# Chapter 3
# Potential Clinical Applications of Three-Dimensional Bioprinting


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**Abstract** Three-dimensional (3D) bioprinting aims to construct complex personalized living tissues mimicking the native tissues. This chapter presents the current advances in 3D bioprinting. Available evidence revealed promising results in potential applications for the regeneration of musculoskeletal, cardiovascular, dermal, and neural tissues. These applications comprise a developing field. However, there are still barriers that hamper further expansion of this technology. Such challenges involve the reliable mechanical properties, size limitations, integration of transplanted grafts, and safeguarding of safety throughout the process of 3D printing and resulting constructs.

**Keywords** 3D bioprinting · Bone · Mesenchymal stem cells · Cartilage · Blood vessels · Cardiovascular tissue

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## 3.1 Introduction

Bioprinting is the process of combining cellular and non-cellular components in bioinks to produce three-dimensional (3D) constructs that can mimic or be used to reconstruct human tissues. This technology is based on 'additive fabrication' of layers to achieve 3D fabrication of tissues that can replicate the hierarchical structure and cell composition of native tissues. Three-dimensional bioprinting as a concept is far superior to currently available tissue engineering approaches that involve the loading of cells and/or growth factors into scaffolds. This technology offers the ability to fabricate 3D tissue structures with high precision, fidelity, and stability at the human clinical scale [1, 2]. The creation of complex tissue architectures with heterogeneous compositions has the potential to revolutionize the transplantation of tissues. Since the medical community realized its potential, 3D bioprinting has captured significant interest, especially over the last decade [3, 4].

In brief, 3D bioprinting uses three common printing technologies, microextrusion, inkjet, and laser-assisted bioprinting methods (Fig. 3.1) [5]. It also involves three distinct steps: (1) pre-processing, (2) processing, and (3) post-processing. Pre-processing involves the creation of a computer-aided design of the tissue. Magnetic resonance imaging (MRI) or computed tomography (CT) scans can be utilized for computer-controlled 3D printing using appropriate bioinks [6]. Cells can be harvested and used fresh or can be manipulated ex vivo. Depending on the tissue of interest, our armamentarium includes a variety of bioinks and hardware. This is often a crucial element that can influence the quality and survival of the graft. Processing is the actual bioprinting of the tissue, while post-processing involves the brief incubation of the tissue or graft in a bioreactor. To minimize the ex vivo manip-

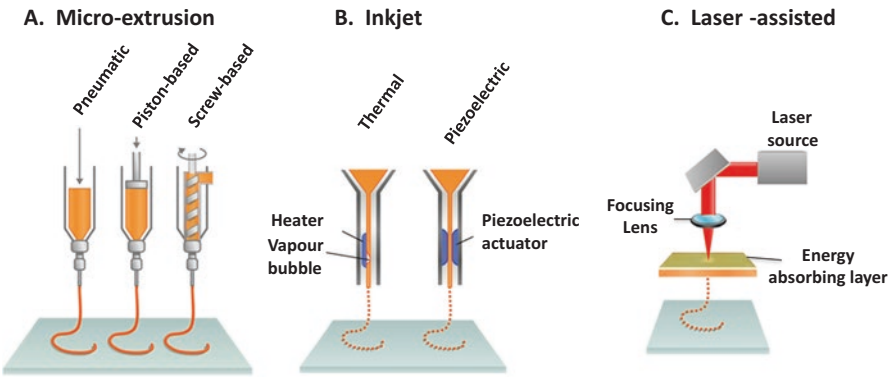


**Fig. 3.1** Three-dimensional bioprinting techniques. The three most common 3D bioprinting techniques: (**a**) microextrusion, (**b**) inkjet, and (**c**) laser-assisted 3D bioprinting. The microextrusion technique can be (1) pneumatic, (2) piston-based mechanical, or (3) screw-based. Inkjet technique can be either thermal or piezoelectric. (**a**, **b**) are adapted from Malda et al. with permission from John Wiley and Sons [5]. (**c**) is adapted from Keriquel et al. [28, 29] with permission from Nature Publishing Group [29]

ulation of these constructs, in situ bioprinting can be performed, i.e. 3D bioprinting directly on the defect site (Fig. 3.2) [7] Using these approaches, a number of tissues have been created with promising results. Tissues of musculoskeletal origin, neural or vascular structures, skin, and other have been developed over the years [8, 9].

In this chapter, we aim to present some of the current and numerous potential clinical applications of 3D bioprinting. Challenges and future developments of 3D bioprinting are also discussed here in this chapter.

## 3.2 Bone

Bone is a unique tissue that provides stability to the whole body and performs other functions like haematopoiesis, locomotion, and homeostasis of important elements of the human body. Bone can be fractured following trauma, and bony defects can occur following severe injuries, tumours, or other pathologies [10–12]. In addition, some fractures and bony injuries fail to heal. It is estimated that 5–10% of long bone fractures will end up in nonunion [12, 13]. Tissue engineering approaches have focused on assisting bone regeneration as an attempt to either upregulate the overall healing process in high-risk cases or provide the required scaffold, cells, and osteoinductive factors in cases of bone loss [10–14]. With the advances made in 3D bioprinting, several attempts to create bone were made. The main challenges remain the selection of materials with optimal rheological properties, biocompatibility,

