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REVIEW ARTICLE

3D bioprinting for vascular grafts and microvasculature

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Abstract

Cardiovascular disease is the world's leading cause of death, and there is a substantial clinical need for transplantable blood vessels. Through tissue vascular engineering technology, large blood vessel grafts with significant clinical effects have been synthesized. However, synthesizing vascular valves, small vessels up to 6 mm in diameter, and capillary networks up to 500 μm in diameter remains challenging due to the lack of precise manufacturing techniques. In particular, constructing a microvascular network in thick tissue is the technical bottleneck of organ transplantation. Three-dimensional (3D) bioprinting is a computer-assisted layer-by-layer deposition method that can deposit cells and biomaterials at a predetermined location, according to an accurate digital 3D model, to build a delicate and complex bionic structure. This review discusses the progress and limitations of 3D bioprinting in preparing large vessels and valves, small-diameter vessels, and microvascular networks. This paper focuses on improved printing technology and innovative bio-ink materials. The future application of 3D bioprinting is prospected in generating artificial blood vessel grafts and vascularized organs with full biological function.

Keywords: 3D bioprinting; Small-diameter vessels; Microvasculature

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1. Introduction

Cardiovascular disease is the leading cause of death in the world, with the annual number of mortalities expected to reach 23.3 million by 2030[1]. The body's vascular system consists of four parts, such as large-diameter blood vessels (diameter 10–30 mm), medium-diameter blood vessels (diameter 6–10 mm), small-diameter blood vessels (diameter 0.5–6 mm), and capillaries (diameter <500 μm).

Macroangiopathy, along with aortic dissection, thoracic aortic aneurysm, and abdominal aortic aneurysm, poses a significant threat to human life and health. These diseases often lead to sudden death of patients due to blood vessel rupture and bleeding[2]. Clinically, the radical surgical approach to significant vascular disease replaces damaged vessels with artificial vessels[3]. The large vessel valve is located at the junction of the cardiac chambers and the large-diameter artery, ensuring unipolar blood flow between the ventricle and the large artery. Narrowing or incomplete closure of the

aortic valves can lead to heart structural and functional abnormalities and, ultimately, heart failure[4]. With the acceleration of the aging population process, the incidence rate of senile degenerative valvular diseases is increasing yearly in China. The current solution is to replace defective valves with mechanical or biological alternatives. However, existing mechanical grafts are predetermined and do not correctly match the patient's aorta shape[5]. Moreover, the patient may have a violent immune rejection reaction to the biological graft and need lifelong anticoagulant therapy[6].

Small-diameter vessels are in great clinical demand, due to three aspects. First, the diameter of the coronary artery is less than 5 mm, which is prone to atherosclerosis and ischemic heart disease. Coronary atherosclerotic heart disease accounts for nearly half of all deaths in developed countries such as Europe and the United States[7]. Secondly, many patients needing hemodialysis must use small-caliber blood vessels to enter the venous dialysis fistula to construct long-term vascular dialysis access. Finally, patients with arterial injuries of more than 2 cm caused by car accidents and falls need to use small-caliber blood vessels for repair. The small-diameter vessels that can be transplanted are autologous vessels and artificial vessels[8]. The great saphenous vein is the most commonly used autograft in coronary artery bypass grafting, but its patency rate is only 60% in ten years[9]. There is no small-diameter artificial vascular graft for clinical operation because of its high incidence of stenosis and occlusion. In coronary artery bypass grafting, the patency rate of artificial blood vessel grafts at 2 years is only 32%[10]. A high survival rate of cells in the vessel wall has yet to be achieved with small-diameter vessel manufacturing techniques, such as electrospinning. In addition, the deficiency of endothelial cells in artificial blood vessels is the leading cause of graft thrombosis[11].

A microvascular network with a diameter of less than 500 microns is the principal site of gas and material exchange in tissues. Oxygen and nutrients can travel along capillary pathways to nourish parenchymal cells of tissues and organs. As a new method in organ transplantation, tissue engineering technology can solve the shortage of organ donors. However, due to the technical bottleneck, using vascular endothelial cells to construct microvascular networks is impossible[12]. The simple diffusion range of oxygen and nutrients is only 100–200 μm. Tissue engineering techniques have successfully produced functional thin skin tissue grafts. However, high-metabolic organs such as the liver, heart, and kidneys, which are fabricated via tissue engineering techniques, are not able to carry out adequate oxygen and nutrient exchange. The lack of biologically functional capillary networks in thick tissues (thickness ≥ 200 μm) has undoubtedly limited the development of tissue engineering in the areas of organ repair and transplantation. How to combine microvascular networks with thick tissues is a hot topic and future development direction.

Bioprinting is the application of three-dimensional (3D) printing technology in regenerative medicine. A typical bioprinting process consists of three parts. First, the pre-printed tissues and organs are imaged to reconstruct the 3D digital models and plan the printing path. Then, according to the pre-printed tissue organs, the matching bio-ink and tissue cells are selected. Finally, bioprinters are used to make the bio-inks containing cells into the desired 3D living tissues/organs according to the 3D model. The terms "3D printing" and "3D bioprinting" should be distinguished here. Both are techniques based on 3D models that print 3D objects layer by layer, but the printing materials differ. 3D bioprinting uses bio-inks containing cells to print living tissues and organs with biological activity directly. General 3D printing uses adhesive polymer materials to print 3D items that do not have cells. This review focuses specifically on bioprinting for vascular tissue containing living cells, so general 3D printing techniques and applications fall outside the scope of this work[13]. Traditional tissue engineering techniques for manufacturing vascular grafts include casting, electrospinning, melting electrowriting, etc. The structural accuracy of the casting process is not precise enough to prepare the complex structure of natural blood vessels. Electrospun fibers have low mechanical properties and cannot accurately form 3D structures. Thermoplastic inks used for melt electrowriting cannot encapsulate cells because high processing temperatures are required. It is worth noting that 3D bioprinting technology can accurately print blood vessel grafts containing living cells with bio-ink under the high precision control of a computer. For the construction of vascular grafts of different diameters and sizes, 3D bioprinting can be excellent. It can build a high-resolution vascular scaffold and provide physical and chemical clues for the adhesion and proliferation of blood vessel wall cells by designing printed patterns and bio-ink components, which is impossible with traditional vascular graft manufacturing technology[14].

Bioprinting technologies for blood vessel manufacturing include droplet-based bioprinting (DBB), extrusion-based bioprinting (EBB), and laser-assisted bioprinting (LAB). The differences between these printing technologies are resolution, printing speed, and adaptive biological inks, among which resolution is the main differentiating factor. Extrusion-based 3D printing, which extrudes bio-ink to form continuous fibers to build blood vessels, is the most common printing method. Its most significant advantage is that it can print a wide range of biocompatible materials. Still, its printing accuracy is relatively low compared with other bioprinting methods, generally at 100 μm. Droplet

bioprinting generates discrete droplet accumulation molding with higher precision. However, due to low driving pressure, inkjet bioprinting cannot print materials with high viscosity or cells with high concentration, which limits its application scope. Photocurable bioprinting, which uses photosensitive materials for photocuring and stacking layer by layer, is a printing method with the highest accuracy and has been used in the study of microvascular networks[14].

