

3D bioprinting technology for regenerative medicine applications

Dhakshinamoorthy Sundaramurthi, Sakandar Rauf and Charlotte A. E. Hauser*

King Abdullah University of Science and Technology (KAUST), Laboratory for Nanomedicine, Division of Biological & Environmental Science & Engineering (BESE), Thuwal, 23955-6900, Saudi Arabia

Abstract: Alternative strategies that overcome existing organ transplantation methods are of increasing importance because of ongoing demands and lack of adequate organ donors. Recent improvements in tissue engineering techniques offer improved solutions to this problem and will influence engineering and medicinal applications. Tissue engineering employs the synergy of cells, growth factors and scaffolds besides others with the aim to mimic the native extracellular matrix for tissue regeneration. Three-dimensional (3D) bioprinting has been explored to create organs for transplantation, medical implants, prosthetics, *in vitro* models and 3D tissue models for drug testing. In addition, it is emerging as a powerful technology to provide patients with severe disease conditions with personalized treatments. Challenges in tissue engineering include the development of 3D scaffolds that closely resemble native tissues. In this review, existing printing methods such as extrusion-based, robotic dispensing, cellular inkjet, laser-assisted printing and integrated tissue organ printing (ITOP) are examined. Also, natural and synthetic polymers and their blends as well as peptides that are exploited as bioinks are discussed with emphasis on regenerative medicine applications. Furthermore, applications of 3D bioprinting in regenerative medicine, evolving strategies and future perspectives are summarized.

Keywords: bioprinting, bioinks, cells, hydrogels, scaffolds, organ transplantation

*Correspondence to: Charlotte A. E. Hauser, King Abdullah University of Science and Technology (KAUST), Laboratory for Nanomedicine, Division of Biological & Environmental Science & Engineering (BESE), Thuwal, 23955-6900, Saudi Arabia; Email: charlotte.hauser@kaust.edu.sa

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1. Introduction

Recent advances in bioprinting technology have opened up new and exciting opportunities for the development of patient-specific medical treatments. The fabrication or printing of biomimetic tissue structures is a prerequisite for the advancement of emerging technologies such as drug testing, tissue engineering, biomimetic sensors and 3D tissue models. Due to the rejection problems associated with allogeneic organ transplantation and scarcity of donors, *ex vivo* methods are being explored for tissue/organ transplantation. These methods involve the expansion of

patient-derived autologous cells and their use as the primary cell source to develop tissues/organs for transplantation. These 3D tissue analogs can be achieved by incorporating native cells with suitable biocompatible materials using a precise and well-controlled fabrication process^[1]. Bioprinted 3D constructs are aimed to mimic the cell density, arrangement, niche and anatomical geometry of the native tissue and hence can be a promising solution for different regenerative medicine applications^[2].

A 3D object can be designed and fabricated using 3D printing techniques. In 3D bioprinting, a layer-by-layer assembly of inks is printed using computer-aided

instructions to develop biological constructs^[3]. Bioprinting can be defined as the use of materials science and fabrication techniques to build biological constructs containing tissues, cells and biomolecules with a particular organization and biological function^[4]. Bioprinting techniques have been recently explored for different biological applications due to their potential to overcome most of the problems associated with the classical tissue engineering methods^[5]. Classical tissue engineering involves the combination of scaffolds, cells and compounds, such as growth factors^[5,6]. Scaffolds are seeded with the cells and compounds that promote tissue regeneration. Tissue engineering strategies have been utilized for the regeneration of various organs such as skin, trachea, bone, esophagus and myocardium^[5]. Though tissue engineering approaches have been shown to be clinically effective, all scaffolds up-to-date lack complex and intricate structures of the native tissue^[7]. In addition, the tissue engineered scaffolds do not mimic the native architecture of the tissues^[8,9].

The key requirements of a tissue engineered scaffold are (1) biocompatibility; (2) biodegradability; (3) adequate porosity; (4) mechanical strength; (5) biomimetic structure and (6) therapeutic activity^[6]. Various fabrication methods such as electrospinning, freeze-drying, phase separation, gas foaming, particulate leaching and solvent casting have been developed to produce tissue scaffolds^[10]. However, tissue engineered scaffolds do not completely mimic the native architecture of the tissues, have difficulties to support the growth of cells in 3D and have problems to deposit different cell types in the scaffolds at specified locations^[8,9,11,12]. Besides, many of these fabrication methods involve the use of organic solvents which impair the cellular growth^[13]. Further, tissue engineered scaffolds do not completely fulfill all the ideal requirements needed for tissue regeneration as discussed above. On the other hand, bioprinting offers an alternative approach solving most of the problems associated with the current tissue engineering methods. Tissue engineering strategies are mainly involved in the development of scaffolds to promote regeneration/repair of tissue defects. While 3D bioprinting methods can also be used to develop whole or parts of organs, the main advantage is its potential to print whole organs for transplantation purposes. Bioprinting can be used to fabricate biological constructs with defined micro/nano architectures combining scaffolds with cells, and bioactive molecules. Bioprinting uses com-

puter-aided design techniques to make structures that closely mimic the anatomical structures of organs/tissues. Based on its ability to produce organ constructs with native tissue biology, bioprinting has received enormous attention in the field of regenerative medicine. Even though bioprinting of a whole organ, suitable for transplantation, is yet to be achieved, this technology is moving fast and could soon satisfy hopes to solve the shortage of organs for transplantation in the future.

In this review article, we will first describe different bioprinting methods such as extrusion-based printing, cellular inkjet printing, laser-assisted printing, integrated tissue organ printing (ITOP) and robotic bioprinting used to develop scaffolds and other biomedical constructs. Secondly, we describe the bioinks available for bioprinting and the challenges involved in developing a suitable bioink that satisfies the critical requirements for printing. Finally, the key applications of bioprinting in regenerative medicine are summarized, and its future directions are outlined.

2. Methods for Bioprinting Tissue/Organs

Bioprinting of a tissue or an organ is a complex process which depends on the inherent properties of the bioinks, printing techniques and cellular systems used for printing. Furthermore, the resolution of the printed structure is controlled by the parameters such as needle orifice size, surface tension and viscosity of the bioink, temperature, and humidity^[14-16]. A typical bioprinting system can dispense bioinks onto a suitable substrate of choice using a cartridge or a syringe. More advanced bioprinting systems contain multiple print heads, and each one can be loaded with the same or different bioinks^[17]. Printing patterns can be generated, modified and printed using computer-aided software such as CAD (Computer Aided Design). The turnaround time taken for making modifications in the CAD files is just seconds to minutes making this process easy and user-friendly^[18]. This is advantageous to bioprint custom made structures such as tissues and organs for transplantation. The prerequisites to develop a bioprinting process comprise characteristics, such as CAD, high resolution to obtain the micro/nanoarchitecture and high-precision to localize cells in a 3D environment. With these design strategies in mind, bioprinting is using biomimicry and 3D tissue generation. The biomimicry approach enables the fabrication of constructs with features that mimic the native architecture of the tissue as close as possible^[19].