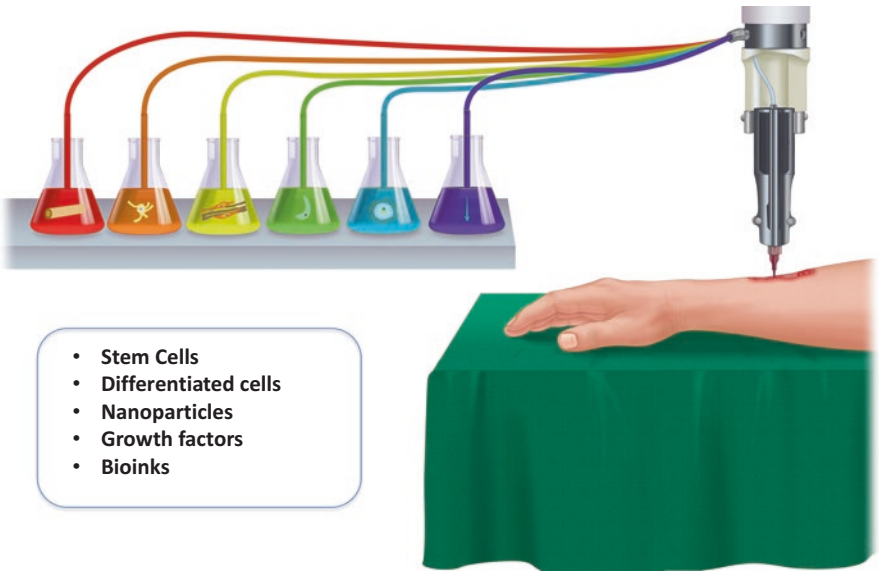


**Fig. 3.2** In situ 3D bioprinting of skin. On-demand personalized bioprinting directly on the defect with bioinks matching the reconstructed tissues

osteoconductivity, and capacity of the graft to be incorporated and remodelled to normal bone [1].

In vitro studies analysed the effectiveness of a number of bioinks for the fabrication of bone (Table 3.1) [1, 6, 15–34]. Some investigators used materials such as poly-ε-caprolactone (PCL) and poly(lactide-*co*-glycolide) (PLGA). Although these are mechanically stable, they have limited osteoconductive properties [19, 20]. A number of alternative materials were explored such as tricalcium phosphates, hydroxyapatite, and bioactive glass [15, 21–23]. In the works of Poldervaart et al., methacrylated hyaluronic acid (HA) with human mesenchymal stem cells (MSCs) was used [15]. The cellular viability was 64% after 21 days of culture, and osteogenic differentiation of MSCs occurred spontaneously in hydrogels. The osteogenic differentiation increased with the addition of bone morphogenetic protein-2 (BMP-2) to the culture medium [15]. In a similar study using gelatin and alginate bioinks with human adipose-derived stem cells, high cellular viability levels were noted with the expression of important osteogenic markers [16]. Similar composite materials were used in a number of studies with favourable results. The combination, for example, of thermo-responsive hydrogels with collagen type I improved the mechanical properties of the constructs [18]. Other investigators used bioactive glass, microcarriers, polymers, and polyethylene, and they obtained similar results when attempting to increase the overall mechanical stability of the 3D printed hydrogels [24–26]. Comparable results were described with other base bioinks, for instance, adding microcarriers to gelatin methacryloyl (GelMA) [19]. A different approach is to load osteogenic growth factors into the bioink. In a study on BMP-2 loaded gelatin, release kinetics and bioactivity showed continuous release of BMP-2 for 3 weeks after bioprinting [17]. Using the aforementioned technologies it was possible to fabricate a whole human mandible as well as calvarial bone, cartilage, and skeletal muscle [Fig. 3.3] [6].

In vivo animal studies have also shown promising results [16, 17]. In a segmental tibial defect model, the application of nano-hydroxyapatite (nHAp)/PCL resulted in the formation of dense bone tissue around the scaffold at 8 weeks postoperatively [22]. In a similar model, of rabbit femoral defects that were treated with poly(D,L-lactide-*co*-glycolide) and β-tricalcium phosphate (β-TCP) nanocomposites, increased bone formation was observed [27]. Less favourable results were though reported when 3D printed constructs composed of TCP and poly(L-lactide-*co*-D,L-lactide) (PLDLLA)-TCP-PCL scaffolds were implanted in ovine segmental defects [1]. At 12 weeks postoperatively, only minor external callus and bone formation were observed, suggesting that adding a biologically active stimulus such as a BMP might be required [1].

In situ 3D bioprinting of bone has been also proposed by a limited number of studies. In the works of Keriquel et al., the feasibility of a laser bioprinter adapted for in vivo use on calvarial defects in mice was studied [Fig. 3.4] [28]. The investigators used nHAp to fill the defects and they followed up the animals for 3 months. The results were mixed with only a proportion of the defects was filled with bone tissue. The same group used this technology employing mesenchymal stromal cells in different arrangements and geometries within a scaffold composed of nHA and

