Chapter

Bioprinting

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Abstract

Bioprinting is an emerging field in the areas of tissue engineering and regenerative medicine. It is defined as the printing of structures consisting of living cells, biomaterials and active biomolecules. The ultimate aim is to produce implantable organs and tissues to replace the use of autografts, which cause donor site morbidity and require two invasive surgeries. Not only is bioprinting aimed at the restoration of tissue, it has significant potential for drug delivery and cancer studies. Bioprinting provides control over cell placement and therefore creates a homogenous distribution of cells correlating to a uniform tissue ingrowth. Another attribute of bioprinting is the production of patient-specific spatial geometry, controllable microstructures and a high degree of reproducibility and scalability between designs. This book chapter will discuss the many parameters of bioprinting; manufacturing techniques, precursor materials, types of printed cells and the current research.

Keywords: inkjet printing, extrusion, stereolithography, laser-assisted, tissue engineering, hydrogel, bioink, additive manufacture, 3D printing

1. Introduction

There are more organ donors and recipients than there have ever been, however, in the UK, up to one in six patients requiring either a liver, heart or lung transplantation becomes too ill or dies while waiting for a donor organ [1]. When a donor organ becomes available, the recipient and clinicians have to make a fast decision whether to accept the organ, which can lead to an improper assessment of the associated risks. Risks associated with the donor organ include the age of the donor, retrieval of the organ after circulatory death and the potential to transmit blood-borne disease or cancer [1].

Bioprinting has the potential to eradicate the problems associated with organ donation and provide implantable organs on-demand. Bioprinting is a subcategory of additive manufacture, a process by which small scale objects are printed from a bottom up approach, through the deposition of successive layers of material. Using this approach enables the production of very precise and accurate designs and shapes to be built using minimal amounts of material. Designs are made on a computer which is then sent to a printer, meaning that designs are highly repeatable. The processes for developing a bioprinted tissue are outlined in **Figure 1**.

Although additive manufacturing uses a variety of materials to fabricate objects with different functions and purposes, bioprinting is specifically the printing of structures consisting of living cells, biomaterials and active biomolecules. First

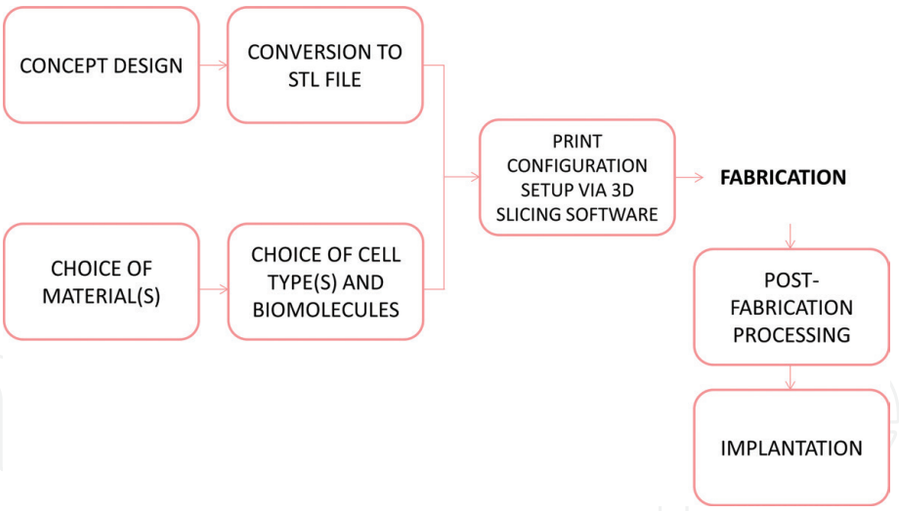

Figure 1.
Flow chart depicting the individual process involved with producing a bioprinted tissue.

patented attempts of bioprinting started with filling cartridges of table-top inkjet printers with bioinks, consisting mainly of cell-laden hydrogels [2]. Although research using inkjet technology was an early adapter for bioprinting, many other additive manufacturing techniques have also been utilized.

Common techniques include inkjet printing, stereolithography and extrusion printing, demonstrated in **Figure 2**. Although these techniques are well established, it is only in the last decade that these processes have been properly developed for applications in the field of regenerative medicine and tissue engineering. Compared to the conventional techniques of producing tissue engineering scaffolds, additive manufacture has the ability to consistently produce highly repeatable designs with a precise, well-defined micro and nanoscale structure [3, 4].

The ultimate goal of bioprinting is to produce an entire complex organ capable of being implanted. This will provide an alternative source of organs so that patients will no longer require long waiting periods to receive a donor organ. Additionally, by being able to fabricate organs on demand, the ethical issues surrounding the supply and use of human or animal tissues is removed.