The generation of 3D tissue structures combines the above mentioned characteristics in order to fabricate constructs of multicellular, anatomical architecture providing vasculature, if needed (Figure 1).

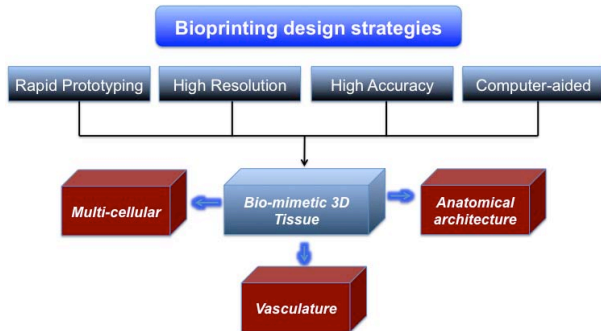


Figure 1. Bioprinting design strategies and approaches to develop 3D tissues and organs (Adopted from Murphy and Atala^[19]).

3. Key Requirements of Bioprinted Tissue/Organs

The key requirements that are preferentially considered for printing tissues/organs are illustrated in Figure 2.

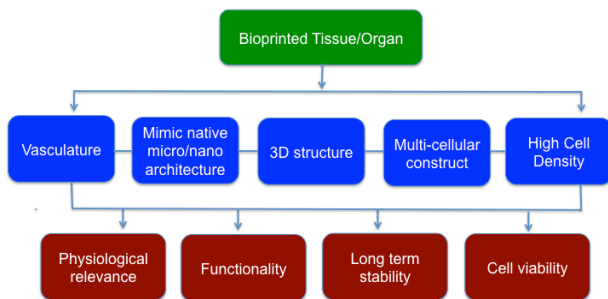


Figure 2. Key requirements of a bioprinted organ.

There are several essential features that need to be considered for developing 3D constructs. The ideal structural features of native tissues such as vasculature, micro/nano architecture, 3D structure, multi-cellular and high cell density are essential to be replicated in 3D printed constructs (Figure 2). These structural parameters are required in a 3D printed construct in order to mimic the native tissues. The structural features of 3D constructs determine the properties of the construct such as physiological relevance, functionality and long term stability. Hence, structural features and their resulting properties are key requirements to develop 3D constructs for regenerative medicine applications.

4. Bioprinting Methods

Bioprinting technology involves the deposition of sca-

fold materials into 3D structures together with viable cells to develop tissues/organs that mimic the native architecture in structure, dimension, and shape. Three different techniques are commonly used for bioprinting that are microextrusion, inkjet printing, and laser-assisted printing^[20]. A comparison between these printing methods is shown in Table 1. In the case of microextrusion method, a computer-controlled mechanism is involved to print different materials onto the substrates using either pneumatic or robotic power. In this method, the material is extruded via a standard extrusion needle and the x, y and z-movements of the stage and extruder are controlled by a CAD-CAM software to produce 3D structures^[21]. Inkjet bioprinters were developed as a bottom-up approach to fabricate biological constructs. Inkjet bioprinters translate a design pattern into structures by printing in a point-by-point fashion (rasterization of a pattern). Different bioinks such as synthetic and natural-derived polymeric solutions can be used for inkjet bioprinting^[22]. Laser-assisted bioprinting is a jet-based printing technique that works on the principle of Laser-Induced Forward Transfer (LIFT). In this method, a pulsed laser beam is used to transfer the bioink onto the substrate^[23]. Among these methods, microextrusion and inkjet printing are the most popular as compared to the Laser-assisted bioprinting which is a relatively newly developed technique. In addition to these three widely used printing methods, integrated tissue organ printer (ITOP) and robotic bioprinting are new methods recently developed to print 3D tissues/organs.

4.1 Microextrusion

Microextrusion is a 3D printing method used for biological and mostly for non-biological purposes. Printers that use the microextrusion method normally utilize a thermo-regulated handling and dispensing system, a piezoelectric humidifier and a stage with provisions for movements along the x, y and z directions^[33]. The deposition area is illuminated with a light source that enables the activation of photoinitiators. A video camera is attached to the xyz stage to monitor and control the printing process^[18,33,34]. Microextrusion technique has been successfully used to print scaffolds for tissue engineering^[34]. The microextrusion head deposits the material onto the substrate as continuous beads based on the instructions from the CAD-CAM software. Initially, the beads are deposited in the x-y direction, then by moving the extrusion head (or) stage in the z-axis, complex 3D structures are

Table 1. Comparison of bioprinting methods (N/A- Data not available)

	Bioprinting Methods				
	Microextrusion	Inkjet printing	Laser-assisted printing	ITOP	Robotic printing
Viscosity	6–30×10 ⁷ mPa/s	3.5–12 mPa/s	1–300 mPa/s	N/A	N/A
Cell density	High	Low < 10 ⁶ cells/mL	Medium, 10 ⁸ cells/mL	High	High
Cell viability	40–80%	85%	>95%	>90%	>90%
Resolution	100 μm - millimeter	75 μm	10 – 100 μm	2–50 μm	N/A
Print speed	100 μm/s	1–10000 drops/s	2–1600 mm/s	N/A	N/A
Nozzle size	20 μm- millimeter	20–150 μm	Nozzle-less	50 μm	N/A
Working principle	Contact	Non-contact	Non-contact	Contact	Contact
Mechanical integrity	High	Low	Low	High	Medium
Purchase cost	Low	Low	Medium	High	High
References	[14,17–19,24–28]	[14,17,19,24,25,27,29–31]	[14,17,19,24,25,27,32]	[69]	[70]

fabricated. Biocompatible polymers, cell spheroids and many hydrogels have been shown to be compatible with microextrusion. Two main dispensing systems that are used to extrude biomaterials are mechanical and pneumatic^[35] (Figure 3). The bioink flow is better managed in mechanical dispensing rather than pneumatic dispensing method^[36,37]. The compressed gas volume in the pneumatic system can delay the ink flow. Pneumatically driven printer systems operate with only air-pressure and are more suited for applying limited force during printing^[20].

Using the microextrusion method a wide range of bioinks with different fluid properties can be operated^[38]. Bioinks in the viscosity range 30 mPa/s to > 60 kPa/s are mostly used for microextrusion based bioprinting.

