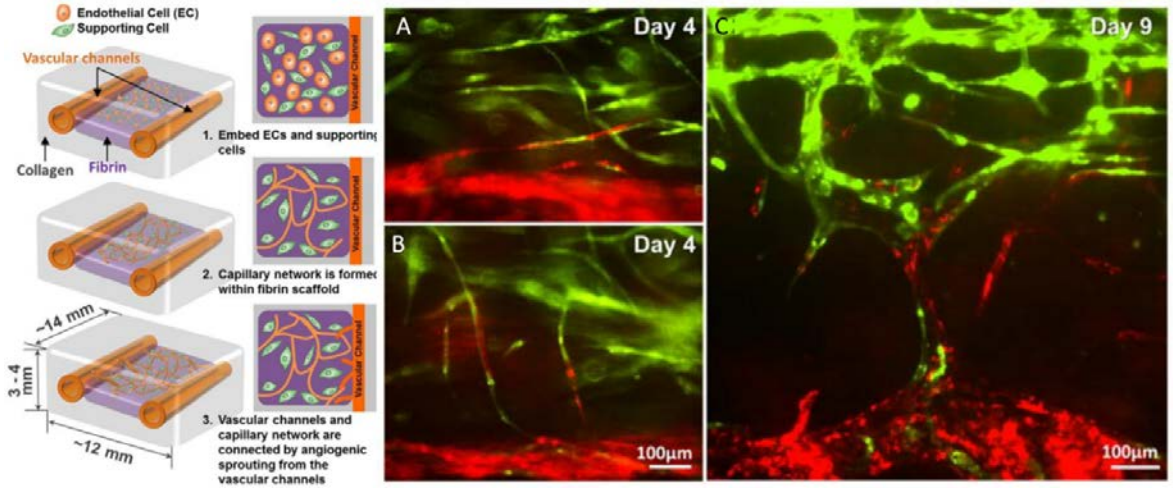

Figure 2. Left: Schematic of a vascularized tissue construct obtained by using a fugitive ink inside a collagen support structure along with deposition of endothelial cells and fibroblasts. The formation of a capillary network was followed by sprouting from the vascular channel into the capillary network in 9 days. Right: (A–C) Images showing the integration of large sprouts from the vascular channel (red) into the capillary network (green) at various stages within their maturation. Adapted from Lee et al. [48] and reprinted with permission.

2.4. Stem cells

Efforts focused on employing stem cells for tissue and organ fabrication are mainly driven by the ability to obtain a multitude of differentiated cells which allows for a more accurate representation of real tissue. Human cardiac progenitor cells, MSCs, and a mixture of both were bioprinted using a decellularized ECM by Jang et al. [50]. The angiogenic response from the mixture bioprinted into a patterned arrangement was the development of neovessels with diameters of approximately 50 μm [50]. Additionally, a study using human adipose-derived stem cells (hADSC) loaded into an alginate-based bioink proved that the viscosity of a substrate was important for cell viability, proliferation rate, and spread [51]. The viscosity can in turn allow for modulation of differentiation. Also among potential stem cell-based bioinks, human nasal inferior turbinate tissue-derived MSCs were encapsulated in a silk fibroin-gelatin hydrogel and showed long-term survivability and viability *in vitro* (up to 1 month), as well as differentiation potential [52].

One challenge associated with stem cells is controlling their differentiation, usually accomplished through biochemical cues. Moreover, attention must be paid to the bioprinting process itself, to ensure the full capabilities of the cells are maintained after the process and are not influenced by other factors, such as mechanical, physical, or other cues [53]. Another issue is with regards to the availability of stem cells. Sourcing cells from the patient would be ideal as the working principle is that organ bioprinting should be done with autologous cells, to avoid immune responses and/or risk rejection of the construct. However, as adult stem cells tend to be tissue-specific and non-abundant [54], this might become a limitation. One direction that could address this issue is the use of induced pluripotent stem cells (iPSCs) which are adult somatic cells reprogrammed back to a pluripotency stage [55].

2.5. Induced pluripotent stem cells

Although rather sparse at the moment, the intersection of the two fields of bioprinting and iPSCs is populated by a few notable advances. In one study, iPSCs were embedded into a thermoresponsive polyurethane hydrogel and deposited layer-by-layer in a feeder-free method [56]. This technique could contribute to the scalability of bioprinting with iPSCs, to reducing costs, and to preventing contaminations by not using different cell lines than the feeder layer to prevent the premature differentiation of the iPSCs.

