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3D Bioprinting Stem Cell Derived Tissues

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Abstract—Stem cells offer tremendous promise for regenerative medicine as they can become a variety of cell types. They also continuously proliferate, providing a renewable source of cells. Recently, it has been found that 3D printing constructs using stem cells, can generate models representing healthy or diseased tissues, as well as substitutes for diseased and damaged tissues. Here, we review the current state of the field of 3D printing stem cell derived tissues. First, we cover 3D printing technologies and discuss the different types of stem cells used for tissue engineering applications. We then detail the properties required for the bioinks used when printing viable tissues from stem cells. We give relevant examples of such bioprinted tissues, including adipose tissue, blood vessels, bone, cardiac tissue, cartilage, heart valves, liver, muscle, neural tissue, and pancreas. Finally, we provide future directions for improving the current technologies, along with areas of focus for future work to translate these exciting technologies into clinical applications.

Keywords—Pluripotent stem cells, Biomaterials, Bioinks, Regenerative medicine, Tissue engineering, Drug delivery, Controlled, Stem cell niche.

INTRODUCTION

The Role of 3D Printing in Tissue Engineering

Tissue engineering combines biomaterial scaffolds, cells, and biomolecules to restore, maintain, or improve injured tissues or even whole organs.[152] The Food and Drug Administration has already approved tissue-engineered skin and cartilage for limited clinical applications.[167] Early tissue engineering strategies included fabricating complex scaffolds followed with cell seeding.[64,95] Current strategies aim to minimize the complexity of the fabrication process, delivering structural support and cells simultaneously through either scaffold-based designs or scaffold-less designs.[46,87,88,126,137,155] 3D printing holds immense promise for the field of tissue engineering as it provides a rapid and robust approach for assembling functional, viable tissue *in vitro*.[42,71,87,88,175] Generating functionally viable tissues-in-a-dish requires a specific niche and micro-architecture that should provide structural and mechanical support, sufficient nutrient supply, the required cell types, and the ability to remodel and integrate with the host once implanted.[94,103,145,151,163] 3D printing can effectively assemble all of these necessary components through the use of biomaterials, printing techniques, and cell delivery methods (Fig. 1).

3D bioprinters create cell patterns within defined spaces while simultaneously preserving cell function and viability.[182] This process usually has two important components namely the 'bioink' or materials which mimic an extracellular matrix (ECM) environment for supporting cell adhesion, proliferation and differentiation, and biopaper.[131] Normally the cells being printed are dispersed throughout the bioink, which is often generated from a hydrogel.[130] 'Biopaper', which serves as the other major component, is the substrate or coating on which the specific patterns are deposited using the bioprinter with the bioink.[130]

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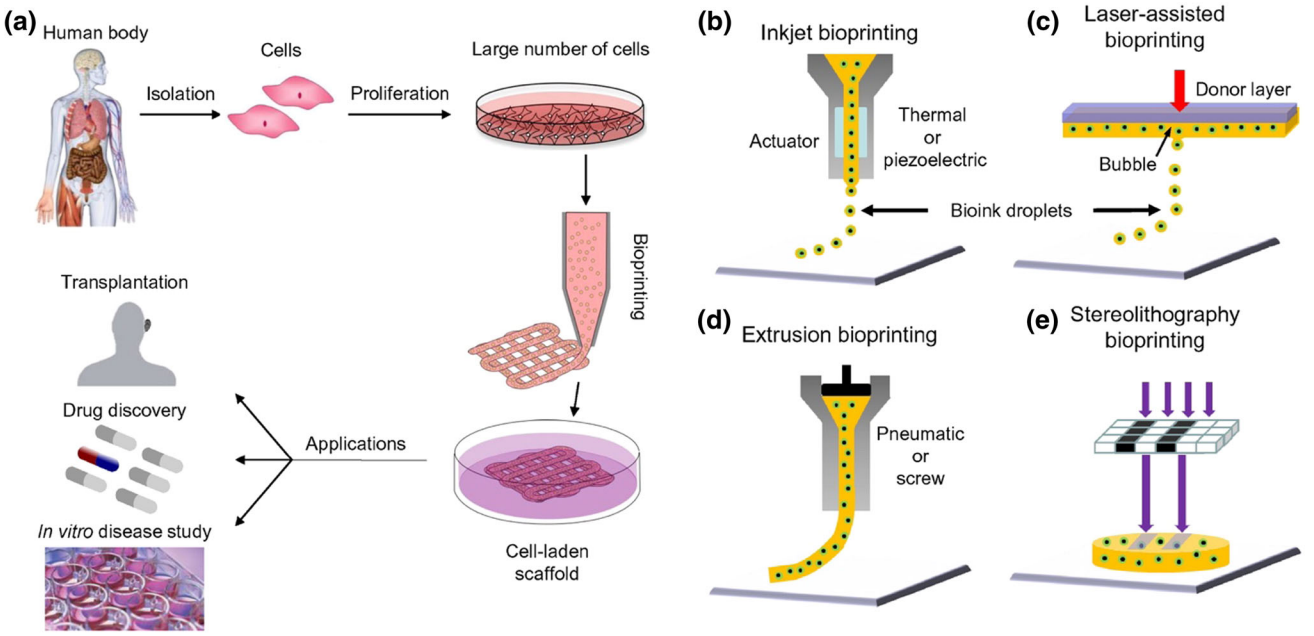

FIGURE 1. Bioprinting process, techniques, and applications. (a) For human therapeutic applications, the typical workflow of bioprinting would involve the isolation and expansion of human cells prior to printing the desired cell-laden scaffold. These scaffolds could then ultimately be used as therapeutic devices themselves, as a testing platform for drug screening and discovery, or as an *in vitro* model system for disease. (b) Inkjet printers eject small droplets of cells and hydrogel sequentially to build up tissues. (c) Laser bioprinters use a laser to vaporize a region in the donor layer (top) forming a bubble that propels a suspended bioink to fall onto the substrate. (d) Extrusion bioprinters use pneumatics or manual force to continuously extrude a liquid cell–hydrogel solution. (e) Stereolithographic printers use a digital light projector to selectively crosslink bioinks plane-by-plane. In (c) and (e), colored arrows represent a laser pulse or projected light, respectively (adapted from with permission from Ref. 121).

Other commonly used techniques for bottom-up assembly of tissue-in-a-dish include 2D inkjet printing,[17] which can generate a variety of tissues *in vitro*, such as skin[190] and nerves.[6,29,65,131] Soft tissue engineering has used both synthetic biodegradable polymers[64,95] and natural polymers[101,185] for printing using layer-by-layer freeform fabrication methods, such as 3D blotting. High concentrations of cells and cell spheroids (i.e. cellular aggregates) have been proposed as bioink for dispension-based printers, relying on the biophysical principles governing cellular self-assembly.[127,137] Other than cells and their aggregates, hydrogels are often used as carriers for the former in formulating bioinks. Many different cell types have been used for 3D bioprinting engineered tissues.[135] In this review, we have chosen to focus on stem cells for 3D printing tissues.

Utility of Stem Cells in 3D Bioprinting

Human stem cells possess the unique potential to develop into different cell types in the body.[72] Furthermore, certain stem cells reside in many adult tissues in our bodies where they serve as an internal repair system, dividing when needed to replenish other cells during disease or injury.[134] When a stem cell divides, each new cell has the potential to either remain a stem cell or become another differentiated cell with a more specialized function, such as a muscle cell, a red blood cell, or a brain cell. Excellent reviews exist in the field that focus on the role of stem cells in research.[132] Since stem cells possess the novel capabilities of self-proliferation and targeted differentiation, they offer significant benefits compared to other cell types used, when bioprinting.[88] Adult stem cells obtained from the patient have the advantage of alleviating immune rejection.

The three commonly used stem cell types in tissue engineering include mesenchymal stem cells (MSCs), embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs). MSCs, which are adult multipotent stem cells often isolated from the bone marrow or adipose tissues, have the potential to differentiate into osteoblasts, adipocytes or chondroblasts *in vitro*. However, they can also become other cell types, including myocytes, tendocytes, ligament cells, smooth muscle cells, endothelial cells, cardiomyocytes, hepatocytes and neural-like cells.[136] Table 1 lists common sources of MSCs. MSCs possesses several advantages, as large quantities of these cells can be obtained from adipose tissues.[115] MSCs derived from the amniotic

TABLE 1. Mesenchymal stromal cells (MSCs) in tissue engineering (adapted from Ref. 141).

