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Advances in 3D Bioprinting

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ABSTRACT

Three-dimensional (3D) bioprinting has emerged as a promising approach for engineering functional tissues and organs by layer-by-layer precise positioning of biological materials, living cells, and biochemical components. Compared with nonbiological printing, 3D bioprinting involves additional complexities and technical challenges owing to the processing of living cells, such as the appropriate biomaterials that fulfill the requirements for both printability and functionality. In this review, we first introduce the development course of 3D bioprinting, highlighting innovative forms of living building blocks and advances in enabling techniques of 3D bioprinting. We then summarize the state-of-the-art advancements in 3D bioprinting for biomedical applications, including macroscale tissue or organ bioprinting, disease modeling, microphysiological systems, biobots, and bioprinting in space. Despite the rapid development of 3D bioprinting over the past decades, most 3D bioprinted tissue or organ constructs are still far from being suitable for clinical translation, and it is necessary for the field of bioprinting to shift its focus from shape mimicking towards functionality development. Therefore, we provide our perspectives on this burgeoning field with an emphasis on functional maturation post printing and translational applications at the bedside.

1. Introduction

Additive manufacturing, also known as three-dimensional (3D) printing, is a bottom-up manufacturing technique that involves depositing and accumulating raw materials with the assistance of software and computer numerical control (CNC) systems to fabricate physical objects [1]. It alleviates the limitations of conventional formative or subtractive processing methods for the fabrication of products with complex geometries and heterogeneous compositions. Additionally, it shortens processing procedure and reduces the cost of small batch or personalized production [2]. To date, 3D printing has been used in numerous areas, including manufacturing, architecture, energy, and healthcare. It has attracted particular attention in the biomedical field, considering the complexity and personalized nature of human tissues and diseases. By modeling a tissue by image reconstruction following computerized tomography (CT) scan and magnetic resonance imaging (MRI), personalized devices, including orthopedic devices and patient-matched implants, can be 3D printed for surgical guidance and restorative purposes [3]. For example, in 2013, an infant with tracheobronchomalacia (TBM) received a 3D-printed bioresorbable external airway splint and recovered [4]. These developments in the 3D printing of nonliving constructs have prompted researchers to develop 3D bioprinting technology. Early work on 3D bioprinting mainly focused on printing acellular structures, on which cells were seeded for in vitro culture or in vivo implantation; however, this approach has some limitations, including low seeding efficiency [5] and difficulty in achieving heterogeneous cellular distribution. Alternatively, living cells can be directly encapsulated into a bioink and presented in the 3D bioprinting process, which in turn limits the direct use of many traditional 3D printing technologies considering the strict biocompatible requirements for both ink materials and processing conditions. Hydrogels are commonly used as cell carriers to formulate a bioink, as they can provide an aqueous environment for encapsulated cells that mimic the extracellular matrix. With the urgent need for 3D printing of functional tissues, the focus of bioink development has recently shifted from providing structural support to invoking tissue functionality [6].

The definition of 3D bioprinting can be described as "the use of computer-aided transfer processes for patterning and assembling living and nonliving materials with a prescribed 2D or 3D organization to produce bioengineered structures serving in regenerative medicine, phar-

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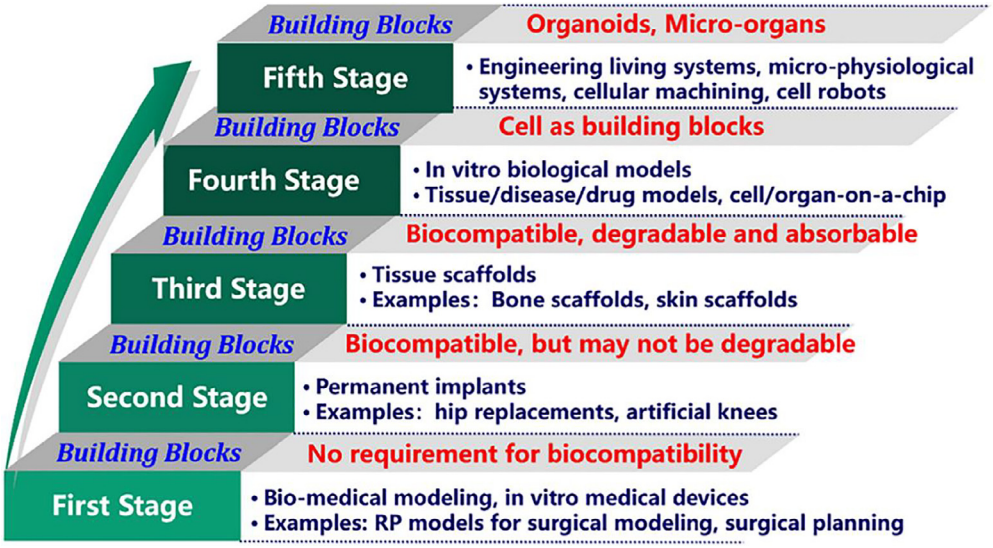

Fig. 1. The development of 3D printing technology in biomedical applications is divided into five stages according to the properties of the building blocks [1]. Reproduced with permission. Copyright 2020, IOP Publishing.

macokinetic and basic cell biology studies" [7]. In the past two decades, different 3D bioprinting technologies have been developed with various materials, speeds, and accuracies [8], which makes 3D bioprinting a versatile and powerful platform technology for numerous biomedical applications, including tissue engineering [9], disease modeling [10], and drug screening [11]. For example, multi-material bioprinting and microfluidic printing have been developed to mimic the heterogeneity and gradients of native tissues [12]. A suspension printing strategy has been proposed to expand the geometrical complexity of printed tissues based on extrusion printing [13]. A volumetric bioprinting strategy has been developed with the ability to print large-scale tissue constructs in seconds, which significantly increases the printing speed of current light projection-based printing [14]. Biomaterial-free bioinks such as cell spheroids and organoids have been developed to achieve physiologically relevant cell density for tissue functionality [15]. In addition, several advanced bioprinting technologies, such as acoustic and magnetic bioprinting, that remotely control cell patterning have also emerged [16].

Despite these considerable advances, most 3D-printed tissues are either in the preclinical state or far from translational application [17]. The bioprinting of clinically relevant functional tissues faces significant hurdles, in particular: achieving tissue heterogeneity with controlled distribution of multiple types of cells and biomaterials at the microscale and macroscale [1]; developing of essential functional elements such as vasculature, innervation, and lymphatics; and achieving their integrating them with host tissues [18]. In this context, our review provides a historical overview of general 3D bioprinting technology, highlighting the introduction of living cells in the manufacturing process. We then introduce the most recent advances in innovative bioinks and bioprinting techniques. Furthermore, we highlight the frontiers in the field towards the next stage, including macroscale tissue or organ bioprinting, disease modeling, microphysiological systems, biobots, and bioprinting in space [1]. Finally, we provide perspectives on the engineering of functional tissues or organs and their clinical applications.

2. Cells as Building Blocks for 3D Bioprinting: Progress of the Last Two Decades

2.1. From nonliving to living building blocks

According to the properties of raw materials and printed products, the development of 3D printing technology in biomedical applications can be divided into five stages, from non-living to living building blocks (Fig. 1) [1]. The first stage is to print implements as surgical models and in vitro medical devices, which do not interact with the human body and thus do not require biocompatibility. The second stage involves 3D printed permanent implants, such as hip replacements and artificial knees, which requires some extent of biocompatibility but not necessarily degradability. The third stage involves typical scaffold-based tissue engineering, where a 3D printed construct serves as a tissue scaffold for cell growth and tissue formation. Ideally, the materials used for tissue engineering scaffold fabrication should be biocompatible, degradable, and absorbable, in line with the formation and infiltration of native tissues. In the fourth stage, living cells are directly printed as building blocks to engineer biomimetic tissues or organs. 3D bioprinting allows for the direct fabrication of in vitro biological products used in regenerative medicine and pharmaceuticals. Recently, 3D printing technology in biomedical applications has advanced into the fifth stage, in which organoids or micro-organs are engineered and assembled to generate complex in vitro living systems or microphysiological systems. This review focuses on the fourth stage and beyond, where the distinctive feature is the presence of living cells in the 3D printing process.

The first documented work on 3D bioprinting dates back to the early 2000s. In 2003, Boland et al. from Clemson University used the inkjet bioprinting technique to pattern protein and cell suspensions on a solid surface by modifying a commercial inkjet printer; their modified inkjet printer is commonly considered as one of the first prototypes of 3D bioprinters [19]. Inkjet bioprinting, driven by either thermal or piezoelectric actuators, has been widely used because of its high resolution, fast speed, and low cost [20]. Laser-induced forward transfer (LIFT) technology is another early example of bioprinting that possesses the capability to pattern living cells with micrometer-scale precision [21]. In 2004, Barron et al. greatly improved the LIFT technique and set up a bioprinter called the biological laser printer (BioLP) to print cell-laden droplets on substrates with a spatial accuracy of 5 μm and almost 100% cell viability [22].

Extrusion-based 3D bioprinting techniques can be used to produce tissue constructs in vitro and in situ and are arguably the most commonly used bioprinting strategies. In 2002, Landers et al. printed a lattice-structure scaffold with agar hydrogel followed by lyophilization; however, no cells were encapsulated in the hydrogel [23]. Tsinghua University [24] and Drexel University [25] pioneered the work of extrusion-based bioprinting with cells (Fig. 2(a)). In 2005, Yan et al. from Tsinghua University published their work on fabricating 3D liver tissue constructs with gelatin/chitosan and gelatin/alginate hydrogels using an indepen-

