Towards artificial tissue models: past, present, and future of 3D bioprinting

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Abstract

Regenerative medicine and tissue engineering have seen unprecedented growth in the past decade, driving the field of artificial tissue models towards a revolution in future medicine. Major progress has been achieved through the development of innovative biomanufacturing strategies to pattern and assemble cells and extracellular matrix (ECM) in three-dimensions (3D) to create functional tissue constructs. Bioprinting has emerged as a promising 3D biomanufacturing technology, enabling precise control over spatial and temporal distribution of cells and ECM. Bioprinting technology can be used to engineer artificial tissues and organs by producing scaffolds with controlled spatial heterogeneity of physical properties, cellular composition, and ECM organization. This innovative approach is increasingly utilized in biomedicine, and has potential to create artificial functional constructs for drug screening and toxicology research, as well as tissue and organ transplantation. Herein, we review the recent advances in bioprinting technologies and discuss current markets, approaches, and biomedical applications. We also present current challenges and provide future directions for bioprinting research.

1. Introduction

When the printing press was first introduced in the 15th century [1], it offered a high-throughput tool to rapidly replicate and widely spread information at a low cost. This approach broke the barriers to free-thinking and gave access to massive amounts of information enabling innovation at an unprecedented rate [2, 3]. Although there have been other revolutionary breakthroughs that enable information transfer since then, the role of printing as a nucleating invention is still significant. Analogously, bioprinting can potentially enable a high-throughput and affordable tool to assemble cells, enabling complex tissue constructs to become broadly available to many researchers and scientists [2–4]. Bioprinting is the process of patterning biological materials (e.g., cells, biomaterials, and biomolecules) to fabricate tissue-mimicking constructs using advanced additive manufacturing technologies [5–8]. During the bioprinting process, biocompatible materials (i.e., bio-inks) are used to facilitate the printing and act as matrices (i.e., bio-papers) for printed cells [5, 9, 10], which can then be grown in perfusion vessels (i.e., bioreactors) for further cellular and functional maturation.

As tissue engineering advances, innovative tools targeting new biological and medical challenges are becoming available. For instance, bioprinting has the potential to create tissue constructs addressing the needs for regenerative and transplantation medicine [11]. Furthermore, bioprinting technologies enable precise fabrication of three-dimensions (3D) heterogeneous functional units for regenerative and developmental biology [12]. In addition, there is evidence that bioprinting might lead to applications for precision medicine mimicking the complexity of native tissues [13, 14]. Translation of bio-inspired materials and their applications in 3D bioprinting aims to yield cellular and extracellular constituents of tissues by mimicking specific functions and structures in cells [15]. For instance, tissue engineering and regenerative medicine promises to create new tissue constructs that

address the need for model systems in drug screening and precision medicine applications [16]. Therefore, as the bioprinting technology arena progresses, more biomedical applications are expected to emerge [15].

Ultimately, bioprinting technology will provide inspiring solutions to address current challenges in tissue engineering and regenerative medicine [17] by utilizing basic science, materials science [18, 19], and robotics [20–22]. Herein, we review the recent technological advances in biomanufacturing focusing on 3D bioprinting platforms for tissue engineering and regenerative medicine. We also highlight the current market, approaches, and biomedical applications of bioprinting as well as provide a future direction for bioprinting research.

2. Bioprinting industry

3D bioprinting is experiencing a rapid transformation from basic research in academic laboratories to an emerging industry due to its potential commercial value in broad fields including pharmaceutical discovery, precision medicine, and tissue transplantation [11, 23, 24]. The market size of 3D printing was approximately $2.2 billion in 2012, and it is expected to reach $10.8 billion by 2021 [25]. In 2014, there were more than 30 companies worldwide having a business directly related to 3D bioprinting and bioprinted products. Products and services provided by these companies are focused on 3D bioprinters and bioprinted scaffolds. Over seven types of 3D bioprinters are available in the market, which target research and development (R&D) users in both academic institutions and biotechnology/biomedical companies (table 1). These bioprinters are mainly based on two types of printing technologies: droplet and extrusion bioprinting. *Tissue Regeneration Systems* provides patient-customized solution to repair skeletal defects and damages using bioprinted polycaprolactone (PCL) scaffolds [26]. The Food and Drug Administration (FDA) has approved this solution in 2013 as the first 3D bioprinted implant for skeletal reconstruction and bone regeneration. In addition, *Organovo* announced the exVive3D™ Liver in 2014 [27]. These bioprinted human liver tissue mimics were maintained functionally and designed to provide drug testing service to evaluate drug toxicity [28]. Although it offers preclinical *in vitro* drug screening service, a reliable, fully functional, and commercially available liver tissue has not been achieved due to many biological and technological challenges that need to be overcome in the liver tissue engineering field [29]. 3D bioprinting companies, their products, and current projects are listed in table 2. Some bioprinting companies also provide professional commercial softwares (e.g., TSIM®, BioAssemblyBot®, and BioCAD®) to design, draw, and print multiscale structures ranging from cells to tissue constructs.

3. State of art bioprinting technologies and materials

3.1. Bioprinting strategies

The 3D printing of biological materials (i.e., cells, growth factors, and hydrogels) is based on three major strategies: laser [30–32], droplet [33–38], and extrusion [39–44] based bioprinting (figure 1). Each strategy provides certain features that influence the printing outcomes including cell viability and functionality post-printing [2–5, 45]. In this section, we discuss in more detail the mechanisms and parameters of bioprinting techniques (table 3).

3.1.1. Droplet bioprinting

The droplet bioprinter (also referred as drop-by-drop or drop-on-demand bioprinter) is one of the earliest used printing methods in the field of tissue and organ biofabriaction [46]. This approach utilizes a non-contact reprographic strategy, where the droplet encapsulated cells and nonliving materials are printed and patterned layer-by-layer on a substrate [23, 47]. The droplet bioprinters can be divided according to the mechanism used to generate the droplets: thermal [3, 33, 48, 49] or piezoelectric (acoustic) [2, 37, 38, 50] modalities (figure 1(a)). Thermal droplet bioprinting function by generating heat within the bio-ink chamber, which results in pulses of pressure that eject picoliter volume droplets at the printer orifice [33]. While the piezoelectric bioprinter utilizes a piezoelectric crystal actuator to generate acoustic waves within the bio-ink chamber that expel the droplets through the printer nozzle. The use of droplet bioprinters has tremendously increased during recent years due to relatively low cost, microscale precision, high printing speed (10 kHz), high cell viability post-printing, and compatibility with nonliving printing materials (e.g., hydrogels) [17, 37, 51]. However, certain drawbacks have hampered its use in tissue biofabrication such as difficulty of printing high viscosity materials (>10 centipoise) and high cell densities (>10 million cells/ml) without resulting in clogging of the printer ejector [5, 52–54]. In addition, the mechanical properties of printed structures are vulnerable due to use of low viscosity bio-inks, which in turn impedes the creation of fully functional 3D constructs [11, 55].

3.1.2. Laser bioprinting

Based on the laser-induced forward transfer method, laser bioprinting has been also utilized for patterning live cells and biofabrication of tissues [56, 57]. In this approach, the cells are printed using a laser beam pulsating at controlled duration of time [58]. The device is composed of a laser beam focused on a ribbon (a glass side which is coated with a laser-absorbing layer made of titanium, gold, or polyimide membrane), where the cells and biological materials are excited and collected on substrate, which is facing the

Table 1. Comparison of 3D bioprinters.

