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Organoid bioprinting strategy and application in biomedicine: A review

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Abstract

Organoids are three-dimensional cell structures cultured *in vitro*. They are self-organizing and can mimic real organs in structure and function. Bioprinting technology breaks through some limitations of organoid manufacturing, making it more widely used in drug screening, regenerative medicine, and other fields. In this review, we first introduce bioinks and bioprinting methods for stem cell and organoid bioprinting, then summarize several vascularization strategies for bioprinting organoids, and present applications in biomedicine. In the future, the development of microfluidic technology and four-dimensional bioprinting technology may be conducive to forming better bioprinted organoids.

Keywords: Bioprinting; Organoid; Tissue engineering

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1. Introduction

Organoids are defined as collections of organ-specific cells. These cells are cultured through *in vitro* three-dimensional (3D) culture systems, self-organized through cell sorting and spatially constrained cell lineage differentiation, and exhibit a high degree of structural and functional similarity to *in vivo* tissues or organs[1]. According to different cell sources, organoids can be divided into tissue-derived organoids and pluripotent stem cell (PSC)-derived organoids. Tissue-derived organoids are composed of tissue-derived cells (TDCs), which include normal stem/progenitor cells, differentiated cells, and cancer cells. These TDCs are typically obtained from biopsy samples of tissue and organs from humans or animals. On the other hand, PSC-derived organoids are generated from PSCs, such as embryonic stem cells (ESCs) or induced pluripotent stem cells (iPSCs)[2,3].

In 2009, Sato *et al.* successfully cultivated the first 3D organoid using mouse intestinal stem cells (ISCs). They cultured intestinal organoids with crypt-villus by planting mouse intestinal adult stem cells into matrix glue *in vitro*, using a serum-free medium supplemented with three growth factors: R-spondin1, EGF, and Noggin[4]. Since then, the field of organoids has developed rapidly. At present, many types of organoids have been successfully constructed, including heart[5,6], kidney[7], liver[8], brain[9], and tumor[10] organoids.

Organoids are widely used in disease modeling, drug development, and personalized treatment because their self-organizing characteristics are similar to biological intrinsic processes, enabling the simulation of the formation process and physiological and pathological state of organ tissues. Since 2020, significant research progress has been made in applying organoids to COVID-19 disease modeling[11,12]. To research how colon and alveolar cells react to SARS-CoV-2 infection, Han *et al.* created human pluripotent stem cell (HPSC)-derived lung organoids (hPSC-LOs) and colon organoids (hPSC-COs). By high-throughput screening of pharmaceuticals that have received FDA approval, they also discovered three medications with antiviral effectiveness against SARS-CoV-2[12].

In order to give full play to the potential of organoid technology, the problems in organoid manufacturing must be solved. The culture method of organoids mainly relies on the traditional 3D culture technology, which utilizes the self-organizing properties of stem cells. However, it has many limitations, such as lack of repeatability, limited size, lack of vascular system, and communication between immune cells and organs[13]. Due to the above-mentioned limitations, 3D bioprinting technology is currently applied to organoid cultivation, replacing manual organoid construction for growing more complex large organoids.

3D bioprinting is derived from 3D printing (also known as additive manufacturing [AM]). Unlike 3D printing, which uses adhesive materials such as powdered metal or plastic, 3D bioprinting uses bioinks as printing materials that are deposited layer by layer spatially to create a tissue-like growth structure. The application of bioprinting technology in organoid manufacturing can control the composition and distribution of bioinks more accurately compared with manual construction; hence, it is expected to realize the stable construction of organoids with high precision, high throughput, and batch automation. In 2021, Lawlor *et al.* used extrusion bioprinting to successfully generate self-organizing kidney organoids with high cell number and viability reproducibility. The produced organs were comparable to manually engineered kidney organoids in terms of morphology, component cell types, and gene expression levels, demonstrating the feasibility of replacing manual organoid engineering with bioprinting methods. In addition, 3D bioprinting can also change the biophysical characteristics of organoids, including volume size, number of cells, and conformational configuration, which has excellent advantages[14].

At present, 3D bioprinting technology is mainly used for bioprinting stem cells for the construction of organoids and then promoting the differentiation of stem cells through growth factors and small molecules, as well as mechanical signaling pathways of extracellular matrix (ECM) materials. Bioprinting of organoids with complex structures remains a challenge[15].

This paper reviews the recent progress and application of bioprinting organoids. Firstly, the bioink and bioprinting methods used in bioprinted organoids are introduced. Secondly, the vascularization strategies of bioprinted organoids are summarized and analyzed in view of the insufficient vascularization of traditional organoids. Then, the applications of bioprinted organoids in drug screening, regenerative medicine, and tumor research are introduced. At the same time, the application of microfluidic technology and more advanced bioprinting methods to solve the defects of existing bioprinting organoids is discussed. Finally, the article concludes with a summary and a look into the possible future directions of developing bioprinting organoid technology (Figure 1).

2. Organoid bioprinting

The selection of bioink and printing methods is critical to realizing organoid bioprinting. Bioink is the necessary condition for the success of organoid bioprinting. A suitable printing method can better play the characteristics of bioink and get a better printing effect. Here, we introduce currently used bioinks and printing methods.

2.1. Bioink for organoid bioprinting

The printing materials used in 3D bioprinting are known as bioinks, and their properties are typically determined by three metrics: printability, biocompatibility, and mechanical properties. Printability refers to the forming characteristics of bioink, which is related to many factors, such as the viscosity of the material and printing parameters. The bioink with good formability shows good flow during printing and can be cured quickly after printing. Biocompatibility requires that the bioinks have an environment similar to the ECM *in vivo*, facilitating the development and communication of cells after printing. Mechanical properties require the bioink to have sufficient strength to support the subsequent culture process[16]. In addition, bioinks for stem cell and organoid bioprinting need to be biodegradable and cell nontoxic. The selection of the most appropriate bioink in bioprinting is usually considered in combination with the specific target tissue, cell type, and bioprinting method[17].

The main components of bioink are cells and biological materials. Hydrogels are 3D network structure gels composed of hydrophilic polymers through crosslinking, which can highly simulate a natural ECM environment *in vitro*. They are the most widely used bioink materials. Hydrogel bioinks usually comprise natural polymers, synthetic polymers, and decellularized extracellular