As an example of a more specific application in tissue engineering, iPSCs were bioprinted within an alginate-based bioink to obtain a “mini-liver” [57]. Cell viability was relatively low but remained stable with time, without any significant changes after 21 days of study. The analysis of markers of differentiation showed that the cells were, as intended, hepatic in nature after 23 days of allowing differentiation *in vitro*. Another group used hiPSC-derived hepatic cells together with endothelial- and mesenchymal-originated supporting cells in a blend of GelMA and glycidal methacrylate-hyaluronic acid hydrogels, to mimic the native architecture and cell composition of the hepatic environment. The cells maintained their viability in a stable construct after 10 days and formed functioning spheroids, showing a level of organization indicative of potential vascularization along the hydrogel lines [58] (Figure 3). Therefore, although this new avenue currently presents several limitations, such as relatively low viabilities of cells in the bioprinted constructs, long periods of times for obtaining cells and subsequently for maturing the organ, etc., it constitutes great promise for the future of 3D bioprinting as our knowledge and technology advance.

The importance of selecting biocompatible cells for tissue engineering applications cannot be understated. With the plethora of 3D bioprinting technologies available, which will be discussed in the following section, a careful selection of cells that can undergo chemical or physical perturbations must be done. Several prevalent sources of chemical or physical damage are: high levels of Pluronic F127 (discussed more later) leading to cytotoxic effects, encapsulation of cells in hydrogels causing osmotic damage, and the removal of some sacrificial molds requiring the use of organic solvents with cytotoxic results [6,59,60]. The 3D bioprinting techniques used should seek to minimize these negative environmental conditions, among others, that cells might be subjected to prevent altering their characteristic behaviors.

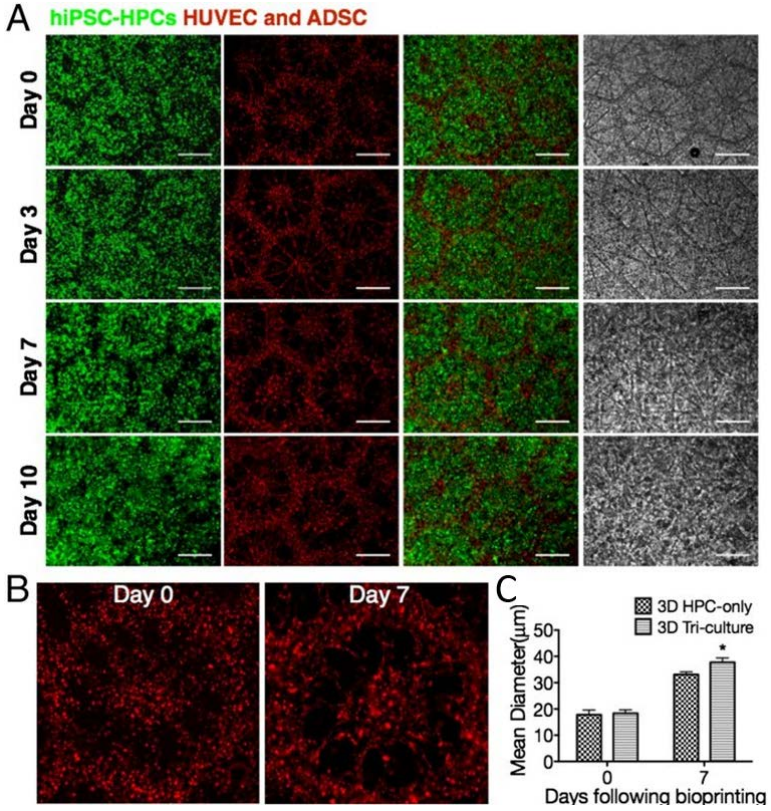

Figure 3. (A) Fluorescent images of a bioprinted construct over the span of 10 days. (B) Fluorescent images of the cell arrangements. (C) Bar graph shows the mean diameters of the spheroids within both HPC-only constructs and triculture constructs on day [58]. Reprinted with permission for non-commercial and academic use.

3. Current 3D bioprinting techniques

Ideally, an artificial tissue would contain not only various cell types characteristic within it but would also have a vascular network allowing for efficient and rapid diffusion of nutrients and oxygen, and control of blood flow. Desirably, this innervated, robust structure would be readily incorporable into the host's own structures. In practice, given the difficulty of printing submicronic capillaries [61], not uncommon in highly vascularized organs, such as kidney or liver, hybrid techniques have been considered [42]. They are mostly based on the principle of the creation of a

macrovasculature, and inclusion of factors to guide angiogenesis and spontaneous formation of capillaries to interconnect, when their fabrication constitutes a technological challenge [45,62,63].