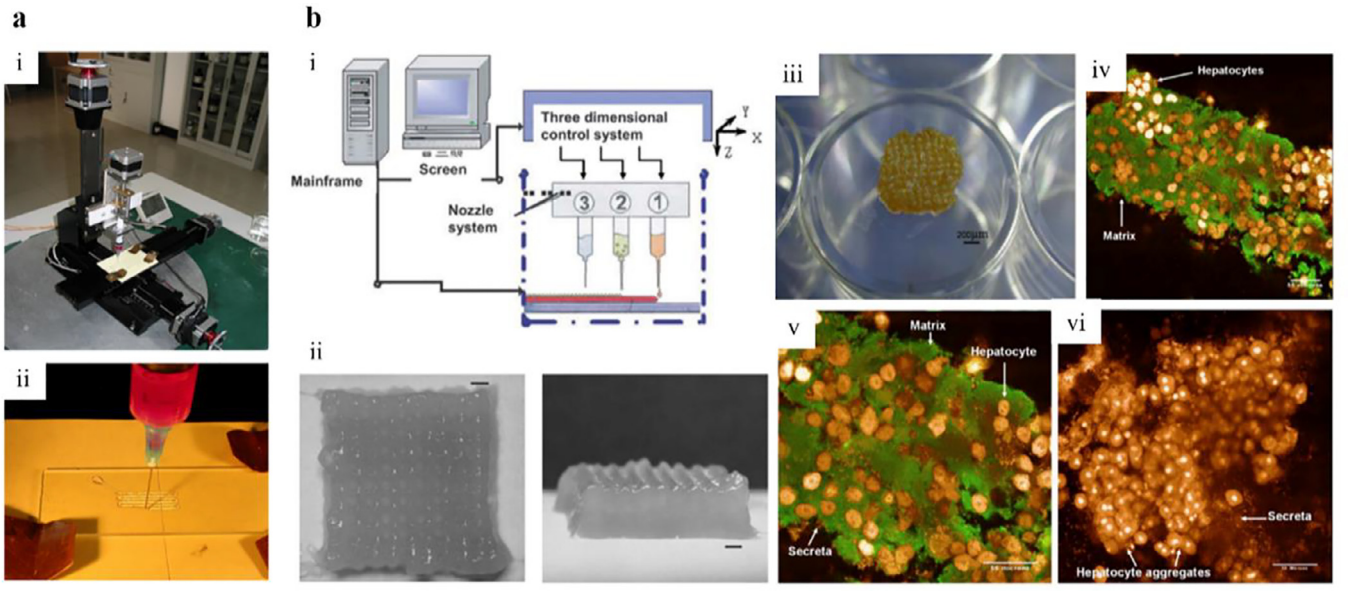

Fig. 2. Representative early work on extrusion-based 3D bioprinting: (a) (i) The first-generation cell assembly printer developed by Tsinghua University in 2002, (ii) the printing process [24]. Reproduced with permission. Copyright 2005, SAGE; (b) 3D bioprinting of liver constructs with high cell viability and considerable biofunctionality using a cell assembly printer: (i) Schematic of the cell assembly system, (ii) top view and side view of a printed lattice structure with hepatocytes, (iii) external view of the printed lattice structure, (iv) immunocytochemistry for rat albumin, (v–vi) magnified view of (iv) [26]. Reproduced with permission. Copyright 2005, Elsevier.

dently developed extrusion-based cell assembly printer (Fig. 2(b)) [26]. High cell viability (approximately 95%) and considerable tissue functionality were observed in this study. In the same year, Khalil et al. from Drexel University developed a multinozzle printer that allowed for the drop-on-demand and continuous deposition of cell-based bioinks [25].

In 2004, Boland et al. modified traditional stereolithography (SLA) technology to print living cells with a resolution of up to 150 μm [27]. In 2006, Itoga et al. developed a novel SLA printing approach called digital light processing (DLP) bioprinting, which used a liquid crystal display projector (LCDP) controlled by a computer instead of an expensive photomask. Different from the point-by-point scanning approach of conventional SLA technology, digital light processing (DLP) bioprinting performs in a layer-by-layer scanning fashion, which greatly increases the printing speed and lowers the cost [28]. Since then, the biofabrication field has witnessed an explosive growth of DLP bioprinting technology. Chen et al. used a digital mirror device (DMD) to print a multilayer complex scaffold that be seeded with cells before or after processing [29].

Since the 2010s, explosive growth in bioprinting has been seen in academia and industry. In 2009, Organovo, Inc. launched the first commercial 3D bioprinter based on the extrusion approach, which continues to be one of the leading players in the field of 3D bioprinting. In the same year, *Biofabrication*, the first peer-reviewed journal in the field of 3D bioprinting and biofabrication, was launched by the Institute of Physics (IOP) Publishing. In 2010, the International Society for Biofabrication (ISBF) was established to accelerate the formation of biofabrication communities. As a representative pioneer in the field, Professor Wei Sun is the Founding Editor-in-Chief of Biofabrication and the Founding President of ISBF. In the past decade, innovations in bioinks and 3D bioprinting technologies have led to the rapid development of the bioprinting of human tissues such as skin [30] and cartilage [31], which have progressed to the preclinical stage. However, the bioprinting of complex tissues such as the heart [32] and liver [33] at clinically relevant scales still faces significant hurdles regarding physical biomimicry (geometrical complexity and heterogeneity) and biological functionality (e.g., vascularization and innervation).

2.2. Innovative forms of living building blocks

A bioink can be defined as "a formulation of cells suitable for processing by an automated biofabrication technology that may also contain biologically active components and biomaterials." A typical bioink used in 3D bioprinting is a cell-laden hydrogel precursor solution, in which single cells are dispersed homogeneously throughout [34]. The polymers used to formulate a bioink can be natural, synthetic, or a combination of both, such as collagen [35], gelatin [36], alginate [37], gelatin methacrylate (GelMA [38]), and polyethylene glycol (PEG [39]). Owing to its high similarity to the native extracellular matrix (ECM), decellularized ECM (d-ECM) has attracted much attention in 3D cell culture and has been directly used in bioprinting as a novel bioink. For example, Choi et al. prepared dECM from muscle tissues and printed it with human skeletal muscle cells in a granular bath to fabricate skeletal muscle constructs that exhibited good repair outcomes in an in vivo experiment with volumetric muscle loss (VML) mice [40]. More recently, Kim et al. developed a new bioink by mixing cardiac dECM with nanoclay and poly(ethylene glycol)-diacrylate (PEG-DA), the latter of which was added to improve the mechanical properties after photocrosslinking [41].

Bioinks equipped with special physicochemical properties, such as electroconductive, self-healing, and shape-changing behaviors, have also attracted considerable attention. For example, gold nanorods have been added to bioinks to obtain stronger electroconductivity for engineering functional cardiac tissues [42]. Rastin et al. formulated a new conductive bioink by dispensing Ti_3C_2 MXenes, a new family of 2D transition metal carbides/nitrides, within hyaluronic acid/alginate hydrogels for extrusion bioprinting [43]. By integrating shape-changing biomaterials, a 4D bioprinting strategy has been used to fabricate constructs with the ability to respond to time or external stimuli. For instance, Kirillova et al. developed a 4D bioprinting approach to fabricate hollow self-folding tubes (average internal diameter of 20 μm) using methacrylated alginate (Alg-MA) and methacrylated hyaluronic acid (HA-MA). Owing to the different swelling ratios of these materials, 3D printed two-layer structures can self-fold into hollow tubes [44]. More recently,

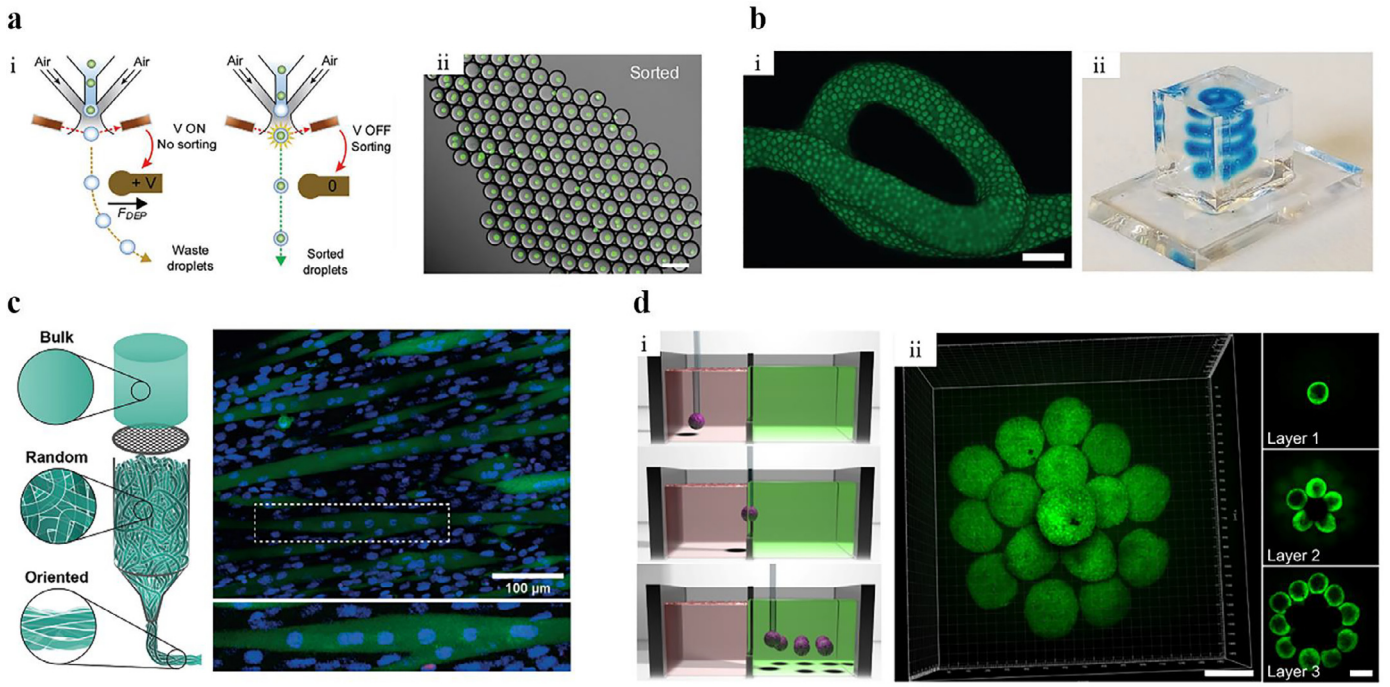

Fig. 3. Representative examples of novel building blocks: (a) High-definition single-cell bioprinting via a miniaturized cell sorter: (i) Illustration of the sorting process, (ii) sorted droplets with >99.5% containing cells [46]. Reproduced with permission. Copyright 2020, Wiley; (b) Densely packed microgels serving as bioink and 3D bioprinting in a supporting hydrogel: (i) Fluorescent image of norbornene-modified hyaluronic acid (NorHA) microgel ink, (ii) printing microgel ink within a shear-thinning support hydrogel [47]. Reproduced with permission. Copyright 2018, Wiley; (c) Entangled microstrands serving as bioink and 3D printed muscle tissues [48]. Reproduced with permission. Copyright 2020, Wiley; (d) 3D bioprinting of cell spheroids in a self-healing hydrogel: (i) Schematic of the printing process, (ii) 3D printed cell spheroid assemblies [15]. Reproduced with permission. Copyright 2021, Springer Nature.

