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Review

Clinical Perspectives on 3D Bioprinting Paradigms for Regenerative Medicine

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ABSTRACT

Three-dimensional (3D) bioprinting is an emerging manufacturing technology that layers living cells and biocompatible natural or synthetic materials to build complex, functional living tissue with the requisite 3D geometries. This technology holds tremendous promise across a plethora of applications as diverse as regenerative medicine, pathophysiological studies, and drug testing. Despite some success demonstrated in early attempts to recreate complex tissue structures, however, the field of bioprinting is very much in its infancy. There are a variety of challenges to building viable, functional, and lasting 3D structures, not the least of which is translation from a research to a clinical setting. In this review, the current translational status of 3D bioprinting is assessed for several major tissue types in the body (skin, bone/cartilage, cardiovascular, central/peripheral nervous systems, skeletal muscle, kidney, and liver), recent breakthroughs and current challenges are highlighted, and future prospects for this exciting research field are discussed. We begin with an overview of the technology itself, followed by a detailed discussion of the current approaches relevant for bioprinting different tissues for regenerative medicine.

KEYWORDS: 3D bioprinting; bioengineering; tissue engineering; heart; bone; cartilage; nervous system; muscle; kidney; liver

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ABBREVIATIONS

ECM, extracellular matrix; PEG, polyethylene glycol; iPSC, induced pluripotent stem cell; HA, hyaluronic acid; MSC, mesenchymal stem cell; PLA, polylactic acid; RGD, arginylglycylaspartic acid; PEGDMA, polyethylene glycol dimethacrylate; GelMA, gelatin methacrylate; SilMA,

silk fibroin methacrylate; BMP-2, bone morphogenetic protein 2; VEGF, vascular endothelial growth factor; TGF-β3, transforming growth factor beta 3; HUVEC, human umbilical vein endothelial cell; HNDF, human neonatal dermal fibroblast; PCL, polycaprolactone; ESC, embryonic stem cell; SMC, smooth muscle cell; PNS, peripheral nervous system; CNS, central nervous system; hNSC, human neural stem cell; PT, proximal tubule; iPSC-HPC, iPSC-derived hematopoietic progenitor cell

INTRODUCTION

By enabling the rapid fabrication of multi-component structures with defined geometries, 3D bioprinting has made a tremendous impact on a wide variety of research fields over the past decade. From *in vitro* modelling of human tissue for drug testing to studying disease in a 3D environment, the applications continue to expand. One particular area of application that has garnered significant interest in recent years is tissue and organ engineering. Using biocompatible materials laden with human or animal cells, researchers world-wide have launched an earnest undertaking to recapitulate the anatomical, biochemical, and functional components of various tissue types—their goal: to fabricate an unlimited supply of customized organs and tissues for replacement in patients who have no treatment option other than donor transplantation. Attaining the ultimate goal would be an unparalleled scientific and medical feat—it would redefine medicine and inevitably generate practical and moral questions on how we choose to renew life.

Compared to the more general technique of 3D printing, bioprinting is much more complex. For instance, in order to truly replicate the complexity of natural tissues, there is the additional need for structurally and biologically compatible materials, flexibility to print a wide variety of cell types, bioprinting conditions to ensure cell viability, and methods to incorporate vasculature and innervation. These challenges are currently being tackled from multiple scientific perspectives, including the engineering disciplines, material science, chemistry, cell biology, biochemistry, and medicine. Many of these challenges remain unaddressed, largely because our own understanding of the biology of regeneration is still evolving, thus explaining the naïve approaches taken in recapitulating nature. In the following, we begin with a summary of the different technologies for 3D bioprinting and materials commonly used, followed by a tissue-by-tissue discussion of the state-of-the-art. Where relevant, the commercial potential of 3D bioprinting is assessed, remaining challenges and research needs are outlined, and future prospects of the field are discussed.

BIOPRINTING TECHNIQUES

There are four major approaches to 3D bioprinting. Thus far, no single technique is capable of fabricating large scale tissues or tissues of all complexities [1]. Each option has its strengths and limitations, and

selecting the most suitable approach is largely dependent on the needs of the specific application. Below we provide the technical background to enable judicious and appropriate selection of bioprinting techniques. Figure 1 illustrates the principles of operation behind all four approaches.

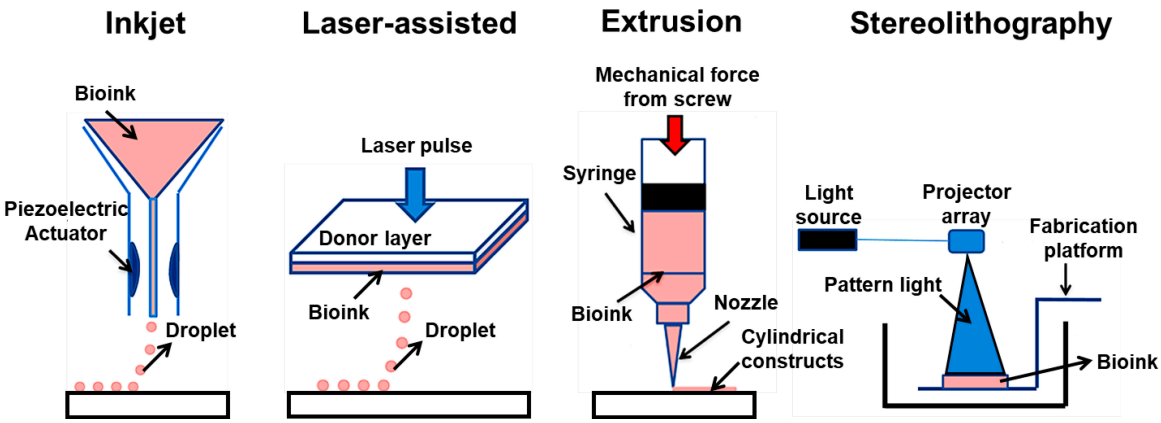

Figure 1. Four main categories of bioprinting techniques. Inkjet bioprinting involves the deposition of bioink droplets through a piezoelectric actuator. Laser-assisted bioprinting uses an energy-absorbing donor layer that responds to laser stimulation, a bioink layer underneath the donor layer, and a collecting layer to form tissue constructs. Extrusion bioprinting uses mechanical force to generate and deposit a continuous cylindrical stream of bioink. Stereolithography bioprinting uses a photosensitive bioink that is cured using precisely controlled light exposure projecting a patterned binary image.

Inkjet Bioprinting

Inkjet bioprinting, introduced by Xu *et al.* in 2005, was the first bioprinting technique to demonstrate success [2]. In an inkjet bioprinting setup, a mixture of hydrogel pre-polymer solution and cells (also known as “bioink”) is stored in a chamber connected to the printer head. During the printing process, the printer head is deformed by a piezoelectric transducer to squeeze out bioink droplets of a desired size (controllable via the bioprinter). Tissue constructs are formed by these spatially defined droplets. The major advantages of inkjet bioprinting are high cell viability and low cost. However, it is beset by several intrinsic problems, including the inability to print viscous material, clogging, and non-uniformity of cell concentration over the bioprinting interval [2,3]. More importantly, it is very challenging to build a free-standing 3D structure that is thicker than one millimeter using a droplet-by-droplet manoeuver [1]. For these reasons, inkjet bioprinting has received less attention in recent years.

Laser-Assisted Bioprinting

Laser-assisted bioprinting originated from laser-induced transfer technology [4]. It is a modified version of inkjet bioprinting that overcomes clogging and compatibility issues. A typical laser-assisted bioprinting setup involves three components: an energy-absorbing donor layer that responds to laser stimulation, a bioink layer underneath the donor layer, and a collecting layer to form tissue constructs [5]. During bioprinting, a

laser pulse is focused on a small area of the top donor layer. Upon energy absorption, this small area in the donor layer vaporizes and creates a high-pressure air bubble at the interface between the donor and bioink layers. The air bubble propels the suspended bioink to form a droplet that is eventually received by the bottom collecting layer. A tissue construct is thereby formed in a droplet-by-droplet manner. Laser-assisted bioprinting is compatible with highly viscous materials and high cell density. In addition, it has been reported that cells maintain high cell viability, over 95%, due to the short period of the laser pulse. However, the generation of pulse laser and the fabrication of a non-reusable donor layer increase costs relative to inkjet bioprinting. Consequently, only a few prototypes of laser-assisted bioprinters exist today [1]. Another significant limitation of laser-assisted bioprinting is the unresolved challenge of building large-scale 3D structures using a drop-by-drop approach.

Extrusion Bioprinting

Extrusion bioprinting is currently the most widely used bioprinting technology [6]. Unlike inkjet bioprinting, extrusion printing is able to print viscous materials. This is made possible through the use of a mechanical screw plunger in place of print heads. The plunger, when applied with a continuous force, can extrude uninterrupted cylindrical lines rather than discrete droplets. In this manner, extrusion bioprinting provides compatibility with highly viscous materials. More importantly, the cylindrical lines can easily be made into large 3D constructs on the centimeter scale. The main drawback, however, is potentially low cell viability due to the high mechanical stress applied during the printing process. Careful optimization of printing conditions (*i.e.*, temperature, bioink composition, applied stress) can address this issue and maintain high cell viability (85% to 90%)[7].

The significant advantages offered by extrusion-based bioprinting have secured this approach as the dominant bioprinting method behind over 90% of commercial bioprinters—including the Bioplotter (EnvisionTec, Gladbeck, Germany), NovoGen (Organovo, San Diego, USA), Biofactory (RegenHU, Villaz-Saint-Pierre, Switzerland), and RX1 (Aspect Biosystems, Vancouver, Canada). It is worth noting that extrusion bioprinting is compatible with almost all bioinks and can be scaled up easily to print multiple materials simultaneously at a reasonable cost [8], opening the doors to fabrication of heterogeneous tissue constructs. However, a major limitation remains in regard to spatial resolution. For most bioinks, the spatial resolution is low, around 200 μm, which is an order of magnitude larger than the dimensions of a single cell (~20 μm). Hence, it is currently infeasible to use extrusion systems for precise definition of the cellular microenvironment.