**Table 3.1** Selected studies showing evidence of successful bone 3D bioprinting

| Author, Year | Printer | Design | Scaffold/Bioink | Cells | Outcome |
|---|---|---|---|---|---|
| Keriquel et al., 2010 [28] | Laser (In situ) | In vivo | Nano hydroxyapatite (nHA) | No cells | • In vivo bioprinting is possible.<br>• No effect to animal's brain. Bone formation only occurred in some defects. |
| Kim et al., 2012 [22] | Extrusion | In vivo | DL-PLGA and β-tricalcium phosphate (β-TCP) nanocomposites | No cells | • Scaffolds integrated with the host bone and were biocompatible. |
| Poldervaart et al., 2013 [17] | Extrusion | In vitro and in vivo | Gelatin loaded BMP-2 and alginate | Goat multipotent stromal cells | • Controlled release of BMP-2 from the scaffold was noted. |
| Du et al., 2015 [34] | Extrusion | In vitro | Methacrylamide gelatin scaffold with collagen microfibers and BMP-2 | MSCs | • BMP-2 was able to be controllably released.<br>• MSCs showed high cell viability (>90%) during printing.<br>• CBD-BMP2-collagen microfibers induced BMSC differentiation into osteocytes within 14 days in culture. |
| Duarte Campos et al., 2016 [18] | Extrusion | In vitro | Collagen type I in polysaccharide-based hydrogels | Human MSCs | • MSC not only survive the 3D-bioprinting process but also maintain the mesenchymal phenotype. |
| Wang et al., 2016 [16] | Extrusion | In vitro and in vivo | Gelatin and alginate | Human adipose-derived stem cells | • Cell viability of 89% on day 1 after printing.<br>• The expression levels of RUNX2, OSX, and OCN were significantly increased on days 7 and 14 after printing.<br>• Bone matrix formation in the 3D bioprinted constructs noted in vivo. |

(continued)

**Table 3.1** (continued)

| Author, Year | Printer | Design | Scaffold/Bioink | Cells | Outcome |
|---|---|---|---|---|---|
| Bendtsen et al., 2017 [30] | Extrusion | In vitro | Alginate-polyvinyl alcohol (PVA)-hydroxyapatite (HA) hydrogel | Mouse calvaria cells (MC3T3) | • Construct remained stable for 2 weeks and high cellular viability was noted. |
| Demirtas et al., 2017 [31] | Extrusion | In vitro | Chitosan solution with nanostructured bone-like hydroxyapatite | MC3T3-E1 pre-osteoblast | • Stable construct that preserved cell viability and allowed osteogenic differentiation in culture. |
| Keriquel et al., 2017 [29] | Laser (in situ) | In vivo (critical size defect) | Nano hydroxyapatite (nHA) and collagen | MSCs | • This technology can print complex structures and favour bone regeneration.<br>• Cell geometries and cell arrangements have a significant impact on bone regeneration. |
| Neufurth et al., 2017 [32] | Extrusion | In vitro | Amorphous microparticles prepared from $Ca^{2+}$ and the physiological inorganic polymer, polyphosphate fortified by mixing with poly-ε-caprolactone | Human bone-related SaOS-2 | • Scaffold was capable of attracting and promoting the growth of human bone SaOS-2 cells. |
| Poldervaart et al., 2017 [15] | Extrusion | In vitro | Methacrylated hyaluronic acid (MeHA) gel | Human MSCs | • Osteogenic differentiation of MSCs occurred spontaneously and further enhanced with the addition of BMP-2. Cell viability remained 64.4% after 21 days of culture. |
| Zhang et al., 2017 [33] | Extrusion | In vitro | β-tricalcium phosphate bioceramic scaffolds containing silver nanoparticles on graphene oxide | Rabbit bone marrow stromal cells | • Excellent antibacterial activity accelerated osteogenic differentiation of the cells. |

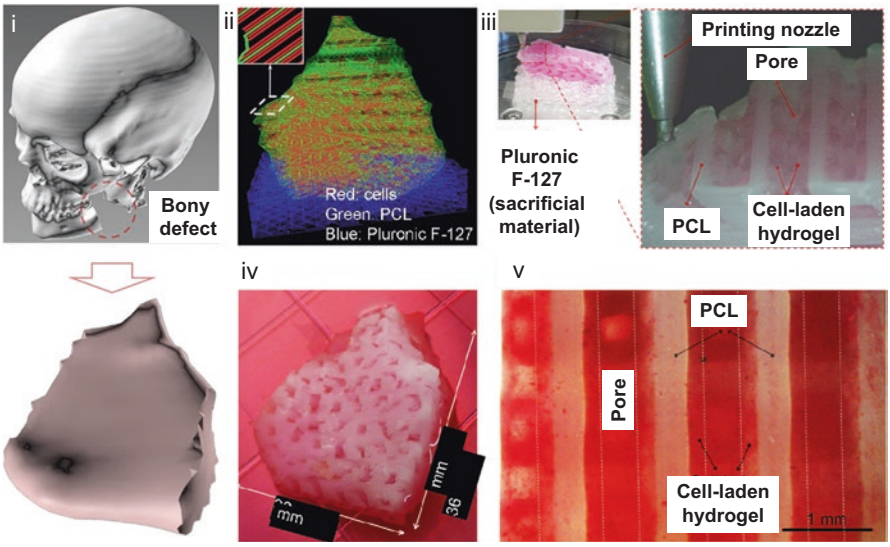


**Fig. 3.3** Reconstruction of a human mandible graft. (**i**) Using CT images a 3D CAD model was created. (**ii**) Reconstruction of the bone defect 3D architecture: Green, blue, and red lines represent the paths used to dispense various inks (PCL, Pluronic F-127, and cell-laden hydrogel, respectively). (**iii**) The patterning of a construct layer using 3D bioprinting. (**iv**) Appearance of the construct in culture after 28 days in osteogenic medium. It was cultured in osteogenic medium for 28 days. (**v**) Calcium deposition following osteogenic differentiation in the printed construct was evident by Alizarin Red S staining. Figure 3.3 was reproduced from Kang et al. [87] with permission from Nature Publishing Group

collagen [29]. The investigators showed that this technology can produce favourable results in the setting of large bone defects. It also demonstrated that a disc configuration with MSCs had the best results in bone healing and regeneration [29].