Lee et al. fabricated 4D bioprinted tissue models with high cell density that can spatiotemporally change shapes during culture [45].

Recently, innovative forms of living building blocks have also been added to bioink palettes. A single cell-laden droplet block is of great interest because it enables the fabrication of precise cell patterns and the study of cellular interactions at the single-cell level. However, it has been challenging to generate and manipulate droplets that contain only one single cell. To address this issue, Zhang et al. integrated a miniaturized cell sorter with microfluidic devices by using a fast and reliable fluorescence-activated cell sorting approach. The ratio of droplets containing one single cell after sorting reached 99.5%, while the ratio without sorting was only 10.0%. This sorter-integrated print technique can also be employed to print droplets containing cell pairs and other numbers of cells (Fig. 3(a)) [46]. The concept of building blocks can also be extended to microgels and microfibers. For extrusion bioprinting, shear-thinning and self-healing properties are crucial to ensure good printability and shape fidelity of the bioink. The newly emerged bioink consisting of densely packed microgels fabricated by microfluidics has demonstrated excellent shear-thinning and self-healing properties. This design strategy for microgel-based bioinks has enabled the direct printing of a wide range of biomaterials with good versatility (Fig. 3(b)) [47]. In addition to microgels, microfibers or microstrands can also serve as building blocks. For example, Kessel et al. fabricated hyaluronic acid microstrands by sizing a bulk hydrogel through a cell strainer, and the generated microstrands naturally formed a porous and entangled formulation (Fig. 3(c)). The printed structure exhibited anisotropy because of the directional arrangement of the microstrands during extrusion through the nozzle, providing a potential way to reconstruct anisotropic tissues such as muscle [48].

Additionally, some recent studies have directly utilized cell spheroids and organoids as building blocks. Ozbolat et al. developed a bioprinting approach that manipulated cell spheroids with the assistance of aspiration force and realized a high resolution of spheroid positions. The authors intended to show that their method can also be extended to other living blocks, such as tissue strands and even single cells [49]. Similarly, Burdick et al. achieved the movement and positioning of high cell-density spheroids in a self-healing hydrogel bath via vacuum aspiration (Fig. 3(d)). Different cell spheroids in the self-healing hydrogel bath fused together into heterogeneous microtissues with high cell density and a predetermined spatial structure, which is of great significance for the development of high-precision models for drug screening and disease modeling [15]. Organoids can self-organize into three-dimensional multicellular structures, and 3D bioprinting technology provides a promising microengineering strategy to direct the morphogenetic processes of organoids. For example, Jonathan et al. successfully fabricated centimeter-scale intestinal tissue through organoid bioprinting, highlighting its great potential in the field of drug discovery, diagnostics, and regenerative medicine [50]. In another example, Kynan et al. used extrusion bioprinting to generate kidney organoids with highly reproducible cell amounts and viability in a high-throughput fashion [51]. Yang et al. printed implantable hepatorganoids in a more clinically relevant example and demonstrated the promotion of mouse survival with liver failure [52]. Progress in the fabrication of heterogeneous tissue models has also been made by printing multiple types of cells, as exemplified by the work of Kim et al., who printed neural cells into skeletal muscle constructs, leading to an accelerated restoration of muscle function [53].

2.3. Advances in the enabling technique of bioprinting

Current enabling techniques of 3D bioprinting can be mainly divided into three categories according to the geometrical dimensionality of the building blocks: zero dimension (point), one dimension (line), and two dimensions (plane) [54]. Bioprinting techniques based on point or droplet building blocks mainly include laser-induced forward, inkjet,

and valve-based bioprinting. Extrusion-based bioprinting and lithography bioprinting are representative techniques that applying line- and plane-shaped building blocks, respectively. In addition, novel bioprinting techniques such as acoustic and magnetic bioprinting have emerged to overcome possible cell damage during the printing process. Tian et al. used acoustic tweezers to precisely manipulate particles and cells, and pattern cells into predesigned arrays, which may contribute to an understanding of cellular interactions at the microscale [16].

In general, extrusion-based and lithography approaches are the most widely used and commercialized bioprinting techniques [25,55]. Extrusion-based bioprinting probably has the broadest applicability to cell aggregates [50], microgels [47], and decellularized materials [41]. Notably, it can print biomaterials with a relatively wide range of viscosities [56]. Furthermore, an extrusion-based approach can be readily used to fabricate heterogeneous structures using a multinozzle setup. Despite its considerable advantages, extrusion-based bioprinting is usually associated with poor printing resolution and potential cell damage induced by shear stress during extrusion. To address these issues, researchers have developed innovative strategies, such as microfluidic printing and suspension printing. Integrating microfluidics with extrusion bioprinting enables the fabrication of more complex and heterogeneous structures. For example, Zhang et al. fabricated 3D hollow microfibrous constructs with low-concentration GelMA as the core phase using GelMA/alginate core-shell coaxial printing, providing an appropriate microenvironment for cell growth [12]. He et al. established a large-scale thick tissue model with vascularized channel networks using coaxial bioprinting [57]. Renaud et al. developed a microfluidic-assisted extrusion-based bioprinting method that can control the cell concentration in real time using a microfluidic dispensing print head [58]. For extrusion-based bioprinting, there are usually opposing requirements for structural printability and biological functionality, resulting in a limited number of available bioinks. Recently, suspension bioprinting has emerged to address this issue by directly printing in a suspension bath that can provide the support for the printed structure. For example, Lee et al. developed a microgel-based supporting bath and printed excellent human heart components with collagen by using freeform revisable embedding of suspended hydrogels, also termed the FRESH technique (Fig. 4(a)) [13].

Lithography bioprinting can be divided into stereolithography (SLA) and digital light processing (DLP), according to the light scanning method [59]. DLP bioprinting has advanced greatly in recent years owing to its fast printing speed, high resolution, and strong adhesion between adjacent layers. For example, Grigoryan et al. fabricated complex intertwined vascular networks within bulk hydrogels using a biocompatible food dye additive as a photoabsorber and developed a stereolithography apparatus for tissue engineering (SLATE) (Fig. 4(b)). Alveolar models with entangled vascular structures and liver implantation models have been established using this open-source bioprinting method [60]. In addition, several prominent in vivo 3D bioprinting studies have emerged in recent years with unique photocrosslinking strategies. Gou et al. developed digital near-infrared (NIR) photopolymerization (DNP)-based 3D bioprinting to obtain macroscale tissue constructs using in vivo noninvasive printing. A human ear-shaped construct encapsulated with chondrocytes was printed inside mice as proof of concept (Fig. 4(c)) [61]. Later that year, Elvassore et al. achieved in vivo 3D bioprinting using two-photon excitation with a near-infrared laser [62]. Additionally, a new form of stereolithography bioprinting, volumetric bioprinting, has been developed, which seems to significantly improve printing efficiency and accuracy. The principle of volumetric bioprinting is to radiate the photocrosslinkable hydrogel precursor, which rotates in a container, by using the DLP method to expose different light patterns at multiple rotation angles. A macroscale tissue model can be fabricated in several tens of seconds (Fig. 4(d)) [14].

3. Moving to the Next Stage: Printing Living Systems

3D bioprinting technologies have flourished in the biomedical field, with pronounced developments in recent years [8]. For example, 3D printing has been widely used to fabricate functional tissues with hierarchical structures similar to their native counterparts, which may address organ shortages [63]. Three-dimensional printing has been used to manufacture drug delivery systems with the potential to make a paradigm shift owing to its advantages over customizing drugs in individually adjusted doses [11]. The convergence of 3D bioprinting technology and space biology may enable researchers to investigate the impact of space-related environments on human health [64]. Therefore, in this section, we introduce the state-of-the-art advancements in 3D bioprinting for biomedical applications, including macroscale tissue or organ bioprinting, disease modeling, microphysiological systems, biobots, and bioprinting in space. The advantages and challenges of 3D bioprinting for different biomedical applications are presented in Table 1.

3.1. Printing large-scale functional tissues or organs

For decades, the field of tissue engineering has been driven by the desire to generate engineered functional tissues or organs that can repair or replace malfunctioning tissues or organs. 3D printing has served as the most promising biofabrication technique owing to its capability to precisely deposit various types of biomaterials and cells [1]. Despite the significant advances in 3D bioprinting for tissue engineering, success in the preclinical stage and clinical translation of 3D-printed living tissues have been restricted to relatively simple tissues such as cartilage, bone, and skin, which consist of relatively simple structures with few cell types [18]. A major roadblock exists in the 3D printing of large-scale functional tissues or organs that mimic the geometrical and anatomical complexities contained in hierarchical vasculatures [17]. Although conventional 3D bioprinting techniques enable a considerable extent of structural complexity in a layer-by-layer fashion, it is often challenging to print complex tissues or organs encompassing geometrical features such as branches, thin walls, and overhangs, as most hydrogels used as bioinks in bioprinting are too soft to provide adequate self-support [34]. To address this issue, researchers have explored polymer frameworks as permanent or temporal supports for printed structures [63]. For example, Kang et al. used polycaprolactone (PCL) and Pluronic-F127 as the framework and sacrificial printing material, respectively, to support the printing of cell-laden composite hydrogels into complex human-tissue-scale constructs such as those for mandibles and ear cartilage [77]. It should be noted that the retained PCL framework with a diameter of hundreds of microns may restrict the space for tissue formation and maturation. Alternatively, the embedded extrusion bioprinting strategy has gained popularity by allowing for the direct deposit of the desired bioink into a suspension medium, with a granular hydrogel as a representative [78]. Owing to its shear-thinning and self-healing behavior, the granular support hydrogel is capable of fluidizing around the traversing writing needle and rapidly solidifying to support the extruded bioinks behind the needle. In addition to printing complex freeform constructs, this strategy also enables the printing of a wide variety of low-viscosity bioinks, such as collagen and dECM, which are highly desirable for the functional maturation of soft tissues [79]. This approach was exemplified by the recent work of Lee et al. [13], in which a collagen ink was printed as shell layers to support the deposition of a high-concentration (3×10^8 mL^{-1}) cardiomyocyte suspension and used to successfully fabricate a cellular contracting model of the heart's left ventricle. Ogle et al. generated a synchronously contracting human chambered muscle pump using the FRESH technique [80]. Conversely, they used the in situ proliferation and differentiation strategy of human iPSCs (hiPSCs) to enhance cellular density and tissue connectivity. Furthermore, Dvir et al. successfully printed a miniaturized cellular heart model containing the major blood vessels by printing dECM-based bioinks in a hybrid support medium of calcium-alginate nanoparticles and xanthan

