3D Bioprinting

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Introduction

The human body has limited capacity to regenerate tissues following injury, and healing is often with the formation of scar tissue [1, 2]. The use of autografts is ideal for replacing lost tissues. However, autologous grafts are limited in their availability, and their retrieval can cause donor site morbidity [3]. These circumstances have triggered a large interest in developing engineered tissues and regenerative therapeutics [4], which aim to find solutions toward this end.

Three-dimensional (3D) bioprinting has been expanding tremendously over the last decade (Fig. 16.1). It aims to develop biomimetic and functional tissues addressing the demand for tissue and organ replacement. Its market share is projected to be about $11 billion in 2021 in comparison with $2.2 billion in 2012 [5]. When compared to other tissue engineering approaches, 3D bioprinting offers several advantages (Table 16.1)

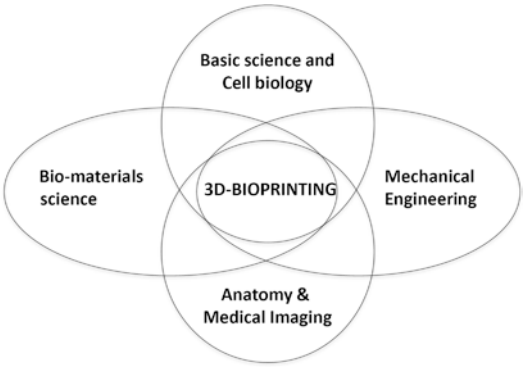

Fig. 16.1 The disciplines contributing to 3D biofabrication of human tissues

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Table 16.1 Comparison of different tissue engineering approaches

Methods	Hanging drop method	Microwell-based method	Microfluidics	Magnetic force-based patterning	Bioprinting
Mechanisms	Cellular spheroids are formed by gravitational force	Microwells are fabricated by nonadhesive materials to form cellular spheroids	Micro-flow mediates stacking cells in layers or forming cell spheroids using trapping	Magnetically labeled cells are compacted in spheroids formed under magnetic forces	Cells are deposited in scaffold-based or scaffold-free manner
Size uniformity	++	+++	+++	+++	+++
Microarchitectural controllability	+	++	+++	+++	+++
Scalability	++	+	+	++	+++
Coculture ability	++	++	++	+	+++
High-throughput capability	+	+++	+++	+++	+++
Low risk of cross-contamination	+	+	++	++	+++

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[6, 7]. More importantly, instead of seeding cells into scaffolds, 3D bioprinting creates a framework for the fabrication of complex cell-laden tissues with specific architectures resembling the target tissue [6, 8]. Provided by a layer-by-layer biofabrication method, cell and growth factor distribution is homogenous, and several biomaterials can be used in the same construct to recapitulate the structure of the target tissue [6, 8, 9]. These advantages have been confirmed by several experimental studies, which show great potential for clinical translation of this technology in the near future [6, 8, 10].

The aim of this chapter is to present the current advances and understanding of 3D bioprinting in the development of viable biomimetic human tissues. The present chapter focuses on the direct bioprinting of such constructs and summarizes the available examples of tissues produced with this technology. The challenges and future perspectives are also discussed.

Three-Dimensional Printing Techniques

Several 3D bioprinting techniques have been developed including extrusion-based bioprinting, light-based bioprinting, and droplet-based bioprinting (Fig. 16.2) [8, 11, 12]. Extrusion-based 3D bioprinting, often referred to as pressure-assisted bioprinting, was developed as a technique for scaffold fabrication. Over the years, the popularity of this technique grew due to its simplicity, diversity, and predictability. Extrusion-based 3D bioprinting can be divided into pneumatic, piston-driven, or screw-driven dispensing [13]. The pneumatic dispensing utilizes air pressure to dispense the biomaterial, while mechanical forces are used for the piston-driven and screw-driven methods [13]. Among the requirements of the bioinks compatible with this technique is a relative viscosity ranging from 30 to 6×10^7 mPa [14]. Factors to consider are the tuning of the viscosity, the state of the bioink prior to bioprinting, and the available biofabrication window [15]. Extrusion-based bioprinting delivers good homogeneity of bioinks, can be performed at room temperature, and can deliver relatively high cell densities. On the other hand, the overall resolution and speed is rather poor compared to other techniques like inkjet bioprinting [14], and some authors have noted deformation of cells and high apoptosis levels [16].

