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Bioprinting Technology: A Current State-of-the-Art Review

Bioprinting is an emerging technology for constructing and fabricating artificial tissue and organ constructs. This technology surpasses the traditional scaffold fabrication approach in tissue engineering (TE). Currently, there is a plethora of research being done on bioprinting technology and its potential as a future source for implants and full organ transplantation. This review paper overviews the current state of the art in bioprinting technology, describing the broad range of bioprinters and bioink used in preclinical studies. Distinctions between laser-, extrusion-, and inkjet-based bioprinting technologies along with appropriate and recommended bioinks are discussed. In addition, the current state of the art in bioprinter technology is reviewed with a focus on the commercial point of view. Current challenges and limitations are highlighted, and future directions for next-generation bioprinting technology are also presented.
[DOI: 10.1115/1.4028512]

Keywords: bioprinting, bioink, tissue engineering

1 Introduction

Since the first successful kidney transplant in 1954 was performed between two identical twins [1], organ transplantation has become a life-saving procedure for many disease conditions that hitherto were considered incurable. In the US, an average of 79 people receive transplants every day; however, the number of donors is much smaller than the number of patients waiting for a transplant [2]. Moreover, infections and rejection of the tissue by the host often make the transplantation process more challenging [3]. Therefore, the ability to make healthy organs would relieve suffering and save lives.

TE is a multidisciplinary scientific field that has rapidly emerged and combines engineering principles with life sciences to replace damaged tissues or restore malfunctioning organs by mimicking native tissues [4–7]. The traditional TE strategy is to seed cells onto a scaffold, a solid support structure comprising an interconnected pore network [8]. The main reasons for the scaffolding approach are to maintain the shape and mechanical properties of the mimicked engineered tissue, to assist in cell attachment, and to provide a substrate for cell proliferation into three-dimensional (3D) functioning tissues [9]. However, there are limitations to this approach. For instance, the lack of precision in cell placement, limited cell density, needs of organic solvents, difficulties in integrating the vascular network, insufficient interconnectivity, inability to control the pore distribution and pore dimensions, and difficulties in manufacturing patient-specific implants can be considered as major challenges and limitations in traditional scaffolding technology [10,11]. In general, the application of scaffolds in TE is straightforward but still subject to some challenges [12,13]. These difficulties have led many groups toward the development of new bioprinting approaches [14–17].

Bioprinting, a biomedical application of additive manufacturing (AM), is a recent innovation that simultaneously writes living cells and biomaterials layer by layer to fabricate living tissue constructs. It is represented by various biologically applied printing systems, such as laser-, extrusion-, and inkjet-based printing systems. Given the huge number of cell types in the body, those biologically applied printing systems and technologies vary in their ability to ensure the deposition accuracy, stability, and viability of cells. In this context, bioprinting deals with science, engineering and technology to generate 2D and even 3D complex biological constructs using living cells and engineered biomaterials [18].

For now, bioprinting of 3D functional complex organs remains in the state of science fiction. However, the field is moving forward. Currently, there is a plethora of research being done on bioprinting technology and its potential as a source for tissue grafts and full organ transplants. A timeline for the evolution of bioprinting technology up to the current state of the art is illustrated in Table 1.

This paper provides a general overview of the current state of bioprinting, describing the broad range of bioprinting technologies now being used to deliver cells and biomaterials in preclinical studies. Distinctions between laser-, extrusion-, and inkjet-based bioprinting techniques along with appropriate bioinks are demonstrated. In addition, the current state of the art in bioprinter technology is reviewed with a focus on the commercial point of view. Moreover, current challenges and limitation are discussed, and future directions are offered to the reader.

2 Bioprinting Techniques

Bioprinting could be defined as a "computer-aided transfer processes for patterning and assembling living and nonliving materials with a prescribed layer-by-layer stacking organization in order to produce bio-engineered structures serving in regenerative medicine and other biological studies" [19]. This process can be divided into three sequential technological steps: preprocessing, processing (actual printing), and postprocessing [20]. Preprocessing is a blueprint of tissue or organ design using imaging and computer-aided design techniques. Accordingly, after the blueprint is designed, the actual printing is processed through a bioprinter. The bioprinted construct must then undergo the process of tissue remodeling and maturation in a specially designed chamber "bioreactor," which accelerates tissue maturation.

The most promising technologies in bioprinting impose the self-assembly and self-organizing capabilities of cells delivered

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Contributed by the Manufacturing Engineering Division of ASME for publication in the Journal of Manufacturing Science and Engineering. Manuscript received April 15, 2014; final manuscript received August 24, 2014; published online October 24, 2014. Assoc. Editor: David L. Bourell.