## 3.3 Cartilage

Cartilage damage and osteoarthritis affect millions of people worldwide. In the USA alone, osteoarthritis affects 37% of the population over 65 causing significant morbidity and reduction in quality of life [35]. Our current approach to the management of osteoarthritis is carrying out joint arthroplasty or fusion, while cartilage regeneration techniques are still in their infancy and with controversial results [36]. However, cartilage is a unique tissue and possibly ideal target for 3D bioprinting applications, as it does not require blood vessels. Many studies currently show that 3D bioprinted cartilage could be a solution to the cartilage loss and may offer better treatment for arthritis [Table 3.2] [37–54].

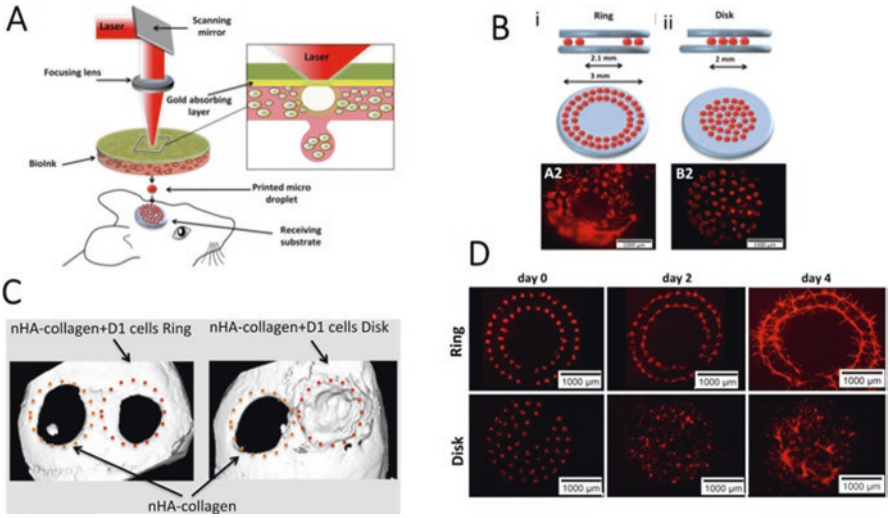


**Fig. 3.4** Laser 3D bioprinting on osteoconductive discs. Osteoprogenitor cells were printed on nHAp collagen discs and subsequently used in the treatment of experimental calvarial defects in mice (**a**). Cells were printed at the peripheral (i) or central (ii) areas of the discs (**b**). Immediately after printing fluorescence images of peripherally (A2) and centrally (B2) printed tomato-positive cells, (**c**) Microtomography (μCT) images, 2 months after surgery, showing increased osteogenic activity for the defects where cells were applied at the central area rather than the periphery. No bone formation was noted in defects where no cells were applied. (**d**) Day 0, 2, and 4 fluorescence images of centrally and peripherally printed tomato-positive D1 cells. Figure is reproduced from Keriquel et al. [28, 29] with permission from Nature Publishing Group

In vitro studies have shown that 3D bioprinted cartilage is feasible [38–40]. Three-dimensional bioprinted cartilage can have long-term stability and mechanical integrity [39, 40]. In addition, the cells used in the bioprinting process have shown acceptable viability levels and remain functional after the fabrication of cartilage [38–40]. Daly et al. compared a number of bioinks for their capacity to support chondrocytes and for ultimately developing hyaline cartilage [42]. They suggest that alginate and agarose hydrogels were superior in developing hyaline-like cartilage as compared to GelMA and BioINK™. The latter resulted in the formation of tissue with cellular phenotype resembling fibrocartilage even though all bioinks supported the cells and achieved high viability. In another model of knee osteoarthritis, silk fibroin with gelatin combined with BMSC-specific-affinity peptide showed promising results [37]. The authors suggested that silk fibroin and gelatin can greatly balance the mechanical properties and degradation rate to match the newly formed cartilage [37]. A different approach involving on-demand personalized biofabrication of grafts was also explored. Di Bella et al. investigated the effectiveness of a hand-held bioprinter on critical size osteochondral defects in sheeps [41]. The bioink comprised gelatin methyacrylamide, HA methacrylate hydrogel, and MSCs [41]. The investigators reported better macroscopic and microscopic appearance of the resulting tissue was comparable to that achieved with microfrac-