The design and development of bio-ink is the key to bioprinting technology. The research, development, and synthesis of new bio-inks that can balance printability, biocompatibility, and mechanical properties are the basis of 3D bioprinting applications. There is currently a shortage of bio-inks with both good printability and angiogenic activity, which is a major bottleneck for using bioprinting systems in blood vessel manufacturing. Hydrogels are the most commonly used bio-inks for constructing vascular stents because of their excellent biocompatibility. Biocompatible hydrogels have a 3D network structure similar to the extracellular matrix (ECM), which can promote cell adhesion and growth. Hydrogels contain natural and synthetic categories. Naturally derived protein bio-inks, such as collagen, gelatin, and fibrin, generally support cell adhesion and have good angiogenesis but are not mechanically sufficient to be used directly as 3D-printed vascular scaffolds[15,16]. Through various chemical modifications, synthetic hydrogels, such as polyethylene glycol and poloxamer, generally have good physical and chemical properties but are bioinert materials that are not conducive to cell adhesion and growth[12]. Multi-material printing is the development trend of 3D bioprinting. Bio-inks with printability and angiogenic activity were produced by selecting different components with different bioactivity and mechanical properties and adjusting their concentrations. By carefully regulating bio-ink formulation, a cellular microenvironment similar to that of natural blood vessels is constructed[17].

According to the classification of vessel diameter size, this paper summarizes the latest progress of 3D printing technology in large-caliber blood vessels and valves, small-caliber blood vessels, and microvascular networks, including the advancement of high-resolution printing technology and the preparation of bio-inks with good mechanical and biological properties. In addition, we briefly review the methods that promote the cultivation and maturation of bioprinted vascular grafts. This review also aims to facilitate the efficient transition of bioprinted grafts from the bioprinting lab to the clinic.

This paper also focuses on constructing a capillary network with biological functions using 3D bioprinting technology and promoting tissue engineering development for organ repair and transplantation. Here, we summarize five prerequisites for 3D printing to construct the microvascular network: good bio-ink performance, high-resolution printing technology, suitable cell source, vascular micropattern guiding angiogenesis, and multi-dimensional vascular network hierarchy appropriate to the host vascular system.

2. 3D bioprinting of large-diameter vessels and valves

2.1. 3D bioprinting of large-diameter vessels

Large-diameter vessels used for vascular replacement require good mechanical properties, consistent host anatomy, and high cell survival. There are still some gaps in the anatomical correlation and cell survival rate of vascular grafts based on electrospinning. 3D bioprinting has an advantage in manufacturing with consistent anatomy and high cell survival rates, as it can rely on computers to accurately model and utilize biocompatible bio-inks.

First, the large-diameter vascular graft used in the patient's surgery should have the same anatomical structure as the host. The grafts mismatched with the patient's blood vessels are more dangerous in the large blood vessel graft, and 36.8% of patients were re-hospitalized within 30 months due to heart failure caused by graft mismatch[18]. The resolution of 3D bioprinting technology depends first on computer modeling and printing path planning. Kucukgul designed a new computer algorithm to accurately print large-diameter blood vessel structures with living cells through a self-supporting "cake-like" printing method[19,20].

Second, vascular wall smooth muscle cells are essential for the long-term stability of the great vessels. Developing bio-ink with good cell compatibility is the key to improving the survival rate of graft cells. The natural ECM contains laminin, fibrin, and cytokines that promote cell adhesion and proliferation. Oropeza *et al.* found that in decellularized ECM (dECM)-printed graft structures, arterial smooth muscle cells could grow for a long time with no dead zone for 24 h. Because of smooth muscle cells, the macrovascular graft can maintain mechanical elasticity for a longer period[21]. Removing the ECM and adding bio-ink can significantly improve the cell survival rate of the graft. Potere *et al.* optimized the de-cellular protocol of the pig's natural aorta and determined the optimal concentration of dECM mixed into bio-inks[22]. The printed structure showed excellent structural stability and elasticity.

2.2. 3D bioprinting of vascular valves

3D printing technology provides a new and effective method for manufacturing biological valves. Cardiac valve

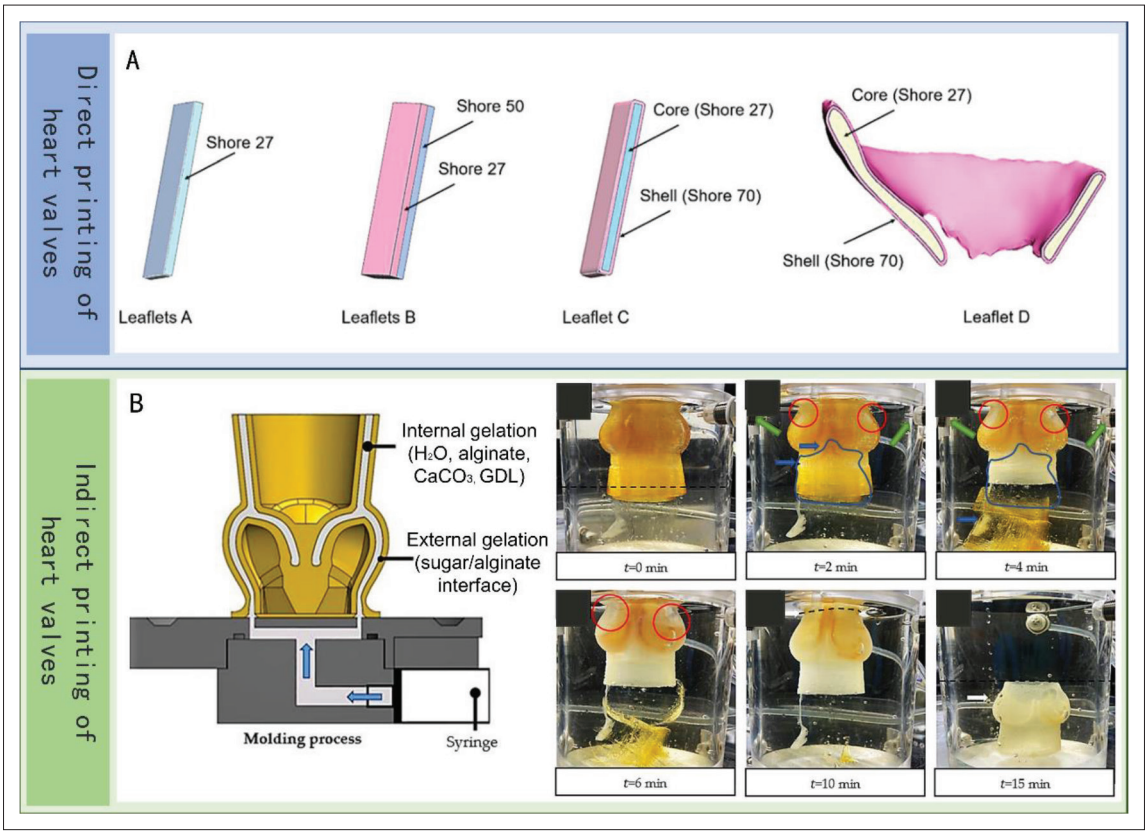

Figure 1. (A) Design drawing of valve multi-level structure modeling[24]. (B) An aortic valve mold made of sugar glass and a time-lapse photo of the mold dissolving[26].

printing methods contain direct extrusion printing and indirect printing.