Table 1 A timeline for the evolution of bioprinting technology up to the current state of the art

Year	Development
1996	Observation that cells stick together and move together in clumps
1996	First use of natural biomaterial in human for tissue regeneration
1998	Invention of cell sheet technology
1999	LDW
2001	First tissue-engineered bladder (using synthetic scaffold seeded with patient's own cells)
2002	Bioprinting using inkjet technology is enabled
2003	Inkjet printing generated viable cells
2004	A modified inkjet printer to dispense cells
2004	3D tissue with only cells (no scaffold) was developed
2006	3D cellular assembly of bovine aortal was fabricated
2007	Digital printing
2008	Concept of tissue spheroids as building blocks
2009	First commercial bioprinter (Novogen MMX)
2009	Scaffold-free vascular constructs
2010	In situ skin printing
2010	Hepatocytes were patterned in collagen using LDW successfully
2012	Applied inkjet printing to repair human articular cartilage
2012	Bipolar wave-based drop-on-demand jetting
2012	Engineer an artificial liver using extrusion-based (syringe) bioprinting
2014	Integrated tissue fabrication with printed vasculature using a multi-arm bioprinter

through application of techniques based on either laser [21–27], inkjet [28–37] or extrusion/deposition techniques [17,38–44]. The reader is referred to Fig. 1 for existing bioprinting techniques, which are discussed extensively in Secs. 2.1–2.3.

2.1 Inkjet-Based Bioprinting. Bioprinting has its roots in inkjet printers, which were first used in offices and then with personal computers in the 1980s. In this technology, droplets of ink are deposited on a piece of paper using narrow orifices. Inkjet printers enable precise control over the locations of droplets and thus give great flexibility to users. In the early 2000s, a small leap was made in this technology, and cells replaced traditional ink in cartridges [33]. For details of the hardware technology, the reader is referred to a recent study by Cui et al. in the *Journal of Visualized Experiments* [45], which demonstrates a clear step-by-step approach in modifying a traditional inkjet printer into a bioprinter and replacing ink with a bioink.

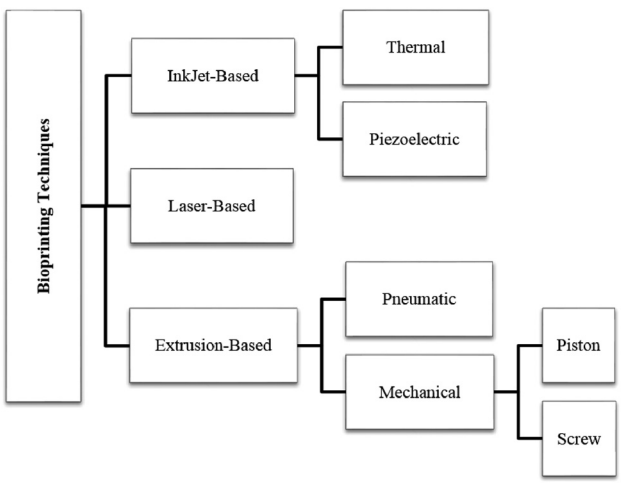

Fig. 1 Classification of bioprinting techniques

In this technique, "bioink," made of cells and biomaterials, is used to print living cells in the form of droplets (each contains 10,000–30,000 cells) by using a noncontact nozzle [46]. Boland and his followers demonstrated both a high level of control over cell dispensing as well as remarkable viability and certain functionality of printed cell patterns using different cell types [35,46–50]. Inkjet printing has since been studied and developed to a quite well-understood process capable of patterning viable cells and biomaterials [51].

Among different types of inkjet printers, thermal or piezoelectric drop-on-demand methods have been adapted in bioprinting. The thermal inkjet system consists of an ink chamber with a small number of nozzles and a heating element (see Fig. 2(*a*)). To generate an ink droplet, a short current pulse is applied to the heat element. Consequently, the temperature of the ink around the heating element increases, forming a bubble that forces the ink out of the nozzle orifice [52]. On the other hand, the piezoelectric inkjet printing uses piezocrystals, which are located at the back of the ink chamber (see Fig. 2(*b*)). An electric charge is applied to the crystals, causing them to vibrate. Inward vibration forces a small amount of the ink out of the nozzle [53].

Several studies have shown that heat and mechanical stresses generated in thermal-based inkjet bioprinters, especially in their orifices, affected cell viability [29,35,54]. Furthermore, specific vibration frequencies and power levels used in piezoelectric-based bioprinters may disrupt cell membranes and cause cell death [35,55]. However, Barbara et al. showed that cells of adult central nervous system (retinal ganglion cells and glia) could be printed using a piezoelectric-based bioprinter without adverse effects on cell viability [56]. They found that printed glial cells retained their growth-promoting properties, which opened the possibility of developing printed grafts for use in regenerative medicine.

Inkjet bioprinters are relatively cheap and can work under mild conditions. In addition, inkjetting is a noncontact technique, which decreases the chance of contamination. For instance, Xu et al. modified an inkjet printing method that allows reproducible and precise arrangement of multiple cell types together with specific matrices to create complex multicell heterogeneous constructs containing three different cell types, which may have clinical implications for building vascularized bone tissues (see Fig. 2(*c*)) [54]. Moreover, Cui and Boland used a thermal inkjet-based bioprinter to simultaneously place human endothelial cells and fibrin (scaffolding material) onto fibrinogen to yield aligned cells in a fibrin channel [57]. When cultured, the cells were placed into channels, proliferated and formed branched tubular structures biomimicking capillaries [57] (see the arrow in Fig. 2(*d*)).

