Bioprinting is a powerful technology in the fabrication of living tissues and organs for tissue engineering and regenerative medicine, transplantation and clinics, pharmaceutics and high-throughput screening, as well as cancer research.

Application areas of 3D bioprinting

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Three dimensional (3D) bioprinting has been a powerful tool in patterning and precisely placing biologics, including living cells, nucleic acids, drug particles, proteins and growth factors, to recapitulate tissue anatomy, biology and physiology. Since the first time of cytoscribing cells demonstrated in 1986, bioprinting has made a substantial leap forward, particularly in the past 10 years, and it has been widely used in fabrication of living tissues for various application areas. The technology has been recently commercialized by several emerging businesses, and bioprinters and bioprinted tissues have gained significant interest in medicine and pharmaceutics. This Keynote review presents the bioprinting technology and covers a first-time comprehensive overview of its application areas from tissue engineering and regenerative medicine to pharmaceutics and cancer research.

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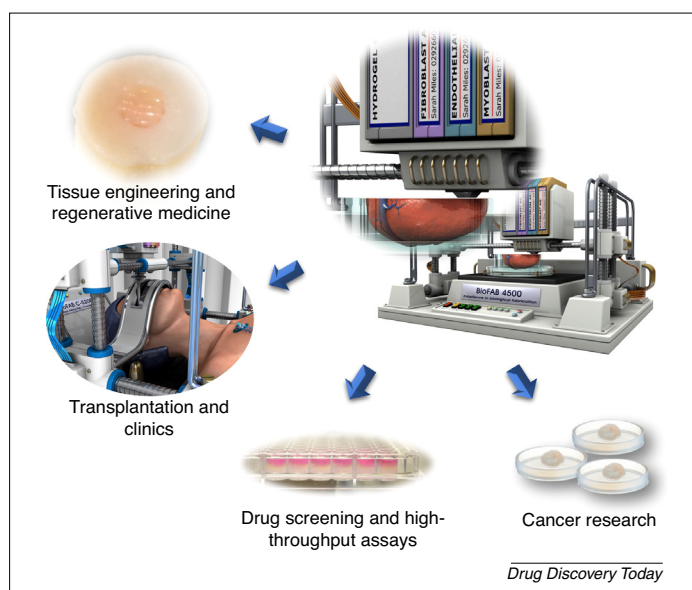
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Introduction

Bioprinting is a growing field that makes a revolutionary impact on medical and pharmaceutical sciences, and has gained significant attention worldwide [1]. Bioprinting can be defined as the simultaneous writing of living cells and biomaterials with a prescribed layer-by-layer stacking organization using a computer-aided transfer process for fabrication of bioengineered constructs [2]. It offers great precision on spatial placement of cells, proteins, DNA, drug particles, growth factors and biologically active particles to guide tissue generation and formation better. This powerful technology appears to be more promising for advancing tissue fabrication toward physiologically relevant tissue constructs, tissue models, tissues and organs and organs-on-a-chip models for medicine and pharmaceutics.

Bioprinting technology has a broad utility in various application areas such as tissue engineering and regenerative medicine [3,4], transplantation and clinics [1], drug screening and high-throughput assays [5] and cancer research [6], as depicted in Fig. 1. Bioprinting for tissue engineering and regenerative medicine fields has been around for more than a decade, and

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FIGURE 1

Application areas of bioprinting technology including tissue engineering and regenerative medicine, transplantation and clinics, drug screening and high-throughput assays, and cancer research (image courtesy of Christopher Barnatt from http://www.explaningthefuture.com).

anatomically correct cell-laden constructs and scaffolds have been fabricated for various tissue types from connective and epithelial tissues to muscle and nervous tissues. With its great advantage in patterning and precisely positioning multiple cell types, bioprinting has circumvented one of the major shortcomings of traditional scaffold fabrication techniques and has enabled fabrication of native-like tissues with a heterocellular microenvironment. Although the vast majority of the effort has been geared toward the fundamental science behind major bioprinting techniques such as laser-, droplet- and extrusion-based bioprinting, a substantial focus has recently been given to bioprinting for functional tissue fabrication [1]. Particularly, considerable work has been dedicated to bioprinting for transplantation, where bioprinted tissues have been implanted to various associated sites *in vivo*. As further progress takes place in biomaterials, cell and transplantation technologies, bioprinting will translate from bench to bedside when approved for human use and has a myriad of advantages in operating rooms in the near future. Before transitioning into clinical practice, bioprinting has already made a great leap in pharmaceutical use because it does not entail any regulatory approvals and there is currently an emerging bioprinting market for tissue fabrication for drug testing and high-throughput assays. With the inclusion of multiple cell types and facilitating a complex heterocellular physiological environment, bioprinted tissue models (e.g. liver) have been used in drug screening. In addition, bioprinting has recently been used in cancer research to investigate cancer pathology, growth and metastasis in a physiologically relevant microenvironment [7]. Here, we present a critical and comprehensive review of the application areas of bioprinting technology and provide an in-depth discussion on successfully bioprinted tissue types in tissue engineering and regenerative medicine, transplantation and clinics, drug screening and high-throughput assays, and cancer research. For each application area, limitations of existing technologies are discussed and future prospects are provided to the reader.

Tissue engineering and regenerative medicine

Bioprinting of functional organs at clinically relevant dimensions remains elusive because there are several challenges such as but not limited to integration of the vascular network from arteries and veins down to capillaries, incorporation of various cell types to recapitulate complex organ biology and limited structural and mechanical integrity and long-term functionality [8]. Despite these difficulties, a wide variety of tissues have been successfully bioprinted such as thin or hollow tissues, for example blood vessel [9], or tissues that do not need vascularization such as cartilage [1].

Bone tissue

Bone tissue engineering has been widely studied using bioprinting because bioprinting has the ability to fabricate anatomically correct patient-specific tissue constructs. In a recent study [10], Gao *et al.* used a thermal inkjet bioprinter to fabricate poly(ethylene glycol) dimethacrylate (PEGDMA) scaffolds. Bone-marrow-derived human mesenchymal stem cells (hMSCs) were co-printed with nanoparticles of bioactive glass and hydroxyapatite (HA) under simultaneous polymerization. Bioprinting in that study enabled uniform distribution of hMSCs compared with manually pipetted hMSCs, which accumulated at the bottom of the scaffold because of gravity (Fig. 2a). The bioprinted constructs encapsulating hMSCs and HA demonstrated the highest cell viability, collagen production and alkaline phosphate activity with increased compressive modulus after 21-day culture *in vitro*. Bioprinting HA particles were also performed for *in situ* bioprinting purposes, where a laser-based bioprinting system was used to deposit HA nanoparticles into mouse calvarial defects in a framework study [11], as detailed below.

In another study, Fedorovich *et al.* bioprinted heterocellular tissue constructs made of Matrigel™ and alginate hydrogels [12]. Endothelial progenitor and multipotent stromal cells were bioprinted in a spatially controlled manner and the bioprinted constructs were subcutaneously implanted into immune-deficient mice. By incorporating osteoinductive biphasic calcium phosphate microparticles, multipotent stromal cells were differentiated into an osteogenic lineage and facilitated bone formation in 6 weeks. In addition to osteoinductive materials, incorporation of growth factors is also crucial in stem cell differentiation in bone tissue engineering. Phillippi *et al.* demonstrated the effect of bone morphogenetic protein (BMP)-2 on stem cell fate [13]. Using inkjet bioprinting of patterned BMP-2 on fibrin-coated coverslips, primary-muscle-derived stem cells were differentiated toward osteogenic lineage on the pattern even if they were treated with myogenic differentiation conditions.

Cardiac tissue

Cardiac tissue engineering has been a growing interest because heart failure is a devastating disease [14]. Although myocardium tissue has a limited regeneration capability because myocyte proliferation rapidly ceases after birth [15], tissue engineering of such a structurally and functionally complicated organ is essential. In the literature, limited attempts have been made in bioprinting of cardiac tissue models. Jakab *et al.* demonstrated extrusion-based

bioprinting of tissue spheroids comprising human vascular endo-thelial cells (HUVECs) and cardiac cells isolated from myocardial tubes of chicken embryos [16]. Tissue spheroids that were adhesive and scaffold-free, and possessed rapid self-assembly capabilities, were bioprinted next to each other on collagen type-I biopaper in a single-layer grid pattern. Upon bioprinting, tissue spheroids were fused together in approximately 70 h and formed a single cardiac tissue patch that could synchronously beat. In addition to the scaffold-free approach undertaken in the above work, scaffold-based bioprinting has been investigated in a few remarkable studies. Xu et al. bioprinted a cardiac tissue construct in a half-heart shape with connected ventricles using inkjet-based bioprint-ing (Fig. 2b1,b2) [17]. In their study, primary feline adult and H1 cardiomyocytes encapsulated in alginate–gelation composite hydrogels and the crosslinker (calcium chloride solution) was selectively sprayed layer by layer. The resulting tissue construct with connected ventricles was electrically stimulated and func-tional excitation–contraction coupling was successfully demon-strated. In addition to these studies, patterning of cells was also applied in cardiac tissue engineering. Gaebel et al. utilized laser-induced forward-transfer (LIFT) to pattern HUVECs and hMSCs in a geometrically defined pattern on polyester urethane urea and the fabricated samples were transplanted to the infarcted zone of rat hearts after LAD ligation [18]. In 8 weeks post-transplantation, samples with LIFT-derived patterns facilitated increased vessel formation compared with randomly bioprinted cells as control groups and the resulted myocardium patch provided significant functional improvement. Besides primary cells, human cardiac-derived cardiomyocyte progenitor cells (hCMPCs) were also bio-printed in a mesh pattern [19]. Bioprinted hCMPCs demonstrated phenotypic properties of cardiac lineage with enhanced expres-sion of early cardiac transcription factors Nkx2.5, Gata-4 and Mef-2c.