Light-based bioprinting technologies include stereolithography apparatus (SLA), digital light processing or projection (DLP), and laser-induced forward transfer (LIFT). Stereolithography is a

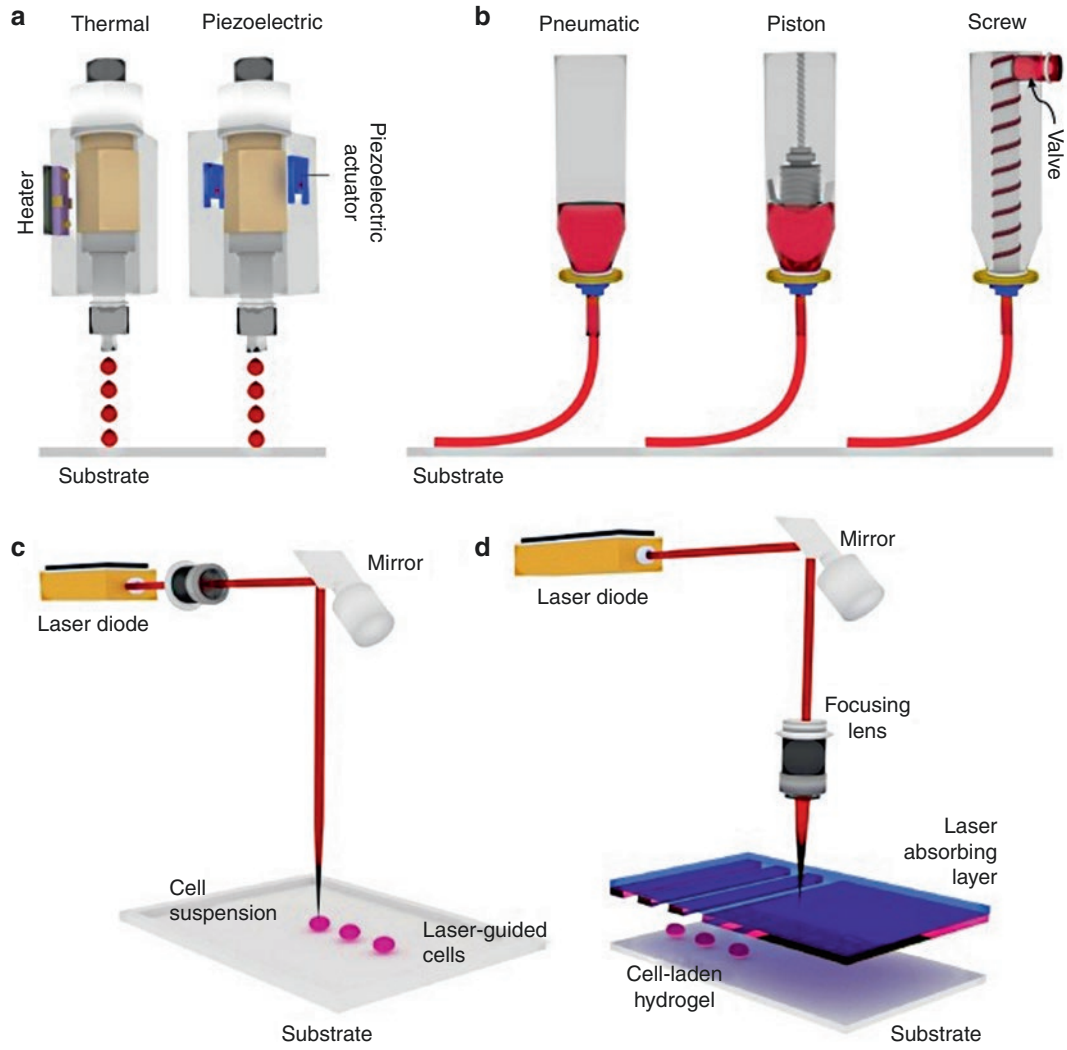

Fig. 16.2 Available 3D bioprinting technologies. (**a**) Thermal and piezoelectric inject printing; (**b**) extrusion bioprinters with pneumatic, piston-, and screw-driven dispensing; (**c**) laser-guided SLA- and DLP-type bioprinter. The difference of SLA and DLP is light sources. While the SLA uses the light source as laser, the DLP uses projector. (**d**) Laser-induced forward transfer (LIFT)-type bioprinter. Laser bioprinter with either driving cells to the substrate or transfer of a vapor bubble containing bioink onto a substrate. (From Knowlton et al. [158]. Reprinted with permission from Elsevier)

light-assisted printing method used to cure light-sensitive bioinks [17, 18]. It involves the curing, i.e., cross-linking, of a cell-laden photo-cross-linkable polymer in a layer-by-layer fashion. Its main advantage is that no printheads are needed, but the printing time is related to the printing resolution and thickness [17, 18]. Gauvin et al. suggested that resolution of 100 μm can be achieved with cell viability higher than 90% [18]. Digital light processing (DLP) utilizes a projector screen to project each print layer [19]. This process is much faster as compared to SLA as it cures the whole layer at once.

Light-based bioprinting technologies also include laser-induced forward transfer (LIFT). Conventional desktop inkjet printing technology

led to the development of inkjet-based 3D bioprinting. It involves a noncontact printing process, which can be further subclassified as drop-on-demand inkjet bioprinting, continuous-inkjet bioprinting, and electro-hydrodynamic jet bioprinting [20]. The overall resolution is around 50 μm, but this technology suffers from failure to sustain continuous flow [21]. For this reason, low-viscosity bioinks are required, with viscosities less than 10 mPa [11, 22, 23]. In addition, despite the fact that the inkjet bioprinting technique is fast compared to other methods, printed cell densities and viability are low [14]. The latter could be attributed to shear and thermal stress that are exerted upon the cells from the high temperatures and pressures reached in the thermal actuator element and piezoelectric actuation systems, respectively [11, 22, 23]. LIFT allows the deposition of either solid or liquid materials in high resolution through the effect of pulsed nanosecond laser energy [24]. Although it creates droplets with the aid of laser and although it is commonly regarded as a light-based bioprinting technology, some researchers consider it as one of the droplet-based bioprinting technologies. Following stimulation, a pressure bubble is created that drives the bioink droplet from the donor film to a substrate plate which contains the bioink [21]. The overall resolution achieved is in the region of 10–50 μm. Important parameters that could influence this technique include the laser energy, speed, and the rheological properties of the bioink [22, 24, 25]. Some researchers highlighted low cell survival rates, probably due to the thermal and shearing stress experienced by cells during the process [26].