Stereolithographic Bioprinting

In addition to the "traditional" bioprinting techniques described, many newer techniques have emerged within the past five years. The most representative is stereolithographic bioprinting, a light-based printing technique that is compatible only with photosensitive bioinks. During stereolithographic bioprinting, a patterned binary image from a projector is used to cure a layer of photo-curable bioink. Only the areas exposed to high-intensity white light receive sufficient energy to cure. In this way, a layer of solid tissue construct is formed. Stereolithographic bioprinting offers several advantages over previous techniques: no matter how complex the pattern is in one layer, the printing time remains constant because the entire pattern is projected over the printing plane. As a result, this technique is faster than extrusion or other point-based bioprinting systems [9]. Stereolithographic bioprinting also provides the highest spatial resolution of all existing bioprinting methods, because the printing resolution is defined by the pixel size of the projector, which is often less than 50 μm [10]. Even higher resolution has been achieved through variations on standard stereolithographic bioprinting. Direct laser bioprinting, for example, which replaces the projector with a high-density laser [11,12], permits ultrafast patterning (under 10 min) of tissue constructs with high resolution around 30 μm [12]. Despite these advantages, one significant challenge of stereolithographic bioprinting is a limited ability to print multiple materials simultaneously.

SKIN TISSUE

Compared to other organ systems, human skin has the simplest layered structure. The outermost layer called the epidermis is 0.5 to 1.5 mm thick and is comprised mainly of keratinocytes (skin cells), between 90% to 95%, the rest being melanocytes (pigment cells), Langerhans cells (immune cells), and Merkel cells (mechanoreceptor cells)[13]. Below the epidermis lies the much thicker dermis, where collagen and elastin form an extracellular matrix (ECM) filled with fibroblasts. The deepest subcutaneous layer contains adipocytes, vasculature, and eccrine (sweat) glands. Embedded throughout all three layers are hair follicles, sweat glands extensions, and nerves. Because of the simplicity of the layered structure, skin was one of the earliest tissues targeted for substitution, and making skin substitutes has been an active endeavor since the mid-1990's, even before the advent of 3D bioprinting. The primary use of skin substitutes in medicine has been to improve the outcome of skin graft surgery, treat ulcers unresponsive to standard wound care, and overcome chronic skin diseases [14]. Currently available non-3D bioprinted skin substitutes can be categorized as follows:

1. Synthetic bilayers composed of a porous collagen matrix and other ECM components. Often these substitutes have a temporary layer of silicone to keep the wound sterile and retain moisture. Commercial substitutes

include Biobrane (UDL Laboratories, Rockford, IL, USA) and Integra (Integra LifeSciences Corporation, Plainsboro, NJ, USA)[15,16].
2. Substitutes derived from human tissue, such as acellular allografts consisting of decellularized skin from a human cadaver dermis that is later cultured with host cells. Examples include AlloDerm (LifeCell Corporation, Branchburg, NJ, USA) and Graftjacket (Wright Medical Group, Memphis, TN, USA).
3. Allogenic dermal substitutes made of human foreskin-derived allogeneic fibroblast cells on a polyglactin scaffold that secretes cytokines to form a metabolically active dermal substitute. Dermagraft (Advanced BioHealing Inc, Westport, CT, USA) developed and commercialized this approach.
4. Composite bilayer substitutes with a functional dermis and epidermis. The most notable is Apligraft (Organogenesis Inc, Canton, MA, USA), which is created by culturing human foreskin-derived neonatal fibroblasts in a bovine type I collagen matrix (dermis) over which human foreskin-derived neonatal epidermal keratinocytes are then cultured (epidermis). Apligraft was approved by the FDA in 1996 to treat foot ulcers that do not heal in response to standard wound care [14].

Although these skin substitutes have found clinical success, they lack vasculature, innervation, sweat glands, pigmentation, and hair follicles—in other words, they do not fully recapitulate all elements of native human skin. Other shortcomings include lack of patient customization, cellulitis, osteomyelitis, and lengthy and costly fabrication [17]. With the advent of 3D bioprinting, several of these challenges have been surmounted in part, such as creating pigmented skin, molding to individual wounds, and reducing fabrication time and cost. However, other elements, such as growing hair follicles and nerves, are not as easily addressed. Perhaps less demanding than creating the perfect human skin substitute for repair are the many other applications to which 3D bioprinting has contributed: *in vitro* disease/drug modelling [18,19] and non-animal testing for the cosmetic industry [13]. In the following, we describe the general process for 3D skin bioprinting, clinical applications and success, and future potential.

3D Bioprinting Skin Tissue

The general approach to 3D bioprinting of skin is outlined in Figure 2. The first step of preprocessing involves choosing relevant cells and biomaterials and designing the construct. There exist multiple stable cell lines for keratinocytes (K38, HEKn, HEKa, HaCaT), melanocytes (HEMa, HEMn), and fibroblasts (HDFa, HDFn, NIH3T3), all of which can be used for skin bioprinting. Other specialized cell types depend on the application but may include stem cells, progenitor cells, epithelial cells, nervous cells, immune cells, or gland tissue. Alternatively, some cell types may be sourced

from a host organism to ensure the construct is autologous and does not induce an immunogenic response, or to model a diseased tissue type. Biomaterials used for skin are similar to other organ systems and include gelatin, collagen, fibrin, sodium alginate, and polyethylene glycol (PEG).

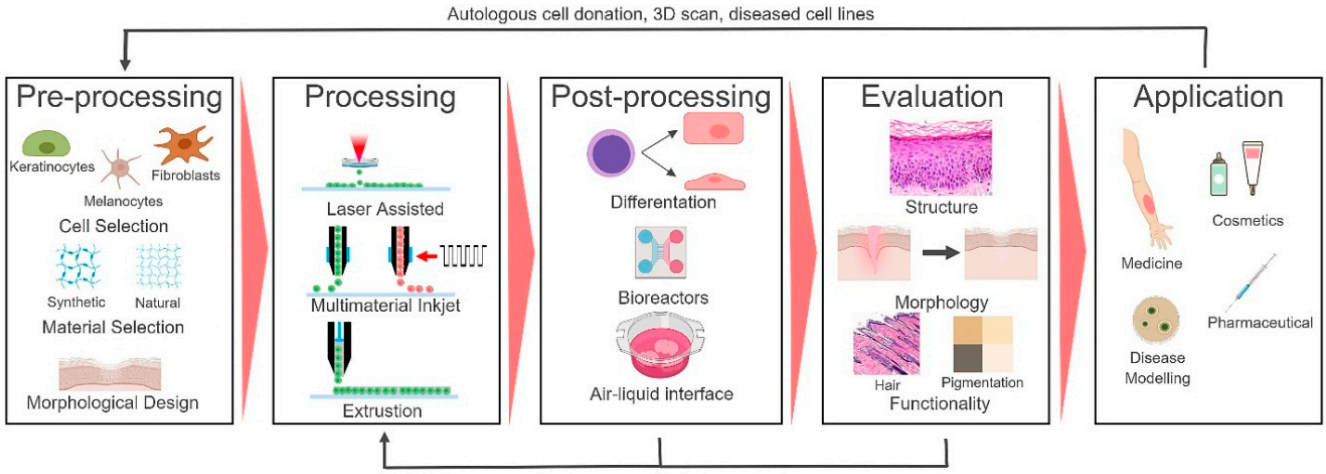

Figure 2. Skin bioprinting pipeline. (**Pre-processing**) Cells are chosen for the application and are cultured *in vitro* to reach a sufficient cell number. Bioinks made of a variety of synthetic and natural polymers are available, and the choice depends upon the application. The 3D design of the printed skin is determined. For wound healing, the skin can be printed to be complementary to the wound**.** (**Processing**) Using one of the available printing approaches, the skin construct is printed. The choice will be influenced by the desired properties of the final construct. (**Post-processing**) Further processing may be required before the skin is ready for use, such as differentiating stem cells, growing in a bioreactor, or maturing cells in an air-liquid interface. (**Evaluation**) The construct is evaluated before application. This may include analysis of structure, morphology, and function. Some applications may require iterations of these three steps—processing, post-processing, and evaluation. (**Application**) The construct is used in its intended application, such as wound treatment, *in vitro* disease modeling, cosmetic testing, and pharmaceutical testing.

The next step is to process the cells via 3D bioprinting. Many different techniques have been applied, including laser-induced forward transfer or laser-assisted bioprinting [20], multi-material inkjet printing [21], and extrusion printing [6]. Unconventional printing techniques have also been proposed for specific applications: electrohydrodynamic bioprinting [22], microfluidic printers [23], and rapid formation of supramolecular polypeptide-DNA hydrogels [24]. After printing, post-processing may be required. This may involve *in vitro* differentiation of stem or progenitor cells, removal of sacrificial layers, and growth and maturation in a bioreactor or via specialized cell culture techniques such as air-liquid interfaces [25]. Prior to use, the constructs must be evaluated for cosmetic appearance and strength, and this is often done *in vitro* via immunohistochemistry and mechanical testing. Disease-relevant phenotypes or biochemical secretions may also be analyzed before the construct is finally accepted for use in its intended application.

Bioprinting Advanced Skin Functionality

It is important to appreciate that while skin substitutes currently play a dominant role in cosmetics, the early pioneering research efforts on making artificial skin were aimed at treating wounds. The priorities at the time were focused on the basics—tissue viability, structural integrity (all three layers present), and preventing graft rejection. As these milestones were attained one by one, researchers began to focus on developing more advanced functionalities found in native skin tissue, as described in the following.

Skin pigmentation depends on the concentration and distribution of melanin produced by melanocytes in the basal membrane of the epidermis and is essential for skin tone and protection against ultraviolet radiation. Previous full-thickness skin grafts engineered for clinical application had issues with mismatched pigmentation against native skin [26,27]. Using 3D bioprinting, which is capable of printing multiple cell-based bioinks simultaneously, skin with human-like pigmentation has been produced. For example, dual-function tyrosinase-doped bioinks in multi-material inkjet printers have incorporated melanocytes into full thickness bioprinted skin [28]. By controlling the location and rate of melanocyte deposition in a two-step, drop-on-demand strategy, Ng *et al.* created a 3D bioprinted construct with a higher degree of resemblance to native skin [29]. Freckle-like pigmentation patterns can also be achieved by alternating between stages of bioprinting and air-liquid interface culture to incorporate melanocytes into a construct [25]. There is also evidence that incorporating induced pluripotent stem cell (iPSC)-derived melanocytes results in superior transfer of melanin to keratinocytes [26]. In summary, 3D bioprinting has successfully enabled the incorporation of melanocytes to provide skin pigmentation.