Cartilage tissue

Current tissue engineering techniques for cartilage regeneration cannot produce cartilage tissue that is indistinguishable from native tissue [20]. Owing to its great potential for precise spatial and temporal deposition of cells and biomaterials with sophisti-cated patterns, bioprinting has recently gained increasing atten-tion for engineering cartilage tissues that can imitate native tissues with zonally differentiated cells and extracellular matrix (ECM) composition. Laser-based bioprinting of stem-cell-differentiated chondrocytes was attempted by Gruene et al., in which a comput-er-aided biofabrication technique was used with the assistance of LIFT [21]. They successfully bioprinted porcine-bone-marrow-de-rived mesenchymal stem cells (MSCs) with high viability, and the cells maintained their functionality and differentiation ability into osteogenic and chondrogenic lineages.

Inkjet-based bioprinting has also been used in cartilage tissue engineering as well as for cartilage defect repair. Cui et al. modified an HP desktop printer, where they were able to bioprint human chondrocytes loaded in PEGDMA hydrogel [22]. The bioprinted cartilage construct had mechanical properties and biochemical composition close to native cartilage. Also, by implanting bio-printed cartilage constructs into articular cartilage defects, inte-gration with the native tissue was observed with enhanced interface strength, which improved the quality of the repaired

cartilage significantly. In another study using the above experi-mental setup, the same group fabricated PEG scaffolds (Fig. 2c1) and investigated the effect of combined transforming growth factor (TGF)-β1 and fibroblast growth factor (FGF)-2 on cell pro-liferation and differentiation capability, and demonstrated that samples treated with TGF-β1 and FGF-2 facilitated the highest glycosaminoglycan (GAG) content [23] and samples without growth factor treatment did not secrete GAG even in 4-week culture (Fig. 2c2,c5). Most recently, Xu et al. created a hybrid bioprinting method to fabricate mechanically improved cartilage tissue constructs by combining 3D bioprinting and electrospin-ning techniques [24]. In that study, electrospinning of polycapro-lactone (PCL) fibers together with inkjet bioprinting of rabbit elastic chondrocytes in fibrin–collagen hydrogel was demonstrat-ed. After printing, cell viability was well maintained and fabricated constructs formed cartilage tissues, with improved mechanical properties, in vitro and in vivo.

In addition to hydrogels used in the abovementioned studies, sodium alginate has been widely used for cartilage tissue bioprint-ing. Ozbolat et al. demonstrated hybrid bioprinting of chondro-cytes loaded in printed alginate filaments in tandem with bioprinting of chondrocyte spheroids to increase the cell density [25]. A multi-arm bioprinter was used to facilitate such a complex hybrid architecture. Using sodium alginate and silver nanoparti-cles, McAlpine's group [26] successfully printed a bionic ear model, which was composed of chondrocyte-loaded alginate in an ear-shape and a conductive coil with the ability to translate sound waves into digital data. Recently, Markstedt et al. demonstrated mixing alginate with nanocellulose, which has outstanding shear-thinning properties that enabled fabrication of anatomically cor-rect ear and meniscus constructs [27].

Despite the great progress in bioprinting for cartilage tissue regeneration, bioprinting of zonally stratified articular cartilage tissues with different structural, biomechanical and biological properties is still a challenge and further progress is needed to achieve articular cartilage tissue constructs with zonal differentia-tion including more horizontal and thinner collagen fibers with high cell density in the superficial zone, and relatively vertical and thicker collagen fibers with less cell density in the deeper zones.

Heart valves

In addition to cardiac tissue engineering, engineering heart valves is also important because heart valves do not possess regeneration capability and dysfunctional heart valves, if the damage or disease is detrimental, need to be replaced by mechanical or biological prosthetic counterparts [28]. Such replacement valves, however, are limited by thrombogenicity and calcification [29]. Despite its crucial role in the cardiovascular system, only a limited amount of work has been carried out in the bioprinting of heart valves. Butcher's group demonstrated the first-time bioprinting of a heart valve [30] using a dual-head bioprinter modified from a Fab@-Home printer [31]. In that work, a dual crosslinking mechanism, consisting of ionic and physical crosslinking, was used to print PEG-diacrylate (PEGDA) mixed with sodium alginate. After print-ing, porcine aortic valve interstitial cells (PAVICs) were seeded and cultured for up to 21 days. In their study, anatomically accurate axisymmetric aortic valve geometries, composed of a root wall and tri-leaflets, were demonstrated. Although the first-time presented
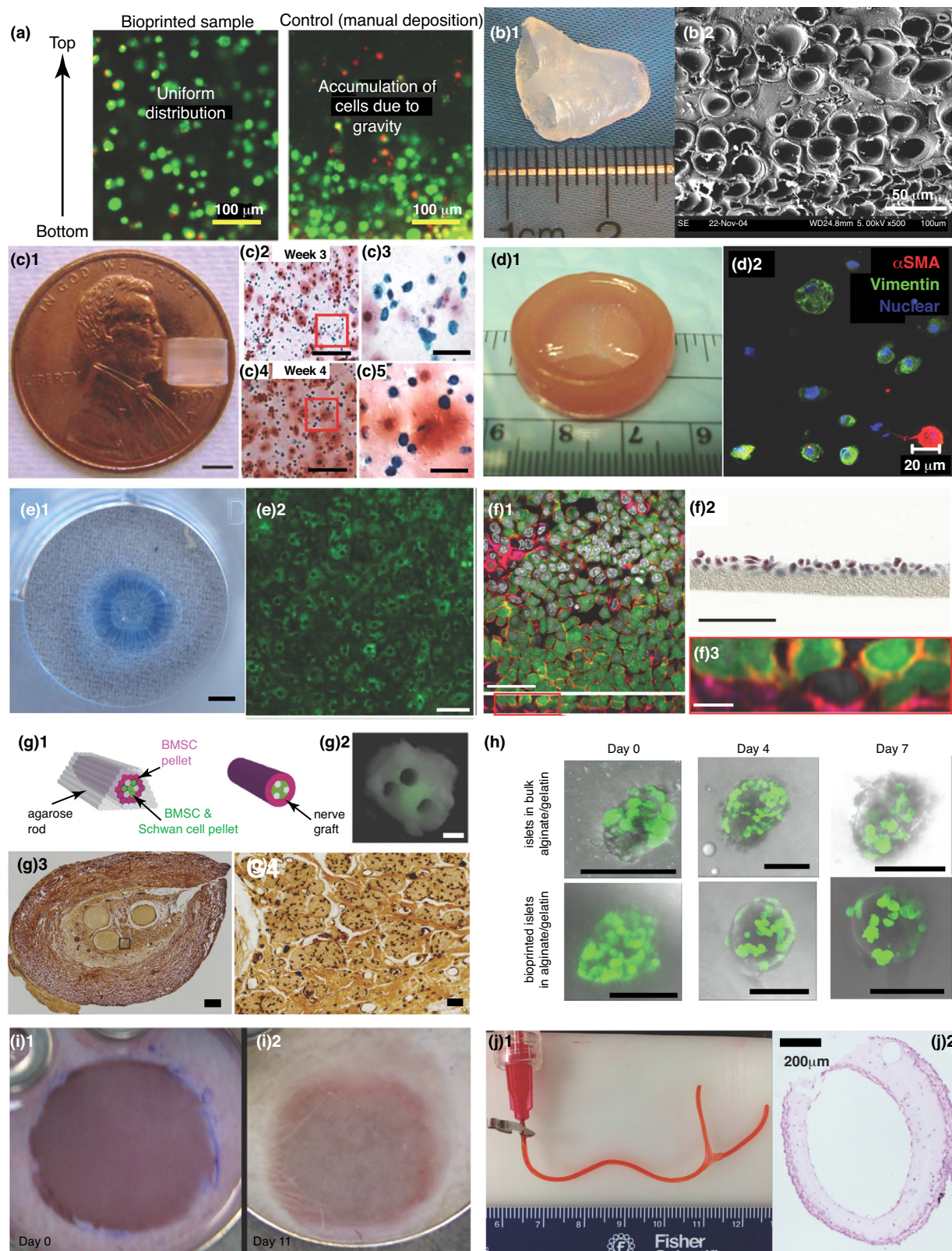
FIGURE 2

Bioprinted tissue constructs for tissue engineering and regenerative medicine. **(a)** Inkjet bioprinting of human mesenchymal stem cells (hMSCs) in hydrogels for bone tissue engineering, where bioprinting resulted in uniform distribution of hMSCs opposed to accumulated hMSCs at the bottom of the scaffold as a result of gravity when hMSCs were manually pipetted; adapted, with permission, from [10]. **(b1)** Bioprinting of cardiac tissue constructs with connected ventricles using a modified-HP printer, **(b2)** SEM image of the cross-section of the scaffold showing loaded cells; adapted, with permission, from [17]. **(c1)** Bioprinting of a 4 mm poly(ethylene glycol) (PEG) cartilage tissue construct with **(c2–c5)** Safranin-O staining with limited glycosaminoglycan (GAG) deposition without transforming

work does not fall under bioprinting because cells were not involved during the printing process, the group later demonstrated bioprinting of aortic valves using different hydrogels and cell phenotypes. In another study by that group [32], dual-nozzle bioprinting of composite alginate–gelatin hydrogel was performed to fabricate thin hydrogel discs. Aortic root sinus smooth muscle cells (SMCs) and aortic valve interstitial cells (VICs) were spatially bioprinted and samples were incubated for a week. The results revealed a cell viability of 81.4 ± 3.4% and 83.2 ± 4.0% for SMCs and VICs, respectively. Acellular aortic valve constructs, by contrast, exhibited reduced modulus, ultimate strength and peak strain. In a recent study [33], the same group presented bioprinting of composite hydrogels using methacrylated hyaluronic acid (Me-HA) and methacrylated gelatin (Me-Gel) loaded with human aortic valvular interstitial cells (HAVICs). Fabricated samples (Fig. 2d1) represented the designed tri-leaflet valve-shape meticulously and the cells bioprinted with increased Me-Gel concentration exhibited better spreading. Human aortic valvular interstitial cells encapsulated within the composite hydrogel expressed alpha smooth muscle actin (α-SMA) and vimentin (Fig. 2d2), and remodeled the ECM by depositing collagen and GAGs.