Bioinks

Bioink is printable formulation for 3D bioprinting, and it is composed of living cells without or with carrier and/or matrix hydrogels. In addition to cells and hydrogels, other additive components such as biomaterials (e.g., bioceramics) and bioactive molecules can be added to the bioink formulation.

Cells

Cells are the main biological component of the bioinks used for 3D bioprinting of functional constructs. Three-dimensional bioprinting should take into account all the different cell types needed to simulate native tissue that needs to be constructed. Accordingly, cells can be of parenchymal type, supportive type, or cells for vascularization. During 3D bioprinting, the cells chosen to be printed will go through a journey that can affect their properties, function, and survival within the newly formed construct [27–29]. This journey begins from their harvesting and extends until their final implantation in vivo, when they are applied for regenerative purposes. Hence, it is essential to minimize the effects from harvesting, handling, culture environment, and media [30]. These cells can be broadly divided into either committed cell types, stem cells [31], and genetically programmed cells [32] to perform specific tasks and functions.

Committed and differentiated human cells could be considered the ideal source for creation of biomimetic tissues. The first issue arising from the use of such cells is the potential host immune reactions in cases of implantation of exogenous cells. Autologous sources are preferred, but donor site morbidity is a potential drawback. In addition, the life span of these cells is limited, and they lose their capacity to proliferate ex vivo. For example, liver cells have been found to have high regeneration capacity in vivo, yet they exhibit poor capacity for expansion in vitro [33]. Except proliferation and survival, the ex vivo manipulation of these cells changes their phenotypic profile. For instance, cardiac valve endothelial cells were shown to express osteogenic markers following isolation [34, 35].

Stem cells can further be subdivided into embryonic stem cells, stem cells from fetal supporting tissues, and adult tissue-derived stem cells. Embryonic stem cells can differentiate in most specialized cell types, and they have an immense capacity to proliferate in an undifferentiated state. There are several drawbacks involved

with the use of these cells. Embryos are destroyed during their isolation, which carries ethical issues [36]. In addition, their use has been related to the development of teratomas [37]. An alternative cell source for stem cells is human placenta and amnion. These cells pose less risk for tumorigenesis and pose minimal ethical concerns but require prolonged freezing and thus investment in the infrastructure for their storage [38].

Adult stem cells are the most studied cell type in the last three decades. Adult stem cells are multipotent precursor cells with tremendous cell renewal capacity [39, 40]. They differentiate toward cell types found in their surroundings following cues derived from tissue trauma [39]. They do not trigger an immune reaction. Often, their endogenous production of cytokines and chemokines diminishes unwanted functions like inflammation and cell death [39–41]. Despite their wide use in research, one important drawback is the lack of sufficient knowledge on the underlying physiology and the mechanisms that control their fates [41, 42].

Induced pluripotent stem cells (iPSCs) are somatic cells that have been transformed to an embryonic stem cell-like state following genetic reprogramming [43]. Genetic reprogramming involves the introduction of genes into the cells that force them toward specific properties similar to those seen in pluripotent cells [42]. This forced genetic expression is introduced through viral vectors, and poor yields of iPSCs are reported [44]. Also, the type of the original cell used to create iPSCs can influence the final functions of the derived cells [45–47].

Biomaterials

Hydrogels are the most utilized biomaterials for bioprinting due to their compatibility with living cells [48]. Several other types of biomaterials can be utilized as additives which can range from soft hydrogels to ceramic [49]. There are specific requirements for achieving successful 3D printing that need to be met by the bioinks as was discussed above.

Hydrogels are three-dimensional polymer networks that can hold a significant amount of water and can mimic the elastic modulus of the majority of human tissues except the calcified bodily structures like bone and teeth [50]. Hydrogels can be further subdivided according to their origin into naturally occurring polymers and their derivatives like alginate, collagen, chitosan, gelatin, and hyaluronic acid or synthetic materials like polyethylene glycol, copolymers, and pluronic F127, which can have adaptable structure, composition, and function [13, 51–53]. Naturally occurring polymers are often favored because of the similarities with human extracellular matrix (ECM) such as collagen and its derivatives. Due to their similarities with tissue environment, these biomaterials are ideal for encapsulating cells [13]. On the other hand, they can cause immune reactions, and they also have relatively poor mechanical properties. Natural polymers can be mixed with synthetic polymers such as polyvinyl alcohol, polycaprolactone (PCL), polylactide (PLA), poly(lactide-*co*-glycolide) (PLGA), and poly(3-hydroxybutyrate) to generate hybrid biomaterial, so as to improve the mechanical properties of hydrogels [9, 54–56]. Also some specific nanomaterials can be added for the improving mechanical strength of hydrogel to obtain functional multicomponent bioinks for the preparation of mechanically demanding tissues – such as bone, cartilage, and tendon [57].