First, direct extrusion bioprinting can directly deposit bio-inks of different concentrations and containing different cell types, which can effectively replicate the mechanical heterogeneity of the valve region. The heart valve is a heterogeneous anatomical region composed of valve ring, valve, and notochord with different mechanical properties. The valve has strong expandability and adaptability and can quickly open and close large blood vessels. The notochord has a high hardness that keeps the lumen open under harsh hemodynamic load. Hockaday *et al.* have successfully produced aortic valves with different mechanical properties by designing two different gel schemes, including a rigid hydrogel for the aortic notochord and a flexible, expandable hydrogel for the aortic valve[23]. The cross-section of the valve is a multi-layer heterogeneous structure. Vukicevic *et al.* compared the mechanical properties of the composite material with the target natural components and constructed the multi-layer structure of the valve using the composite material with different concentration ratios (Figure 1A)[24]. Duan *et al.* wrapped smooth muscle cells directly in the root of the valve and aortic stromal cells directly in the valve lobules. On day 7 of graft culture, the strength and stiffness of the cell material were significantly higher than that of the cell-free scaffold[25].

Second, indirect printing provides a relatively simple and highly reproducible method for valve printing. Rioux *et al.* used low-cost sugar glass to print the stent mold and then filled it with sodium alginate hydrogel, crosslinking it with $CaCl_2$. A complete aortic valve is produced after the glass-lined mold dissolves (Figure 1B)[26].

3. 3D bioprinting of small-caliber blood vessels

The research and development of small-diameter vascular prostheses has been a hot topic in the past decade, but formal clinical products have not yet emerged. The ideal small-diameter artificial blood vessel has a high forming resolution, cell integration rate, and mechanical strength. 3D bioprinting allows for the successful construction of perfect small-caliber blood vessels by combining new bio-inks, well-designed printing strategies, and multiple manufacturing methods.

3.1. Bio-inks with good biomechanical properties

Research in 3D bioprinting focuses on developing bio-inks suitable for printing small-diameter blood vessels. By mixing different kinds of natural and artificial compounds, adjusting the formula concentration can effectively improve the biomechanical properties of biological ink.

Zhou *et al.* introduced alginate lyase into natural ink to improve the biological activity of biological ink and gradually degrade alginate, which played a supporting role in the substrate[27]. The wall structure printed by the ink mixed with lyase contains a more porous structure. Endothelial cells and smooth muscle cells in the vascular wall have higher nutrient exchange efficiency and larger adherent proliferation space. The growth rate of smooth muscle cells in the lysozyme group was significantly faster than in the non-lysozyme group. The density of smooth muscle cells and endothelial cells encapsulated in the bio-inks is 1 million per mL[27]. After adding an acellular ECM from the great saphenous vein to bio-ink, Kamaraj *et al.* found that dECM induced the differentiation of human umbilical cord mesenchymal stem cells (UMSCs) into vascular smooth muscle cells and enhanced alpha-smooth muscle actin (α-SMA) expression[28]. They constructed the vascular structure with a high cell integration rate successfully, and the density of UMSCs encapsulated in the bio-inks is 10 million per mL[28].

To enhance the mechanical properties of bio-inks, Gold *et al.* blended gelatin methacrylate (GelMA), polyethylene glycol diacrylate (PEGDA), and two-dimensional nano-silicate to develop a new high-viscosity bio-ink[29]. Nano-silicates enhanced the compression elasticity of blood vessel walls, showed high printability regardless of cell density, and protected encapsulated cells from high shear forces during bioprinting. The authors selected a cell density of 2.5 million cells per mL for developing this vascular model[29]. Li *et al.* also found that doping the bio-ink with carbon nanotubes could improve the mechanical strength of the stent[30]. The authors successfully made engineered blood vessels with an inner diameter of 3 mm from bio-ink containing carbon nanotubes with a cell concentration of 4×10^5 cells per mL[30]. Liu *et al.* designed a double-network hydrogel by chemically crosslinking bio-inks, using calcium ion-crosslinked alginate to form the first network of a two-network hydrogel[31]. They used polyacrylate and PEGDA as polymer crosslinkers to prepare the secondary network structure of double-network hydrogels. The vascular structure printed by the double-network hydrogel has high toughness and elastic properties[31]. Freeman *et al.* combined fibrinogen and gelatin, using gelatin's excellent rheological properties to convert non-printable fibrinogen into a printable biomaterial[32]. Tissue-engineered vascular grafts use cell-laden (containing 3×10^6 cells per mL) bio-ink composed of 7.5% (w/v) gelatin and 10 mg/mL fibrinogen[32].

3.2. Sophisticated printing strategy

Designing a sophisticated printing strategy can effectively improve the printing precision of small-diameter blood vessels. Zhou *et al.* developed an interfacial diffusion printing (IDP) technique to control the thickness and diameter of the tube wall structure by controlling the time of gel crosslinking inside and outside the tube[33]. Jin *et al.* developed a method for making hollow blood vessels without relying on sacrificial materials and used a two-step crosslinking method[34]. The semi-solid wall with a quarter of lumen size was prepared by crosslinking in the first step, and then the concave structure with a 3/4 lumen size was made by covering the GelMA not crosslinked in the second step. Two separate parts constructed a complete tubular structure with a two-step crosslinking. Using no sacrificial material avoids damage to existing vascular structural accuracy and cell activity during removal. This method provides a new way to improve the printing precision of tubular structures[34]. Instead of printing layer by layer, Zhang *et al.* used a robotic arm with six degrees of freedom (6-DOF) to build a new 3D bioprinting system[35]. Composed of six 360° rotating joints, the manipulator can print routes from all directions in 3D space, significantly improving the ability to bioprint complex anatomical structures. They also successfully used the dual-robot platform to print a stent similar to the heart coronary artery network complex shape (Figure 2)[35].