Source	Advantages	Disadvantages
Bone marrow	Relatively easy acquisition *In situ* recruitment Well-characterized	Donor morbidity Limited proliferative potential Fewer cells compared to other sources Cell number Related to age and health of donor
Adipose tissue	Easy acquisition Well-characterized	Donor morbidity (due to anesthesia)
Oral cavity MSCs (dental pulp, periodontal ligament)	Abundant Easy acquisition	Not well-characterized
Skin	Abundant Minimal donor morbidity	Not well-characterized
Periosteum	Well-characterized *In situ* recruitment Can be co-seeded with bone marrow-derived stem cells	Cell number and activity related to donor age

fluid can be cultured for a sustainably long period *in vitro*.[156] Unlike ESCs and iPSCs, they do not show tumorgenicity *in vitro*, meaning undifferentiated MSCs can be used for allograft implantation.[36,156] Moreover, undifferentiated MSCs possess immunomodulatory properties that can reduce the risk of graft rejection following transplantation.[58]

Both ESCs and iPSCs can differentiate into somatic cells of all three germ layers: ecto-, meso- and endoderm.[188] Thus, they offer greater multipotency than adult stem cells. ESCs originate from within the inner cell mass of a blastocyst while iPSCs represent somatic cells that have been reprogrammed into pluripotency.[161,162] Concerns with ESCs include the controversy over their derivation from embryos and the limited number of cell lines, which could induce an immune response depending on the patient. iPSCs offer a feasible alternative to ESCs without the ethical and immunogenic drawbacks of the latter.[188] Yamanaka *et al.* were the first to generate iPSCs from fibroblasts by introducing four transgenes using retroviral transfection: Oct 3/4, Sox2, Klf4, and c-Myc.[162] More complex cocktails of proteins, peptides, chemicals and other factors have been developed for greater reprogramming control in recent years. The clinical use of both ESCs and iPSCs has been challenging due to the risk of *in vivo* teratoma formation, resulting from the presence of residual undifferentiated cells. Removing undifferentiated cells prior to implantation can help improve the anticipated outcome.[128] The use of iPSCs is also linked to carcinoma generation, due to the genomic integration of a lentivirus. Virus free iPSCs are being developed to make them a more feasible, safer option.[154] Nonetheless, iPSCs have driven a paradigm shift in tissue engineering and the modelling of human disease in a dish.[70,139] Additionally, the ability to reprogram patient-specific cells can enhance our understanding of disease mechanisms and phenotypic variability. 3D bioprinting has been successfully performed using multiple stem cell types of different lineages and potency.[139,140]

Bioinks Used in 3D Bioprinting

An ideal 3D printed construct promotes growth while attracting cells, allowing them to migrate and proliferate to form functional tissues. The micro-environmental niche serves as the foremost important factor for influencing cell fate, as seen in developmental biology.[122] The ECM delivers mechanical and chemical cues, which can be assembled bottom–up *in vitro*. Hydrogels, which are commonly used as bioinks, possess several of these micro-environmental cues required for cell adhesion and growth. Bioinks distinguish themselves from traditional biomaterials such as polymer networks and foam scaffolds, due to their ability to be deposited as filaments during an additive manufacturing process. However, unlike other additive manufacturing materials such as thermoplastic polymers, ceramics and metals, they are processed under milder conditions, retaining their properties to prevent the degradation of bioactive molecules and the viability of living cells.[189] An ideal bioink possesses high shear thinning ability (printability), viscosity, gelation kinetics, biocompatibility, hydration ability and viscoelasticity.[48] Most bioinks are often adopted from existing hydrogel biomaterials, being derivatives of natural polymers like gelatin, alginate and fibrin.[48] Hydrogels are degradable, absorbable and most importantly biocompatible, providing them with significant advantages over other materials.[38,119] These materials exhibit epitopes (e.g. the peptide sequence—RGD) that promote cell adhesion and other related effects. For example, Das *et al.* cultured tissue-derived mesenchymal progenitor cells encapsulated in a unique silk fibroin–gelatin based bioink. The effect of optimized rheology, favorable amino acid sequences

of silk–gelatin bioink known to promote cell adhesion, temporally controllable gelation strategies and printing parameters led to maximum cell viability and multi-lineage differentiation of the encapsulated human mesenchymal progenitor cells.[34]

Alginate hydrogels have been used extensively as bioinks for 3D bioprinting.[90] However, native alginates possess limited biodegradation capability. Accordingly, Jia *et al*. explored the applicability of oxidized alginates with controlled degradation in bioprinting of human adipose derived stem cells (hADSCs).[90] They investigated the effects of two key material properties (i.e. viscosity and density) of alginate solutions on their printability to identify a suitable range of material properties to be applied to bioprinting. Four different formulations of alginate solutions with varied biodegradability were printed with into lattice-structured, cell-laden hydrogels. As a result, these alginate-based bioinks could modulate proliferation and spreading of hADSCs without affecting the structure integrity of the lattice structures. This research laid a foundation for the development of alginate-based bioinks for tissue-specific applications.[90] Wust *et al.* formulated a hydrogel composite including alginate and gelatin precursors which included different concentrations of hydroxyapatite (HA). It was characterized in terms of rheology, swelling behavior and mechanical properties to assess the versatility of the material towards bioprinting.[183] Human mesenchymal stem cells (hMSCs) mixed into these bioinks showed high cell viability of ~ 85% viability after 3 days of subsequent *in vitro* culture with the inclusion of HA, making it useful for tissue engineering applications.[183]

Other bioinks with unique thermogelling properties, including carboxylated agarose, allow the fabrication of scaffolds with tunable mechanical properties. Tunable bioinks can reproduce natural mechanical aspects of functional tissues.[50] Similarly, peptide bioinks containing lysine-hexapeptides self-assembled into stable, nanofibrous three-dimensional hydrogels with high stiffness values.[116] These biocompatible scaffolds were shown to support the three-dimensional culture of human stem cells. Recently, Anil Kumar *et al.* used a novel visible light induced crosslinkable gelatin-based bioink for engineering cardiac tissue.[3] The integration of such naturally derived and biodegradable polymers and stem cells with existing bioprinting techniques offers immense opportunities for tissue repair, organ transplantation, therapeutics and diagnosis.[89,110] While most bioinks are hydrophilic, Campos *et al.* developed a hydrophobic high-density fluid, perfluorotributylamine (C(12)F(27)N) bioink, which allowed hMSCs and MG-63 cells placed within 3D constructs to remain viable allowing for cell proliferation and production of ECM.[23] Thus, a wide range of biomaterials have been developed for bioprinting applications.

Current Status and Translational Concerns in 3D Bioprinting

Bioprinting combines cells with various natural and synthetic bioinks to fabricate scaffold-based or scaffold-free constructs. The desired tissue geometries are generated using computer aided design, which is then realized by using various bioprinting modalities including droplet-, extrusion-, or laser-based bioprinting to create tissue constructs. Further, some of these techniques, such as laser-based bioprinting, allow the printing of uniform sized embryoid bodies (cell clusters) that are necessary for targeted differentiation of ESCs.[37,142] Laser-induced jet formation used for bioprinting of MSCs showed high viability and high resolution of encapsulated cells.[2] Microfluidic/valve-based extrusion bioprinting methods can produce uniform sized cell aggregates.[43,186] A microvalve-based bioprinting system for the manufacturing of high-resolution, multi-material 3D-structures was reported by Blaesar *et al.*[16] This fluid-dynamics based system controlled the shear stress at the nozzle site as different levels of shear stress influenced cell viability and proliferation potential during bioprinting.[16] Distinct 3D patterns of ESCs were achieved using a combination of dielectrophoresis and stereolithographic techniques.[5] Computer assisted biofabrication of fully functional living tissue for regenerative medicine provides unique possibilities for the deposition of different living cells and biomaterials in a well-defined 3D structure.[98] The application of robotics in 3D printing allowed direct delivery of cells submerged in a hydrogel bioink using an automated robotic dispensing system which resulted in them adopting an elongated morphology in alignment with the patterned grooved substrate.[13] Since each specific bioink has its own set of advantages and disadvantages,[26] various materials and techniques are combined to maximize the benefits. Researchers have been successful in bioprinting cartilage, bone, cardiac, nervous, liver, and vascular tissues. We have chosen not to discuss skin in this review as it is often considered to be a 2D construct. One major limitation to clinical translation is building large-scale vascularized constructs. Many challenges must be overcome before this technology becomes routine in a clinical setting.[25,28,54,83,86,88,102,163–165] Here, we review specific, relevant examples of 3D printed tissues using stem cells.

DIFFERENT TYPES OF TISSUES ENGINEERED BY BIOPRINTING STEM CELLS

Adipose Tissue

Adipose tissues, the loose connective tissue that store lipids and fat in the body, contains a significant amount of stem cells as discussed previously.[51] As 3D tissue culture mimics the natural tissue architecture more accurately, 3D bioprinted adipose tissue using stem cells, can provide a deeper insight into metabolic diseases and can potentially serve as a replacement for damaged tissues. A group of German researchers used laser-assisted bioprinter (LaBP) to generate 3D grid-shaped grafts using hADSCs and an alginate-blood plasma bioink, which had no negative effect on stem cell behavior, proliferation, and differentiation, opening up the possibility for autologous tissue replacement.[66] These engineered tissues were kept in culture for 21 days and they produced lipids and expressed the adipogenic markers LPL, aP2 and PPAR-γ 2. The ability of a direct-write, pen-based bioprinter to fabricate cellular spheroid constructs using multipotent adipose-derived stromal vascular fraction (SVF) cell has also been studied.[180] These spheroids were created by crosslinking printed droplets of alginate bioink. The gauge of the needle and pressure of the direct-write printer system can be tuned to precisely control the size of the generated spheroids. The adipose SVF cell population maintained their viability and integrity for 16 days in suspension. This direct-write bioprinter can also print more complex structures by varying the needle size and the extrusion pressure, forming tube-like structures in cell suspension culture. Using 3D bioprinting to generate cell laden constructs derived from mouse MSCs and a bioink consisting of nanocellulose, alginate, and hyaluronic acid (HYA) can produce functional adipose tissues.[74] This process yields more mature tissues compared to traditional 2D culture methods. The cells showed increased lipid accumulation and gene expression of adipogenic markers PPARγ and FABP4 in comparison to cells cultured using conventional 2D methods. Adipose tissue occupies a large percentage of the salivary glands.[157] Patients suffer from dry mouth or xerostomia when the salivary glands do not function properly and it is particularly severe after radiotherapy for head and neck cancers.[47,174] These glands contain stem cells capable of producing adipose tissue as well as organoids knows as salispheres.[148] Further improvement in nanotechnology based bioprinting can develop therapeutic approaches for repair of damaged salivary glands.