Product	Company	Website	Country	Technology	Materials	Resolution	Speed
Life-Printer 'X'	Bio 3D Technologies	http://bio-3d.com	Singapore	Extrusion, nano-jetting	Biomaterials	10 μm	1–400 mm s^{-1}
BioScaffolder 2.1	GeSiM	http://gesim.de	Germany	Pressuredriven 3D printing; piezoelectric nanolitre pipetting; pneumatic extruders and piezodispensers	Hydrogels, biopolymers (e.g., collagen and alginate), bone cement paste, biocompatible silicones and polymer pastes	Step width: 2 μm in X/Y, 10 μm in Z	Up to 500 mm s^{-1}
TE subseries of nScrypt's Tabletop series	nScrypt	http://nscrypt.com	USA	Extrusion printing	A few centipoise to 1 million centipoise; thermal plastic biopolymer materials, gels and pastes in terms of form for the materials	X/Y accuracy: 12 μm; Z accuracy: 6 μm	X/Y max speed: 100 mm s^{-1}; Z max speed: 50 mm s^{-1}
CellJet Cell Printer	DigiLab	http://digilabglobal.com	Japan	—	Ordinary cell media with watery consistency and viscous solutions such as hydrogels	X, Y motor resolution of the synQUAD is 1.5 μm and positional accuracy is $\pm$ 10 μm at 95% confidence	—
3D-Bioplotter	EnvisionTEC	http://envisiontec.com	Germany	Extrusion printing	Soft hydrogels over polymer melts up to hard ceramics and metals	Axis resolution (X-Y-Z): 0.001 mm; minimum strand diameter: 0.100 mm	0.1–150 mm s^{-1}
Fab@Home Model 3	Seraph Robotics	http://seraphrobotics.com	USA	Extrusion printing	Wide range of materials	Position accuracy: 100 μm	Max: 80 mm s^{-1}; typical: 10 mm s^{-1}
BioFactory	RegenHU	http://regenhu.com	Swiss	Thermopolymer extruder, Ink-Jet head; direct dispenser	Medium with a viscosity up to 10 000 mPa s^{-1}	10 μm	Depend on the material type

Table 2. 3D bioprinting companies and products.

Company	Website	Founded year	Country	3D printer	Project/Products
Cyfuse Biomedical	http://cyfusebio.com	2010	Japan	Regenova: needle based spheroids printing	Cartilage and subchondral bone; Tubular tissue; Liver tissue;
TeVido Biodevices	http://tevidobiodevices.com	2011	US	Cellatier: droplet printing technology	Breast cancer; Bone;
Regenovo Biotechnology	http://regenovo.com	2013	China	Extrusion bioprinting	Blood vessel; Liver;
Aspect Biosystems	http://aspectbiosystems.com	2013	Canada	Microfluidic based bioprinting technology	Engineer tissues for Pharma R&D;
RegenHU	http://regenhu.com	2007	Swiss	BioFactory	Printed dermis equivalent; BioTrack®, a three-dimensional optical biopsy unit
Osteopore International	http://osteopore.com.sg	2003	Singapore	Polycaprolactone printing	Osteoplug; Osteomesh
Organovo Holdings	http://organovo.com	2007	US	NovoGen MMX bioprinter	exVive3D™ Liver Testing
Tissue Regeneration Systems	http://tissuesys.com	2008	US	TRS Scaffold printing	Bone reconstruction and regeneration (FDA approved)
Next 21	http://next21.info	2000	Japan	Scaffold printing	Custom-made artificial bone implants
3D Bioprinting Solutions	http://bioprinting.ru	2014	Russia	3Dbio	Mouse thyroid

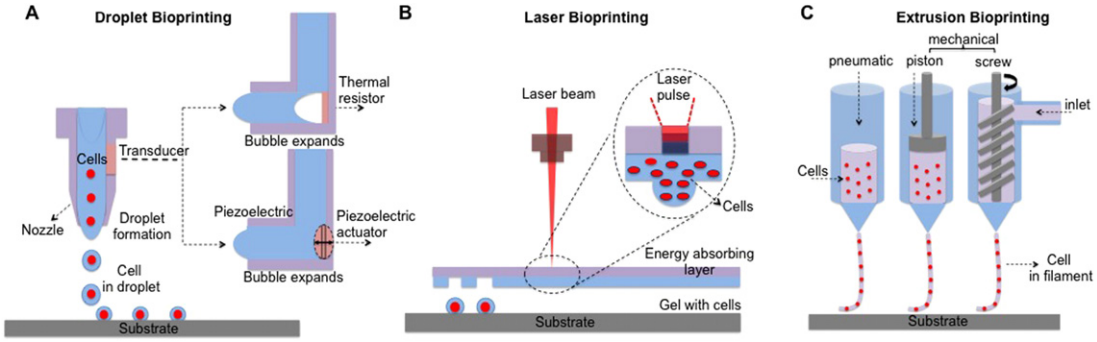

Figure 1. Schematic illustration of bioprinting approaches. (A) Thermal and piezoelectric droplet bioprinting setups, (B) laser bioprinting setup, and (C) pneumatic and mechanical extrusion bioprinting setups.

Table 3. Summary of bioprinting technologies.

	Laser bioprinting	Droplet bioprinting	Extrusion bioprinting
Fabrication resolution (droplet size)	>20 μm	50–300 μm	200 μm
Spatial resolution	Medium-high	Low	Medium
Preparation time	Medium-high	Low	Low-medium
Fabrication speed	Medium-fast (200–1600 mm s^{-1})	Fast (1–10 000 droplet/s)	Slow (10–50 μm s^{-1})
Throughput/Scalable production	Low-medium	High	Medium
Material viscosity	1–300 mPa s^{-1}	3.5–12 mPa s^{-1}	30–6 $\times$ 10^7 mPa s^{-1}
Cell viability	>95%	>90%	>40–95%
Single cell control	High	Low	Low
Cell density	Medium	Low	Medium-high
Multiple cells and Material delivery	Medium	Low-medium	Medium
Cost	High	Low	Medium

ribbon [58, 59] (figure 1(b)). The laser bioprinting is a nozzle-less approach, which allows printing high cell densities and high viscosities of bio-inks without the conventional challenges associated with the droplet and extrusion bioprintings such as clogging and increasing the temperature of ejectors [60–62]. However, several shortcomings (e.g., high-cost of the printer and inability to print large constructs due to the limited width of the laser beam) have hindered the successful translation of this approach [2]. In addition, the laser bioprinting has a relatively low flow rate due to the requirement of fast gelation kinetics to obtain high shape fidelity for high resolution [2]. Moreover, in many cases the final tissue-engineering construct includes some of the metallic residue, which occurs during the printing process as a result of the evaporation of the metallic laser-absorbing layer [5, 52].