**Table 3.2** Selected studies showing the current evidence on 3D bioprinting of cartilage

| Author, Year | Printer | Design | Scaffold/Bioink | Cells | Outcome |
|---|---|---|---|---|---|
| Cui et al., 2012 [38] | Inject | In vitro | Poly(ethylene glycol) dimethacrylate (PEGDMA) | Human chondrocytes | • High cellular viability with preservation of function.<br>• Promising anatomic cartilage engineering using 3D bioprinting technology. |
| Schuurman et al., 2013 [49] | Extrusion | In vitro | Gelatin-methacrylamide with/without ε-polycaprolactone or HA | Human chondrocytes | • When gelatin-methacrylamide is combined with HA and/or a reinforcing support structure, such as PCL, gelMA can be fabricated into layered hydrogel structures, which could aid in the engineering of human cartilage. |
| Xu et al., 2013 [40] | Hybrid (inkjet and electrospinning system) | In vitro and in vivo | Polycaprolactone fibers and chondrocytes suspended in a fibrin-collagen hydrogel | Rabbit chondrocytes | • 80% viability 1 week after printing was noted.<br>• Cells proliferated and maintained their basic biological properties.<br>• Constructs formed cartilage-like tissues both in vitro and in vivo as evidenced by the deposition of type II collagen and glycosaminoglycans.<br>. |
| Kundu et al., 2015 [39] | Extrusion | In vitro and in vivo | Polycaprolactone and chondrocyte cell-encapsulated alginate hydrogel | Human chondrocytes | • Enhanced cartilage tissue and type II collagen fibril formation at 4 weeks following implantation in vivo were observed. |
| Markstedt et al., 2015 [50] | Extrusion | In vitro | Nanofibrillated cellulose | Human chondrocytes | • Cell viability of 73% and 86% after 1 and 7 days, respectively, was noted. |

(continued)

**Table 3.2** (continued)

| Author, Year | Printer | Design | Scaffold/Bioink | Cells | Outcome |
|---|---|---|---|---|---|
| Costantini et al., 2016 [51] | Extrusion | In vitro | Gelatin methacrylamide, chondroitin sulphate amino ethyl methacrylate, and HA methacrylate. | Human MSCs | • Enhanced viability and chondrogenic differentiation of BM-MSCs was noted. |
| Ren et al., 2016 [52] | Extrusion | In vitro | Collagen type II hydrogel | Rabbit chondrocytes | • ECM production was positively correlated with the total cell density. |
| Daly et al., 2016 [42] | Extrusion | In vitro | Agarose, alginate, GelMA, and BioINK™ | MSCs | • High viability levels with all bioinks were reported.<br>• GelMA and BioINK™ resulted in developing a more fibrocartilage-like tissue. |
| Nguyen et al., 2017 [53] | Extrusion | In vitro | Nanofibrillated cellulose composite with alginate or HA | Human chondrocytes | • Cell viability, pluripotency, and function were maintained. |
| Shi et al., 2017 [37] | Extrusion | In vitro and in vivo | Silk fibroin and gelatin | MSCs | • Superior performance for cartilage repair in a knee joint as biomaterial matches mechanical properties of the newly formed cartilage. |
| Apelgren et al., 2017 [54] | Extrusion | In vivo | Nanofibrillated cellulose and alginate | Human chondrocytes and human MSCs | • Chondrocytes showed good proliferation ability.<br>• In constructs comprising a mixture of chondrocytes and stem cells, an additional proliferative effect was observed involving chondrocyte production of glycosaminoglycans and type 2 collagen. |

tures or equivalent bench-based printed scaffolds. A higher amount of newly regenerated cartilage was noted with the absence of subchondral deformation or collapse.

Although the aforementioned studies proposed that the use of cartilage 3D bioprinting for the treatment of focal defects will be feasible soon, it is unclear whether a holistic approach will be required when the whole joint is affected by osteoarthritis. This argument is supported by current evidence suggesting that osteoarthritis results in extensive changes, which are not limited to the cartilage but involve the entire joint including the subchondral bone [43]. Hence, a number of researchers focus on the production of osteochondral constructs rather than cartilage patches [44–48]. Woodfield et al. suggested that anatomically shaped, 3D bioprinted constructs with designed mechanical properties might offer alternatives for the reconstruction or restoration of congruent articulating surfaces [45]. In their study, 3D constructs loaded with chondrocytes were evaluated in vitro and in vivo (in rabbits). Fully functional chondrocytes were observed and the integration of the constructs with the bone was seen. Weight-bearing and functional joints were noted. In a similar study, a rabbit proximal humeral joint was captured with laser scanning and a scaffold was bioprinted layer-by-layer using HAp powder and PCL [46]. This scaffold was infused with transforming growth factor β3 (TGFβ3). The investigators reported that TGFβ3-infused bioscaffolds were fully covered with hyaline cartilage in their articular surface [46]. Similar approaches were also used by other researchers in animal models of femoral head and temporomandibular defects [47, 48].

## 3.4 Skin

Human skin is a complex structure having a variety of layers and cellular components. Skin loss from trauma and burns has been one of the earliest motivations of tissue engineering. Despite significant advances in skin tissue engineering, the designs often simplify considerably the structure of the skin to two main components (dermis and epidermis). Alternatively, 3D bioprinting has the potential to produce structures of higher complexity [Table 3.3] [55–67].