Blood Vessels

Precisely patterned vascular networks transport oxygen, nutrients, and waste associated with cell metabolism.[124] Pre-vascularization can maintain tissue viability during fabrication and minimize hypoxia in tissues or organs being transplanted. Although the body's vessel system will usually invade a transplant, the time scale needed to invade lengths greater than the diffusion limit of oxygen is inadequate and thus leads to necrosis on thicker tissues.[96] The following sections focus on current vascularization methods and advances that 3D bioprinting has had on tissue engineering, which are summarized in Table 2. Figure 2 demonstrates how to create embedded channels in a matrix by printing a fugitive ink into the scaffold, which is then removed to form topographical features. The resulting hollow channels were then seeded with endothelial cells (ECs). Fugitive inks must be compatible with one another under ambient conditions and the fugitive ink removal process must not harm any of the surrounding cells or ECM. Pluronic-F-127, an aqueous triblock copolymer, is amongst one of the most commonly used sacrificial inks because it undergoes thermo-reversible gelation at around 4 °C.

TABLE 2. A summary of the vascularization techniques and cells used to promote both vasculogenesis and angiogenesis using 3D bioprinting technology.

Technique	Cells	Study	References
Pluronic F-127 fugitive ink extrusion bioprinting	hUVECs	Formation of microvascular network	99,100
Laser-Assisted Bioprinting	hUVECs, hBMSCs	Endothelial cell migration	19
3D multicellular array by laser-assisted bioprinting	ASCs, ECFCs	Cellular response to 3D environment	66
Blend bionk using double-nozzle extrusion	hUVECs, MSCs	Creation of perfusable vasculature	91
Fugitive porcine gelatin bioink with fibrin matrix	hUVECs, Normal Human Lung Fibroblasts	Capillary network formation	107
3D perfused microstructure with biologically inspired smart release nanocoating	hUVECS, hMSCs	Smart-release system for defect reconstruction	33
Tubular channel construction using coaxial nozzle extrusion bioprinting	CPCs	Formation of cell-laden and perfusable tubular channels	194

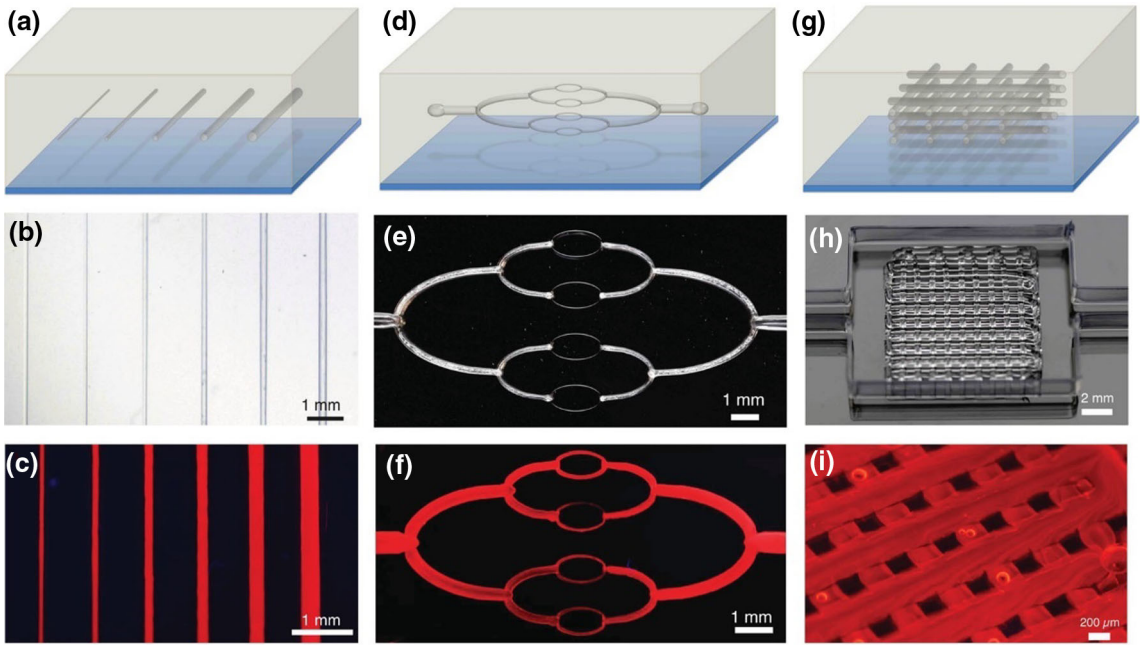

FIGURE 2. Hollow channels can be created in multiple geometries using a fugitive bioink and then subsequently perfusing the system with a liquid or cell-laden suspension (adapted from with permission from Ref. 99).

For example, the fugitive ink generated a vasculature channel system within a gelatin methacrylate (GelMA) ECM. In contrast to Pluronic-F-127, GelMA undergoes thermo-reversible gelation at 23 °C. The difference in these properties allows GelMA to hold cells while in gelatin form while Pluronic-F-127 is liquefied and removed upon cooling, allowing for construction of 100μm channels to larger diameters.[99] Due to the limitations in constructing thick tissues for clinical applications, Kolesky *et al.* have advanced this process to engineer vascularized tissues with 1 cm channels that can be perfused for over 6 weeks.[100] Cell-laden, silicone, and fugitive inks are co-printed to construct these vascularized tissues on 3D perfusion chips. The silicone ink was printed and cured, creating a custom perfusion chip where other inks are printed. Pluronic-F-127 and thrombin were printed in the desired geometry for a vascular network followed by the casting of an ECM consisting of gelatin, fibrinogen, cells, and other biological material. The deposited thrombin crosslinked these materials. The Plurionic-F-127 layer was then removed followed by seeding with ECs, forming a perfusable vessel network.

Bourget *et al.* co-printed human umbilical vein endothelial cells (hUVECs) and human bone marrow mesenchymal stem cells (hBMSCs) onto a collagen biopaper using a LaBP for vascularization applications.[19] Specifically, the study focused on the influence of cell dispersion relative to islets of deposited ECs and MSCs. The cells were printed either alone or as a 1:1 mixture in collagen droplets about 250 μm apart to form a composite 1000 μm line. ECs printed homogeneously spread throughout the collagen matrix after 24 h. Bioprinted MSCs showed negligible migration in the collagen gel. hUVECs stayed in the printed area instead of migrating when a suspension of mixed cell types was printed with the same parameters. These results suggested that ECs in their printed area would eventually form capillaries due to the stabilization of MSCs on capillaries. In another study, a multicellular array of adipose derived stem cells (ADSCs) and human endothelial colony-forming cells (ECFCs) were printed on a HYA-fibrinogen hydrogel using a LaBP.[67] Adipose stem cells migrated toward the ECFCs after 3 days, making contact. The ECFCs formed vascular networks and matured into large networks after a week. A vascular tree formed due to these cell–cell interactions. The flexibility of this bioprinting technique enables the investigation of multicellular arrays on other cell types.

Smart release systems that intelligently regulate angiogenesis and osteogenesis have been developed using 3D fabrication techniques followed by functionalization procedures to create vascularized and dynamic bone growth.[33] A bone model with blood vessels was 3D printed using a fused deposition modeling printer. Recombinant human vascular endothelial growth factor was coated on the top 5 layers and recombinant human bone morphogenetic protein was deposited on the bottom 15 layers, followed by

crosslinking to enhance the bioactivity of the scaffold. This growth distribution ensured that blood vessel formation occurred before osteogenic differentiation when co-culturing hMSCs and hUVECs, and it successfully induced angiogenesis and osteogenesis.

The flexibility of 3D bioprinting can use coaxial nozzles to create tubular constructs when engineering blood vessels.[35,76,195] Yu *et al.* demonstrated proof of principle by co-printing cartilage progenitor cells in alginate around a coaxial crosslinker material that stabilizes a hollow cylindrical structure upon printing.[194] Not only did the structures have a homogenous structural integrity, but they could be perfused with oxygenated media with no leakage. These observations can be extended to a triaxial nozzle to construct larger vessels by surrounding an inner endothelial layer with smooth muscle cells. However, this technique cannot form vessel-like structures in a submicron or nano scale. Additional challenges now lie in refining these techniques for clinical settings.

Bone

Bone tissue, known as osseous tissue, consists of osteocyte, osteoblast, and osteoclast cells forming both cortical (outer) and cancellous (inner) layers of the bone, respectively.[18,141] The interactions between these cells regulate maturation, differentiation, remodeling, and resorption of the bone tissue. The endosteum separates the cortical layer from cancellous bone with the periosteum covering the outside surface. Postnatal bone maintains its mechanical properties through renewal and remodeling.[120] 3D printing techniques can generate novel scaffolds by incorporating cells and growth factors with spatiotemporal diffusion for bone tissue engineering applications.[92] For example, inkjet bioprinting patterned bone morphogenetic protein (BMP)-2 onto a fibrin surface for directing stem cells into bone.[146] Wenz *et al.* studied polymer solutions based on GelMA and methacrylated hyaluronic acid (HYAMA) containing hydroxyapatite particles (HAp).[178] They determined that modified HAp-containing bioinks can be printed into relevant 3D geometries with micro-extrusion bioprinting (Fig. 3), improving the remodeling of the hydrogels in bone matrix development.

