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Additive Manufacturing of Biomaterials, Tissues, and Organs

Three-Dimensional Printing of Tissue/Organ Analogues Containing Living Cells

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Abstract—The technical advances of three-dimensional (3D) printing in the field of tissue engineering have enabled the creation of 3D living tissue/organ analogues. Diverse 3D tissue/organ printing techniques with computer-aided systems have been developed and used to dispose living cells together with biomaterials and supporting biochemicals as pre-designed 3D tissue/organ models. Furthermore, recent advances in bio-inks, which are printable hydrogels with living cell encapsulation, have greatly enhanced the versatility of 3D tissue/organ printing. Here, we introduce 3D tissue/organ printing techniques and biomaterials that have been developed and widely used thus far. We also review a variety of applications in an attempt to repair or replace the damaged or defective tissue/organ, and develop the *in vitro* tissue/organ models. The potential challenges are finally discussed from the technical perspective of 3D tissue/organ printing.

Keywords—3D tissue/organ printing, Bio-inks, 3D tissue/organ analogues, Tissue engineering and regenerative medicine, 3D *in vitro* tissue/organ models.

INTRODUCTION

Since the application of three-dimensional (3D) printing in the field of tissue engineering and regenerative medicine about two decades ago, 3D printing-based approaches have been widely expanded and have developed reliable strategies for the fabrication of 3D biomaterial matrices (known as scaffolds). To date, diverse 3D printing techniques have been applied directly or indirectly to fabricate 3D scaffolds, and their capabilities have been demonstrated with a large variety of biomaterials by overcoming the inherent limitations in the process of traditional techniques.[24,26,28,66,67,76,95] 3D printed scaffolds using various biomaterials have also been revolutionized by biological, chemical and mechanical modification methods in various applications.[12]

3D printing is an additive manufacturing technique fundamentally based on computer-aided design and computer-aided manufacturing (CAD/CAM). In the 3D printing process, a two-dimensional (2D) pattern with a defined thickness is printed by selectively adding the desired materials, and 3D structures are built by piling up 2D patterns in layers. This automated additive process of 3D printing allows 3D scaffolds to have precisely controlled architecture (external shape, internal pore geometry, and interconnectivity) with highly reproducibility and repeatability.[27,29,40,68] Another important advantage of 3D printing is that the use of medical image data such as magnetic resonance imaging (MRI) and computerized tomography enables the creation of patient-specific implants that have the geometric shape and size of the defective part.[24,67,76,95]

Recently, technical advances in the 3D printing-based approach have enabled living cells to be included in the printing process itself with all of advantages of 3D printing; therefore, the spatial positioning of living cells together with desired biomaterials and supporting biochemical factors within a 3D structure has become possible.[4,10,35,57,60,77] Diverse 3D tissue/organ printing techniques such as dispensing, droplet, and stereolithography (SLA) techniques have been developed and applied to reproduce the complex micro-architecture, components of the extracellular matrix (ECM) and multiple cell types in sufficient resolution. In this regard, 3D tissue/organ printing involves additional

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strategies to encapsulate and print living cells, and this is directly related to the viabilities of printed living cells and eventually the specific functions of 3D printed tissue/organ analogues.

3D tissue/organ printing fundamentally begins with the design of the 3D tissue/organ model using 3D CAD software. The medical image data including geometric and anatomical information about the target tissue or organ can provide a useful guide for the structural (external shape and internal architecture) and biological design of a 3D tissue/organ model. A 3D CAD model of the defective tissue or organ that have been created by mirroring the configuration of normal tissue/organ anatomy can also be used to design a 3D tissue/organ model on demand. Considering the available 3D printing techniques, the cell types (differentiated, pluripotent, or multipotent stem cells), biomaterials (synthetic or natural polymers and decellularized ECM (dECM)), and supporting biochemical factors are then selected, and the configuration of these printing components is constructed within the 3D tissue/organ model. These printing components are integrated with the 3D printing system, and the pre-designed 3D tissue/organ model eventually starts being printed according to the fabrication code containing the printing strategies, including the printing path and conditions, to realize the configuration of each printing component.

3D tissue/organ printing has demonstrated its remarkable potential in the creation of 3D tissue/organ analogues that show competent functionality for tissue regeneration and other applications. In particular, recent developments and applications of dECM-derived hydrogels as a new printable biomaterial have expanded the versatility of 3D tissue/organ printing.[71,72] Here, we review the different types of 3D tissue/organ printing techniques, including their basic principles and respective features, as well as the printable biomaterials that have been widely used so far. We then describe representative applications, the development of 3D tissue/organ analogues for tissue engineering, and *in vitro* 3D tissue/organ models for testing or screening systems. We finally discuss the current limitations of 3D tissue/organ printing and future perspectives.

3D TISSUE/ORGAN PRINTING TECHNIQUES

3D tissue/organ printing employs living cells in the printing process together with the inherent advantages of 3D printing-based approaches. Diverse techniques have been developed to create 3D tissue/organ analogues, and each technique has advantages and disadvantages in terms of the range of available biomaterials, resolution, and the printing speed.[35,60,77] Representative techniques can be primarily categorized into three types according to the printing modality (Fig. 1).