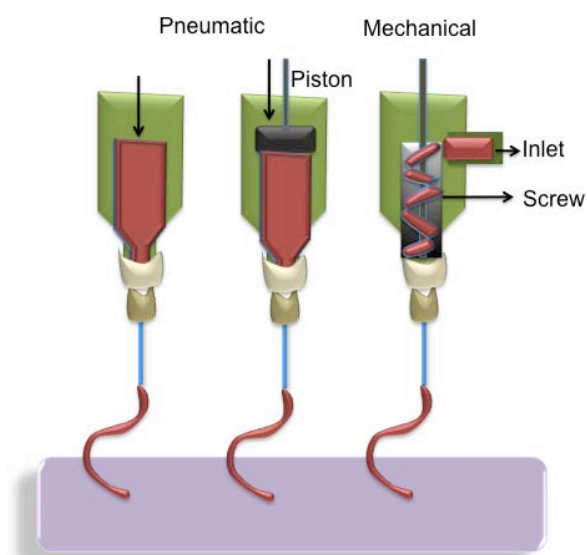


Figure 3. Microextrusion bioprinting using pneumatic and mechanical methods (Adopted from Murphy and Atala^[19] and^[21]).

Bioinks capable of shear thinning and thermal cross-linking have been used for microextrusion bioprinting^[39]. For example, cell spheroids that can self-assemble into 3D structures can be subjected to microextrusion to develop 3D spheroid tissues. Microextrusion printing has been utilized to develop aortic valves^[40], tumour models^[41] and vascular tissues^[42]. Printing high-resolution complex structures using microextrusion requires a longer time, however, the microarchitecture is well developed in the printed constructs. In addition to this, the cell viability has been reported to be over 90% in the biological constructs developed using microextrusion methods^[5].

4.2 Inkjet Bioprinting

Inkjet printers are referred to as drop-on-demand printers since these printers can reproduce digital information by printing small bioink drops onto the predefined location in a suitable substrate^[43]. These printers are widely used for many biological and non-biological applications^[44]. The cartridges can be refilled with bioinks, and the substrate is controlled by an electronic stage to enable z-axis movements^[45]. Nowadays, custom-designed inkjet printers are available that can use different bioinks with enhanced speed, accuracy and resolution^[45]. Inkjet-based printers utilize acoustic and thermal forces to eject bioinks on the substrate^[5]. In the case of acoustic forces based printers, a piezoelectric material is fixed to the needle that generates an acoustic wave to break the ink into small droplets at pre-determined intervals^[46]. When a voltage is applied, the piezoelectric material rapidly undergoes shape transformations which produce adequate pressure to eject bioink from the needle orifice (Figure 4)^[47]. Some

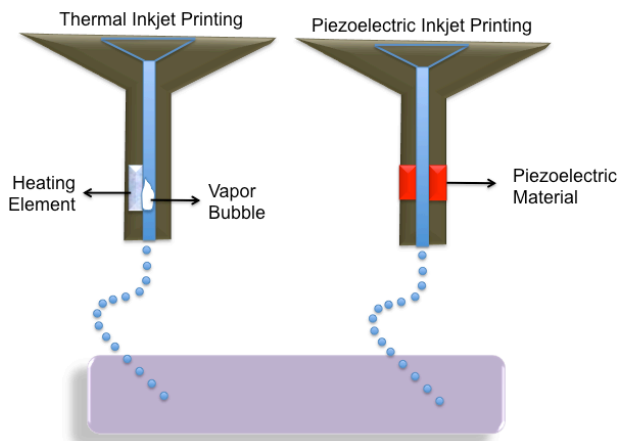


Figure 4. Electrical heating thermal inkjet printer and acoustic inkjet printer that uses piezoelectric material (Adopted from ref.^[19,41]).

inkjet printers use acoustic radiation coupled with an ultrasonic sound to pump out the ink^[19]. In this method, the parameters of ultrasound such as amplitude, time and pulse can be varied to control the rate and size of the ejected droplets^[19]. Further, the desired ink droplet size can be easily generated and monitored. In this method, cells containing bioinks are not subjected to pressure and heat, hence better cell viability^[48]. In addition to this, nozzle-less print heads can be used to avoid exposing cells to shear stresses which may also improve cell viability^[49]. However, an important problem involved in this type of printing is the use of 15-25 kHz frequencies to eject ink, which causes cell membrane damage^[50]. Also, it is hard to use bioinks with high viscosity^[24].

In thermal inkjet printers, a pulsed pressure is generated to eject the ink by applying electrical heat to the print head. Various reports have demonstrated that the heating of the print head is localized and has no effect on the stability of the bioinks or the cell viability after printing^[50,51]. The main advantages of thermal inkjet printers are their low cost and enhanced print speed. However, clogging, variable droplet sizes, less directionality and poor cell encapsulation are some of the disadvantages of thermal inkjet printers. The resolution of the inkjet printers is in the range of 20–100 μm . These printers can print the droplets of up to picolitre volume to achieve higher resolution; however, the time taken for printing can be longer depending on the size of the droplet^[20,52]. In the case of bioinks, picoliter droplets are difficult to achieve due to the high viscosity. Also, the mechanical integrity of the biological constructs could be weak post-print^[5,53].

4.3 Laser-assisted Bioprinting

Biological constructs developed using laser-assisted bioprinting can yield resolution at a single cell per droplet^[21]. The tissue organization and cell population can be easily controlled in laser-assisted bioprinting, which makes it a potential technique to develop tissue equivalents having similarities in both structure and function of the native tissue^[54]. This technique is based on the principle of laser-induced forward transfer which was initially used to print inorganic or organic structures with micrometer scale resolution but now successfully used to print bioinks such as DNA, cells, and peptides^[55]. When compared to other bioprinting methods, laser-assisted bioprinting was not widely used in earlier days, but it has been increasingly popular nowadays for the fabrication of engineered tissues for regenerative medicine applications^[56]. Laser-assisted bioprinting system consists of a pulsed laser beam (to induce the transfer of bioink), a focusing system (to align and focus laser), an absorbing layer (ribbon- made of gold or platinum), and a substrate for the bioink layer. During printing, the laser pulse is focused on the ribbon layer that generates a high-pressure bubble from the bioink layer which transfers the bioink onto the substrate (Figure 5).