3D bioprinting can treat complex elbow fractures as reviewed recently.[192] 3D printing bone has potential advantages compared with the conventional surgery, such as lower blood loss, shorter surgical duration and higher elbow functionality. In a relevant example, 3D bioprinted structures consisting of 5% GelMa were generated with perfusable blood vessels to enhance cell survival and proliferation during *in vitro* maturation into bone over 21 days.[22] 3D bioprinting technology can produce biomimetic bone matrix for studying the interaction between hBMSCs and breast cancer (BrCa) cells. This model showed that the BrCa cells prevented proliferation of MSCs while MSCs improved the growth of BrCa cells.[196] In other work, a 3D Bio-

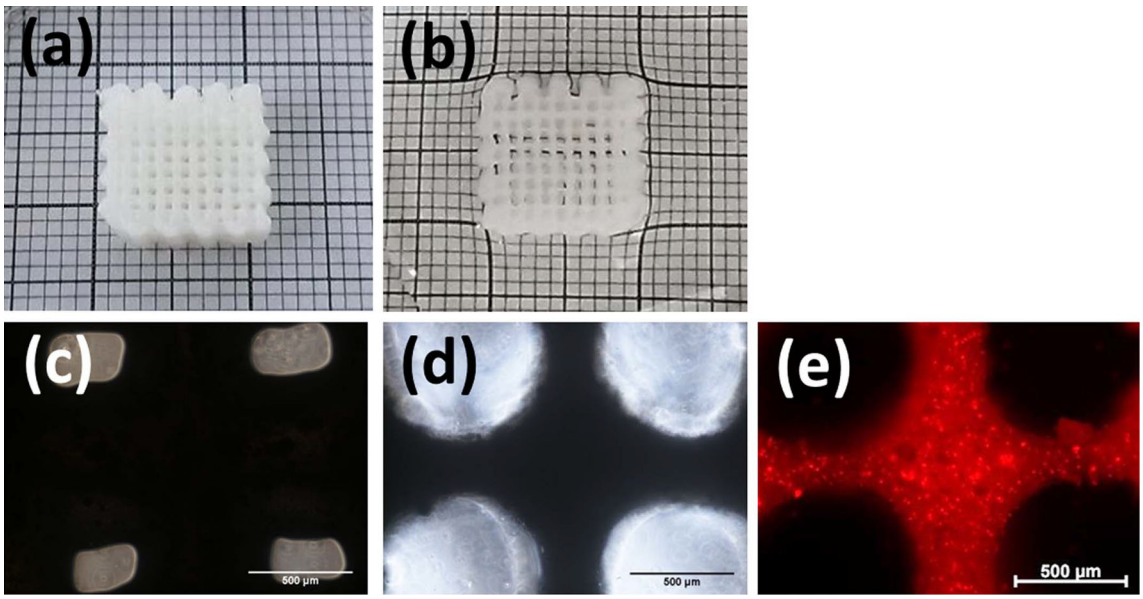

FIGURE 3. Microextrusion-printed grid structures based on cell-laden HAp-bioink. 3D grid structure with a height of 5 mm and edge length of 10 mm; (a) printing with an extrusion-based printing system and (b) after 24 h incubation in DMEM under physiologic conditions. Light microscopic evaluation of a cross-section of the grid structure; (c) directly after printing and (d) after incubation. (e) Fluorescence microscope evaluation of cell distribution in the printed structure after 24 h of culture (adapted from Ref. 178).

plotter dispensing system printed hADSCs into 3D bone tissue constructs.[176] High levels of cell viability and noticeable formation of bone matrix were observed in these 3D constructs.

3D scaffolds with highly interconnected microvascular channels and supporting structures have been successfully printed to enhance vascular cell growth and regeneration of the osteogenic bone.[79] Cross-communication of bone progenitor cells and ECs enabled the construction of a mature vascularized bone.[9] For instance, culturing human hMSCs, together with ECs in collagen gels, promoted the formation of the vasculature bone.[168] 3D bioprinting was used to fabricate function-control modules into stem cell laden methacrylamide gelatin scaffolds with high cell viability (> 90%) post printing.[39] hMSCs were printed layer-by-layer in polymerized poly(ethylene glycol) (PEG) -GelMA to develop a mechanically strong bone.[56] Acrylated peptides and PEG hydrogels were printed simultaneously with hMSCs using photopolymerization, and the resulting constructs were able to form bone tissue.[57]

Thermal inkjet bioprinting has shown promise for bone tissue engineering. hMSCs suspended in poly(-ethylene glycol) dimethacrylate (PEGDMA) were printed with bioactive ceramic nanoparticles with accurate locations in the resulting 3D structure.[55] In a different approach, bioprinted constructs consisting of MSCs encapsulated in poly(lactic acid) (PLA) and GelMA-gellan gum bioinks cultured inside spinner flasks, yield viable constructs.[111] Indirect 3D printing creates complex structures by injecting liquids into powder biomaterials, yielding biomimetic scaffolds.[105] These scaffolds supported high levels of MSC proliferation. Inkjet-based bioprinting can generate accurate low-dose presentation of modified protein patterns for directed differentiation of stem cells. For example, presentation of BMP-2 using acellular dermal matrix directs osteoprogenitors, resulting in complex bioprinted osseous structures.[158] 3D printing of drug releasing particles, such as those that deliver BMP-2, promotes osteogenic differentiation of stem cells both *in vitro* and *in vivo*.[147]

In another study, patterning human osteoprogenitors and nano-HA using LaBP was performed to study the effect of this bioprinting method on the cell micro-environment.[24] LaBP printing did not alter the physio-chemical properties of the scaffold and cell viability. Grafts containing MSCs were generated using laser-induced forward transfer (LIFT) technique.[68] These printed MSC grafts differentiated into cartilage and bone with high levels of viability. Inkjet bioprinting technology can mimic naturally occurring stem cell micro-environments, including growth factors.[146] Using this approach, printed patterns of BMP-2 seeded with muscle-derived stem cells (MDSCs) were able to differentiate into osteogenic lineages. Overall, bioprinting enables the replication of important parameters, like the distribution of mineralized and non-mineralized regions, protein fibers formation, and the interaction between interface cells, resulting in the generation of complex structures found in bone.

Cardiac Tissue

Cardiovascular disease remains the leading cause of morbidity and mortality in the world despite recent advances in cardiology and cardiac surgery.[10] Myocardial infarction, the most common cause of heart failure, occurs when plaque build-up within the arteries and veins constricts blood flow to the heart wall, resulting in ischemia and then necrosis of the cardiac muscle cells.[170] Cardiac cells possess a limited ability to regenerate, making the loss of these cells particularly devastating in terms of reducing function. These events also produce a collagenous scar tissue, compromising the contractile ability of the heart. Accordingly, there is huge interest in developing regenerative medicine that restores normal cardiac function following heart damage. Organ transplantation remains the most popular method for the treating heart disease, but donor organ supples remain limited. Mechanical methods, like ventricular assist devices, can restore the lost function, but poses the risks of strokes, excessive bleeding, infection, and device malfunction over the lifespan of the implant. Cardiomyoplasty, the process of injecting viable cells into the damaged region of the myocardium to replace necrotic cardiomyocytes, had achieved significant improvement in cardiac performance, but the lack of retention and vascularization and the migration of cells from the target location limits cell viability.

3D bioprinting can address these limitations associated with the current treatment options. Some of the most commonly used natural hydrogels for cardiac tissue regeneration include collagen, fibrin and Matrigel, while synthetic macromolecules such as PEG, polyacrylamide, poly(2-hydroxyethyl methacrylate) are also widely used.[113] The advantages of both natural and synthetic hydrogels can be combined by blending them both. Cardiac patches, 3D biomaterial scaffolds seeded with viable cells, are implanted *in vivo* over the defective region of the heart. These patches can be created from sheets of interconnected cells or the suspension of cells in a scaffold that mimics the ECM, regenerating the myocardial tissue.[15] 3D printing can be used to generate such cardiac patches (Fig. 4). Hollow fibrin gel tubes populated with neonatal cardiomyocytes exhibited normal cardiac functions suc-