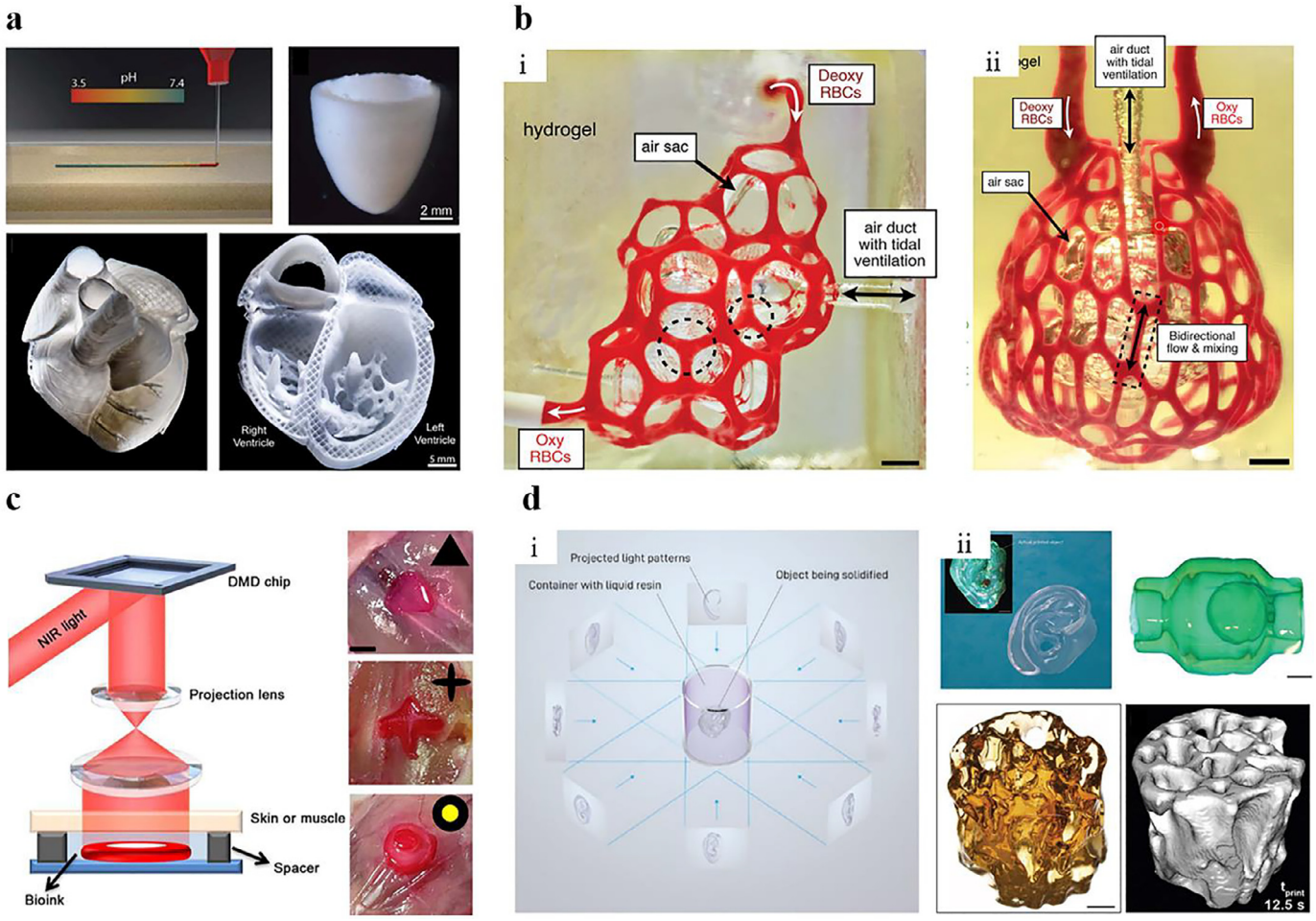

Fig. 4. Representative progress in enabling techniques of 3D bioprinting: (a) FRESH technique: 3D bioprinting of collagen to rebuild components of the human heart [13]. Reproduced with permission. Copyright 2019, AAAS; (b) SLATE technique: 3D complex structures printed by a digital light processing technique that can mimic human alveoli: (i) Photograph of a printed hydrogel during red blood cell (RBC) perfusion, (ii) photograph of a printed hydrogel containing the distal lung subunit during RBC perfusion while the air sac was ventilated with O_2 [60]. Reproduced with permission. Copyright 2019, AAAS; (c) Noninvasive in vivo bioprinting: 3D bioprinting of a human ear-shaped construct using digital near-infrared (NIR) photopolymerization [61]. Reproduced with permission. Copyright 2020, AAAS; (d) Volumetric stereolithography bioprinting: complex tissue models produced by curing the hydrogel precursor in a rotating container with multiple light patterns: (i) Schematic of 3D bioprinting of the human auricle model by tomographic projections, (ii) complex structures by volumetric printing [14]. Reproduced with permission. Copyright 2019, Wiley.

Table 1
Advantages and challenges of 3D bioprinting for different biomedical applications.

Applications	Advantages	Challenges	Refs No.
Printing large-scale functional tissues or organs	Precise depositing of biomaterials and cells; high degree of structural complexity and heterogeneity	Achieving entirely physiological biomimicry and functionality; obtaining clinically relevant numbers of cells	[35, 63, 65]
Printing disease and cancer models	Heterogeneous microenvironment; high throughput	Fully recapitulating the disease dynamics; maintenance of parental cell phenotype and characteristics during in vitro culture; reproducibility	[66, 67, 68]
Printing microphysiological systems and organ-on-a-chip	Cost-efficient; fast fabrication process; precise location of multitypes of cells	Achieving high resolution as soft lithography does; developing bioinks with biocompatibility and mechanical robustness	[69, 70, 71]
Constructing biohybrid robots	Multimaterial depositing; complex macroscale structure; direct combination of nonliving and living materials	Tailoring micro- and nanoscale structure; maintaining a longer lifetime	[72, 73, 74]
Bioprinting in space	Personalized product; fast fabrication process	Miniaturization and automatization of a 3D bioprinter in a space station	[64, 75, 76]

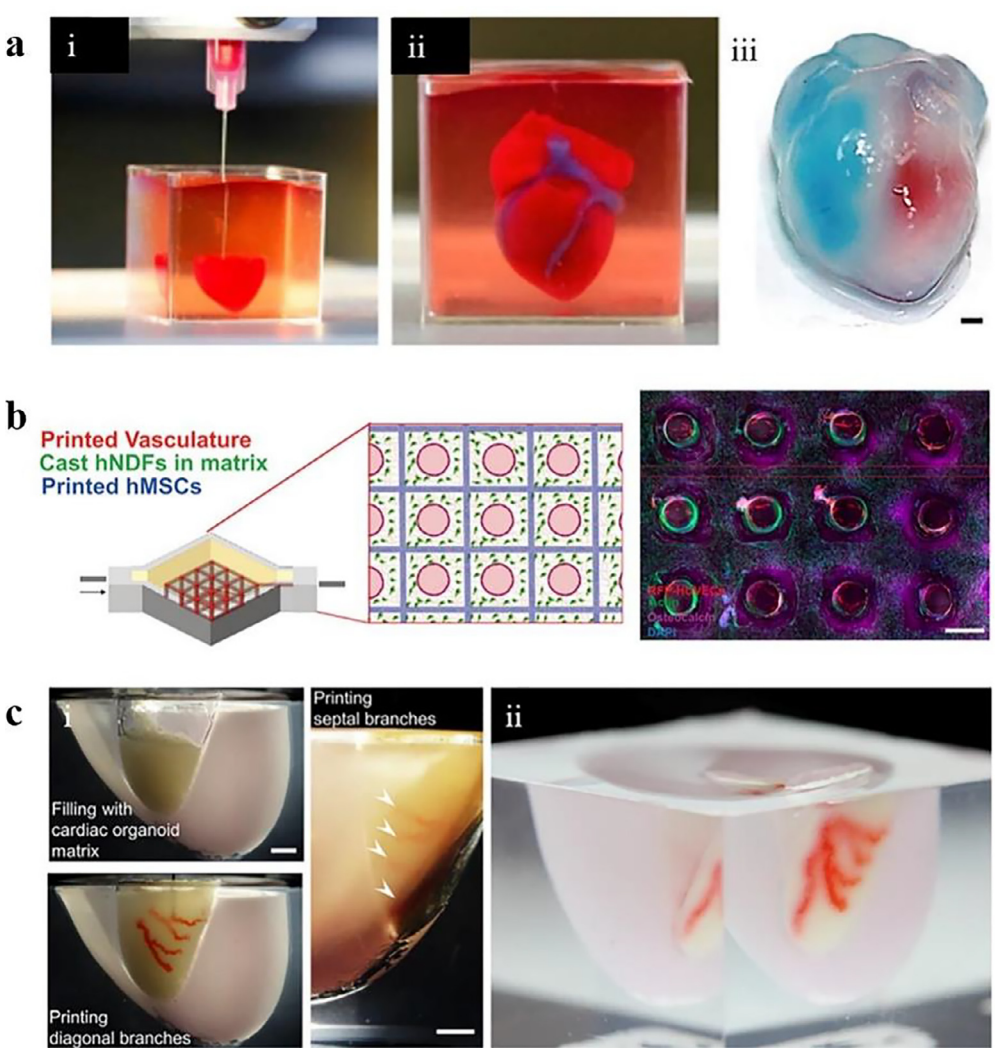

Fig. 5. 3D printing of large-scale functional tissues or organs: (a) (i) 3D printing of a miniaturized cellular human heart model in a suspension bath, (ii) 3D printed heart model with the major blood vessels, (iii) perfusion of red and blue dyes into the left and right ventricles, respectively [81]. Reproduced with permission. Copyright 2019, Wiley; (b) 3D vascularized tissues fabricated by simultaneously printing cell-laden bioink and sacrificial bioink [87]. Reproduced with permission. Copyright 2016, National Academy of Sciences; (c) (i) 3D printing of cardiac tissues in suspension media consisting of cardiac organoids, (ii) 3D printed cardiac tissues with high cellular density and vascular networks [88]. Reproduced with permission. Copyright 2019, AAAS.

gum (Fig. 5(a)) [81]. It should be noted that the printed heart construct lacked the anatomical complexity of the native heart and was still far from clinical application.