The setup of inkjet bioprinters facilitates the integration of multiple print heads, which enables deposition of multiple cell types. This feature allowed Weiss and his coworkers [58] to develop a multihead inkjet-based bioprinting platform and fabricate heterogeneous structures with a concentration gradient changing from the bottom up. On the other hand, Boland et al. made use of thermosensitive gels to generate sequential layers for cell printing by using a commercially available inkjet printer [33]. The printer used by Wilson and Boland [33] could put up to nine solutions of cells or polymers into a specific place and then print 2D tissue constructs.

Recently, Xu and his coworkers developed a platform-assisted 3D inkjet bioprinting system to fabricate 3D complex constructs such as zigzag tubes [59]. They fabricated vascularlike alginate tubes with a hemibranching point using drop-on-demand inkjet printing [60]. This study also examined the bioprinting rheology along with the underlying physics [61] and is considered as a cornerstone in efficient fabrication of viable 3D vascular constructs with complex anatomies and vascular trees. Inkjet-based bioprinting technology has also been used to print various biological compounds, such as nucleic acid, proteins [62], growth factors [63,64] and biological cells [28,31–33,35,65].

Despite the great progress in inkjet-based bioprinting, this technique still faces some limitations. One of the main restrictions is

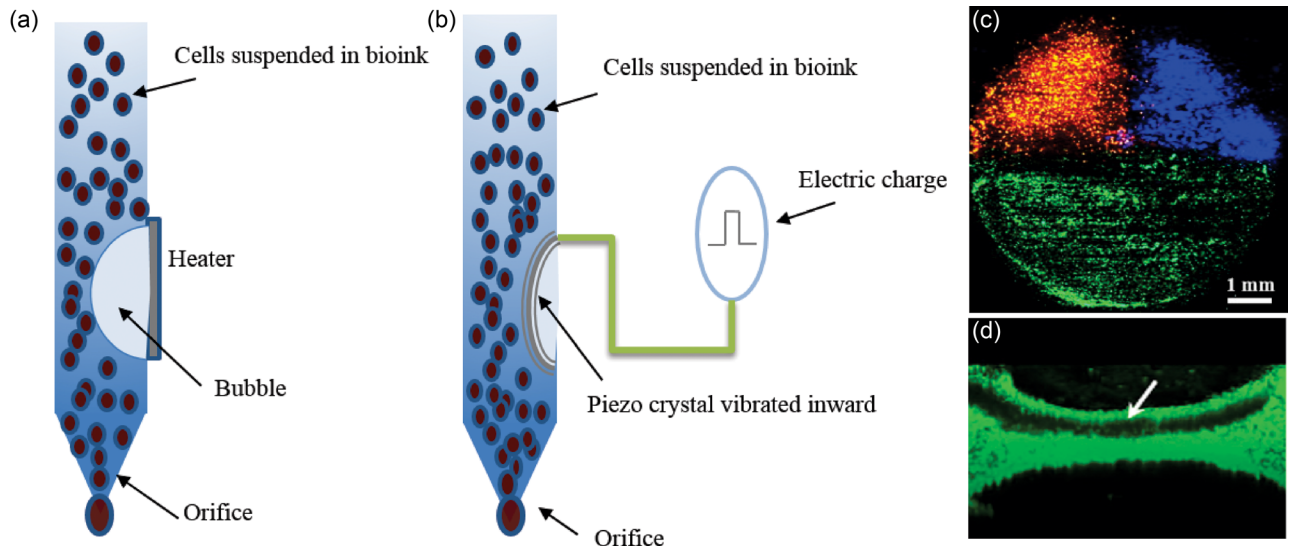

Fig. 2 Inkjet-based bioprinting: (*a*) a schematic of thermal-based inkjet bioprinting, (*b*) a schematic of piezoelectric-based inkjet bioprinting, (*c*) microscopic top views of a complete 3D multicell "pie" construct using an inkjet-based bioprinter (courtesy of Elsevier [54]), and (*d*) a tubular structure of the printed human microvasculature using an inkjet-based bioprinter (courtesy of Elsevier [57])

the low upper limit for viscosity of bioink, which is on the order of 0.1 Pa s^{-1} [66]. This makes the deposition of highly viscous hydrogels and extracellular matrix (ECM) components more complicated [42]. Moreover, material throughput, reproducibility of droplets, the range of shear forces within the nozzle, cell aggregation and sedimentation in the cartridge reservoir, clogging of the nozzle orifice, and the number of fluids that may be printed during a single experiment are other limitations and challenges associated with this technique. This technology has a great potential to solve and break through some of the intrinsic problems present in TE, providing the capacity to eject very small droplets of several inks on demand.

2.2 Laser-Based Bioprinting. A laser-based system was first introduced in 1999 by Odde and Renn to process 2D cell patterning [67]. In this technology, laser energy was used to excite the cells and give precise patterns to control the cellular environment spatially.