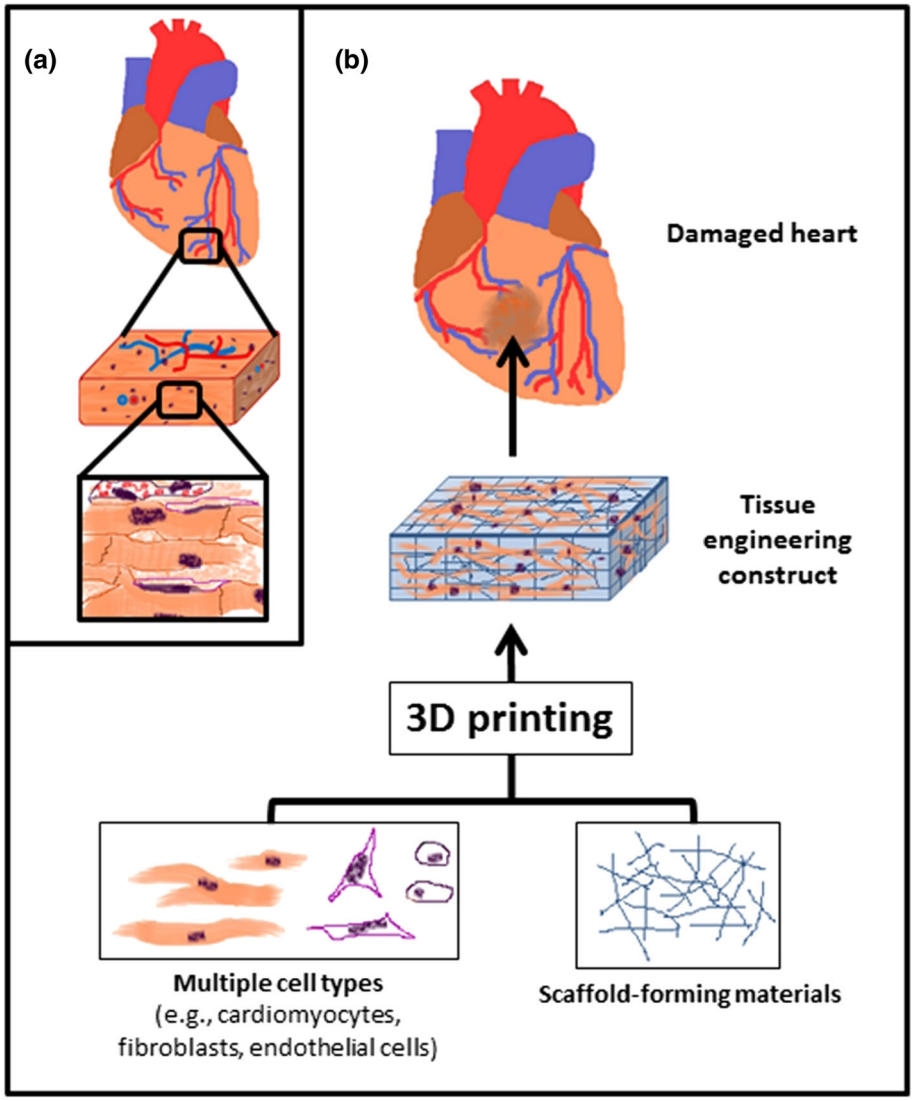

FIGURE 4. Schematic representation of 3D printing for tissue engineering applications, such as for cardiac tissue engineering. (a) Tissues are composed of multiple types of cells assembled into hierarchal structures. (b) 3D printing can be utilized to assemble functional tissue from cells and scaffold-forming materials (adopted with permission from Ref. 150).

ceeding a few weeks after implantation.[15] Huang *et al.* showed that fibrin gel infused with rat cardiomyocytes retained their contractility up to two months with a normal pacing ability.[14,73,82] Human induced pluripotent stem cells (hiPSCs) have the potential to develop engineered cardiac patches and also avoid the ethical concerns that pertain to ESCs. These cells can be differentiated into several cell types including cardiomyocytes, smooth muscle cells (SMCs), and ECs found in healthy cardiac tissues.[8,144] Ong *et al.* successfully bioprinted a scaffold-free 3D cardiac tissue by assembling multicellular hiPSC-derived cardiospheres containing cardiomyocytes, human cardiac fibroblasts (FBs) and hUVECs. These patches spontaneously beat after implantation into immunocompromised rat hearts.[140] They also showed evidence of vascularization and engraftment as indicated by confocal microscopic analysis. Currently, the density and thickness of these engineered tissues are restricted by the transport of nutrients to the cells. Only thin tissue engineered structures remain viable after implantation that can integrate with the host network.[21,166] These issues will have to be addressed before larger 3D bioprinted patches can be successfully printed.

Cardiac Valve Replacements

The aortic valve ensures the smooth and proper functioning of the cardiovascular system, including the heart, by directing the flow of blood through the aorta

and coronary arteries. Common heart valve ailments include valvular stenosis and insufficiency. Stenosis occurs when one more of the heart valves do not fully open due to fused or stiff leaflets, resulting in extra strain on the heart when pumping blood that leads to heart failure. Vascular insufficiency occurs when heart valves do not close fully resulting in regurgitation or the backward flow of blood. Trans catheter aortic valve replacement serves as the most popular treatment strategy for people suffering from these issues. However, paravalvular leakage can occur if the prosthetic implant does not perfectly fit. Efficiency of valve performance depends on its complex anatomical geometry and heterogeneous tissue biomechanics.[21] Congenital defects and acquired valve diseases can compromise the valve shape and tissue mechanics resulting in stenosis or regurgitation. Although surgical repair of aortic valves is possible, prosthetic replacement remains the only viable option for a majority of patients suffering from aortic valve disease.[49,173] Tissue engineering of the aortic valve offers an alternative strategy to provide a living valve replacement that can grow and also effectively integrate with the host tissue.[53] Hockaday *et al.* produced tissue engineered bioprinted valves using encapsulated MSCs that were evaluated using *in vitro* testing and mechanistic studies.[78] One of the main challenges of developing a valve tissue is the lack of biomaterials that can effectively withstand the physiologic pressure while providing a micro-environment that can promote cell survival and growth.[40,77] Although cardiac valve tissue engineering has a lot of exciting potential, several technical challenges must be overcome before clinical translations. It is not yet possible to fabricate a cell laden heart valve, especially ones with thin leaflets that can withstand the physiological pressures that a native heart is subjected to, even after static culture. There needs to be significant effort in this field to successfully develop a 3D bioprinted heart valve.

Cartilage

Cartilage tissues possess a limited regenerative capacity, often requiring significant healing time with larger injuries and degenerative osteoarthritis treatments requiring prosthetics or grafts.[129] The current accepted treatment for osteoarthritis and chondral injuries uses autologous cartilage implantation where cartilage tissues or cells are harvested from the patient and implanted at the treatment site.[133,138] Articular cartilage provides a smooth surface for joint movement that maintains low friction even when load bearing, allowing the tissue to withstand compressive and shear forces.[30,138] 3D bioprinting enables the manufacturing of cartilage replacements with precise geometry, like replacement joint pads or superficial constructs.[106,129]

Hwang *et al.* showed ESC derived embryoid bodies expressed more cartilage and bone related transcription factors when cultured in 3D hydrogels compared to monolayer cultures.[85] Articular cartilage-derived progenitor cells (CPCs), found at low concentrations in the cartilage, exhibit higher rates of self-renewal than chondrocytes. Levato *et al.* bioprinted GelMA hydrogels seeded with CPCs, MSCs, and chondrocytes alone and in combination to generate cartilage.[112] The CPC-derived tissues contained more cells than the MSC samples after 56 days. All cell types underwent chondrogenic differentiation indicated by glycosaminoglycan (GAG) production with MSCs outperforming the chondrocytes and, to a lesser extent, the CPCs.

Gao *et al.* overexpressed the transcription factor NR2F2 in MSCs to promote chondrogenesis.[59] These 3D bioprinted constructs containing NR2F2 over-expressing cells along with control tissues were implanted subcutaneously in mice. Safranin O staining showed higher proteoglycan production in the NR2F2 over-expressed constructs, which were also much stiffer than the controls on day 21, due to the accumulation of cartilage ECM. Stichler *et al.* functionalized poly(glycidol) (PG) scaffolds with HYA to generate a novel bioink for printing the cartilage tissue.[160] The hydrogel formulations showed to promote cartilage specific ECM production, including cartilage specific collagen II and aggrecan. Soybean oil epoxidized acrylate, a novel bioink with many desirable properties, is biocompatible and photo-curable without heating or the addition of photo-initiators, making it easy to bioprint. MSCs have higher attachment and proliferation in the soybean bioink than the more commonly used PEG-based bioinks.[125] Pati *et al.* used a novel decellularized ECM (dECM) bioink to print MSCs for generating cartilage.[143] The dECM bioink was printed along-side pre-printed 200 μm polymeric supports to produce the desired structure, capable of withstanding the compressive forces subjected on cartilage. The GAG and collagen II decreased after decellularizing cartilage ECM due to increased trypsinization time for the denser matrix. The group speculated that factors were still present that triggered chondrogenesis due to the morphology of seeded MSCs and their tendency to create a mesh structure. The bioinks produced with dECM were designed heat sensitive to gel as temperature increased from working temperature (15 °C) to body temperature (37 °C).[143]

Costantini *et al.* used GelMA based bioinks, either solely or modified with HYAMA or chondroitin sulfate amino ethyl methacrylate (CS-AEMA), along with Ca^{2+} crosslinkable alginate to print their 3D chon-

drogenic constructs.[30] Alginate allowed for rapid gelation at the time of extrusion. while GelMA was cured after printing to create stable hydrogels for long-term culture. The alginate in the constructs dissolved after a few days, leaving the covalently crosslinked GelMA to persist. While all the constructs maintained good cell viability, the bioink consisting of alginate, GelMA and CS-AEMA had the highest collagen type II/collagen type X ratio and equal expression of collagen type I to type II, making it the most suitable for chondrogenesis. In other work, Gao *et al.* demonstrated the production of cartilage tissues from hMSCs using PEG in a modified inkjet bioprinting system. Their constructs showed homogeneous cell distribution, good mechanical properties, chondrogenic differentiation and ECM production.[60] PG has a similar structure to PEG but has additional side groups that can be functionalized to improve its properties as a bioink.[160] PG, like PEG, is bio-inert and must be modified to support cell adhesion. Stichler *et al.* functionalized PG scaffolds with HYA to increase the bioactivity of the gel, producing mechanically stable scaffolds that supported MSC viability for 21 days post print.[160] Nguyen *et al.* used nanofibrillated cellulose (NFC) in combination with alginate and HYA as a collagen biomimetic bioink to support the differentiation of iPSCs into cartilage.[133] NFC/HYA bioinks induced iPSC differentiation while the NFC/alginate bioink maintained their pluripotency after printing. The cells differentiated into cartilage tissue after 5 weeks of culture.[133] Möller *et al.* printed NFC/alginate constructs seeded with hMSCs and implanted these constructs subcutaneously into mice.[129] The implanted constructs were integrated into the subcutaneous tissues after 14 days and show improved stiffness after day 60. These samples expressed GAGs and collagen II production, indicating production of cartilage.