Approaches to induce vascularization in implanted tissues/organs include building constructs enriched in pro-angiogenic factors such as vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF), platelet-derived growth factor (PDGF), or transforming growth factor (TGF) either on their own or co-delivered to support the creation of new vessels by the host [64,65]. In other approaches, researchers aim to create pre-vascularized tissue, obtained by encapsulation of endothelial cells and other supporting elements *in vitro* prior to implantation [63,65,66]. While obtaining bioactive inks containing pro-angiogenic factors (such as VEGF or FGF) is more straightforward, pre-vascularized constructs show better anastomosis [67].

3.1. Sacrificial techniques

Carbohydrate glass was recently found to be a biocompatible sacrificial material for use in 3D bioprinting by Miller et al. [59] and others have further expanded upon this approach [68,69]. This technique was developed to overcome two main issues in engineering vascular networks: having a sacrificial material that is stiff enough to support a 3D lattice and being readily dissolved by cells [59,69]. 3D printing of carbohydrate glass is a multifaceted tool for tissue engineering because of its ability to be patterned layer-by-layer or in 3D freeform paths [69]. The use of a 3D printer with thermal extrusion of the carbohydrate glass allows for reproducible geometries and configurations to be made [59] that can be freestanding [69]. Mirabella et al. [68] further used this technique to 3D-print vascular patches for grafting onto ischemic tissue to restore perfusion and rescue damaged muscle/tissue. Their results showed not only that the vessels of the patch became integrated with the host but that enhanced angiogenesis was observed in the ischemic tissue [68]. This research could offer a viable alternative to treating ischemia in patients using 3D-printed vasculature. Gelber et al. [69] designed a high-precision 3D printing approach which extended direct-write assembly of isomalt to very intricate and freeform geometries. In this approach, the isomalt melt was extruded through a nozzle and solidified upon extrusion at ambient conditions. This 3D space translation promoted the fabrication of sparse, freestanding networks of cylindrical filaments, and showed potentials in additive manufacturing of carbohydrate glasses in tissue engineering and microfluidics.

Hydrogels, as mentioned earlier, have also been another route used to enhance the vascularization of tissue constructs. Typically employed hydrogels include: fibrin, gelatin, alginate, and agarose, among others [6,15,60,70]. Kolesky et al. [60] used GelMA, a form of denatured collagen, to compose the ECM and Pluronic F127 to fabricate the vessel structure in 3D constructs. Pluronic F127 ink undergoes thermally reversible gelation, which facilitates its bioprinting and also its removal from the tissue construct once its structural purpose has been fulfilled. Under 4°C the Pluronic F127 becomes liquid while the GelMA remains stiff [60]. Recently, success has been achieved with bioprinting vascularized tissue with thickness greater than 1cm using the same technology [21], where HUVECs, human neonatal dermal fibroblasts (HNDFs), and human bone marrow-derived MSCs were co-cultured (Figure 4). The culture time was in excess of 6 weeks whereas many earlier attempts could only be cultured up to 14 days. Additionally, Kang et al. [10] developed an integrated tissue-organ printer (ITOP) that used a bioink based on polycaprolactone (PCL), cell-laden hydrogels, and Pluronic F127 as fugitive ink. They bioprinted

bone, ear, and muscle structures, which were implanted *in vivo* and showed evidence of vascularization without necrosis [10].

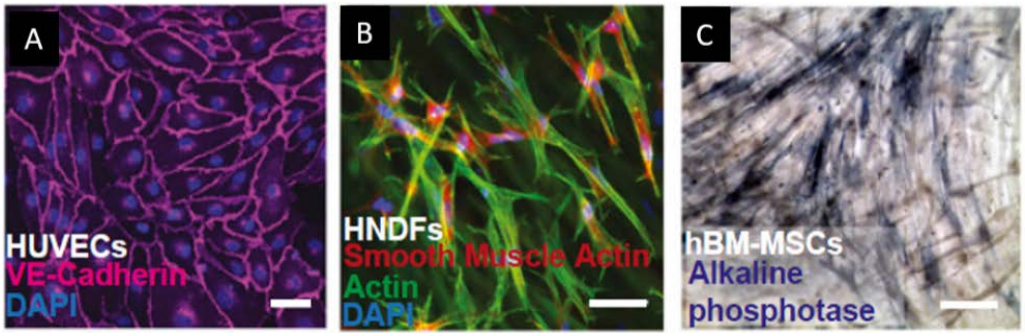

Figure 4. The gelatin-fibrin matrix supports multiple cell types including human umbilical vein endothelial cells (HUVECs), human neonatal dermal fibroblasts (HNDFs), and human bone marrow-derived mesenchymal stem cells (hMSCs), scale bars: 50 μm) [21].