Liver tissue

Although liver has excellent regenerative and recuperative properties, liver tissue engineering has been a growing interest because liver failure, in association with failure of multiple organs, is a significant cause of morbidity and mortality [34]. Engineering liver tissues stands as a promising direction for future organ transplantation needs in addition to the great potential of bioprinted liver tissue models in drug testing and high-throughput screening because liver tissue is highly sensitive to drug toxicity. Faulkner-Jones et al. demonstrated the first-time bioprinting of human-induced pluripotent stem cells (hiPSCs), where bioprinted hiPSCs were stimulated and differentiated into hepatocytes for liver micro-organ engineering [35]. The presented work systematically analyzed the effect of the bioprinting process on stem cell fate, and the influence of pressure and nozzle length on the viability of hiPSCs and human embryonic stem cells (hESCs), and concluded that the utilized inkjet bioprinting process was gentle enough to maintain viability and pluripotency of cells, and directed their differentiation into a hepatic lineage. A dual-head valve-based inkjet bioprinter was used to deposit sodium alginate and calcium chloride to fabricate multilayer tissue constructs (Fig. 2e1) and the

results demonstrated that bioprinted stem cells differentiated into a hepatic lineage successfully after a 17-day differentiation period and expressed hepatocyte markers including hepatocyte nuclear factor 4 alpha (HNF4α), albumin and zonula occludens-1 (ZO-1), where albumin secretion peaked on day 21 (Fig. 2e2).

In addition to bioprinting liver tissue constructs, liver carcinoma HepG2 immortal cells were bioprinted in larger tissue models. Bertassoni et al. utilized a modified NovoGen MMX Bioprinter[TM] and bioprinted HepG2 cells and fibroblasts within gelatin–methacrylamide (GelMA) hydrogel strands along with agarose strands. Upon printing, agarose solidified immediately as a result of a rapid drop in the temperature and UV light was applied to photo-crosslink GelMA precursor under 6.9 mW/cm^2 of UV light (360–480 nm) for up to 1 min [36]. After complete gelation of GelMA, agarose was completely removed to create perfusable channels. The study revealed that cells preserved their viability for up to 8 days. The work was then extended using other hydrogels such as star poly(ethylene glycol-co-lactide) acrylate (SPELA), PEGDMA and PEGDA hydrogels at different concentrations [37]. This section presents liver tissue bioprinting attempts for tissue engineering only and further discussion on bioprinting of liver tissue models for drug testing and high-throughput screening will be provided below.

Lung tissue

Bioprinting for lung tissue engineering is new and there is only one recent attempt studying the fabrication of a lung tissue model using bioprinting. Hovath et al. demonstrated bioprinting of an in vitro air–blood barrier model using BioFactory® by regenHU [38]. In this regard, they bioprinted a zonally stratified tissue construct in a few layers. First, a thin layer of Matrigel[TM] was bioprinted as a basement membrane followed by bioprinting of a single layer of EA.hy926 endothelial cells to facilitate attachment of cells on the Matrigel[TM] layer. Later, on day 2, a new layer of Matrigel[TM] was bioprinted on top of the previously built construct followed by bioprinting of a single layer of A549 epithelial cells. Manually deposited layers were also constructed as control samples. On day 5, the samples were fixed for characterization, and cell viability of >95% and ≥86% was achieved for epithelial and endothelial cells, respectively. As can be seen in Fig. 2f1 and Fig. 2f3, epithelial and endothelial cells were uniformly distributed with epithelial cells on the top and endothelial cells at the bottom. Sagittal histological sections also confirmed formation of thin, packed and uniform

growth factor (TGF)-β1 and fibroblast growth factor (FGF)-2 treatment even if high density (20 million cells per ml) was used; adapted, with permission, from [23]. **(d1)** A bioprinted heart valve with encapsulated human aortic valvular interstitial cells (HAVICs) and **(d2)** a representative immunohistochemistry image showing α-SMA (green) and vimentin (red) expression; adapted, with permission, from [33]. **(e1)** A bioprinted 40-layer alginate ring-shape construct, where **(e2)** hESCs differentiated toward hepatocyte-like cells showing positive for albumin expression; adapted, with permission, from [35]. **(f1)** Bioprinted four-layer lung tissue model with highly organized distribution of a549 cells (green) and endothelial cells (labeled with VE-cadherin in pink), where F-actin and nuclei are labeled in red and white, respectively. **(f2)** Histological cross-section stained with Masson–Goldner trichrome coloration showing uniform thickness of a tissue sample. Cytoplasm, collagen fibers and cell nuclei are stained in red, green and dark brown, respectively. **(f3)** Sagittal cross-section demonstrates uniform epithelial layer on the top and endothelial cell layer at the bottom; adapted, with permission, from [38]. **(g1)** Scaffold-free bioprinting of nerve tissue using bone marrow stem cell (BMSC) pellet and co-culture of BMSC and Schwann cell pellet, where cell pellet was bioprinted within printed agarose mold for aggregation of cells **(g2)** for multi-luminal nerve grafts, **(g3,g4)** Bielschowsky's staining of histological sections showing axons as black dots; adapted, with permission, from [41]. **(h)** Green fluorescent protein (GFP)-transduced islets plotted in alginate–gelatin scaffolds kept their morphology for over a week in culture compared with the encapsulated counterparts in bulk hydrogels (control); adapted, with permission, from [43]. **(i1)** A bioprinted skin substitute consisting of 20-layer of fibroblasts and 20-layer of keratinocytes on top of Matriderm® was implanted on the dorsa of a mouse **(i2)** and resulted in complete wound closure on day 11; adapted, with permission, from [51]. **(j1)** Bioprinted vascular tissue constructs using a co-axial nozzle apparatus, where **(j2)** loaded human umbilical vein smooth muscle cells (HUVSMCs) generated smooth muscle matrix under 6-week differentiation media, particularly in the luminal and outer surface of the tissue constructs; adapted, with permission, from [98].

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tissue layers (Fig. 2f2) compared with manually pipetted control samples. The barrier quality, such as the tightness of the constructs, was investigated through measuring the translocation of blue dextran molecules from the apical to the basolateral compartment of the samples after 3 days of culture and results revealed that the tightness of the bioprinted samples was better than that of the manually pipetted samples.

Neural tissue

Engineering nervous system tissues offers tremendous promise to replace diseased, aged or injured components of nervous system; however, there is limited work done in the context of bioprinting for neural tissue fabrication. Lee et al. studied the effect of vascular endothelial growth factor (VEGF) release on proliferation and migration of murine neural stem cells (C17.2) [39]. In their study, C17.2 cells were bioprinted on a collagen layer next to a fibrin disk loaded with VEGF. The study showed that neural cells migrated toward VEGF-releasing fibrin gel and proliferated successfully in contrast to the cells that could not proliferate within the collagen matrix. Recently, Hsieh et al. demonstrated bioprinting of a thermoresponsive polyurethane hydrogel with tunable stiffness and gelation ability at 37°C without the need for a crosslinker [40]. They showed the effectiveness of the bioink by loading it with neural stem cells and injecting it into a zebrafish embryo neural injury model. The results revealed that the injected gel rescued the function of the impaired nervous system in 6 days.

Owens et al. used a scaffold-free approach, where a pellet of Schwann cells (SCs) and bone marrow stem cells (BMSCs) were extruded within a 3D-printed agarose mold [41] (Fig. 2g1 for the schematic of the process). Cells in the agarose mold aggregated and formed a nerve tissue graft with three lumina in each as shown in Fig. 2g2. The fabricated grafts were then implanted into mice, and their histology (Fig. 2g3,g4) and functionality was evaluated 10 months after implantation, and compared to the functionality of autologous grafts and hollow collagen grafts. Although the number of samples were not enough to draw a definitive conclusion about the performance of the 3D bioprinted grafts with respect to commercially available collagen grafts, the presented case demonstrated a proof-of-concept for bioprinting nerve grafts.

Pancreas tissue

Because primary pancreatic β-cells do not easily survive in vitro and only a very few attempts have taken place to differentiate β-cells from human stem cells, regeneration of pancreas tissue is primarily embodied to the extent that β-cells from mouse lines or insulinoma cells have been used to fabricate pancreatic islets [42]. A limited amount of work has been done in the context of bioprinting for pancreatic tissue fabrication. Recently, Marchioli et al. encapsulated human and mouse islets as well as rat insulinoma INS1E β-cells within alginate or alginate–gelatin hydrogels and bioprinted them in dual-layer scaffolds [43]. The scaffolds were later implanted in diabetic mice and explanted 7 days thereafter. Although the viability and morphology of islets was not impaired by encapsulation and bioprinting processes in alginate and alginate–gelatin hydrogels (Fig. 2h), bioprinted islets and INS1E β-cells lost their functionality in 7 days because they were not responsive to the change in glucose level. This can be attributed to the high level of calcium (Ca^{2+}) ions within crosslinked alginate because

transmembrane calcium ion-gradient, mediated by voltage-gated calcium channels, stimulated higher insulin secretion at low glucose level [44]. In addition, recent work demonstrated the microfabrication of scaffold-free tissue strands (with strong expression of insulin) for extrusion-based bioprinting [45], where tissue strands were made of rat fibroblasts and mouse insulinoma TC-3 β-cells in the core and shell, respectively. The authors envisioned to use the demonstrated tissue strands for scale-up tissue bioprinting purposes.