The Multi-head Tissue/Organ Building System, an extrusion based bioprinter, offers multiple dispensing heads, which are individually controlled to allow the printing of various biomaterials in one structure.[106] Temperature controlled extrusion heads make printing support polymers, like poly(caprolactone) (PCL), along with cell-seeded hydrogels possible.[106,143] Lee *et al.* used the system to print a PCL support structure as well as PEG sacrificial layers, producing the complex geometry of the outer-ear.[106] The PEG layers created a negative of the ear shape for the PCL. The PEG is then dissolved, leaving the ear structure onto which chondrocyte and adipocyte laden alginate hydrogels were bioprinted, to create the cartilage and lobe fat of the ear respectively. Both chondrogenesis and adipogenesis occurred with the tissues being confined to their deposition location.[106]

Several research groups have developed handheld printing "biopens" for *in situ* bioprinting cartilage.[41,138] Biopens can immediately repair cartilage without knowing the geometry of the injury site before-hand. Surgeons can free form the new cartilage to whatever shape required during the operation.[138] One caveat is that the printed cartilage needs to withstand the forces imposed on native cartilage tissues while maintaining cell viability. O'Connell *et al.* designed a biopen capable of extruding two bioinks side by side *in situ*. The prints, made using a GelMA/(HYAMA) bioinks, and ADSCs, remained stable for up to 6 weeks in culture with high levels of cell viability.[138] Duchi *et al.* designed a co-axial extrusion biopen for printing cartilage tissues (Fig. 5).[41] The crosslinked shell provided mechanical strength to withstand forces at the print site with the cells to be encapsulated in a softer non-crosslinked hydrogel, providing a better environment that increased cell viability.

Graham *et al.* used droplet bioprinting to produce millimeter scale cartilage tissues consisting of bovine MSCs in an agarose bioink.[63] These constructs were cultured in the presence of TGFβ3 and expressed SOX9, an early chondrogenic differentiation factor, after 3 days. Gao *et al.* modified an HP Deskjet 500 thermal inkjet printer and cartridges, generating a bioprinting system.[60] Like droplet-based printing, inkjet printing can precisely deposit cells, scaffolds and growth factors in 2D and 3D arrangements. The group used the modified inkjet printer to print constructs of hMSCs in PEG diacrylate (PEGDA) with high levels of viability. The cultures were maintained for 4 weeks and showed increased proteoglycan production, indicative of chondrogenesis. Overall, an extensive amount of work has been done in the field of bioprinting cartilage.

Liver

The liver, an essential organ made up mainly of hepatocytes, metabolizes foreign chemicals, produces proteins, and generates bile. Liver failure often results from drug induced toxicity.[44,108,118] Better liver tissue models would significantly lower the risk of drug induced side effects going undetected in pharmaceutical development.[44,108] Hepatocytes make up most of the cells in the liver, performing most of its functions. Sinusoidal ECs line the hepatic sinusoid, providing filtration. Kupffer cells, macrophages present in the liver, mediate inflammatory response. Hepatic stellate cells contain an abundance of intracytoplasmic fat droplets and branched processes which, along with ECs create the sinusoid lining where they regulate sinusoid contraction.[97] Liver biofabrication often fo-

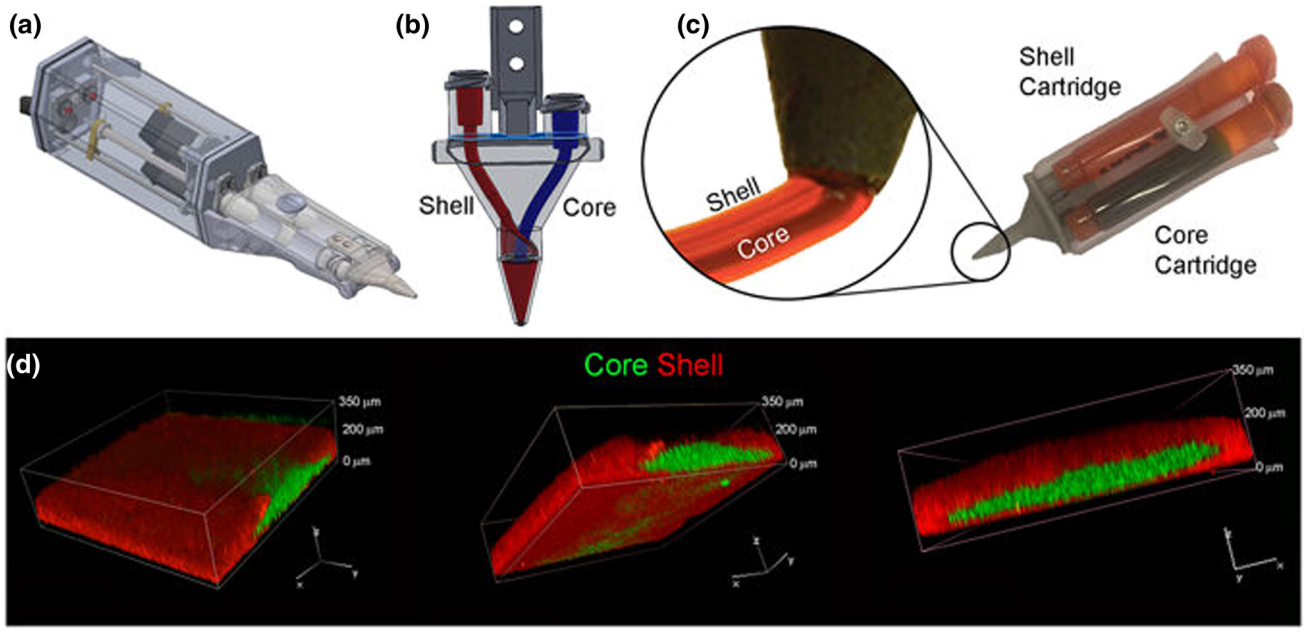

FIGURE 5. Handheld biopen developed for *in situ* cartilage tissue printing. (a) Schematic illustration of handheld biopen. (b)Schematic representation of coaxial nozzle. (c) Picture of the cartridges dedicated to Core and Shell loading in the printer, with relative magnification of the nozzle during co-axial deposition. (d) Representative 3D rendered confocal images of Core/Shell printed sample labeled with fluorescent beads (adapted from Ref. 41 licensed under http://creativecommons.org/licenses/by/4.0/).

cuses on hepatocytes as they perform the most functions and are the most abundant cells in the organ.

Lee *et al.* used a dECM bioink for printing liver tissues.[108] The constructs consisted of this bioink seeded with BMSCs as well as liver cancer cells (human hepatocellular carcinoma) along with PCL for structural support with control constructs prepared with a collagen bioink. The dECM group had higher expression of liver specific transcription factors (HNF1A, HNF3B, HNF4A, HNF) particularly HNF4A. However, low expression of the HNF6 marker was observed for both bioinks. Analysis of liver-specific functions of these constructs by assessing albumin and urea levels showed that the dECM bioink enhanced the cell functions.[108] Ma *et al.* developed 3D models consisting of mature hiPSC-derived hepatic progenitor cells (HPCs) cultured with endothelial and mesenchymal derived supporting cells.[118] The different cells types were arranged by a digital light processing 3D printer in precise patterns through masking and unmasking areas based on photo-polymerization of a GelMa based bioink. The patterns created vascularized liver lobule like prints. Higher expression of mature hepatic markers (HNF4A, TTR, ALB) was found in 3D multicellular model in comparison to 2D and 3D HPC only cultures. The multicellular constructs maintained the highest levels of albumin and urea production, making it physiologically similar to native liver tissues.

Faulkner-Jones *et al.* differentiated hiPSCs and hESCs into hepatocyte-like cells (HLCs) using a nanolitre droplet dispensing system to generate 3D constructs.[44] The viability of printed human hESCs and hiPSCs remained high post printing. hiPSCs viability was comparable to the un-printed control with hESCs viability higher than the control. The differentiation process was not disrupted by the printing process as the constructs expressed hepatic markers. The group then printed taller constructs consisting of hESC-derived HLCs in 20 or 40-layer tubes. Viability decreased in the first 24 h, resulting in low cell density in the prints. These 3D printed constructs took longer to differentiate in comparison to 2D culture.[44] In conclusion, 3D printed liver tissues would greatly improve drug testing models as they would provide an accurate response to toxicity as chemicals are metabolized. However, significant issues remain when engineering such tissues.

Muscle Tissue

Skeletal muscle tissue engineering holds great promise for replacing diseased or injured muscle tissue.[45] Researchers used inkjet printing to print scaffolds using recombinant human BMP immobilized in fibrin as a bioink for differentiating MDSCs into muscle.[146] Another group of researchers fabricated a 3D muscle tissue construct containing multiple cell types including

canine SMCs, hAFSCs, and bovine aortic ECs and an alginate bioink using ink-jet printer.[187] The cells were separately mixed with calcium chloride ($CaCl_2$) which acts a crosslinker and loaded into three different ink cartridges and printed layer-by-layer, creating a sodium alginate-collagen composite. 3D ink-jet printing has the ability and mechanism to overcome the challenge of incorporating vasculature while printing thick tissues as it allows organizing multiple cell types as well as different growth factors inside an appropriate scaffold. The research group cultured the cells in the printed construct for three days and then implanted the samples into the backs of three mice for up to 18 weeks.[187] The printed cells proliferated, retained phenotypic characteristics and physiological properties within the 3D constructs *in vitro* and survived with adequate vascularization when implanted *in vivo*.