In comparison, Bertassoni et al. [6] found that several types of hydrogels could be cast onto a 3D-bioprinted agarose template and photo-crosslinked through UV light exposure (Figure 5). One novel aspect of their research was the finding that agarose fibers did not bond to the hydrogels which enabled their removal from the template leaving behind perfusable microchannels. This technique eliminated the need to dissolve the template or to encapsulate cells. Bioprinting the fibers individually allows for the inclusion of multiple sizes of vessels (250, 500, and 1000 μm) in the same construct in which HUVECs could then be cultured (Figure 1). There are several issues with this method including that individually aspirating each microchannel could prevent closed-loop networks and as the size of the construct increases the path length of the UV light increases which could lead to variable crosslinking throughout the construct [6]. While different techniques and materials were used, both researchers saw the value in crafting hollow, perfusable channels through the use of removable materials encased in the ECM.

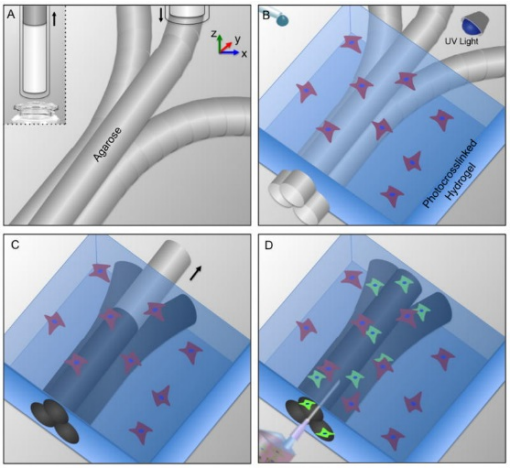

Figure 5. A representative schematic of the bioprinting technique used by Bertassoni et al. [6]. A template of agarose fibers was removed to achieve the formation of microchannels. Reprinted with permission.

Freeform reversible embedding of suspended hydrogels or "FRESH" bioprinting takes advantage of the thermoreversible properties of hydrogels to serve as supports during the synthesis of vasculature. The bioprinted hydrogels were deposited into a thermoreversible gel bath at 22 ± 1℃. The gel bath acted as a Bingham plastic providing little mechanical resistance to the moving nozzle but enough rigidity to the deposited hydrogel that it retained its shape and placement. The gel bath was subsequently melted away at 37℃. The ability to freeform-bioprint structures eliminates limitations of previous layer-by-layer deposition techniques and allows for more variation in synthesized structures [71]. More recently, Suntornnond et al. [72] were able to develop a hydrogel that could be bioprinted at 30 ± 3℃, considerably less harsh temperatures than earlier attempts which used bioprinting temperatures as high as 80℃ [6] or as low as -28℃ [15]. Additionally, their technique made use of a bioprinted and support material which were both in the gel phase. Pluronic monocarboxylate (Plu-MP) and gelatin GelMA were combined to create the hydrogel used for bioprinting, Plu-GelMA. Pluronic F127 was used as the support material. This method was able to support the viability of HUVECs for 10 days [72].

3.2. Scaffold-based and scaffold-free techniques

Several 3D bioprinting innovations over the last few years have paved the way for creating vascularized tissue constructs and 3D organs. Lee et al. [73] were able to construct perfused vascular channels using 3D-bioprinted thick collagen scaffolds. The process of bio-fabrication used necessitated constant perfusion but allowed for many cell types to be seeded into the porous matrix to form the desired tissue structures. This technique used a layer-by-layer approach beginning by printing a collagen precursor into the bottom of a flow chamber. By using a perfusion setup, the HUVECs along the channel subjected to a flow were found to elongate along the channel in the direction of shear and become smoother and straighter with no branching observed. The perfusion flow allowed for a cell viability of > 90% in the first three days with a maximum survival distance of 5mm from a channel. The main issue with this technique was that cell survivability was limited to 2 weeks [73].

Zhao et al. [15] investigated ways to improve upon the thin tissues cultured *in vitro*. They noted that successful organ manufacturing techniques required a combination of natural ECM and synthetic polymers to enhance the stability of the cell environment. Their experiments focused on ADSCs as they are easily harvested from patients and have minimal immune rejection potential. Synthetic poly(lactic-co-glycolic acid) (PLGA) was squeezed out of a syringe to form interconnected channels through a layer-by-layer deposition technique at -28℃. The minimum achievable resolution of the deposition technique was found to be 10 μm. It was found that the maximum thickness of the fibrin/collagen hydrogel had to be limited to 10 mm to ensure that nutrients were adequately dispersed. Once the hydrogel reached a thickness of 6mm, cell activity decreased in excess of 50%. The ADSCs were found to be able to differentiate into ECs, which could then be surrounded by differentiated smooth muscle cells, similar to the intima and media layers of blood vessels [15].