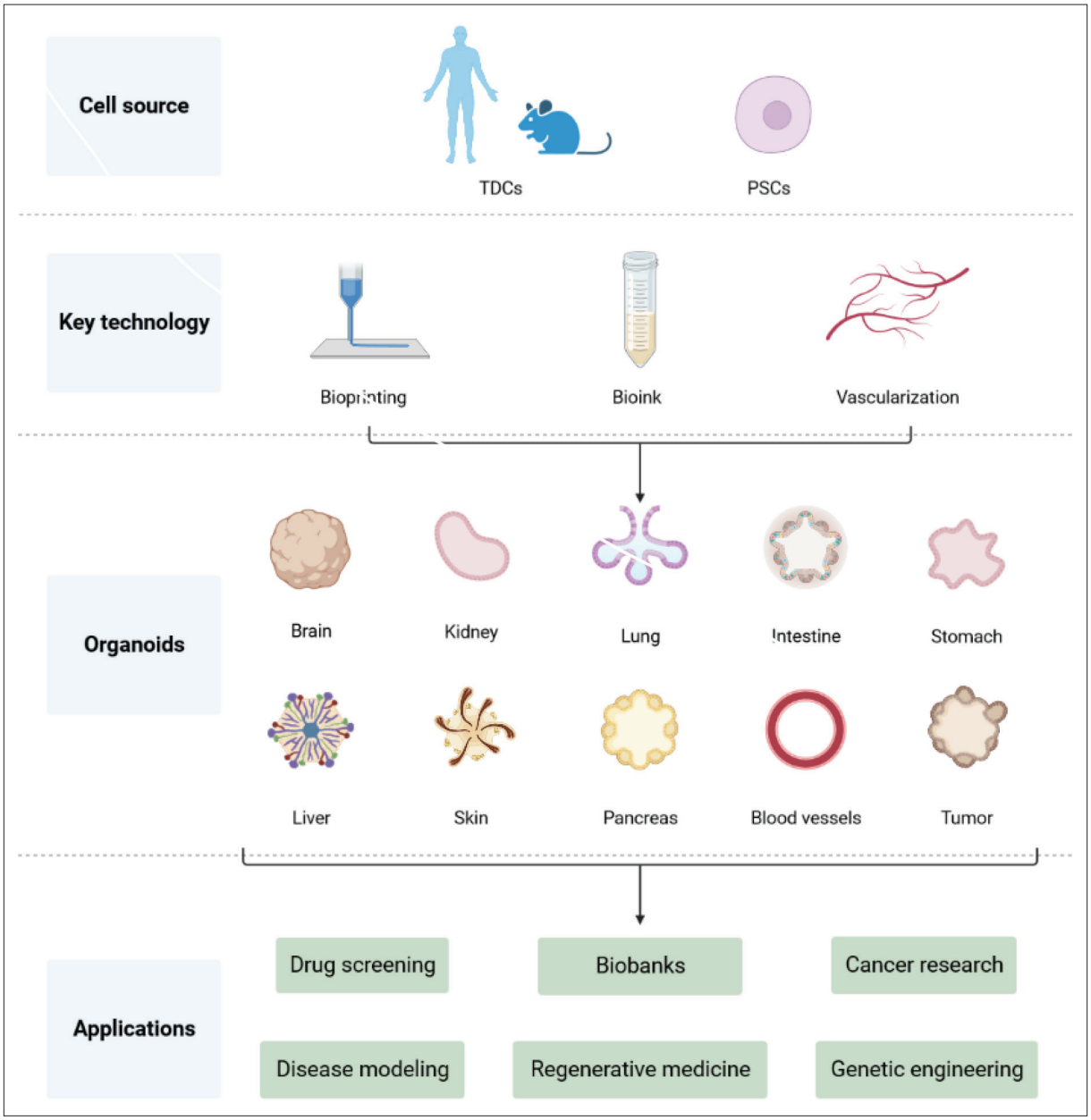

Figure 1. Key technologies and typical applications of bioprinted organoids. Organoids can be realized through bioink, bioprinting methods, and vascularization strategies. Applications of bioprinted organoids include but are not limited to drug screening, biobank, cancer research, disease modeling, regenerative medicine, genetic engineering, etc. Created using BioRender.com.

matrix (dECM)[17]. Natural bioinks include alginate, gelatin, fibrin, collagen, and hyaluronic acid (HA). Synthetic bioinks include polyethylene glycol (PEG), Pluronic, and other types (Table 1).

Natural bioinks have good biocompatibility but generally have problems such as poor printability, which could be less conducive to printing complex tissue structures. Synthetic bioinks provide good printability but poor biocompatibility and may produce toxic degradations[15]. By improving bioink characteristics, using compound bioink, or developing new bioink materials, bioink performance can be effectively improved.

Oxidative reactions have demonstrated controlled degradation of alginate. Jia *et al.* explored the application of oxide-alginate in bioprinting[36]. They used hADSCs/oxide-alginate as bioinks for bioprinting through a piston-driven deposition system. The results show that oxidized alginate bioinks with a specific density and viscosity can maintain

Table 1. Comparison of properties of bioinks for stem cell bioprinting

Type	Bioink	Benefits	Drawbacks	Bioprinting cell types	Reference
Polysaccharides	Alginate	Fast gelation, low cost, good stability	Limited biodegradation	Human iPSCs, human neural stem cells	[18,19]
Polysaccharides	HA	Good rheology, high viscosity	Poor stability, poor mechanical properties	Adipose-derived stem cells, iPSCs	[20,21]
Protein-based	Gelatin	Good biodegradability, low antigenicity, easy to process	Inherent low viscosity, poor mechanical properties	Human adipose tissue-derived stem cells (hASCs), umbilical cord-derived mesenchymal stem cells, and endothelial cells	[22,23]
Protein-based	Silk fibroin	Good mechanical properties, high elasticity	Poor printability	Human inferior turbinate tissue-derived mesenchymal stem cells (hTMSCs), bone marrow mesenchymal stem cells	[24,25]
Protein-based	Fibrin	Promotes angiogenesis and induces cell attachment and proliferation	Poor mechanical properties	Human dental pulp stem cells (hDPSCs), human amniotic fluid stem cells	[26-28]
Protein-based	Collagen	Rich in RGD sequences, promoting cell attachment	Slow gel rate, poor mechanical properties	hASCs, rat bone marrow-derived stem cells	[29-31]
dECM-based	dECM	Provides a natural extracellular matrix environment for cells rich in cell growth and differentiation factors	Low viscosity, poor mechanical properties, fast degradation rate	hASCs, hTMSCs	[32]
Synthetic polymer-based	PEG	Customizable and strong mechanical properties, no cytotoxicity or immunogenicity	Bioinert, not conducive to cell attachment	Bone marrow-derived human mesenchymal stem cells	[33,34]
Synthetic polymer-based	Pluronic	Good printability, temperature-sensitive gel	Poor biocompatibility, poor mechanical properties	Human mesenchymal stem cells	[35]

uniform cell suspension during printing, providing higher printing resolution and better cell viability. It also proves that oxidized alginate bioinks can effectively regulate stem cells' proliferation and diffusion behavior without affecting printability and structural integrity. Wu *et al.* proposed that sodium citrate is also an effective method to improve the insufficient degradation of alginate[37]. They used human corneal epithelial cells (HCECs)/collagen/gelatin/alginate hydrogel as bioinks and bioprinted using extrusion bioprinting technology. The controlled degradation of alginate was also achieved by using a culture medium containing sodium citrate, which resulted in the better proliferation and expression ability of specific marker proteins in the printed HCECs. To eliminate the inherent low viscosity of gelatin, He *et al.* used reversible quadruple-hydrogen-bonded ureido-pyrimidinone (UPy) and enzyme-responsive tyramine moieties (Tyr) sequence to chemically modified gelatin and developed a new type of gelatin bioink Gel-UPy-Tyr[38]. They used human bone marrow mesenchymal stem cells (hBMSC)/Gel-UPy-Tyr as bioinks for bioprinting by extrusion bioprinting, demonstrating that Gel-UPy-Tyr has temperature-programmable viscosity and enzyme-curing properties, providing better printability while maintaining higher cell viability and promoting the proliferation of hBMSC.

Due to excellent biocompatibility and good printability, composite bioinks have been widely used in stem cells and organoid bioprinting. Li *et al.* created a novel type of bioink called GelMA/alginate/PEGDMA/xanthan gum hydrogel bioink which can be printed at room temperature, by incorporating PEGDMA and xanthan gum into gelatin methacrylate (GelMA)/alginate-based hydrogels. Among them, the GelMA provides good biocompatibility and is helpful for cell adhesion and growth; the fast alginate gelation ensures structural integrity after printing; PEGDMA improves mechanical properties; and xanthan gum is a viscosity enhancer to improve printability[39]. They used hMSCs/GelMA/alginate/PEGDMA/xanthan gum as bioinks, combined with extrusion bioprinting technology to print, and the cells still had strong vitality and proliferation ability after printing. Yu *et al.* synthesized KEGC bioink from keratin methacrylate (KEMA) and glycol chitosan methacrylate (GCMA), wherein keratin provides biological

function. Glycol chitosan improves mechanical strength and biocompatibility[40]. Using hASCs/KEGC as bioinks, they were bioprinted by extrusion bioprinting technology, and the printed cells remained highly viable and could be continuously cultured. In addition, nanoparticles are widely used in bioinks because of their excellent properties. It can interact with polymers to adjust their properties and can also be used to transmit cellular signals. Alcala-Orozco *et al.* developed Sr-GelMA nanocomposite bioink consisting of strontium carbonate (Sr) nanoparticles and GelMA, where GelMA provides good biocompatibility, and Sr improves printability[41]. They bioprinted hMSC/Sr-GelMA with extrusion bioprinting technology, and the printed cells maintained high viability (>95%). In addition, Sr also promoted osteogenic differentiation of hMSCs. In addition, studies have shown that introducing solid micro scaffolds into composite bioinks can also improve the cell viability of organoid bioprinting[42].