Another possibility for bioprinting is to provide an alternative to animal testing. The potency and dosages of pharmaceuticals can be tested on bioprinted organoids or on an "organ-on-a-chip". This could prove to be a more efficient and effective

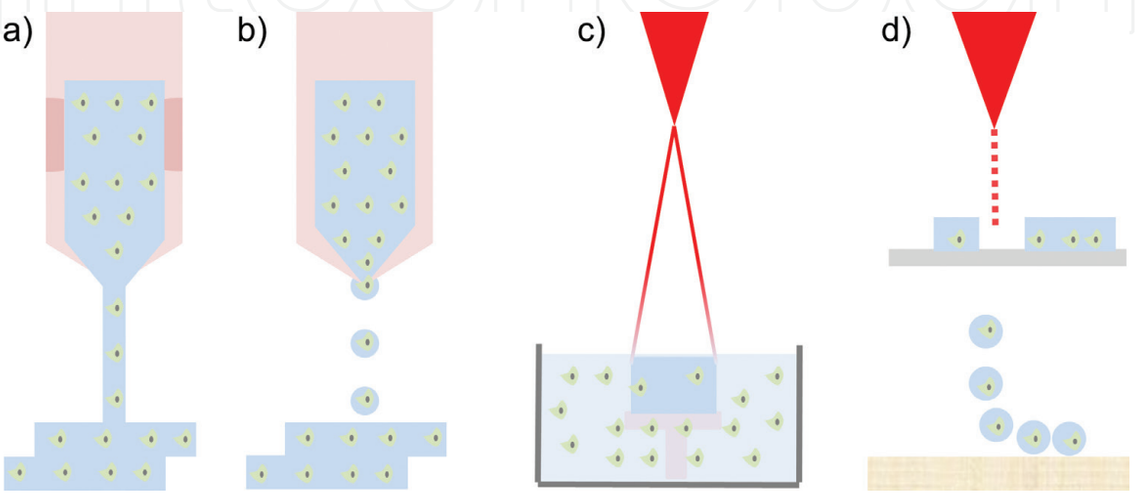

Figure 2.
Bioprinting techniques: (a) extrusion-based, (b) inkjet-based, (c) stereolithography and (d) laser-assisted.

method for the testing of pharmaceuticals, as the translation from an animal model in to humans is not without its flaws, as an animal system cannot fully replicate a human system.

Common tissue engineering manufacturing techniques are capable of producing tissue specific scaffolds; however they require to be seeded with cells post-fabrication. Cell seeding post-fabrication can lead to a poor distribution of cells, especially in larger scale 3D scaffolds. Homogeneous distribution of cells is not a problem with bioprinting, as printing can be used to control the positioning of cells during fabrication [5].

Simple tissues have been repaired in a clinical setting using tissue engineering scaffolds, such as the larynx [6], bladder [7] and urinary tract [8], however through bioprinting, the ability to build a more complex tissue scaffold for implantation becomes a greater prospect.

2. Printing techniques

2.1 Inkjet printing

Inkjet printing is a non-contact method of fabrication, which limits the risk of contamination during printing. Scaffolds are constructed using the deposition of droplets and can achieve excellent resolution ranging between 20 and 100 μm [9–11]. Droplets are ejected from a printhead via thermal, piezoelectric or microvalve processes. Thermal inkjet printing uses the nucleation of an air bubble to induce droplet formation, but a localized temperature between 100 and 300°C could inflict damage on cells and limits its use for printing natural polymers [10, 12]. Piezoelectric-based inkjet printing uses acoustic waves to eject a droplet, however this limits the use of viscous and therefore more concentrated inks, as these will dampen out the acoustic waves before they can expel a droplet [13]. Microvalve printing uses pneumatically controlled pistons to eject droplets. Inkjet printing can print multiple inks at once that can be used to interact with each other to control scaffold properties and create complex structures with multiple types of cells [14–16].

2.2 Extrusion

Bioink is extruded from a micro-nozzle to build a three-dimensional structure in layer-by-layer fashion. Extrusion-based printing is either mechanically (piston or screw) or pneumatically driven [9]. This fabrication technique uses highly viscous bioink and does not require any chemical additives to enable the curing of the material [9]. As inks are viscous, and therefore more concentrated, fabrication rates are relatively quick. However, the more viscous a bioink is, the higher the induced shear-stress during extrusion, which can result in a higher cell apoptotic activity. Out of the different extrusion bioprinting techniques, pneumatic systems have demonstrated the best cell viability post printing [17]. Extrusion-based techniques are promising as they create scaffolds with high structural integrity using bioinks containing high cell densities and can achieve resolutions around 200 μm [9, 18].