A biodegradable and biocompatible single crosslinking system has been developed which achieves a highly elastic and robust hydrogel for cell bioprinting. Xu et al. [70] synthesized a triblock biodegradable polymer of PCL-poly(ethylene glycol)-PCL (PCL-PEG-PCL) using PEG in order to initiate a ring-opening polymerization using a visible-light water-soluble initiator, followed by the acrylation process to obtain the PEG-PCL-DA. The highly elastic and flexible PEG-PCL-DA hydrogel was formed by photopolymerization as shown in Figure 6. The desired elastic properties of

this hydrogel are capable of transmitting the correct mechanical stimulation to cells and could potentially improve tissue adaptation to the biomechanical environment [70].

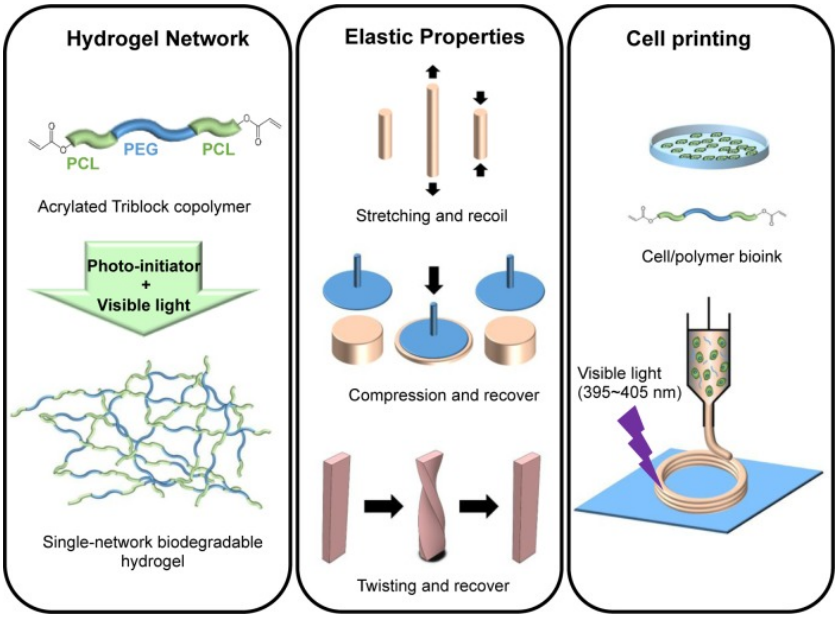

Figure 6. The single-network 3D bioprinting technique developed by Xu et al. [70]. This technique allows for crosslinks to be formed in the visible light range and leads to desirable mechanical properties. Reprinted with permission.

Very recently, Qilong et al. [74] reported a novel programmed shape-morphing scaffold by integrating a shape memory polymers with electrospun nanofibrous membrane for 3D endothelialization (see Figure 4 from their paper). This endothelial cell-laden scaffold can effectively achieve the programmed deformation from temporary 2D planar shapes to 3D closed-hoop tubular shapes at 37℃. This new approach could be applied to the preparation of small-diameter vascular grafts with improved endothelialization and to building biomimetic endothelium models for drug screening.

Apart from the previous approaches, scaffold-free techniques have also made headway in obtaining vascularized constructs. Norotte et al. [40] used multicellular spheroids and cylinders to build linear and bifurcated vessels, which enabled the fabrication of the tubular or non-tubular vascular grafts with intricate and complex construct structures. Moreover, Yu et al. managed to obtain scaffold-free tissue strands that self-assembled and did not require a liquid delivery medium during extrusion, which might allow the integration of a vascular network in the bottom-up fabrication of organs [75]. A similar approach to 3D bioprinting of scaffold-free vessels was proposed by Xu et al. [76] that employed inkjet printing of zigzag cellular tubes.

3.3. 3D bioprinting with microfluidics

Recently, hollow tube bioprinting has emerged as a strategy for single-step generation of perfusable vascular structures. In this method a co-axial, multi-layered printhead is used, where the bioink (usually containing alginate) is delivered through the sheath flow while the crosslinking agent,

a calcium chloride solution, is delivered through the core. This leads to immediate physical crosslinking of the bioink that assumes a tubular structure (Figure 7A,B) [75,76]. The hollow tubes can be patterned into different shapes with bioprinting and can allow for perfusion, which mimics the function of the blood vessels. Moreover, by further integrating cell-instructive components, such as GelMA into the bioink formulation, vascular cells could be encapsulated to eventually form a biologically relevant perfusable blood vessel [49].