Vasculature is essential to maintaining viable tissue, and most cells need to reside no farther than 200 μm from a blood vessel for sufficient oxygen and nutrient supply [30]. To achieve nutritive diffusion in bioprinted skin, 3D micro channels have been printed. These channels may be created using a sacrificial gelatin layer [31], hollow channels [32], fibrin [33], or hyaluronic acid (HA)[34]. To achieve true vascularization, however, stem cell-derived endothelial cells are required. One can take the approach of pre-vascularizing skin patches, which Kim *et al.* successfully adopted using endothelial progenitor and adipose-derived stem cells [35]. A different approach is de novo vasculogenesis, which Zhang *et al.* adopted to create stable constructs over a six-week period by encapsulating human umbilical vein smooth muscle cells in sodium alginate with smooth muscle matrix and collagen [36]. These proof-of-concept studies demonstrate the feasibility of generating functional vasculature in 3D bioprinted skin.

Innervation to restore native touch, temperature, and pain sensitivity is an important trait for artificial skins, especially those grafted to human patients. Even for drug screening, accurate *in vitro* representation of nerves is useful when testing allergic drug reactions. Thus, incorporation

of sensory neurons into complex skin models is desirable. Limited success has been achieved in several non-bioprinting approaches. In one report, some nerve regeneration, primarily limited to the dermis, was demonstrated in mice following burns by embedding keratinocytes in a collagen hydrogel [37]. Improved innervation was seen in another study where dorsal root ganglia-derived mouse sensory neurons were seeded into tissue-engineered skin; nerve fibres grew into the epidermis and were studied for two months [38]. While neither study utilized 3D bioprinting methods, bioprinting has been proposed for guiding nerve growth via laminin-filled micro channels [38]. The promise of bioprinting nerves has yet to be explored fully and incorporated for creating fully functional human skin.

Commercial Success

There are many commercial products for artificial skin, most of which are not formed via 3D bioprinting. To compete with these products, 3D bioprinted skin must have additional properties, mainly functional, or reduced costs to overcome competition in the market. Not surprisingly, the first successes for 3D bioprinted skin were in the cosmetics industry, which had hitherto been an underexplored market. L'Oréal recently has partnered with the French biotech company Poietis to 3D bioprint skin with hair follicles for cosmetic testing [39]. L'Oréal has also partnered with American bioprinting company Organovo to use Organovo's proprietary NovoGen Bioprinting Platform [40]. In 2015, Procter and Gamble invited Singapore researchers to submit a proposal for their £27.4 million fund for 3D bioprinted skin to test their products [41,42]. Beyond the cosmetic industry, 3D bioprinted skin has made modest inroads due to competition from other artificial skin manufacturing methods that have been firmly in place for decades.

Future Development of Skin Bioprinting

Despite some success in adding skin pigmentation and vascularization via bioprinting, innervation and the incorporation of hair follicles remain significant challenges. Thus, achieving fully functional 3D bioprinted skin remains a distant goal. Another critical barrier to skin bioprinting is the associated cost. Current burn treatment costs on average USD $88,000 per patient [43], and scaled production of bioprinted skin must cost less than this threshold in order for it to become a viable option given the existing competition on the commercial front. To assume a dominant position in solving the problem of artificial skin fabrication, 3D bioprinting must demonstrate superior functionality, reduced cost/time requirements, and improved application outcomes. A cohesive and interdisciplinary effort will be required in future years before this exciting technology can be translated to the clinic on a large scale.

BONE AND CARTILAGE TISSUES

Bony tissue is distinct from other tissues in the body by nature of its hardness, and it is this mechanical property that enables the many functions of the skeletal system (e.g., movement, protection, support). Cartilage, an elastic but stiff connective tissue found in many areas of the skeletal system, also relies on its mechanical properties for proper function (e.g., connecting bones, load-bearing, fluid joint movement). Because proper function is contingent on proper mechanics, bone and cartilage regeneration is focused largely on restoring the salient mechanical properties of each tissue type. Both conventional 3D printing and bioprinting are poised to assist in bone and cartilage repair; however, the strategies involved are very different. In the following, we review current strategies for bioprinting functional bone and cartilage, recent *in vivo* studies, clinical translation, and future capabilities.

3D Bioprinting Bone and Cartilage

In contrast to creating a non-living bone substitute for providing solely mechanical support, the aim of bioprinting cell-laden bone scaffolds and mimicking the *in vivo* cellular microenvironment is to achieve proper function and restore tissue-level integrity [1]. Regeneration utilizes mainly stem cells with osteogenic potency, since human osteoblast cell lines are limited in availability. Amongst different stem cell sources, human mesenchymal stem cells (MSCs) have shown excellent potential for bone regeneration. Other than choosing the appropriate cell type, three other major factors also need to be considered: **biomaterials, soluble biomolecules**, and **cell-cell interactions**. A variety of biomaterials have been assessed, including printed collagen-hydroxyapatite scaffolds seeded with human MSCs that have shown osteogenic outcome *in vitro* and enhanced bone repair in a rabbit model [44]. Following this work, Heo *et al.* developed a scaffold that did not rely upon expensive animal sources and was much more scalable: a printable synthetic hydrogel made from polylactic acid (PLA) and arginylglycylaspartic acid (RGD) conjugated nanoparticles. The PLA hydrogel imparted improved mechanical properties, while RGD nanoparticles promoted cell adhesion and osteogenic stem cell differentiation [45]. Other researchers have reported that adding agarose (a stiff thermo-responsive hydrogel) to collagen (a natural cell-adhesive hydrogel) significantly improved the mechanical properties and promoted osteogenic differentiation of human MSCs [46]. Taken together, these advances highlight the importance of considering both mechanical and biological properties of biomaterials for facilitating osteogenesis.

In vitro bioprinted models are just as well established for chondrocytes as they are for osteocytes. One of the earliest studies on cartilage bioprinting was conducted in 2012—chondrocytes were bioprinted in poly(ethylene glycol) dimethacrylate (PEGDMA), an FDA-approved

synthetic material [47]; several parameters, including stiffness and methods of crosslinking, were optimized to promote chondrocyte function as indicated by the glycosaminoglycan content. Later studies were performed to examine systematically the effects of bioinks for printing chondrocytes. Alginate-nanocellulose [48,49], HA [50], and gelatin methacrylate (GelMA)[51,52] all showed superior performance for maintaining long-term viability and functionality of chondrocytes *in vitro*. The key to designing suitable biomaterials, as was done in these studies, was to closely mimic the mechanical properties of cartilage (~300 kPa) and allow good cell adhesion. A pilot study presented in 2015 showcased a human MSCs-laden RGD-functionalized PEGDMA hydrogel system—encapsulated cells successfully differentiated into chondrocytes [53]. Subsequently, Daly *et al.* benchmarked a range of widely used biomaterials (agarose, alginate, GelMA and PEGDMA) for differentiating human MSCs into chondrocytes [54]. They found that cellular activity and cartilage formation were dependent upon the biomaterial type and its mechanical properties, especially, the Young's modulus, which is in good agreement with the studies listed above [48–52]. Recently, a new type of biomaterial named silk fibroin methacrylate (SilMA) was developed and applied in 3D bioprinting of cartilage-like constructs [55]. Compared to the natural and synthetic materials described above, SilMA delivered comparable strength but also allowed for tunable stiffness. Furthermore, SilMA demonstrated suitable cell adhesion, providing a promising solution for fabricating hard tissue scaffolds. In summary, biomaterials are the basis for bioinks and dominate the mechanical properties of the bioink. Selecting a biomaterial that closely mimics the *in vivo* microenvironment of target tissues is a critical design aspect for bioprinting and regenerating bone and cartilage.

Biomolecules, such as growth factors, and their proper integration also play an important role in tissue regeneration. However, few studies have investigated their role in 3D bioprinting. In 2015, Park *et al.* studied the effect of spatially defining growth factors [56]. Using extrusion bioprinting, the authors spatially defined the distribution of bone morphogenetic protein 2 (BMP-2) and vascular endothelial growth factor (VEGF) to promote bone differentiation and angiogenesis, respectively. Printed scaffolds with these spatially defined growth factors were seeded with human dental pulp stem cells, which have osteogenic and vasculogenic potential, and implanted in mice and analyzed after 28 days. A significant extent of newly formed microvessels in areas containing VEGF was observed, with better bone regeneration seen in BMP-2 scaffolds. This idea was extended to engineering bone-cartilage interfaces, which were spatially controlled by a gradient of transforming growth factor beta 3 (TGF-β3)[57]; gradient-dependent differentiation towards a cartilaginous or ligamentous phenotype was achieved. In addition to growth factors, a recent study examined the effects of encapsulated plasmid DNA on osteogenesis [58]. Therapeutic genes encoding for BMP were mixed with

RGD-functionalized alginate and HA. Enhanced bone matrix deposition and mineralization were observed with the help of plasmid DNA both *in vitro* and *in vivo*. These studies highlight the importance of incorporating proper biomolecules in the bioprinting process and releasing them in a controlled manner in order to achieve biomimetic heterogeneity and authentic tissue regeneration.