2.3 Laser-based printing

There are two laser-based techniques used for bioprinting. The first is stereolithography (SLA), which is a nozzle-free technique that depends on photocuring with ultraviolet light (UV). The UV energy is directed at a reservoir of photosensitive polymers to formulate 3D structures in a layer-by-layer fashion. This technique

is known to have quick fabrication times as its speed depends on the height of the print rather than on the print complexity [19]. A resolution ranging between 5 and 300 μm can be achieved using SLA [20, 21]. This technique is not limited by the viscosity of the bioink, however, photoinitiators are often added to the bioink to improve polymer photosensitivity, and can affect the viability of the cells. The type and concentration of photoinitiator will influence cell viabilities differently [19]. Another concern is UV-induced damage to the cells, and has led to the development of alternative visible light-based and initiator-free techniques [22, 23]. Collectively, the short fabrication time and the absence of shear stress-induced apoptosis make SLA a good candidate for bioprinting.

Another laser-based bioprinting technique is laser-assisted bioprinting (LAB), which is a scaffold-free and nozzle-free technique where droplets of biomaterial/cells/peptides are propelled from a donor material slide onto a receiver material. Laser energy induces a vaporization effect used to transfer material from the donor material slide onto the receiver. This technique can achieve a micro-scale resolution with multicell positioning [24, 25]. The accurate positioning of multiple cell types using this technique has great potential in creating biologically relevant complex designs, yet the main drawbacks of this technique are its low stability and scalability [24].

3. Bioinks

Bioink is a term given to the precursor material used during printing. The main component of the bioink usually consists of a hydrogel. Hydrogels are used as they are highly hydrated, enable cell encapsulation and provide a cushioning effect during fabrication. Furthermore, hydrogels have adaptable rheological properties making them suitable for processing and printing [15]. Hydrogels used in tissue engineering applications are either naturally derived or synthetically derived polymers.

3.1 Naturally derived hydrogels

Collagen, gelatin (denatured collagen), silk, alginate and chitosan are the most commonly used naturally derived polymers in bioprinting with collagen being used in 26% of the bioprinting-relevant literature [26]. Natural polymers provide great bioactivity and high adhesiveness, resulting in high cell viability and proliferation. However, a lack of mechanical competence and reproducibility limit their use in a non-composite form.

3.2 Synthetically derived hydrogels

Aliphatic polyesters and poly(ethylene glycol) are the most commonly used synthetic polymers in bioprinting [27]. Synthetic polymers have a high printing fidelity and controllable degradation kinetics and mechanical properties. However, their inert properties result in low cell viability and proliferation rate [28]. Aliphatic polyesters used in tissue engineering are usually poly(lactic acid), poly(glycolic acid) and poly(caprolactone). Refer to **Table 1** for a comparison of their different properties.

3.3 Characteristics

When processing bioinks, three main parameters must be considered: viscosity of the precursor hydrogel, gelation and network stiffness post-processing [30].

Aliphatic polyester	Hydrophilicity/hydrophobicity	Degradation time	Young's modulus [29]
PCL	Very hydrophobic	Lowest	0.4 GPa
PLA	Hydrophobic	Medium	2.7 GPa
PGA	Hydrophilic	Fast	7.0 GPa
PLGA	Ratio of different polymers will influence rate of degradation and water affinity; PLA percentage means mixture has lower degradation rate and is more hydrophobic.		Amorphous

Table 1.
Correlation between water affinity and degradation rate for aliphatic polyesters.

The latter two points can affect cell viability, proliferation, migration and even differentiation. The three parameters are mainly affected by the type of polymer, its concentration and molecular weight. Other factors that can have an adverse effect on cells are the shear stress induced during printing and the time period during which the cells are exposed to non-physiological conditions (fabrication time).

Rheology of the bioink, particularly viscosity, is an important consideration for printing. Viscosity affects printing fidelity and also influences the shear stresses induced during printing. High shear stresses during printing can damage cells in nozzle-based techniques leading to higher cell apoptotic activity. Each printing technique requires a different viscosity range; inkjet-based printers require low viscosity solutions [31], extrusion-based printers require a high viscosity [9], while laser-assisted bioprinting requires a medium viscosity [5]. However, stereolithographic techniques are not as limited by hydrogel viscosity ranges. Recent research has shown that the sheer stresses induced by the applied pressure in extrusion printing can be alleviated if the bioinks are prepared as isotonic solutions [32].

The objective to have a 3D structure laden with cells that are viable, proliferative and differentiative, along with a structure that exhibits mechanical competence, stability and biologically relevant complexity is dependent on the bioink and the printing process. Ultimately, the type of polymer, its chemical properties, viscosity, gelation, stiffness and fabrication time will affect the cells' status and the print fidelity.