The resolution of the laser-assisted bioprinting system depends on the laser energy, air gap between the absorbing layer and substrate, nature of the substrate surface, surface tension and viscosity of the bioink^[57]. It is a nozzle-free printing method, and hence clogging of bioink/cells can be completely avoided. However,

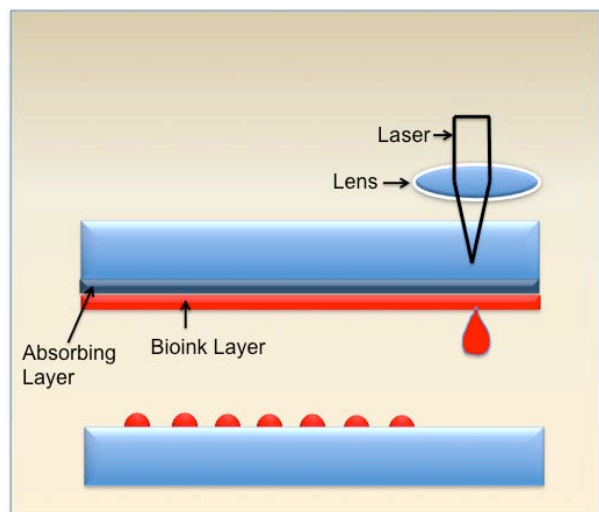


Figure 5. Laser-assisted printers use an absorbing layer to create laser pulse pressure that creates droplet ejection from the bioink layer (Adopted from ref.^[19,41]).

this type of printers require bioink of fast gelation kinetics to develop constructs with good shape fidelity, and this may hinder the flow rate during printing^[25,58]. Also, preparing multiple cells containing ribbon or absorbing layer is a time-consuming process. The constructs that are fabricated by laser-assisted bioprinting are often found to contain traces of contamination (comes from the absorbing layer). To avoid such contaminations absorbing layers made up of non-metallic substances are being used^[59]. Furthermore, it is hard to focus the laser spot and to precisely locate the cells during printing. To overcome this difficulty, the “aim and shoot” technique is used^[60]. Here, the laser beam will scan and choose the region of interest, in order to locate specific cells and to eject one cell per laser pulse^[60]. Using this printing method bone constructs and skin with cells for implantation have been successfully fabricated^[61,62]. Table 2 and 3 show some of the constructs fabricated by different bioprinting methods and points to their advantages and disadvantages, respectively.

4.4 Integrated Tissue Organ Printer (ITOP)

A major challenge for existing 3D bioprinting methods is the decrease in cell viability in the core regions of the tissue constructs due to the lack of nutri-

tion and oxygen. Recently, ITOP (Integrated Tissue Organ Printer) bioprinting method has been reported for the fabrication of complex human tissues with good viability and vasculature^[69]. This approach demonstrated the printing of various polymers and cell types in a single tissue construct using multi-dispensing modules. ITOP uses pneumatic-actuated microextrusion method but differ in dispensing systems, hardware and software as discussed below. ITOP method uses air pressure to control dispensing volume and a three-axis motorized stage for 3D patterning. The 3D patterns employed in ITOP method were generated from computed tomography (CT) and magnetic resonance imaging (MRI) data of human organs/tissues. This data was finally converted into 3D patterns using a computer-aided design (CAD) software. It was proposed that ITOP method can offer many advantages over existing 3D bioprinting methods such as better carrier materials for cell delivery, the high-resolution nozzles (2 μm for biomaterials and 50 μm for cells), post-print cross-linking of cell-laden hydrogels and simultaneous printing of supporting polymers and acellular sacrificial hydrogels^[69].

In ITOP method, synthetic polymer (for mechanical support) and a sacrificial polymer (without cells) together with hydrogels (with cells) are used for

Table 2. Examples of the biological constructs developed using bioprinting methods

Bioprinting methods	Bioink used	Construct developed	Features	Applications	References
Microextrusion	Gellan-alginate blend with biocartilage particles	Composite cartilage graft	Proliferation of chondrocytes and deposition of cartilage ECM	Bioactive cartilage scaffold	[63]
Microextrusion	Gellan gum-RGD (RGD-GG)-peptide modified polymer	Brain-like branched neuronal network	3D architecture and high neuronal cell viability	<i>In vitro</i> model to study neuronal disorders	[64]
Microextrusion	Alginate	Porous alginate gel with Human fetal cardiomyocyte progenitor cells (hCMPCs)	Cell growth and viability in 3D with native gene expression	Patch to treat myocardial infarction	[65]
Inkjet	Poly(ethylene glycol) dimethacrylate (PEGDMA, PEG) and gelatin methacrylate (GelMA)	Layer-by-layer assembled PEG-GelMa containing hMSCs	Good cytocompatibility. Osteogenic and chondrogenic differentiation	Suitable for cartilage and bone tissue engineering	[66]
Inkjet	Fibrin, collagen and thrombin	Graft with amniotic stem cells and MSCs	Accelerate wound closure and re-epithelialization	Scaffold for stem cells delivery	[67]
Inkjet	Poly(ethylene glycol) dimethacrylate (PEGDMA)	Layer-by-layer assembly of PEGDMA and chondrocytes	Promote chondrocyte growth and ECM deposition	Cartilage scaffold	[68]
Laser-assisted	HMSCs	Highly defined cell patterning	3D with high resolution	Could create complex tissue structure	[61]
Laser-assisted	Matrigel®	Skin fibroblasts and keratinocytes positioned in Matrigel	Functional skin cells in the construct	3D skin for wound regeneration	[62]

Table 3. Advantages and disadvantages of bioprinting methods

Microextrusion	<u>Advantages</u> Slightly viscous bioinks (cell spheroids, hydrogels, copolymers) can be used Create tissues with high cell density Print vascular tissue constructs <u>Disadvantages</u> Cell viability affected while applying more extrusion pressure Difficult to enhance print speed and resolution
Inkjet printing	<u>Advantages</u> High resolution Concentration gradient of cells, and growth factors in the construct Electronic control of drop size and ejection rate <u>Disadvantages</u> High viscous bioinks cannot be used Weak mechanical integrity of the construct
Laser-assisted printing	<u>Advantages</u> Nozzle-less printer setup Microscale resolution Compatible with broad range of viscous bioinks <u>Disadvantages</u> Time consuming ribbon layer preparation Costly Difficult to position cells

bioprinting^[69]. The sacrificial polymer can be dissolved post-print once the tissue construct achieved sufficient strength to retain a proper shape. Also, the dissolution of sacrificial hydrogel leaves a lattice of a network throughout the construct that permits rich oxygen and nutrient supply. Simultaneous printing of supporting polymer, cell-laden hydrogels, and sacrificial polymer provides good mechanical integrity to the constructs and may help to overcome the current limitations of 3D printing methods^[69]. The calvarial bone construct was developed via ITOP using PCL and tricalcium phosphate. A calvarial bone disc of 1.2 mm thick and 8 mm diameter was printed with human amniotic fluid derived stem cells as cell support. This disc was cultured in osteogenic differentiation media for 10 days and then implanted in cranial bone defect created in sprague drawley rats. After 5 months of implantation, new bone formation was shown to be higher in cranial defects treated with bioprinted calvarial disc^[69].