Self-assembling peptides are highly similar to the ECM, both structurally and mechanically, and have been applied in bioprinting as a novel bioink material[43]. Cofiño *et al.* developed a bioink blend of self-assembling peptide RAD16-I with methylcellulose (MC). RAD16-I is not immunogenic and cytotoxic, and can support the attachment, growth, maintenance, and differentiation of various cells. MC is added to enhance the viscosity of the bioink[43]. They used hMSCs/RAD16-I/MC as bioinks, and the printed structure has high shape fidelity and stability while maintaining high cell viability. Alhattab *et al.* developed two kinds of ultrashort peptide bioinks using Ac-Ile-Ile-Cha-Lys-NH2 (IIZK) and Ac-Ile-Cha-Cha-Lys-NH2 (IZZK) peptide sequences, respectively, and combined with human bone marrow mesenchymal stem cells (hBM-MSCs) for bioprinting. The cells showed high activity after printing, and the two ultrashort peptide bioinks promoted the chondrogenic differentiation of hBM-MSCs[44].

dECM refers to the remaining ECM after the removal of cellular components from tissues through decellularized technology[45]. Although dECM bioinks have limitations such as low viscosity, poor mechanical properties, and fast degradation rate, they also have many outstanding advantages compared with natural and synthetic bioinks. DECM has excellent tissue-specific functions, provides cells with a natural ECM environment, and is rich in cell growth and differentiation factors and various proteins. Due to these properties, dECM bioinks are gradually being widely used in bioprinting[46]. The limitations of dECM can be improved by mixing dECM with other bioink materials. Xu *et al.* developed a novel bioink in which porcine intestinal dECM provided an ECM environment, and photosensitive GelMA provided rapid gelation and good photocuring properties. Other components of the bioinks serve different functions; for instance, the photoinitiator lithium phenyl (2,4,6-trimethylbenzoyl) phosphinate (LAP) triggers chemical crosslinking between polymers, and HA improves biocompatibility and viscosity[47]. By printing the dECM-HA bioink of mixed mouse crypts and GelMA/LAP pregel, and seeded with submucosal cells, they successfully established a co-culture system of submucosal cells and intestinal organoids and found that it enhanced the function and proliferation of ISCs. Zhang *et al.* combined dECM with silk fibroin protein to develop SF-dECM bioink, in which dECM was derived from natural cartilage tissue and provided a matching ECM environment for bone marrow mesenchymal stem cells (BMSCs). Silk fibroin improves mechanical strength[48]. The structure printed by this bioink mixed with BMSCs can support the proliferation of BMSCs and promote cartilage differentiation (Figure 2).

2.2. 3D bioprinting technology for organoid bioprinting

With the integration of organoids and 3D bioprinting technology, more and more printing methods have been applied to the bioprinting of stem cells or organoids. According to different principles, the commonly used printing methods are divided into three categories: extrusion-based bioprinting, droplet-based bioprinting, and photocuring-based bioprinting[49]. In addition, more new bioprinting technologies have been gradually developed, such as coaxial bioprinting[50], acoustic bioprinting[51], and magnetic bioprinting[52] (Table 2, Figure 3).

2.2.1. Extrusion-based bioprinting

The extrusion-based bioprinting (EBB) technology consists of two main parts: a fluid distribution system for extruding and an automatic robotic system for printing. The fluid distribution system is driven by pressure-assisted pneumatic, piston, or screw systems, and the bioink is extruded from the nozzle and deposited in the form of cylindrical silk[53]. EBB technology can be used to print biomaterials with viscosity ranging from 30 to 6×10^7 mPa/s, suitable for bioinks with high viscosity. Its characteristics of continuous deposition of filaments can provide better structural integrity for bioprinting, so EBB technology has been widely applied in organoid bioprinting. However, EBB technology also has many limitations. Firstly, EBB technology's resolution can only reach about 100 μm, which reduces printing accuracy and limits the function of printing tissue. Secondly, high shear stress caused by extrusion of high-viscosity bioink reduces cell vitality, and the survival rate of cells after EBB technology printing is usually between 40% and 86%. It is significantly lower than

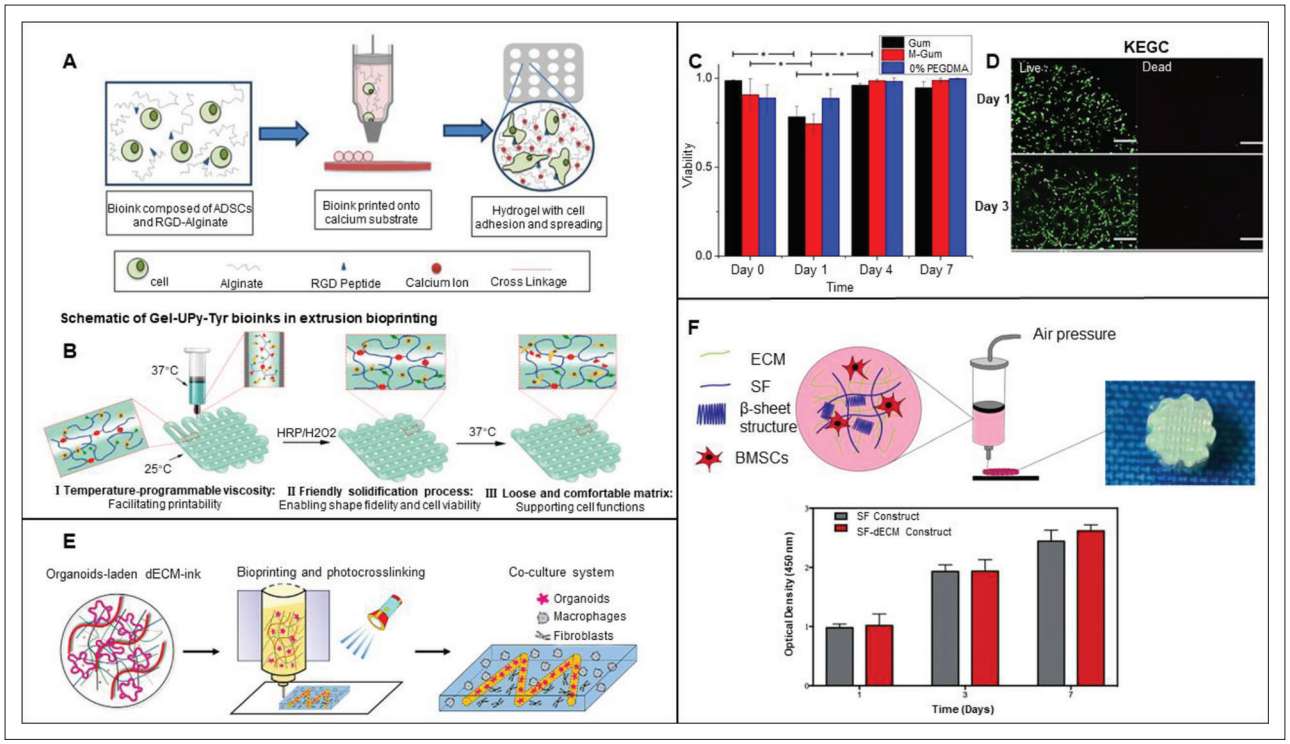

Figure 2. Bioinks for stem cell and organoid bioprinting. (A) Oxidized alginate and ADSCs for bioprinting as bioinks. Reprinted with permission from ref.[36]. Copyright 2014 Elsevier. (B) Gel-UPy-Tyr bioink for extrusion bioprinting. Reprinted with permission from ref.[38]. Copyright IOP Publishing. (C) hMSCs activity in printed bioinks, 0% PEGDMA (10% GelMA + 1.25% alginate + 0% PEGDMA + 3% gum), Gum (10% GelMA + 1.25% alginate + 2% PEGDMA + 3% gum), M-Gum (10% GelMA + 1.25% alginate + 2% PEGDMA + 3% crosslinked gum). Reprinted with permission from [39]. Copyright 2022 Elsevier. (D) hASCs live and dead staining in KEGC mixed bioink printed showed that living cells were stained green, with a high cell survival rate and good migration ability. Reprinted with permission from [40]. Copyright 2023 ACS Publications. (E) Preparation of intestinal organoids and submucosal cell co-culture system using bioprinting and dECM bioink (from ref.[47] licensed under Creative Commons Attribution 4.0 license). (F) Bioprinting using SF-dECM bioinks and BMSCs activity in print structures measured by CCK-8. Reprinted with permission from ref.[48]. Copyright 2023 Elsevier.

other bioprinting technologies[16]. Reducing the size of the nozzle is an effective method to improve the resolution. However, the decrease in the size of the nozzle is prone to blockage and increases the extrusion pressure, which leads to a decrease in cell survival rate. Therefore, when using extrusion-based bioprinting technology, appropriate parameters should be set in combination with multiple factors such as viscosity, printability, and cell survival rate of bioink[54]. In light of this, developing new EBB technologies can effectively improve the areas where the limitations of traditional EBB technologies are found.