4. Bioprinted tissues

4.1 Neural

Injuries of the central and peripheral nervous system can be challenging to repair. The central nervous system does not regenerated under normal conditions, and any injuries it sustains can lead on to neurogenerative disease. Damage to the peripheral nervous system is mostly treated with autografts and allografts, however motor and sensory recovery rates are poor. In a review of studies recording sensory recovery after digital nerve repair, only 67% of patients reported good sensory repair or better with a nerve graft, which increased to 79% of patients with artificial conduits [33]. This result indicates the potential for synthetically made nerve guidance conduits and therefore the use of bioprinting.

Some of the initial work involving bioprinting of mammalian cells was performed using Chinese hamster ovary (CHO) and rat embryonic motoneuron cells [34]. It was established that using an inkjet printer to print the cells did not affect cell proliferation or the polarized morphology of the motoneuron cells. However,

evaluation of cell lysis immediately after printing indicated that ~8% of the cells were damaged due to the printing process. Further work with rat primary embryonic hippocampal and cortical neurons demonstrated a cell viability of 74.2 ± 6.3%, as well as the maintenance of basic function, phenotype and electrophysiological properties [35].

Lorber et al. inkjet printed cells from the mature adult nervous system [36]. Monitoring the cells during droplet ejection, the cells did not undergo major distortion and hence destruction caused by shear forces. It was shown that printed glia cells retained their ability to support the growth of the seeded retinal ganglion cells, which is an essential interaction for maintaining proper nerve function. Tse et al. printed a combination of neuronal and glial cells [37]. High cell viabilities >86% for neuronal cells and >89% for Schwann cells were achieved. Not only did the printed cells exhibit early neurite growth in comparison to non-printed cells, neurites also grew longer in length.

Using an extrusion printing process, neural stem cells (NSCs) have been printed embedded in polyurethane (PU) [38]. The bioprinted scaffolds restored nerve function when used to treat Zebra fish with a traumatic brain injury. Owens et al. developed the extrusion bioprinting process to produce purely cellular nerve grafts [39, 40]. Mouse bone marrow stem cells (BMSCs) and Schwann cells (SC) were extruded alongside agarose rods. The agarose rods were removed after a 7 day maturation period leaving a tubular cell structure. Implanted to repair a sciatic nerve injury in rats, both motor and sensory function was restored after 40 weeks.

SLA has been used to print human neural stem cells (hNSCs) [41]. Scaffolds were made containing GelMA and graphene nanoplatelets. GelMA content had a negative effect on cell proliferation rates, while the presence of graphene, known to stimulate hNSCs into neural differentiation [42], improved the cellular response. By monitoring glial fibrillary acidic protein (GFAP) expression, which is associated with the development of ependymal cells, and ß-tubulin III, which is associated with neurons, it was determined that the printed cells had differentiated over a 14 day period post-printing.

4.2 Skin

In the repair of deep partial thickness and full thickness burns a new epidermal barrier needs to be established, this is usually achieved through transplantation [43]. Current treatment techniques, including split thickness grafts, lack the inclusion of structures such as hair follicles, subcutaneous glands or sweat glands and do not provide the full restoration of sensory and motor neurons [43]. This can cause problems for the patient, especially the lack of sweat glands, which can prevent proper thermo-regulation. Bioprinting can be used to develop a complex tissue as multiple inks can be printed at once. Although the production of replica human skin has yet to be achieved, there have been many promising advances for the treatment of full thickness wounds.

Laser-assisted bioprinting was tested for the printing of different skin cells: fibroblasts and keratinocytes, as well as human mesenchymal stem cells (hMSCs) [25]. Printing was shown not to induce apoptotic activity and cell viabilities were measured as >90%. The hMSCs maintained their phenotype, implying that the transfer process did not induce differentiation of the cells.

Kim et al. bioprinted a skin model using both extrusion and inkjet technologies [44]. A collagen/PCL ink was extruded to form the basis of the scaffold, followed by the inkjet printing of primary human dermal fibroblasts (hFBs) and primary human epidermal keratinocytes (hKCs). Using inkjet printing to deposit the cells provided a controllable and even distribution. When placed in cell culture over a

14 day period, the bioprinted construct developed biological characteristics such as elongated hFB, and dermal and epidermal layers.

A PEG based skin model was produced by Rimann et al. [45] using a microvalve droplet system. Alternate layers were printed of a PEG-based ink followed by the hFBs ink suspension. Each layer of the PEG based ink was immediately polymerized using UV light. Due to the use of UV light the cells were analyzed for UV damage. By comparing printed and unprinted cells, it was shown that the cell DNA was not damaged during the printing process. The cells adhered to the structure, maintaining a typical morphology and were well dispersed within the polymer matrix. After a 3-week period, the cells continued to proliferate and produce their own extra cellular matrix, however, subsequent seeding of hKCs on top of the printed scaffolds did not achieve a fully stratified epidermis when exposed to the air-liquid interface for 14 days.