Ear cartilage and skeletal muscles were also bioprinted using ITOP. The ear cartilage (3.9 cm × 1.6 cm × 0.9 cm) was printed with rabbit ear chondrocytes as cell support. This cartilage construct was cultured in chondrogenic differentiation media for 5 weeks and then implanted in dorsal subcutaneous space of athymic mice for 2 months. After 2 months, the construct was shown to have mature chondrocytes with native expression of ECM markers such as glycosaminoglycans and collagen II. Skeletal muscle construct (15 mm × 5 mm × 1 mm) was implanted in the muscle adjacent to common peroneal nerve (CPN) of hind limbs of

nude rats. The dissected CPN was embedded inside the construct to enable proper nerve impulse. After 2 weeks of implantation, the printed muscle construct was shown to have organized myofiber orientation with native myoblast markers expression such as alpha-bungarotoxin and acetyl choline receptor. All these three printed tissue constructs showed good maturation and organization both *in vitro* and *in vivo*^[69].

4.5 Robotic Bioprinting of Organs

Robotic bioprinting of 3D tissues using cell spheroids is an emerging technique that can improve the success of regenerative medicine. Automated robotic systems are employed to achieve precise printing and scalability of organ bioprinting. Robotic printing enables direct self-assembly of tissue spheroids to develop large scale tissues/organs^[70]. Robotic bioprinting uses pneumatic-actuated microextrusion printing method but differ in dispensing systems, hardware and software as discussed below. In this approach, a robotic dispensing system is used to direct the tissue structure alignment (layer-by-layer assembly) using a suitable bioink (cell spheroids) onto biopapers (hydrogel sheets). Also, an Organ Biofabrication Line (OBL) is required to fabricate complex human organs. OBL has many components such as stem cell bioreactors, perfusion bioreactors, tissue spheroids, encapsulator and a robotic bioprinter^[70]. Different OBL systems such as “Fabber”(a robotic printer developed by Cornell University, USA), 3D dispensing laboratory printer (LBP) developed by MUSC bioprinting research centre, Charles-

ton, SC and 3D-Bioassembly Tool (BAB) developed by Sciperio, Orlando USA have been developed to construct 3D tissues/organs. Though BAB is still in its infancy, this method can evolve as a promising solution to create patient-specific tissue constructs for regenerative medicine applications^[33,70,71]. However, lack of scalability and problems with precise printing are the major drawbacks of the current robotic bioprinters. Recently, Advanced Solutions (Kentucky, USA) has developed a six-axis robotic dispensing bioprinter that can efficiently handle curves and allows precise printing of the structures. The main advantage of this method is its software, TSIM (TSIM-Tissue Structure Information Modeling) that can perform an MRI scan of human tissue and convert it into a printable 3D shape. Robotic bioprinters and tissue spheroid encapsulators are well developed commercially available OBL components. However, high-performance perfusion bioreactors are yet to be developed to improve organ printing. The existing technological challenge is to develop a complete and perfect OBL to print organs at a larger scale for regenerative medicine applications.

5. Bioinks for 3D printing

The 3D printing technology was initially developed for many non-biological applications that involve the use of high temperature and toxic organic solvents. These harsh conditions are not suitable for printing biological cells and other biomaterials. Hence, it is essential for printing to find suitable bioinks with desired functional and mechanical properties in order to come close to native tissue. Both natural polymers (such as collagen, gelatin, alginate, fibrin, hyaluronic acid and chitosan) and synthetic polymers (such as polyethylene glycol (PEG), poly(L-lactic acid) (PLA) and poly(ϵ -caprolactone)(PCL)) are predominantly used as bioinks. Ultrashort peptides that can self-assemble into nanofibrous structures have recently been proposed as novel bioinks and are attractive candidates for bioprinting due to biocompatibility and processability^[72]. This newly developed bioink contains helical fiber structures that strongly resemble collagen fibers in topography and diameter^[72].

Printability is an important feature of an ideal bioink. During printing, the bioink should be accurately deposited in the construct providing the desired temporal and spatial resolution. Bioinks should also enhance the cell viability post-printing and must have desired physico-chemical properties to suit the printing needs. For

example, thermal inkjet printers require bioinks of lesser thermal conductivity to improve the cell viability^[73]. Biocompatibility is another important facet of a biological construct aimed for regenerative medicine applications^[74]. Bioinks should be biocompatible and provide a favorable milieu to the cells. The degradation profile of the constructs should be in tune with the regeneration of new tissues^[75] and the degradation products of the constructs should not cause any adverse reactions. Most importantly, bioinks should provide mechanical support and structural support to the growing cells to maintain 3D microenvironment. Bioinks should have biomimetic properties to have a positive influence on the cell adherence, proliferation and other functionalities^[76]. An ideal bioink should also possess tunable gelation properties and easy to make chemical modifications to improve the biological functionalities^[1]. These attributes are essential for an ideal bioprinting material/bioink. The following section will give detailed descriptions about bioink materials.

5.1 Natural Polymers

(1) *Alginate*

Sodium alginate (alginate) is a raw material extracted from brown seaweed. Alginate is a polysaccharide and anionic in nature. It is a linear block copolymer having M (β -D mannuronic acid monomers) and G (α -L-guluronic acid blocks) domains. Alginate structure has a mixture of M and G domains. G-blocks can form ionic bonds when interacts with divalent cations and become gels in solutions. Biomimetic structure, suitable viscosity, gelation at ideal temperatures and high biocompatibility are some of the properties of alginate that makes it suitable for bioprinting^[77-81]. Cell-laden 3D alginate hydrogels were prepared using inkjet printing^[81]. Although this hydrogel provides biocompatibility and mechanical strength, it lacks cell recognition motifs. Moreover, bioprinting alginate constructs of thick tissues with well interconnected pores is yet to be achieved.

(2) *Collagen and Gelatin*

Collagen is a naturally occurring protein in tissues which constitutes largely of amino acids such as hydroxyproline, proline, glycine and trace amounts of sulfur containing amino acids and aromatic amino acids. Hydroxyproline and proline maintain the tertiary structure of the collagen. Collagen is a major extracellular matrix (ECM) protein and controls all the cellular fate processes^[82]. It is used as a scaffold material for various tissue engineering applications; however, its poor

mechanical properties limits its suitability in bioprinting. Gelatin is a denatured form of collagen and hence has less tertiary structures^[83]. The presence of RGD motifs in gelatin makes it a suitable candidate for a broad range of applications in tissue engineering^[84].