3.1.3. Extrusion bioprinting

The extrusion bioprinting approach is one the most explored and affordable bioprinting systems. The method is increasingly applied in tissue biofabrication [63]. The extrusion bioprinting system is composed of a dispensing (ejector or multiple ejector) system and an automated three-axis (x–y–z) robotic stage controlled by stage controller [11, 52]. In this approach, a pneumatic or mechanical (piston or screw-based) dispenser is used to deposit the bio-ink with suspended cells on a building substrate [11, 52] (figure 1(c)). The major advantages of the extrusion systems are the ability to print high viscosity materials with high cell densities at a relatively high speed with an acceptable cell viability post-printing [64, 65]. However, this approach provides a relatively low fabrication resolution (~200 μm) [2, 52]. The system allows for a continuous filament of a viscous bio-ink rather than a droplet. Direct and more spatial control over the material flow can be achieved using the mechanical approach; however, this approach can result in a shear stress-induced cell deformation [66]. The pneumatic approach provides less deformation of the cells; however, precise control over the material flow is challenging as result of delay of compressed gas volume in this system [2]. Furthermore, an acoustic nozzle-less bioprinting approach was designed for precise cell encapsulation in bio-fluids at picoliter scale with high viability, which could eliminate the issues associated with nozzle clogging in conventional ejector-based method [3, 43, 44]. The extrusion bioprinting approach has the potential to enable various innovative applications including the ability to preserve cells for transfusion medicine [67, 68] and investigate cells at a single-cell level for regenerative medicine and omic applications [69].

3.2. Patterning and assembly-based approaches

Patterning or assembly of cells enables constructing 3D tissue architectures. These patterning and assembly

techniques allow us to address some of the current challenges in 3D bioprinting technology by providing innovative experimental platforms based on high-throughput, rapid, and directed assembly of cells, cell encapsulating microgels, and spheroids. Patterning and assembly strategies [13, 70–75] target obstacles arising from structure and heterogeneity associated with large-scale applications. Development of macro-scale structures is particularly required for clinical applications. Recent additive advances in assembly techniques such as magnetic [21] and acoustic assembly [76] may allow generation of new designs and geometries, which can further lead to ordered and complex structures (figure 2) [77]. Advanced additive assembly technologies, such as self assembly [78], robotic assembly [21], Faraday acoustic assembly [76, 77, 79], bio-acoustic levitational assembly [80], and magnetic assembly [70, 72, 81–86] enable the user to organize, reorganize, and regenerate basic units to form the 3D tissue architecture. Its reconfigurable feature provides flexibility. Furthermore, it could lead to development of rapid and easy-to-use bioprinters and scaffold-free cell printing technologies [87].

3.2. Bioprinting materials/bio-inks

Mimicking the native tissue architecture and composition using 3D bioprinting approaches is quite challenging, because balancing the physical and chemical cues of the cell hosting biomaterials requires understanding of cell physiology and cell–extracellular matrix (ECM) interaction [2, 60]. Engineered 3D microenvironments can be achieved by utilizing natural (e.g., collagen, fibrin, hyaluronic acid (HA), hydroxyapatite, and alginate) and synthetic (e.g., PCL, polylactide (PLA), polyglycolide (PGA), poly(lactic-*co*-glycolic acid) (PLGA), and polyethylene glycol (PEG)) polymers (table 4) or hybrid biomaterials that merge natural and synthetic materials together [2, 15, 53, 88–96].

3.2.1. Natural materials

Chemical and physical compositions of the natural hydrogels can be tuned according to target tissue and cell types [97]. Physical properties of biopolymers such as stiffness, viscosity, and porosity play a critical role in the survival and functionality of generated constructs [2]. Most cell types can hydrolyze natural hydrogels and secrete their own cellular matrix, allowing space for cell growth and migration. Moreover, these matrices can be designed to contain tissue specific growth factors and chemokines such as transforming growth factor beta (TGF-β), epidermal growth factor, insulin-like growth factor as well as matrix metalloproteinase, which help to restore the chemical cues of the microenvironment [98, 99]. One of the limitations of natural hydrogels is the batch-to-batch variability, which may affect the validation of the engineered microenvironment as different batches may have slightly different compositions [2].

Collagen- and fibrinogen-based hydrogels are naturally-derived matrices and are widely used in tissue engineering [100, 101]. Collagen type I is the most abundant component of the native ECM and provide a favorable 3D environment for cell adhesion and proliferation [101]. Photocrosslinkable gelatin methacrylate (GelMA)-based hydrogel combines biological features permitting integrin-mediated cell adhesion and proteolytic degradation [2]. GelMA is broadly used in engineering artificial tissues due to its ease of manipulation and photocrosslinking properties [21]. Alginate is highly biocompatible natural polymer that can be easily crosslinked in calcium solutions; therefore, often used as bioink in 3D bioprinting applications [102–104].

Generation of 3D artificial tissues can also be achieved by utilizing reconstituted basement membranes from mouse tumors like Matrigel™ and Cultrex® into bioprinting process [105]. Basement membranes are composed of ECM containing proteins like fibronectin, laminin, and collagen IV that play important roles in cell adhesion and spatial organization [88]. These natural membranes can be applied in embedded and overlay culture to promote 3D cellular organizations. Introducing basement membrane in a bioprinting polymer solution has shown to sustain the geometrical architecture and enhance the cell function [106].

3.2.2. Synthetic and semi-synthetic materials

To overcome the drawbacks of biologically derived biomaterials such as batch-to-batch variability, a library of semi-synthetic and synthetic biomaterials have been engineered. These matrices offer a relevant alternative as they have native ECM components together with tunable material properties, resulting in better reproducibility and comparability between different studies. Thus, biocompatible synthetic polymers such as PCL, PEG, PLA, PGA, and PLGA, are frequently used in bioprinting applications [107–111]. PEG hydrogels are one of the most popular synthetic matrices used in tissue engineering [112]. PEG based hydrogels can be prepared by chemical or UV light crosslinking of the functionalized polymers with reactive chain ends, allowing cell encapsulation with high viability [72]. Biomimetic PEG hydrogels can be synthesized via the incorporation of a variety of ligands from oligopeptides to whole protein growth factors to restore matrix-derived biochemical signaling. For example, the inclusion of arginine–glycine–aspartic acid (RGD) peptides enables integrin-mediated cell adhesion and promotes migration. However, synthetic matrices are not as biologically relevant as naturally derived 3D matrices, and do not contain the signaling molecules (e.g., peptides) provided by native ECM [113]. Self-assembled peptide hydrogels are also widely used to generate 3D microenvironments for cell culture. Peptide scaffolds are made of peptide sequences that allow the scaffold to self-assemble