A more complex bioprinted skin model has been achieved by printing multiple cell types [16, 46, 47]. Using a microvalve droplet system, initial investigatory work used both hFBs and hKCs suspended in cell media that were printed in-between layers of a printed collagen gel [46, 47]. Using a droplet based technique enabled the precise placement of cells, with a recorded 68 ± 13 hKCs per droplet and 93 ± 13 hFBs per droplet for each respective ink suspension. Compared to skin tissue scaffolds that had used a manual seeding technique, the bioprinted scaffolds retained their shape better and developed denser epidermal layers. The epidermal layers formed tight junctions between neighboring cells, an important feature of the epidermis for providing an effective barrier.

Further work included pigmentation of the printed skin model by printing human epidermal melanocytes (hMCs) into the epidermal layer [16], demonstrated in **Figure 3**. The hKCs reached a terminal differentiation to form a stratum corneum-like structure. hMCs started producing melanin which accumulated at hKC interfaces, causing a light pigmentation. When the hMCs were printed as a uniform layer, they formed nevus-like structures (freckles), however it was suggested that this could be prevented by controlling hMC cell densities to create a more even spatial distribution.

A collagen skin scaffold containing hFCs and printed human dermal microvascular endothelial cells (hMVECs), that was subsequently seeded with neonatal hKCs mixed in collagen, was compared to a commercial graft (Apligraf®) for treating full thickness skin wounds in mice [48]. After 14 days, the bioprinted scaffolds had adhered to the wound while the Apligraf® had dried out and detached from the implant site, although no infections were recorded for any of the test groups. Complete closure of the wound occurred first for the bioprinted scaffold after 21 days, followed by the Apligraf® after 28 days. Wound sites treated with either the commercial graft or the bioprinted graft developed epidermal and dermal

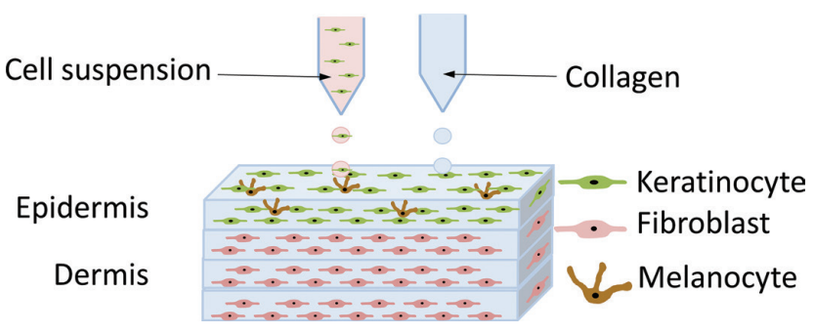

Figure 3.
Bioprinted pigmented skin model fabricated using a microvalve droplet technique [16]. Fibroblasts are used for a dermal layer, and keratinocytes and melanocytes for an epidermal layer.

layers, however the bioprinted skin graft was shown to have the most histologically similar appearance to native skin, and developed a micro-vessel network.

Skardal et al. successfully demonstrated *in-situ* bioprinting to repair full thickness skin wounds on the backs of mice [49]. Amniotic fluid-derived stem (AFS) cells and bone marrow-derived mesenchymal stem cells (MSCs) were separately suspended in fibrinogen-collagen solutions and printed into the wound sites. The fibrinogen-collagen solution was crosslinked by printing a thrombin solution between each layer. Printing the skin graft directly into the wound site achieved complete coverage of the wound and a tight seal between the skin and graft. At all points during the study, wound contraction and re-epithelialization were significantly higher in wound sites treated with bioprinted cells. Printed scaffolds exhibited re-epithelialization of up to 89% which had organized, well defined layers, while the control only exhibited a 51% re-epithelialization with a poor structural quality.

4.3 Cardiovascular

Cardiovascular disease is one of the major causes of death globally. According to World Health Organization (WHO), cardiovascular disease accounts for more than one third of the total deaths each year [50, 51]. Depending on severity, treatment solutions may require replacement of damaged vital vasculature, or even heart transplantation. Problems such as organ shortages or requirements for multiple invasive surgeries mean that alternative solutions need to be found.