As 3D environment accelerates smooth muscle cell differentiation, extrusion-based bioprinting is a potential technique for printing muscle tissue construct. Another group bioprinted hADSC-derived smooth muscle cells evenly mixed with a gelatin bioink using an extrusion-based 3D bioprinter.[193] Their results showed enhancement in smooth muscle cell differentiation and maintained more robust cell viability and cell proliferation compared to other conventional bioprinting methods. Higher expression of smooth muscle actin and smoothelin was observed after 3 days and western blotting analysis confirmed the differentiation of smooth muscle cells. Though skeletal muscle tissue has the ability of self-healing in case of small injury, it cannot restore the function of lost tissues after severe injury. For this reason, skeletal muscle tissue engineering holds great promise for the replacement of severely diseased or injured muscle tissue. In other work, an innovative microfluidic-enhanced high-resolution 3D bioprinting technique for printing skeletal muscle tissue with functional morphologies was developed.[31] They custom-built a bioprinter with microfluidic nozzle head coupled to a coaxial needle extruder for printing aligned hydrogel fibers since aligned muscle cells generate efficient contraction in order to promote enhanced myogenic differentiation from myoblast cells to parallel aligned long-range myotubes formation. They also optimized the 3D bioprinting process combining the rheological behavior of the extruded bioink consisting of PEG-fibrinogen and alginate biopolymer laden with muscle precursor cells (C2C12). The 3D bioprinted structures with C2C12 cells were cultured up to 21 days forming highly aligned multinucleated myotubes *in vitro*. After 7 days of culture, some of the constructs were implanted in the backs of mice along with bulk-hydrogel constructs used as controls for *in vivo* studies and both types of grafts were collected from the bodies after 28 days of implantation. After analyzing both kinds of samples, the bioprinted samples revealed a better organized muscle-like tissue compared to bulk hydrogels. These samples were completely striated confirming the advanced degree of myotube maturation and partially degraded and substituted by arising myofibers as well. A similar study was done by another group [93] where they used a different bioprinting technique known as an integrated tissue–organ printer (ITOP) for fabricating human-scale tissue constructs of any shape and structural integrity. They performed both *in vitro* and *in vivo* studies using supporting PCL layers, cell-laden hydrogel, pluronic F127 as a sacrificial material and thrombin solution as a cross-linker. After 7 days of *in vitro* culture the differentiated aligned myotube formation was observed. These bioprinted tissues promoted adequate innervation of implanted muscle tissue *in vivo*.

Decellularized ECM (dECM) derived from porcine skeletal muscle tissue can also be used as a bioink for printing skeletal muscle.[27] This bioink remained at solution state at temperatures below 15 °C and transformed into a gel state after incubating at 37 °C for 30 min. They printed parallel, diamond and chain-types of 3D cell-printed muscle constructs using the muscle dECM bioink and the 3D bioprinting system. The results of the experiments revealed that the bioink had sufficient printability for creating various geometries of 3D muscle constructs, with mechanical properties and which exhibited good cell viability, proliferation, myogenic differentiation and myotube formation. Thus, such bioinks show promise for bioprinting physiologically relevant muscle tissues.

Neural Tissue

Accurately reproducing the high complexity of the brain and neural micro-environments requires sophisticated 3D printing techniques. Currently, no long term cures exist for most central nervous system diseases and injuries despite over 50 million people suffering from them.[80] Neurodegenerative diseases are prevalent in elder populations, resulting in cognitive, memory and motor impairments.[62] Cell therapies have shown promising results for neural diseases and injuries such as Alzheimer disease,[117] Huntington's disease,[52] Parkinson's disease,[171] amyotrophic lateral sclerosis,[123] stroke,[7] and spinal cord injury.[4] Researchers have been able to develop 3D printed neural constructs with specific characteristics using different combinations of biomaterials and stem cells that have allowed a better understanding of cell behavior, environment, tissue formation and architecture (Fig. 6). The Shu group printed mouse neural stem cells (NSCs) using a thermo-responsive and biodegradable bioink

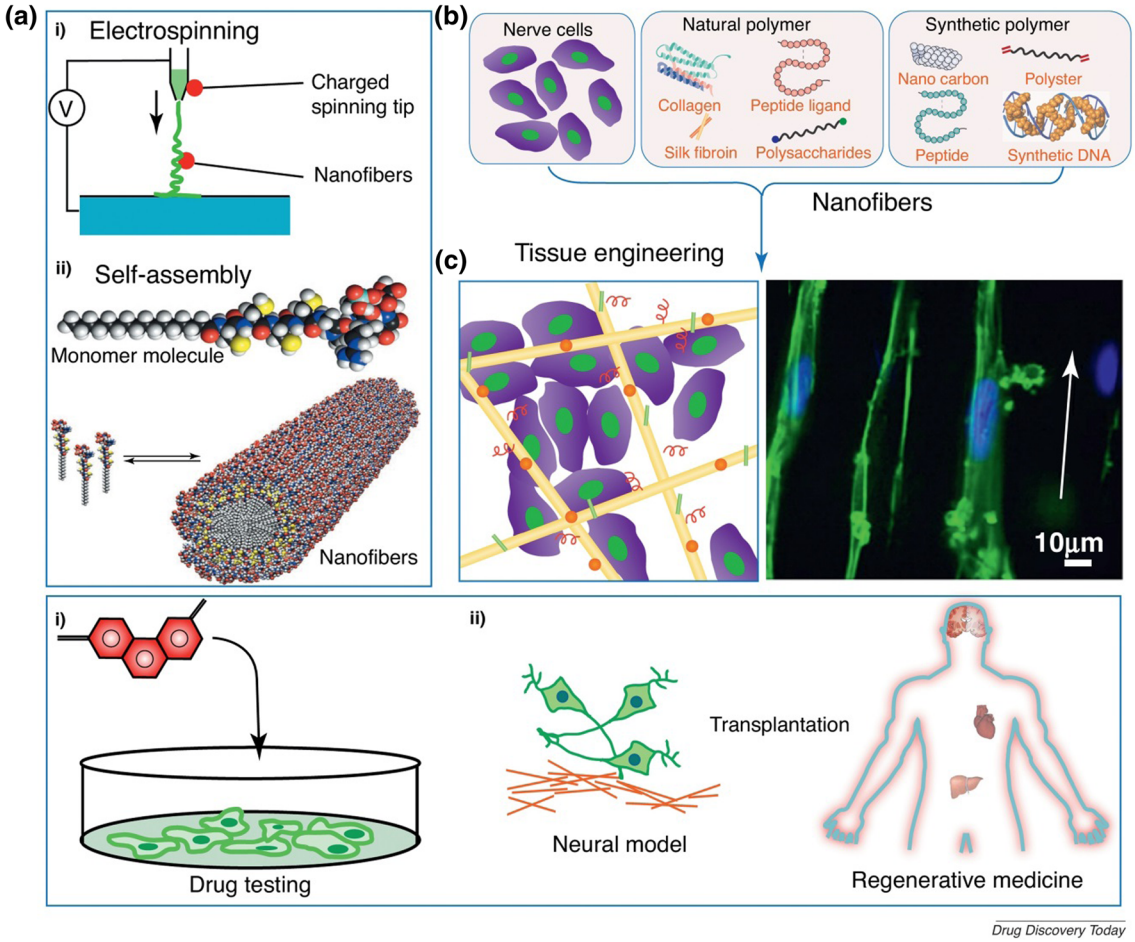

FIGURE 6. Fabrication of biomimetic nanofibrous scaffolds for neural tissue engineering. (a) Strategies of electrospinning and self-assembly used for nanofiber fabrication. (b) A general pathway of engineering neural tissues with the use of nerve cells and nanofibers composed of natural polymers and synthetic polymers. (c) Schematic illustration and image of neural cells on nanofibrous scaffolds (adapted with permission from Ref. 181 licensed under http://creativecommons.org/licenses/by/4.0/).

made out of polyurethane (PU) without any cross-linker using fused deposition manufacturing.[81] These PU nanoparticles gelled upon heating and their properties were tuned to obtain similar modulus and stiffness to the neural tissue. NSCs were encapsulated in the 25% PU hydrogel before gelation and these constructs consisted of eight printed layers (approximately 1.5 mm thickness) of fiber stacks were printed. Cell viability greater than 50% was observed 24 h after printing along with increased expression of β-tubulin III after 72 h. These printed tissues restored function when transplanted into a zebrafish model of traumatic brain injury. Hsu *et al.* developed a hybrid thermosensitive waterborne and biodegradable PU bioink with improved properties using the thermoplastic plant-derived polymer- soy protein isolate (SPI) in combination with the PU dispersion (PU/SPI).[114] NSCs were directly encapsulated in the hybrid hydrogel and then bioprinted in a continuous layer-by-layer deposition with dimensions of 20 mm x 20 mm and thickness of 10 mm. This hybrid PU/SPI hydrogel showed to be a permissive environment for NSC survival, proliferation and metabolic activity for 3D printing neural tissue. Frequently, multiple materials must be combined to achieve the desired specific properties for a bioink. Gu *et al.* used three different materials in their bioink to 3D print neural tissue. Functional 3D neural mini-tissues were printed using a combination of 5% (w/v) alginate, 5% (w/v) carboxymethyl-chitosan, and 1.5% (w/v) agarose containing human NSCs.[69] These tissues consisted of mainly GABAergic neuronal and glial cells. The functionality of the tissue was evaluated by measuring bicuculline-induced calcium response of neurons, as a result, spontaneous calcium spikes were observed indicating the functionality of the tissues.

ECM molecules can serve as scaffolds for 3D printing neural tissue as studied by Lee *et al.*[104] They used a type 1 collagen gel as bioink for printing astrocytes and neurons from day 18 embryonic rats

using a layer-by layer 3D printing A total of eight layers of collagen were printed for the multilayer-cell constructs by previously crosslinking the hydrogel precursor layer with sodium bicarbonate, a nebulized crosslinking solution. The cells were suspended and printed on the partially-crosslinked hydrogel and then coated with the sodium bicarbonate solution with the cells showing high levels of viability post printing. Immunostaining was also performed after 12 days, indicating the presence of neurons and astrocytes.