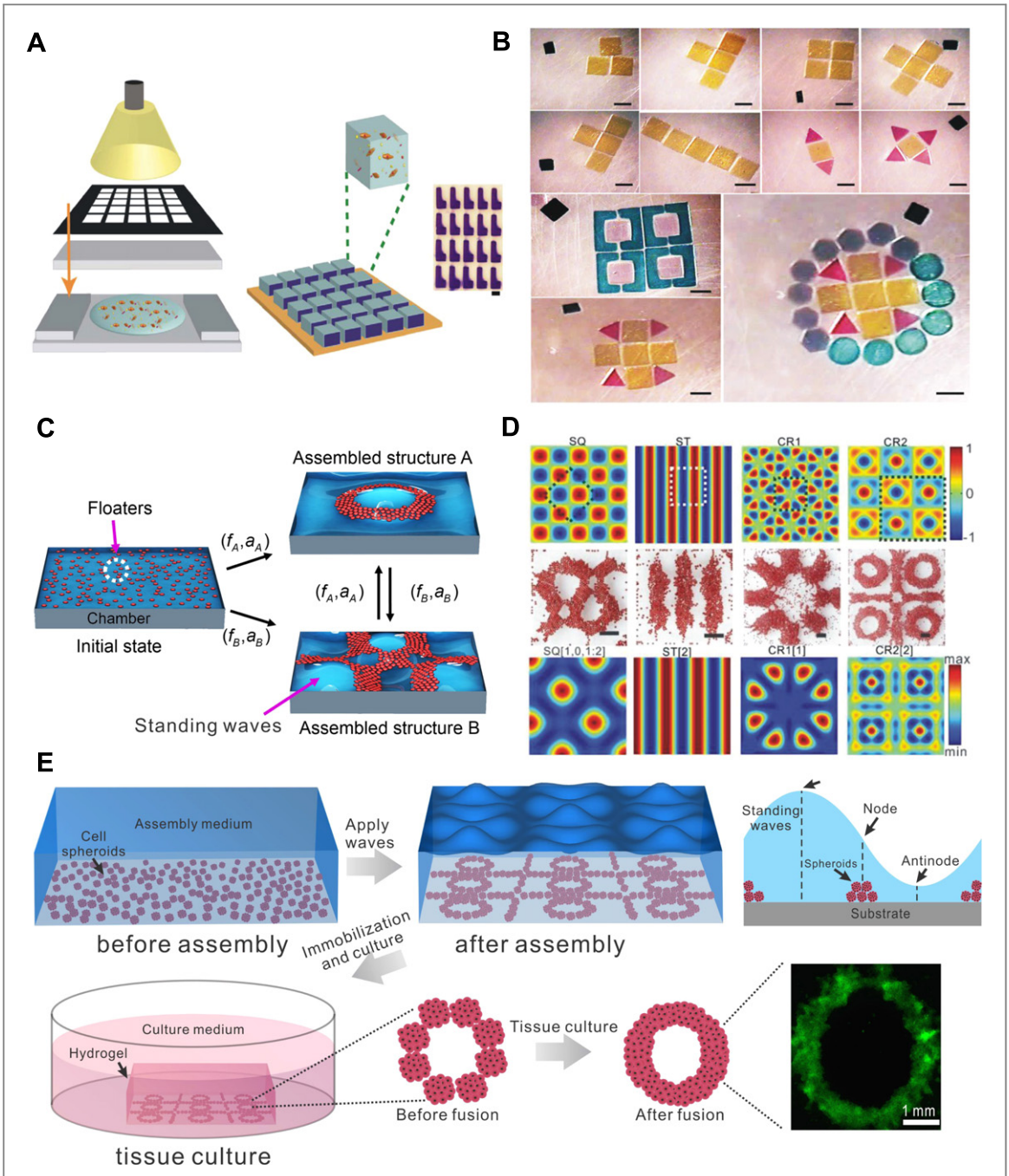

Figure 2. Patterning and assembly-based approaches. (A) Schematic illustration of cell encapsulation via hydrogel formation based on UV photocrosslinking, (B) configuration/reconfiguration of hydrogels via assembly (scale bar: 1 mm). Reprinted by copyright permissions from [21]. (C) Schematics of dynamic reconfiguration of the assembled structures on a liquid template, and (D) numerical simulation of waveforms and assembled structures via liquid-based templated assembly (scale bar: 2 mm). Reprinted by copyright permissions from [76]. (E) Schematics of acoustic node assembly of cell spheroids into a predefined patterns and formation of 3D microtissue from assembled spheroids via spheroid fusion. Reprinted by copyright permissions from [149].

under certain physiological conditions, permitting cell encapsulation in the hydrogel [88]. For instance, BD™ PuraMatrix™ is a hybrid peptide hydrogel [114]. The composition of BD™ PuraMatrix™ is similar to other semi-synthetic hydrogels as it contains 99% water and only 1% amino acids. Such engineered matrix allows the control over the composition of growth factors, cytokines, ECM proteins, and hormones.

One of the most recent approaches to engineer a native-like 3D microenvironment is to use cells that can generate the natural ECM [115]. This strategy is based on initial seeding of the porous graft with death programmed sacrificial cells that can secrete ECM and be induced to apoptosis. The devitalized graft can be further stored as an off-the-shelf product until seeded with autologous cells [115].

Bioprinting materials/bio-inks can also be designed to fulfill the mechanical and biological aspects of target tissues [116, 117]. In this regard, the selection and design of bio-ink requires critical evaluation of the physiology of native organ. For example, in load bearing organs, such as bone [118], the

Table 4. Synthetic and natural materials used in biofabrication.

Material	Type	Gelation method	Cytocompatibility	Cell type
Agarose	Natural	Thermal/chemical	<95%	HUVEC, 10T1/2
Alginate	Natural	Thermal	<95%	HeLa, RatSMC, NIH 3T3 fibroblast
Collagen	Natural	Thermal	95−75%	RFMF
Fibrin	Natural	Enzymatic	85−70%	HMVEC
Gelatin	Natural	Thermal/chemical	75–95%	Hepatocyte
Matrigel	Natural	Thermal	High, <95%	HUVEC, 10T1/2
PEG	Synthetic	Thermal/photo	High, <95%	NIH 3T3 fibroblast
PHEMA	Synthetic	Thermal/photo	Not studied	HepG2/C3A
PCL	Synthetic	Thermal	<95%	hMSC, L929 fibroblast
PLGA	Synthetic	Thermal	<95%	hMSC, L929 fibroblast

bioprinted graft should be stable enough to provide mechanical strength especially for bone tissue culturing. PCL has shown to be a good candidate as bio-ink in orthopedic applications [2, 116, 119, 120]. Composite of PCL with osteoinductive hydroxyapatite particles improves the osteogenic features of the constructs and brings them closer to the native stiffness of target tissues [121]. Hydroxyapatite particle based bio-inks can also be sequentially bioprinted with cell-loaded alginate to form complex 3D osteogenic structures [122]. Thus, the potential use of bioprinted biocompatible synthetic polymers such as PCL and PCL matrix reinforced with hydroxyapatite can enhance the mechanical and biological performance of 3D scaffolds for biomedical applications [116, 117].

4. Biomedical applications

4.1. Bioprinting for tissue regeneration and regenerative medicine

Different bioprinting approaches showed potential of additive manufacturing in which generated tissues hold promise to create tissue constructs mimicking many tissues and organs such as liver, kidney, heart, skin, neuron, and vascular systems. In this section, we highlight major approaches for generating 3D printed tissue constructs while maintaining cellular functions.

4.1.1. Vascular grafts

Tissue engineering is currently limited by vascularization challenge, which creates issues in nutrient perfusion, oxygen diffusion, and mass transportation for *in vivo* systems. Vascular grafts are well-studied models of 3D bioprinting technologies since they are directly integrated with the solution of vascularization problem. A 'scaffold-free' bioprinting approach was utilized for fabrication of vascular grafts (figure 3) [40, 123]. Tubular constructs were generated using cell spheroids and agarose templates (figures 3(a) and 4(b)). Multicellular spheroids were used as building blocks, and spheroids were assembled into tubular structures by using agarose templates (figure 3(c)). Additionally, vascular grafts were printed by using three different cell types: human aortic smooth muscle cells, human aortic endothelial cells, and human dermal fibroblasts [124]. At the post-printing stage, cells are allowed to fuse [124, 125] as shown in figure 3(d), and vascular constructs were moved into a bioreactor for a maturation step designed to improve physical and mechanical properties of vascular grafts. In another approach, formation of vascular channels was achieved by combining 3D bioprinting technology with perfusion techniques (figure 4) [126]. In this study, the collagen hydrogel precursor was used for bioprinting of human umbilical vein endothelial cells (HUVECs) while vascular grafts were formed using gelatin (figure 4(a) and (b)). The printed constructs were then integrated into a polycarbonate flow chamber to form fluidic vascular grafts. Further, viability assays (figure 4(c)) confirmed that the viability of printed vascular grafts was higher under dynamic flow conditions.