Different methods have been explored for the bioprinting of a 3D vascular network. Endothelial cells have been successfully printed with a high degree of accuracy using an inkjet printer. It was shown that during printing, a precise number of cells were ejected per drop [52]. Boland et al. used inkjet printing to print a crosslinking agent (calcium chloride) into an alginate/gelatin solution to produce vascular structures [53]. By printing a crosslinking agent into the polymer solution, a structure was formed out of hollow shells providing a microscopic porosity that proved beneficial for cell migration.

Cui et al. used inkjet printing to encapsulate human microvascular endothelial cells (hMVEC) in a fibrin hydrogel [54]. Scaffolds were fabricated with micron-sized fibrin channels that had similar mechanical properties to other tissue engineered blood vessels. In cell culture, the cells aligned along the channels in a confluent lining, and had formed a ring-shaped microvasculature that sealed the fibroin channel with a high level of integrity.

Extrusion bioprinting has been used to print heart valve conduits [55]. Scaffolds were fabricated using a bioink of methacrylayed hyaluronic acid (Me-HA) and methacrylated gelatin (Me-Gel) with suspended human aortic valvular interstitial cells (hAVIC). In cell culture, cells adhered and formed a monolayer on the surface of the bioprinted structure while the encapsulated cells below the surface started to remodel the hydrogel after 3 days.

Initial work into printing cardiac tissue was performed using alginate hydrogel and primary feline adult and human H1 cardiomyocytes [56]. Inkjet printed in a half heart shape, scaffolds had a 1 cm inner diameter and two connected ventricles. Due to the printed porosity, the large structure enabled the transportation of nutrients, and meant that the cells remained viable when placed in cell culture. Using electrical stimulation, the cardiac cells were observed to contract rhythmically which in turn caused the whole structure to beat periodically.

The generation and use of human pluripotent stem cell (iPSCs) is of growing interest for many applications as they can differentiate into multiple cell lineages [50]. Zhang et al. demonstrated a bioprinting methodology to fabricate

endothelialized myocardium using iPSCs [57]. GelMA and iPSC scaffolds were extruded and seeded with cardiomyocytes to induce the formation of myocardium. The fabricated scaffolds were capable of spontaneous and synchronous contraction using low level electrical stimuli.

4.4 Bone

An aging population and the use of bone grafts to augment bone for repair and regeneration, have led to an increasing demand of functional bone grafts. The current gold standard of treatment involves autologous bone retrieved from the patients' iliac crest, however this requires multiple surgery sites, is expensive to perform and increases the associated risks involved with a second surgery site [58]. Therefore an alternative solution, such as the development of bioprinted bone grafts, presents an attractive alternative.

Using SLA, stem cells derived from mouse bone marrow stromal cells (OP-9 cells) were embedded in crosslinked PEGDA hydrogels [59]. After 24 h, the cells remained viable and subsequent seeding of mouse mesenchymal stem cells (mMSCs) onto the scaffold showed intensive mineralization.

Catros et al. used LAB to position osteosarcoma cells (MG63) onto poly(caprolactone) (PCL) scaffolds [60]. Cells were printed either between layers of electrospun PCL or only as a final layer. *In vitro* testing initially showed no difference in proliferation rate between the two groups, however, after 14 days, the scaffolds that included printed cells between layers of PCL exhibited a significantly higher proliferation rate. When implanted in calvarial defects in mice, significant bone tissue ingrowth was observed that had an organized structure. Extruded PCL scaffolds containing decellularized bone matrix and human adipose-derived stem cells (hASCs) used for craniofacial regeneration in mice, showed improved bone regeneration in comparison to a pure PCL scaffold [61].

Using a bioink consisting of either a matrigel or matrigel and alginate, endothelial progenitor cells (EPCs) and multipotent stromal cells (MSCs) were extruded into bone tissue scaffolds [62]. Implanted into subcutaneous dorsal pockets of mice, the scaffolds without the inclusion of alginate performed better, as they developed a bone-like tissue structure.

Zhou et al. employed a table-top stereolithographic printer to create biomimetic bone matrices to study metastasis of bone cancer in bone tissue [63], as shown in **Figure 4**. Scaffolds containing osteoblasts and hMSCs suspended in hydrogels containing different concentrations of GelMA and nanocrystalline hydroxyapatite, were subsequently seeded with breast cancer cells (BrCas). Placed in cell culture, osteoblast proliferation rates were observed to decrease, while BrCas proliferation rates increased. Interactions between the cell types within the bioprinted model led to the conclusion that the osteoblasts and MSCs secrete macromolecules that promote BrCas growth.

4.5 Cartilage

Because of its avascular nature and the presence of low cell densities, cartilage defects will not completely self-regenerate [64]. It is therefore important that different treatment possibilities are investigated to initiate its' regeneration.