The shear stress generated by the extrusion of bioinks with low viscosity is minor and will not significantly impact cell activity, but the complex organizational structure cannot be firmly maintained after printing[55]. Suspension bioprinting (also known as embedded bioprinting) enables the printing of low-viscosity bioinks that effectively improve cell survival. Unlike the traditional extrusion printing method, which deposits bioink in cylindrical silk on a flat surface, suspension printing introduces a suspension medium, which deposits bioink in a support bath containing the suspension medium. A suspension medium is a yield stress material, showing solid and liquid properties according to the critical stress. When no external force is applied, the suspended medium behaves like a solid under critical stress. When the printing nozzle moves in the suspended medium, the generated force exceeds the critical stress, above which the suspended medium flows like a liquid. When the nozzle passes through, the suspended medium quickly returns to the solid form in a self-healing manner[56,57]. The characteristics of the suspended medium support the printing of low-viscosity bioink, maintain the stability of the printing structure, and realize all-around printing, free from the constraints of construction direction, complex geometric shape, and other factors[57]. Lee *et al.* developed a freeform reversible embedded suspended hydrogel (FRESH) technology, which uses gelatin particles as the suspension medium to support the printing process. When heated to 37°C, the suspension medium will melt and release the printed structure[58]. Using collagen as bioink and human embryonic stem cell-

Table 2. More 3D bioprinting for stem cell or organoid bioprinting

3D bioprinting technology	Basic principle	Benefits	Drawbacks	Bioprinted stem cells or organoids	References
Coaxial bioprinting	One of EBB technologies, concentric nozzles are stacked to form coaxial nozzles for printing a variety of bioinks.	Enables the construction of layered tubular structures with adjustable biological/mechanical properties, with cell viability dependent on extrusion speed and nozzle diameter	Low resolution, 7 mm–210 μm	Mesenchymal stem cells (MSC)	[49,50,74]
Acoustic bioprinting	One of the DBB technologies, bioinks are ejected in droplet form by force generated by sound waves.	High resolution (~37 μm) and cell viability (>90%), high printing speed	The movement of the print head interferes with the droplet ejection and is not suitable for high-viscosity bioinks.	Bladder tumor organoids	[51,61,75]
Electrohydrodynamic jetting (EHDJ) bioprinting	One of the DBB technologies that use a high-voltage-driven electric field to pull the bioink out of the nozzle hole in the form of a droplet	With a better resolution of 2–5 μm, cell viability >90%, suitable for printing high-viscosity bioinks	Difficult to print complex structures and cell viability may be affected in the long term after printing, making it unsuitable for precise bioprinting applications	Human adipose stem cells	[49,60,61,63,64]
Microvalve bioprinting	A DBB technology in which a microvalve controls the opening of the nozzle hole and the bioink is sprayed out of the open nozzle hole in the form of a droplet when the air pressure is high enough	Accurate cell localization, high cell survival rate >86%, fast printing speed	The size of the nozzle hole limits the printing of bioinks within a specific viscosity range (1 to 200 mPa/s).	Human embryonic stem cells	[61,76,77]
Magnetic bioprinting	By magnetizing cells with magnetic nanoparticles, the magnetic force is used to control the spatial pattern of the desired morphology of the cell aggregates.	Cell vitality >90%, reasonable spatial control, synthesis of endogenous ECM, can quickly print a variety of tissue structures	The cytotoxicity of magnetic nanoparticles may cause adverse effects on cells.	Salivary glands organoids	[52,78] [79,80]

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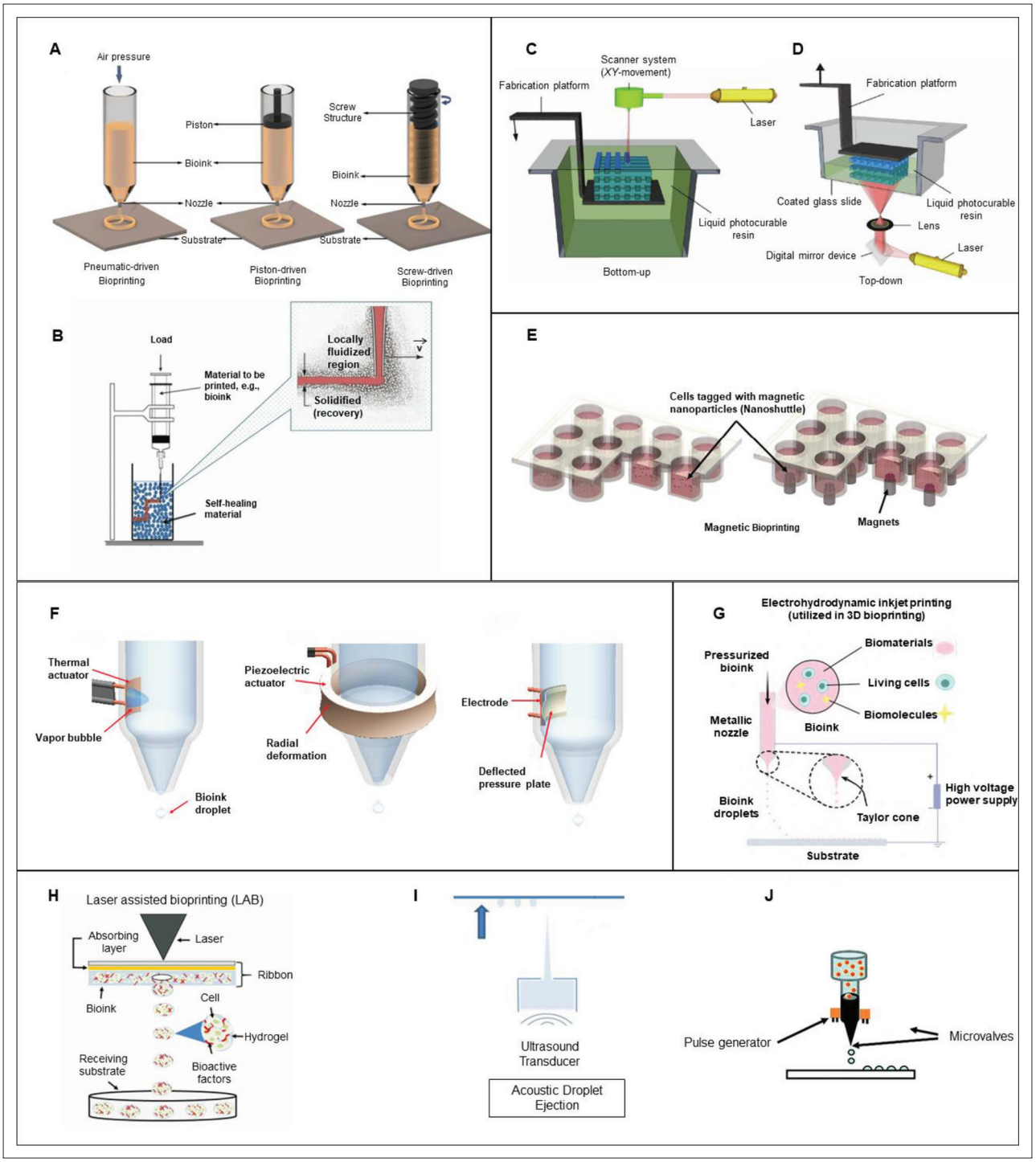

Figure 3. Bioprinting technology. (A)–(B) Extrusion-based bioprinting: (A) Extrusion bioprinting (pneumatically driven, piston-driven, screw-driven) (from ref.[16] licensed under CC BY-NC-ND 4.0). (B) Suspension bioprinting (from ref.[59] licensed under Creative Commons Attribution 4.0 license). (C)–(D) Photocuring-based bioprinting: (C) Stereolithographic bioprinting (from ref.[69] licensed under Creative Commons Attribution 4.0 license). (D) Digital light processing bioprinting (from ref.[69] licensed under Creative Commons Attribution 4.0 license). (E) Magnetic bioprinting. Reprinted with permission from ref.[78]. Copyright 2020 Elsevier. (F)–(J): Droplet-based bioprinting: (F) Inkjet bioprinting (thermal inkjet, piezoelectric inkjet, electrostatic inkjet). Reprinted with permission from ref.[61]. Copyright 2016 Elsevier. (G) Electrohydrodynamic jetting bioprinting. Reprinted with permission from ref.[64]. Copyright 2023 Springer Nature. (H) Laser-assisted bioprinting (from ref.[65] licensed under CC BY-NC 4.0). (I) Acoustic bioprinting (from ref.[79] licensed under CC BY-NC 4.0). (J) Microvalve bioprinting (from ref.[76] licensed under Creative Commons Attribution 3.0 license).

derived cardiomyocytes (hESC-CMs), they achieved high-resolution bioprinting of cardiac organoids with systolic function using FRESH technology. Suspension bioprinting also has some limitations[59]. The first limitation is that the integrity of the printing structure may be compromised when the structure is extracted from the suspended medium. The second limitation is that the suspended medium restricts the printing process. For example, the bioink cannot be bioprinted at the temperature that the suspended medium cannot accommodate.