The last ingredient for mimicking the *in vivo* native microenvironment for effective tissue regeneration is cell-cell interaction. Incorporating cell-cell interaction in 3D bioprinting requires access to multiple cell types and a complicated multi-nozzle bioprinter, which has made this area of research the most difficult to explore. Kolesky *et al.* utilized a multi-nozzle bioprinter to fabricate a thick vascularized bone construct using human MSCs, human umbilical vein endothelial cells (HUVECs), and human neonatal dermal fibroblasts (HNDFs)[8]. HUVECs were founds to improve vascularization and permeability for enhanced diffusion, which facilitated long-term (45 days) culture of the construct *in vitro*. The HNDFs served as a binding layer for connecting HUVECs and human MSCs, and with the help of HUVECs and HNDFs, human MSCs underwent osteogenesis in a highly permeable, integrated 3D environment and even expressed upregulated osteogenic markers, including osteocalcin and collagen I. Another multiple cell type bioprinting endeavor used a low-cost strategy to facilitate cell-cell interactions via cell-laden cylindrical construct [59]. By stacking the cylinders together, a bone-like construct was built where HUVECs were placed in the middle, surrounded by human MSCs to mimic the interactions between bone marrow and cortical bone. In this multi-step process, the need for a multi-nozzle bioprinter was eliminated but at the expense of increased handling complexity. The heterogeneous construct was cultured for 21 days *in vitro* and significant osteogenesis was observed, as indicated by upregulated Alizarin Red S staining and gene profiling of osteogenic markers. These studies highlight the importance of cell-cell interactions for long-term tissue maturation and functionality. Figure 3 illustrates 3D bioprinting for both bone and cartilage.

In Vivo Evaluation of Bioprinted Bone and Cartilage

Systematic *in vivo* evaluation of bone and cartilage bioprinted constructs gained momentum only recently. In one 2016 study, human MSCs-laden polycaprolactone (PCL) scaffolds were implanted subcutaneously in nude mice to assess the feasibility for proper vascularization [60]. Their results indicated that printed millimeter-scale constructs with proper lumen channel could undergo a level of vascularization *in vivo*, even without the support of growth factors, at 12 weeks post-implantation. However, the methodology of the study was not anatomically ideal as bony tissues were implanted subcutaneously. In a different 2016 study, the feasibility of using extrusion bioprinting to perform *in situ* bone repair was investigated in a rabbit model [61]. This

research built on previous work that used BMP-2 and TGF-β to spatially define vascularized bone constructs [56]. The study concluded that vascularized bone constructs had a stable host-guest response *in vivo* for 8 weeks and significant reconstruction of damaged knee joints, thus proving the potential for regenerating bone tissues *in vitro* and *in vivo*.

Assessment of bioprinted cartilage constructs *in vivo* is likewise quite recent. In 2017, Apelgren *et al.* quantitatively examined the proliferative capacity and cartilage-formation ability of mono- and co-cultures of chondrocytes and human MSCs [62]. It was found that after 60 days post-implantation, 17.2% of the surface area was covered with proliferating chondrocytes, which produced glycosaminoglycan and type 2 collagen, demonstrating functional cartilage regeneration. However, the transplantation site was sub-optimal, being in subcutaneous fat and not defective cartilage. More recently, an *in situ* transplantation model was reported on cartilage repair using bioprinted cartilage [63]. The authors developed a miniaturized bioprinter, which was essentially a handheld extrusion bioprinting system, and demonstrated it could significantly benefit surgical procedures. More importantly, chondral defects created in a sheep model demonstrated, for the first time, the ability of bioprinting to successfully repair cartilage in a large animal model. Preliminary results revealed that the hand-held bioprinter could successfully regenerate cartilage constructs with layers of subchondral bone and calcified cartilage. Taken together, these preliminary transplantation studies demonstrate long-term biocompatibility of printed bone or cartilage constructs, but future research needs to impose a greater focus on *in situ* transplantation models for studying functional regeneration.

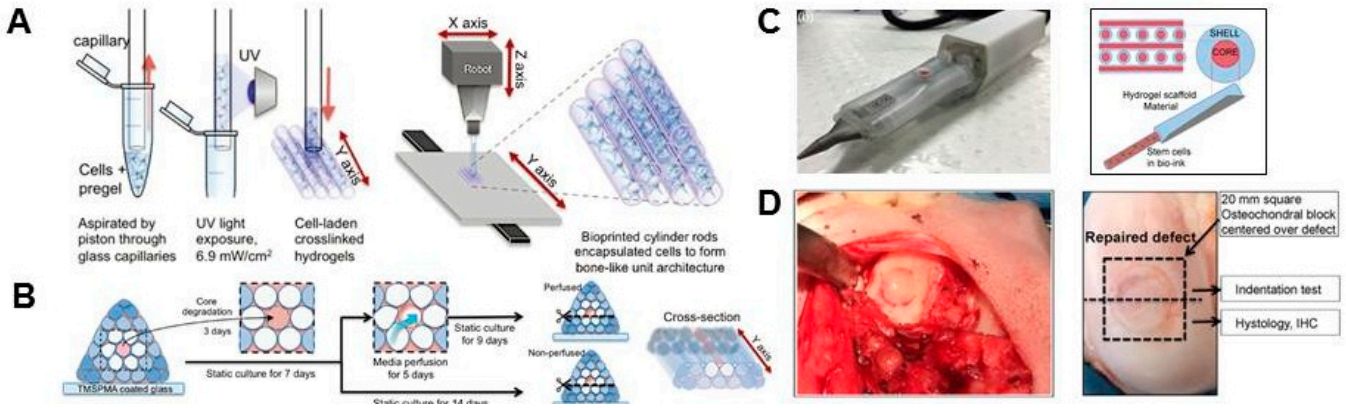

Figure 3. Bone and cartilage bioprinting. (**A**) Reconstructing heterogeneous bone tissue using a multi-material printing strategy. Curing photocurable bioink in glass capillary was used to create cylindrical rods that were deposited to a substrate by a 3D robotic deposition system. (**B**) Perfused culturing system. (**C**) *In situ* repair of defected cartilage in large animal models using bioprinting. Photograph and illustration of a portable extrusion bioprinter, the Biopen. (**D**) Photograph of the circular defects generated in sheep model before and after repair. A,B is adapted with permission from [59], copyright © 2017 John Wiley and Sons. C,D is adapted with permission from [63], copyright © 2018 John Wiley and Sons.

Clinical Translation

Currently, no searchable clinical trial using bioprinted *cell-laden* bone or cartilage constructs exists, although several trials using non-3D printed cell-laden constructs are underway (NIH: ClinicalTrials.gov). A phase 1 study completed by the Tehran University of Medical Sciences and involving 6 patients had implanted human MSC-laden scaffolds to regenerate articular cartilage (ID: NCT00850187). No results were presented and no downstream studies were continued, thus suggesting a greater barrier for cell-laden constructs to receive clinical approval. In contrast, cell-free scaffolds for bone repair have achieved greater success in clinical trials (ID: NCT03057223). This particular study aimed to print a patient-specific titanium jaw for surgery (N = 10)[64]. According to their published results, the intraoperative success rate was 100% and no major adverse events were observed 6 months after transplantation. Motivated by these positive findings, several large-scale, phase 2 trials are underway for 3D bioprinted solutions for bone defects (ID: NCT03185286, NCT03166917).

Future Development of Bone/Cartilage Bioprinting

Bone and cartilage tissues have a simple anatomical structure and minimal vascularity compared to other organ systems, rendering them an ideal target for regeneration using 3D bioprinting. The success of animal models described in this section showcases the feasibility of bioprinting for bone and cartilage reconstruction. However, a major current challenge is the translatability of research outcomes to therapeutic products. For example, many cell culture mediums (*i.e.*, fetal bovine serum) and biomaterials (*i.e.*, collagen) involve animal-derived components and may cause severe immune response after transplantation [65]. The use of such materials is strictly regulated in clinical studies and must be accounted for in these trials. Another source of concern is that various types of stem cells have the potential to form teratomas, a type of stem cell cancer which can be fatal to the patient [66]. The long-term integrity of the bioprinted tissue and its integration with native host tissues is also a concern, although a recent study has achieved encouraging results to the contrary [67]. In this study, bioprinted calvarial bone constructs were implanted *in situ* in mice, and upregulated growth of new bone was observed in the constructs after five months. This demonstrated the possibility of proper, long-term integration between bioprinted and native constructs to from functional new tissue. The challenges summarized here are not unique to bone and cartilage regeneration. However, compared to the other tissue types we will examine later, 3D bioprinted bone and cartilage are arguably the closest to clinical translation. Yet, even with abundant proof-of-concept characterization completed, considerable translational work remains to bring state-of-the-art bone and cartilage tissue bioprinting techniques, and any tissue bioprinting approaches, from the bench-top to the clinic.

CARDIOVASCULAR TISSUE

Compared to skin, cartilage, and bone tissue, 3D bioprinting heart tissue is a much more complex problem from both a biological and engineering perspective. Yet, cardiac tissue stands to reap the most benefit from 3D bioprinting technology simply due to a paucity of treatment alternatives. The adult heart is incapable of repairing properly due to the low regenerative capacity of cardiomyocytes, and the only treatment currently available for patients with end-stage heart failure is heart transplantation. In many cases, the patient will not survive long enough to receive a donor heart [68,69]—and the need for a readily available heart substitute is very real. Perhaps due to the urgent nature of this clinical condition, the application of 3D bioprinting in the cardiovascular domain has seen rapid progress in recent years. Several animal studies have already shown that 3D bioprinted cardiac patches have the ability to reduce fibrosis, hypertrophy, and infarct extent [70–75]. The impact will extend beyond transplantation to include disease modeling and drug studies, as we have seen for other tissues such as skin. In this section, we review efforts on heart patch and valve bioprinting approaches that incorporate multiple cell types and organized spatial patterning [76].

The biomaterials, or bioinks, used to deliver cells play a major role. These materials must be able to support cell growth, proliferation, differentiation, and also provide the requisite biomechanics and electrical integration with host tissue [69,77]. The most common bioinks used in cardiovascular applications to date include gelatin, collagen, alginate, and fibrin—these materials offer strong mechanical properties and cell adhesion [76,78]. Other bioinks currently in development emphasize the use of multiple components in order to enhance cell migration, organization, and alignment [79]. To truly mimic nature, bioink made from decelllularized ECM has also been investigated, and significant benefits for stem cell function and differentiation were shown where other bioinks have failed [80,81]. Regardless of the bioink material chosen, however, a number of common obstacles remain: non-homogeneous cell distribution, cell sedimentation during printing, and cell damage due to high shear stress. Measures to address these challenges were described recently in a novel mixing-induced two-component hydrogel containing alginate and engineered protein; this system was developed to maintain homogeneous cell distribution and provide protection and hydration during the bioprinting process [82]. Therefore, not only is the biochemistry of the bioink important, but also its mechanical properties must be carefully adjusted for optimal support, distribution, and integration of new cardiac tissue.