Gelatin usually exists in the coiled form at 40° C and when cooled it can regain triple helix form^[83]. This transition property is necessary for a bioink to improve the integrity of the constructs post-print. Gelatin was blended with methacrylamide to obtain gelatinmethacrylamide, a photoactive polymer, that can form stable 3D structures after UV crosslinking^[85]. This cross-linking stabilizes the construct post-print^[86]. Various chemical functionalization methods have been employed to control the gelling behavior, cross-linking behavior, and viscosity of gelatin in solution. Though gelatin bioink has shown cellular compatibility, its highly viscous nature limits its applications in bioprinting.

(3) *Hyaluronic acid*

Hyaluronic acid is a linear polysaccharide made of (β -1,3) β -1,4-linked D-glucuronic acid and N-acetyl-D-glucosamine disaccharides. It is a viscoelastic, biodegradable and highly biocompatible polymer. Hyaluronic acid is an interesting candidate for bioprinting, but its high hydrophilicity limits its application^[87]. Chemical cross-linking methods and derivatization of hyaluronic acid with hydrophobic side chains have been attempted to reduce hydrophilicity but still not successful in bioprinting^[87]. Blending hyaluronic acid with some photocrosslinkable materials such as DEXHEMA have been shown to improve the cell viability of chondrocytes^[88]. Further, the physical blends of gelatin-alginate^[89], fibrin-collagen^[67], gelatin-hyaluronic acid^[88] have also been used as bioinks.

(4) *Silk fibroin*

Silkworm (*Bombyx mori*) derived fibrous protein called silk fibroin is an amphiphilic block copolymer. The main heavy chain of silk fibroin has twelve repeating domains with frequent occurrence of G-X-G-X-G-X where G is glycine and X may be serine or alanine. The repeating units are separated by hydrophilic peptides that have eleven amorphous regions. Silk fibroin has high tensile property and also good biocompatibility^[90]. The addition of weak acids such as methanol will cause a transition of molecular organization between random coils to aggregation and β -sheets formation. This property makes silk fibroin suitable for bioprinting^[90]. However, printing silk fibroin alone can cause needle clogging due to aggrega-

tion (crystallization of β sheets). Silk fibroin physically blended with gelatin will improve the ink flow. Also, gelatin can incorporate RGD motifs in silk fibroin which in turn improve the cellular compatibility. Silk fibroin-gelatin scaffolds promote the redifferentiation of chondrocytes and multilineage differentiation of human nasal inferior turbinate tissue derived mesenchymal cells^[91,92].

5.2 Synthetic Polymers

Natural polymers containing cell adhesion motifs have been used to mimic the native extracellular matrix. Synthetic polymers offer biocompatibility, strong mechanical properties, degradation profile and allow chemical modification to alter the structure and function of the polymer. The ease of processability has made synthetic polymers as a good candidate for bioprinting applications. Bioactive molecules can be incorporated to modify these polymers to induce specific cellular responses^[93]. Some of the synthetic polymers used for bioprinting are discussed as follows.

(1) *Poly(lactide-co-glycolide) (PLGA)*

PLGA is a copolymer of lactide and glycolide, synthesized via ring opening polymerization mechanism. It can be synthesized with different copolymer ratios, and their degradation rates can be controlled. PLGA has been successfully used as bioink to create 3D vascular networks. Human umbilical vein endothelial cells (HUVECs) were deposited on the PLGA based biopaper by using biological laser printing method^[94]. 3D tissues were created by stacking the PLGA sheets containing HUVECs^[94]. Hydrolytic degradation behavior and fast solvent evaporation of PLGA makes it a promising bioink for printing various types of tissue structures.

(2) *Poly(ethylene glycol) (PEG)*

Poly(ethylene glycol) (PEG) is a biocompatible and a hydrophilic polymer used for various biomedical applications. PEG has been employed in various applications such as nanoparticle coating to prevent aggregation, bioink for printing scaffolds and encapsulation of cells^[42]. It is soluble in water but require chemical modification to form gels. Moreover, tissue engineered scaffolds were surface modified with PEG to improve cellular compatibility and protein adsorption^[42]. This polymer can easily form physical or chemical crosslinked networks after acrylation. Photoinitiators are employed to crosslink PEG under UV exposure. Acrylated PEG has been used as bioink to print vascular grafts^[42]. PEG blended with dimethacrylate

(PEGDMA) was successfully used to print cartilage construct containing human articular chondrocytes^[95]. In this study, PEG has been utilized as 3D biopaper to make constructs that repair osteochondral plugs^[95].

(3) *Poly(L-lactic acid) (PLA)*

PLA is an aliphatic polymer with glass transition temperature of 60°C and an excellent mechanical strength. It is a biodegradable, biocompatible and semicrystalline polymer used for various tissue engineering applications. As a bioink, PLA is less viscous in nature and can be easily ejected through the needle. After printing, PLA exhibits faster evaporation and can provide structural integrity to the construct. Recently, an acrylonitrile butadiene styrene-PLA blend was used as a bioink to produce a cartilage graft^[96]. Nucleus pulposus and primary articular chondrocytes cultured on this scaffold maintained their native phenotypes over three weeks^[96].

(4) *Poly(ε-caprolactone) (PCL)*

PCL is a synthetic polyester which is semicrystalline, biocompatible and biodegradable. It is an easily processable bioink due to its excellent properties such as low melting point, thermoplastic behavior, hydrolytic degradation and excellent mechanical properties^[97]. Initially, PCL being a viscous solution had difficulties in printing because of the requirement of large diameter nozzle and high pressure. To overcome this problem, an electrohydrodynamic jet technique was used to print PCL bioinks. Applying electrohydrodynamic forces created a temperature gradient in the ink and high resolution (10 μm) 3D constructs were formed^[97]. However, PCL cannot be used as cell-laden bioink due to its high melting point (60°C). Instead PCL can be used to provide supporting structure in 3D constructs and also to reinforce stability to the fabricated scaffolds^[9,97]. Though synthetic polymers offer many advantages in bioprinting, further developments are required to improve the biocompatibility and degradation behavior of this class of polymers.

5.3 Ultrashort Peptides

Hauser and co-workers have recently reported that distinct peptides selected from the earlier discovered class of self-assembling ultrashort peptides can be used as bioinks for bioprinting applications^[98-100]. These ultrashort peptides have an innate tendency to self-assemble into hydrogels with a nanofibrous topography that closely resemble collagen and thus mimicking the native architecture of tissue ECM^[72,98]. As an example, the biocompatibility of these ultra-

short peptides based hydrogels was demonstrated by maintaining the organotypic culture of intestinal epithelial cells (Caco2) and 3D culture of stem cells. It was shown that embryonic stem cells encapsulated within these ultrashort peptide hydrogels can retain their pluripotency, using Tra-I-60, Tra-I-81, Oct4 and Nanog as pluripotency markers^[72]. Furthermore, human mesenchymal stem cells encapsulated in these peptide based hydrogels differentiated into adipogenic lineage under defined culture conditions. It was proposed that these peptide hydrogels can offer a suitable nanotopography and 3D microenvironment to support organotypic culture of primary cells (gastrointestinal and skin cells) as well as 3D culture of stem cells^[72,98]. Bioinks made from these ultrashort peptides exhibit interesting properties that could be useful for the development of 3D organotypic cultures for drug screening and biological constructs for tissue engineering applications in the future^[100].