Three-dimensional bioprinting of human skin involved mainly loading of fibroblasts or/and keratinocytes into hydrogels of collagen, gelatin, or alginate [55]. The results of many studies showed that the resulting 3D engineered skin achieved high cellular survival and its histological appearance resembles that of human skin. In a slight deviation of most of the available studies, Koch et al. has added MSCs to the bioink [59]. They reported that MSCs retained high survival during the printing process and did not become apoptotic following the construction of the graft. The aforementioned approaches often result in low stability of the construct; hence crosslinking is required. To overcome this drawback, Min et al. printed fibroblasts, melanocytes, and keratinocytes onto collagen hydrogel crosslinked through neutralization using sodium bicarbonate [63]. The authors reported that the resulting melanocyte-containing epidermal layer showed freckle-like pig-

**Table 3.3** Selected studies showing the current evidence on 3D bioprinting of skin

| Author, Year | Printer | Design | Scaffold/Bioink | Cells | Outcome |
|---|---|---|---|---|---|
| Lee et al., 2014 [55] | Extrusion | In vitro | Collagen type I | Keratinocytes and fibroblasts | • 3D printed skin tissue had histological similarities to the human skin tissue |
| Lee et al., 2009 [58] | Extrusion | In vitro | Multilayer hydrogel | Keratinocytes and fibroblasts | • Highly viable proliferation of each cell layer was observed.<br>• Organo-typic skin tissue culture is feasible. |
| Binder et al., 2010 [65] | In situ skin printer | In vivo (mice) | | Keratinocytes and fibroblasts | • Acceptable survival rate of cells after printing was noted.<br>• Fast healing rate of the skin defects occurred. |
| Koch et al., 2010 [60] | Laser-induced forward transfer | In vitro | Alginate hydrogel | Keratinocytes and fibroblasts and human MSCs | • High cells' survival of the printing process.<br>• All used cell types maintained their ability to proliferate after printing.<br>• Skin cells and hMSC showed no increase of apoptosis or DNA fragmentation. |
| Skardal et al., 2010 [66] | In situ extrusion | In vivo (mice) | Fibrin-collagen gel | Amniotic fluid cells and bone marrow-derived MSCs | • The graft resulted in higher re-epithelialization with increased microvessel density and capillary diameters.<br>• The secreted trophic factors could be responsible for the favourable effect, rather than direct cell–cell interactions. |
| Albanna et al., 2012 [67] | In-situ extrusion | In vivo (porcine) | Fibrogen/ collagen solution | Fibroblasts and keratinocytes | • In situ skin bioprinting is a viable option for treatment of large skin defects.<br>• The utilization of autologous cells outperformed in healing potential compared to allogeneic cell use. |

(continued)

**Table 3.3** (continued)

| Author, Year | Printer | Design | Scaffold/Bioink | Cells | Outcome |
|---|---|---|---|---|---|
| Koch et al., 2012 [59] | Laser-assisted | In vitro | Collagen type I | Keratinocytes and fibroblasts | • Laser-assisted bioprinting is an outstanding tool for the generation of multicellular 3D resampling human skin. |
| Michael et al., 2013 [61] | Laser-assisted | In vitro and in vivo (mice) | Collagen type I | Keratinocytes and fibroblasts | • In vitro experiments showed proliferative cells, but they were in the whole epidermis.<br>• Printed fibroblasts produced collagen.<br>• In the mice, some blood vessels could be found to grow from the wound bed and the wound edges in the direction of the printed cells. |
| Cubo et al., 2016 [56] | Extrusion | In vitro and in vivo (mice) | Bioinks containing human plasma fibrin | Keratinocytes and fibroblasts | • Generated skin was similar to human skin.<br>• Skin was indistinguishable from bilayered dermo-epidermal equivalents. |
| Liu et al., 2016 [64] | n/a | In vivo (mice) | Gelatin-alginate scaffold | No cells used | • 3D printed scaffold accelerated wound healing. |
| Kim et al., 2017 [62] | Inject and extrusion | In vitro | Gelatin and collagen I | Keratinocytes and fibroblasts | • Keratinocytes were uniformly distributed into the engineered dermis.<br>• Maturation of a skin occurred.<br>• Favourable biological characteristics including a stabilized fibroblast-stretched dermis and stratified epidermis were noted. |

(continued)

**Table 3.3** (continued)

| Author, Year | Printer | Design | Scaffold/Bioink | Cells | Outcome |
|---|---|---|---|---|---|
| Pourchet et al., 2017 [57] | Extrusion | In vitro | Gelatin (Bovine) | Keratinocytes and fibroblasts | • Immuno-staining and electronic microscopy presented all characteristics of human skin.<br>• The printability of large skin objects is demonstrated with the printing of an adult-size ear. |
| Min et al., 2018 [63] | Extrusion | In vitro | Collagen hydrogel crosslinked through neutralization using sodium bicarbonate | Fibroblasts, melanocytes, and keratinocytes | • Melanocyte containing epidermal layer showed freckle-like pigmentations at the dermal-epidermal junction, without the use of external ultraviolet light or chemical stimuli. |

mentations at the dermal-epidermal junction, without external ultraviolet light or chemical stimuli [63].