3.3. Combined multiple manufacturing technologies

In the past, single processing methods were used to manufacture small-caliber blood vessels, but the blood vessels fabricated by these processes, when used alone, were unable to mimic the complex structure and function of the natural small-caliber blood vessels. Integrating multiple manufacturing technologies into a single biofabrication platform can effectively compensate for the limitations of a single processing method. The current 3D bioprinting technology is limited to a resolution of close to tens of microns. It cannot effectively print the nanoscale ECM structure, thereby hindering the reproduction of the microenvironment for blood vessel cells. Electrospinning uses high pressure to produce nanoscale diameter fibers with a large specific surface area that can mimic the physical function of the natural ECM, providing many attachment points for cell adhesion and growth. Fazal *et al.* developed a hybrid device that combines bioprinting and electrospinning[36]. The device, which has a bioprinting head and two electrospinning heads, is capable of

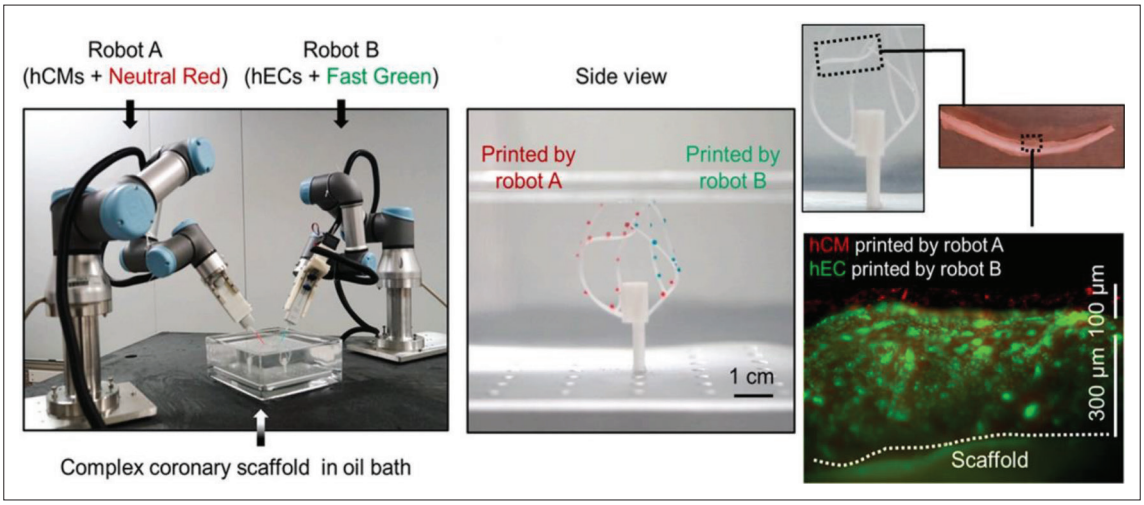

Figure 2. The blood vessel model was printed from multiple angles by two six-degree-of-freedom robotic bioprinters. The image at the bottom-right side shows the double-layer cross-section of the blood vessel wall[35].

producing tubular structures with electrospun nanofibers and layered hydrogel structures, which not only effectively improve the mechanical properties of the hydrogel but also simulate the double-layer structure of natural blood vessels[36]. Jin *et al.* employed two methods, electrospinning and bioprinting, to print double-layer blood vessel structures in sequence[37]. Electrospinning was first used to create a scaffold for the inner layer of blood vessels, which helps endothelial cells adhere and proliferate. The authors used bioprinter to evenly distribute hydrogels containing smooth muscle cells on the outer layer of the scaffold, and successfully constructed a double-layer structure of small-caliber blood vessels[37].

Electrowriting is a novel biomanufacturing technology combining electrospinning and 3D printing principles. It effectively compensates for the limitation of electrospinning technology, which is the inability to form a stable 3D structure. Melt electrowriting can print sub-micron-sized 3D designs with good mechanical properties through thermoelectric fluid power injection. Größbacher *et al.* combine fused electrowriting with bioprinting to print a composite tubular structure with a patterned fiber network[38]. The microfiber network of fused electrowriting enhanced the hydrogel tubes' bending, bursting, and tensile strength[38]. Cao *et al.* successfully produced a tubular scroll scaffold with anisotropic internal morphology by printing 20 μm oriented poly (ε-caprolactone) (PCL) fibers on the surface of a four-dimensional (4D)-bioprinted hydrogel using melt electrowriting technology[39]. PCL fibers promote cell adhesion and proliferation, but homogeneous hydrogels do not have this function[39].

Using 4D printing technology to prepare vascular stents is one of the future development directions. A remarkable feature is the self-deforming printing material that can respond to external stimuli. 4D printing technology can improve the accuracy and resolution of blood vessel prints. After bioprinting the vascular stent, post-processing reduces the diameter of the 3D structure, thereby enhancing the resolution of the vascular print. Kitana *et al.* used a hydrogel with a vertical expansion gradient to print shape-controlled vascular network elements[40]. By precisely controlling the 3D printing parameters, hydrogel concentration, and crosslinking parameters, they successfully prepared the hollow tube structure as a scalable T-joint with a diameter of 2–15 mm[40]. When swollen, the 3D-printed photo-crosslinked alginate flake gel rolls and forms a roll-like tube. Kirillova *et al.* controlled the tubular structure's diameter by controlling the photo-initiator's crosslinking density, exposure time, and concentration[41]. The self-coiled tubular structure has uniform cell colonization and avoids cell damage caused by extrusion printing[41].

4. 3D bioprinting of microvasculature

The microvascular network is the main place of oxygen and nutrient exchange in tissues and organs. The current tissue engineering techniques are still not able to construct thick tissues with rich vascular networks, and the artificial tissue fabricated containing a thick parenchymal cell layer is susceptible to ischemic necrosis.

This section discusses the prerequisites of 3D printing in built microvascular networks, including bio-inks with good performance, high-resolution printing techniques, suitable cell sources, micropatterns that induce angiogenesis, and multi-diameter vascular printing.

4.1. Bio-inks with good performance

Making a printable pro-angiogenic bio-ink includes mixing multiple hydrogels and chemically modifying a single hydrogel. Substances that improve rheology or elasticity are added to compound bio-inks to optimize the mechanical properties of naturally derived hydrogels. Wu *et al.* synthesized a shrinkage glycyrrhizin-methacrylate silk (SilkMA) with excellent mechanical properties and mixed it with GelMA and sodium alginate[42]. They found that the higher concentrations of GelMA (7.5%) and SilkMA (20%) were better suited for cell adhesion and proliferation, and by using this method, they rapidly generated an inch-long blood vessel construct in as little as 3 days[42]. Xanthan gum is a microbial extracellular polysaccharide produced by fermentation engineering, which has good rheology and biocompatibility. Muthusamy *et al.* utilized xanthan gum as a collagen thickener and found that collagen bio-ink with 10 pc xanthan gum added has the best printing adaptability, shape fidelity, and good capillary network generation[43]. Laponite is a montmorillonite nanoclay suspension that self-assembles to form a reversible shear-dilute gel. Laponite and alginate nanocomposite showed improved rheological properties, printing compatibility, and higher recovery rate after shear under higher clay concentration, and the constructed bone tissue scaffold showed *in vitro* angiogenesis ability[44]. Poly (itconic acid-co-citrate-co-octanediol) and poly (octanediol-co-maleic anhydride-co-octanediol) are synthetic elastic polymers that can be mixed with hydrogels to improve their stability and elasticity. Cell traction or body movement prevents the resulting blood vessels from irreversible deforming[45].