Various types of stem cells, including embryonic stem cells (ESCs), iPSCs, and MSCs, have been incorporated in 3D bioprinting cardiovascular applications [68,69], as shown in Figure 4 [83]. Non-stem cell types that have been investigated include cardiac progenitor cells, cardiomyocytes, endothelial cells, and many more [68]. As with all complex tissues, one key

challenge in cardiac tissue regeneration is recreating the native multi-cellular environment. Does one adopt the approach of seeding all the different cell varieties, or should one recruit the pluripotency of stem cells to create the necessary cell types for proper cardiac function? The answer remains open, but 3D bioprinting, with its ability to bioprint multiple cell types, is perfectly suited to exploring this question.

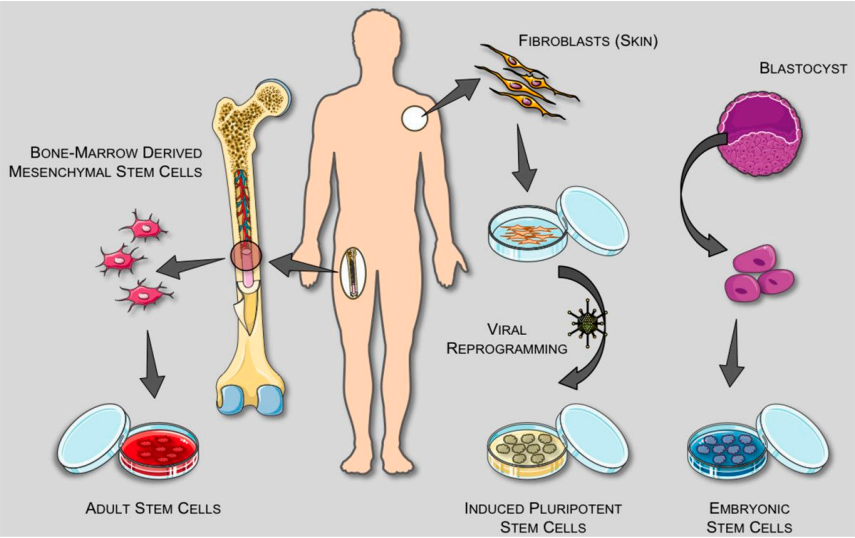

Figure 4. Stem cells for 3D bioprinting in cardiovascular tissue repair. MSCs harvested from the patient's bone marrow are developed into adult stem cells. Fibroblasts harvested from the patient's skin are reprogrammed *in vitro* to produce iPSCs. ESCs harvested from a blastocyst can differentiate into any cell type in the body. Reproduced from paper [83], copyright © 2011 BioMed Central.

3D Bioprinting Cardiac Patches

Bioprinted cardiac patches are one of the most promising cardiac tissue regeneration treatments currently under investigation. Bioprinting has been very successful in this application, because it allows for precise control over structure, design, and incorporation of multiple cell types. Recently, Atala's group designed a functional 3D bioprinted cardiac patch composed of a fibrin-based bioink and printed in a sacrificial hydrogel that supported the cell-laden bioprinted construct [84]. Ventricular cardiomyocytes isolated from neonatal rats were used to seed the construct, and the result was a cardiac patch capable of mimicking the biomechanical and physiological nature of native myocardium. Immunofluorescence imaging showed the development of heart muscle bundles that later developed into aligned muscle fibers. Other groups have approached cardiac patch design using different materials. For example, *in vivo* implantation of alginate and PEG-fibrinogen patches containing HUVECs and iPSC-derived cardiomyocytes has shown successful integration with host tissue and the formation of vasculature [85]. This work is seminal for future cardiac regeneration strategies, because it is the first report to show the formation of blood vessels using endothelial cells in a multicellular, spatially organized construct. There is also evidence

that biomaterials are not absolutely necessary to creating a cardiac patch. In a 2017 study, human iPSC-derived cardiomyocytes, fibroblasts, and endothelial cells were cultured and aggregated into cardiac spheroids [86]. These spheroids were able to produce a structurally intact tissue layer that was implanted onto the rat myocardium. Vascularization and integration with the host heart were both demonstrated, pointing to the potential of a biomaterial-free 3D bioprinted cardiac patch technology. All these efforts on 3D bioprinted cardiac patches contribute essential knowledge on a broader level to advance the field of cardiac regeneration.

3D Bioprinting Cardiac Valves

Patients who have valvular disease currently undergo valve replacement surgery to receive a mechanical or bio-prosthetic valve. While the treatment itself is not without major risks, including durability, compatibility and functionality [69], efficacy diminishes rapidly for young children, who would need several valve replacement surgeries as they grow. Therefore, the need for a biologically-derived valve that can grow with the child is a question that 3D bioprinting is well poised to answer. There currently exist reports on bioprinted valves made from a variety of materials and cells types. For example, alginate-gelatin and collagen encapsulated with aortic valve interstitial cells and smooth muscle cells (SMCs) have shown great promise in recapitulating biomechanical and physiological properties [69]. Duan *et al.* also established mechanical and structural integrity and high viability with their 3D bioprinted alginate/gelatin valves [87]. In addition to selecting an appropriate type of biomaterial, choosing the correct biomaterial concentration can also have a significant impact on structural performance and viability. In bioprinting a tri-leaflet valve, for instance, increasing the concentration of methacrylated gelatin was shown to decrease stiffness and increase viscosity, thereby improving cell distribution and other physical attributes [88]. Future efforts in this arena should also give consideration to the differential mechanical forces seen on different sides of a valve and at different valvular locations in the heart.

Bioprinted *versus* Non-Bioprinted Cardiac Models

The results obtained to date on 3D bioprinted cardiac muscle and valves are encouraging, rivaling popular alternative approaches. For example, seeding cells on decellularized constructs and organs has been investigated for several decades and is based on similar principles as bioprinting: cells and scaffold. However, the decellularized ECM approach does not allow one to define precisely the spatial distribution of one cell type, let alone multiple cell types [68]. A different type of non-bioprinting technology is a novel platelet fibrin patch that can be sprayed onto an infarct region without requiring a major surgery [89]. *In vivo* testing confirmed the spray did not cause damage to living cells and was able to restore some function in the myocardium. However, this technology does

not allow for the delivery of new cells to the ischemic region, only biomaterial. On its own, the biomaterial may be inadequate in restoring full cardiac function. Yet another non-bioprinting alternative is injecting modular cardiac constructs into the infarct zone to deliver new cardiac cells [90]. Although promising, similar to stem cell injections, these modular constructs are difficult to deliver, and ensuring that the cells are released in the region of interest is a huge obstacle, one that 3D bioprinted patch solutions can easily overcome.

Future Development of Cardiac Bioprinting

Regenerating cardiac tissue is a complex endeavor, and there are many unanswered questions that need to be addressed with 3D bioprinting, including: proper vascularization, electrical integration, mechanical contractility, and biocompatibility. Cell and biomaterial orientation and patterning are major factors to consider, as it has been shown that patterned stem cells and cardiomyocytes provide improved function and structure [71,91]. Furthermore, research must focus on not only the biomaterial and cell types employed but also appropriate growth factors that are conducive to driving regeneration. A ubiquitous example is incorporating VEGF, which is known to promote vascularization and has been demonstrated specifically in the heart [81,92]. Although 3D bioprinted cardiac tissue engineering strategies are far from entering clinical trials, they have already made meaningful advances in the research lab. With continued research and development, bioprinted cardiac tissue may become a viable treatment option for patients with heart failure and other types of heart disease within the next two decades.

CENTRAL AND PERIPHERAL NERVOUS SYSTEMS

The peripheral nervous system (PNS) and central nervous system (CNS) are one of the most difficult tissues to regenerate and repair. Bioprinting, with its *customizable* platform to create accurate 3D culture models and better performing scaffolds, has a vast potential to advance nervous system repair [93], despite the fact its application in PNS and CNS regeneration is still very new. Different materials that mimic endogenous brain or nervous tissue can be used, biochemical cues that promote nerve regeneration can be integrated to direct tissue development, stem cells or derived neural cells can be incorporated, and precise orientation of the 3D geometry of the device or scaffold can be achieved. Another advantage of using bioprinting to make constructs is the reproducibility and consistency enabled by an automated or semi-automated process. There are already many examples to demonstrate the utility of the bioprinting platform and the potential for developing commercial products.

The potential applications of 3D bioprinting in CNS and PNS regeneration can be broadly divided into two categories: (1) *in vitro* models of the nervous system and (2) scaffolds for nervous system tissue repair. Bioprinting is useful for creating *in vitro* 3D models that most

faithfully represent their *in vivo* counterparts; these models can be used to recreate layered structures that resemble the complexity of brain architecture or multiple components of peripheral nerves. Scaffolds for nervous system regeneration can also be developed using 3D bioprinting. These scaffolds can be designed to incorporate biochemical gradients, multiple cell types, and 3D geometries to improve the regeneration of PNS or CNS tissues. Figure 5 summarizes where these applications currently reside along the development stages toward clinical or commercial use.