In addition to structural complexity, another hurdle in engineering large-scale functional tissues or organs is the lack of a hierarchical vascular system that ensures a constant supply of essential nutrients and oxygen to individual cells and the removal of waste [35]. Although endothelial cells can self-organize into rudimental capillaries and integrate with host vasculatures, the spontaneous process usually takes days or weeks and fails to satisfy the metabolic demand of engineered tissues [82]. For this reason, the engineering prevascularized tissues that enable rapid perfusion after printing has become the consensus of the biofabrication field [83]. The past decades have witnessed a variety of engineering techniques to accomplish this goal. Among them, the sacrificial templating strategy has become increasingly popular for engineering 3D vascularized tissues [84]. In this strategy, sacrificial or fugitive materials, such as Pluronic F127, gelatin, and carbohydrates, are fabricated into temporal templates with predefined geometries, which are subsequently cast with parenchyma cell-laden hydrogels [85]. Upon completion of the fabrication process, the cellular constructs are crosslinked while the templates are dissolved or liquefied to generate hollow channels, which are further seeded with endothelial cells and perfused with oxygen- and nutrient-rich culture media. This strategy is exemplified by the work of Miller et al., who printed carbohydrates as sacrificial templates [86]. Alternatively, Lewis et al. proposed a sacrificial printing strategy by printing cell-laden bioink and sacrificial bioink simultaneously to create vascularized tissues (Fig. 5(b)) [87]. It should be stressed that these approaches rely on post-EC seeding to form the endothelium, which might result in nonuniform cell distribution, especially for large-scale tissue constructs. To circumvent this difficulty, Dvir et al. encapsulated ECs within a sacrificial bioink and generated a vascularized 2D cardiac patch by coprinting an iPSC-CM-laden ECM-based bioink and an EC-laden gelatin bioink [81]. Furthermore, Ouyang et al. incorporated a void-free bioprinting process with an in situ endothelialization approach to fabricate thick tissue constructs incorporated with 3D interconnected endothelialized channels [35].

Another strategy that enables the generation of vascular channels is omnidirectional printing in suspension baths. One major advantage of omnidirectional printing is its capability to generate vascular channels with higher degrees of biomimicry by overcoming the layer-by-layer deposition fashion of conventional 3D bioprinting. This strategy possesses a higher printing speed for large-scale vascularized tissues, as the bulk tissue does not need to be printed line-by-line. Adapting this strategy, Skylar-Scott et al. introduced a printing technique termed “sacrificial writing into functional tissue” (“SWIFT”) by developing a suspension medium mainly consisting of embryoid bodies from iPSCs as organ building blocks (OBBs) [88]. The OBB-ECM slurry with unique shear-thinning and self-healing behavior enabled the printing of sacrificial gelatin bioinks into cell-laden suspension media in an arbitrary and discrete manner. The granular OBBs fused into tissues and gelatin bioinks were liquefied to generate a channel system after curing the matrix by thermal gelation of the ECM solution at 37 °C (Fig. 5(c)). The

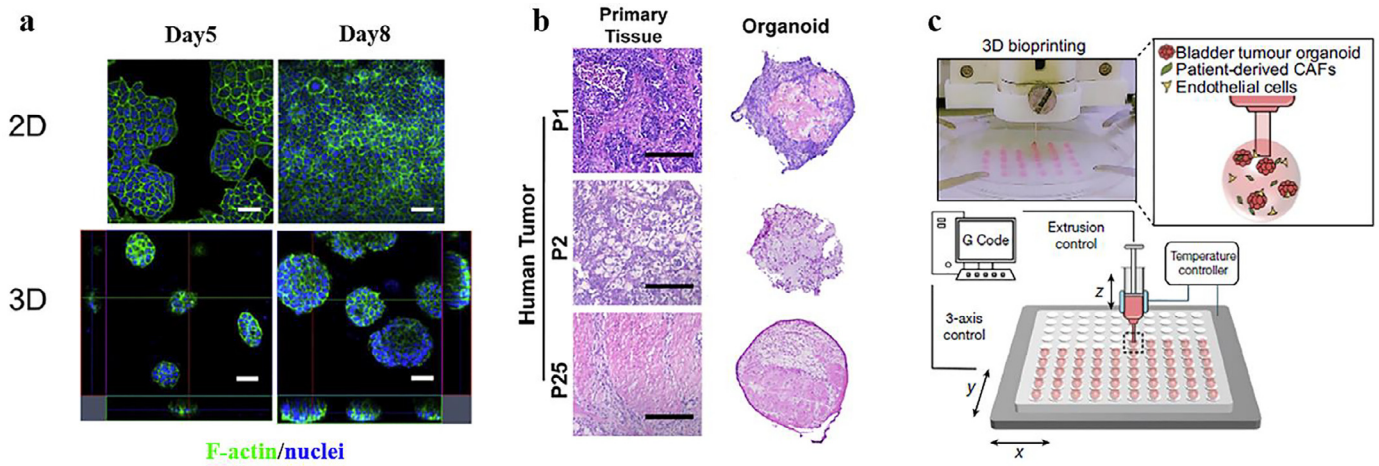

Fig. 6. 3D bioprinting of disease and cancer models: (a) Cytoskeleton staining on day 5 and day 8 in a 2D planar culture and 3D bioprinted models [94]. Reproduced with permission. Copyright 2014, IOP Publishing; (b) Histological images of organoids derived from human lung (P1), liver (P2), and gastric (P25) tumors, and a comparison with their parental tumors [97]. Reproduced with permission. Copyright 2020, Elsevier; (c) Schematic of 3D bioprinting bladder tumor assembloids [68]. Reproduced with permission. Copyright 2020, Springer Nature.

resulting channels were perfusion seeded with endothelial cells to form an endothelial layer on the lumen, which greatly enhanced cell viability in the core of the engineered tissues. This approach could probably address the enduring challenge of bioprinting regarding the engineering of tissues with a high cell density of physiological relevance.

3.2. Printing disease and cancer models

To date, clinical trials are still the most effective way to study the pathophysiological characteristics of a disease. Developing a new therapeutic drug usually takes years and millions of US dollars and is restricted by ethical and safety regulations when using animal models. Therefore, it is particularly important to create alternative preclinical disease models in vitro. In recent years, 3D in vitro disease models based on human cells have proven their potential to simulate the 3D in vivo microenvironment, reproduce the interaction between pathological cells and normal cells, and regulate cellular growth and tissue function. In early 2009, Li et al. from Tsinghua University introduced an approach to construct 3D vascularized liver tissues by multinozzle bioprinting, which served as an in vitro model for drug testing and mechanistic studies [89]. Cancer is one of the most endangering diseases and is discussed in this section. The occurrence, progression, and migration of tumors [90] involve a series of complex processes, such as spatiotemporal changes, cell-microenvironment interactions, cell–cell interactions, and intracellular signal transmission [91,92], which makes the in vitro modeling of tumors extremely challenging.

By accurately positioning cells, active molecules, and biomaterials, 3D bioprinting provides an opportunity to construct in vitro complex tumor models with highly heterogeneous microenvironments [66,93]. In 2014, Zhao et al. [94] used an extrusion-based bioprinter to print cervical tumor models using gelatin, sodium alginate, and fibrinogen. The results revealed great differences in tumor cell morphology, protein expression, and drug resistance between 2D monoculture and 3D bioprinted conditions (Fig. 6(a)). In 2019, Pang et al. [95] tracked and monitored the epithelial-mesenchymal transformation (EMT) of tumor cells in cervical tumor models constructed by 3D bioprinting. EMT was successfully inhibited in the 3D-printed construct after treatment with disulfiram and the EMT pathway inhibitor, C19. In another example, Langer et al. [67] bioprinted a scaffold-free model to study the tumor-stromal interactions. Cells within bioprinted models can self-assemble and deposit matrix proteins, exhibiting heterogeneous responses to therapeutic and extrinsic signals. Despite this progress, the cells commonly used in tumor models are immortalized cell lines, which have lost the genetic phenotype and function of the original tumor. In 2016, the National Cancer Institute of the United States decided to “retire” NCI-60 (60 types of human tumor cell lines grown in culture medium) from its drug screening program and updated the cancer model library with fresh patient samples [96].

Primary tumor cells derived from patients retaining most of the physiological characteristics of tumor cells in vivo have gained popularity. Using patient-derived tumor cells, a 3D bioprinted tumor model can reconstruct the in vivo tumor microenvironment and maintain the tissue and structural characteristics of the original tumor tissue. Compared with the patient-derived tumor xenograft (PDX) model, the 3D bioprinting tumor model is more cost-efficient and more suitable for extensive drug screening. Specifically, the 3D bioprinting tumor model can (1) reproduce the phenotype and function of parental tumor tissue [67], facilitate physiological and histological detection, and guide patient prognosis; (2) be used to evaluate and predict the effect of a treatment on patients before clinical trials [93]; and (3) establish a patient-derived cell bank [97] and track the progression of patients in real time. In 2019, Yi et al. [98] used 3D bioprinting technology to construct a glioblastoma (GBM)-stroma chip consisting of patient-derived tumor cells, vascular endothelial cells, and a decellularized extracellular matrix. The concentric-ring chip with a radial oxygen gradient and high heterogeneity reconstructed the complex ecology of native tumors in vivo. In addition, the patient-specific GBM chip model showed resistance to the drug combination that exhibited superior tumor killing capabilities, reproducing the clinically observed results of the patient to radiotherapy and temozolomide treatment. In 2020, Jiang et al. [97] developed an automated platform that could rapidly generate and pattern uniform organoids with heterogeneity using 3D bioprinting technology combined with the microfluidic method (Fig. 6b). The diameter of the tumor organoids was approximately 500 μm, and drug screening could be completed within 1 week in a high-throughput fashion. The evaluation of 31 types of anticancer drugs in 21 patients revealed a screening accuracy rate of 81.0%, indicating that bioprinted tumor organoids highly reproduced the drug response among individual patients. Alternatively, Eunjee et al. [68] developed a tumor assembloid model using 3D bioprinting technology, which could accurately simulate mature organ architecture and associated tissue microenvironments by 3D reconstruction of stem cells and stromal components (Fig. 6(c)). These assembloids exhibited similar cellular composition and gene expression to mature organs and could mimic the in vivo regenerative response of normal tissues to injury. Such assembloids could be used in per-

sonalized cancer treatment for individual patients, providing a unique paradigm for the next generation of drug discovery and screening.