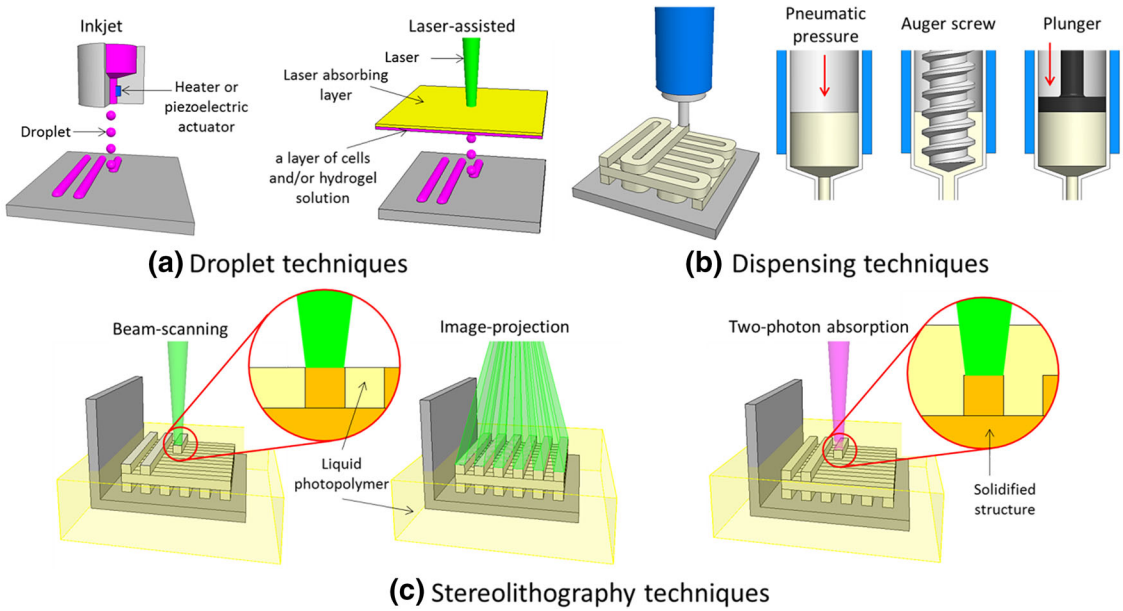

FIGURE 1. 3D printing techniques to create 3D tissue/organ analogues. (a) Droplet techniques: inkjet and laser-assisted. (b) Dispensing techniques: pneumatic pressure, auger screw, and plunger. (c) Stereolithography techniques: beam-scanning, image-projection, and two-photon absorption.

Droplet Technique

In the droplet technique, a droplet of cell encapsulated hydrogels or cell slurries are generated and jetted to pre-defined locations on the substrate (Fig. 1a). This technique allows the direct printing of cells with spatially well-organized patterns in high resolution.[2,7,9] Droplet techniques can be classified into three primary categories: inkjet, pneumatic pressure-assisted and laser-assisted printing.[5,21,42,61,63,65]

The inkjet technique is commonly used in 3D tissue/organ printing. In this technique, droplets can be generated *via* thermal or piezoelectric forces, and ejected from the inkjet-head nozzle (Fig. 1a). The thermal inkjet technique employs local heating in the range 200–300 °C inside the printing head to produce and eject the droplets. The piezoelectric technique uses a piezo-crystal pulse actuator to generate a pulse for the ejection of small droplets at regular intervals. The mechanisms in these two techniques are the most widely used in commercially available inkjet printers. Many researchers have modified commercial inkjet printers by replacing the ink in the cartridge with biological materials containing living cells, and the paper with the elevator stage.

The pneumatic pressure-assisted technique uses a set of electromechanical micro-valves, and the droplets can be produced by opening the micro-valve under constant pneumatic pressure. This technique can use various types of liquid biomaterials with viscosities of up to 200 Pa s, and control the droplet volume by controlling the pressure to the fluidic pathway and valve gating time (up to 200 μs of opened and closed duration).

The laser-assisted technique uses the principles of laser-induced forward transfer. The donor layer, composed of a laser-absorbing layer and a layer of cells and/or hydrogel solution, is the critical part of this technique. The focused laser pulse stimulates a small area of the laser-absorbing layer, and a high-pressure bubble is generated as the response to laser stimulation. A droplet of cell-containing hydrogel is then propelled towards a substrate and subsequently cross-linked. The laser-assisted technique is less common than other techniques because of the expense of using laser sources and the complexity of the laser pulse control; however, its applications have been increasingly expanded with its advantages of high resolution, throughput, and cell viability.[60]

The droplet technique can achieve cell patterns with very high resolution; however, this technique can only use low-viscosity or liquid bio-ink materials, and printed tissue/organ analogues cannot maintain their shape without sufficient mechanical strength.[77] The printing of 3D volumetric constructs is also limited with this technique.

Dispensing Technique

The dispensing technique, which originated from fused deposition modelling (FDM), can manufacture complex 3D structures by selectively stacking layers of 2D patterns that represent the cross section of the 3D structure (Fig. 1b). The systems for the dispensing technique consist of a three-axis motion stage and dispenser including heating controller, with one or both capable of movement according to x, y, and z axes. A few systems adopt independently controlled multiple heads to dispense multiple biomaterials. The motion stage with high resolution (accuracy and repeatability of less than $\pm$ 3 μm) is used to precisely control the location of the micro-sized nozzle and allows the printed structure to have the 3D micro-environment of the target tissue/organ.[33,81] The common types of dispenser are mechanical extruder dispenser (piston and screw) and pneumatic pressure dispenser.[30,60,81] Mechanical dispensing systems provide relatively higher accuracy and direct control over the biomaterial flow, particularly for high viscosity biomaterial, with respect to the dispensing volume.[30] Pneumatic dispensing systems have a simple drive-mechanism and have the advantage of leaving little discarded biomaterial remaining inside the syringe.[81] Commonly used biomaterials (synthetic polymers and hydrogels) in dispensing techniques should have thermo-plasticity, thermo-stability, proper viscosity, high fidelity, and formability to stack 3D constructs.

Dispensing techniques have the advantage of handling a wide range of fluid properties. Higher-viscosity biomaterials such as synthetic polymers provide structural support to mimic the shape of the 3D constructs, and lower-viscosity materials such as hydrogels provide a suitable environment for maintaining cell viability and function throughout cell encapsulation.

Dispensing techniques also facilitate directly printing cells with high cell densities. Cell-laden hydrogels or cell spheroids in syringes are directly printed in the desired position through a micro-sized nozzle.[58,80,81] Many research groups fabricate multicellular cell spheroids to replicate functional tissue scaffolds for the regeneration of target tissues such as branched vascular trees, single vessels, and aortic valves.[13,56,64,80] They print cell spheroids in a line to fuse with each other through a self-assembling strategy.[56,80] In addition, 3D tissue/organ printing studies that utilize cell-laden hydrogel have been investigated.[58,80] Compared with cell spheroids, cell-laden hydrogels, in which a variety of cells are encapsulated in hydrogels, maintain cell viability and effectively induce target tissue formation with proper mechanical properties and function because the hydrogel serves as a barrier and 3D micro-environment similar to the ECM of tissue.[60,80]

Moreover, synthetic polymers that have relatively high mechanical strength have been employed to reinforce printed 3D tissue/organ analogues for the dispensing technique.[43,80,81,89] Some researchers dispense PCL in advance to fabricate a porous framework, and print cell-laden hydrogels into the spaces in the framework on the same layer. In this manner, 3D tissue/organ analogues that can maintain their shape are completely printed.[43,89] However, this strategy has limited applications in soft tissue regeneration.