The controlled release of morphogens and bioactive proteins serves as an important tool when engineering neural tissue from stem cells.[179] The combination of collagen and vascular endothelial growth factor (VEGF) releasing fibrin scaffolds for 3D printing applications was studied by Lee *et al.* Changes in cell morphology and migration were observed for VEGF-releasing fibrin gels, including elongated neurite-like shapes after 2 days of culture. Scaffolds containing higher collagen concentrations showed more proliferation. Furthermore, increased cell viability was observed at the lowest collagen concentration. Zhang *et al.* combined graphene and GelMA, producing a novel nano-bioink called "G-GelMA" for 3D printing neural tissue.[197] A stereolithography based 3D bioprinter was used to 3D print neural constructs containing NSCs. These tissues contained neurons as indicated by β-tubulin III expression and formed neurite extension. Huang *et al.* prepared a bioink consisting of PU and graphene for 3D printing adult murine NSCs.[84] Constructs with dimensions of 15 mm $\times$ 15 mm $\times$ 15 mm (W $\times$ D $\times$ H) were printed using a Regenovo printer. The composite bioink of PU with a low graphene concentration of 25 ppm resulted favorable for 3D printed NSCs survival and differentiation into neurons and astrocytes.[84]

3D printed tissues can be combined with other technologies to improve the functionality of these tissues, such as low level light therapy (LLLT).[1,169] LLLT, also known as 'biostimulation'', has beneficial effects on cell proliferation, metabolic processes, wound healing, inflammation and neurological disorders.[1] Accordingly, Zhang *et al.* exposed 3D printed mice NSCs to LLLT to promote cellular proliferation and differentiation.[177] They used a self-developed 3D printer based on the Printrbot® rapid prototype platform and a combination of 10% GelMA and 15% PEGDA for the bioink, generating a transparent scaffold enabling light transmission.[198] The bioprinted tissues were stimulated for different periods of time with a 15 s exposure resulting in the highest proliferation rate with 60s and 90s reducing cell proliferation rates. Increased reactive oxygen species (ROS) synthesis was observed following light stimulation in comparison with non-stimulated cells. β-tubulin III expression increased for the stimulated cells.

Electrospun fibers can also be combined with 3D printing. One group 3D printed hydrogels containing PCL electrospun fibers and NSCs as a way to engineer neural tissue.[109] They used a stereolithographic based 3D printing platform in combination with the Printerbot. Electrospun fibers were placed at the bottom of the dish, and then the neural tissue was bioprinted on top of these scaffolds. The addition of PCL and PCL/gelatin fibers onto the printed scaffold increased the Young's modulus and the attachment of NSCs. Increased neuronal differentiation was observed for tissues grown on PCL/gelatin fibers after 11 days. This group also presented the greatest degree of neurite alignment with the electrospun fibers.

Interestingly, 3D printed constructs can serve as tumor models for screening chemotherapy agents for brain cancer treatment. Xu *et al.* 3D printed SU3 glioma cells using gelatin, alginate, and fibrinogen bioink to mimic the *in vivo* tumor environment.[184] These tumor spheroids replicated their *in vivo* functions, including secretion of VEGF and expression of nestin, a glioblastoma marker. These tissues were then exposed to temozolomide, one of the most common brain tumor chemotherapeutics with the 3D printed glioma cells being more resistant to this drug than 2D cultures. These results show the importance of the 3D architecture and physiology in making such models more relevant for drug screening applications.[184] Mimicking the real architecture and complexity of the brain and spinal cord enables the construction of accurate artificial neural tissues for disease modeling, performing drug screening and tissue transplantation. 3D printing can be used to generate such neural tissues as detailed in this section.

Pancreas

The pancreas is composed of many cell types in a complex, vascularized 3D structure, which performs both endocrine and exocrine functions. Pancreatic islets contain the endocrine cells of the pancreas, α, β, δ, γ, and ε-cells, which all function to produce required hormones. α and β-cells control blood glucose levels by the secretion of glucagon and insulin respectively. δ-cells produce somatostatin which inhibits α and β-cell action. ε-cells release ghrelin to regulate energy consumption and appetite.[149] Type 1 diabetes results in the loss of beta cells, the insulin producers in the islets of Langerhans. Current treatment strategies require the transplantation of β-cells or pancreatic tissues. Associated challenges include a lack of donor cells and tissues and immune response causing the cells to stop functioning.

3D printing offers the ability to create physiologically relevant pancreatic models with precise cell and spatial arrangements. Xu *et al.* printed ADSCs using a gelatin, alginate and fibrinogen bioink with pancreatic islets added post printing to generate an *in vitro* model of pancreatic tissue.[185] Pancreatic progenitor cells (PPCs) can differentiate into cell types in the pancreas, both endocrine and exocrine. Cell–cell contact may improve the function of PPCs, so the cells are often cultured in small aggregates to allow sufficient nutrient diffusion to the interior. Yang *et al.* developed a high throughput 3D printing method to control the size of generated cell clusters to produce transplantable β-cells.[191] Tobacco mosaic virus (TMV) particles, modified with arginine-glycine-aspartate (RGD) tripeptides, were patterned using an inkjet printer. The group seeded PPCs on to their printed TMV constructs and showed controllably and reproducibly sized clusters. The RGD improved cell adhesion to the 3D printed TMV constructs. Song *et al.* bioprinted hESCs encapsulated in fibrin gel, then embedded in a PLA scaffold as a way to generate stem cell derived beta cells (SC-β).[159] *In vivo* experiments were run to assess the level of stimulated insulin secretion. SC-β seeded devices were implanted into mice; insulin secretion was measured at 0 and 30 min after mice were fasted for 16 h then injected with glucose. Mice with the devices implanted had detectable human insulin with glucose injections increasing levels of human insulin approximately 2.5 times. Overall, 3D printed pancreatic tissues provide much needed models for pathology response during drug testing as well as for studying pancreatic disorders like metabolic syndrome. This technology can be applied to high-throughput generation of transplantable β-cells for the treatment of Type I diabetes.

FUTURE WORK AND OUTLOOK

This review has summarized a large body of work detailing the use of 3D printing techniques for generating tissues from stem cells. These techniques include fuse deposition modeling, inkjet bioprinting, and extrusion-based printing methods. Current methods are limited in terms of the types of materials that can be printed, the resolution of the printed constructs, and the number of materials being incorporated into a construct. The need to incorporate vasculature structures serves as one of these important issues to be addressed when engineering stem cell-based constructs. These limitations can be addressed by using more sophisticated printing technologies. For example, the Feinberg group at Carnegie Mellon University developed a novel 3D printing method using the freeform reversible embedding of suspended hydrogels (FRESH).[75] This novel printing process generates intricate structures that mimic the properties of native tissues found *in vivo*, including the structures found in bone and brain. Another interesting new bioprinting technology has been developed by the Vancouver based company—Aspect Biosystems. Their novel Lab-on-a-Printer system enables rapid switching between materials and cell types.[11,12,20] They currently market a novel engineered tissue—the 3D Bioring Airway—which replicates the physiological function of this muscle tissue. Often, maintaining cell viability and ensuring proper differentiation of stem cells can be challenging. One possibility for addressing these challenges is through the fabrication and characterization of more sophisticated bioinks that deliver the necessary cues for promoting cell survival and the desired differentiation. Thus, bioink development remains a high priority for future studies. In particular, the use of decellularized matrix provides an interesting opportunity for additional research.

Organ damage can result from genetic conditions, accidents, and diseases, representing a critical medical problem in our society.[32] Current treatment for major organ damage depends completely on organ transplants, a complex and expensive process. There is an acute shortage of organ transplantation as there is not enough donor to satisfy the needs of the patients and also the huge cost of transplant surgery which is more than $300 billion in 2012.[32] Again, organ transplantation involves the risk of immune-rejection, meaning the patients must find a donor who is a tissue match.[153] These issues could be addressed by 3D bioprinting the tissues or organs using stem cells taken from patient's own body to build a replacement of the damaged organ. Thus, 3D bioprinting holds great promise providing excellent outcomes to treat various serious diseases in a simple and cost-effective way. Bioprinting of layer-by-layer organs or tissue is possible using cost-effective bioprinter and bioink materials. Markets and Markets reported in April 2016 the overall 3D printing market will reach $30.19 billion by 2022.[153] Many of the modern 3D printers cost about $2,500 to $3,000, and even simpler models might be bought for as little as $300 to $400.[172] Therefore, compared to drugs and surgical therapy, bioprinting organs from stem cells would become more cost-effective in a few decades.

Finally, the advent of 3D bioprinting has also raised certain ethical issues.[61] These issues include considering whether there should be limitations placed on what structures can be generated and examining the key risks of significant harm associated with testing 3D bioprinting for humans. Additional concerns will require the investigation and development of appropriate clinical trial paradigms for testing 3D bioprinter tissues along with developing a specific framework for the regulation and testing of 3D bioprinting treatments. Also, analysis

should be performed that examines the ethical questions of irreversibility, loss of treatment opportunity and replicability associated with 3D bioprinting. Overall, the use of 3D bioprinting using stem cells to generate tissues has grown in popularity and future work will translate these promising technologies for a wide variety of applications from drug screening to clinical use.

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ETHICAL APPROVAL

This article does not contain any studies with human participants or animals performed by any of the authors.

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