3.3. Printing microphysiological systems and organ-on-a-chip

Organ-on-a-chip, a combination of in vitro cell culture techniques with microfluidic chips, has provided a new way to create dynamic human physiological tissue models. A small number of tissues/cells can be isolated, cultured, manipulated, and detected in an integrated system to yield biomimetic outcomes [99,100]. Typically, hundreds or thousands of cellular samples, including spheroids or multicell constructs, can be contained on one chip, and multiple sets of assay data can be obtained simultaneously, which is more cost-efficient and faster than traditional methods such as animal models. Micro functional devices, including channels, chambers, valves, and pumps, can be further fabricated and integrated within chips using micro- and nanofabrication techniques [100]. A tiny volume of fluid (10^{-9}–10^{-8} L) flows in microchannels (1–1000 μm) with mass transfer mainly by diffusion. Controlled fluid flow enables precise parametric control of multiple systems with physiological resemblance, including chemical gradients [101], gas gradients [102], fluid shear [103], cell spatial distribution [104,105], interfaces [106], and cell interactions [107]. Furthermore, by combining with external stimuli, microchips provide a powerful platform for the in vitro simulation of complex microphysiological microenvironments [108], recapitulating drug responses at the tissue level [109] and modeling disease and physiological malfunctions [110]. To date, many human organs, including the liver [111], lung [110], intestine [112], kidney [113], blood vessels [114], and heart [115], have been modeled using microfluidic chips. In 2022, the size of the microfluidic chips market is expected to reach $117 million; eventually, the market may reach a multibillion-dollar size in the future.

To date, the most widely used technique for organ-on-a-chip fabrication is soft lithography, which provides a convenient microfabrication method for bioengineers [69]. By establishing an organ-on-a-chip with a 3D co-culture of hepatocytes, stellate, Kupffer, and endothelial cells, Roth et al. from Roche Pharma. found that the liver-on-a-chip exhibited better functionality than a 2D culture of single hepatocytes for drug screening and hepatotoxicity detection [116]. Stelzle et al. constructed a liver microarray with strip arrangement of hepatocytes and perfused fluid on both sides of the flow channels to facilitate hepatocyte survival with stable functionality for up to 7 days [117]. Ingber et al. designed a multilayer microfluidic kidney chip separated by a porous membrane, with the upper layer simulating the flow of renal tubular fluid and the lower layer as static fluid. Compared with the in vitro models under static conditions, the kidney chip better simulated the fluid shear force on renal tubular epithelial cells, and human proximal tubular epithelial cells cultured in the kidney chip demonstrated better physiological performance [118]. To model the dynamic absorption process of the intestine, Ingber et al. designed an intestinal chip composed of two microfluidic channels and a porous PDMS membrane, in which the PDMS membrane was covered with human intestinal epithelial cells and an extracellular matrix [119]. Despite this progress, there remain some challenges to be addressed: (1) most of the current organ-on-a-chip systems simulate only one type of tissue/organ [120], (2) the most widely used PDMS is hydrophobic and nonbiodegradable, making it challenging for cells to adhere to it [109]; (3) micrographs can only be patterned on the 2D plane of microfluidic chips to regulate the growth and spatial positioning of cells [121], whereas it is difficult to change the cell arrangement in the height direction [114].

Considering these pressing problems, 3D printing microfluidic chips have gained popularity [70]. The 3D printing technique provides a novel processing approach that requires no complicated steps and specific molds with economic and efficiency improvements. Shallan et al. used microstereolithography to fabricate concentration generators with a 5-layer structure, which greatly simplified the preparation process compared with the conventional lithography process [122]. Moreover, 3D printing technology enables the integrated formation of a microfluidic chip consisting of a PDMS base material, hydrogels, and sacrificial materials. For instance, Lind et al. simultaneously printed six functional materials to enable the integration of soft strain sensors with microarchitectures that can guide the self-assembly of physio-mimetic laminar cardiac tissues (Fig. 7(a)) [123]. Lee et al. fabricated integrated endothelialized liver microarrays using 3D bioprinting. This printed chip demonstrated a significantly lower uptake of small molecule proteins than that of the conventional PDMS chip (Fig. 7(b)) [124]. Homan et al. successfully constructed a renal proximal tubule (PT) chip using a four-nozzle jet 3D bioprinter, exhibiting cell polarity, epithelial barrier function, and albumin uptake (Fig. 7(c)) [125]. Alternatively, Ouyang et al. fabricated hydrogel-based microfluidic devices in PDMS chambers by void-free 3D printing and in situ endothelialization, which allowed for an extended perfusion culture (Fig. 7(d)) [36]. Furthermore, 3D printing enables crosstalk between multiple organ-specific cells in printed microphysiological systems. Skardal et al. printed microarrays containing three organ-specific cells (liver, heart, and lung), demonstrating that the multiorgan chip simulated the metabolic processes of drugs more accurately and could be used to detect drug toxicity that could not be detected by a single-organ chip (Fig. 7(e)) [71]. In summary, 3D printing technology has revolutionized the field of organ-on-a-chip more conveniently and efficiently, which would greatly facilitate the translational applications of organ-on-a-chip systems.

3.4. Constructing biohyrid robots

With the rapid development of biomanufacturing technologies and advances in tissue engineering, biohybrid robots (biobots), and intelligent miniaturized robots constructed by integrating living cells with soft materials have attracted extensive attention and research interest [126]. In 2005, Xi et al. created the first walking biobot driven by cardiomyocytes, with a walking speed of up to 38 μm/s [127]. Since then, inspired by nature, researchers have designed various biobots with biomimetic behaviors and functions by employing biological components with contractile cells as actuators. To date, various types of living cells and tissues, including cardiomyocytes, skeletal muscle cells, and optogenetic neuromuscular tissues, have been used as biological actuators for biohybrid robots [128]. Typically, a single cardiomyocyte with a length of approximately 100 μm can generate a contraction force of at least 1 μN, making them suitable for actuating biobots [129]. For instance, Yoon et al. designed microcylinder bioactuators by capitalizing on the spontaneous contractility of neonatal rat ventricular cardiomyocytes [130], while Shang et al. created a swimming guppy-shaped biobot driven by cardiomyocytes [131].

3D bioprinting and microfabrication techniques, including micromolding and etching approaches, have offered great capabilities for tailoring soft substrates and materials with specific shapes to reduce potential hydraulic resistance and microstructures to guide cellular alignment [72]. For instance, Bashir et al. developed a walking machine with skeletons fabricated via stereolithographic 3D printing technology to mimic the in vivo musculoskeletal system (Fig. 8(a)) [132]. The biomimetic skeleton consisting of a flexible beam with pillars at both ends was positioned in a customized mold filled with C2C12 cells and a hydrogel matrix to form mature skeletal muscle strips. Under electrical pulse stimulation, the 3D printed walking machine was powered by C2C12-derived muscle strips with a passive tension force of 1.7 mN and an active tension of 300 mN. Furthermore, by tailoring the substrate with specific surface morphologies or chemical gradients, the alignment of cardiomyocytes and muscle cells could be induced to enhance their contractile force and thus the performance of biobots [133]. For instance, Parker et al. printed an instrumented cardiac microphysiological device with parallel microgrooves of a PDMS substrate to effectively promote the self-assembly

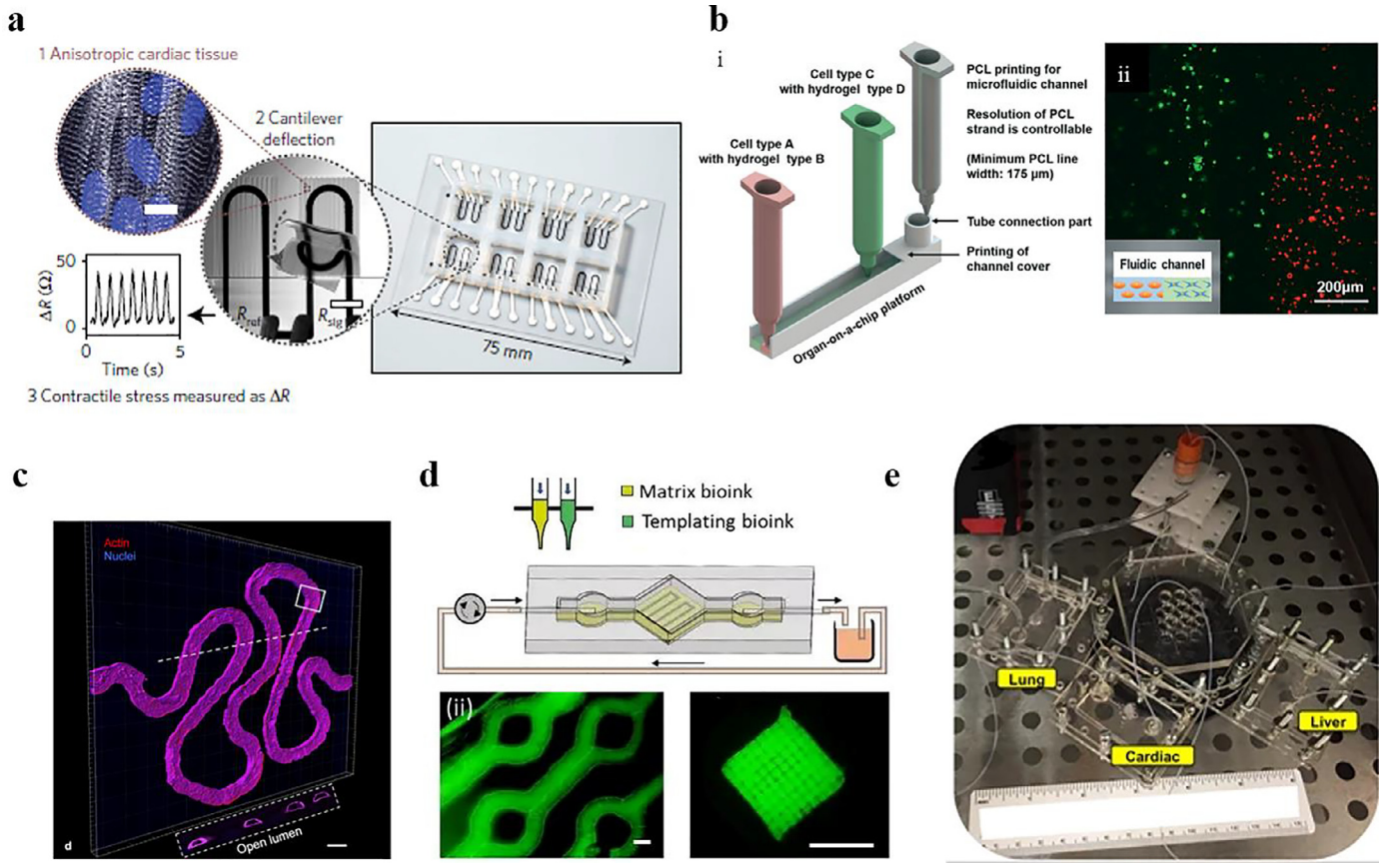

Fig. 7. 3D printing of an organ-on-a-chip: (a) 3D printed cardiac microphysiological device with a strain sensor [123]. Reproduced with permission. Copyright 2017, Springer Nature; (b) (i) 3D printing of endothelialized liver microarrays, (ii) heterogeneous cell distribution shown by the fluorescent image [116]. Reproduced with permission. Copyright 2016, Royal Society of Chemistry; (c) 3D printed renal proximal tubule chip [125]. Reproduced with permission. Copyright 2016, Springer Nature; (d) Void-free 3D printing for in situ microfluidic perfusion [36]. Reproduced with permission. Copyright 2020, Wiley; (e) Organ-on-a-chip system with a liver, cardiac, and lung module [71]. Reproduced with permission. Copyright 2017, Springer Nature.

and contractile performance of cardiomyocytes [134]. Alternatively, through microcontact printing, the PDMS film could be patterned with fibronectin lines, on which cardiomyocytes were guided to form discrete muscle fibers with uniaxial alignment [135].