Dispensing techniques have the advantage of high cell densities with homogenous distribution throughout the 3D analogues, as well as being less time consuming for effective tissue formation and organization.[12] This techniques can achieve up to 95% cell viability by considering the dispensing pressure, the nozzle size, and the cross-linking characteristics of the hydrogels.[8,81,83] However, the printing conditions of the cell-laden hydrogel can be limited due to high shear force. Relatively low resolution is also disadvantage for the dispensing technique.

Stereolithography Technique

SLA is the oldest 3D printing technique, developed in the 1980s, and enables the fabrication of 3D complex structures with very high resolution and accuracy compared to other 3D printing techniques.[54] SLA uses the spatially controlled irradiation of light such as ultra-violet (UV), infrared, or visible light to solidify a 2D patterned layer through selective photo-polymerization in the liquid photopolymer (Fig. 1c). A 3D structure is then built by piling up solidified 2D patterned layers *via* a layer-by-layer process whereby the building platform moves stepwise in the z-axis after the solidification of each layer.

Photo-polymerization in SLA can be induced by either single-photon or two-photon absorptions; it is highly dependent on the light intensity, irradiation time, and the photo-initiator concentration.[4,41] A 2D patterned layer can be solidified *via* two different methods in single-photon-based SLA: beam-scanning and image projection.[1,26,28,45,66,68] The beam-scanning method employs the selective scanning of a focused laser beam to draw a pattern, and a 2D pattern starts to be solidified in the scanning path of the laser beam. In the Image-projection method, a 2D pattern image generated with an image generation device such as digital micro-mirror device is projected into the liquid photopolymer, and one entire 2D layer is solidified by a single projection of the pattern image. This printing process enables a significant decrease in printing time. Single-photon-based SLA has evolved into a micro-stereolithography (MSTL) using specific light systems and optics to print a 3D structure with micro-scale resolution.[26,28,66,68]

Two-photon-based SLA, called a nano-stereolithography (NSTL), uses simultaneous two-photon absorption.[39,84] Two photons, absorbed at the same time and point in the liquid photopolymer, acts as one photon with double the wavelength, and photo-polymerization can be induced within a very small region without affecting other regions inside the liquid photopolymer. The movement of the focal point then enables the solidification of a 3D structure without the use of the building platform inside the liquid photopolymer. This technique can achieve spatial solidification with a resolution of up to 100 nm, which is the highest resolution among all 3D printing techniques.

MSTL and NSTL have generally been applied to print 3D scaffolds directly or indirectly in the field of tissue engineering and regenerative medicine[4,26,28,54,66,68,84]; the application of SLA to create 3D tissue/organ analogues has become possible with the development of water-soluble photopolymers that are compatible with living cells and visible light-based SLA without damaging the cellular DNA.[1,11,47,90] However, it is still an inherent limitation that the SLA technique can only use photopolymerizable and biocompatible materials.

BIOMATERIALS FOR 3D TISSUE/ORGAN PRINTING

Polymers are widely used as biomaterials for the printing of scaffolds or cell-laden constructs due to their distinctive advantages with respect to biocompatibility, versatility of chemistry, and biological properties.[53] Biomaterials for 3D tissue/organ printing have been extensively developed and used in various tissue engineering applications along with various cell types (Table 1). Biopolymers can be classified into several types according to their structural, chemical, and biological characteristics. There are two primary categories of biopolymers that are mostly used in 3D tissue/organ printing. The first is synthetic polymers that are used to fabricate frameworks along with other materials or produce 3D scaffolds for mechanically robust constructs. The other is hydrogel; to print living cells and supporting biochemical factors, the encapsulating material should be used to protect cells from the external environment during printing process. This material is called a "bio-ink" and has to meet several physicochemical requirements.[64] In this section, we provide a comprehensive overview of printable biomaterials used in 3D tissue/organ printing, and several other biomaterials that have been developed.

Synthetic Polymers

Biodegradable synthetic polymers are widely used in biomedical fields because of their tailorable material properties. The physicochemical and mechanical properties of synthetic polymers can be easily modified for enhancing tissue engineering outcomes, and these materials can be produced at low cost and there is no risk of there being pathogens in a printed construct.[76] Some synthetic polymers are extensively used in tissue/organ printing, including polycaprolactone (PCL), poly(Lactic-co-Glycolide) (PLGA), polyethylene glycol (PEG), and Poloxamer 407 (Pluronic F127)). Blends of synthetic and natural polymers can result in combinational effects in an attempt to improve cellular responses.[69,73,79]

PCL is the most widely used in micro-extrusion technique because it has a low melting temperature (59–64 °C) that allows easy printing processing. PCL is also non-toxic, biocompatible, and has hydrolysis-induced bulk erosion/biodegradation profile so that the shape of the structure can be maintained prior to degradation. In addition, PCL is thermally stable with proper rheological characteristic to dispense 3D constructs with great resolution (around 10–50 μm).[91] Traditionally, PCL is used for fabricating tissue engineering scaffolds; however, by shifting the paradigm from the construction of scaffolds to 3D tissue/organs, the new role of PCL in printed constructs is a supportive framework to provide printed cell–laden constructs with shape fidelity.[81]

PLGA is a thermoplastic, biocompatible, and has controllable degradation by adjusting the polymerization ratio between the PLA and PGA. The most popular use of PLGA in 3D tissue/organ printing is as a stackable biopaper substrate on which to stack cells to create high-resolution 3D tissue constructs using a 3D biological laser printing technique.[74]

PEG is a hydrophilic, biocompatible, and Food and Drug Administration (FDA)-approved polymer that is intensively used in biomedical applications. In particular, PEG has a role as a representative sacrificial material for fabricating complex 3D constructs because it has water-soluble properties.[13] For the use of PEG as a bio-ink, the polymer should be chemically modified prior to forming physical or chemical networks. The key element for achieving gel formation is acrylation, and the chemically modified PEG is generally crosslinked *via* photoinitiator (PI)-induced polymerization under UV exposure.[99]

Pluronic 127 has a characteristic of thermo-reversible gelation that is advantageous in 3D tissue/organ printing.[92] The thermo-reversible characteristic of Pluronic 127 can be observed at 20–30% w/w concentration in solution. Pluronic 127 is a liquid under 4–5 °C and turns into a gel at over 16 °C. The formed gel is permanently reversible.