Laser-based direct writing (LDW) is one of the leading methods in laser-based bioprinting techniques [68]. In LDW, a laser pulse guides an individual cell from a source to a substrate. The laser pulse is used to transfer the suspended cells in a solution from a donor slide to a collector slide. The laser pulse creates a bubble, and shock waves generated by the bubble formation eventually force cells to transfer toward the collector substrate (see Fig. 3(*a*)). The most popular techniques in LDW are laser-induced forward transfer (LIFT) and matrix-assisted pulsed laser evaporation direct writing (MAPLE DW). MAPLE DW is schematically similar to LIFT; however, MAPLE DW utilizes a lower powered pulsed laser compared to LIFT. These techniques allow precise deposition of cells in relatively small 3D structures. For instance, Lothar et al. used LIFT to print skin cell lines (fibroblasts/keratinocytes) and human mesenchymal stem cells (hMSC) [69]. In their study, they evaluated the influence of LIFT on the cells. They stated that the skin cells survived with a rate of 98% ± 1% and with 90% ± 10% for hMSC. On the other hand, Nathan et al. deposited patterns of human dermal fibroblasts, mouse myoblasts, rat neural stem cells, human breast cancer cells, and bovine pulmonary artery endothelial cells by using MAPLE DW to study aspects of collagen network formation, breast cancer progression, and neural stem cell proliferation. They have shown that MAPLE is an effective and reproducible technique for processing idealized cellular constructs that incorporates many cell types, such as dermal

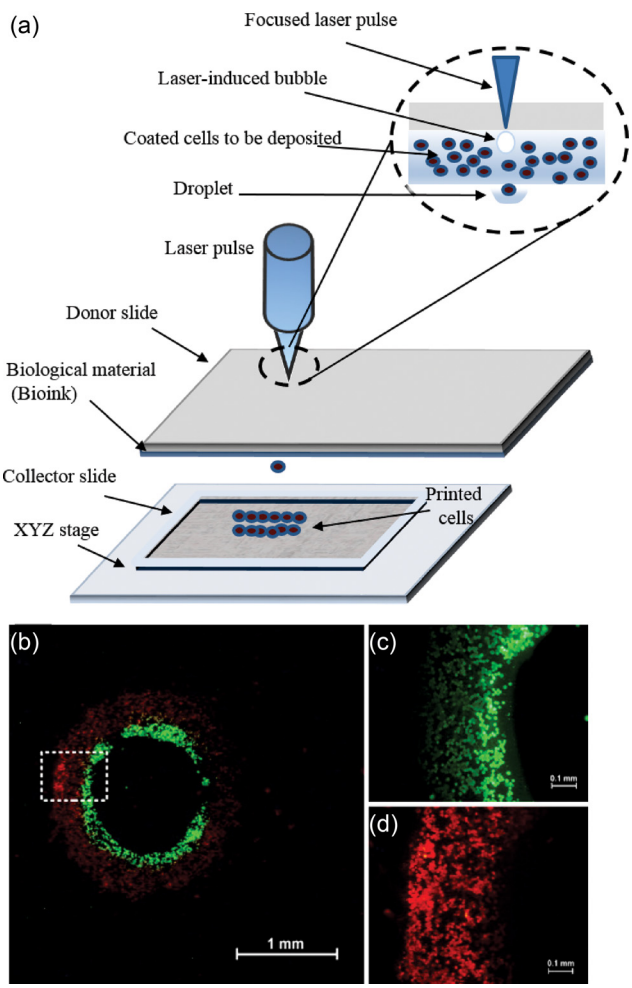

Fig. 3 Laser-based bioprinting: (*a*) a schematic of laser-based bioprinting (LDW and LAB), (*b*) different cell types printed in close contact to each other with a high cell concentration (courtesy of Elsevier [74]), (*c*) chondrocytes stained with Calcein, and (*d*) osteoblast cells stained with DiI-LDL

fibroblasts, myoblasts, neural stem cells, bovine pulmonary artery endothelial cells, and breast cancer cells [70]. Microscale cell patterning can be achieved through optimizing the viscosity of the bioink, laser printing speed, laser energy and pulse frequency [71]. Writing of multiple cell types is also feasible by selectively propelling different cells to the collector substrate.

The nozzle-free nature of this method enables the usage of high-viscosity bioink, unlike inkjet and extrusion-based bioprinting techniques. Moreover, given the high precision characteristic, lasers may offer the most benefit for bioprinting the smallest features of an organ. For instance, Barron et al. demonstrated the ability to print mammalian cells onto a hydrogel substrate as either stacks or individual cells via LDW [72]. Their results showed that it was possible to deposit cells and build 50–100 μm thick cellular stacks. In 2005, Nahmias et al. patterned hepatocytes in collagen and Matrigel, forming a 3D cellular structure [73]. Guillotin et al. demonstrated that a laser-based bioprinter could deposit cells with a microscale resolution. They successfully printed miniaturized tissue constructs with high cell density and microscale organization [74] (see Figs. 3(*b*)–3(*d*)). On the other hand, Gaebel et al. used a laser-based bioprinting technique to fabricate a cardiac patch made by polyester urethane urea seeded with human umbilical vein endothelial cells and hMSCs [27].

Conversely, this technology, as any other technology, has a number of limitations. The heat generated from laser energy and/or laser light may damage cells or affect the ability of cells to communicate and aggregate in the final tissue construct. In general, cell viability in laser-based bioprinting is lower than that of inkjet-based bioprinting. Moreover, gravitational and random setting of cells in the precursor solution, prolonged fabrication time, limitations in printing in the third dimension and the need for photocrosslinkable biomaterials are other limitations in laser-based bioprinting [7]. In addition, a recent study by Gudapati et al. demonstrated that gelation of hydrogels during the process is also vital in preserving high cell viability in laser-based bioprinting [75].