Endothelial cells can regulate vascular inflammation and thrombosis, and rapid endothelialization of early vascular grafts can effectively prevent the blockage of small-diameter vessels. The biological properties of bio-inks are essential for the rapid endothelialization of vascular grafts. To improve the ability of hydrogel scaffolds to promote the formation of microvascular networks, substances that can induce the adhesion and proliferation of endothelial cells can be added to the composite bio-ink. Natural alginate is biologically inert but can be modified with cell adhesion peptides to customize its biological activity with excellent performance, precision, and control. To promote vascular morphogenesis, Barrs *et al.* modified alginate scaffolds to promote vascular morphogenesis using RGD (an integrin-binding peptide for cell adhesion) and vascular endothelial growth factor (VEGF) mimic peptides (Figure 3A)[46]. *Paeonia lactiflora* extract is a compound derived from the herb peony that regulates the inflammatory microenvironment in skin wounds. Combining 3% paeoniflorin with a 3D mesh structure of the scaffold can promote the secretion of collagen by epithelial cells and stimulate the formation of vascular buds in the wound surface[47]. Bacterial cellulose is an organic compound biosynthesized by *Gluconacetobacter xylinus*. After dehydration, the bacterial cellulose matrix can generate a microchannel structure for cell adhesion and proliferation in the vascular scaffold and promote the construction of a smooth muscle layer in the vascular wall (Figure 3B)[48]. De-cellularization of tissues is a promising technique that allows the removal of cellular components while preserving ECM structure and composition. By mixing pig ovary dECM solution with seaweed gelatin mixture solution, the printed ovarian 3D scaffold showed more positive signals for new angiogenesis, cell proliferation, and survival (Figure 3C)[49]. ECM not handled thoroughly poses a threat to the body's immune response or pathogen transfer. Oliveira *et al.* solved this problem using a cell assembly extracellular matrix (CAM) synthesized from normal skin fibroblasts *in vitro*[50]. The thick structure printed by CAM can support the survival and maturation of capillary networks and successfully connect with the host circulatory system to establish active perfusion[50].

Sacrificial bioprinting is a classic indirect bioprinting method. Sacrificial bioprinting of vascularized tissue removes soluble bio-inks, usually through temperature change or enzymatic ablation, leaving behind a perfusable channel. Indirect printing is usually bioprinting based on extrusions with low resolution and minimum characteristic sizes larger than 100 μm, making them less suitable for fabricating capillary structures. How to improve the resolution of sacrificial bioprinting is a new research direction. Li *et al.* combined a heat-sensitive polymer (n-isopropyl acrylamide) with biocompatible GelMA to form a heat-responsive hydrogel[51]. In cell culture, the thermo-responsive hydrogel underwent significant volume shrinkage, which effectively triggered the production of smaller microscale vasculatures, with a minimum diameter of 50 μm[51]. Thomas *et al.* have proposed a two-component biomaterial system using a photo-crosslinked methacrylate hyaluronic acid (HAMA) and GelMA[52]. With highly-resolution enzyme-digested photo inks based on hyaluronic acid and the complementary enzyme, they printed vascular structures using stereolithography 3D bioprinting[52].

4.2. High-resolution printing technology

Currently, most microvascular networks are constructed by an uncontrolled spontaneous induction because previous printing techniques have not been able to accurately print microvessels with an internal diameter of fewer than 500 microns. The vascular network formed by self-assembly, which is less efficient in material exchange

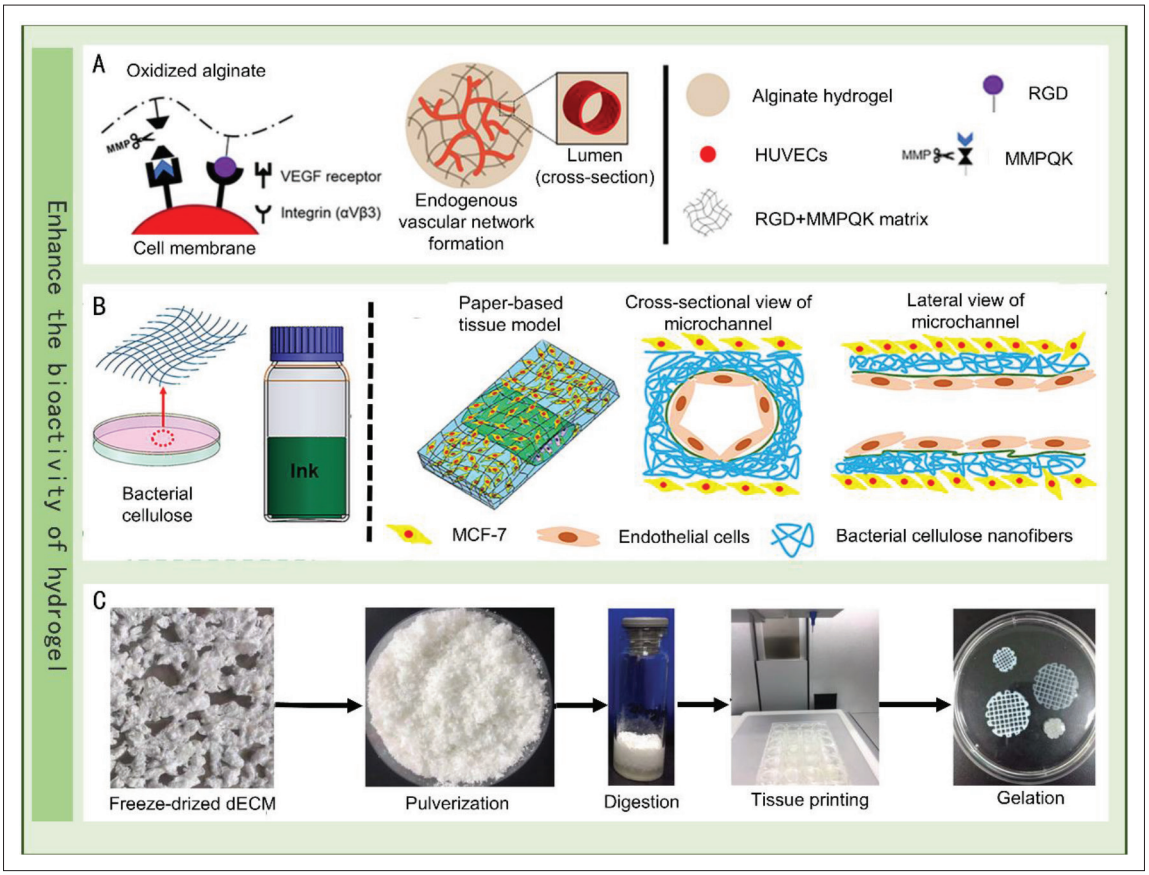

Figure 3. Bio-inks with good performance[40]. (A) Alginate scaffolds modified with RGD and MMPQK[46]. Reprinted (adapted) with permission from American Chemical Society. Copyright © 2021, American Chemical Society. (B) A microchannel structure formed by bacterial cellulose in a scaffold[48]. Reprinted (adapted) with permission from American Chemical Society. Copyright © 2019, American Chemical Society. (C) Mixing the ovarian extracellular matrix in bio-ink[49].

and more susceptible to thrombosis, differs significantly from the natural blood vessels. Therefore, despite being a complex endeavor, developing high-resolution 3D printing technology is a reliable research direction with the aim to realize printing of microvascular networks in future.

Due to high shear force, extrusion bioprinting impels cell activity while a micron-size pipe structure is being prepared. Kirillova *et al.* used advanced 4D bioprocessing methods to print hollow self-folding tubes, which had high-resolution control of diameter and structure, with shapable hydrogels. The self-folding tube's diameter depends on the hydrogel film's polymer concentration and the crosslinking time. The hydrogel film printed under low shear force contains cells with good activity and successfully produces tubular structures with a diameter of as low as 20 microns[41].