In addition, the research proves that mixed printing with other bioprinting technologies was also an effective measure to improve the limitations of traditional EBB technology. Yeo *et al.* developed a hybrid bioprinting technology combining traditional EBB technology and electrohydrodynamic jetting. Human adipose stem cells in the cell load structure printed by this technology could still maintain high cell vitality. It has been confirmed that the hybrid bioprinting technique can achieve rapid and stable bioprinting of cell-loaded structures without loss of cell viability[60].

2.2.2. Droplet-based bioprinting

Droplet-based bioprinting (DBB) technology is printed by stacking independently separated droplets with higher resolution than EBB's technology. According to the different principles of droplet formation, bioprinting technology can be divided into inkjet bioprinting, laser-assisted bioprinting (LAB), electrohydraulic dynamic jet (EHDJ) bioprinting, acoustic bioprinting, and microvalve bioprinting. Among these, inkjet bioprinting and LAB are currently more widely used.

Inkjet bioprinting, in which bioink is ejected as a droplet from a nozzle when a pressure pulse is generated, may contain multiple print heads, each equipped with a fluid chamber containing bioink and one or more nozzles with a minimum diameter of 18 μm. According to the driving mechanism of the pressure pulse, inkjet bioprinting can also be subdivided into thermal inkjet, piezoelectric inkjet, and electrostatic inkjet[61]. Inkjet bioprinting has good performance, based on: Firstly, it can generate droplets at a high-speed rate (up to 30 kHz), and the size of the droplets generated is tiny, resulting in a very high resolution (about 50 μm). Secondly, inkjet bioprinting has good cell activity, and the cell survival rate is generally 80% to 95%[62]. As inkjet bioprinting is a non-contact printing technique, the droplets are not subjected to any harm by the print head moving after injection. Additionally, inkjet bioprinting has a print head with multiple nozzles that can print multiple bioinks simultaneously, enabling the creation of multicellular tissues or organs[63]. Nevertheless, inkjet bioprinting has significant limitations. First, droplet spatter is inevitable in the injection process, leading to the decline of resolution[64]. In addition, due to the limitation of nozzle size, inkjet bioprinting is only suitable for bioinks with low viscosity and cell density. Otherwise, the nozzle blockage is likely to happen during the printing process, resulting in poor durability of the print head[63].

LAB techniques, including laser-guided direct writing (LGDW) and laser-induced forward transfer (LIFT), are more commonly used due to their excellent performance. The LIFT device for bioprinting consists of three parts: A laser source (mainly nanosecond laser), a target plate, and a receiving substrate. The target plate consists of clear glass, an absorbing layer (made of metal), and a bioink layer. The laser energy vaporizes the absorbing layer of metal to produce high-pressure bubbles that squeeze the bioink out as droplets. The absorbent layer can also avoid direct contact between the bioink and the laser, protecting it from the laser[65]. LIFT has significant advantages over inkjet bioprinting, as it is a nozzle-free printing method hence no nozzle clogging problem, making it suitable for printing bioinks with high viscosity and cell density, with a resolution of up to micron, 95% cell viability after printing, and normal cell proliferation[66]. Sorkio *et al.* used LIFT technology to print limbal epithelial stem cells (hESC-LESC) and hASCs derived from human embryonic stem cells to simulate natural corneal tissue structure, and the cells maintained good vitality after printing[30]. However, LAB still has limitations despite its advantages over others. The vaporization of the metal absorption layer may lead to metal residue in the structure of bioprinting, causing pollution. In addition, it is necessary to manufacture multiple target plates when printing multiple bioinks, necessitating longer manufacturing time and higher cost[67].

2.2.3. Photocuring-based bioprinting

Photocuring-based bioprinting uses light to solidify bioinks. The technique can be subdivided into stereolithography (SLA) and digital light processing (DLP) according to different ways of curing.

SLA uses a UV point light source and point-to-point irradiation of bioinks to cure layer by layer selectively and eventually form complex structures[68]. SLA has no nozzle limitation compared to extrusion-based bioprinting technology, so it has higher resolution (generally less than 100 μm) and cell viability (up to 85%). It should be noted that UV light sources may cause damage to cells during the printing process, resulting in reduced cell viability[69]. Grix *et al.* used SLA technology combined with HepaRG and human stellate cells and successfully achieved accurate bioprinting of liver organoids. The printed liver tissue equivalent was found to maintain cell viability for at least

14 days, confirming the potential application of SLA technology in organoid bioprinting[70].

Unlike SLA technology, DLP uses an ultraviolet surface light source. The light projected from a digital micromirror device illuminates an entire layer of bioink, curing an entire layer at a time. Hence, DLP has a much faster printing speed than SLA[71]. In addition, DLP has a high resolution (less than 20 μm) and cell viability (more than 90%). DLP technology is widely used in bioprinted organoids due to its excellent performance. Xie *et al.* bioprinted hydrogel microspheres (MSs) containing BMSC using DLP technology, which maintained good cell viability after printing and successfully constructed callus organoids after cartilage induction[72]. However, DLP technology still has room for improvement. Currently, DLP printing technology with different light sources is being investigated to further reduce the impact on cells and improve cell viability. Additionally, DLP technology has a high demand for bioinks. While meeting traditional requirements, bioinks also need to ensure the photo-polymerization speed and the fidelity of the printing structure[73].

3. Tissue vascularization strategies

The lack of vascularization is a significant drawback of large-scale tissue construction, as the natural diffusion of oxygen and nutrients can only support tissue growth up to a size of 150 μm. This is also true for organoids, which face similar challenges. As traditionally-constructed organoids grow beyond a certain size, the exchange of oxygen, nutrients, and metabolites can no longer be achieved through natural diffusion, thereby resulting in the loss of cell vitality and the development of necrotic cores[81]. Przepiorski *et al.* used a rotating bioreactor to generate hiPSCs-differentiated kidney organoids. They found that the core cells of kidney organoids with a diameter greater than 700 mm were significantly reduced. The cell viability was not as good as kidney organoids with a smaller diameter[82]. Therefore, it is essential to realize the vascularization of organoids. Bioprinting technology has been widely used in vascularization due to its excellent performance. Various techniques are now available to achieve the vascularization of bioprinted organoids, including growing the organoids directly onto 3D-printed vascular structures, using growth factors to encourage angiogenesis, and combining endothelial cells with stem cells or organoids to create organoids with blood vessels[15] (Figure 4).

The printing of blood vessel structures can be divided into direct and indirect printing. Direct bioprinting mainly uses mixed bioinks, while indirect printing uses sacrificial bioinks.

3.1. Direct bioprinting vascularization

Direct printing involves using bioink and bioprinting technology to print out tubular structures. Coaxial bioprinting technology is often used in the direct printing of blood vessels. However, without a supporting material, the printed blood vessel structure is prone to deformation or collapse during the printing process[83]. As a result, the direct printing of blood vessels demands high-quality bioink with better printability, mechanical properties, and angiogenic potential[84]. Jia *et al.* developed a hybrid bioink consisting of GelMA, sodium alginate, and PEGTA, with excellent printability, biocompatibility, and mechanical properties that support the survival and proliferation of vascular cells. By using this cell-loaded hybrid bioink and coaxial bioprinting technology, they have successfully achieved direct bioprinting of perfused vascular structures[85]. Hong *et al.* also applied gelatin to the printing of blood vessel structures. They synthesized gelatin-PEG-tyramine (GPT) mixed bioinks, in which tyramine has rapid crosslinking properties, and PEG acts as the spacer material between gelatin and tyramine to promote rapid gelation. They demonstrated one-step bioprinting of perfumable vascular structures using GPT bioinks and coaxial bioprinting[86]. In addition to mixed bioinks, chemically modified natural bioinks can also be used for vascularization direct bioprinting. Barrs *et al.* developed a novel peptide-functionalized alginate hydrogel bioink using RGD (integrin-binding state for cell adhesion) and a vascular endothelial growth factor (VEGF)-mimetic peptide with matrix metalloproteinase cleavable linker (MMPQK) for modification of oxidized alginate. Direct bioprinting of vascularized tissue units (VTUs), consisting of the blood vessel and tissue-specific components, was successfully achieved using RGD/MMPQK bioink for vascular components and tissue-specific bioink for tissue-specific components[87].

3.2. Indirect bioprinting vascularization

Indirect printing involves the use of sacrificial bioinks to print vascular structures. These bioinks can be removed physically or chemically after printing, leaving a perfusive and endothelialized hollow lumen. It should be noted that sacrificial bioinks are usually bioprinted with extrusion-based bioprinting technology. However, the lack of resolution in this technology limits the application of sacrificial bioinks in the bioprinting of small-diameter blood vessels[84]. Additionally, the complexity of indirect printing technology may affect the size and function of the resulting vascular structures. Kolesky *et al.* adopted Pluronic F127 as the sacrificial bioink and GelMA as the bioink. Pluronic F127 appears in a solid state, GelMA ink in a liquid state when the temperature is higher than 22°C, and Pluronic F127 in a liquid state and GelMA ink appear in a solid state when the temperature is lower than 4°C.