Natural Polymers

Natural polymers are widely used in a hydrogel foam as printable materials that encapsulate and print living cells due to their similarity to the native tissue microenvironment.[85] It can also provide tissue-specific biochemical and physical stimuli to guide cellular behaviors including migration, proliferation, differentiation, and maturation.[20,60]

Alginate is an anionic polysaccharide derived from algae. This material is composed of two repeating monosaccharides (i.e., L-guluronic and D-mannuronic acids) so that the hydrogel can be formed using multivalent cations including Ca^{2+}, Ba^{2+}, and Fe^{3+}.[44] Alginate can be easily modified for a variety of tissue engineering applications. Crosslinked alginate has a similar structure to native ECM, excellent biocompatibility, and easy rapid gelation makes it attractive for 3D tissue/organ printing.[14]

Collagen contains a large quantity of glycine, proline, and hydroxyproline residues. A variety of ECMs are significantly constituted of this material, and there are numerous collagen-mediated physiological interactions that participate between cells and ECMs.[31] Collagen facilitates simple crosslinking *via* thermo-responsible gelation under physiological conditions, which can be a major advantage of using collagen in 3D printing.[80]

Gelatin is also derived from denatured collagen. This material is widely used as a gelling agent in foods, pharmaceuticals, and cosmetic manufacturing.[88] The gelation mechanism of this material is to coil its molecular structure at temperatures above 40 °C in aqueous conditions; and it reversibly forms an alpha helix structure below 30 °C. Gelatin has abundant proteins including fibronectin, vimentin, vitronectin, and RGD peptides, which promote cell adhesion *via* integrin receptors.[46]

Fibrin is formed by the interaction between fibrinogen and thrombin that is known as a blood coagulation mechanism. Fibrin plays an important role in the wound healing process, and it is widely used as surgical glue due to its rapid gelation property.[87] There are abundant cell adhesion motifs that provide encapsulated cells with cytocompatibility.[93] However, the mechanical stability of a fibrin structure fabricated *via* inkjet-based 3D printing technology has been shown to be soft and fragile, and it is difficult to ensure it maintains its 3D shape.[62]

Hyaluronic acid (HA) is a linear polysaccharide component of the ECM, and is a widely used material in biomedical applications. This material has excellent

TABLE 1. Biomaterials, cell types in 3D tissue/organ printing, and their tissue engineering applications.

3D printing technique	Biomaterials	Cell source	Applications	Refs.
Dispensing	Alginate/PEG-diacrylate	Porcine aortic valve interstitial cells	Heart valve tissue construction	23
	Alginate	Human nasal septum chondrocytes	Osteochondral tissue regeneration	81
		Human articular chondrocytes		15
		Human adipose derived stem cells	Cartilage tissue engineering	43
		Human nasal septal chondrocytes	Cartilage tissue engineering	37
		Human cardiac-derived cardiomyocyte progenitor cells	Cardiac tissue regeneration	17
	Alginate/chitosan	Cartilage progenitor cells	Vasculature fabrication	96
	Collagen	Mouse pre-osteoblasts	Bone tissue engineering	80
	Nanofibrillated cellulose-Alginate	Human nasoseptal chondrocytes	Cartilage tissue engineering	51
	Gelatin-Hyaluronic acid	Human cardiac-derived progenitor cells	Cardiac tissue engineering	18
	Gelatin-Methacrylate	Human umbilical vein endothelial cells	Vasculature fabrication	36
		Human neonatal dermal fibroblasts		
	Gelatin/Alginate	Human aortic root smooth muscle cells	Heart valve tissue construction	13
		Aortic valve leaflet interstitial cells		
	Decellularized ECM	Human inferior turbinate-tissue derived mesenchymal stromal cells	Cartilage tissue engineering	25,72
		Human cardiac progenitor cells		
		Human adipose-derived stem cells	Adipose tissue engineering	71,72
Droplet	Poly(ethylene glycol) dimethacrylate (PEGDMA)	Human articular chondrocytes	Osteochondral tissue engineering	
	Collagen	Human skin fibroblasts	Skin tissue engineering	42
SLA	Poly(ethylene diacrylate) (PEGDA)	Murine OP-9 marrow stromal cells	Bone tissue engineering	47

biocompatibility, viscoelasticity, hydrophilicity, and biodegradability for 3D tissue/organ printing applications.[19] HA is commonly modified by chemically conjugating methacrylate groups to form a gel *via* free radical polymerization under UV exposure.[32]

Recently, dECM has been spotlighted for its ability to recapitulate a tissue-specific microenvironment in printed 3D tissue/organ analogues.[82] There are a variety of proteins, proteoglycans and glycoproteins, which can mimic native tissue-like ECM compositions. In particular, printable tissue-specific dECM bio-ink has been reported and the printed structure using this material improves stem cell differentiation.[72] Further advances in 3D tissue/organ printing using dECM bio-ink have been actively investigated.[25,71]

Bio-ink is typically printed through tapered conical needles to reduce its time-dependent shear thinning property. High viscosity provides the yield stress of the bio-ink, which is related to the shape fidelity of the printed construct. However, the viability of the encapsulated cells can be reduced by increasing the viscosity due to the tightly connected environment. In this regard, the viscosity should be carefully controlled for printability as well as cell viability.[50] After fabricating a 3D cell-laden structure, an adequate stabilization process should be conducted to provide mechanical properties, which is performed using proper crosslinking methods. In addition, the swelling and contraction characteristics of the bio-ink have to be considered so that deformation of the final construct can be prevented *via* the proper selection of the bio-ink type.

APPLICATIONS

3D tissue/Organ Printing for Tissue Regeneration

Cartilage and Osteochondral Regeneration

3D tissue/organ printing has been widely applied in cartilage regeneration. Cartilage, which is a flexible connective tissue, is very important for elastic and smooth motion in daily human activities.[43] There are three types of osteochondral (OC) (articular cartilage) in the human body: hyaline, fibro, and elastic cartilage. Cartilage tissue has poor self-repairing capabilities due to the low restoration of chondrocyte and its avascularity; therefore, cartilaginous tissues should be preserved *via* repair processes. Much effort has been reported in the reconstruction or regeneration of neocartilage tissue with 3D tissue/organ printing techniques.