4.1.2. Skin

Bioprinting of a skin tissue is one of the most promising examples of bioprinting for several applications, such as development of topical drugs, wound healing studies, and dermal toxicology research. Various approaches have been developed to engineer cellular microenvironment and physiology of human skin. For instance, to develop skin substitutes, fibroblasts and keratinocytes were printed on a stabilizing matrix (Matriderm) via laser assisted bioprinting technique [127]. Both *in vitro* and *in vivo* trials showed that neovascularization could be achieved by employing this technique. In another study, a direct-forward bioprinting technique was used to print amniotic fluid-derived stem cells (AFSCs) for wound healing and skin regeneration in a mouse model [128] (figure 5). This technique used a mixture of AFSCs and bone marrow-derived mesenchymal stem cells (MSCs) that were suspended in fibrin–collagen matrix. At week 2, formation of a well-defined and ordered epidermal layer was observed for MSC and AFSC treated wounds (figures 5(b) and (c)), while epidermal layer formation was poor for the wounds treated with gel only (figure 5(a)). However, based on fluorescence labeling results (figures 5(d) and (e)) cell migration did not occur for MSC and AFSC printed samples, which

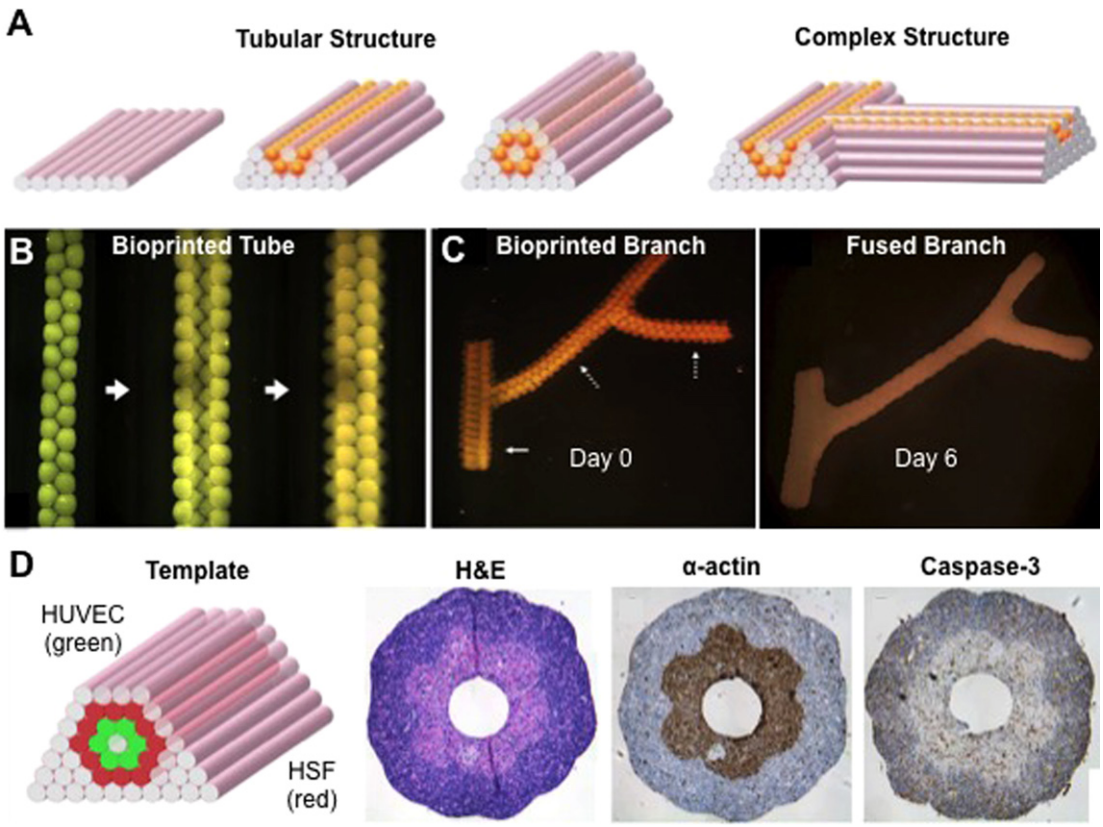

Figure 3. Vascular graft printing. (A) Model agarose template for smallest diameter vascular graft and a more complex agarose template for branched vascular grafts. (B) Assembled spheroids according to the tubular model in (A). (C) Assembled spheroids according to the complex model in (A) and fusion of spheroids after 6 days of deposition. Arrows represent large (1.2 mm; solid arrow) and small (0.9 mm; dashed arrow) branches. (D) Printing multicellular vascular tissue of human umbilical vein smooth muscle cells (HUVEC, green) and human skin fibroblasts (HSF, red) and subsequent immunohistochemistry examination using hematoxylin–eosin (H&E), α-actin, and caspase-3 stainings following 3 days of fusion. Reprinted by copyright permissions from [40].

suggested that printed cells did not penetrate into regenerating tissue.

4.1.3. Neuron

3D bioprinting of neural cells to construct brain-like or neuronal structures is highly desirable to model neuronal pathogenesis, regeneration, and networks, as well as provide a platform to investigate new therapeutic treatments (e.g., Alzheimer and Parkinson's drugs) [41, 129]. However, one of the main challenges of this approach is the difficulty to recapitulate the brain multi-layers, specifically in the medial or sagittal plane (figure 6(a)) [129, 130]. In addition, patterning neuronal tissues that are functional post-printing remains a challenge. Recently, an extrusion 3D bioprinter was used to create distinct layers of primary neural cells encapsulated in a natural hydrogel [130] (figure 6(b)). In this study, the authors built a 3D structure with multilayered architecture that mimics brain cortical tissue. The hydrogel used in this study was based on a natural RGD peptide-modified gellan gum. Confocal microscopy demonstrated that printed cells formed 3D neural networks after 5 days in culture (figures 6(c) and (d)). In addition, cell viability results demonstrated that there was no significant difference between the printed group compared to the control (non-printed) group for up to 5 days post-printing (figures 6(e) and (f)). In another study, a piezoelectric droplet bioprinter was used to pattern two adult rat central nervous system cells [131] (i.e., retinal ganglion cell (RGC) neurons and retinal glia cells). No significant differences in cell viability and neurite outgrowth were observed between the printed and non-printed control cells. However, when glia cells were used as a substrate for printed and control cells, the RGC neurite outgrowth was increased significantly. Recently, a novel bioacoustic levitation assembly (BAL) approach was developed to engineer 3D brain-like constructs [80] (figure 6). Random suspended human neural progenitor cells (hNPCs) were levitated to the nearest nodes of acoustic standing waves in a fibrinogen solution by acoustic radiation force and assembled into a multilayered cell construct (figure 6(g)). The interlayer spacing can be flexibly tuned by acoustic frequency in tens of seconds. The levitated hNPCs were immobilized in the fibrin hydrogel construct via gelation and differentiated into neural cells in the 3D microenvironment to form both inter-and intra-layer neural connections (figure 6(h)). BAL provides a simple, rapid, and biocompatible method to bioengineer multilayer tissue constructs for