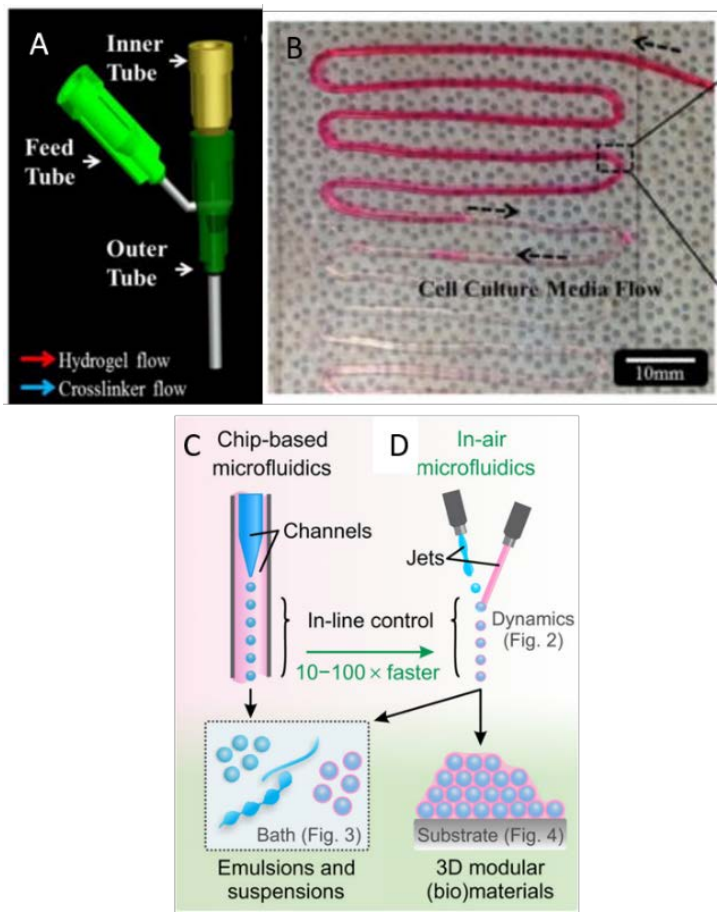

Figure 7. 3D model of the coaxial nozzle(A) and perfusion of cell culture media through zigzag patterned channel (B) developed by Zhang et al. [77]. Adapted and reprinted for non-commercial and academic use from Zhang et al. [77]. Right: IAMF approach presented by Visser et al. [78] (C) The printing mechanism of the chip-based microfluidic is shown where the co-flow (pink) is used to transport droplets (blue). (D) The new method proposed by Visser et al., whereby the IAMF relies on jet ejection and combination of the co-flow and droplets in air. This figure was not modified from the original copyrighted print and was reprinted with permission under the following public license: https://creativecommons.org/licenses/by-nc/4.0/legalcode. Refer to the disclaimer of warranties in the public license for additional information.

Visser et al. [78] has more recently developed an in-air microfluidic (IAMF) that has several advantages over the chip platform such as a higher throughput. In this approach, the microfluidic channels were replaced by micrometer-sized jets of liquid which combine in the air (Figure 7). Using

piezoeletric vibrating elements, monodisperse droplets can be achieved. This type of additive 3D bioprinting is relevant for tissue engineering applications. A pre-vascular network was achieved within one week of culture utilizing a co-culture of human endothelial cells and MSCs embedded in a fibrin gel [78].

3.4. Stereolithography and laser-assisted techniques

Stereolithography (SLA) is a technique that involves crosslinking a selectively patterned area in a reservoir of photo-sensitive pre-polymer using an array of light beams with controllable intensities [79]. Alternatively, laser-based techniques can use either pulse or continuous laser light to stimulate an absorbing layer that creates a high-pressure bubble at the interface of the bioink, propelling droplets of it onto a receiving substrate and crosslinking it in the process [80]. A simplified schematic of both of these methods are shown in Figure 8. While many papers report using such methods for fabrication of various tissue constructs, such as bone [81], vessels [82], cartilage [83], cornea [84], and adipose tissue [85] with good speeds and resolution, some concerns remain regarding the cost and the effects that the high interface pressure could have on mechanical properties and viability of bioprinted cells.