Xu et al. fabricated a loadbearing cartilage scaffold using an inkjet printer to bioprint rabbit articular chondrocytes suspended in a fibrin–collagen hydrogel onto an electrospun PCL matrix [65]. It was found that by including a PCL fibrous network within the scaffold, mechanical properties were significantly increased. Scaffolds implanted subcutaneously in mice developed a dense, well organized

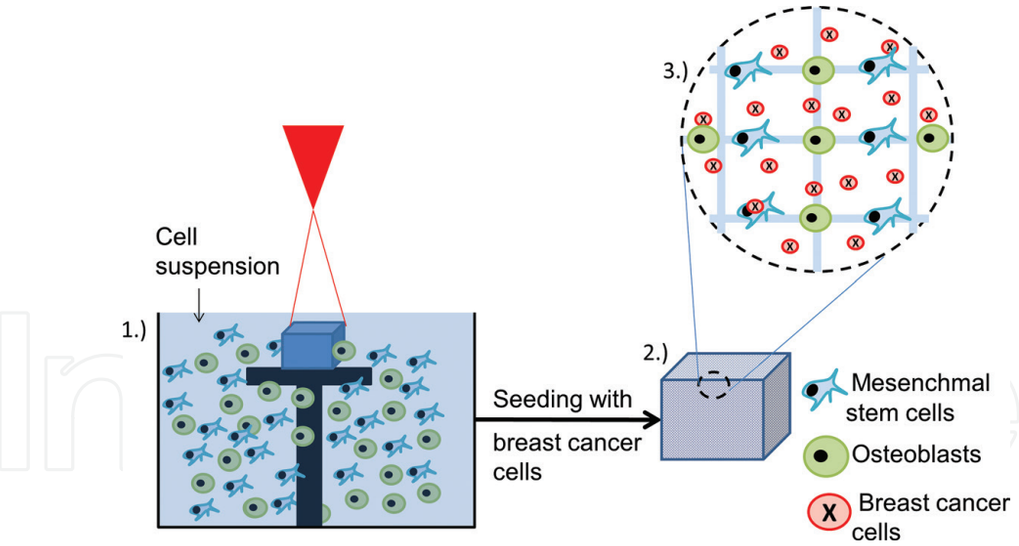

Figure 4.
Stereolithographic process to fabricate a cell-laden bone model to study breast cancer metastasis. (1) Homogenous suspension of MSCs and osteoblasts in a photocurable hydrogel; (2) cell-laden 3D structure that is seeded with breast cancer cells; (3) a close-up of homogeneously adhering MSCs/osteoblasts and a suspension of breast cancer cells.

collagen, not present in the control group (unseeded scaffolds). The cartilage tissue formation within the bioprinted scaffolds appeared histologically similar to normal elastic cartilage.

The use of PCL to provide structural support was also employed by Schuurman et al., who extruded bioprinted GelMA embedded with chondrocytes [66]. For improved cartilage development, scaffolds were also fabricated with a GelMA/hyaluronic acid hydrogel. The inclusion of hyaluronic acid improved cell viability *in vitro*, however when implanted in mice, both scaffolds types achieved similar levels of cartilage tissue formation. Other extruded PCL-alginate gel scaffolds that contained chondrocytes also reported the formation of cartilage after being implanted into the dorsal subcutaneous space of mice [67].

In situ bioprinting has been investigated by Cui et al. using human chondrocytes suspended in a poly(ethylene glycol) dimethacrylate (PEGDMA) solution [68, 69]. Scaffolds were printed directly into osteochondral plugs that had been harvested from bovine femoral condyles. Compared to scaffolds fabricated outside of the defect, the *in situ* bioprinted scaffolds had enhanced tissue integration. Another *in situ* bioprinting method has been developed by O'Connell et al. [70]. Based on an extrusion bioprinting technique, an "*in-situ* biopen" has been made comprising of two ink chambers and an extruder nozzle with a UV source that enables direct application of a bioink during surgery. Using the biopen, a human infrapatellar fat pad adipose stem cell (IPFP) laden gelatin-methacrylamide/hyaluronic acid-methacrylate (GelMA/HAMa) hydrogel scaffold was fabricated for *in vitro* testing. It was observed that the viability of biopen printed cells was 97% after 7 days.

4.6 Muscle

Bioprinting has shown great potential for building highly hierarchically organized cellular structures that comprise muscle tissue. Miri et al. created hierarchical cell-laden structures to mimic multicellular tissues [71]. Using extrusion-based bioprinting, multiple bioinks were deposited onto microfluidic chips. Structures resembling musculoskeletal junctions were printed using poly(ethylene glycol) diacrylate (PEGDA) and GelMA that were loaded with NIH/3T3 fibroblasts and C2C12 skeletal muscle cells. *In vitro*, the chips retained their printed interfaces

and demonstrated adequate proliferation rates. PEGDA-framed chips that had a concentration-gradient of methacrylated gelatin (GelMA) ranging from 5 to 15%, were implanted subcutaneously in rats. After 30-days, enhanced cell proliferation was reported for regions containing 10% GelMA, signifying a great potential for angiogenesis. This study demonstrates the possibility to create hierarchically cell-laden structures to mimic multicellular tissues.