By combining advanced biomanufacturing techniques with artificial materials, various biobots, including crawlers, swimmers, jumpers, and rollers, have been developed thus far, showing great potential for a wide range of applications [173]. Sun et al. developed a caterpillar-shaped biohybrid robot driven by primary cardiomyocytes and used it as a potential platform for drug screening [136]. The spontaneous beating of cardiomyocytes deformed the soft substrate, leading to the crawling of biobots and structural color variation. By incorporating external stimulations such as electrical [137], magnetic [138], and optical stimulations [139], biohybrid bots can perform various biological behaviors and missions with flexibility and adaptability. For instance, Parker et al. presented a novel tissue-engineered jellyfish-like biobot with biomimetic propulsion actuated by cardiomyocytes under an electric stimulation [140]. Furthermore, they created an artificial ray fish with controllable locomotion using optogenetic cardiomyocytes and optical manipulation (Fig. 8(b)) [74]. Cardiomyocytes were engineered via the lentivirus method to allow the expression of light-responsive proteins, that is, channelrhodopsin-2 (ChR2). Therefore, guided by light, the artificial ray fish could propel itself by the forward thrust due to the undulatory motion of fins and even swim through obstacles at a high speed. This work laid the foundation for developing autonomous and adaptive biohybrid robots, which possess great potential as miniaturized medical robots in minimally invasive surgery for sensing and tracking that can guide the operation of surgeons. Compared with traditional soft robots, biohybrid robots have several advantages, including miniaturized features, intrinsic softness, remarkable energy conversion efficiency, and environmental compatibility. Despite the significant progress made in the past decade, the field of biohybrid robots is still in its infancy, with challenges such as relatively short lifetimes.

3.5. Bioprinting in space

With the development of space technology, space has become an important strategic high ground that many countries are competing to seize. Space exploration has also become a new engine of economic development. Long-term and long-distance exploration missions to land or even emigrate to the Moon or Mars are widely regarded as the next important goal of human space exploration. However, long-distance space flight still faces significant safety and health risks due to limited ground support. Considering that life can self-replicate and self-repair, it is expected that organisms can be used as raw materials to produce food, fuel, customized tools, smart fabrics, and even organs in orbit through bioprinting in the future. For example, with cells isolated from astronauts and expansion in vitro, 3D bioprinting can be used to construct transplantable tissues/organs for space accidents, which is beneficial for providing alternative treatment options for an accidental injury that may occur during a long-distance interstellar exploration mission [75]. To date, commercial companies such as 3D Bioprinting Solutions, Techshot, and nScrypt have conducted a series of 3D bioprinting exper-

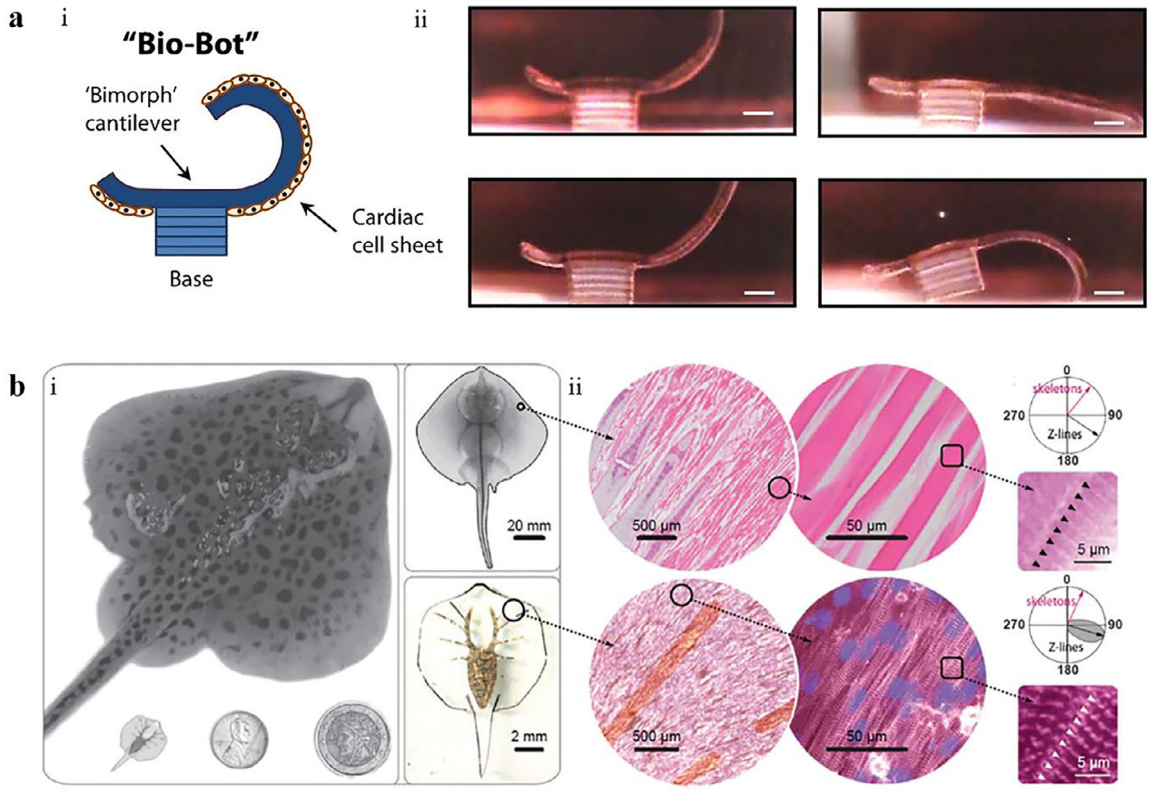

Fig. 8. Representative examples of biohybrid robots: (a) (i) Schematic of a miniaturized walking biohybrid robot fabricated via stereolithographic 3D printing technology, (ii) walking behavior of a biohybrid robot driven by cardiomyocytes [132]. Reproduced with permission. Copyright 2012, Springer Nature; (b) (i) An artificial stingray with a 4-layer structure controlled by optical stimulation, (ii) cell alignment and nanostructure of an engineered stingray [74]. Reproduced with permission. Copyright 2016, AAAS.

iments in space, which mainly used extrusion bioprinting and magnetic levitation strategies [141]. Magnetic levitation bioprinting is an emerging scaffold-free assembly technology. Under microgravity conditions in space, the obstructive effect of gravity on magnetic particles is significantly reduced. Therefore, a magnetic levitation biological assembly can be achieved using a lower concentration of toxic paramagnetic media [142]. At the end of 2018, Russian astronauts used a 3D bioprinter from Invitro Medical to print human cartilage tissue and rodent thyroid tissue in orbit for the first time at the International Space Station (ISS) (Fig. 9(a)) [143]. The printer supported the cultivation of a variety of organs in orbit, including those that are sensitive to radiation, to evaluate the adverse effects of cosmic radiation, which laid the technical foundation for 3D bioprinting of human organs in space.

The direct use of animal cells for bio-3D printing to make food in space promises to address the problem of food shortage during long-distance space travel [64]. In 2019, Aleph Farms cooperated with 3D Bioprinting Solutions, and two other British companies to successfully fabricate the first artificial meat by space bioprinting in a magnetic suspension (Fig. 9(b)) [144]. On Earth, gravity prevents molecules from uniformly making contact and nucleating to form large molecular weight crystals. 3D Bioprinting Solutions used a magnetic trap at the center of a magnetic 3D bioprinter to generate a gradient magnetic field to induce the creation of high-quality protein crystals (Fig. 9(c)) [145]. Most of the crystals obtained exhibited a regular form, ranging in size from 300 to 1500 μm. This research will help scientists create more effective drugs that affect viruses and bacteria at the molecular level. The NASA-affiliated BioServe Space Technologies Research Center and the University of Colorado have also launched a research project called "Magnetic 3D Cell Culture". Researchers have used the magnetic levitation method to print nanoscale gold atoms into a polymer matrix containing human lung cancer cells. As gold atoms can quickly adhere to the cell membranes of lung cancer cells and can be controlled by a magnetic field, they can be used to manipulate the cells and to form complex patterns with precise positioning, which is of great significance for ongoing research on the International Space Station [146]. In addition, Russian researchers used magnetic levitation bioprinting to study the aggressiveness of bacteria and their resistance to antibiotics under space conditions [147]. Extrusion 3D printing has advantages such as simple equipment, wide applicability of biomaterials, and good cytocompatibility. In January 2020, Techshot, a US microgravity equipment manufacturer, successfully printed heart structures using cardiac cells on the ISS, which was the first time human organs such as the heart and lungs were manufactured in space [148]. A human body that is subjected to an extended state of weightlessness will experience many problems, such as poor blood flow, muscle atrophy, and bone mass reduction. A research team at the Technical University of Dresden (TUD) recently collected autologous stem cells of astronauts and added calcium phosphate cement as a structural support material to print skin and bone tissues using extrusion bioprinting in space [76]. In addition to engineering human tissue constructs for medical use, 3D bioprinting in space can also be used to study the effects of microgravity and cosmic radiation on human tissues, which is of great significance in expanding our understanding of space travel. Despite very limited studies and reports, we believe that the field of 3D bioprinting in space will continue to gain popularity in the future.