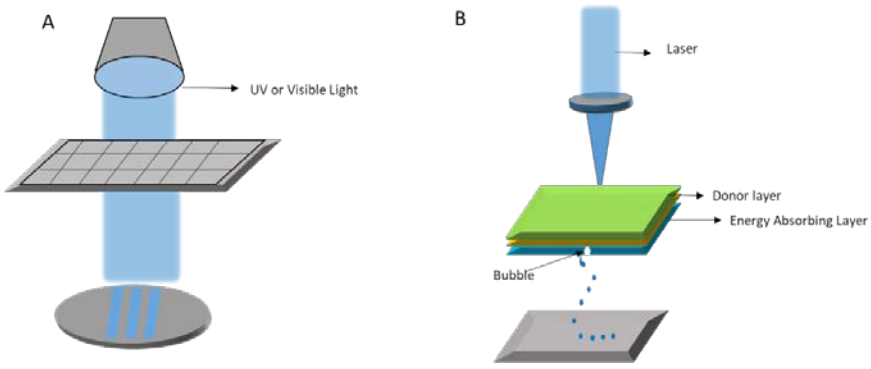

Figure 8. (A) Schematic representation of bioprinting by SLA and (B) laser-assisted methods. Redrawn from Foyt et al. [86].

In this sense, a hybrid platform, Hybprinter, that uses both molten material extrusion (MME), and digital light processing-based stereolithography (DLP-SLA) is a one-stop-shop type of bioprinting method. This allows the user to obtain a complex, rigid, and soft scaffold. The process was developed by Shanjani et al. [87], whose use of visible light induced less damage than UV-based techniques and improved the speed of bioprinting of a pre-vascularized construct comprising a porous scaffold with an embedded perfusable conduit.

Additionally, Aguilar et al. [88] have recently developed a 3D electrophoresis-assisted lithography (3DEAL) platform. The system is capable of creating molecular patterns within 3D hydrogels using electrophoresis to precisely position molecules within a hydrogel (Figure 9). This technique creates patterns on the micro-meter scale using biological molecules such as: immunoglobulin G, elastin, and fibronectin. Fibronectin columns patterned into polyacrylamide-collagen gels were able to support cell spreading up to 100 μm along 800 μm long columns and displayed an actin cytoskeleton. The inability of cells to spread along the full length of

the columns was attributed to the small pore size of the gel [88]. While studies of vasculature have not been conducted in this system, the ability to create a scaffold with biological molecules using a process similar to gel electrophoresis offers a promising new pathway for tissue engineering.

As for the laser-based systems, an idea challenging the set paradigm was proposed by Hribar et al. [89], who used an NIR-laser to pattern channels in a gold nanorod-collagen cell-laden hydrogel, achieving a writing speed of 2mm/s while maintaining over 90% cell viability. Moreover, a method named "direct laser bioprinting" was developed by Wang et al. [90], which uses direct laser illumination to crosslink a patterned area of the bioink, maintaining cell viability and mechanical properties. Keeping in mind with all the work done towards mitigating some of the drawbacks of laser-based systems, and their high resolution and impressive speeds, laser-assisted bioprinting is expected to be more and more prevalent as the cost of lasers and diodes decreases.

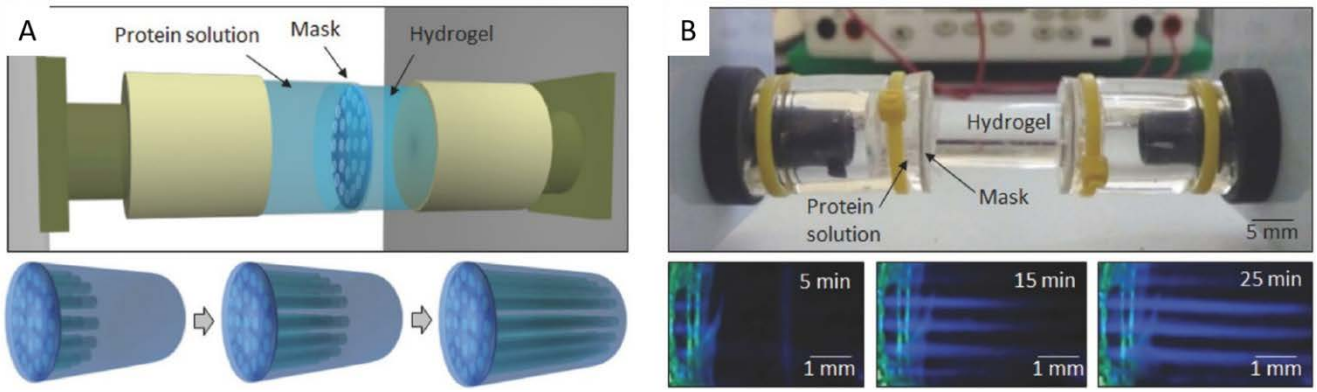

Figure 9. (A) Schematic of the 3DEAL system showing the path of molecules along the direction of electric field during printing. A dialysis membrane serves as the mask and allows for the permeation of buffer and water molecules but constrains printing molecules to a set pattern. (B) Actual setup of system with fluorescent imaging of the molecule movement during printing. Adapted from Aguilar et al. [88] and reprinted with permission.