In addition to natural and synthetic hydrogels, hydrogels can also be developed from decellularized tissues to create tissue-specific bioinks. For instance, tissues including bone, cartilage, liver, and heart have already been shown to create tissue-specific bioinks [58, 59]. Here, after decellularization of the tissue, it is enzymatically digested and solubilized to form a viscous bioink which, in turn, allows for the encapsulation of cells. Bioinks from decellularized tissue inherently show *thermal gelation*, resulting in gelation (solidification) at body temperature.

Biomolecules

Multifunctionalization of biomaterials [60] is a critical process in tissue engineering. It involves the inclusion of agents that can help in the regulation of cell fates and function through their interactions with cells within the 3D bioprinting construct. These molecules can direct cells in the engineered tissue constructs toward a specific phenotype and guide their migration, proliferation, and differentiation – and they can also influence native cells toward processes such as vascularization of the graft or in situ regeneration [61]. Alternatively, modifications of the biomaterials through the incorporation of bioactive cues, recognition sites, and adhesion molecules have been used [5, 62]. The choice of the biomolecules is dependent on the target tissue that one aims to treat. For bone regeneration, for example, molecules that improve angiogenesis – such as the *vascular endothelial growth factor* (VEGF), *osteogenesis-like growth factors* belonging in the TGF-β superfamily (TGF-β), or the *bone morphogenetic proteins* (BMPs) – have been used [54, 63–65]. Similarly, in nerve regeneration, *neurotrophic factors*, such as the nerve growth factor, *neurotrophin-3*, and *ciliary neurotrophic factor*, have been used [66]. These molecules are the steering forces giving cues to the cells to adopt specific function leading to the healing and incorporation of the graft.

Computer-Aided Design and Manufacturing for Tissue Modeling

The fabrication of biomimetic tissues can be achieved through the use of computer-aided design (CAD) and computer-aided manufacturing (CAM) techniques. CAD is defined as the computer software aiming to design target tissue structure, while CAM is referred as the software used to control the printer during 3D printing. Due to the complexity of tissue anatomical and structural organization, information on the tissue composition at the microscale level is essential. Computed tomography (CT) and magnetic resonance imaging (MRI) can provide information on the geometries and brief structure of calcified and soft tissues [67]. Once this information becomes available, histological 3D sections are designed based on the underlying anatomy of the target tissue. The thickness of these sections depends on the printer's resolution and can range from 100 to 500 μm depending on the machine and material used [67]. CAM technologies are equally important for the creation of the CAD models. CAM takes into account the properties of the underlying tissue and bioinks and aims toward successful creation of target structures. Bioink deformation, stiffness, fusion, nozzle clogging, and viscosity are controlled through CAM [68, 69]. In addition, CAM controls the survival and properties of the cellular components of bioinks [8, 70]. In essence, while CAD is critical for the reproduction of biomimetic tissues, CAM safeguards the quality of the 3D printing process.

Applications

The potential of 3D bioprinting has been shown in a number of applications. The fabrication of biomimetic tissues including bone, cartilage, nerves, cardiovascular tissue, and others has become possible through this technology.

Bone and Cartilage

Bone and cartilage regeneration have been important areas that tissue engineering has addressed over the last decades. Among the challenges mostly faced are the need of recreating the complex organization of these structures, the optimization of the rheological properties, biocompatibility, osteoconductivity, and realizing the potential of implanted grafts to be integrated and remodeled [71, 72].

Evidence from a wide range of 3D bioprinted constructs for bone regeneration has been promising [54]. Some bioinks were found capable of yielding stresses and Young's modulus similar to that of the human bone [57]. It is well recognized that mechanical stability alone is not the only

desirable feature of bone constructs; the chosen biomaterials should allow high viability while preserving the osteogenic capacity of osteoprogenitor cells printed within. In fact, some authors highlighted that although materials like PCL and PLGA are mechanically stable, they are not enough to support osteogenesis [73, 74]. On the contrary, other biomaterials, for example, decellularized bone matrix with PCL, were associated with upregulation of osteogenic genes of human adipose-derived stem cells [75]. Similarly, Campos et al. compared the effect of the addition of thermo-responsive agarose in a collagen bioink. This addition improved the mechanical stiffness of the construct [76]. The addition of bioactive glass particles has been shown to improve the mechanical performance while allowing for the construction of a porous construct, mimicking the pores of native human bone [77, 78].

Constructs which allow for the controlled release of molecules that either improves cell viability, angiogenesis, or osteogenesis could be a potential option [79, 80]. Du et al. created a 3D bioprinted gelatin-based bioink encapsulating MSCs and microfibers containing BMP-2. The addition of BMP-2 induced a stronger osteogenic phenotype following culture [80]. In a similar study, incorporating BMP-2 and VEGF to the construct resulted in increased expression of osteoblast-related genes Col1a1, Runx2, and Osx [79].