Skin tissue

A myriad of tissue engineering approaches have been applied in skin tissue fabrication and tissue substitutes including autologous split-thickness skin graft (gold standard) [46], allografts [47], acellular dermal substitutes and cellularized graft-like commercial products [47] [i.e. Dermagraft® and Apligraf® (Organogenesis)] [48]. Recently, bioprinting technology has been adopted for skin tissue fabrication as well. Lee et al. presented bioprinting of skin tissue using an eight-channel valve-based bioprinter, where a 13-layer-tissue construct was bioprinted using collagen hydrogel [49]. Keratinocytes were bioprinted on top of alternating layers of human foreskin fibroblasts and acellular collagen layers, and the resulted constructs demonstrated densely packed cells in epidermis layers as opposed to the dermis with low density of cells and less ECM deposition. In addition to droplet-based bioprinting, laser-based bioprinting has also been used for biofabrication of skin tissue substitutes [50]. Cells from a human immortalized keratinocyte cell line and NIH 3T3 fibroblasts were bioprinted in collagen matrix in alternating layers on a sheet of Matriderm™. Histological results demonstrated high density of keratinocytes and fibroblasts with expression of laminin protein, which is a major component of basement membrane in skin. The same group extended their work [51] and demonstrated implantation of the tissue constructs on dorsa of mice as shown in Fig. 2i1. Results revealed that the bioprinted tissues were engrafted with the hosts in 11 days (Fig. 2i2) with stratified epidermis with early signs of differentiation and formation of the stratum corneum as well as some blood vessels. Control samples at the air–liquid interface in in vitro culture, by contrast, demonstrated proliferation of cells with limited differentiation. In a recent study [52], Boland's group demonstrated the effect of bioprinting endothelial cells within skin substitutes on formation of macrovasculature during new tissue remodeling. In this regard, they encapsulated neonatal human dermal fibroblasts and epidermal keratinocytes (NHEKs) in collagen, and laid down the dermis layer followed by patterning human dermal microvascular endothelial cells (HMVECs) on the dermis layer of the construct by selectively bioprinting thrombin-laden HMVECs on the manually deposited fibrinogen layer. The process was completed by covering the fibrin layer with collagen-laden NHEKs. Then, the fabricated skin substitutes were implanted on the dorsa of mice and compared with commercially available skin substitutes (control). The results revealed that bioprinted HMVECs formed microvessels and the implanted constructs barely generated contraction compared with the control groups. In the abovementioned studies, tissue constructs were bioprinted in vitro and implanted to a host; however, Skardal et al. [53] demonstrated in situ bioprinting of stratified skin substitutes by alternating layers of fibrinogen–collagen and thrombin loaded with amniotic-fluid-derived stem (AFS) cells owing to

their lack of immunogenicity. With *in situ* bioprinting, skin substitutes were 3D bioprinted directly onto full thickness wounds on pigs and recapitulated the native skin more closely than control groups including the bioink loaded with MSCs and acellular hydrogels. Despite the efforts in skin tissue bioprinting, biofabrication of skin substitutes that virtually mimic native skin is still a challenge because integrating sweat glands and hair follicles has remained elusive [54].

Vascular tissue

Bioprinting of scale-up tissues and organs vitally depends on vascularization because integration of vascular network will essentially provide oxygen and media supply to cells for their survival and function [2]. Vascular tissue fabrication has been performed by various bioprinting modalities including extrusion- [55–58], droplet- [59,60] and laser-based bioprinting [61]. In extrusion-based bioprinting, a wide variety of extrusion techniques have been utilized. Ozbolat and co-workers used coaxial-nozzle extrusion, where hydrogels including sodium alginate and chitosan were bioprinted directly in tubular form with encapsulated cells [55,57]. During coaxial (core-shell) flow, ejected crosslinker (flowing through the core) contacted the precursor hydrogel solution (flowing through the shell), and facilitated rapid gelation and formation of tubular constructs (Fig. 2j1). Six-week cultured HUMSMC-laden samples demonstrated deposition of smooth muscle matrix (Fig. 2j2). That approach enabled direct bioprinting of vascular constructs in a practical manner. In addition, there are other direct vascular tissue bioprinting approaches, such as bioprinting droplets of cell-laden hydrogels layer-by-layer using inkjet-based bioprinting performed by Nakamura and his co-workers [62], Huang's group [59] and Blaeser *et al.* [60]. Utilizing bottom-up construction, inkjet-based bioprinting enabled branched tubes built in horizontal and vertical directions. A similar approach was also performed using laser-based bioprinting demonstrated by Huang's group [61]. In the abovementioned studies, scaffold-based approaches were utilized; however,

Forgacs and co-workers followed a scaffold-free approach in bioprinting vascular tissues, where tissue spheroids were bioprinted one by one and self-assembled into larger tissue units [58]. Because agarose is inert to cell adhesion, the printed agarose mold facilitated rapid fusion of tissue spheroids and maturation of the tissue. In addition to direct bioprinting of tubular vascular tissues, indirect bioprinting of perfusable tissue constructs has been performed using various hydrogels including fibrin [63], collagen [64] and GelMA [36]. In an indirect approach, a fugitive-ink – that is dissolvable or reversibly crosslinkable such as agarose [37], sugar [65], Pluronic® [66] or gelatin [64] – was used to create open channels. Upon removing the fugitive ink, endothelial cells were perfused and glued to create endothelium within open channels [65]. This approach enables bioprinting of highly complex vascular constructs that can be perfused over a long time depending on the degradation profile of the matrix. Although both approaches can be utilized, the former is more appropriate in generating vascular grafts for transplantation and the second is more appropriate for fabrication of perfusable channels for *in vitro* tissue engineering applications [1].

Composite tissues

In addition to single tissue types, efforts have been geared toward bioprinting composite tissues to recapitulate the complex biology, anatomy and functionality of organ-level structures. Merceron *et al.* recently demonstrated fabrication of muscle-tendon units by bioprinting hybrid constructs using a multi-head nozzle assembly [67]. In this regard, PCL and polyurethane (PU) were 3D printed to construct a frame to support cellular constructs, where half of the unit was printed using PCL and the other half was printed using PU (Fig. 3a1–a4). A composite hydrogel-based bioink, comprising 3 mg/ml hyaluronic acid, 35 mg/ml gelatin and 25 mg/ml fibrinogen in calcium-fee high-glucose Dulbecco's modified eagle medium (DMEM), was used to 3D bioprint 3T3 fibroblasts and myoblasts into the PCL and PU frames to construct tendon and muscle units, respectively. The results revealed cell
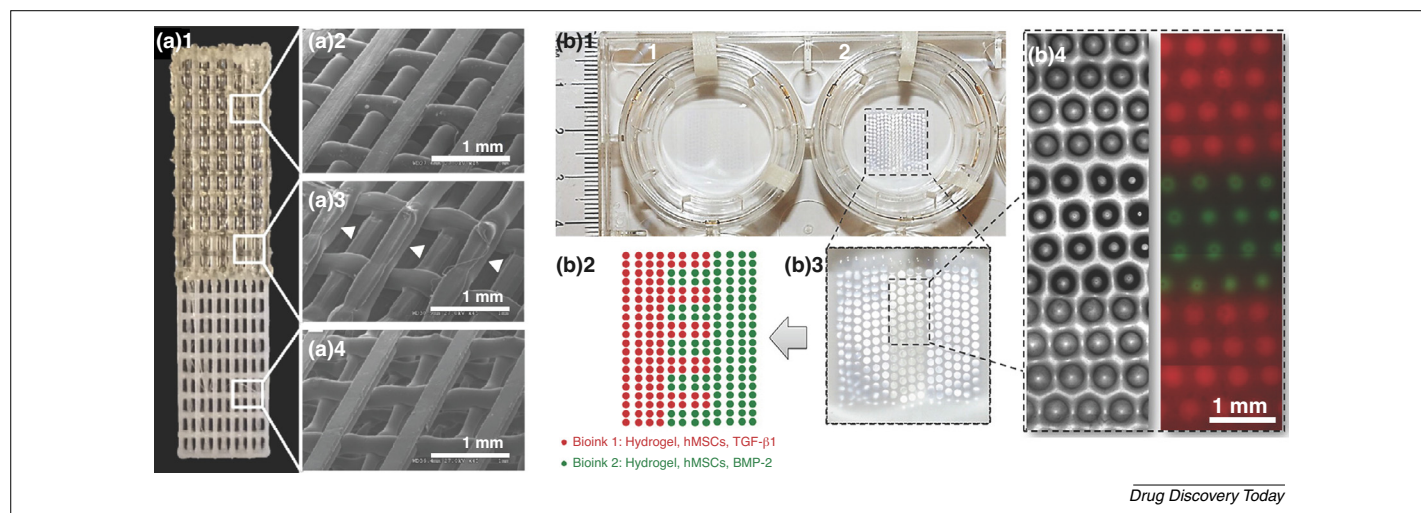

FIGURE 3

Bioprinted composite tissue constructs. **(a1)** A muscle-tendon unit frame made of PU (upper-half) and polycaprolactone (PCL; lower-half) at a higher magnification view of **(a2)** printed PU filaments, **(a3)** filaments at the interface and **(a4)** printed PCL filaments; adapted, with permission, from [67]. **(b1)** Bioprinted fibrocartilage samples **(b2,b3)** patterned by selectively depositing human mesenchymal stem cell (hMSC)-laden methacrylated gelatin (Me-Gel) droplets loaded with either transforming growth factor (TGF)-β1 or bone morphogenetic protein (BMP)-2. **(b4)** Bioprinted precursor gel droplets were photocrosslinked and droplets were demonstrated using Rhodamine B (red) and Dextran-Alexa Fluor 488 (green); adapted, with permission, from [71].