4. Perspectives

4.1. Time matters: 3D bioprinting for better growth of organs

Although 3D bioprinting has significantly advanced in the past decades such that 3D tissue-mimetic in vitro structures can now be fab-

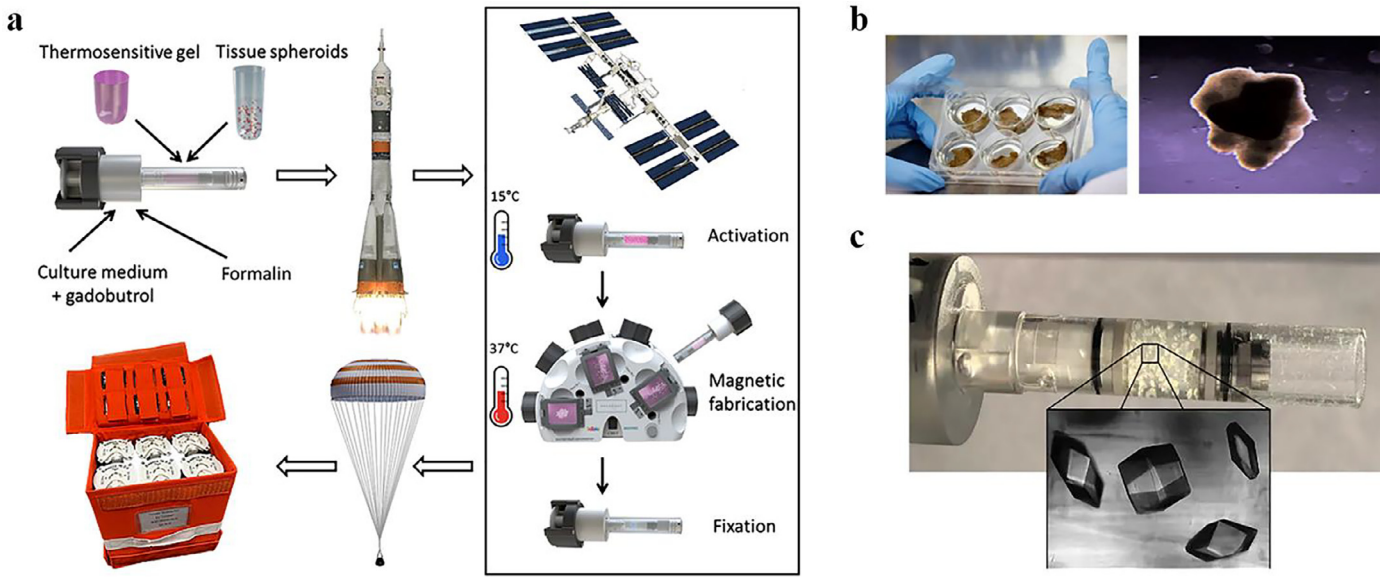

Fig. 9. Bioprinting in space: (a) 3D printing of human cartilage tissue in orbit at the International Space Station [75]. Reproduced with permission. Copyright 2020, AAAS; (b) 3D bioprinted artificial meat in space [144]. Reproduced with permission. Copyright 2019, Frontiers; (c) 3D printed high-quality protein crystals by magnetic suspension assembly [145]

ricated, obstacles remain to be overcome to achieve the ultimate goal of printing an entire organ. An organ is inherently complex owing to its hierarchical organization of multiple types of tissues and cells, whose microenvironment involves dynamic and heterogeneous cell–cell and cell-ECM interactions. Tissue stem cells are responsible for tissue maintenance and regeneration through a balance of proliferation and differentiation that is influenced by the local ECM microenvironment, which is defined as their niche [149]. The orchestrated cellular behaviors of stem cells that are located in their niche are regulated by neighboring cells as well as the tissue-specific ECM [150], which provides both physical and biochemical cues. Moreover, during dynamic morphogenesis and organogenesis, time is critically emphasized in addition to solely considering the 3D spatial location of the cells and the ECM and is referred to as the fourth dimension (4D). It would be of expectation that, in the future, an organ could be fabricated in vitro by combining bioprinting and culturing. Therefore, the current progress focuses on integrating a 4D culture into 3D bioprinting to improve tissue growth and maturation after depositing cells in the desired spatial location.

Several emerging strategies target seeded cells and bioink materials with time tunability. Together with predominant cells, stromal cells could be added to the bioink to secrete and remodel the ECM during coculture postprinting [151]. Cell aggregates and organoids formed through self-assembly and cell sorting from single cells could be applied as modular bioprinting building blocks and could further grow over time to form an engineered system [152]. The bioink can be designed with tunable spatiotemporal properties that aid dynamic cellular processes while forming tissue constructs. The dynamic properties of the bioink could either be passive or active; moreover, a bioink could be engineered to have passive reversible cross-links by using the Schiff-base reaction [153], a boronate ester [154], the Diels–Alder reaction [155], and oxime ligation [156], which results in a bioink that exhibits self-healing and stress relaxation behavior versus time [157]. Such properties are believed to promote cell-ECM interactions through mechanotransduction signaling via integrin sensing [158]. In addition, attempts have been made to cross-link hydrogels with matrix metalloproteinase (MMP)-degradable peptides to regulate the embedding hydrogel stiffness by passive cellular degradation [159]. The bioink can also be modified to actively respond to a series of external physical factors, including light, sonic, thermal, electric, and magnetic fields [159]. In response to physical factors as triggers, the bioink can spatiotemporally crosslink, degrade, release, or bind specific biochemical cues such as growth factors, peptides, and bioactive molecules [160]. Photoresponsive hydrogels that undergo adjustments over time can support cellular processes, and their behaviors have been explored [161]. Although the current resolution of the commonly used extrusion-based bioprinting could not meet the requirements for constructing a stem cell niche to generate proliferative tissue, it is hoped that a preliminary tissue construct could be fabricated through conventional bioprinting approaches based on the responsive bioink and then further engineered or micropatterned with external stimuli (e.g., light).

4.2. *Challenges: from bench to bedside*

Over the past decades, bioprinting has considerably advanced the engineering of tissues and organs; however, the majority of these endeavors have been conducted in vitro. In this context, 3D tissue constructs are bioprinted and cultured in vitro for maturation before implantation in vivo. Recently, the concept of patient-specific in situ or in vivo bioprinting, in which constructs are directly printed in vivo at the injury site, has gained popularity [162]. Defect imaging, data analysis, preoperative planning, and bioprinting are performed consecutively in a surgical setting. The in vivo bioprinting of tissue substitutes into the target sites is highly beneficial, as the patient's body serves as an ideal bioreactor, and there is no need for an in vitro postprinting maturation process [163]. Recently, researchers have achieved in situ repair of bone and cartilage defects using 3D scanning and in vivo printing [164]. By using minimally invasive procedures or using a miniaturized printing platform, noninvasive in vivo printing has become feasible for the in situ repair of internal injuries [165]. For example, Xu et al. developed a micro bioprinting platform that can be installed onto an endoscope that is then conveyed into the human body and successfully printed patterned cellular structures at a gastric wound site [166]. Alternatively, near-infrared photopolymerization-based stereolithography has emerged as a promising noninvasive in vivo bioprinting strategy as it allows for the curing of subcutaneously injected cell-laden bioink using patterned NIR irradiation [61]. Despite rapid progress, the field of in vivo bioprinting is still in its infancy and faces many challenges associated with translational applications, such as vascularization and integration with host tissues.

There are substantial challenges associated with the cost-effectiveness and regulation of bioprinted tissues for personalized

therapy. To date, efficient cell expansion techniques are still needed to generate clinically relevant numbers of cells [167]. For instance, the adult human heart consists of approximately 4 billion cardiomyocytes (CMs), which possess a very limited self-renewal capacity. Although highly efficient differentiation protocols of hiPSC-CMs have been established in 2D culture, scaling up to 10^9 cells would be time-consuming and labor-intensive (at least a hundred of 100-mm dishes are needed). Alternatively, 3D suspension differentiation platforms such as microcarriers are more space efficient; unfortunately, the complicated procedures for attaining a highly pure CM culture are particularly costly. Despite the easy accessibility, expandability, and capability of iPSCs to give rise to almost any cell type, in vitro culture and expansion may accompany changes in iPSC phenotypes and even deadly mutations [168]. The potential of iPSC tumorigenicity remains to be addressed before translational application. Alternatively, human leukocyte antigen (HLA)-matched hiPSC tissue banks could be a valuable source for therapeutic transplantations that can overcome immune rejection by the host [169]. However, the development of an allogeneic cell bank would require concerted efforts from the international society to obtain sufficient tissue repositories, considering the complexities of ethnic diversity. The translational application of 3D bioprinted tissues also faces regulatory challenges, as they are intrinsically different from conventional clinical products owing to the complexity of the therapeutic mechanism and as-yet-unknown long-term effects in the human body [170]. Therefore, there is an urgent need for standardization and quality control guidelines for the bioprinting process covering biomaterials (i.e., bioinks), cell sources, and cell culture media to ensure the safety, efficacy, and reproducibility of 3D bioprinted tissues. Additionally, the cryopreservation and transportation of 3D bioprinted tissues should be taken into consideration. Despite all the challenges mentioned above, we believe that 3D bioprinting of functional tissues and organs will continue to develop with rapid progress.

5. Conclusions

In summary, 3D bioprinting has served as a promising approach for engineering complex tissues, offering precise control over cellular building blocks to recapitulate the intricate hierarchy and complexity of native human tissues. Recent advances in the innovative forms of living building blocks and enabling bioprinting techniques have greatly expanded the capability to engineer more complex and functional tissues and further extend their applications in engineering disease models, microphysiological systems, and biobots. However, many challenges still need to be addressed before translational applications, including, but not limited to, low printing resolution and speed, lack of vascularization and innervation, and poor functionality. With the convergence of advanced microfabrication techniques, stem cells, and smart biomaterials, we anticipate an accelerated development of 3D bioprinting for engineering complex living systems, which may not necessarily possess the exact anatomical structures of native tissues or organs but should possess the required tissue functionalities.

CRediT authorship contribution statement

Yongcong Fang: Writing - original draft, Writing - review & editing, Visualization, Funding acquisition. **Yuzhi Guo**: Writing - original draft, Writing - review & editing. **Tiankun Liu**: Writing - original draft. **Runze Xu**: Writing - original draft. **Shuangshuang Mao**: Writing - original draft. **Xingwu Mo**: Writing - original draft. **Ting Zhang**: Funding acquisition, Writing - review & editing. **Liliang Ouyang**: Conceptualization, Supervision, Funding acquisition, Writing - review & editing. **Zhuo Xiong**: Conceptualization, Supervision, Funding acquisition, Writing - review & editing. **Wei Sun**: Supervision, Writing - review & editing.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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