Cartilage regeneration in tissue/organ printing techniques should consider proper cell sources, proper hydrogels, and growth factors (GFs) to induce chondrogenesis.[15,43,81] Cells commonly used in cartilage reconstruction are mesenchymal stem cell (MSC), adipose derived stromal stem cell (ASC), and chondrocyte harvested from OC, septal, and auricular cartilage.[15,37,43,51] Various hydrogels such as collagen (type I and II), gelatin, hyaluronic acid, and alginate hydrogel are also widely used for providing encapsulated cells for similar 3D environments for cartilage in tissue/organ printing techniques.[15,37,43] In addition, GFs such as transforming growth factor-β1 (TGF-β1), basic fibroblast growth factor, and insulin-like growth factor-1 are also used for effective chondrogenesis in printed cells according to the release period.[16,37,49,70,86]

Many research groups that fabricate 3D analogues have focused on tissue formation without mimicking the whole shape for articular cartilage regeneration.[43,89] Chondrocytes or stem cells encapsulated in hydrogel solutions were also directly printed using commercialized printers. Human articular chondrocytes suspended in Poly(ethylene glycol) dimethacrylate (PEGDA) solution were printed using an inkjet printer and, simultaneously, photo-polymerized by long-wave UV light to the defects in OC plugs.[6] Through *in vitro* analysis, it was confirmed that printed OC structure has good ECM close to the native articular cartilage. Fedorovich *et al.* co-printed a heterogeneous structure with human MSC- and human articular chondrocyte-laden alginate hydrogels using a dispensing-based 3D printer, and demonstrated the possibility of creating viable structured tissues for OC regeneration.[15]

Recently, 3D tissue/organ analogues with considering the anatomical architecture of the target tissue shape have been printed with synthetic polymer and/or cell-laden hydrogel containing GFs.[15,37,43,51] 3D cartilage analogues that facilitate not only tissue formation but also both histological and external architecture mimesis were developed to accelerate chondrogenesis and maintain the shape of the structure against external forces.[43,89] Markstedt *et al.* developed bio-ink with nanofibrillated cellulose (NFC)/alginate hydrogel to maintain printing fidelity (Fig. 2a), and printed human nasoseptal chondrocyte-laden bio-ink to demonstrate the potential use of NFC in the fabrication of living cartilage tissue.[15,51] Visser *et al.* developed a new approach to fabricate complex-shaped structures with PVA, PCL, and alginate hydrogel as sacrificial materials (Fig. 2b), and manufactured various 3D structures with complex shapes *via* sacrificial procedures.[89] They also mentioned the possibility of using tissue/organ analogues for OC tissue regeneration.[15,89] The final analogues in these studies have the advantages of tissue formation with external shape; however, it might seem difficult to maintain their shape between bones due to their low mechanical properties.

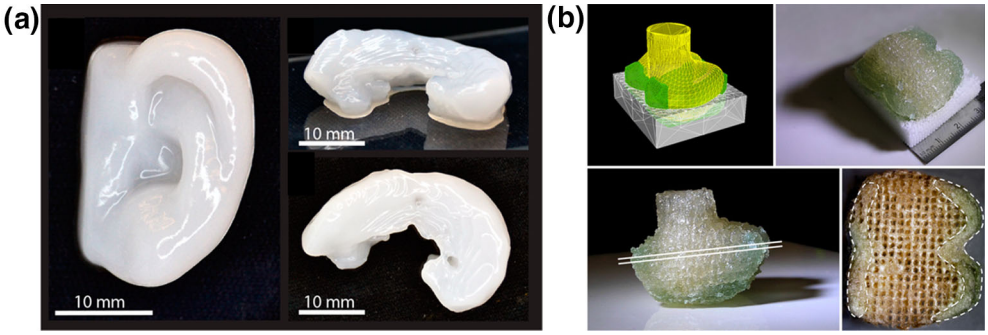

FIGURE 2. 3D tissue/organ analogues for cartilage tissue regeneration. (a) Markstedt *et al.* printed 3D hydrogel structures with ear and meniscus shape, and represented the potential to regenerate neo-cartilage with complex-shaped scaffold-free structures. (b) Visser *et al.* developed a new approach using PVA, PCL, and alginate hydrogels to fabricate complex-shaped structure by printing only cell-laden hydrogel for osteochondral tissue formation. Reproduced with permission.[51,89]

For this consideration, synthetic polymer biomaterials with good mechanical properties were used to fabricate a porous framework that contains cell-laden hydrogel in their pores. In this manner, 3D analogues were fabricated with synthetic polymer and cell-laden hydrogel, and it was enough to endure external forces.[37,43,81] Kundu *et al.* fabricated a 3D cartilage analogue with PCL and nasoseptal chondrocyte and TGF-β1 encapsulated in alginate hydrogel using an in-house-developed multi-heads deposition system (Fig. 3a), and proposed the proper fabrication conditions to form chondrogenesis.[37] Lee *et al.* fabricated various structures with complex shapes using a sacrificial layer process with biomaterial, and heterogeneous structures with two ASC-laden alginate hydrogels for ear regeneration (Fig. 3b). They demonstrated the possibility of cartilage tissue formation and the outward shape maintenance of heterogeneous analogues with complex shape.[43] These cell printed structures induced chondrogenesis closer to the native cartilage because of the accelerating tissue formation caused by the high densities of the printed cells.

Cardiovascular Regeneration

Cardiovascular disease (CVD) is a class of diseases that involve the heart or blood vessels, and are a predominant cause of death globally.[59] CVD includes coronary artery diseases such as angina and myocardial infarction, which are a major cause of ischemic heart diseases.

Cell therapy has been widely applied to repair CVD, but most injected cells are lost *via* extrusion (50–90%), and over 90% of engrafted cells die due to the hostile environment.[59] In this regard, a tissue engineering approach can be an alternative and can offer additional physicochemical support directly to injured cardiovascular tissues.