6. Applications of Bioprinting

Bioprinting makes use of novel bioinks and 3D printing techniques to fabricate closely resembling organs/tissues for regenerative medicine applications. Bioprinting techniques make it possible to print cells in the constructs in specific locations which is important for mimicking native tissue architecture^[19]. As discussed in section 2, there are several structural and functional features that are considered ideal for developing 3D constructs. Among the structural features, vasculature is one of the important factors that determine the success of bioprinted constructs by improving cell viability^[48]. The vasculature of 3D constructs is essential to improve nutrient delivery, tissue ingrowth, and regeneration^[53]. Cells in tissues are mostly found within 100-200 μm away from adjacent blood vessels. Cells that are present within this limit of 100-200 μm receive nutrition and oxygen through diffusion from the nearby capillaries. Hence bioprinted 3D constructs need to be prevascularized to overcome this diffusion limit and also to mimic the native tissue^[69]. Several bioprinting approaches have been shown to stimulate vascularization of scaffolds for tissue engineering applications^[19,41,53]. For example, a 3D microvascular construct was printed using human microvascular endothelial cells and fibrin as bioinks^[53]. In the case of cell viability, numerous studies have demonstrated that there was no difference in cell viability between non-printed and printed cells^[48,101]. Cell viability and vasculature are some of the important para-

meters that need to be considered to develop 3D constructs for regenerative medicine applications. Some of the applications of 3D bioprinting in regenerative medicine are listed in Table 4.

Existing skin grafting techniques and commercially available skin grafts do not meet all the requirements that are needed for aesthetic skin regeneration. Bioprinting methods have been employed to construct complex stratified layers of skin that may be used in skin grafting applications^[62]. Lee *et al.*^[102] have engineered skin tissue constructs through a layer-by-layer assembly of collagen, dermal fibroblasts and epidermal keratinocytes. The printed 3D skin was proposed to be useful as a skin substitute to treat full thickness skin damages^[102].

Bioprinted cartilage may mimic some of the properties of the native cartilage and could be useful as a scaffold for the repair of cartilage damages such as joint injuries^[96,103]. For example, bioink made of PEG and alginate can form an interpenetrating network^[103]. Additional crosslinking of this hybrid polymer using calcium sulfate allowed higher cell encapsulation and also showed toughness (1500 J m^{-2}) greater than native cartilage^[96,103].

Critical-sized bone defects require graft assistance for healing. Although tissue engineered scaffolds offer solutions to the existing problems associated with non-healing bone defects, an ideal scaffold that can restore the native functions of the bone is yet to be identified^[104]. Bioprinting methods may provide an alternative method to the development of bone scaffolds that closely mimic the native functions of the bone. For example, PEGDMA hydrogel developed via photopolymerization method had a compressive mod-

ulus of $>500 \text{ mPa}$ and used to print bone constructs^[104]. In another study, Cooper *et al.*^[105] have developed a 3D printed bone made from DermaMatix and BMP-2 (Bone Morphogenetic Protein-2). This construct showed an effective healing of a calvarial defect in a mouse model^[105].

Bioprinting can be employed to develop neural stem cells constructs to treat central nervous system (CNS) diseases such as Huntington's disease, Parkinson's disease, and Alzheimer's disease. Hsieh *et al.*^[106] printed 3D neural tissue constructs of thermoresponsive polyurethane containing neural stem cells. These neural stem cells-laden 3D printed polyurethane scaffolds rescued traumatic brain injury in a zebrafish model. Here, bioprinting of neural stem cells was demonstrated to improve neural stem cells encapsulation and viability.

Aortic valves or prosthetic heart substitutes are developed for regenerative medicine applications^[65,40]. These biological structures have complex architecture and also contain multiple cell types. 3D bioprinting methods may offer ways to develop aortic valves/heart substitutes with native structure and viable cells. In a recent study, alginate–gelatin aortic valve was fabricated using 3D bioprinting^[40]. This bioprinted valve was claimed to closely match the native anatomy of aortic valve. In addition to the structural similarity, the fabricated aortic valve also has viable aortic smooth muscle cells and aortic valve leaflet interstitial cells. These studies demonstrate that it is possible to create aortic valves using 3D bioprinting^[40].

Pluripotent stem cells and embryonic stem cells (ES cells) are cell sources for patient-specific treatments and hence attractive for 3D bioprinting of constructs

Table 4. Applications of bioprinting in regenerative medicine

Application	Bioink	Printing method	Cell type	Inference	Ref.
Cartilage	Alginate-Polyethylene glycol	Microextrusion	Bone marrow derived hMSCs	Tougher mechanical integrity like native cartilage	[103]
	Acrylonitrile butadiene styrene (ABS) and polylactic acid (PLA)	Microextrusion	Primary articular chondrocytes and nucleus pulposus	Porous scaffold for cartilage and intervertebral disc tissue engineering	[96]
Skin	Layer-by-layer assembled collagen	Microextrusion	Human skin fibroblasts and human skin keratinocytes	Skin matrix that resembles structural and biological features of native skin	[102]
Bone	DermaMatrix™ human allograft with bone morphogenetic protein-2	Inkjet	Mouse C2C12 progenitor cells	Osteogenic differentiation of C2C12 cells and promotes calvarial bone healing	[105]
Nerve	Polyurethane	Microextrusion	Neural stem cells	Recovery from CNS neural injury in zebra fish	[106]
Heart	Alginate-gelatin	Microextrusion	Aortic root sinus smooth muscle cells (SMC) and aortic valve leaflet interstitial cells (VIC)	Cell encapsulated aortic valve retain anatomic complexity	[40]
Liver	Alginate	Microextrusion	Human induced pluripotent stem cells	Post-print differentiation into hepatocyte lineage	[107]

for various biomedical applications^[107,108]. These stem cells can differentiate into specific lineages and can offer potential cell sources for *in vitro* tissue models. Bioprinting tissues containing stem cells could be a potential strategy to develop patient-specific tissue constructs for regenerative medicine applications. Tasoglu *et al.* have described in detail the applications of bioprinting in stem cells research^[108]. Human induced pluripotent stem cells (iPSCs) printed with alginate hydrogels were allowed to differentiate into hepatocyte-like cells using differentiation factors. These iPSCs showed better cell viability and also differentiated into hepatocyte-like cells^[107]. These differentiated cells were positive for hepatocyte phenotypes such as albumin secretion and morphology. This approach may be helpful to generate patient-specific 3D liver constructs using iPSCs for drug screening and organ transplantation^[107].