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viability of >80% with differentiated cells at the end of a 7-day culture. The final muscle-tendon units were elastic on the PU-C1C12 muscle section with an elastic modulus of 0.39 ± 0.05 MPa and stiff on the PCL-3T3 tendon side with an elastic modulus of 46.47 ± 2.67 MPa.

In addition to muscle-tendon units, bioprinting of osteochondral models has been an interest in tissue engineering. Fedovorich et al. demonstrated bioprinting of MSCs and chondrocytes using alginate in a mesh pattern with two different cell types bioprinted within two opposite ends of the scaffold [68]. Mesenchymal stem cells were co-extruded with osteoinductive biphasic calcium phosphate particles, HA and β-tricalcium phosphate. The bioprinted samples were cultured with a mixture of chondrogenic and osteogenic medium for 21 days. The results revealed that bioprinted osteochondral tissue constructs demonstrated differentiated characteristics of cells into osteogenic and chondrogenic lineages along with related ECM deposition in vitro and in vivo. A similar approach was performed by Park et al., where a systematic analysis was performed to understand the effect of native ECM components on the fate of osteoblasts and chondrocytes [69]. They bioprinted osteoblasts in collagen type-I and chondrocytes in hyaluronic acid, and compared the performance of bioprinted osteoblasts and chondrocytes when they were bioprinted in hyaluronic acid and collagen type-I, respectively. Fourteen-day culture in vitro suggested that osteochondral tissue regeneration could be successfully attained when the proper hydrogel type was selected. The same group demonstrated another osteochondral model by depositing alginate hydrogel loaded with human chondrocytes and human MG63 osteoblasts within a PCL frame [70]. Osteoblasts and chondrocytes were supplemented with osteogenic and chondrogenic growth factors loaded in hydrogels for differentiation purposes. Another approach was conducted using acoustic-based bioprinting for the same purpose, where bioprinted nanodroplets of MSCs in TGF-β1 and BMP-2 patterns (Fig. 3b1–b4) resulted in localized differentiation of MSCs toward osteogenic and chondrogenic lineages shown by the gene expression study [71].

In addition to osteochondral models, Yu et al. demonstrated hybrid bioprinting of macrovascularized stromal tissue, where scaffold-free tissue strands made of fibroblasts were assembled around a perfusable macrovasculature loaded with smooth muscle cells extruded through a co-axial nozzle unit [72]. Tissue strands quickly fused and assembled around the macrovasculature in a week, which can be further scaled-up by extending the macrovascular network.

Other tissue types

In addition to the presented tissue types, there is some other work at the early fundamental study level for bioprinting of retinal and brain tissues. Lorber et al. presented piezoelectric inkjet bioprinting of retinal ganglion cells (RGCs) and glia, and investigated the effect of bioprinting parameters on the viability of cells and their growth-promoting properties [73]. They concluded that inkjet bioprinting did not adversely affect the cell viability and RGC neurite outgrowth, rather RGCs demonstrated further neurite growth when bioprinted on a glial substrate. Recently, Lozano et al. presented manual deposition of primary cortical neuron-laden gellan-gum-RGD for brain-like tissue fabrication [74]. Three-layer constructed tissue models, with cortical neurons encapsulated in the top and bottom layers, demonstrated axon growth and penetration toward

the cell-free middle layer in 5 days. Although no computer control motion system was applied, the presented work unveiled the first time demonstration of layer-by-layer fabrication for brain tissue engineering.

Transplantation and clinics

Bioprinting of living tissue and organ constructs has been widely studied and performance of these constructs was assessed via animal transplantation. Several bioprinted tissue types, including nerve [41], cardiac [18], blood vessel [9], bone [11] and skin [52], have been implanted into associated locations on animals to evaluate their functionality, neovascularization and anastomosis and engraftment with the host [1]. In addition, various tissue constructs were bioprinted and implanted subcutaneously to assess in vivo differentiation of cells and functionality of implanted tissue constructs [68]. Despite these attempts, none of the bioprinted tissues has been clinically used for humans because no approval has been granted from the FDA yet. There are no regulations laid down for bioprinters or bioprinted products; however, with the increasing global interest and emerging businesses in the growing bioprinting market, the success with the first technology going through FDA regulations will be exemplary for preceding technologies and products. For details of the regulatory concerns of bioprinting, the reader is referred to our recent article [75].

Although bioprinting technology is still in its infancy in clinics, 3D-printed plastic, ceramic or metallic implants for bone tissue replacement [76] have been successfully transplanted into humans. In addition to permanent implants, a recent work published in New England Journal of Medicine [77] demonstrated a unique case of transplantation of a 3D-printed bioresorbable airway splint into an infant. The institutional review board of the University of Michigan consulted with the FDA and approved the use of a 3D-printed device under the emergency-use exemption and the written consent of the patient's parents. No unforeseen problems have been observed with the splint and full degradation of the device is expected to take around 3 years. This was an exemplary case for clinical use of 3D-printed scaffolds and hopefully will lead to similar success with bioprinted tissues and organs. Despite the accomplishments in bioprinting research, bioprinting for transplantation in a clinical setting for humans requires further advances and translational efforts. Organs and tissues that do not need significant vascularization (i.e. skin and cartilage) are expected to be translated into clinical use sooner. Tissues and organs that are metabolically highly active (i.e. heart, pancreas and liver) are immensely challenging. No bioprinting technology so far facilitated the fabrication of a vascular hierarchical network spanning arteries and veins down to capillaries. Because it is difficult to bioprint capillaries at the submicron scale using the current technology, an alternative could be to bioprint macrovasculature and then leave nature to create capillaries by itself. Bioprinted vascular networks should be designed and fabricated in a way that they can be easily sutured to a blood vessel in a host, and possess certain properties such as enough mechanical strength to satisfy burst pressure, sufficient intactness of endothelium to prevent thrombosis and a high patency rate to support occlusion-free circulation [78].

The other approach in facilitating vascularization is in situ bioprinting of tissue and organ constructs directly into the defect sites in surgery settings rather than bioprinting tissue constructs,
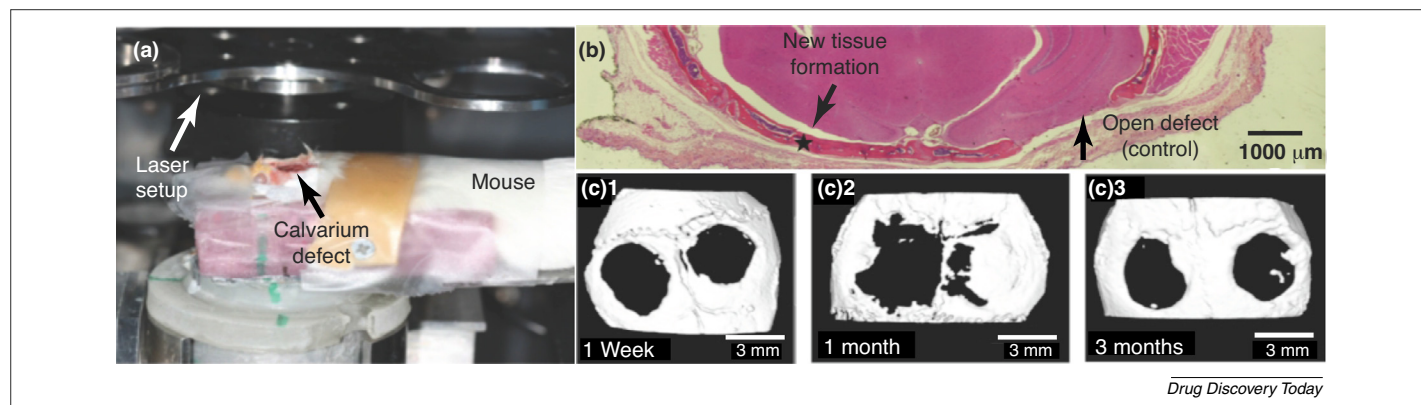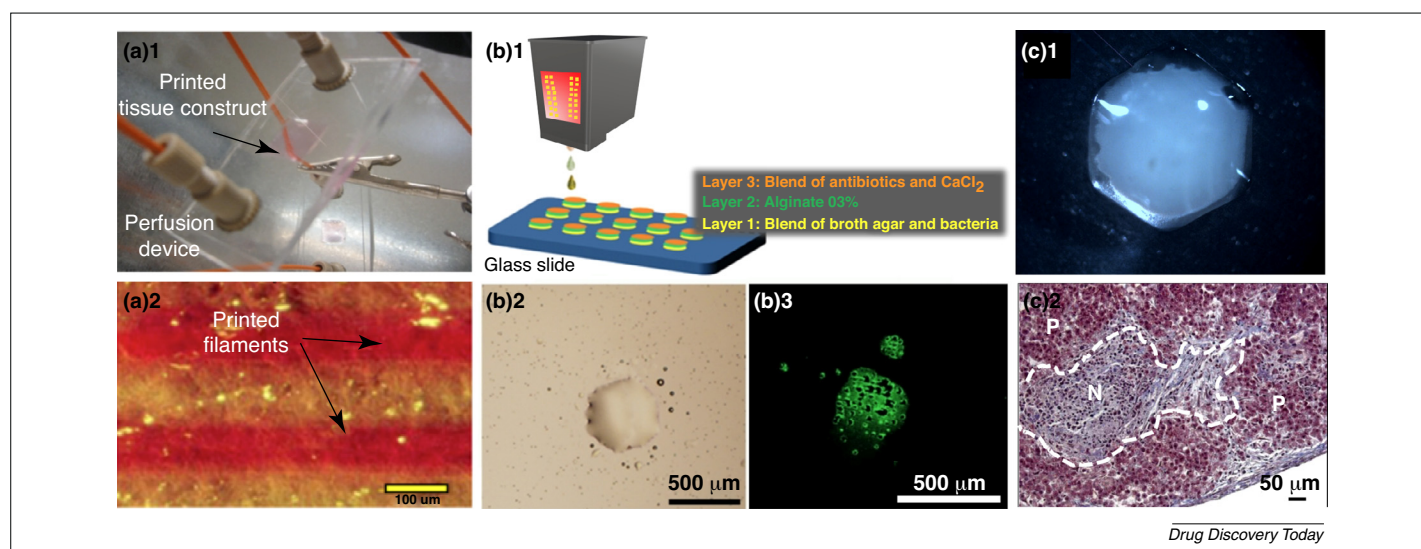
FIGURE 4