A different approach to the aforementioned studies was reported by three different investigators exploring on-demand in situ 3D bioprinting [65–67]. In the treatment of a full-thickness skin defect model, Binder et al., 3D applied printed constructs containing keratinocytes and fibroblasts [65]. The resulting skin was similar to normal skin and complete wound healing was reported [65]. Skardal et al. used a full-thickness skin wound model where 3D printed amniotic fluid cells and bone marrow-derived MSCs suspended in fibrin-collagen gel were placed on the defects [66]. This approach resulted in higher levels of re-epithelialization and increased microvessel density and capillary diameters. Due to the fact that the printed cells did not permanently integrate with the surrounding tissues, authors concluded that the secreted trophic factors could be responsible for the favourable effect, rather than direct cell–cell interactions. In the third study, an experimentally induced 10x10cm skin defect in a porcine model was created [67]. The investigators explored the healing potential of fibroblasts and keratinocytes suspended in fibrogen/collagen solution and compared the overall potential of autologous versus allogeneic cells [67]. The results showed that this technique is a viable option for the treatment of large skin defects with the autologous cells outperform the use of allogeneic cells in terms of healing potential.

## 3.5 Neural Tissues

At present, the ideal approach for nerve repair is the precise microsurgical implantation of a healthy autologous nerve graft. This is the closest resemblance to the original microstructure of the missing nerve [68]. Although this is our current gold standard approach, the technique is associated with poor nerve function, donor site morbidity, and the formation of neuromas [68]. It has been proposed that 3D bioprinting could offer great potential in fabricating the precise cellular structures for nerve tissues.

For clinical scenarios where nerve transection occurs without nerve loss, the application of a 3D bioprinted fibrin scaffold created by extruding fibrinogen solution into thrombin solution and utilizing HA and polyvinyl alcohol, was found to mimic the natural fibrin clot that forms between injured nerve ends and encapsulated Schwann cells, thus providing natural guidance of neurite growth [69]. In cases of nerve damage with loss of neural tissue, approaches of 3D bioprinting could be divided in those aiming to construct hollow nerve guidance conduits or constructing more complex tissues with cells within complex bioinks. Three-dimensional printed hollow nerve conduits can be of natural or synthetic materials, single lumen or multilumen [70–72]. In vivo experiments have shown that these materials could promote nerve regeneration [70–72]. Alternatively, more complex 3D printed constructs using cells have also been found to promote nerve regeneration in experimental animal studies [73–75]. In particular, using 3D bioprinted scaffold-free conduits made from human normal dermal fibroblasts in an experimental animal model of transacted sciatic nerve, Yurie et al. reported favourable outcomes in the regeneration of the nerve [74]. Similar results were reported in an experimental tibial nerve injury model in rats [75]. Bioprinted cryopolymerized GelMA (cryoGelMA) gel was cellularized with adipose-derived stem cells. This graft could support the re-innervation across a 10 mm sciatic nerve gap in rats, with results close to those obtained with the use of autografts in terms of functional and histological characteristics [73]. In a different approach, a 3D printed layer-by-layer cylindrical structure loaded with cell suspension composed of 90% MSCs and 10% Schwann cells was used in the treatment of experimental sciatic nerve defects in rats [74]. The investigators in this concept study reported favourable results, recognizing the importance of several adjustments that need to be made. These adjustments include the removal of agarose rods from the construct lumina prior to implantation or using a hydrogel with faster degradation time in vivo, adjusting the number of lumina and modifying the cell types used or adding growth factors [74].

## 3.6 Blood Vessels

One of the major challenges in tissue engineering is the fabrication of vasculature or vascularized tissue. It has been previously noted that cells can survive at a distance of 200–400 μm from a blood vessel as the farthest [76]. To overcome the lack of vasculature, tissue engineering approaches have employed the addition of angiogenic factors such as the vascular endothelial growth factor (VEGF) to promote vascular migration from the host or employing surgical vascularized flaps [12, 77].

Several studies have evaluated the construction of vasculature through 3D bioprinting technologies [78–87]. Large blood vessels such as aortic tissue construct was 3D bioprinted layer-by-layer using mouse embryonic fibroblast cell aggregates and hydrogels. Smaller blood vessels were also constructed using 3D bioprinting. Tubular structures with 300 μm wall thickness, inner diameters of 1–2 mm, and defined pores with a constant diameter of approximately 100 or 200 μm mimicking the structure of blood vessels were also 3D printed [83]. In the work of Zhao et al., robotic 3D cell printing technology with a mesoscopic fluorescence molecular tomography imaging system was used to construct perfused collagen scaffolds with endothelial lining [78]. The authors imaged both the fluid flow and fluorescent-labelled living endothelial cells at high rates, with high sensitivity and accuracy [78] Finally, a more sophisticated construct was presented by the Atala group [87]. The authors documented the development of an integrated tissue-organ printer that can produce human-scale tissue constructs of various shapes and incorporating microchannels that allow for the diffusion of nutrients to printed cells. These tissues could be sustained for long periods, enabling the differentiation of cells into various lineages [82].

## 3.7 Muscle

Injuries to the skeletal muscles are debilitating and they result in extensive scaring which leads to functional impairment. Advances in 3D bioprinting showed significant potential for application in muscle regeneration [6, 88–91].