2.3 Extrusion-Based Bioprinting. The pressure, or extrusion-based, method has been used for quite a long time. However, since TE emerged, this technology has also started to be investigated as a promising technique for creating living tissue constructs. Extrusion-based bioprinting is a combination of a fluid-dispensing system including a pneumatic or mechanical (piston or screw-driven) one and an automated robotic system for extrusion and writing [76] (see Fig. 4(*a*)). Piston-driven deposition generally provides more direct control over the flow of bioink through the nozzle. Screw-driven systems may give more spatial control and are beneficial for dispensing bioinks with higher viscosities. However, screw-driven extrusion can generate larger pressure drops along the nozzle, which can potentially be harmful for loaded cells. Thus, screw design needs to be carefully performed in order to be used in bioprinting setups. The main advantage of using a pneumatically driven system is the various types and viscosities of bioinks that can be dispensed by adjusting the pressure and valve gating time. During bioprinting, bioink is dispensed by a deposited system, under the control of a computer, and then crosslinked by light, chemical or thermal transitions [7], resulting in precise deposition of cells encapsulated in cylindrical filaments of desired 3D custom-shaped structures. Therefore, this technique provides relatively better structural integrity due to continuous deposition of filaments. Moreover, this method can incorporate computer software such as CAD software, which enables users to load a CAD file to automatically print the structure [77]. Thus, it is considered as the most convenient technique in producing 3D porous cell-laden structures. For instance, Yan et al. used extrusion-based bioprinting to deposit different cell types loaded in a wide range of biocompatible hydrogels [78]. They used hepatocytes and adipose-derived stromal cells together with gelatin/chitosan hydrogels to engineer artificial liver tissue

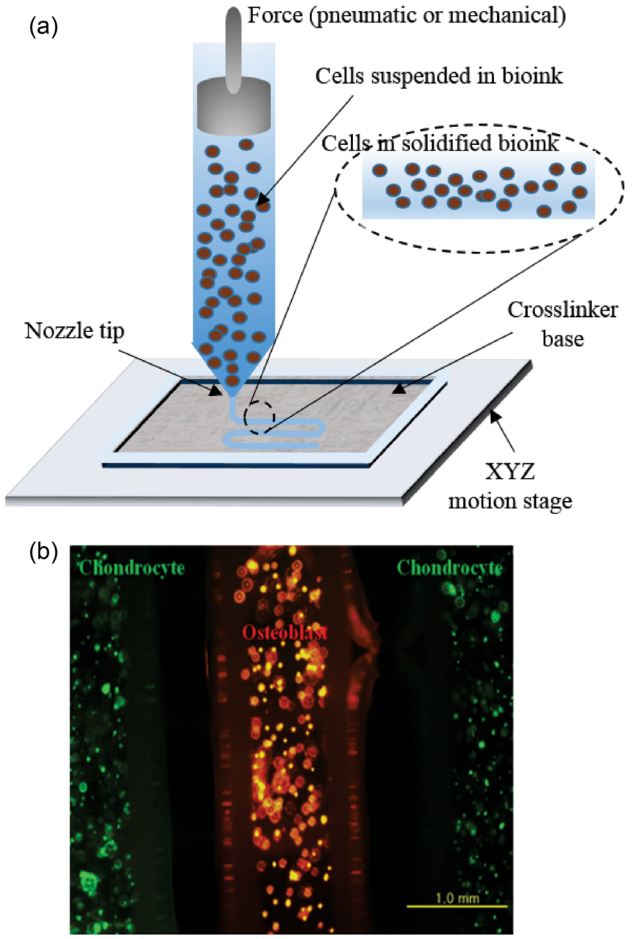

Fig. 4 Extrusion-based bioprinting: (*a*) a schematic of extrusion-based bioprinting technique, (*b*) a cell-laden structure consisting of chondrocytes and osteoblasts was produced using an extrusion-based bioprinter (courtesy of Jin-Hyung Shim)

constructs. On the other hand, Sun and his coworkers developed a multinozzle low-temperature deposition system with four different micronozzles: peumatic microvalve, piezoelectric nozzle, solenoid valve, and precision extrusion deposition nozzle [79]. Moreover, Sun et al. and Lee et al. fabricated multilayered cell-hydrogel composites by using an extrusion-based technique [79–82]. Their rheology study and cell viability assay were performed to investigate cell damage due to mechanical stress during the printing process [83]. They found that cell viability was influenced by material concentration, dispensing pressure, material flow rate and nozzle geometry. Furthermore, Shim et al. produced a cell-laden structure consisting of a polycaprolactone (PCL) framework, chondrocytes, and osteoblasts using their extrusion-based bioprinter (see Fig. 4(*b*)) [84]. Extrusion-based bioprinting has been recently used in bioprinting vascular network toward thick tissue construct fabrication either through direct bioprinting of vasculature network using coaxial nozzle configuration [85–87] or indirect bioprinting by utilizing a fugitive ink that is removed by thermally induced reverse crosslinking leaving a vascular network behind [88,89]. In the last couple of years, several researchers have attempted to create branching vascular network using the latter technology including Lewis and her coworkers [89,90], Chen's group [91], Khademhosseini's group [92,93], and Dai's group [94,95], which have successfully achieved angiogenesis by sprouting endothelial cells within fibrin network loaded with other supporting cells [96]. With the advancements in these technologies, vascularization providing an efficient media exchange system will be enabled for thick tissue fabrication in the near future.