In most bioprinting systems, bio-inks have no self-supporting structure during printing. The structure-forming rate of softer biomaterials is low because of the gravity drop[12]. To solve this problem, embedded bioprinting is used to design a printing carrier filled with a supporting matrix. Hydrogels with weak mechanical properties, such as collagen, can print stably in the supporting matrix, avoiding the pattern distortion caused by sagging gravity[53].

Granular hydrogel is an excellent support medium for embedded 3D bioprinting[54]. Hinton *et al.* first proposed the free-form reversible embedding (FRESH) printing method of suspended hydrogels by extruding bio-ink from a thermally-reversible support bath composed of gelatin particulate slurry[55]. Then, Lee *et al.* developed a greatly improved second-generation suspended hydrogel free-form reversible embedding (FRESH v2.0) 3D bioprinting technology[56]. The technology can print collagen silk with a diameter of 20–200 μm, an order of magnitude higher than the first-generation FRESH, and has been demonstrated to successfully print a fully perfused vascular network of 8–50 μm[56]. Although liquid media allow low-viscosity inks, water structures constructed in viscous oils

are unsuitable for cell culture. Zhang *et al.* demonstrated a bioprinting method that uses cells to simulate two-phase water systems (ATPS)[54]. The new ATPS used poly-lysine (PLL) aqueous solution as ink and oxidized bacterial cellulose (oxBC) aqueous solution as a cell-containing medium. When the PLL ink loaded with cells was deposited into the oxBC medium phase, oxBC and PLL formed a condensed complex through electrostatic interaction at the water–water interface, successfully constructing a 3D cell interconnection network lined with perfusion channels[54].

Laser-assisted technology has exceptionally high resolution and can print feature sizes of less than 10 μm. Two-photon polymerization (TPP) is a laser direct writing technique that uses near-infrared femtosecond lasers to induce crosslinking reactions in monomer solutions, enabling nanoscale resolution[57]. Dobos *et al.* tested various TPP parameters on the small-diameter channel structure, including voxel size, layer spacing, etc., which improved the accuracy and throughput of the printing process, and successfully built a microvascular network with a diameter of 10–30 μm[58].

Stereolithography (SLA) allows patterning in photoreactive hydrogels and fabrication of blood vessels at the scale of millimeters and micrometers at high printing speeds. Xue *et al.* used the system to manufacture a variety of bracket architectures, ranging from regular geometries such as serpentine, spiral, and fractal shapes to more irregular/complex geometries such as bionic trees and capillary networks, with channel widths ranging from tree trunks (width >1100 μm) to small branches (about 17 μm in width)[59]. Thomas *et al.* used SLA to construct perfusable endothelialized blood vessels successfully[52].

4.3. Suitable sources of cells

A layer of endothelial cells usually forms the capillary wall. Adding endothelial cells directly to the bio-ink for printing is the primary way to create hollow endothelial tubes. Three common endothelial cell types used for tissue engineering include human umbilical vein endothelial cells (HUVECs), human microvascular endothelial cells (HMVECs), and induced pluripotent stem cell-derived endothelial cells (iPSC-ECs). The globular aggregates of cells better mimic the function of living tissue and promote the formation of microvascular networks, compared to the dispersed individual cells. The spherical culture chips made by Anada *et al.* can produce 500 spheroids per device at a time and allow for the collection of spheroids in a quick and non-invasive manner (Figure 4A)[60]. Liu *et al.* extracted small balls of early vascular cells (EVCs) from human embryonic stem cells (hESCs) to construct microvascular networks. When the spherical vascular cells were mixed into the hydrogel, the spherical vascular cells underwent angiogenesis at a rate significantly faster than individual EVC cells (Figure 4B)[61].

Support cells around blood vessels, such as perivascular cells, mesenchymal stem cells, and fibroblasts, provide mechanical support for blood vessel wall cells, shape the microenvironment around blood vessels, and promote angiogenesis. Therefore, adding support cells to vascular printing, which utilizes hydrogels, is a recommended strategy.

As perivascular support cells, adipose-derived stem cells (ASCs) express various angiogenic factors that stimulate endothelial and smooth muscle cell proliferation. For 7 days, Benmeridja *et al.* co-cultured ASCs with HUVECs, forming a network of capillaries in the printed adipose tissue[62]. Using three-culture spheres containing HUVECs, human preputial fibroblasts (HFF), and adipose tissue-derived mesenchymal stem cells (ADSC), de Moor *et al.* found that HUVECs spontaneously organized into a capillary-like network throughout the sphere[63].

The vascular wall comprises endothelium, smooth muscle, and outer membrane. When natural blood vessels are damaged, vascular stem cells that are located in the outer membrane of blood vessel wall differentiate into smooth muscle cells or endothelial cells that repair themselves.

To fully mimic the natural vascular wall hierarchy, Dogan *et al.* used human iPSC-derived mesodermal progenitor cells (hiMPCs), instead of mature endothelial or smooth muscle cells, to print the vascular network[64]. Using hiMPCs, they induced *de novo* generation of small and large containers with multi-walled structures that possess the inner, medium, and outer membrane-like layers[64].

4.4. Micropatterns that induce angiogenesis

Endothelial cells form the vascular networks in artificial tissues mainly through self-assembly. Biomaterials and biomaterial inks that precisely control cell adhesion are essential for creating functional microvasculature systems. Since the lumen formation of endothelial cells depends on cell–matrix interactions, the vascular system formed by self-assembly is often subject to hemodynamic disorder. By designing high-resolution patterns, 3D bioprinting can exert a degree of exogenous control over the self-assembly process of blood vessels. It creates biological cues that guide the formation of blood vessels by depositing endothelial cells and collagen fibers in artificial tissue[65]. The printing of the microvascular network contains two aspects: endothelial cell mapping and extracellular matrix mapping. The arranged and formed cord of endothelial cells defines the structure of neovascularization *in vivo*. The patterned matrix structure can give mechanical clues to endothelial cells' adhesion and proliferation and promote vascular network formation.