(a) Laser-assisted particles into critical-size calvarium defects in mouse model. **(b)** Histology results of 3-month *in vivo* culture revealing new tissue formation (marked with star) as opposed to an empty defect. **(c1)** X-ray microtomography images of bone tissue formation **(c1)** in 1 week, **(c2)** 1 month and **(c3)** 3 months; adapted, with permission, from [51].

and maturing and assessing them *in vitro* before transplantation. With *in situ* bioprinting, bioprinted tissue constructs can recruit endothelial cells from the host and facilitate neovascularization followed by anastomosis of newly formed vessels with the vascular network of the host. Therefore, *in situ* bioprinting has a great advantage over the traditional two-step bioprinting approach and can be applied for regeneration of a wide array of tissues and organs (e.g. maxillo- and cranio-facial reconstruction, plastic surgery, skin tissue and flap tissue). *In situ* bioprinting has been recently used in skin regeneration for large wounds on porcine models [53] and calvarium defects in rodents [1,11]. Fig. 4a demonstrates laser-assisted printing of n-HA particles into crucial size defects on a mouse model, where one of the defects remained empty for the control group. The histology and microtomography results for bone regeneration were not consistent (Fig. 4b,c), which

was because of immobilization of printed n-HA particles within the defects. Although the ink in the demonstrated work did not comprise any biologics, it unveiled the application of laser-based bioprinting systems in operating rooms, which will enable translation of bioprinting technologies from bench to bedside.

One of the controversies of the clinical translation potential of bioprinted tissues and organs originates from the bioprinting technology itself because bioprinting involves living cells in bioink and the use of patient-specific cells is fairly new in bioprinting. Stem cells, such as embryonic stem cells and induced-pluripotent stem cells [79], have been potential unlimited sources of patient-specific cells for fabrication of tissues and organs. Patient-specific cells can be differentiated and then bioprinted or bioprinted and then differentiated toward multiple lineages to fabricate tissues and organs that will contribute minimum immunogenicity risk.

FIGURE 5

Bioprinted tissue models for drug testing and high-throughput screening. **(a1)** Bioprinted liver tissue model loaded into a perfusion chamber; adapted, with permission, from [83]. **(a2)** Where HepG2-laden alginate filaments were patterned allowing media flow for drug testing; adapted, with permission, from [84]. **(b1)** A schematic showing inkjet-bioprinting of three-layer constructs, where agar and bacteria were bioprinted on a glass slide followed by printing of alginate and a blend of antibiotics and CaCl$_2$, **(b2)** light-microscopy and **(b3)** fluorescence imaging of the bioprinted samples; adapted, with permission, from [85]. **(c1)** A 3 × 3 mm bioprinted liver tissue model for drug testing with **(c2)** hematoxylin and eosin stain showing parenchymal (P) and nonparenchymal (N) regions (image courtesy of Organovo).

Pharmaceutics and high-throughput screening

Drug discovery entails a time-consuming and costly endeavor that requires substantial investment in financial and human resources. Despite continuing efforts to improve the productivity of the drug development process, only one out of an estimated 10,000 new chemical entities and one out of ten drug candidates entering clinical trials reaches the final approval stage and enters the market [80]. Improving the ability to predict the efficacy and toxicity of drug candidates earlier in the drug discovery process will speed up the translation of new drugs into clinics. Recent attempts in 3D *in vitro* assay systems is an ideal way to resolve this bottleneck because 3D tissue models can closely mimic the native tissue and have the capability to be used in high-throughput assays because they can be fabricated in microarrays. Among various methods for engineering 3D *in vitro* systems [81], bioprinting has superiorities such as controllability on size and micro-architecture, high-throughput capability, co-culture ability and low-risk of cross-contamination. Bioprinted tissue and organ models have been increasingly considered for the potential of pharmaceutics use such as drug toxicology and high-throughput screening [75].

In the literature, liver and tumor tissue have been a primary focus in fabrication of tissue models for pharmaceutics. Sun's group investigated the fabrication of a bioprinted liver micro-organ model for drug metabolism [82] (Fig. 5a1). In that study, an automated syringe-based direct cell-writing process was applied for extrusion of hepatocyte (HepG2)-encapsulated alginate strands as demonstrated in Fig. 5a2. Polydimethylsiloxane (PDMS) elastomer soft lithography was combined with a micro-molding technique to fabricate 3D microfluidic chambers housing aforementioned constructs. The presented 3D liver micro-organ with a sinusoidal flow pattern was an *in vitro* 3D micro-fluidic, micro-analytical, micro-organ (3DM) device for simulation of the physiological liver response to drug administrations and toxic chemical exposure. Effective drug metabolism in the micro-liver chamber was demonstrated by metabolizing a nonfluorescent pro-drug, 7-ethoxy-4-trifluoromethyl coumarin (EFC), to an effluent fluorescent metabolite 7-hydroxy-4-trifluoromethyl coumarin (HFC). Additionally, they schemed and created dual-microtissue microfluidic chips, which were connected to facilitate multicellular interaction and downstream effects of metabolism on the target tissue [5,83]. Epithelial cells and hepatocytes encapsulated in Matrigel™ were used to show the path of drug diffusing from the bloodstream to the tissue because epithelial cells are the cells lining the lumen through which the drugs pass from bloodstream to the target, where hepatocytes were used as the target cell type. The anti-radiation drug amifostine was used as a pro-drug, which can be converted to an active form by epithelial cells. The results showed that the percentage of radiation-damaged cells for the single-tissue was more than twice the dual-tissue. Based on the presented tissue model, Tourlomousis and Chang developed a computational macroscale model for such *in vitro* tissue models using a convection-diffusion-cell kinetics numerical framework, which is helpful for future research in 3D micro-organ pharmacokinetics and toxicity [84].

Emerging microengineering technologies enable versatile fabrication of 3D cell-based microarrays including soft lithography, surface patterning, microfluidic-based manipulation and bioprinting [81]. Among them, bioprinting technology has the advantage of generating co-culture and single-cell array with precise control on cell density in a high-throughput manner. A recent study developed a novel inkjet-based bioprinting method for assembling a high-throughput miniature drug-screening platform as presented in Fig. 5b1 [85]. The authors applied a modified HP model 5360 compact disc printer with picoliter per droplet resolution and bioprinted *Escherichia coli*-laden alginate to array a chip on coverslips (Fig. 5b2,b3). Droplets of three antibiotics were printed on the spots of cells in a layer-by-layer fashion. Results demonstrated similar cell viability, functionality and antibacterial effects of antibiotics in inkjet bioprinted and micropipetted samples, which confirmed that inkjet-bioprinted high-throughput arrays is an effective method to minimize the duration of typical drug screening tests. Demirci's group presented cell-based biosensors (CBBs), where acoustic-based bioprinting, studied by the same group earlier for high-throughput screening purposes with various cells (including mouse embryonic stem cells, fibroblasts, AML-12 hepatocytes, human Raji cells and HL-1 cardiomyocytes) [86], was used to create a microarray of smooth muscle cell droplets in collagen [87]. The bioprinted microarray was then stimulated with different environmental conditions (e.g. temperature) and the effect of the applied stimuli as well as the bioprinting process parameters on cell viability was evaluated. With the bioprinted CBBs, the effects of analytes, such as pathogens, contaminants, toxins and drug candidates, on living systems can be obtained [87]. Besides 3D bioprinting of high-throughput cell microarrays, controlled delivery of drug candidates into cell microarrays is also a key factor for successful drug screening. Various methods have been developed for controlled drug delivery onto cell microarrays, including drug patterning, drug stamping, aerosol sprays and microfluidic drug loading [81].

Recently, bioprinting companies have developed bioprinted tissue models for high-throughput drug screening [88,89]. In November 2014, Organovo began offering its 3D-printed exVive3D™ liver tissue models to screen drugs for liver toxicity. A scaffold-free tissue bioprinting approach was performed using NovoGen MMX Bioprinter™, where pellets of co-cultured human hepatocytes, hepatic stellate and endothelial cells were bioprinted into a temporary mold structure with building units in a hexagonal shape (Fig. 5c1) [90]. After bioprinting, cells further aggregated and the tissue construct matured toward a native-like tissue model (Fig. 5c2), which maintains normal function for at least 42 days. Formation of microcapillaries took place at certain locations. The bioprinted tissues were characterized by ATP production and secretion of liver-specific albumin protein. The results revealed that ATP production increased over time during a 4-week culture period and ELISA results showed that albumin secretion increased after fabrication and stabilized in 21 days. One pharmaceutical partner recently employed Organovo to test two of its drugs within the same class. One of the drugs has been used for years and was considered safe; the other failed in a late Phase III clinical trial owing to liver toxicity that did not show up in either cell culture or animal studies. Organovo's system demonstrated very clear signals confirming the commercial drug's safety and the failed drug's toxicity. Other companies, including Aspect Biosystems from Vancouver, Canada, and Texas-based Nano3D Biosciences, are also developing technology for a similar purpose.