Although this technology is considered as the most convenient technique for the scale-up tissue and organ fabrication process, it suffers from several limitations, such as shear stress and limited material selection due to the need for rapid encapsulation of cells via gelation. A higher dispensing pressure can allow ejecting highly viscous bioinks, but this could increase the shear stress, which reduces cell viability and increases cell damage [7]. In this regard, Yu et al. demonstrated the capability of direct fabrication of cell-laden conduits using an extrusion-based bioprinting technique [97]. They revealed that the bioprinting process could induce quantifiable cell death due to changes in dispensing pressure, nozzle geometry and bioink concentration. Moreover, Tirella et al. systematically investigated the effect of shear stress on cell viability and functionality endured during the deposition process [98]. Therefore, using optimum process parameters such as bioink concentration, nozzle diameter, pressure and speed, one can overcome these limitations and challenges to some extent.

As stated before, each bioprinting technique has its advantages and disadvantages with respect to printing capabilities, resolution, deposition speed, scalability, bioink, and material compatibility, ease of use, printing speed and price, as well as fundamental aspects of biocompatibility. Therefore, it would not be appropriate to consider these techniques and technologies as competing technologies. The reader is referred to Table 2 for a detailed comparison of bioprinting techniques.

3 The Bioink

Biomaterial development can be considered as one of the most challenging aspects of bioprinting. Inclusion of cells within biomaterials (i.e., ink) to form a "bioink" is considered the cornerstone of creating complex biologically relevant 3D tissue constructs. Therefore, properties of the ink need to fulfill the biological requirements necessary for cells from one side and the physical and mechanical needs of the printing process itself from the other side [122]. In the literature, hydrogel and hydrogel-free cell aggregates have been used in printing artificial tissues and tissue constructs.

3.1 Hydrogels. Hydrogels are broadly used in TE and biomanufacturing fields because they contain several features of natural ECM components and allow cell encapsulation in a highly hydrated, mechanically supportive 3D environment. Hydrophilicity is one of the main factors that determines the biocompatibility of hydrogels, thus making them attractive for application in the fabrication of tissue constructs [123]. As a result, they can provide embedded cells with a 3D environment similar to that in many natural tissues. Hydrogels in TE are generally classified as either naturally derived polymers such as alginate, collagen, chitosan and fibrin [124–126] or synthetic polymers like pluronics and polyethylene glycol [38,127–129]. Naturally derived hydrogels are generally cell friendly; therefore, they have been used in TE and bio-applications more frequently than synthetic hydrogels, which often lack biofunctionality.

In bioprinting, hydrogels are used as bioink materials or as cell delivery vehicles. Many types of cells can be viable when encapsulated within hydrogels, such as fibroblasts, chondrocytes, hepatocytes, smooth muscle cells, adipocytes, and stem cells [130]. During bioprinting, a hydrogel with suspended cells is processed into a specifically defined shape, which is successively fixed by gelation. Gelation is usually a crosslinking reaction initiated by physical, chemical or a combination of both processes [99]. Physical crosslinking is a reversible interaction that depends on meshes of high molecular polymer chains, ionic interactions and hydrogen bridges [99]. This type of crosslinking is compatible with biological systems such as growth factors and living cells. One example is ionotropic gelation by interaction between anionic groups on alginate (COO) with divalent metal ions (Ca^{2+}). Another example is gel formation due to aggregation of helix upon cooling a hot solution of carrageenan. However, poor mechanical properties are considered as the main drawback of this crosslinking reaction. Therefore, postprocessing crosslinking and/or an additional crosslinking agent is required. In contrast, chemical crosslinking forms new covalent bonds, which give relatively high mechanically stable constructs compared to physical crosslinking [99]. For example, the crosslinking of natural and synthetic polymers can be achieved through the reaction of their functional groups (i.e., OH, COOH, and NH_2) with crosslinkers such as aldehyde (i.e., glutaraldehyde, adipic acid dihydrazide). However, this type of crosslinking may involve exposure of constructs to irradiation, which affects the embedded cells [99]. Moreover, it gives 3D mesh networks that are smaller than cells, which limits the mobility and migration of encapsulated cells [131]. Conversely, degradation sites can be incorporated into hydrogels, permitting migration and proliferation of cells [132–134].