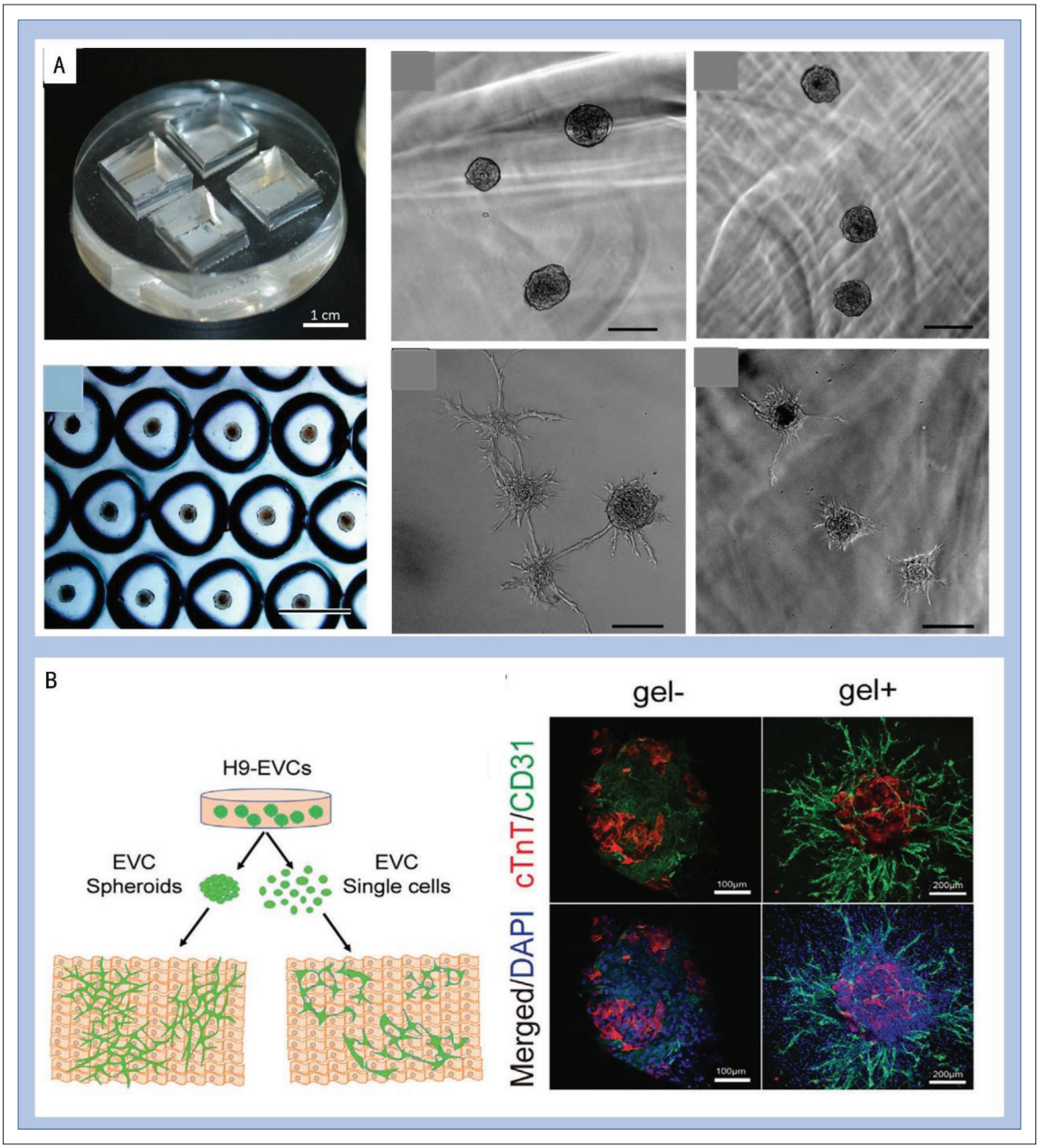

Figure 4. Suitable sources of cells. (A) A culture chip for a sphere of cells. Self-assembling spheres of endothelial cells form microvessels[60]. (B) Left: Early vascular cell (EVC) spheres can generate vascular networks more rapidly than single EVCs. Right: Fluorescent image of a microvascular network growing out of an EVC sphere[61].

The endothelial cells arranged in different patterns have different activities of generating vascular networks. Mirabella *et al.* designed three different vascular-patterned print structures[66]. Compared with the vascular patch without the pattern, the vascular patch with the geometric pattern had better perfusion after implantation in the distal limb of the host. This result suggests that the geometric design of the vascular channel in the patch affects its ability to salvage perfusion[66]. Kérourédan *et al.* designed and printed different patterns of endothelial cells on bone calcium defects in mice, including a "ring" pattern, a "disk" pattern, and a "cross-circle" pattern. The "disc" and "cross-circle" patterns had the highest local endothelial cell density and significantly increased blood vessel formation in the body[67].

While the natural cellular microenvironment is anisotropic, the cellular and matrix components in bio-ink

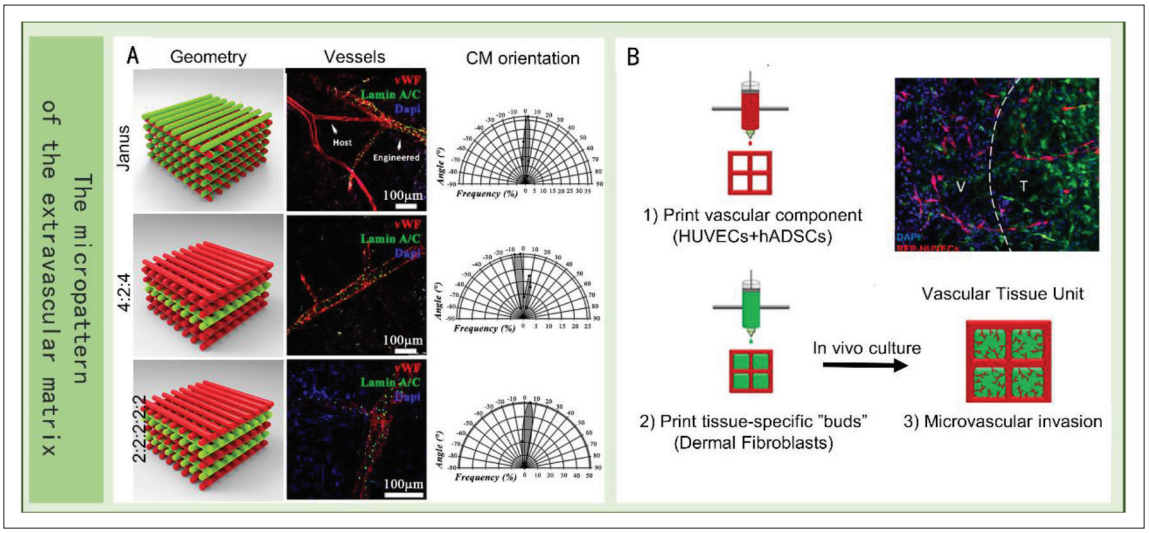

Figure 5. Vascular micropatterns that guide angiogenesis[64,66]. (A) Three different printing ratios of endothelial cells and cell-matrix[69]. (B) A vascularized tissue unit (VTU) containing both vascular and surrounding non-vascular tissue components[46]. Reprinted (adapted) with permission from American Chemical Society. Copyright © 2021, American Chemical Society.

are homogeneous. Chansoria *et al.* achieved high control over cell arrangement patterns in GelMA substrates through ultrasound-assisted bioprinting driven by volume sonic waves[68]. The patterned GelMA matrix can arrange the adherent endothelial cells along the patterned array and successfully construct a biomimetic vascular network with structural and mechanical anisotropy[68]. Different cell distribution also affects the efficiency of vascular network generation. Maiullari *et al.* designed three heterogeneous structures based on the proportion of HUVECs and cell-matrix in the printed tissue[69]. The two layers of HUVECs alternated with two (2:2:2:2:2) or four (4:2:4) layers of cell-matrix (Figure 5A). The Janus model constructs the most developed vascular network of the three models, and it is most clearly distributed in the direction of induced pluripotent stem cells (iPSC)-derived cell-matrix (angular spacing between -10° and +10°), revealing a potentially beneficial interaction between HUVECs and cell-matrix in the organization[69]. Barrs *et al.* have designed a vascularized tissue unit (VTU) containing vascular and surrounding nonvascular tissue components[46]. Only heterogeneous VTUs structures contained tissue components capable of angiogenesis. Moreover, there was no angiogenesis in the mixture of the vascular and non-vascular elements. This result indicates increased vascularization in heterogeneous, compartmentalized tissue structures compared to uniform, mixed tissue structures (Figure 5B)[46].