Bajaj et al. used stereolithography to bioprint muscle tissues composed of mouse embryonic stem cells (mESCs) and mouse myoblast cell line (C2C12), embedded in PEGDA [72]. Live/dead staining showed a slight decrease in cell viability for the bioprinted scaffolds, possibly due to the non-adhesive properties of PEGDA.

4.7 Periodontal

Periodontitis is a chronic inflammatory condition resulting in total destruction of the periodontium consists of alveolar bone, cementum, gingiva and periodontal ligaments and if left untreated, can result in tooth loss [73, 74]. Periodontal ligament stem cells (PDLSCs) have been shown to support the regeneration of periodontal tissues [75]. Ma et al. investigated cell viability of extruded PDLSCs encapsulated in a GelMA/PEG hydrogel [76]. It was shown that cell viabilities were around 94% after 24 hours post printing. Extrusion printing has also been used to reconstruct the maxillary bone in a 12-year-old dog, and is believed to be the first case of maxillary bone reconstruction using a 3D printed scaffold [77].

4.8 Corneal

Diseases of the cornea have a significant impact on visual health worldwide. Corneal opacity is the fourth leading cause of bilateral blindness globally [78, 79]. A total of approximately 50,000 corneal transplantations were performed in the United States in 2013 [80–82], yet drawbacks to this procedure include a reduced quality of visual recovery due to an early endothelial cell loss, detachment of the posterior lamellar grafts and vascular in-growth into the lamellar plane [79]. Using sodium alginate and methacrylated type I collagen mixed with corneal keratinocytes, Isaacson et al. extruded corneal scaffolds [83]. To produce a concave structure, gelatin was used as a support material and had a hollowed out shape. Cell viability of cornel keratinocytes were 90% after 1 day post-fabrication which dropped to 83% after 7 days.

The limbus borders the cornea and provides it with limbal epithelial stem cells (LESCs) for regeneration. Damage caused to it by disease or injury can impair a person's vision [84]. Using laser-assisted bioprinting Sorkio et al. printed human embryonic stem cell derived limbal epithelial stem cells (hESCs-LESCs) and hASCs for the repairing of the limbus [85]. *Ex vivo* assessment of the structures was performed on porcine corneal tissue, with results compared to commercially available acellular Matriderm® sheets as a control. The bioprinted scaffolds exhibited strong adhesion with the host tissue enabling hASCs migration, while control group had a more limited response.

5. Conclusion

Bioprinting has been used to create a variety of complex tissues and has demonstrated great potential as an alternative to autologous, allogeneic and xenogeneic organ and tissue transplantation. Progress towards a complete implantable organ is at various stages of development depending on the organ or tissue to be fabricated.

Some of the greatest developments have been with neural and skin tissues. It has been shown that neuronal cells can be printed without affecting their function or phenotype. In one investigation using an inkjet printer, the printed neuronal cells demonstrated faster and longer neurite growth in comparison to non-printed cells [37]. When implanted, bioprinted neural scaffolds have been able to restore nerve function [38–40].

Complex structures such as skin have been fabricated that include both dermal and an epidermal layers. By using bioprinting technology, it is possible to create a skin model with pigmentation [16]. Bioprinted skin scaffolds have been successfully applied via direct *in situ* printing [48]. Used to repair full thickness wounds in mice, *in situ* bioprinted scaffolds have regenerated skin tissue with a histologically similar appearance to native skin.

A potential use for bioprinted tissues could be in modeling diseased tissues. Research has already been conducted studying the metastasis of bone cancer in a bioprinted bone tissue model [63]. Using bioprinted tissue models it could be possible to gain a greater understanding of the interactions between infected and diseased cells with healthy tissues. By doing so, new treatments can be produced and tested to determine the most effective solution.

Each bioprinting technique has demonstrated the potential to generate fully functional organs, which in the future can be used for applications such as: direct implantation, to fill the gap for much needed donor organs; provide a model system for pharmaceutical testing, and replace the necessity for animal testing; or be used for disease modeling and enable different treatments to be easily explored. Overall, bioprinting is gaining substantial interest in the field of tissue engineering and regenerative medicine due to its capability to produce complex organs and tissues.

Conflict of interest

The authors of this book chapter have no conflicts of interest to declare.

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