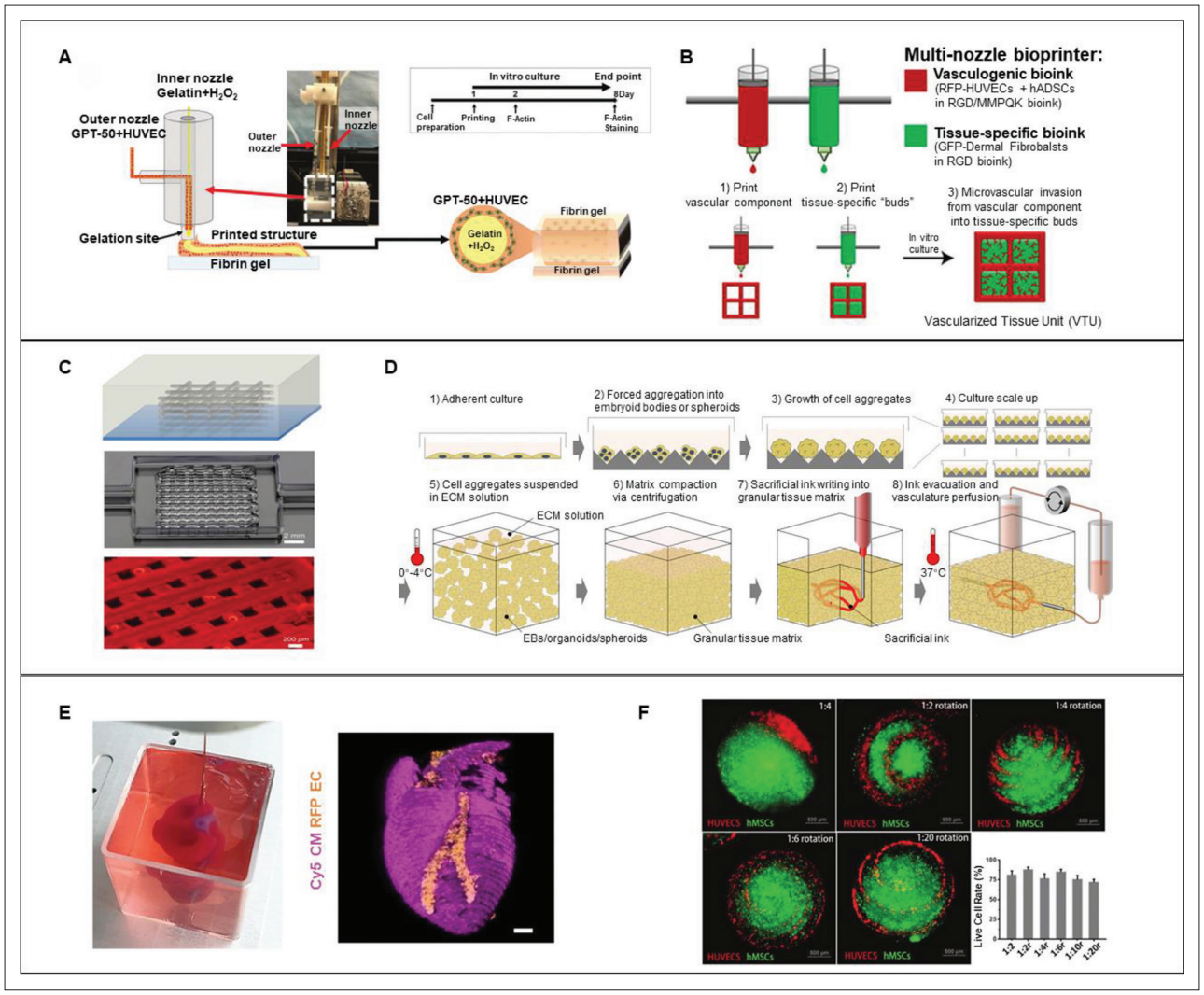

Figure 4. Vascularization of bioprinted organoids. (A)–(B) Direct bioprinting vascularization: (A) Coaxial bioprinting of GPT bioinks containing HUVEC. Reprinted with permission from ref.[86]. Copyright 2013 Royal Society of Chemistry. (B) Direct bioprinted vascularization tissue units. Reprinted with permission from ref.[87]. Copyright 2021 American Chemical Society. (C)–(D) Indirect bioprinting vascularization: (C) Pluronic F127 as a sacrifice bioink to print 3D microvascular network. Reprinted with permission from ref.[88]. Copyright 2014 John Wiley & Sons. (D) Schematic diagram of SWIFT technical process (from ref.[89] licensed under the CC BY-NC 4.0). (E) DECM personalized hydrogel bioink-printed heart containing blood vessels and its 3D confocal image (CMs, induced pluripotent stem cell (iPSCs) derived cardiomyocytes, ECs, endothelial cells) (from ref.[93] licensed under Creative Commons Attribution 4.0 license). (F) Airflow-assisted bioprinted helical vascularization structure. Reprinted with permission from ref.[94]. Copyright 2018 John Wiley & Sons.

As a result, the sacrificial bioinks can be easily removed at low temperatures, and Pluronic F127's bioenergy allows it to be printed and removed without damage to cells. After removing the sacrificial bioinks, they successfully obtained perfusive channels and realized vascular structure after endothelialization culture[88]. Using patient-specific induced iPSC-derived organoids as organ building blocks (OBB) and the sacrificial write functional tissue (SWIFT) methodology, Skylar *et al.* created a unique method for producing vascularized organ-specific tissues with high cell density and maturation. They used heart organs as OBBs and gelatin as sacrificial bioinks. At 37°C, the gelatin sacrificial bioinks were removed upon melting, and a perfusable vascular channel was successfully achieved in the heart tissue[89]. Bioprinted kidney organoids have been proven to have better maturity. The combination of bioprinted organoids as OBBs and SWIFT technology may realize the construction of centimeter-level kidney organoids with blood vessels[90].

3.3. Vascular growth factor

Vascular growth factors, such as vascular endothelial growth factor (VEGF), primary fibroblast growth

factor (bFGF), and others, can promote angiogenesis and development. Compared with other bioinks, dECM has been identified as a promising bioink material for vascular bioprinting due to its rich composition of proteins and growth factors. In fact, bioinks based on dECM have been successfully used in vascular bioprinting. Wang *et al.* combined pancreatic extracellular matrix (pECM) with hyaluronic acid methacrylate (HAMA) to develop a new bioink. The study confirmed the ability of dECM to promote the formation of new blood vessels[91]. However, the preparation of dECM requires a decellularization process, during which many growth factors may be lost. Therefore, Wang *et al.* modified the above bioinks by combining GelMA, pECM, and platelet-rich plasma (PRP) to prepare a new bioink that is readily available and rich in various growth factors. At the same time, it also has good printability. The improved bioink also showed better angiogenic ability[92]. Islet organoids printed by the above two bioinks and DLP technology may have potential application value in islet transplantation. Noor *et al.* prepared personalized hydrogel bioink using dECM derived from the omental tissue of patients and successfully printed the vascularized heart structure in the supporting material. The printed structure was extracted from the supporting material through enzymatic hydrolysis or chemical degradation, and this extraction method did not significantly compromise the cell vitality[93].

In addition, studies have shown that combining endothelial cells with stem cells or organoids is also an effective method of organoid vascularization. Zhao *et al.* have developed a new airflow-assisted bioprinting technique to print multifunctional helical microstructures inside microspheres. Using this technique, they successfully created osteoblast-like organs with vascularized spiral structures, which involved printing endothelial cell spiral structures into bone marrow MSC microspheres. *In vitro*, bone marrow MSCs were induced to differentiate into osteoblasts, and endothelial cells produced vascularized cells[94].

4. Biomedical applications of bioprinted organoids

With appropriate bioink, bioprinting, and tissue vascularization strategies, complex organoids that are highly similar to tissues and organs in the body can be constructed. To date, bioprinting has been utilized extensively in drug screening, regenerative medicine, tumor research, and many other areas. Several organoids, including hearts, livers, and kidneys, have been created using this technique (Figure 5).