To facilitate cell retention, survival, and integration into the host heart tissue, patch-type 3D cardiac tissue constructs have been investigated widely.[94] The construct includes engineering heart tissue,[98] a sponge-like macro porous structure,[48] and cell sheet-based scaffold-free structures,[52] and it can be applied through the epicardial delivery method. After implanting the patch-type constructs, left ventricular dysfunction can be attenuated *via* mechanical support and direct cell and biological material delivery. Various biomaterials can be used to fabricate 3D patch-type structures such as natural, synthetic, and dECM-based polymers. Recently, various 3D printed patch-type structures have been studied. Gaetani *et al.* printed 3D lattice-shaped structures using a human cardiomyocyte progenitor cells (hCMPCs) encapsulated alginate bio-ink (Fig. 4a).[17] This structure contains a porous architecture so that oxygen and nutrients are well supplied to cells in the printed structure. However, the low cell adhesion capability of the alginate prohibits cellular activity in the printed construct. As a follow-up study, they developed a hyaluronic acid and gelatin (HA/Gel) mixed bio-ink to improve the therapeutic potential of 3D printed structures with embedded hCMPCs (Figs. 4b and 4c).[18] This structure showed enhanced cell attachment, proliferation, and differentiation *in vivo* for up to 1 month (Fig. 4d).

3D printing is also beneficial in the creation of vasculature in engineered tissues *via* the indirect printing of sacrificial materials or the direct printing of vascular cells. The former technique facilitates a hollow network in 3D printed constructs after removing the sacrificial materials followed by seeding with endothelial cells for endothelialization. For example, Wu *et al.* printed 3D biomimetic omnidirectional microvascular networks using Pluronic F127 as a sacrificial bio-ink (Fig. 4e).[92] This technique can fabricate a vascular network inside a large-volume construct with desired architecture. Miler *et al.* generated vascular networks *via* the rapid casting of printed vasculature in 3D large-volume tissues.[55] They used

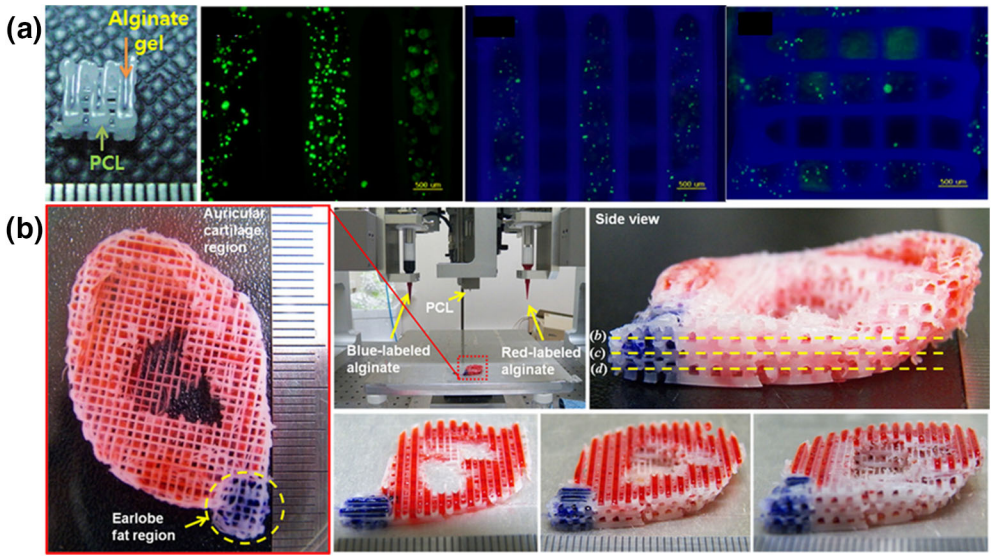

FIGURE 3. 3D tissue/organ analogues with PCL and cell-laden hydrogel to maintain the whole shape loaded by external forces for cartilage and ear tissue regeneration. (a) Kundu *et al.* fabricated a 3D cell printed structure with PCL and nasoseptal chondrocyte and TGF-β1 encapsulated in alginate hydrogel, and confirmed chondrogenesis *via in vivo* tests. (b) Lee *et al.* fabricated an acellular structure with an ear shape using PCL, alginate hydrogel and PEG as a sacrificial layer process, and demonstrated the possibility of cartilage tissue formation and the outward shape maintenance of heterogeneous cell printed structures with complex shapes. Reproduced with permission.[37,43]

water-soluble bioglass to print a 3D filament network, coated the network with biopolymer, and then cast it into a 3D ECM hydrogel. The bioglass was removed by flowing media through the filament network (Figs. 4f and 4g). Meanwhile, numerous studies for the direct printing of blood vessel networks are also being actively conducted. Kolesky *et al.* created 3D vascularized, heterogeneous cell-laden tissue constructs using three different cell sources.[36] A major advantage of this direct printing method is a high scalability, which allows the programmable arrangement of each cell with desired architecture (Fig. 4h). Zhang *et al.* introduced versatile printing methods using a co-axial nozzle to fabricate a vessel-like microfluidic channel, which enabled the direct printing of a vascular network in the form of a hollow tube (Fig. 4i).[96]

3D printing can fabricate the anatomical geometry and microstructural complexity of a heart valve, which allows intrinsic biomechanical and hemodynamic functions. To replace a calcified aortic valve, Hockaday *et al.* printed an engineered aortic valve with complex 3D anatomy and heterogeneity using alginate/polyethylene glycol-diacrylate (PEG-DA) bio-ink (Fig. 4j).[23] The printed valve achieved great shape fidelity but the cell adhesion affinity was not sufficient. In this regard, Duan *et al.* developed a more biocompatible bioink by conjugating hyaluronic acid and gelatin separately with methacrylate (Me-HA and Me-Gel) (Fig. 4k).[13] These materials were then mixed together and they found the optimal combination of these two materials to achieve the desired shape fidelity.