To improve the printability of hydrogels, polymer concentration has to be increased [135,136], which increases the stiffness of hydrogels. However, cells need an aqueous environment in which their migration and mobility is not limited by a dense polymer network. In this regard, Murphy et al. [137] evaluated the

Table 2 Comparison of bioprinting techniques

	Laser-based [67,72,74,99–105]	Inkjet-based [28,31–33,35,99,106–111]	Extrusion-based [38,42,79,99,112–121]
Resolution	High	Medium	Medium–low
Droplet size	>20 μm	50–300 μm	100 μm–1 mm
Accuracy	High	Medium	Medium–low
Materials	Cells in media	Liquids, hydrogels	Hydrogels, cell aggregates
Commercial availability	No	Yes	Yes
Multicellular feasibility	Yes	Yes	Yes
Mechanical/structural integrity	Low	Low	High
Fabrication time	Long	Long–medium	Short
Cell viability	Medium	High	Medium–high
Processing modes	Optical	Thermal and mechanical	Mechanical, thermal, and chemical
Throughput	Low–medium	High	Medium
Control of single-cell printing	High	Low	Medium
Hydrogel viscosity	Medium	Low	High
Gelation speed	High	High	Medium
Advantages	High accuracy, single cell manipulation, high-viscosity material	Affordable, versatile	Multiple compositions, good mechanical properties
Disadvantages	Cell-unfriendly, low scalability, low viscosity prevents build-up in 3D	Low viscosity prevents build-up in 3D, low strength	Shear stress on nozzle tip wall, limited biomaterial used, relatively low accuracy

characteristics and properties of twelve hydrogels to determine their suitability for bioprinting applications. They found that Extracel-UV performed the best among other tested hydrogels in terms of crosslinking time, UV irradiation, and biocompatibility.

3.2 Cell Aggregates. During the embryonic maturation process, cells from multiple sources are assembled and organized into tissues and organs without a need for scaffolding [138]. Tissues and organs are self-organizing systems, where cells undergo biological self-assembly and self-organization without any external influence in the form of instructive, supporting and directing rigid templates or solid scaffolds. Consequently, closely bioprinted cell aggregates undergo tissue fusion and self-folding. As nature takes over, the cell aggregates slowly fuse together. Individual cells organize into multicellular subunits, creating the final tissue construct [14–17]. This is called self-assembly [139,140]. Self-assembly approaches demonstrate that fully biological tissues can be engineered with specific compositions and shapes by exploiting cell–cell adhesion and the ability of cultured cells to grow their own ECM, thereby helping to reduce and mediate inflammatory responses.

The success of aggregation depends on the use of nonadhesive substrate or using a substrate that has reduced adhesion (removal of cellular attachment molecules from the substrate), and the use of more nutrient factors in liquid culture overlay than in the substrate plate. In bioprinting, a substrate is usually used as a bioprocessable scaffolding material, contributing as a biological and structural support for cells to attach, proliferate and differentiate. Ideally, it must be biocompatible, nontoxic, dispensable, capable of rapid solidification and functional with growth factors to enable high cell viability. Thus far, naturally derived hydrogels such as collagen and soy agar gel have been used as a substrate material, and various stem cell types (i.e., embryonic stem cells, human bone marrow stem cells, and adipose-derived stem cells (ASCs)) could be patterned onto substrates in a high-throughput manner with the goal of achieving encapsulated stem cells of interest for genomic analysis [141,142].

Cell-aggregate-based bioinks can be homogeneous, containing a single-cell type, or heterogeneous, prepared by coculturing several cell types. The cell-aggregate-based bioinks typically used are tissue spheroids, cell pellets and tissue strands.

Tissue spheroids (see Fig. 5(*a*)) can be considered as "living materials" with certain measurable, evolving and potentially controllable material properties [138]. However, these spheroids must be maximally standardized in their size in order to make them processable or dispensable through a bioprinter nozzle or other means without clogging problems and without being destroyed. Thus, standardization of the tissue spheroid dimension is desirable for continuous dispensing. Multiple methods have been described to prepare tissue spheroids [17,138,144,145]. The method of tissue spheroid biofabrication must not induce significant cell injury and/or damage. In this regard, Rezende et al. discussed the main competing methods of tissue spheroids [146]: (1) the modified handing drop method, (2) molded nonadhesive hydrogel technology, and (3) digital microfluidic technologies. Instead of triggering cells to form spheroids, the cell pellet can be directly used as a bioink; they can be loaded into a syringe unit or a pipette for printing (see Fig. 5(*b*)). Although the cell pellet possesses very high cell density and does not bring challenges with scale-up bioprinting technologies or nozzle clogging, it is in liquid form and cannot be solidified immediately by any means. Thus, printing a support material that is inert to cells, such as a thermal-sensitive hydrogel (i.e., agarose), is essential to create a mold for the cell pellet [147]. Upon fabrication, the cell pellet in hydrogel mold further aggregates and maturates and eventually generates a solid tissue. The cell pellet can also be molded into tissue strand form (by culturing or coculturing) prior to the bioprinting process (see Fig. 5(*c*)). By applying the essential growth factors and culture conditions, capillarization can also be achieved, and tissue strands can be grown. They can then be loaded into a custom-made nozzle unit for the extrusion-based bioprinting process. The main advantages of tissue strands are that they can be printed without a need for printing a mold, which enables large-scale bioprinting.

In addition to hydrogels and cell aggregate-based bioinks, decellularized matrix components have been recently considered as a new bioink type. Dong-Woo Cho's group used decellularized adipose, heart and cartilage tissue matrix components and printed them along with PCL framework to support tissue analogues in 3D [148]. In general, bioinks differ from each other, in that different bioinks have different mechanical properties, gelation methods and other bioprintability characteristics. Thus, it is important to be aware of these characteristics in order to implement the appropriate bioink for various bioprinting techniques.