4.5. Multi-diameter vascular printing

Creating a systematic blood vessel network has become the future goal of vascular engineering. Blood vessel diameters in the network of natural blood vessels range from 10 microns (in capillaries) to 5 millimeters (in arterioles). 3D bioprinting can construct a similar hierarchical vascular network.

Son *et al.* first produced perfusable endothelialized channels (hundreds of microns in diameter) by printing sacrificial bio-inks containing endothelial cells and selectively removing the sacrificial material[70]. Then, they controlled the growth direction of vessels sprouting from the endothelialized channel by applying a gradient of angiogenic factors. Using the chemotaxis of endothelial cells, they employed the designed multi-cellular structure to create a multi-scale microvascular system consisting of a user-designed capillary network with good perfusion capacity[70]. Nie *et al.* constructed endothelial cell channels with diameters of 10, 20, 40, 100, 150, 250, and 500 μm by adjusting the printing speed and squeezing pressure to control the size of blood vessel diameters precisely, realizing the construction of the entire vascular system including large vessels and capillaries[71]. Szklanny *et al.* utilized 3D printing technology to construct a network of blood vessels inside tissues from a microscopic perspective and integrated the network of blood vessels, called "VascFold," from a mesoscopic perspective. The artificial tissue containing the vascular network can be anastomosed directly to the host vasculature through the small-diameter blood vessels[72].

5. Post-processing of bioprinting

Promoting the maturity of small-caliber blood vessels and microvascular networks is required in the construction of vascular grafts with complete biological functions.

Blood vessels' development and maturation process is very complex, involving the precise regulation of multiple biological, chemical, and physical stimuli in chronological order. Currently, bioprinting can only be used to create a static angiogenic environment and print blood vessel grafts containing pro-angiogenic factors. In addition, the vascular cells in grafts lack dynamic biochemical and mechanical stimuli that help recapitulate the physiological complexity of native blood vessels.

To address this challenge, the post-processing of bioprinting is significant. A bioreactor is a device specifically designed to cultivate and stimulate the development of engineered tissues. McFetridge *et al.* developed a modular bioreactor and perfusion system that allows vascular structures to grow and increase during an extended culture period[73]. Perfusion bioreactors can provide a constant supply of nutrients for blood vessel development and accurately replicate the hemodynamic stimulation of natural blood vessels, including pressure, strain, flow rate, and wall shear stress.

The physical environment in which blood vessel cells live is crucial for cell maturation. Hemodynamic shear stress is an essential determinant of endothelial function and phenotype. Wang *et al.* designed an *in vitro* flow adjustable vascular bioreactor system (VesselBRx) and demonstrated that under low-flow culture conditions, the intima of small-caliber vascular grafts thickened 2.5 times within 7 days, accompanied by a loss of 80% lumen area[74]. In contrast, under high-flow culture conditions, no neointima was observed. Under low-flow conditions, the endothelial cells no longer formed a single layer but extended into the cavity as a multi-layer structure. Under high-flow conditions, the endothelial cells physiologically remained as a monolayer with a vascular structure comparable to natural controls. This result indicates that the traditional static culture method cannot effectively maintain the normal physiological state of blood vessel cells, and bioreactors can provide sufficient hydrodynamic stimulation for vascular development and inhibit abnormal intimal hyperplasia[74]. Syedain *et al.* used pulsed fluid stimulation to promote the proliferation of fibroblasts in vascular grafts[75]. After 9 weeks of bioreactor culture, vascular grafts could withstand bursting pressures in the range of 1400–1600 mmHg, and their compliance was comparable to that of autologous arteries[75]. In addition, artificial blood vessels produced from this bioreactor have been transplanted surgically in ten patients with end-stage renal disease requiring hemodialysis access[76]. In light of the above, in-depth investigations into the cultivation and maturation of small-caliber vascular grafts in bioreactors are instrumental in effectively translating the fundamental bioprinted samples into the real-life clinical applications.

6. Conclusion

Cardiovascular diseases, a collection of non-infectious diseases with the highest mortality rate globally, put forward huge clinical demand for functional vascular grafts. As the technical bottleneck of organ transplantation, how to construct a microvascular network for material exchange in thick tissue is also the research focus of tissue engineering technology. 3D bioprinting uses computer modeling to accurately make complex pre-designed anatomical structures from cells and biological materials, providing a new way to overcome the shortage of blood vessel donors and the bottleneck of organ transplantation technology. In this review, we summarize and discuss functional vascular grafts and thick tissues prepared by 3D bioprinting technology from three aspects: large vessels and valves, small vessels, and microvascular networks, according to the anatomical caliber classification of the human vascular system. The large blood vessel grafts manufactured by 3D bioprinting can effectively solve the problems of inconsistent anatomy between electrospun grafts and host, and low cell survival rate. The complex heterogeneous structure of the valve is closely related to its special physiological functions. With the help of computed tomography scanning, computer modeling, and 3D bioprinting technology, it is possible to prepare biocompatible valves with good mechanical properties. 3D bioprinting technology can directly and accurately deposit vascular endothelial cells and smooth muscle cells to construct endothelialized small-diameter grafts with good biological properties, which effectively solve the high occlusive rate of small-caliber vascular grafts. High-resolution 3D bioprinting technology can design vascular micropatterns that guide angiogenesis and multi-dimensional vascular network layers that adapt to the host vascular system, which are the key differences between 3D bioprinting technology and other tissue engineering technologies. Combined with bioactive inks, 3D bioprinting technology can provide effective physical cues and biological stimuli for angiogenesis in thick tissues.

In the future, high-resolution printing technology and biological ink with good biological and mechanical properties will remain the research hotspot and key to 3D bioprinting technology. The emerging artificial intelligence can further improve the accuracy and resolution of bioprinting technology. In addition, the rise of smart materials has spawned the concept of 4D printing. Compared with the static structures generated by 3D bioprinting, 4D bioprinting allows 3D-printed structures to change their configuration or function over time in response to external stimuli, such as temperature, light, and water, thus bringing 3D printing to life. Endowing vascular grafts with the ability to respond to natural vascular environmental stimulation through innovative materials is also a possible future research direction for bioprinted

vascular grafts. Along with further improvement of existing biomaterials and molding processes, bioreactors can effectively promote the maturation of printed blood vessel, and fabrication of blood vessel grafts as well as thick, vascularized tissues and organs with complete biological functions will become possible.

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Conflict of interest

The authors declare no conflict of interest.

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