Cartilage is another important tissue, and its regeneration may benefit from 3D bioprinting. It is a specialized form of elastic connective tissue constituting parts of joints, the outer ear, and the nose. *Articular* cartilage draws most interest as its loss (e.g., in arthritis) is a major cause of morbidity and disability worldwide. Articular cartilage is not vascularized; hence, it is an ideal target for regenerative therapy using 3D bioprinting. However, the ideal cell carrier for chondrocytes is not yet identified, and available suitable materials lack enough mechanical integrity to enable successful function in high-load-bearing sites [81].

Tellisi et al. compared hydrogels, ceramics, and meshes for cartilage tissue engineering [82], and they found that chondrocyte proliferation was more in hydrogels as compared to ceramics and mesh. Daly et al. have also compared a wide range of commonly used hydrogel that included BioINK™, GelMA, alginate, and agarose [81]. The results showed that the choice of bioink can direct the cells to different functions. More specifically, alginate and agarose hydrogels resulted in the formation of tissue rich in type II collagen, i.e., supported the development of hyaline-like chondral tissue. On the other hand, GelMA and BioINK™ led to the development of a more fibrocartilage-like tissue. The combination of nanofibrillated PLGA [83], cellulose, or PLA nanofibers with cell-laden alginate hydrogel was also explored [84, 85]. These approaches were reported to result in improved cell density and better reinforcement of the mechanical strength of the constructs. Another study reported that high-density collagen is an ideal bioink for reconstruction of cartilage due to its capability of maintaining appropriate cell growth and for having mechanical stability [86].

Finally, in situ 3D bioprinting is presenting an attractive option [10]. For example, Di Bella et al. developed a handheld 3D bioprinter in an experimental animal model of critical size cartilage defect [87]. This printer was capable of on-demand filling of these defects with MSCs together with gelatin methacrylamide and HA methacrylate hydrogel. Improved macroscopic and microscopic appearances of the resulting tissue were noted when compared to conventional approaches. A higher amount of newly regenerated cartilage was seen with no signs of subchondral collapse or deformation [87].

Clinical evidence has shown that during the development of arthritis, changes to the underlying bone coexist with loss of cartilage. Therefore, a combined approach might be required. A number of researchers have worked on this principle, aiming for the development of osteochondral constructs rather than bone or cartilage patches [88–92]. In these studies, 3D bioprinted constructs with predesigned mechanical properties were created for potential clinical applications ranging from femoral head to temporomandibular defects [88, 89, 91, 92]. In an experimentally induced proximal humeral defect in rabbits, a customized layer-by-layer 3D bioprinted con-

struct containing transforming growth factor β3 (TGF-β3), HAp powder, and PCL was applied following capture with laser scanning [90]. The authors suggested that the entire articular surface of the synovial joint could regenerate without the addition of cells. It was hypothesized that the regeneration of complex tissues could occur by homing of endogenous cells.

Neural Cells

Nerve injury is the cause of significant disability and represents a clinical challenge due to the poor regenerative capacity of neural tissues. Three-dimensional bioprinting could be applied to nerve regeneration. For example, England et al. created a 3D bioprinted fibrin-based scaffold to guide neurite growth by encapsulating Schwann cells [93]. In cases of nerve loss, hollow nerve conduits composed of either synthetic or natural materials found to promote nerve regeneration [94–96]. Some authors suggested that cells in the bioink enhance the healing potential [97, 98]. In an experimentally created tibial nerve transection with 10 mm gap in rodents, Adams et al. used engineered nerve conduits utilizing fibroblasts and embryonic rat nerve cells [98]. They showed adequate distal motor nerve conduction velocity and large axons within the repaired nerve segment. In a similar study on the sciatic nerve defects in rats, cylindrical layer-by-layer 3D printed grafts were created. The cylinders contained MSCs (90%) and Schwann cells (10%) [97]. In this proof of concept study, the authors showed that this construct performed better than the standard collagen tubes and highlighted the complexities and the numerous adjustments needed to optimize the performance of such grafts.

Blood Vessels

The primary goal of tissue engineering is to create functional structures which could be incorporated into the host after implantation and can withstand the demands of the target tissue. Having complex structures without a vascular network to support the printed cells can lead to failure, because cells can survive on diffusion, only at a farthest distance of 200–400 μm from a feeding blood vessel [99]. In many studies, the lack of vasculature within the graft is surpassed by addition of angiogenic factors to promote angiogenesis. However, there is often a long process before angiogenesis is established; therefore, implanted graft survival is at risk [100].