Additionally, Miri et al. [91] initiated the integration of a microfluidic device with a digital micromirror device (DMD)-based bioprinter to provide a multimaterial stereolithographic bioprinting platform. This novel microfluidic device has fast switching between different (cell-loaded) hydrogel bioinks so that it provides a faster printing speed than the other existing stereolithography and/or DMD-based platforms. This high-speed feature indicated the great potential of this platform in bioprinting of high-fidelity multimaterial microstructures in tissue engineering, regenerative medicine, and biosensing, which cannot be easily achieved with conventional stereolithographic biofabrication platforms.

4. Perspectives

The recent advances in 3D bioprinting have increased the success in obtaining vessels, as well as vascularized constructs, such as functional *in vitro* vascular channels with perfused open lumens [42] or 3D multilayer perfusable constructs [49]. However, the limitations in spatial distribution, mechanical properties, and anastomosis are remaining challenges to be tackled [92].

Some of these limitations stem from the subpar resolution of techniques currently employed and the tradeoff between the size of the construct and the printing resolution; while extrusion-based techniques are faster and can employ several types of bioinks, their resolution is relatively limited [93]. Inkjet and laser-based techniques have higher resolutions but are at the moment restricted to obtaining small structures which are not clinically applicable [67,94].

Bridging large vessels with capillaries could establish a connected multiscale vascular network and recent research has been aimed at this task. For instance, Lee et al. [48] designed a microvascular bed contained between two fluidic vessels. The bed was connected to the vessels through angiogenic sprouting from the channel edge. This provided a feasible solution to connect the capillary network to the large perfused vascular channels. Gao et al. [95] demonstrated a novel approach to create multiscale vessel systems using coaxial printing on a rod template. The fusion of adjacent hollow filaments allowed for the formation of a macro-channel in the middle of the cylindrical template with micro-channels on either side of it. Further developments are still required to increase the complexity of vascular network and to eventually control flow-relevant factors.

Besides the integration of biofabricated vasculatures, fabricating clinically relevant vascularized tissue constructs is still challenging: in multilayer vascular structure, the vessels (e.g., arteriole, venule, artery, and vein) should be able to maintain barrier function, vasodilation, and vasocontraction [96]. While current technologies allow the bioprinting of biomimetic constructs [97,98], which are useful for *in vitro* drug assays [99] or studying vasculature in the context of diseases such as cancer [100] or ischaemia (insufficient blood supply to an organ or tissue) [68], both the printing speed as well as the resolution would need to be improved to obtain larger volumes.

In terms of bioink development, the main limitation stems from the myriad of types of cells that need to be included in the material. As different types of cells require different environments (mechanical cues, biochemical cues, etc.), the ideal formulation for bioprinting of vascularized constructs/organs is still under development [101,102]. Promising progress in the stem cell (e.g., iPSC) field [58,103,104] has brought new capabilities to the field of 3D bioprinting. Because of their autologous nature and pluripotency, they could enable the presence of different specific cell types characteristic to the tissue as well as to the microvasculature, provided their differentiation is supported correspondingly during the maturation period. Obtaining and maintaining iPSCs is a complex and expensive process, limiting their mass production [105], however, recent advances in gene editing are helping to overcome these challenges [106,107]. In addition, advances in our understanding of embryonic development, especially angiogenesis, can potentially aid in the application of 3D bioprinting in tissue engineering [108–110]. For bioprinting, it is imperative to develop more biocompatible bioinks that can sustain high cell viability during the printing and retain functionality and mechanical integrity during long-term culture [96]. Viscosity-tunable bioinks and materials that are capable of easy phase transition or multistep phase transitions could be employed to improve the printing results [108]. Combining the current existing bioprinting techniques could be another avenue which can further enhance the bioprinting capability. Recent studies indicated that bioprinting systems can be equipped with extrusion, nozzle, and photopolymerization to effectively produce macro- and microstructures using a single system [96,109,110]. Furthermore, building upon the recent advances in 3D bioprinting, it is possible to make breakthroughs in organ printing by tackling challenges such as selection of viable cells, preparation of bioinks, and choice of 3D bioprinter. For a more comprehensive look at tissue engineering beyond vascularization the reader is referred to a recent review by Aljohani et al. [111].

In summary, 3D bioprinting is still an emerging and developing field that has made great headway towards biofabricating constructs and organs for tissue engineering, as showcased in this review. With the advent of new breakthroughs in 3D bioprinting technologies, cell manipulation and improved knowledge of clinical requirements of engineered tissue, 3D bioprinting is expected to evolve as a scalable method to obtain functional organs.

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Conflict of interest

All authors declare no conflicts of interest in this paper.

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