In another study, 3D bioprinting technology was used to create ES cells into 3D hydrogel spheroids to maintain the stem cell pluripotency^[109]. These spheroids were made from gelatin and alginate. In this method, ES cells laden hydrogel spheroids with controlled size and uniform pluripotency were bioprinted using an extrusion-based 3D bioprinter. The cell spheroids were shown to retain pluripotent stem cell markers such as Oct 4, SSEA-1 and Nanog^[109]. In another study, a novel bioink made from ultrashort peptide hydrogels were used to bioprint 3D structures encapsulated with human embryonic stem cells. It was shown that embryonic stem cells encapsulated within these ultrashort peptide hydrogels can retain their pluripotency, using Tra-I-60, Tra-I-81, Oct4 and Nanog as pluripotency markers^[72]. This bioink was shown to have very good applications in 3D bioprinting of tissue constructs and organoids for applications such as drug screening and tissue engineering.

7. Emerging Strategies in Bioprinting

Recent advancements in 3D printing methods and bioink materials will enable further improvements in the 3D bioprinting technology. Modified and new printing methods are being employed to design better quality bioprints with improved properties suitable for organ engineering. Novel bioink materials such as ultrashort peptides and hybrid polymeric materials are promising candidates for 3D bioprinting of tissues/organs. For instance, Shanjani *et al.*^[110] developed a hybrid bioprinting method using polymer con-

taining a soft part (hydrogels for loading growth factors/cells) and a hard part (rigid and porous for mechanical integrity). In this study, poly-ethylene glycol diacrylate (PEGDA) and poly-(ϵ -caprolactone) (PCL) were used as model materials for soft hydrogel and rigid scaffold, respectively. This bioprinting method involves digital light processing-based stereolithography (DLP-SLA) and molten material extrusion based techniques for soft and rigid materials, respectively. It was demonstrated that the properties of this hybrid hydrogel can be easily tailored using DLP-SLA method and the resultant bioprint had a compressive modulus (6 MPa) greater than many hydrogels. This hybrid bioprint was reported to exhibit good cell viability and vasculature^[110].

Bioprinted constructs containing native ECM components may help to improve the cellular functions such as proliferation, maturation and differentiation. To achieve this, the bioink materials can be modified/functionalized with ECM components. In a recent study, collagen films were first grinded using a crushing-particle desk crusher and passed through a 38 μ m mesh to get collagen microfibers of length $22 \pm 13\mu$ m^[111]. These collagen microfibers were linked with bone morphogenetic protein-2 (BMP2) that contained collagen-binding domain (CBD-BMP2). The CBD-BMP2 was printed onto bone marrow mesenchymal stem cells-laden methacrylamide gels. It was reported that these bone marrow mesenchymal stem cells differentiated into osteocyte cells due to the presence of ECM components such as collagen and BMP-2 in CBD-BMP2^[111].

Cells are subjected to a mild stress (thermal or mechanical) during bioprinting that may affect the cell viability in the printed constructs. New strategies that can minimize cell stress during printing are needed to further improve cell viability in bioprinted constructs. As an example, Blaeser *et al.*^[112] have developed a fluid-dynamic model to control the shear stress while printing by optimizing nozzle diameter, bioink viscosity, and extrusion pressure. In another study, microfluidics-based platform and bioprinting technology were combined to print constructs using low-viscosity bioink (a blend of alginate and gelatin methacryl (GelMA)), which resulted in visible cell viability via minimizing the shear stress during bioprinting^[112].

Extra-hepatic transplantation of islets cells using biomaterials may be useful in glycemic correction of insulin dependent diabetic patients^[114]. Marchioli *et al.*^[114] bioprinted 3D structures using a bioink solution

containing a mixture of islets of Langerhans and alginate. This construct showed sufficient oxygen and nutrient permeability due to the suitable porosity of the printed construct. The islets in the bioprinted construct were found to be viable and also showed native morphology^[114]. This bioprinted hydrogel may be used as islets carrier for transplantation^[114].

In vivo bioprinting is another emerging and promising technology that may bring improvements in the field of regenerative medicine. *In vivo* bioprinting is thought to be an alternative to the existing *in vitro* bioprinting methods^[115,116]. *In vivo* bioprinting technology is a medical intervention to directly print new tissue constructs at the defect site. During surgical procedures, the probe of the bioprinter is inserted (minimally invasive) into the defect site to reconstruct the damaged tissues *in vivo* by printing relevant structures. Keriquel *et al.*^[116] have recently shown a proof-of-concept study to repair osteochondral bone in a mouse model using *in vivo* bioprinting of n-hydroxyapatite into the defect region.

8. Conclusions and Future Directions

Bioprinting is one of the tools for rapid prototyping to develop 3D constructs for clinical applications. The main goal of 3D bioprinting is to develop 3D organs that fully mimic the native tissue architecture and functions. An additional goal of 3D bioprinting is develop novel methods like *in vivo* bioprinting to be used in clinics to directly print structures at the damaged tissues in patients to promote regeneration. 3D bioprinting technology offers a broad range of applications in the biomedical field from tissue models for drug screening studies to the fabrication of organ transplants for regenerative therapies. This technology allows printing of cells, biomolecules, and ink materials and controls their precise localization in the 3D construct. However, bioprinting of complex, multicellular and 3D native tissue structures remain a major challenge though there are few attempts to achieve this goal. In addition, bioprinted structures do not exactly match the native mechanical strength of the tissues/ organs. Hence, further improvements are required to overcome these challenges. 4D bioprinting is an emerging field, where time is integrated as fourth dimension with 3D bioprinting. In 4D bioprinting, the printed structures are capable of changing their shapes with time when an external stimulus is imposed. This technology can enable the reorganization of materials and cells after printing to improve effective cell pat-

ternerng. Though, this field is in its infancy, 4D bioprinting may help to overcome some challenges in 3D bioprinting^[117].

Vasculature is one of the important factors that determine the success of an organ transplant since it is responsible for nutrients delivery and oxygen supply. Though several researchers have been focusing on developing vascularized constructs using bioprinting, further developments are required in this area of research. Simple handheld bioprinters can also be fabricated in the future to address various clinical challenges. Bioinks and fabrication technologies could be further improved to generate fully functional tissues/organs for regenerative medicine applications in the future.

Authors' Contributions

All authors have equal contribution in the preparation of the manuscript.

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