Although currently the biofabrication of vascularized tissue has not been achieved, several authors evaluated ways to create and incorporate blood vessels into 3D printed grafts [101]. Some authors focused on the creation of large constructs like aortic tissue. One approach involved the use of embryonic fibroblasts and hydrogels printed in a layer-by-layer fashion to form an aortic tissue construct [102]. Another group utilized decellularized ECM with the use of separate layers of human smooth muscle cells, endothelial cells, and fibroblasts to recreate the media, intima, and adventitia layers through perfusion into the corresponding location of the supporting scaffold [103]. The fabrication of smaller blood vessels can be constructed as tubular structures with defined pores of 100–200 μm mimicking the structure of native vasculature [104]. Biomaterial selection is a key aspect. The production of sophisticated human-scale constructs of various sizes and shapes and incorporating microchannels allowing the diffusion of nutrients have been attempted [67, 105].

Muscles and Tendons

Musculoskeletal injuries are common and can result in significant morbidity [106]. Several authors have thus far explored the potential of musculotendinous regeneration through 3D bioprinting. The fabrication of isolated muscle units composed of myotubes and myoblasts resulted in contraction following electrical stimulation like in native muscles [67, 107, 108]. Kang et al. created skeletal muscle units of 15 × 5 × 1 mm which were stretched along the longitudinal axis and responded to stimulation preserving their

structural stability [67]. In regard to tendons, only limited groups have developed biomimetic tendon constructs [109]. The main challenge has been defining the ideal bioink, which could achieve structural stability equivalent to that of native tendons. Attempts to develop complex muscle-tendon units mimicking functional human muscle are also available. A two-layer construct composed of thermoplastic polyurethane co-printed with C2C12 cell-containing hydrogel and PCL co-printed with fibroblast-containing hydrogel offered elasticity for muscle development and stiffness for the development of the tendon [110].

Skin

Skin loss can be the outcome of trauma, skin diseases, and burns. Autografts are of limited availability, and substitutes often fail to achieve acceptable outcomes [111, 112]. Tissue engineering with the use of 3D bioprinting could provide an alternative approach, creating multilayered biomimetic structures to serve as skin substitutes (Fig. 16.3). The simplest option is the seeding of cells such as fibroblasts, keratinocytes, and melanocytes in predefined concentrations and layers into biomaterials, mimicking native human skin [113, 114]. The results have shown that these

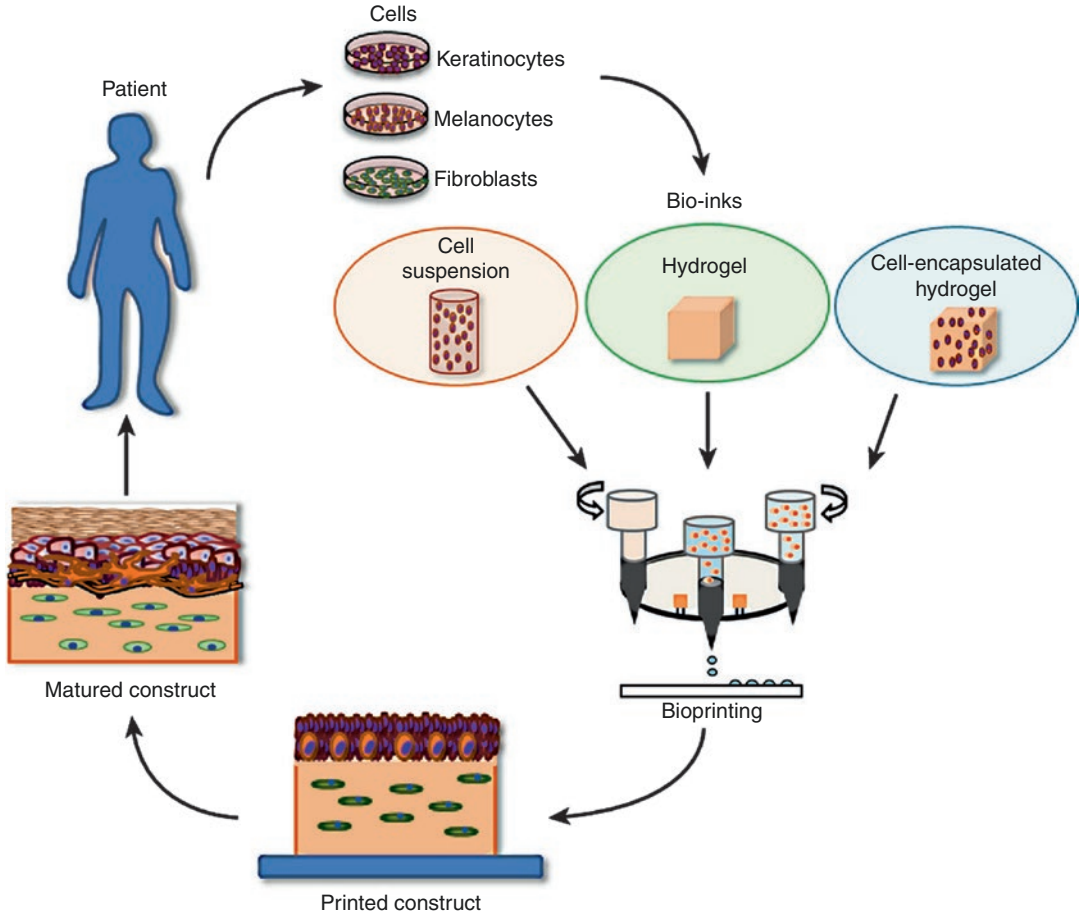

Fig. 16.3 3D bioprinting of skin. Following collection of cells, ex vivo expansion of the cells is commenced. Then 3D bioprinted biomimetic skin is constructed and once matures it is implanted to the patient. (From Ng et al. [159]. Reprinted with permission from Elsevier)

cells survive the printing process, and once implanted in experimental models, they form structures which have histological similarities to normal skin [113, 114].