4.1. Drug screening

The development of new drugs usually requires a large amount of cost. Before clinical trials, drugs must be screened for various aspects, such as activity, toxicity, metabolism, efficacy, side effects, and dose response[95]. Two-dimensional (2D) cell culture models and experimental animal models are often used in traditional drug screening models, but there are many problems with traditional drug screening models. The 2D cell culture model supports the observation of cell morphology at the cellular level, but it lacks the interaction between cells and does not simulate the complex *in vivo* environment. Animal models improve complexity, but differences between animal and human genomes make animal models inaccurate predictors of drug response, and there are ethical and moral controversies associated with animal models[95,96]. Taken together, evaluating a new drug usually takes 12–15 years, and 50% of new drug development will fail due to unpredictable toxic reactions[97]. Therefore, the research of an accurate drug screening model is essential.

Bioprinted organoids, highly similar to tissue organs *in vivo*, are widely used in drug screening as an alternative to traditional drug screening models. As many drugs can cause varying degrees of damage to the liver and kidney, these organs, which are crucial for human drug metabolism and detoxification, are also important for toxicity tests. In addition, bioprinting can also support the application of high-throughput screening technology and improve drug screening efficiency[97].

Lawlor *et al.* successfully constructed kidney organoids using extrusion bioprinting technology and tested them for the toxicity of aminoglycosides. This all-purpose antibiotic can heal infections brought on by Gram-negative germs, but it frequently causes acute tubular necrosis, which damages the kidneys[14]. Lawlor *et al.* treated printed kidney-like organs using aminoglycosides, and cell viability was estimated by ATP (adenosine triphosphate, a direct source of energy for vital activities) content after 72 h. The results showed that the activity of the treated cells decreased, and the decrease showed concentration-dependent characteristics, which confirmed that bioprinted organoids are practical tools used in toxicity test. Bouwmeester *et al.* created liver structures using intrahepatic bile duct cell organoids (ICOs) and extrusion-based bioprinting techniques. After printing, the organoids in the structure maintained relatively stable cell viability for 10 days. They exposed organoids in the bioprinted structures to acetaminophen (APAP, a hepatotoxic compound) on the 7th day after printing. After 72 h of exposure, organoid cell activity decreased to 21%–45%, and the organoid shape was damaged, indicating cellular stress. It demonstrated that liver structures obtained by combining organoids

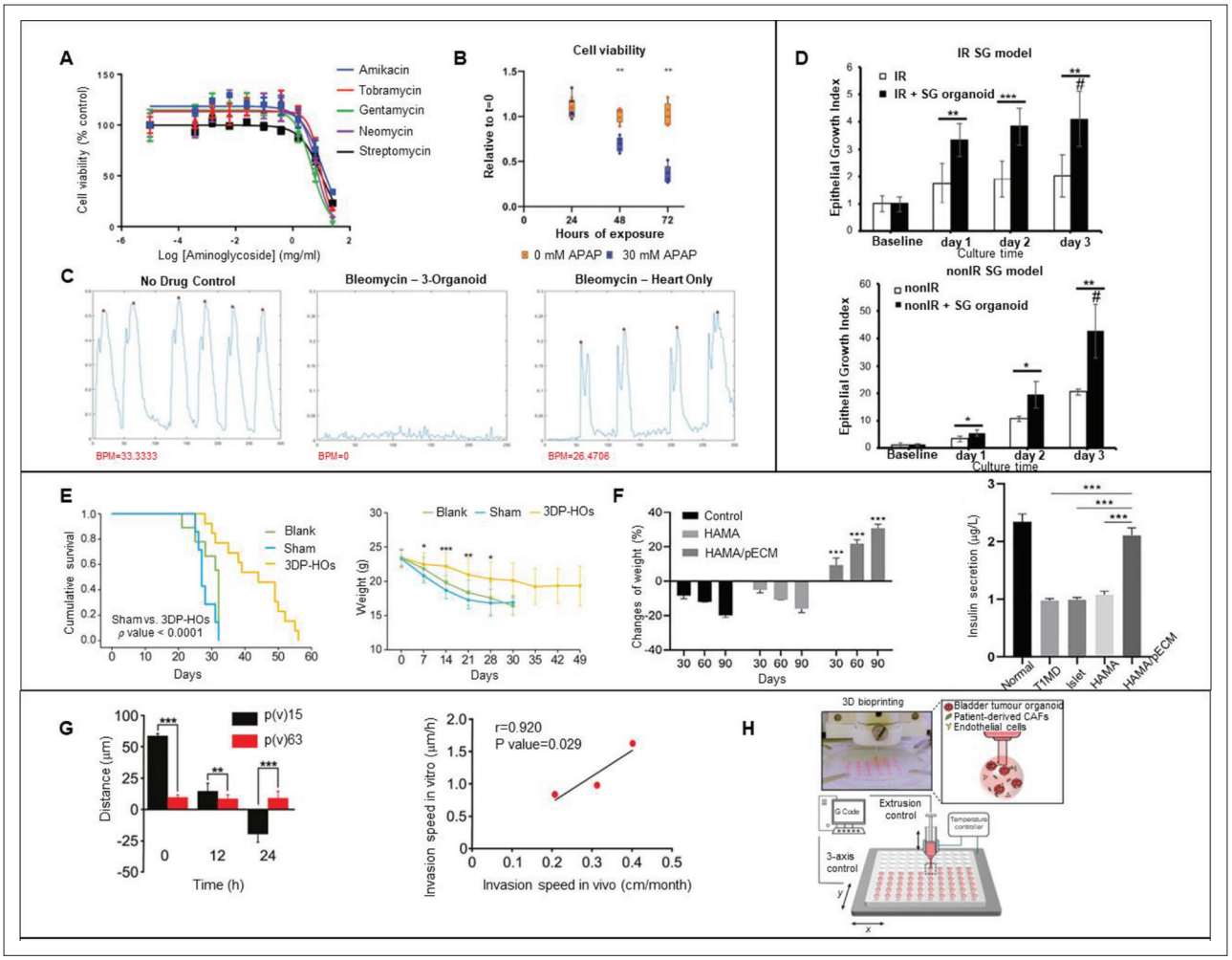

Figure 5. Biomedical applications of bioprinted organoids. (A)–(C) Drug screening: (A) Cell viability of bioprinted kidney organoids after 72 h treatment with aminoglycosides. Reprinted with permission from ref.[14]. Copyright 2020 Springer Nature. (B) Cell viability of organoids in bioprinted liver structures exposed to acetaminophen (APAP) from day 7 post-printing (from ref.[98] licensed under Creative Commons Attribution 4.0 license). (C) The beating of cardiac organoids after adding bleomycin to three types of bioprinted organ systems (from ref.[99] licensed under Creative Commons Attribution 4.0 license). (D)–(F) Regenerative medicine: (D) Epithelial growth index of SG after bioprinted SG organoids were transplanted into radiation injury and health SG models. Reprinted with permission from ref.[52]. Copyright 2018 Elsevier. (E) Survival curves and body weight changes of mice after bioprinted liver organoids were transplanted into F/R mice. Blank (control group), Sham (sham operation group), 3DP-Hos (organoid transplantation group) (from ref.[101] licensed under the CC BY-NC 4.0). (F) Changes in mice's body weight and serum insulin levels after bioprinted islet organoids were transplanted into mice. From left to right were normal mice, diabetic mice, simple islet transplantation group, HAMA ink islet transplantation group, and HAMA/pECM ink islet transplantation group. Reprinted with permission from ref.[91]. Copyright 2022 Elsevier. (G)–(H) Tumor studies: (G) Acoustic bioprinting PDMs simulated tumor invasion, changes in invasion distance, and correlation between tumor invasion rate and primary tumor spread rate (p(v)13 and p(v)15 represented patient source numbers 13 and 15). Reprinted with permission from ref.[103]. Copyright 2022 John Wiley & Sons. (H) Construction of bladder tumor assembly by bioprinting. Reprinted with permission from ref.[105]. Copyright 2020 Springer Nature.

and bioprinting techniques could be used to predict drug-induced liver injury[98].

The environment inside the human body is complicated, and tissues and organs are highly interconnected, so the evaluation of drugs by a single organoid model is not comprehensive enough. In order to better simulate the complex reactions and interactions between tissues in the drug screening process, the study of a multi-organoid system is of great significance. Skardal *et al.* developed a lung–liver–heart three-organ platform using bioprinting technology and validated bleomycin, a drug known to treat some cancers that cause severe pulmonary fibrosis and inflammation[99]. Cardiac organoids remained unaffected when treated with bleomycin alone. However, when the three-organ platform was treated with bleomycin, cardiac organoids stopped beating, suggesting bleomycin might induce other tissues in the system to produce factors that

affect the functionality of cardiac organoids. It is confirmed that the single organoid model may miss unanticipated toxicity and side effects in drug screening.