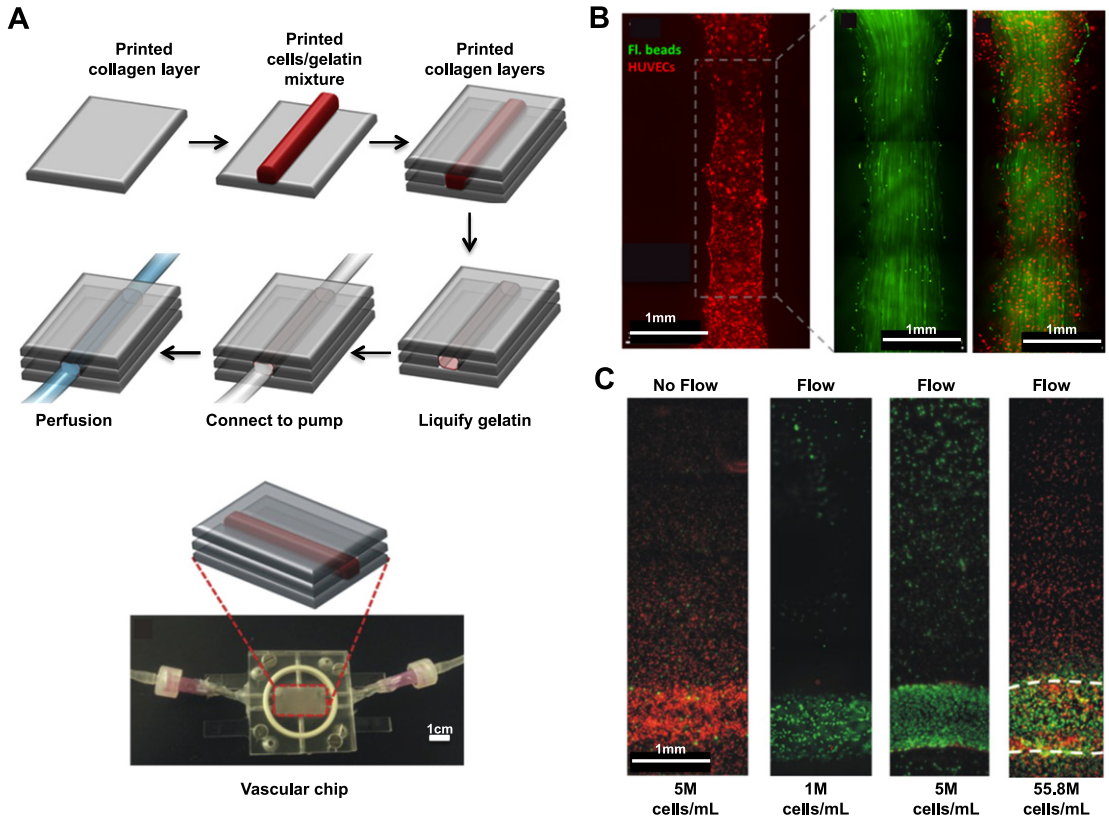

Figure 4. Vascular graft construction. (A) Fabrication steps of vascular channel using gelatin-cell mixture. (B) Fluorescence micrographs showing the vascular channel a following 1 day of dynamic flow culture (endothelial cells: red and fluorescent beads: green). (C) Cell viability (green: live and red: dead) of constructed vascular tissue under static and dynamic flow conditions. Reprinted by copyright permissions from [126].

a wide array of applications including neuroscience, cardiovascular, and cancer biology.

4.1.4. Bone

The studies in bone regeneration often encounter two major problems: graft porosity and vascularization [132–136]. Gene therapy represents an ideal approach for bone regeneration while delivering regenerative molecules to the specific tissues [137]. A new technique has been developed [138], which integrates effective printing strategies with gene therapy for bone regeneration studies. As an addition to bioprinting technique, plasmid DNA encoding bone morphogenetic protein-2 (BMP-2) is added to the constructs to induce osteogenic differentiation. The performance of porous constructs was better than the non-porous ones in means of BMP-2 production. Porosity and high BMP-2 production enhanced the rate of osteogenic differentiation. Expression of osteogenic markers, alkaline phosphatase, and osteocalcin were higher for porous and BMP-2 induced constructs suggesting that porosity combined with gene induction provided a suitable microenvironment for bone regeneration. Recently, another approach has been reported, which incorporates bioprinting technology with a nanoliter gel droplet system to create biomimetic fibrocartilage microenvironment [37]. Human MSCs were encapsulated in bio-ink, which is gelatin-based metacrylated hydrogel. In addition, BMP-2 and transforming growth factor β1 (TGF-β1) were added to mimic the native fibrocartilage phase of the bone. Quantitative RT-PCR analysis supported that upregulation of osteogenesis and chondrogenesis occured in 3D fibrocartilage model, thus making this approach as a functional tissue model system.

4.1.5. Liver

Primary hepatocytes and stem cell derived hepatocytes are heavily used for the tissue engineering of liver tissues [29, 139–146]. However hepatocytes lose their functionality easily under culture conditions [29]. To sustain long-term hepatocyte functionality, a new technique has been reported [147], which utilizes bioprinting technology to form hepatic cord like tissues called 'canaliculi'. Rat hepatocytes were cultured and canaliculi formation was observed starting from day 3 with collagen matrix. Expression of the apical marker MRP2 and basolateral marker CD147 verified the functionality of hepatocytes.

A biomimetic ECM system has been used for evaluation of hepatocyte function on different ECM-based hydrogels [148]. Primary human hepatocytes were cultured in liver ECM extracts, which was combined with Collagen Type I, HA or