Early studies of 3D bioprinted myoblasts onto micro-sized cantilevers showed fusion of myoblasts to mature myotubes in 4 days of culture [89]. Alternatively, 3D printing of fibronectin stripes onto biodegradable L-lactide/trimethylene carbonate copolymer (PLLA-TMC) films with murine myoblast induced cell alignment and improved myotube formation [90]. In another study by Peele et al., muscle mimicking the function of musculature was printed using layer-by-layer stereolithography technique at high resolutions of 37 μm [91]. Merceron et al. have 3D printed a muscle-tendon unit resembling a functional human muscle [88]. This construct was developed in two layers. The first layer was composed of thermoplastic polyurethane co-printed with C2C12 cell-laden hydrogel-based bioink for elasticity and muscle development. The other layer was composed of PCL co-printed with

NIH/3T3 cell-laden hydrogel-based bioink for stiffness and tendon development on the other [88]. It exhibited high cell viability and allowed cellular differentiation [88]. Finally, muscle tissue that can respond to electrical stimulation in vivo was created by Kang et al. [87]. In this study, skeletal muscle constructs with the size of 15 mm × 5 mm × 1 mm were created by printing cell-laden hydrogels with biodegradable polymers in integrated patterns and anchored on sacrificial hydrogels.

## 3.8 Cardiovascular Tissue

Cardiovascular disease is one of the main causes of death worldwide [91, 92]. Tissue engineering approaches have focused on the regeneration of myocardium and the replacement of cardiovascular structures such as heart halves [93, 94]. The main aim of this technology is to fabricate biocompatible and non-immunogenic cardiac tissues having morphological and functional properties of the human heart.

In myocardial regeneration, 3D printed patch fabricated by using nano-reinforced hybrid cardiac patch laden with human coronary artery endothelial cells, methacrylated collagen micropatterning, and an alginate matrix was found to allow significant cellular proliferation, migration, and differentiation [95]. In a similar study, the fabrication of a cardiac patch composed of human cardiac-derived progenitor cells (hCMPCs) in a HA/gelatin (HA/gel) based matrix lead to the preservation of cardiac performance in myocardial infarction model in mice [96] In another study, cell-laden hydrogel printed with a sacrificial hydrogel resulted in the formation of cardiac tissue constructs that exhibited spontaneous synchronous contraction in culture. This implies in vitro cardiac tissue development and maturation [97].

In heart valve repair, literature has shown that 3D bioprinting technology is a promising tool in constructing valves to meet the biomechanical and haemodynamic requirements [98–100]. Hockaday et al. presented a novel simultaneous 3D printing/photo-crosslinking technique for rapidly engineering complex, heterogeneous aortic valve scaffolds [98]. The investigators proposed that these constructs can be fabricated rapidly and when they were seeded with porcine aortic valve interstitial cells, these cells maintained a nearly 100% viability over 21 days of culture [98]. High cellular viability was also reported in a similar study of 3D bioprinted alginate/gelatin hydrogel valve conduits with anatomical architecture and direct incorporation of aortic root sinus smooth muscle cells (SMC) and aortic valve leaflet interstitial cells in a regionally constrained manner [99].

## 3.9 Other Tissues

Three-dimensional bioprinting has found applications in several other fields of regenerative medicine. Human tissues such as liver, trachea, and retina were also created. Printing of constructs that resemble human liver and allow heterotypic

cellular interactions within the resulting structures was proposed [101, 102] Three-dimensionally printed PCL mimicking human trachea was also reported [103]. The investigators highlighted the importance of cells in this technique, as severe inflammation and an unorganized structure occurred when it was implanted in rabbits [103]. The latter complication was reduced significantly when the graft was cultured in the omentum for 2 weeks [102]. Other applications of 3D printing include also the construction of human ear or auricular cartilage, meniscal tissues, and other tissue analogues [104–107]. Extensive research is currently underway exploring the feasibility of fabricating of human retina [108–111]. A 3D printing of retinal and glial cells as a retina model was demonstrated by Lorber et al. [108]. The printed cells seemed to retain their growth-promoting properties and their viability, unaffected by the piezoelectric printhead [108] The differentiation of retinal cells seemed influenced by the extracellular matrix [109] More specifically, it seems crucial to recapitulate the extracellular environment of these cells, so it can mimic the stiffness of the human retina, which seems to promote cell differentiation [108, 110, 112]. To this end, 3D-bioprinting of HA hydrogels with the addition of retinal progenitor cells was found to have favourable results [110].

The fabrication of pathological tissue models for research is also possible using 3D bioprinting. An in vitro cervical tumour model using Hela cells and gelatin/alginate/fibrinogen hydrogels was constructed [113]. Zhou et al. developed a biomimetic bone matrix using 3D bioprinting technology to investigate the interaction between breast cancer cells and fetal osteoblasts or human bone marrow MSCs [114]. The authors suggested that this was a suitable model to study the interactive effects of cells in the context of an artificial bone microenvironment and may thus serve as a valuable tool for the investigation of post-metastatic breast cancer progression in bone [114]. Finally, the development of a perfusable vascularized 3D tissue resembling liver tissue was used to study drug toxicity in vitro [115].

## 3.10 Conclusions

Three-dimensional bioprinting has evolved rapidly over the last decade as a promising tool in tissue regeneration. Its main advantages are the high precision of tissue fabrication and a fast construction speed. At present, there is an abundance of studies showing the potential of this technology in vitro and in several animal models. It is indisputable that in comparison with other tissue engineering approaches, 3D bioprinting holds most ground as it enables the fabrication of biomimetic tissues. Several challenges can be identified including the biomechanical control, the selection of scaffolds, and the safeguarding of safety throughout the process until the implantation of the constructs into the patient takes place. Other challenges include the vascularization of the 3D printed constructs and the overall survival in the body. These challenges will hopefully be overcome soon through collaborations between medics, biologists, bioengineers, and physicians.

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