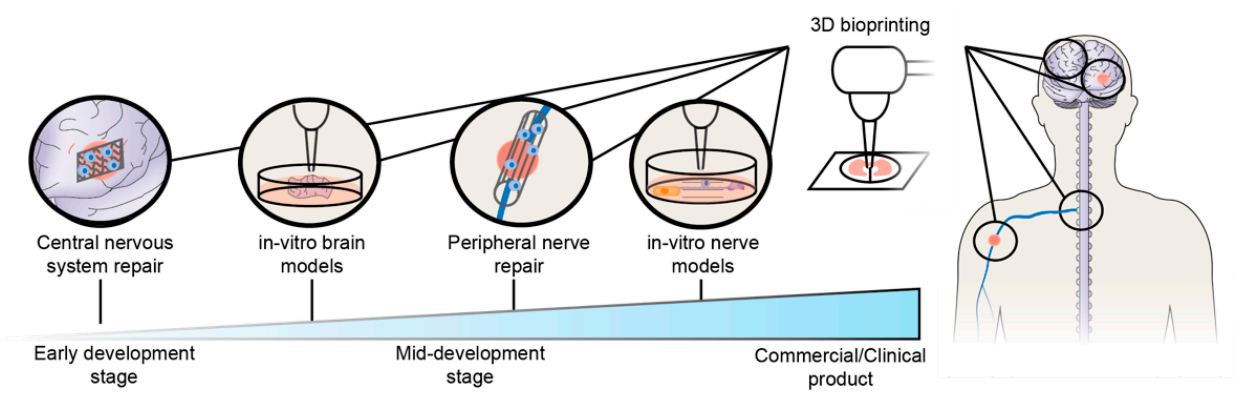

Figure 5. Different stages of development for 3D bioprinting of brain and nerve tissues. 3D bioprinting has been investigated for multiple nervous system tissue regeneration applications. On the left, scaffolds for central nervous system repair are still at a very early stage in development, as are 3D printed *in vitro* brain models. 3D printed scaffolds for peripheral nerve repair are slightly further in development, as are *in vitro* models of peripheral nerves.

3D Bioprinting *in Vitro* Nervous System Models

In modeling the PNS, a main challenge is the incorporation of multiple cell types into specific patterns. Strategies such as using electrospun fibres to align neural cells [94] or patterning microgrooves to align axon growth have been investigated [95]. Johnson *et al.* created an *in vitro* device for studying the PNS using a 3D printed nervous system on a chip [96]. This 3D printed device contained three chambers for PNS cells, Schwann cells, and terminal cells in addition to microgrooves for axon alignment. The authors demonstrated the ability to track a viral infection as it spread from one cell type to another within multiple chambers of the model PNS device. Another application of 3D bioprinting is to model one specific aspect of the nervous system for detailed *in vitro* analysis. Myelination of nerves is a good example, as it is critical for signal propagation and normal nervous system function. Espinosa-Hoyos *et al.* created a 3D bioprinted platform containing synthetic axons to study how different physical, geometric, and surface chemistries could alter nerve myelination [97]. These examples demonstrate the versatility of 3D bioprinting for creating *in vitro* devices to model the PNS by incorporating multiple cell types in specific spatial locations, or modeling one specific aspect of nerve development.

Modeling the CNS *in vitro* has far-reaching implications for studying brain and spinal cord function that would otherwise be difficult to examine *in vivo*. Since the first example of 3D bioprinted neural cells over a decade ago [98–100], researchers have explored how to use 3D bioprinting to create more representative 3D *in vitro* models for studying neural development. Recently, Gu *et al.* created a bioink that incorporated human neural stem cells (hNSCs) and allowed the cells to be printed into 3D structures [101]. By printing hNSCs into 3D structures and then differentiating them into mature neural cells, higher expression of genes associated with differentiated neural cells were obtained compared to 2D cultures. The authors built upon this work to demonstrate the ability to print human iPSCs into 3D structures and either maintain the iPSCs or differentiate them into neural cells [102]. The arrangement of stem cells within a 3D framework again resulted in greater expression of differentiated neural cell markers than the same process carried out in 2D culture. These results indicate that creating a 3D environment for both iPSCs and hNSCs allows them to differentiate more efficiently, or in a more uniform manner, compared to 2D culturing. Bioprinting in 3D also permits the creation of complex structures that recapitulate important aspects of brain structure. For example, the human cortex contains a distinct six layered structure. Recapitulating this layered structure in a controlled manner is difficult to do manually but can be achieved efficiently through 3D bioprinting. To achieve exactly this layered structure, Lozano *et al.* utilized bioprinting to create bioinks capable of printing multiple distinct layers containing neural cells [103]. Although still at an early stage of development, these examples highlight the potential of 3D bioprinting for creating complex 3D *in vitro* models of the brain.

The spinal cord is the other major component of the CNS that researchers are starting to investigate. Conventional *in vitro* platforms for studying the spinal cord are limited due to challenges with fabricating scaffolds that can form both the structure and cellular composition of the spinal cord. To address this challenge, 3D printed scaffolds have provided a means to seed multiple neural cell types at precisely controlled spatial locations to mimic the spinal cord [104]. This method can be used to create scaffolds that are on the scale of tens of millimeters, and when multiple cell types (neural and glial progenitor cells) are printed into the scaffold, both axon development and functional calcium dependent signalling are seen. This *in vitro* model of the spinal cord demonstrates the ability of 3D bioprinting to address challenges faced in fabricating complex 3D tissue models of the CNS. In the future, refinement of the construction, cell differentiation, and stricter functional evaluation may allow for this model to be used commercially for studying the spinal cord.

3D Bioprinting for Nervous System Regeneration

Bioprinting is currently being explored as an alternate method to create scaffolds for PNS and CNS repair. Printing in 3D confers the ability to

incorporate multiple materials, cell types, and biochemical cues in a natural geometry to promote the regeneration of healthy nervous tissue. For the bioprinted scaffold, a variety of materials with different structural properties have been evaluated for both PNS and CNS repair, including polyurethane hydrogel [105], photocurable poly(ethylene glycol) resin [95], methacrylate gelatin hydrogel [106], and poly (ethylene glycol) diacrylate [107,108]. Within these scaffolds, different cell types have also been evaluated, including stem cells [109,110], Schwann cells [111], and dermal fibroblasts [112]. Similarly, different biomolecules have been assessed: agents to promote adhesion such as polydopamine [106], carbon nanotubes [113], and chemotractants [114] to guide axon development. The flexibility of 3D bioprinting for creating different biomaterial compositions and for on-demand fabrication makes it an appealing option for both PNS and CNS repair.

Peripheral nerve damage occurs relatively frequently from disease and trauma, with over 200,000 nerve repair procedures performed annually in the United States alone [115]. Traditional methods for repair include grafting replacement nerves from other sites in the body or using decellularized grafts. These approaches have drawbacks such as the requirement for multiple surgeries to harvest the nerve, chronic pain, and limitations on graft size and geometry. A solution to these hurdles is possible through 3D bioprinting, which allows the creation of nerve guidance conduits that can vary in size, shape, and material composition, as well as the inclusion of cell types and biochemical cues to enhance nerve repair. These bioprinted conduits can be augmented with other therapies, such as low level light therapy [116] and electrical stimulation [113], to further enhance repair. Because bioprinting solutions practically eliminate the need for surgical harvesting of nerve graft and can create grafts of any size and shape, numerous groups have focused their attention on developing different 3D printed peripheral guidance conduits and testing them in pre-clinical models.

One approach in bioprinting peripheral nerve guidance conduits has been to incorporate biochemical cues, such as growth factors, within the bioink to improve nerve regeneration. For example, a 3D printed conduit designed from scanning the injured nerve site has enabled the first step of creating a guidance conduit the exact size and shape required, but it was the incorporation of biochemical gradients within the material that promoted sensory and motor nerve growth towards their respective paths [114]. A branching nerve guidance conduit made of gelatin methacrylate hydrogel is 3D printed containing a gradient of nerve growth factor down one branch and glial cell line-derived neurotrophic factor down the other branch, to guide sensory and motor nerve growth, respectively. In both *in vitro* cell migration studies and an *in vivo* peripheral nerve injury model, the conduits containing the biochemical gradients improved axon development down their respective pathways, providing an advantage in nerve repair over conduits that contained no growth factors.

Another effective strategy is the incorporation of numerous cell types into 3D bioprinted peripheral nerve guide conduits. Mesenchymal stem cells [109,110], Schwann cells [111], neuronal rat cells [95], human dermal fibroblasts [112] and adipose derived stem cells [110] have all been attempted with a certain level of success. An interesting adaptation on the cellular approach is to form spheroids from cells and use the spheroids as the bioink to form nerve guidance conduits [109,112]. Zhang *et al.* did this by taking human gingiva-derived MSCs, forming them into spheroids, and differentiating them into Schwann cells or neuronal cells [109]. These spheroids were then 3D printed onto a needle array in the form of a tube with a diameter of 9 mm and length of 3 mm. This was further cultured in a bioreactor to form a cellular nerve graft. The cellularized 3D printed graft was compared to an autograft and silicone tube conduit in a rat model of facial nerve injury. On both functional assays and histology, the 3D printed graft performed similarly to the autograft and better than the silicone tube for peripheral nerve repair.

Bioprinting scaffolds for CNS repair is less advanced compared to those for PNS repair. A substantial hurdle is that most bioinks available do not match the mechanical properties of native CNS tissue or require high temperature or toxic crosslinkers. To address this difficulty, Hsieh *et al.* developed a thermo-responsive polyurethane hydrogel bioink containing stem cells [105]. The mechanical properties of the hydrogel can be tuned based on the composition of two polyurethane monomers that crosslink to form a gel at body temperature. *In vitro* studies confirmed stem cell viability and neuronal cell differentiation in the printed hydrogel scaffold. The polyurethane hydrogel containing neural stem cells was then tested against the neural stem cells alone in two zebrafish models of CNS injury. In both injury models, the 3D printed neural stem cell-containing hydrogel out-performed neural stem cells alone. These *in vivo* results firmly demonstrate the potential of 3D bioprinting for creating CNS scaffolds for repair.

Future Development of CNS/PNS Tissue Bioprinting

Bioprinting has the potential for creating *in vitro* models and regenerative scaffolds for the PNS and CNS. Many different materials, cell types, and biochemicals can be accommodated and arranged in precise geometries and fabricated with an automated system. However, several significant challenges exist, and at the present moment, no 3D bioprinted scaffolds are being clinically evaluated for either CNS or PNS repair. For the CNS, the structure and function of the brain is very complex and not well understood. Therefore, in contrast to other tissue types in the body, using 3D bioprinting to build *in vitro* models or regenerative scaffolds is extremely challenging, because the basis for location-specific function of the organ, despite a spatially homogeneous composition, remains largely unknown. Bioprinted scaffolds for CNS also need to be tested in more representative models of human disease, such as in rodent models of CNS

injury. By comparison, the PNS is simpler and better understood. Current *in vitro* 3D bioprinted models of the PNS have the potential to be used more widely if they were tested and more thoroughly validated. Bioprinted guides for peripheral nerve repair are the closest to clinical evaluation [117]. To move them into the clinical space, they need to be evaluated head-to-head with clinically used guides and autografts. They could also be explored in instances where autografts fail or are challenging to use, such as cases when the distance of the peripheral nerve injury is further than a few millimeters.