In Vitro Tissue/Organ Models

Although the developed humanized or transgenic mice with specific gene alterations have great potential for investigating the fundamental modes of prevention, current studies show that the underlying mechanism is not particularly similar between animal and human models.[75] In this regard, physiologically relevant 3D tissue or disease models are necessary to gain a better understanding of the pathophysiology.[3] 3D printing technology has been highlighted as a cutting-edge technique for creating highly complex 3D architecture. 3D printed *in vitro* tissue models can be utilized to test a variety of drugs, because this technology can offer the ability to form highly controllable tissue models. In particular, 3D printed cancer models are advantageous for furthering our understanding of pathogenesis and metastasis compared to the use of 2D cancer models. Zhao *et al.* developed 3D printed *in vitro* cervical tumor models using fibrinogen, gelatin, and alginate–mixed hydrogel with HeLa cells (Figs. 5a–5c).[97] Results obtained from 2D and 3D tumor models showed different cellular activities such as proliferation, matrix metalloproteinase protein expression, and the chemoresistance of the cells (Figs. 4d and 4e). These differences can originate from the differences of cell–cell and cell–matrix interactions between 2D and 3D culture

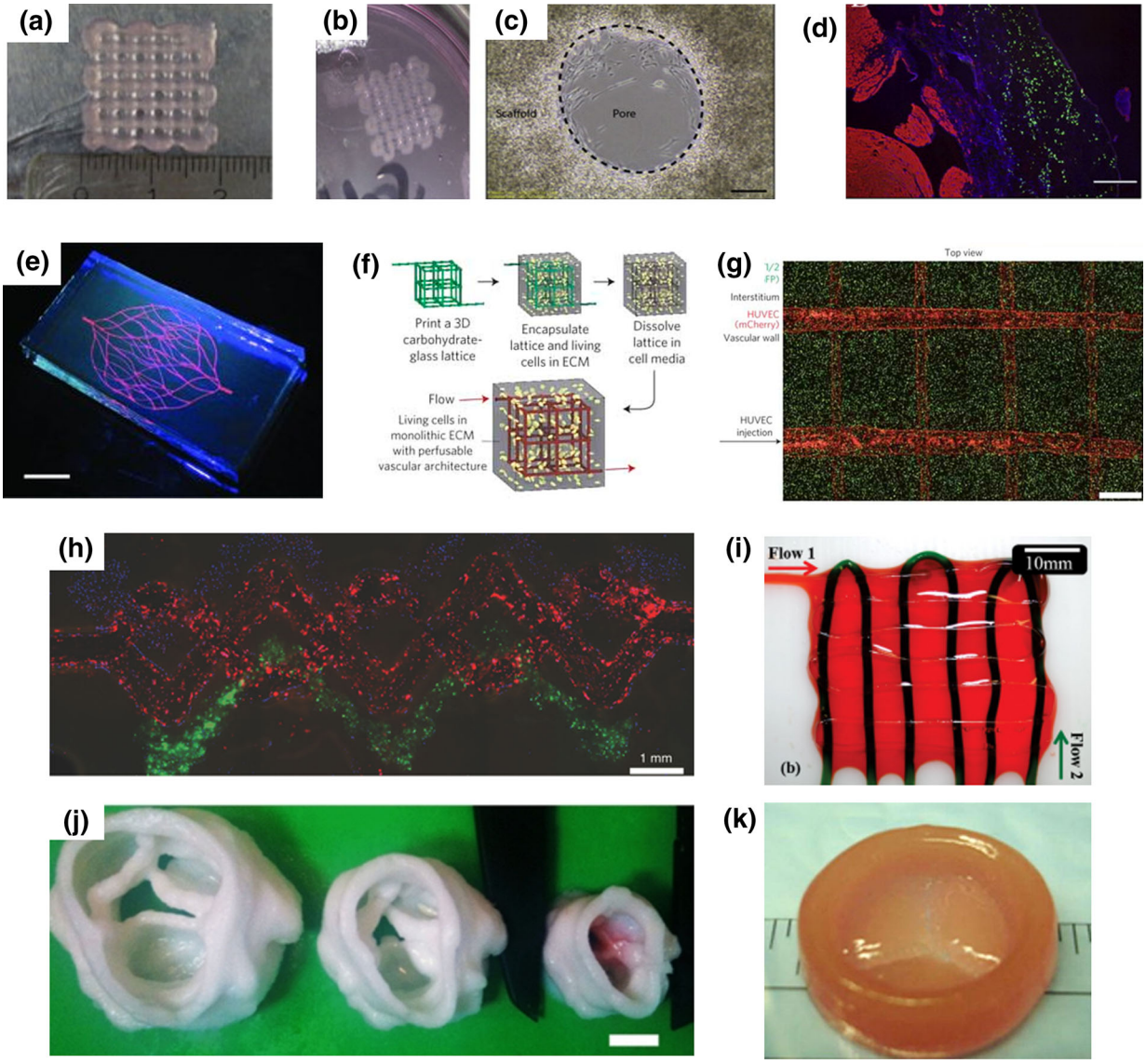

FIGURE 4. Representative examples of cardiovascular disease applications. (a) Printed hCMPCs in 10% alginate scaffold. (b) 3D tissue printing of hCMPCs and (c) homogenously presented CMPCs in the structure 1 day after encapsulation, scale bar 200 μm (d) Immunostaining of in vivo grafted patch 4 weeks after transplantation. (red: cardiac troponin I, greed: human lamin A/C, blue: DAPI) (e) Fluorescent image of a 3D microvascular network fabricated omnidirectional printing of a fugitive ink (dyed red) within a photopolymerized Pluronic F127-diacrylate matrix (scale bar = 10 mm). (f) Schematic overview. An open, interconnected, self-supporting carbohydrate-glass lattice is printed to serve as the sacrificial element for the casting of 3D vascular architectures. (g) Control of the interstitial zone and the lining endothelium of vascularized tissue constructs (scale bar = 1 mm). (h) Composite structure of the 3D printed tissue construct using three different fluorescent channels. (i) Dual layers of printed alginate channels with multidirectional media flow. (j) Scaffolds were printed with 700 MW PEG-DA at different scale for fidelity analysis, where the inner diameters (ID) were 22, 17 and 12 mm. (k) Bioprinting of heart valve conduit with encapsulation of HAVIC within the leaflets. Reproduced with permission.[13,17,18,23,36,55,92,96]

conditions. In addition, King *et al.* studied 3D printed breast cancer tissue for testing new anticancer therapies.[34] To recreate the breast tumor stroma, breast cancer cells were dispensed with adipocytes, endothelial cells, and fibroblasts in spatially distinctive patterns without supportive polymeric frameworks. 3D printing also enables the investigation of cancer progress, including tumor heterogeneity, leaky tumor vasculatures, cancer metastasis, and patient specific anticancer drug testing.