Min et al. attempted to recreate a multilayer structure of fibroblasts on a collagen hydrogel, which was then covered with layers of melanocytes and keratinocytes [115]. Following histological analysis, the authors reported a distinct skin layer, the presence of pigmentation, and the presence of the outmost layer of normal skin (the stratum corneum). Three-dimensional bioprinting technologies allowing in situ bioprinting have also been developed [10]. In situ 3D bioprinting provides a platform for the creation of fully customized biomimetic structures printed exactly at the site of injury or defect [10]. A number of authors have developed handheld devices capable of ejecting multiple bioinks and demonstrated satisfactory cell survival and fast healing of skin defect [116–118].

Cardiovascular Tissue

Cardiovascular diseases are highly prevailing, and they represent one of the most common causes of death worldwide [119]. Tissue engineering has attempted to identify treatment options to facilitate the prompt repair of the affected tissue, mainly through the implantation of stem cells. Unfortunately, only a small fraction of these cells survive the effects of cytokines, free radicals, and lack of nutrients [120, 121]. Several attempts to create the hierarchical structure of the native myocardium through 3D bioprinting have been described [122, 123]. For example, Zhang et al. developed an endothelialized myocardial tissue by first aligning endothelial cells along the periphery of microfibers [123]. Then endothelial tissue was covered by cardiomyocytes. This construct had features of functional myocardium and expressed rhythmic beating. In a similar study using MSCs, Tijore et al. created microchanneled gelatin hydrogel that promotes human MSC myocardial commitment and supports native cardiomyocyte contractile functionality [122]. The feasibility of creating biomimetic cardiac tissue was also confirmed by Wang et al., who developed cardiac tissues formed with uniformly aligned, dense, and electromechanically coupled cardiac cells expressing the cardiac markers like α-actinin and connexin [124]. Three-dimensionally printed patches for myocardial regenerations were also explored [125, 126]. These patches were composed of human coronary artery-derived endothelial cells, methacrylated collagen, and an alginate matrix. They were found to upregulate cellular proliferation, migration, and differentiation in the damaged myocardium.

In addition to the regeneration of myocardium, the replacement of heart valves can be feasible utilizing 3D bioprinting technology. The construction of aortic valves capable of withstanding the hemodynamic requirements was proposed. Hockaday et al. used photo-cross-linked bioink loaded with porcine interstitial cells to show the feasibility of creating rapidly biomimetic aortic valve tissues with excellent cellular viability and cell engraftment capabilities [127]. Other groups have showed similar results, with some highlighting that the technique used to improve mechanical strength of the construct can adversely affect the viability of the cells [128, 129].

Retina and Cornea

Corneal and retinal diseases are the most important causes of blindness worldwide. At present, there is extensive research exploring the feasibility of engineering structures of the human eye including the cornea, retina, and lens. Isaacson et al. used extrusion 3D printing to fabricate a corneal-like cell-laden structure [130]. In a similar study, Sorkio et al. created a cornea-mimicking tissue using human stem cells and laser-assisted 3D bioprinting [131]. Printed constructs were examined for their microstructural properties, cell viability, and proliferation and for the expression of key proteins (Ki67, p63α, p40, CK3, CK15, collagen type I, VWF) [131]. As far as the retina is concerned, Lorber et al. created a 3D bioprinted construct containing retinal and glial

cells [132]. These cells retained their growth-promoting properties and exhibited higher than 70% viability [132]. Other authors highlighted the importance of the ECM as a determinant of cell differentiation [8, 133]. It is crucial the ECM should mimic the characteristics and stiffness of the human retina [8, 133]. In a scaffold-free approach, Masaeli et al. utilized an inkjet 3D bioprinting system to create (with precision) a construct made of photoreceptor cell layer lying on top of a bioprinted retinal pigment epithelial layer [134]. The cells expressed structural markers including opsin B, opsin R/G, MITF, PNA, rhodopsin, and ZO1 and released large amounts of human vascular endothelial growth factor (hVEGF).

Tissue Models

The development of tissue models for studying tissue and organ function, studying disease states, and testing drugs and chemicals represents another important potential application of 3D bioprinting [135]. This can help to overcome the limitations of current in vitro models which rely on the use of two-dimensional (2D) cell cultures. It is argued that 2D models cannot represent appropriately native tissues [136]. In this relation, Maden et al. developed a 3D bioprinted model of human intestinal mucosa mimicking the function and the biochemical and histological characteristic of the native human tissue [137]. Vascularized perfusable liver tissue has been also created. Drug toxicity on 3D printed tissue was also conducted, with the authors suggesting the advantages of this approach for the evaluation of drug-induced liver injury [138]. Commercially available 3D printed liver and kidney tissue is currently available for research purposes [139].