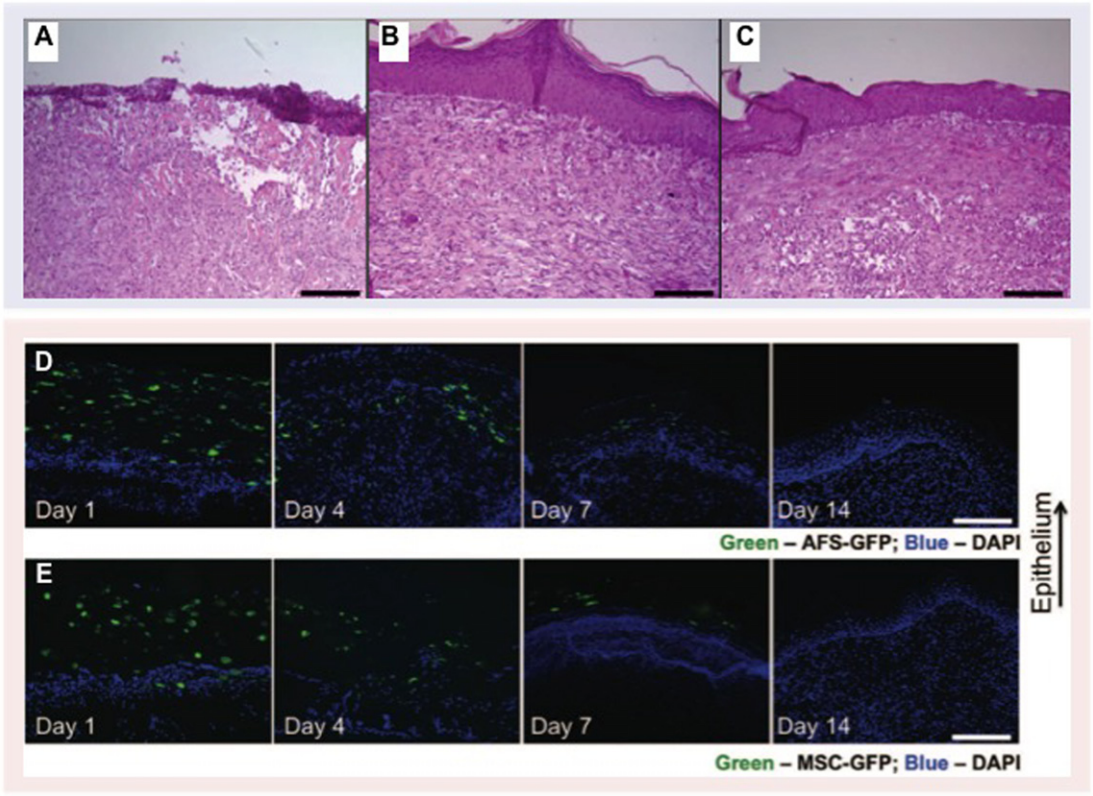

Figure 5. Re-epithelization of skin tissue. Histological examination of gel-only, (B) bone marrow-derived mesenchymal stem cell (MSC) treated, and (C) amniotic fluid-derived stem cell (AFSC) treated skin tissues (scale bars 50 μm). Fluorescence micrographs of (D) AFSCs and (E) MSCs showing that both cells are transient in wound regeneration process (green: GFP expressing MSC and AFSC; blue: nuclear staining/DAPI, scale bars 50 μm). Reprinted by copyright permissions from [128].

heparin-conjugated HA (HP). Primary human hepatocytes were maintained in sandwich-like hydrogels for 4 weeks. Increased hepatocyte metabolism, steady levels of secreted albumin, and urea confirmed that customized ECM-based hydrogels might be a suitable cell expansion platform for cell therapy, toxicology experiments, and further drug screening studies.

Another approach has been recently reported [149] for the biofabrication of *in vitro* 3D liver model. Multi-layered tissue constructs were formed by stacking rat and human hepatocyte cells with endothelial cell layers. This work showed that multilayered cellular architecture could be successfully used as a liver analogue for drug studies and other biomedical applications. In addition, a recent scaffold-free acoustic node assembly method was developed to bioengineer 3D *in vitro* tissue model. Thousands of tissue spheroids in a fluidic environment can be rapidly assembled into a predefined architecture in seconds. The assembled architecture can be dynamically reconfigured to diverse patterns by altering the wave frequency. Assembly of hepatocyte spheroids was demonstrated for generation of 3D liver model. Formation of bile canaliculi and hepatocyte gap junctions was observed in the bioengineered liver model via anti-MRP2 and anti-Connexin 32 immunostaining after 6 days in culture. This acoustic node spheroid assembly strategy represents an efficient and rapid way to generate 3D tissue models with a control over the predetermined geometric patterns. It also allows switching between patterns in a matter of seconds using high throughput assembly of spheroids guided by vibrations.

4.2. Bioprinting for various biomedical applications

Recent advances in cell manipulation and bioprinting technologies have allowed for various biomedical applications, including cryoprinting of cells for biobanking [67] and generation of 3D models for drug screening [40, 123–129].

4.2.1. Drug discovery

Development of *in vitro* tissue models holds a great promise for drug discovery studies [143]. Drug discovery is likely to be one of the most promising and growing area in medical applications of 3D bioprinting while technologies for artificial organs may incur delays due to regulatory hurdles. Recent technological advancements have accelerated drug design and discovery significantly, but the number of approved experimental platforms is still insufficient due to employment of 2D monolayer systems. Miniaturized 3D tissue models are valuable experimental platforms for design, discovery, and development of new generation drugs, since they mimic native microenvironment