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REVIEWS

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TABLE 1

Applications of bioprinting technologies

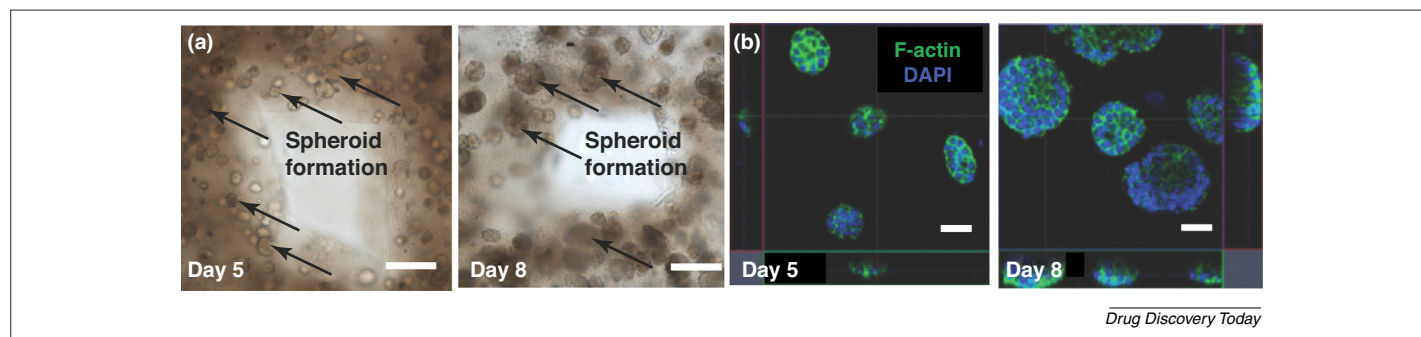| Application	Tissue type	Cell types bioprinted	Bioink or substrate used	Bioprinting modalities used	Bioprinters used	Remarks
Tissue engineering and regenerative medicine	Bone	Bone-marrow-derived human mesenchymal stem cells [10]; endothelial progenitor and multipotent stromal cells [12]; primary-muscle-derived stem cells [13]	PEGDMA [10]; nano-HA slurry [11]; Matrigel™ and alginate [12]; BMP2 and fibrin (substrate) [13]	Thermal inkjet [10]; laser-induced droplet ejection [11]; extrusion-based (pneumatic) [12]; piezoelectric drop-on-demand [13]	Hewlett-Packard deskjet [10]; HT-BioLP workstation [11]; Bioplotter [12]; MicroJet™ [13]	Bioprinting bone tissue for critical-size defects is currently feasible but bioprinting of scale-up vascularized bone tissues still remains elusive
	Cardiac	Cardiac cells and HUVECs [16]; primary feline adult cardiomyoctyes and HL1 cardiac muscle cells [17]; HUVEC and hMSC [18]; human cardiac-derived cardiomyocytes progenitor cells [19]	Tissue spheroids and collagen type-I (biopaper) [16]; alginate [17,19]; PU [18]	Extrusion-based bioprinting (mechanical) [16,19]; thermal inkjet [17]; LIFT [18]	nScrypt [16]; HP DeskJet 550 printers [17]; custom laser bioprinter [18]; BioScaffolder [19]	Because cardiac cells do not have proliferation capability, scaffold-free bioprinting with high cell density is advantageous
	Cartilage	Human chondrocytes [22]; rabbit elastic chondrocytes [24]; bovine articular chondrocytes [25]; calve articular chondrocytes [26]; human nasoseptal chondrocytes [27]	PEGDMA [22]; fibrin-collagen type I [24]; alginate [25,26]; alginate/nano-cellulose [27]	Thermal inkjet [22]; solenoid inkjet [24]; extrusion-based (pneumatic) [25]; extrusion-based (micro-valve) [27]; extrusion-based (mechanical) [26]	HP Deskjet 500 printer [22]; XYZ plotter [24]; MABP [25]; Fab@home [26]; regenHU [27]	Considerable work has been performed; however, zonally stratified articular cartilage is still a challenge and a great need in clinical use
	Heart valve	Aortic root sinus smooth muscle cells and aortic valve interstitial cells [32]; aortic valvular interstitial cells [33]	PEGDA and alginate [30]; alginate and gelatin [32]; Me-Gel [33]	Extrusion-based (mechanical) [30,32,33]	Fab@home [30,32,33]	Although anatomically accurate tissue models have been bioprinted, no performance evaluation has been done *in vivo*
	Liver	hiPSCs and human embryonic stem cells [35]; HepG2 [36]	Alginate [35]; GelMA [36]	Valve-based inkjet [35]; extrusion-based (mechanical) [36]	Custom cell printer [35]; NovoGen MMX Bioprinter™ [36]	Limited progress has been made in bioprinting of liver tissues for regenerative medicine, and patient-specific cells with long-term viability is still a concern
	Lung	Endothelial and epithelial cells [38]	Matrigel™ (substrate) [38]	Valve-based inkjet [38]	BioFactory® [38]	Although lung is hollow and reasonably easy regarding survival compared with other organ types, human airway models for cytotoxicity testing seem to be on the near horizon
	Neural	Murine neural stem cells [39,40]; Schwann cells and BMSCs [41]	Collagen type-I (substrate) [39]; polyurethane [40]; cell pellet and agarose (support) [41]	Microvalve-based inkjet [39]; extrusion-based (mechanical) [40,41]	Custom 4-head dispenser [39]; NovoGen MMX Bioprinter™ [41]	Nerve grafts are commercially available for short damages but bioprinting has the capability to generate longer counterparts
	Pancreas	INS1E β-cells, mouse islets and human islets [43]	Alginate and alginate/gelatin [43]	Extrusion-based (pneumatic) [43]	BioScaffolder [43]	Beta cell source, its long-term functionality and viability and availability of associated cells are still a challenge
	Skin	Human foreskin fibroblast and HaCaT keratinocytes [49]; HaCaT keratinocyte cells and NIH 3T3 fibroblasts [50,51]; human dermal microvascular endothelial cells [52]; amniotic fluid-derived stems [53]	Collagen type-I [49]; collagen type-I on Matriderm™ (substrate) [50,51]; thrombin [52]; collagen/fibrinogen and thrombin [53]	Microvalve-based inkjet [49,53]; LIFT [50,51]; thermal inkjet [52]	Custom 8-head dispenser [49]; LaBP [50,51]; Modified HP Deskjet 340 [52]; skin printer [53]	Great progress has been made in skin bioprinting but advancements are needed for further improvement in scarless tissue formation and integration of sweat glands.
	Vascular	HUVSMCs [58,98,99]; chondrocytes [55,56]; 3T3 mouse fibroblasts [59]; HUVEC [63,64] and normal human lung fibroblast [63]; human skin fibroblasts [58]	Alginate [59,98]; alginate and chitosan [55,56], alginate with carbon nanotubes [99]; fibrin [63]; collagen [64]; GelMA [36]; tissue spheroids [58]	Co-axial nozzle extrusion [55,56,98,99]; piezo inkjet [59]; valve-based inkjet [63,64]	Nordson [55,56,98,99]; Microfab [59]; custom multi-head dispenser [63,64]; NovoGen MMX Bioprinter™ [36,58]	Long-term *in vivo* efficacy of bioprinted blood vessels has not been tested yet. For organ fabrication, enabling technologies are needed to bioprint vascular network in multiscale
	Composite	3T3 fibroblasts and myoblasts [67]; MSCs [68,81] and chondrocytes [68]; osteoblast and chondrocytes [69,70]; HUVSMCs and fibroblasts [72]	Hyaluronic acid/gelatin/fibrinogen and PU [67]; alginate [68,70,72]; collagen type I and hyaluronic acid [69]; tissue strands [72]	Extrusion-based (pneumatic) [67–70,72]; acoustic-based droplet [81]	Custom multi-nozzle head [67,68,81]; MtoBS [69,70]; MABP [72]	Bioprinting of composite tissues is highly vital and a substantial progress is needed to generate organ-level constructs by integrating tissues such as bone, muscle, tendon, nerve, blood vessels and skin together