In addition to healthy tissue models, a number of pathologic tissue models based on 3D bioprinting currently exist. For example, such models can be valuable tools for gaining in-depth understanding of tumor progression and invasion – as well as for the study of the interaction between different cell types and treatment of chemotherapeutic drugs [140]. The clinical scenarios are diverse, and models should be designed accordingly. In metastatic bone disease, Zhou et al. developed a biomimetic bone matrix analyzing the interactions between breast cancer cells, fetal osteoblasts, and human bone marrow MSCs [141]. In another study, 3D bioprinted microtissue, recapitulating the in vivo environment of tumor cells in pituitary adenoma, was found to be an excellent model for cancer research [142]. Similarly, uterine cervical tumor models, lung cancer, neuroblastoma, and breast cancer models exist [143–146].

Other Applications

It has to be noted that the potential targets of 3D bioprinting are not limited to the aforementioned applications. At present, numerous other applications based on 3D bioprinting are being explored – especially ones involving the creation of biomimetic soft or solid human tissues. Such structures include the kidney, liver, and trachea. Ali et al. created 3D bioprinted renal constructs exhibiting structural and functional features of the native renal tissue [147]. Lee et al., on the other hand, 3D printed human liver which mimicked the cellular interactions seen within human liver [148]. Hard structures such as the human trachea were printed using PCL, and the constructs were then placed in omentum culture prior to transplantation [149]. This approach facilitated the rapid re-epithelialization and revascularization of the scaffold and prevented postoperative luminal stenosis [149]. Other potential applications of such 3D bioprinting approaches in hard tissue engineering include the creation of knee meniscal tissues, human ear, and auricular cartilage [85, 150–152].

Current Limitations and Future Prospects

Despite the significant advantages seen in 3D bioprinting over the last decades, at present, this technology has several limitations, which prevents its further expansion. These challenges fall

into three main categories: *(a)* decoding of human anatomy and physiology, *(b)* manufacturing issues, and *(c)* creation of viable constructs that will integrate and function in vivo.

With regard to decoding human physiology, despite a gross understanding of the structure of human tissues, the underlying interactions at a cellular level are largely obscure. Not infrequently, our understanding of the composition, organization, and interactions occurring within human tissues is based on animal in vivo models and then extrapolated to explain our knowledge gap in humans. Animals are different species, and thus we often see complications and adverse effects to drugs in humans despite the safe results obtained from experimental studies [153].

In terms of manufacturing, several technical difficulties should be overcome. Attempts to improve the resolution of the printed tissues (probably at a cellular level) will open new avenues to 3D bioprinting. This resolution should be maintained throughout the bioprinting process, and drawbacks – like nozzle clogging with highly homogenous bioinks maintaining their viscosity and shear-thinning properties – should be addressed. Further work on developing new biomaterials for 3D bioprinting is required identifying the ideal material for a given tissue and maintaining stability and mechanical rigidity. In cases when hard tissues (such as bone) are to be created, the bioink should maintain mechanical stability to withstand the demands but, at the same time, should allow the migration, proliferation, and differentiation of osteoprogenitor cells to enable the incorporation and remodeling of the newly formed bone.

Another major challenge of 3D bioprinting is the creation of viable and functional constructs. One of the main challenges is to recreate vascularity. It is well known that cells should be in close proximity to the capillaries; otherwise, increased cell death can follow [154]. It can be hypothesized that improving the vascular networks within these structures will facilitate the functionality and integration of these structures to the host. Studying critical size bone defects has shown that the larger the defect is, the longer the *time* is required for healing, and beyond a critical size, healing by regeneration may *not* occur [155]. This time does not purely correspond to the time required for the bony ends to heal, but instead it correlates with time it takes to achieve revascularization of the graft and the incorporation to the host.

Reflecting on the current growth rate of 3D bioprinting and the intensity of research activity, we envision that, in the near future, customized medical applications will be introduced into clinical practice [156, 157]. Complex constructs mimicking native tissues will emerge. This would require extensive knowledge of biomaterials and the capacity to incorporate bioinks of different properties during the same bioprinting session. These materials should be loaded with the exact cell layers and growth factors to develop microenvironments that may closely mimic that of the target native tissue. Further development in the incorporation of a functional vascular tree in printed constructs is an important factor required to achieve success. Despite the fact that all aforementioned challenges are important, decoding and understanding human anatomy and physiology is the most vital element that will unleash the capabilities of 3D bioprinting.

Conclusions

Today, 3D bioprinting is a rapidly evolving technology for tissue engineering. It enables the fabrication of biomimetic tissues in a fast manner and with high precision. Despite the increasing number of studies presenting its potential role in clinical practice, several challenges are still facing the manufacturing process. The selection of bioinks suitable for a given target tissue, the lack of a vascular tree to support the cellular elements, and the final integration of a functional replacement to the host are among the most important challenges. These challenges will ultimately be overcome via coordinated work that involves biologists, bioengineers, and clinicians.

Conflict of Interest No benefits in any form have been received or will be received from a commercial party related directly or indirectly to the subject of this chapter.

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