4.2. Regenerative medicine

Donor shortage and immune rejection have been major medical challenges, with only 10% of the global demand for organs currently reported to be met[100]. At the same time, the uncertain outcomes of organ transplantation increase the risk of infection. Therefore, how to construct biological tissues and organs *in vitro* to improve portability and limit the risk of immune response is an urgent challenge to be addressed[100]. Bioprinted organoids, which can replicate the structure and function of native tissues, have broad application prospects in regenerative medicine and can be used as organ transplant donors.

Xie *et al.* induced callus organoids generated by bioprinting *in vitro* and transplanted the callus organoids into a 5 mm × 4 mm cylindrical defect at the distal end of the rabbit femur. Four weeks after implantation, they observed that the callus organoids promoted the formation of new bone, and the newly generated bone tissue almost filled the defect site. Compared to the 2–3-month recovery time required in traditional tissue engineering, bioprinting offers a faster solution for bone repair, achieving rapid bone healing[72]. Adine *et al.* used magnetic bioprinting technology to print salivate glands (SG) organoids and transplanted SG organoids into an *in vitro* model. After transplantation, SG organoids were found to significantly stimulate the epithelial and neuronal growth of damaged SG, which is of great significance for treating dry mouth syndrome[52].

Yang *et al.* bioprinted liver organoids using HepaRG cells, and the printed organoids obtained liver functions such as albumin secretion, drug metabolism, and glycogen storage 7 days after differentiation. They transplanted liver organoids into F/R mice (a modulated model of liver damage that inhibits immune rejection, where deletion of the Fah gene leads to the accumulation of toxic tyrosine metabolites in liver cells that cause liver damage). They found that the liver organoids matured further and formed functional blood vessels. After transplantation, the survival period of mice was significantly prolonged, and the degree of weight loss was reduced, confirming that the transplantation of liver organoids could alleviate the liver failure of animals and has the potential to be used as organ transplant donors[101]. It is worth mentioning that the bioprinted liver organoids contain functions such as protein secretion, which may have potential applications in the production of biological agents. Wang *et al.* implanted islet organoids based on HAMA/pECM bioink bioprinting into diabetic mice. After 12 weeks of implantation, the islet organoids were still alive, and the mice could recover regular blood sugar within 60 min after meals, and their weight also showed a rising trend[91].

4.3. Cancer research

Cancer is a severe disease that poses a threat to life, and its morbidity and mortality are increasing. However, the mechanisms by which cancer occurs and its treatment are still poorly understood. Patient-derived 2D tumor culture and xenotransplantation have been widely used to simulate tumor invasion and therapeutic response. However, 2D models cannot fully summarize biochemical and biophysical signals of the tumor environment, and xenotransplantation also has problems such as genetic variability[102,103]. An ideal model for studying tumors is an organoid that mimics the histology, immunohistochemistry, and genetic heterogeneity of tumors but does not contain the mesenchymal cellular elements and intercellular connections found in the actual tumor tissue. In this regard, bioprinting technology can precisely control the distribution of cells and biomaterials, mimicking the spatial and chemical distribution of natural tissues, and therefore has great potential for applications in building complex tumor models and reconstructing tumor microenvironments[104].

Gong *et al.* generated bladder tumor organoids with acoustic bioprinting and murine-derived bladder tumor cells. The printed organs had a survival rate of more than 85% and maintained an immune milieu comparable to the original tissue for 2 weeks. In addition, after 2 days of co-culture of printed organoids and autologous lymphocytes, they observed the generation of tumor-specific T cells that can kill tumor organoids, suggesting that bioprinted organoids can be used for individualized immunotherapy[51]. Chen *et al.* used the same printing technique to print patient-derived microtissues (PDMs, consisting of colorectal tumors and healthy organoids derived from colorectal cancer (CRC) patients), simulated tumor invasion in CRC patients, and demonstrated the application of PDMs in drug screening[103]. In drug screening, two drugs (5-FU and Erbitux, the drugs for the treatment of colon cancer disease) were selected, and organoids were tested under different drug concentrations. Then, the distance between the tumor and healthy organs served as a measure of tumor invasion potential. The results showed that organoids were more sensitive to Erbitux. As long-term cultured bladder organoids lack vital components of a normal bladder (such as tissue matrix and muscle layer), Kim *et al.* proposed the assembly concept to tackle the problem. They successfully constructed bladder tumor assembly through bioprinting technology, combined with patient-derived bladder tumor organoids, endothelial cells (HULECs), and patient-derived cancer-related fibroblasts

(CAFs), which could accurately simulate the pathological characteristics of tumors *in vivo*. The study proved that bioprinted tumor assemblies have the same drug response as manually constructed tumor assemblies, and at the same time can be used for high-throughput drug screening[105].

5. Future outlook and summary

Organoid growth factors are mainly produced from patient-derived induced pluripotent stem cells and human stem cell differentiation. Due to their excellent ability to simulate human development and diseases, organoids have great application potential in drug testing and future organ replacement. However, organoids cultivated by traditional methods have size, complexity, and maturity limitations. Its more comprehensive application has been severely limited[106]. Bioprinting is regarded as a promising biomanufacturing technology because it can accurately deposit bioink materials and cells in space.

Bioprinting has been shown to improve the size limitations of traditional organoid culture techniques. Bioprinting-Assisted Tissue Emergence (BATE) technique prints stem cells or organoids into an ECM that encourages spontaneous self-organization using extrusion-based bioprinting technology and microscopy. This enables the construction of centimeter-scale intestinal organoids by controlling geometry and cell density[107]. Bioprinting also enables repeatable, consistent construction of organoids. A new immersion bioprinting technology prints organoids in a support bath with HA as the suspension medium, avoiding the influence of orifice wall on printing during the manufacture of high-throughput organoids, and the printed organoids have a high degree of consistency in volume and geometry[108].

However, there are still many shortcomings in bioprinting organoids. Firstly, in terms of bioinks, organoid bioprinting has high requirements for bioinks. An ideal bioink material should possess the characteristics of biocompatibility, mechanical and structural integrity, biodegradability, non-cytotoxicity, and immunogenicity, as well as the ability to provide a highly biomimetic environment for cells. Besides that, it should be commercially available[109]. These requirements have greatly limited the development of bioinks, so it is urgent to create new formulations of bioinks. Recently, a new glycerohydrogel bioink has been proposed that demonstrates outstanding bacteriostatic properties and long-term shape fidelity of printed tissues, as well as cytoprotection ability during printing, cryopreservation, and transportation[110]. The new bioinks could have great applications in organoid bioprinting. Secondly, in terms of bioprinting technology, high-cell-density bioinks allow the cells in the printed tissue to be close enough for cell-to-cell communication. However, most current bioprinting methods are unsuitable for high-cell density bioinks, and resolution and printing speed are limited. Finally, insufficient vascularization of organoids is also a problem that needs to be addressed. Microfluidic technology and four-dimensional (4D) bioprinting technology may be the effective ways to solve the above problems.

The application of microfluidic technology to the real-time distribution of cell concentration can successfully assist bioprinting to realize the construction of tissues and organoids with high cell density[111]. Microfluidics can also be applied to the vascularization of organoids by printing microfiber scaffolds to construct vascularized tissues[112]. Since the microenvironment of 3D bioprinting structure may not elicit appropriate biological responses, which will limit the application of 3D bioprinting, recently, 4D bioprinting technology has been investigated to solve the abovementioned problems. 4D bioprinting is a technology that combines 3D bioprinting with stimulus-response materials, also known as innovative materials, which can change their properties according to stimuli. Therefore, the structure of 4D bioprinting can more accurately simulate native tissues[113]. Photocured silk fibroin (Sil-MA) hydrogels can deform in a typical cell culture medium, and the tracheal structures obtained by combining with DLP bioprinting technology have great application potential in regenerative medicine[114]. However, 4D bioprinting is still at an early stage of development, and more research is needed in the future. More recently, a strategy to apply artificial intelligence (AI) to organoid bioprinting has also been proposed, with the potential to build more standardized organoids with an improved resolution by leveraging AI's monitoring and verification capabilities[115].

Since single organoid bioprinting cannot fully predict the crosstalk between organs, organoid bioprinting may develop into multi-tissue organoid bioprinting in the future with the development of bioinks and bioprinting techniques.

In summary, this paper introduces bioprinted organoids, reviews the progress of bioinks, bioprinting techniques, and tissue vascularization strategies, and demonstrates the application of bioprinted organoids in biomedicine. Although there are still many shortcomings in bioprinting organoid technology, it is believed that with the development of research, this technology will be more mature.

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Conflict of interest

The authors declare no conflict of interest.

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Writing - review & editing: Chen He, Jiasheng Yan

Ethics approval and consent to participate

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Consent for publication

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Availability of data

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