SKELETAL MUSCLE

Skeletal muscle comprises nearly half of our body weight and is involved in supporting the skeletal system, providing movement, and even regulating metabolism [118]. Aside from the structural and functional changes that evolve naturally with aging, the need for skeletal muscle replacement may arise from myopathies, accidents, or surgery. In the United States alone, 4.5 million reconstructive surgeries are performed every year [118]. Traditionally, the most promising treatment option for skeletal muscle has been multiple cell injections into the damaged area. Although this approach has yielded some positive results, it has not been translated into clinical applications due to massive cell death upon injection and low engraftment rates [119], challenges also seen in cell injection therapies in the heart. Furthermore, fully functional skeletal muscle constructs have not been achieved *in vitro*; specifically, the mechanical forces generated by engineering muscle remain very low compared to normal baseline [120]. With the advent of 3D bioprinting, however, many of the hurdles associated with conventional muscle engineering may be overcome. In the following, we review recent efforts and strategies for bioprinting skeletal muscle and future prospects.

3D Bioprinting Skeletal Muscle

One of the most difficult limitations facing conventional muscle engineering solutions is achieving precise 3D spatial organization of cells. Bioprinting can easily address this hurdle by enabling very high precision in cell deposition. A variety of cell types have been investigated for 3D bioprinting musculoskeletal tissue and have shown efficacy, including MSCs, muscle-derived stem cells, and myoblasts [121]. Myoblasts have been used quite frequently due to their ability to proliferate indefinitely and to be directed along the differentiation pathway into multinucleated myotubes [122]. Muscle-derived stem cells have been incorporated in several applications for their ability to differentiate into myogenic lineages [68]. These constructs have shown not only high viability and favorable mechanical properties but also positive responses to electrical stimuli [68,122].

Equally important is precise deposition of matrix materials that can support cell proliferation and differentiation. Both natural and synthetic

polymers have been used: natural materials for their cell-supportive properties and synthetic polymers for mechanical strength and tunability. Alginate, collagen, cellulose, agarose, and gelatin are natural bioinks commonly employed in skeletal muscle bioprinting, while PCL, PEG, PLGA, and polyurethane are common synthetics [118]. Composite biomaterials are also popular, as they combine the advantages of both natural and/or synthetic materials. Gelatin methacryloyl (GelMA)-alginate-methacrylate composites or GelMA-poly(ethylene glycol) diacrylate composites are such examples, as they can sustain high viability of encapsulated cells while providing structural integrity, which is key to solving the problem of low engraftment [123]. In another report, a gelatin-PCL bioink was used to encapsulate human muscle progenitor cells [119]. This construct was assessed both *in vitro* and in a mouse leg injury implantation model and observed for 28 days. The bioprinted muscle showed a highly organized multi-layered bundle and aligned myofiber-like structure; *in vivo*, 82% functional recovery of the tibialis anterior muscle was observed. Comparison with non-3D bioprinted muscle tissue constructs seeded with the same cell type revealed that the 3D-printed construct not only yielded better cell organization and tissue regeneration but also had developed sustainable vascularization and nerve integration with the native tissue. Naturally, the most biomimetic material is decellularized ECM, which is tissue-specific and contains all the proteins and cytokines to direct differentiation and maturation. In a recent endeavor, Choi *et al.* used a decellularized ECM bioink to encapsulate myoblasts and created a 3D bioprinted muscle construct, which was compared with one made from a much more common substrate, collagen [124]. The ECM scaffold developed larger myotube lengths, widths, and surface area when compared with the collagen scaffold. Muscle fiber alignment was present along with high viability, proliferation, and myogenic differentiation. Despite these promising results, a known drawback of ECM bioinks is batch-to-batch variation and potential for immunogenicity.

Future Development of Skeletal Muscle Bioprinting

Skeletal muscle has a more complex multicellular anisotropic structure compared to most other tissues, a property that naturally lends itself to 3D bioprinting processing. Since geometry plays a key role in creating functional muscle, special attention needs to be given to the spatial organization of bioprinted cells and their microenvironment [125]. Future research also needs to focus on establishing adequate vascularization as well as successfully incorporating mechanical and electrical integration [126]. In order to translate the technology into the clinic, one must enable patient customization and scale up to produce the quantity required. Unfortunately, of the many cell sources available, such as iPSCs and perivascular cells, very few can be expanded *in vitro* to large quantities in the hundreds of millions. To address the point on patient customization, Kang *et al.* recently developed a novel 3D bioprinting technique to design

patient-specific constructs [67]. Data collected via computed tomography and magnetic resonance imaging was incorporated into the 3D bioprinting software to build human-scale muscle tissue, thus allowing the construct design to be tailored to the specific form of muscle injury in individual patients. With continued research in this area, beyond making skeletal muscle substitutes, 3D bioprinting has relevance in other areas, such as disease modeling and advanced 3D *in vitro* drug testing [127].

RENAL TISSUE

Kidneys play a very crucial role in human health. From filtering waste products and reabsorbing nutrients to maintaining essential endocrine, metabolic, and immunological activities, life cannot be sustained without normal renal function. Many kidney conditions, including diabetes, chronic kidney disease, and renal cancer, can lead progressively to renal failure, which currently necessitates donor transplantation. Other treatment alternatives may be suitable in some cases, such as a bio-artificial kidney [128]. However, many of these approaches have limitations, specifically, the failure to replace all essential renal functions. The possibility of 3D bioprinting to restore renal function is an attractive one, and as metabolic syndromes continue to rise in incidence, so has the attention on bioprinting approaches to regenerate the kidney. While bioprinting whole human kidney for transplantation is a very difficult endeavor, the application of 3D bioprinting to building kidney tissue models for testing drug toxicity is simpler and equally important, since nephrotoxicity is the primary reason underlying attrition in drug development. The following describes current progress on 3D bioprinting *in vitro* renal tissue models for disease modeling and drug testing. Bioprinting efforts on regenerating the kidney have yet to be reported.

3D Bioprinting *in Vitro* Renal Tissue Models

The human kidney consists of approximately 1 million nephrons, and each can be subdivided into five sections: glomerulus, proximal tubule, loop of Henle, distal convoluted tubule, and collecting duct. Active solute transport takes place in the proximal tubule (PT), where essential ions and proteins are reabsorbed and environmental chemicals and drugs are removed. Due to the high exposure to toxic agents, PT cells are more exposed to hypoxia and harmful chemicals than other nephron segments [129]. Therefore, most *in vitro* renal models have focused on recreating PT cell function.

Homan *et al.* reported the first bioprinted platform of 3D convoluted human renal PT [130]. They embedded the tubules within a perfusable ECM on customized perfusion chips to promote the formation of a tissue-like epithelium with improved phenotypical and functional properties (Figure 6). They were further able to show that Cyclosporine A, a nephrotoxin, disrupted the epithelium in a dose-dependent manner. A similar but more recent advance in this area is a proprietary Organovo 3D

bioprinting platform for creating a fully cellular human *in vitro* model of the PT interstitial interface [131]. A layered structure comprised of endothelial cells and fibroblasts was built to support a monolayer of primary human renal epithelial cells. The resulting 3D tissues demonstrated histologic and functional features of the PT. Furthermore, tissues exhibited cisplatin-induced nephrotoxicity and were protected from this damage when the cationic uptake transporter OCT2 was inhibited. These studies exemplify how an accurate PT kidney model can greatly advance bioprinting-assisted drug discovery.

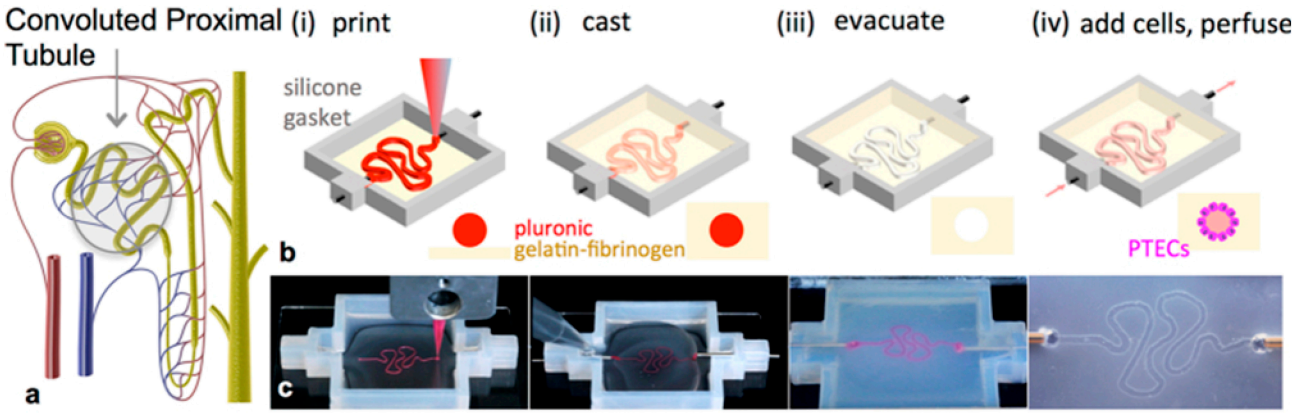

Figure 6. *In vitro* renal model of the convoluted proximal tubule. The different steps of fabricating 3D convoluted, perfused proximal tubules (PT). (**a**) Schematic of a nephron. (**b**,**c**) corresponding schematics and images of different steps in the fabrication. A fugitive ink is printed on a gelatin-fibrinogen ECM (**i**). Additional ECM is cast around the printed feature (**ii**). The fugitive ink is evacuated to create an open tubule (**iii**). PT endothelial cells (PTEC) are seeded within the tubule and perfused for long time periods via an external peristaltic pump (**iv**). Reproduced from [130], an open access article distributed under the Creative Commons Attribution 4.0 International License (http://creativecommons.org/licenses/by/4.0/).