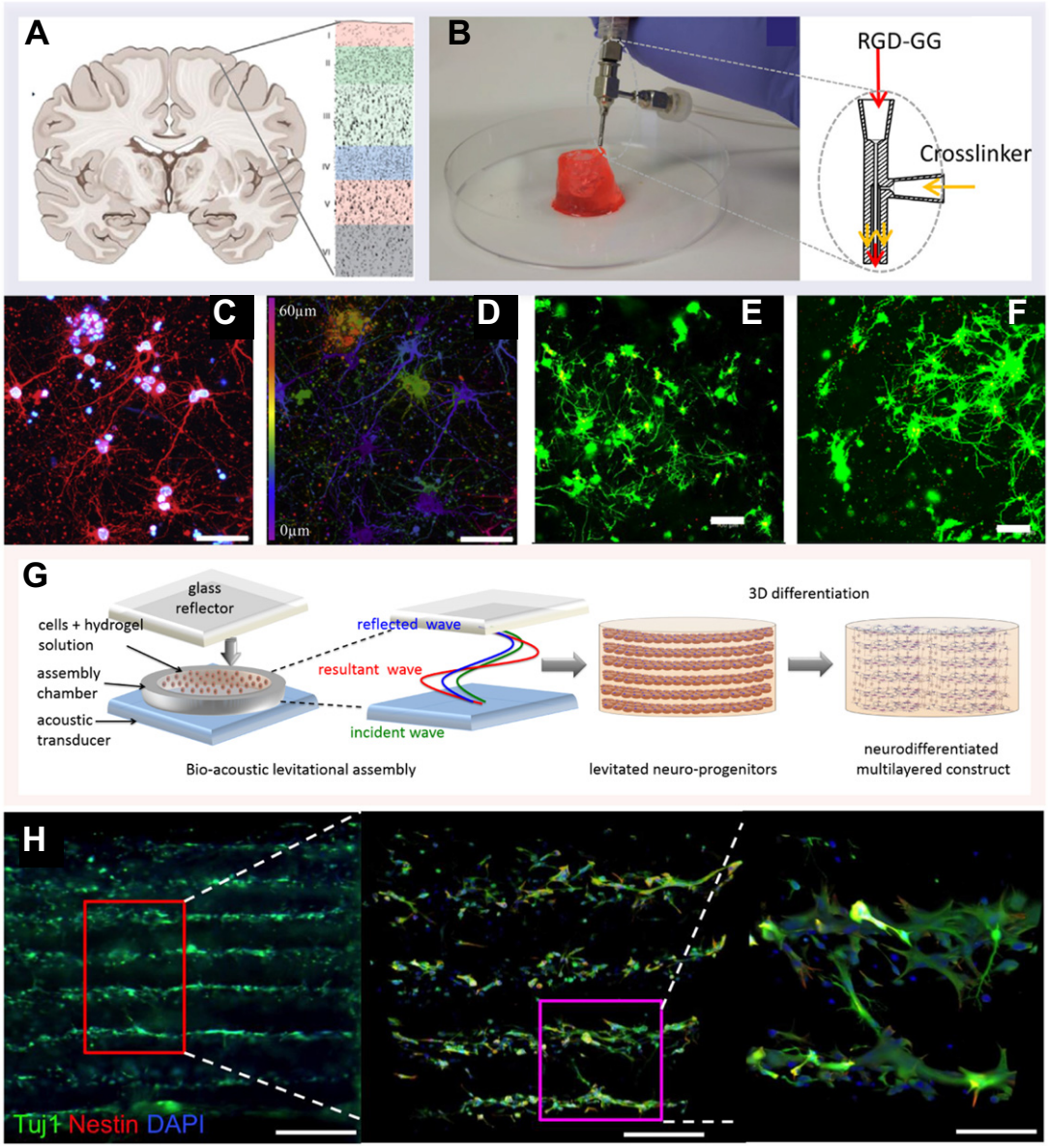

Figure 6. Neuron bioprinting and assembly. (A) Schematic illustration of the six layers of brain cortex. (B) Printed 3D structure and illustration of the extrusion-based bioprinter. (C), (D) Cortical neurons encapsulated RGD peptide-modified gellan gum after 5 days in culture. (C) β-III tubulin (red) and DAPI (blue) stainings for cortical neuron and nuclei, respectively. (D) Confocal micrographs showing the 3D neuronal model after 5 days in culture. (E), (F) Cell viability of non-printed (control, (E)) and printed (F) primary cortical neurons encapsulated in RGD peptide-modified gellan gum after 5 days in culture. Scale bars represent 50 μm (C), (D) and 100 μm (E), (F). Reprinted by copyright permissions from [130]. (G) Schematic illustration of acoustic levitation to fabricate 3D multilayered neural constructs. (H) 3D multilayer neural constructs assembled by acoustic levitation and differentiated in media for 8 days. Confocal micrograph showing the interaction between immunostained (nestin, red; Tuj1, green; DAPI, blue) cells in different layers. Scale bars represent 500 μm (left panel), 250 μm (middle panel), and 100 μm (right panel). Reprinted by copyright permissions from [80].

of the tissues more accurately than 2D cell cultures [83, 150–154]. 3D tissue models have a high potential to evaluate and predict success or failure of drug candidates in preclinical stages [155, 156]. These microsystems offer more predictive and cost-effective alternatives to *in vitro* or animal-based preclinical drug screening [155]. The applications of 3D printing in drug discovery can provide many other benefits such as customization and personalization of medical products and drugs, fast and precise drug response, and increased output of drug discovery while decreasing preclinical trial costs.

Many drug screening studies have been performed by using different bioprinting platforms such as cell spheroids [157–160], cell encapsulation [161, 162], and microfluidic systems [163, 164]. Recently, an array type drug screening platform was used as a droplet bioprinting technology to produce sub-nanoliter droplets [165]. It describes the microfabrication of CYP3A4 based protein array for multiplexing studies. In another approach, a valve-based bioprinter was utilized to fabricate a 3D lung model for drug evaluation [166]. The 3D architecture was generated by layer-by-layer approach for high-throughput drug screening.

Employment of 3D tissue constructs could provide better results in drug development and evaluation due to their ability to mimic cellular structures, while reducing the number of animals utilized for drug development studies [152].

4.2.1. Biopreservation

Conventional cryopreservation methods use bulk volumes that impede proper cooling and rewarming, causing detrimental alteration of cells during biopreservation [167–172]. Utilizing bioprinting technology to transform a bulk sample of cells into micro- and nano-liter droplets can address the challenges associated with conventional cryopreservation (e.g., ice crystal formation) [167]. Recently, a bioprinting approach in conjunction with innovative bio-inspired cryo-inks was utilized to vitrify red blood cells (RBCs) [67]. The bioprinter generates considerably smaller volumes of cell-encapsulated droplets (<0.15 nl), with the aim of reducing the unfavorable effects on RBCs by eliminating the need for high cryoprotectant concentrations to achieve vitrification, and it provides ultra-high cooling and rewarming rates that enable preserving cells minimizing ice crystal formation. The cells were also loaded with a bio-inspired ectoine-based cryo-ink, which acts as a non-toxic cryoprotectant, to eliminate the toxic effect associated with conventional cryoprotectants (e.g., dimethyl sulfoxide and glycerol) [167, 173]. Following rewarming, the biopreserved cells maintained their unique morphology, mechanics, and function. The bio-inspired bioprinting approach has the potential to create new avenues to biopreserve cells (e.g., stem cells, lymphocytes, and oocytes) for regenerative and reproductive medicine applications [7, 68, 174].

5. Future perspective and challenges

In this review, we have illustrated current guiding principles for 3D bioprinting in tissue fabrication, as well as recent advances and technological developments. The speed at which our knowledge has advanced with additive manufacturing and automated printing systems shows a promise to expand our basic science and engineering capabilities towards addressing healthcare problems.

One of the major milestones in 3D bioprinting is to fabricate and mimic cellular microenvironments from molecular to macroscopic scales in an autonomously ordered manner for tissue engineering and regenerative medicine. Recently developed 3D bioprinting technologies provide multiple approaches for biofabrication of tissue constructs. In particular, innovative technologies that engineer 3D cell microenvironment hold promise to facilitate the artificial tissue fabrication, and eventually to enhance our understanding from cellular interactions to tissue formation. By cooperation of various cutting-edge technologies, such as microfluidic systems [147, 149], biopatterning [37], and layer-by-layer assembly [149, 175] biomanufacturing of micro-tissue constructs within scaffolds or scaffold-free environments has been achieved. Despite the great progress of biomaterial development for tissue engineering, there are still certain challenges that need to be overcome. For instance, vascularization is one of the most important limiting parameters in tissue engineering and bioprinting [176, 177]. Due to restricted structure and vascularization challenges, hypoxia, apoptosis, and immediate cell death occurs, and thus, there is an urgent need to developed innovative solutions for vascular networks. Up to now, meeting this challenge has been mostly attempted by fabricating porous scaffolds [178], which provides sufficient space for vascularization. However, this approach cannot overcome the vascularization challenge completely due to the diffusion of cells and other materials into these porous structures [179]. Evidently, advances in bioprinting technologies will provide potential solutions to overcome this problem while forming interconnected, well-defined vascular structures during biomanufacturing process. On the other hand, mechanical strength and stability in 3D tissue engineering are among the key requirements [180]. For instance, in regeneration of hard (e.g., bone) and soft (e.g., vascular grafts) tissues, elastic modulus is an important parameter that needs to be optimized in those tissue constructs [181–183]. Furthermore, the development of a fully closed bioprinting system that integrates printing and post-printing processes such as *in vitro* culture and maturation of tissue constructs is still a challenge. Once the challenges mentioned above are addressed, scaling up bioprinting technologies will potentially improve rapid clinical solutions and advance medical implants. Further, we envision that the integration of cells and biomaterials through bioprinting with microfluidic technologies are likely to create unique microenvironments for various applications in cancer biology, tissue engineering, and regenerative medicine [172, 184–188]. Additionally, developments on high-throughput biomanufacturing of 3D architectures will pave the way for further advancements of *in vitro* screening and diagnostic applications, potentially enabling complex organ constructs.

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