CURRENT CHALLENGES AND FUTURE PERSPECTIVES

Diverse 3D tissue/organ printing techniques have been developed, and their potential has already been

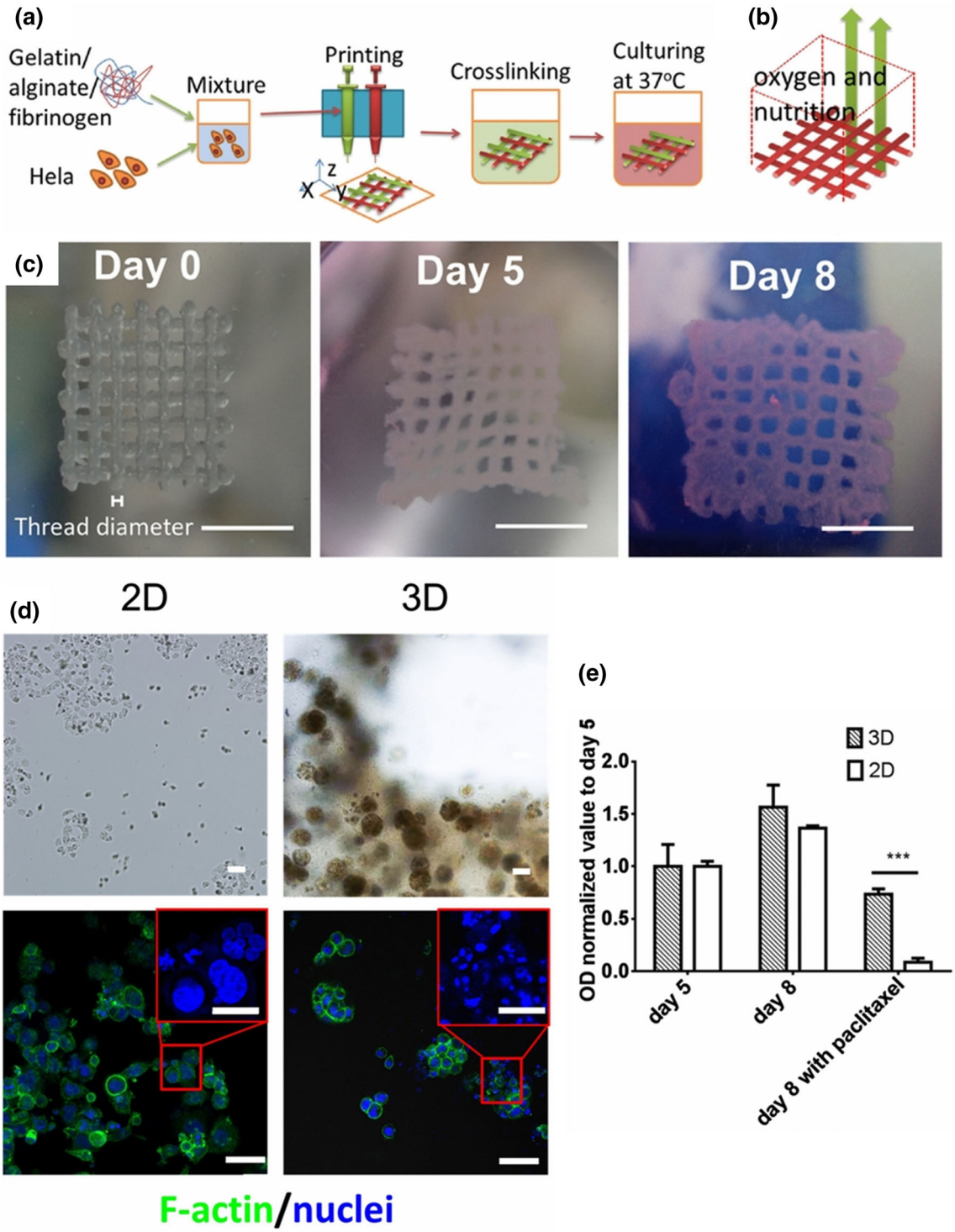

FIGURE 5. 3D printed cancer models. (a) Schematic of the 3D printing of cervical cancer model. (b) Design of the 3D HeLa/bioink constructs. (c) 3D printed HeLa/bioink constructs on day 0, 5, and 8. (d) Cell morphology after anti-cancer drug treatment in 2D and 3D models (green: F-actin; blue: DAPI). Scale bar, 50 μm (enlarges images, scale bar, 20 μm). (e) Cell metabolic activity test after treating anti-cancer drug (paclitaxel) in 2D and 3D models. Reproduced with permission.[97]

demonstrated by creating several tissue-like constructs. Different types of living cells have been successfully incorporated into biological constructs with the precise control of their locations. However, current 3D tissue/organ printing has to address many technical challenges to increase the resolution, printing speed and

flexibility with relevant biomaterials for creating more complex and composite tissue/organ structures at clinically relevant sizes.

All 3D tissue/organ printing techniques are based on a layer-by-layer process to reproduce the complex micro-architecture of the tissue or organs. However, this unique process often requires much time as the number of printing components increases, and a prolonged printing time can result in adverse effects on the cell viability and the functionality of printed analogues of clinically relevant sizes. This challenge can be addressed by optimizing the printing paths of each component with the minimum stage movement or integrating different printing techniques to facilitate the printing of each relevant component. In this regard, Shanjani *et al.* recently developed a hybrid printing system able to run a combined process of two different techniques: dispensing and SLA techniques.[78]

Another strategy can be the development of new or additional printing techniques, and the application of fabrication techniques other than layer-by-layer. A variety of fabrication techniques have been extensively developed for using a wide range of biomaterials even though living cells were not incorporated in the fabrication process.[22,38] The successful application of reliable fabrication techniques can improve the compatibility and flexibility of relevant biomaterials in 3D tissue/organ printing, and eventually achieve further advance for realizing the potential of 3D tissue/organ printing.

CONCLUSION

3D tissue/organ printing is an emerging field that encompasses specific technical, material and cellular aspects, and is at an early stage. However, this technology has already demonstrated its remarkable potential for the development of 3D volumetric and functional tissues or organs, and its versatility has been expanded to other applications, such as *in vitro* tissue/organ models for various research studies. Although challenges still remain in this research field, further multidisciplinary research to advance printing techniques and printable bio-ink materials can address the current challenges and realize the emerging potentials of 3D tissue/organ printing.

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CONFLICT OF INTEREST

The authors have no financial conflicts of interest.

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