Cancer research

Two-dimensional tumor models have been widely used in cancer research; however, they do not represent the physiologically relevant environment because they lack cell–cell and cell–matrix interactions in 3D. Thus, bioprinting has offered great advantages to recapitulate the cancer microenvironment to precisely locate various cell types and microcapillaries to study cancer pathogenesis and metastasis; however, bioprinting for cancer research is new and only a few research procedures have been performed in this emerging application area [7]. Demirci's group was the first to demonstrate bioprinting of tumor tissue models for *in vitro* assays [91]. In their study, human ovarian cancer (OVCAR-5) cells and MRC-5 fibroblasts were bioprinted using an inkjet-based bioprinting platform with dual-ejectors. Multiple cell types were spontaneously bioprinted on Matrigel™ to form multicellular acini in a high-throughput and reproducible manner with a spatially mediated microenvironment with controlled cell density and cell–cell distance. The presented approach did not only demonstrate a tool for cancer research but also provided a great platform for high-throughput screening. Sun's group recently demonstrated bioprinting of HeLa cells to form cervical tumor models [92]. In this regard, HeLa cells were extruded and bioprinted in a gelatin-alginate-fibrinogen composite hydrogel in patterned-form with >90% cell viability. In 5–8 days, HeLa cells migrated toward each other and formed cell aggregates within hydrogel filaments (Fig. 6) as opposed to the control groups, where cells in 2D culture formed cell sheets with lower chemoresistance and lower level expression of metalloproteinase. Although these two studies presented biofabrication of tumor spheroids using bioprinting technology, fabrication of larger tissue models to study cancer cell migration and metastasis is also important. Huang et al. [93] demonstrated a laser-based 3D projection printing system to bioprint HeLa cells and noncancerous 10 T1/2 fibroblasts in PEGDA along with microvascular network with channel widths of 25, 45 and 120 μm to reflect blood vessel diameters. The results revealed that bioprinted fibroblasts were not affected by the morphology of the channel width; however, HeLa cells migrated significantly when the channel diameter decreased. In addition to scaffold-based approaches, Organovo demonstrated scaffold-free bioprinting of a breast cancer model using the NovoGen Bioprinting™ platform, where cancer cells were surrounded by a physiologically relevant stromal milieu comprising MSC-differentiated adipose cells, mammary fibroblasts and endothelial cells [94]. Histomorphological analysis revealed that bioprinted neotissues were viable for 2 weeks *in vitro* with a clear compartmentalization of adipose, stromal and epithelial components with localized signs of microcapillary formation. The effects of the chemotherapeutic drug tamoxifen were assessed for viability by ATP luciferase assay and it was concluded that isolated 2D cancer cells were more susceptible to tamoxifen-induced toxicity than cells incorporated into 3D tissue models when treated with the same dose of the drug for the same period *in vitro*. Table 1 summarizes the application areas of bioprinting technology and lists the tissue types that have been successfully bioprinted along with the information of cell and bioink types, bioprinting modalities and bioprinters used in fabrication of each tissue type.

TABLE 1 (Continued)

Application	Tissue type	Cell types bioprinted	Bioink or substrate used	Bioprinting modalities used	Bioprinters used	Remarks
Pharmaceutics and drug testing	Liver	HepG2 [82,83]; epithelial cells and hepatocytes [5]; human hepatocytes, hepatic satellite cells and endothelial cells [90]	Alginate [82,83]; Matrigel™ [5]; cell pellet [90]	Extrusion-based (valve) [5,82,83]; extrusion-based (mechanical) [90]	Multi-nozzle system [5,82,83]; NovoGen MMX Bioprinter™ [90]	Bioprinted liver tissue models have a great potential in early drug discovery but a standard model is yet to be developed
	Cell droplets for high-throughput arrays	*Escherichia coli* [85]; primary smooth muscle cells from rat bladder [87]; mouse embryonic stem cells, fibroblasts, AML-12 hepatocytes, human Raji cells and HL-1 cardiomyocytes [86]	Alginate and soy agar (substrate) [85]; collagen [87]; sucrose and dextrose [86]	Thermal inkjet [85]; acoustic-based [86,87]	Modified-HP [85]; a custom acoustic bioprinter [86,87]	Picoliter size of droplets can be generated with high accuracy in droplet size and location, which is highly efficient for high-throughput arrays for drug testing
Transplantation and clinics	Bone, cartilage and skin	AFS and bone-marrow-derived MSCs [53]; bone marrow stem cells [1]	PCL [77]; nHA [11]; collagen-fibrin [53]; alginate and Pluronic®-collagen [1]	Laser-based printing [77]; laser-based bioprinting [11]; piezo-inkjet [53]; extrusion (pneumatic) [1]	EOS P 100 Formiga system [77]; HT-BioLP workstation [11]; custom inkjet printer [53]; MABP [1]	Only transplantation of a splint (using a non-bioprinting technique) into a human and *in situ* bioprinting on animal models has been achieved in operating rooms
Cancer research	Ovarian cancer	Human ovarian cancer cells and MRC-5 fibroblasts [91]	Matrigel™ (substrate) [91]	Solenoid-valve ejection [91]	A custom dual-head bioprinter [91]	Only a technological platform has been demonstrated so far but bioprinting of a biomimetically developed ovarian cancer model is yet to be researched
	Cervical cancer	HeLa [92,93]; 10 T1/2 fibroblasts [93]	Gelatin/alginate/fibrinogen [92]; PEGDA [93]	Extrusion-based (mechanical) [92] and laser-based projection printing [93]	Cell assembly system I [92]; DMD-PP [93]	Only a very few attempts, at the basic research level, have been made for bioprinting of cervical cancer models
	Breast cancer	MSC differentiated adipose cells, mammary fibroblasts and endothelial cells [94]	Cell pellet [94]	Extrusion-based (mechanical) [94]	NovoGen MMX Bioprinter™ [94]	Further substantial development is needed to use the bioprinted breast cancer model for cancer screening and drug testing

Abbreviations: PEGDMA, poly(ethylene glycol) dimethacrylate; BMP2, bone morphogenetic protein 2; hMSC, human bone marrow-derived mesenchymal stem cells; LIFT, laser-induced forward transfer; MABP, multi-arm bioprinter; PEGDA, poly(ethylene glycol) diacrylate; hiPSCs, human-induced pluripotent stem cells; GelMA, gelatin methacryloyl; BMSCs, bone marrow stem cells; NIH, The National Institutes of Health; HUVSMCs, human umbilical vein smooth muscle cells; MSCs, mesenchymal stem cells; HepG2, human liver carcinoma cells; PCL, polycaprolactone; nHA, nano-hydroxyapatite; MRC, human fetal lung fibroblast cells; DMD-PP, digital micromirror device-projection printing.

FIGURE 6

Bioprinted tissue models for cancer research. Cervical tumor model: **(a)** phase-contrast images showing bioprinted HeLa cells forming spheroids within gelatin-alginate-fibrinogen on day 5, where spheroids got larger with further aggregation and proliferation of cells on day 8; **(b)** immunofluorescence images showing f-actin and (4′,6-diamidino-2-phenylindole) DAPI (nuclei) of the forming aggregates on days 5 and 8; adapted, with permission, from [92].

Concluding remarks and future outlook

This paper has introduced bioprinting technology and presented a first-time comprehensive review of its application areas including basic research in tissue engineering, regenerative medicine and cancer pathogenesis, tissue bioprinting for transplantation and clinics, and some recent efforts in beginning translational technologies in pharmaceutics for drug testing and high-throughput screening. Currently, there are around 15 different tissue types under experimentation in the bioprinting field; however, there exist several other tissues in the human body that have not been touched yet. In this regard, diverging the focus into other tissue and organ models that are challenging but can revolutionize medicine is necessary in the near future. This also depends on the advances in the tissue engineering field because bioprinting research vitally depends on our understanding of unexplored tissue types. In addition, bioprinting of new types of organs, such as bionic organs or organs that do not exist in nature, will be one of the new directions in bioprinting.

The majority of bioprinting research has evolved around homo-cellular tissue construct using a single cell type; however, native tissues have a heterocellular nature with multiple cell types patterned in a highly complex anatomy. Although bioprinting-simplified tissue models are relatively acceptable in basic research, functional tissue fabrication for clinics or pharmaceutics necessitates inclusion of multiple cell types because some of the functionality of cells can be enabled or further boosted by cell–cell interactions. Bioprinting multiple cell types requires further understanding of the optimum culture conditions including the right medium and reagents to support their growth and behavior.

Current efforts in translation of bioprinting to transplantation are mainly limited to murine models. Murine models are small and physiological conditions of the animals are not closely relevant to that of humans. For example, a small representative pancreatic tissue with bioprinted islets can be implanted into a diabetic murine model and can regulate insulin secretion, but the average volume of islets in a mouse pancreas is approximately 100,000 times smaller than that in a human pancreas [95,96]. Therefore, bioprinting of scale-up tissues and organs is highly important to fabricate them at clinically relevant dimensions. Larger animals such as a porcine model can be a transitional step toward trials on humans because these models represent human physiology more closely than small animals [97]. To scale-up bioprinted organs for clinical use, bioprinting of hierarchical vascular networks is vital in addition to their mechanical strength and elasticity, and long-term structural stability [98,99]. In addition, integration of nerve tissues and establishing innervation is a vital step toward functional tissue and organ fabrication because tissues such as cardiac, muscle and skin need innervation, which is currently a challenge in bioprinting. The majority of bioprinting research entails the use of hydrogel-based bioinks as a result of their favorable environment for 3D growth of cells [100]; however, hydrogels are weak in mechanical properties when they are used at low concentrations to support cell proliferation. In addition, biodegradable materials such as thermoplastics can be used as a supporting frame for the scale-up of tissues and organs. Current biomaterials using synthetic polymers are strong but their degradation takes a prolonged time and does not match with the tissue regeneration process closely. Therefore, new biomaterial development is crucial for the advancement in bioprinting of tissues and organs for clinical use. In addition to biomaterials, selection of the right cell source is also a crucial factor in translation of bioprinting technologies into clinics and pharmaceutics. For personalized medicine it is challenging to acquire different primary cell types. Stem cells stand as a promising source of cells and further advances are needed to establish standard protocols for differentiation of stem cells into various stable and functional cell lineages.

Miniature 3D-array platforms such as tissue-on-a-chip or organ-on-a-chip models are a prominent way of tissue fabrication for high-throughput screening. Combination of bioprinting technology with other technologies such as microfluidics is needed for fabricating physiologically simulated, complex and long-term viable target micro-organ arrays. Technologies generating picoliter droplets with low process induced cell damage with single cell sorting capabilities should be encouraged in bioprinting of cell microarrays. Controlled drug delivery onto cell microarrays should be elaborately considered and implemented.

Despite the recent attempts in engineering cancer microenvironment using bioprinting technology, further advancements are needed for fabrication of a physiologically relevant complete microenvironment, comprising tumor site, healthy site and microvascular network in between, to study cancer metastasis research. Tumor tissue models should be precisely placed within bioprinted vascularized parenchymal tissues to study tumor growth, and tumor cell intravasation and extravasation.

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