In vitro 3D renal models can also play a significant role in studying kidney function and disease modeling. In a recent study by Lin *et al.*, a 3D vascularized PT model composed of conduits lined with PT epithelium and vascular endothelium, and embedded in a permeable ECM, was bioprinted [132]. Using this model, the authors investigated albumin uptake and glucose reabsorption, and they also studied the effect of hyperglycemia on PT cells and their rescue by a glucose transport inhibitor. This 3D renal tissue model provides a valuable platform for future renal physiological and pharmacological studies.

3D Bioprinting Renal Tissue for Regeneration

As for all complex 3D organs such as the heart, kidney, liver, and lungs, bioprinting faces unique challenges, as these organs possess highly organized multicellular structures and require an intact vascular network that can be connected to the systemic circulation upon transplantation. The kidney is one of the most complex organs, with over 30 different cell types and intricate compartmentalization. Bioprinting is considered to be

one of the only two viable approaches (the other being decellularization/recellularization technology) to bioengineer the whole kidney [133]. At present, there is no report on successes in bioprinting the kidney at the organ level.

Future Development of Renal Tissue Bioprinting

Despite promising results in bioprinting renal PT models, building a complete and fully functional kidney remains a distant goal. One of the greatest hurdles that must first be solved is finding appropriate biomaterials to support the complex and diverse anatomical and physiological differences in different regions of the kidney [133]. In this regard, acellular kidney ECM-derived bioink may provide kidney-specific instructional cues to printed cells. Another challenge is to recapitulate the hierarchical structure of the kidney; achieving this goal requires the spatial resolution of the 3D bioprinter to be an order of magnitude higher. Vascularization must be incorporated, as for all complex tissues. Finally, to make the whole kidney bioprinting problem more tractable, we recommend first reconstructing the other renal structures beyond the PT, with the long-term aim of integrating all parts.

LIVER TISSUE

The liver is a vital organ involved in metabolism, detoxification, and homeostasis. Hepatocytes, the dominant cell type, are the key driver for most of the liver's functional activities. As a vital organ, the liver needs to function properly in order to sustain life. Yet, it is susceptible to many diseases, including hepatitis B, cirrhosis, and hepatocellular carcinoma, all of which can eventually lead to liver failure [134]. High rates of liver-related morbidity and mortality are also seen in drug-induced hepatic injury. In fact, many pharmaceutical products undergoing clinical studies either fail or are withdrawn because of liver toxicity [135–137]. Thus, in addition to the goal of replicating the complex micro-architecture and cell diversity for liver regeneration, there is an equally important impetus to develop reliable *in vitro* liver models for drug testing and studying disease [135,138].

3D Bioprinting *in Vitro* Liver Tissue Models

Current *in vitro* liver models using human iPSCs derived from hepatic cells are largely limited to 2D or simple 3D culture. Unfortunately, these fail to recreate the structural and cellular composition of the native liver [139], and many liver-specific functions are not seen *in vitro* due to differences between native and culture environments [140]. In an effort to replicate the native environment more faithfully, Ma *et al.* proposed a tri-culture model bioprinted in a hydrogel system [141]. Human iPSC-derived hematopoietic progenitor cells (iPSC-HPCs), HUVECs, and adipose-derived stem cells were embedded in a hexagonal micro-architecture.

Compared with 2D monolayer culture and a 3D HPC model, the 3D printed tri-culture model exhibited both phenotypical and functional enhancements in the iPSC-HPC population. Specifically, the biomimetic environment improved morphological organization and increased metabolic product secretion. Figure 7 details the 3D bioprinting scheme for this tri-culture liver model, which has significant potential for pathophysiological studies with a patient-specific platform and early drug screening. In a different report, 3D bioprinted liver decellularized ECM scaffolds were created with tailorable mechanical properties to mimic regional stiffness in the cirrhotic liver [142]. Using this model, the authors showed that HepG2, a human liver cancer cell line, displayed stromal invasion from the nodule with cirrhotic stiffness. These examples clearly illustrate the value of bioprinted liver models for studying physiology and disease.

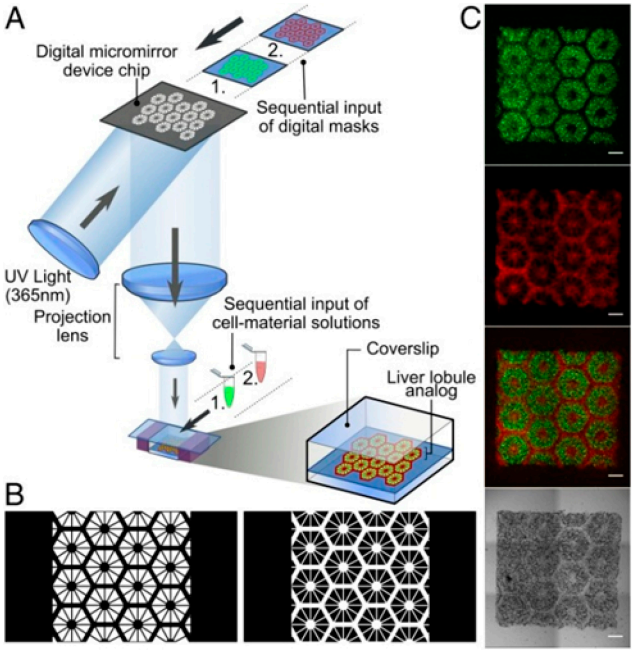

Figure 7. 3D bioprinting of hydrogel based hepatic construct. (**A**) Schematic of a two-step 3D bioprinting approach in which hiPSC-HPCs were patterned by a digital mask, followed by patterning via a second mask. (**B**) Grayscale digital masks for polymerizing lobule structure (**left**) and vascular structure (**right**). (**C**) Fluorescent images (5×) show patterns of fluorescently labeled hiPSC-HPCs (green) and supporting cells (red) on day 0. (Scale bars, 500 μm.). Reproduced from paper [141], copyright © 2016 the National Academy of Sciences of the United States of America (NAS).

3D Bioprinting Liver Tissue Constructs for Regeneration

Whereas the main purpose of liver models is to study pathophysiology and perform drug screening, the purpose of building liver constructs is to repair and replace diseased liver. These regenerative *in vivo* models are necessarily more complex, but a few existing reports demonstrate promise in this new field. The first report was made by Faulker-Jones *et al.* in 2015, where human iPSC-derived hepatocyte-like cells and human ESCs were

bioprinted in an alginate hydrogel matrix [143]. The cells were found to secrete albumin and display morphological similarities to hepatocytes. Jeon *et al.* also used an alginate scaffold to reconstruct liver tissue but used instead liver-derived HepG2 cells [144]. They observed that the 3D culture system gave rise to more accurate liver architecture, greater cell repopulation, and increased liver-specific gene expression. Angiogenesis has also been incorporated into bioprinted liver tissue using a multi-head tissue/organ building system [145]. Polycaprolactone was used as a framework biomaterial because of its ideal mechanical properties. By infusing cells into the canals of the framework, they were able to form capillary-like networks, which facilitated liver cell growth. The co-cultured 3D microenvironment of three cell types (hepatocytes, HUVECs, and human lung fibroblasts) lent itself to heterotypic cell interactions, resulting in normal function of hepatocytes (albumin secretion and urea synthesis) and increased survivability. In the most recent 2019 report, a continuous 3D bioprinting technology was employed to work with decellularized tissue-specific ECM bioinks [146]. Human iPSC-derived hepatocytes were shown to maintain higher viability and maturation in tissue-matched ECM, and spontaneous cellular reorganization was possible through cues from patterned lobular liver structures.

Future Development of Liver Tissue Bioprinting

The past decade has seen progress in 3D bioprinting of liver tissue both for regeneration and for *in vitro* models for disease studies and drug screening. The platform for drug testing is particularly promising and is likely to have significant impact within the current decade. While 3D bioprinted liver tissue for regeneration is a longer endeavor, it is important to remember that the liver has an intrinsic ability to regenerate and only the liver mass, not its shape, needs to be replaced. Therefore, engineering healthy liver tissue does not require the entire organ to be recreated, thus reducing the complexity of bioprinting and the challenge of scale-up. Amongst the various soft tissue organs, the 3D bioprinted liver may very well be the first to see a clinical role in regenerative therapy.

CONCLUSIONS

Bioprinting shows promise for future clinical and commercial applications in regenerative medicine. The key advantages to using 3D bioprinting are automated tissue fabrication and the flexibility of incorporating many different materials and cell types in precise anatomical 3D geometries. In theory, this capability allows for the structural, mechanical, biochemical and cellular components of different tissues and organs to be recapitulated simply by using different bioinks and printing methods. For structurally simple tissues such as skin, 3D bioprinting is close to becoming a clinically relevant method for producing skin grafts and is already being used in the cosmetic industry. For most soft tissue organs such as the heart and kidney, reproducing a

heterogeneous composition and structure and emulating diverse functions at the tissue level is very challenging and far from clinical translation. Despite these hurdles toward the ultimate goal of bioprinted organs for transplantation, 3D bioprinted *in vitro* tissue models have found more immediate relevance in drug screening and disease studies. These small tissue models still require accurate recapitulation of structure and function; however, the bioprinting task is simpler as the need to scale up and mimic organ-level heterogeneity is eliminated. *In vitro* models of bioprinted liver and peripheral nerve tissues are excellent examples where value is gleaned from tissue models that help us identify new drug candidates or better understand tissue development. The field of 3D bioprinting is rapidly advancing and bringing exciting new discoveries. Breakthroughs in this field will very likely require a truly cohesive effort amongst engineers, cell biologists, materials scientists, and physiologists.

AUTHOR CONTRIBUTIONS

All authors wrote the manuscript draft: SL on cardiac, BRK on the nervous system, ZW on bioprinting techniques and bone/cartilage tissue, DNP on skin tissue, and MT and HLMC on muscle, kidney, and liver. HLMC revised the manuscript. All authors approved the final version.

CONFLICTS OF INTEREST

The authors declare that they have no conflicts of interest.

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