4 Bioprinters: The State of the Art

In 2000, Rolf Muelhaupt's group at Freiburg Materials Research Center introduced an AM fabrication technique using 3D plotting of thermoreversible gels in a liquid medium. This group was the first to report the deposition of living cells using an extrusion approach [149,150]. Afterward, in 2002, an evolution in bioprinting took place when Nakamura realized that the ink droplets in an inkjet printer were the same size as human cells [151]. Consequently, he decided to adapt the technology, and by 2008, he had created a functional bioprinter that could print out a biotube mimicking a blood vessel.

The ideal bioprinter has specific system requirements, which include—but are not limited to—high resolution, high throughput, ability to dispense various biomaterials simultaneously, ease of use, nontoxicity, cell viability, affordability, and the ability to control dispensing of multiple bioinks with different viscosities [152]. Therefore, for precise dispensation of a bioink, a bioprinter

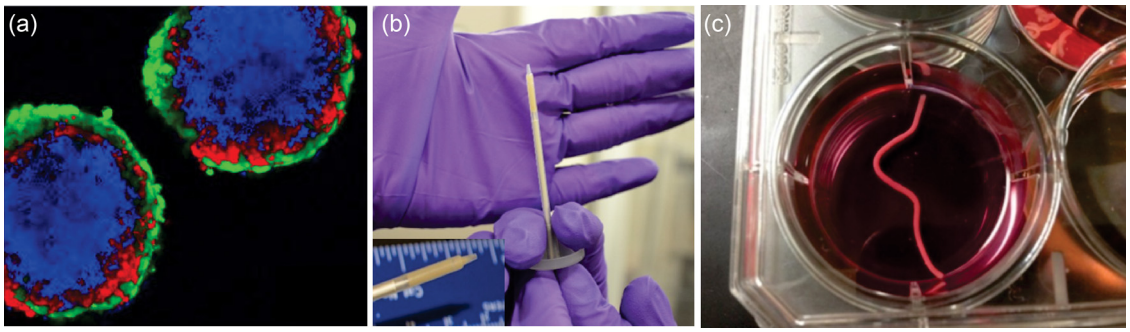

Fig. 5 Cell-aggregate-based bioink: (*a*) tissue spheroids (150 μm in diameter): human primary brain endothelial cells (outermost layer), human primary pericytes cells (middle layer), human primary astrocytes cells (hpAs) (innermost layer), and the complete spheroid composed of all three cell types (courtesy of Elsevier [143]), (*b*) cell pellet in a syringe, and (*c*) tissue strands

should include three essential elements: an x–y–z robotic motion system, bioink dispensers and computer-based software-enabled operational control to print bioink with satisfactory resolution [138,153,154]. In this regard, a bioprinter with three print heads was used to fabricate functional blood vessels and cardiac tissue constructs [155]. The first two heads dispense cardiac and endothelial cells, while the third dispenses a collagen scaffold to support the cells during the printing process. Another bioprinter, called the Palmetto printer [156], dispenses spheroid bioink in 3D and was designed and developed by Medical University of South Carolina (MUSC) and Clemson University (see Fig. 6(*a*)). The printing head was designed with three interchangeable dispensers for placing biomaterials. To build 3D tissue constructs, biocompatible materials and cellularized spheroids can be printed using an x–y–y positioning robot.

Until 2005, all 3D printers were expensive, proprietary and in industrial scale. Therefore, the high cost and closed nature of the 3D printing industry limited the accessibility of the technology to the exploration that could be done by end-users. To change this situation, the Fab@Home project was initiated as the first multimaterial 3D printer available to the public [157]. Since its open-source release in 2006 [158], it has created a versatile and low-cost printer to accelerate technology innovation and its migration into the consumer space.

For dispensing a wide range of relevant biomaterials, Shim et al. developed a multihead tissue/organ building system (MtoBS) possessing six dispensing heads to fabricate 3D tissue constructs [84] (see Fig. 6(*b*)). With the MtoBS, 3D customized porous structures for osteochondral tissues were fabricated. They used sequential dispensing of PCL and two alginate solutions with and without two different live cells, such as osteoblasts and chondrocytes [84]. Song et al. developed a bioprinting system that consists of three axes for the motion control stage as well as an additional axis for injection syringe control. Their injection syringe system can deposit a wide range of hydrogel solutions with various material properties [159]. Almost all of the existing bioprinters in the literature only have one arm and use multiple heads to print bioinks one at a time. Ozbolat et al. recently developed a bioprinter with two independent arms, which may be able to speed up the process and brings higher flexibility (see Fig. 6(*c*)) [160]. This bioprinter, named the multi-arm bioprinter (MABP), can lay down multiple bioinks simultaneously. For example, one arm can print a vascular network while the other one lays down tissue strands between vascular conduits.

An affordable and high-resolution bioprinter that can control the dispensing of multiple bioinks with different viscosities is an important accomplishment and will definitely enable and enhance further developments in biofabrication technology.

4.1 The Commercial Viewpoint. The emergence of commercially available bioprinters is probably one of the most remarkable developments of the past decade. The explosive growth of different variants of bioprinting technology resembles the early development phase of AM technology two decades ago, when many competing technologies were developed but not all of them were successfully commercialized. Robotic bioprinters are already commercially available, whereas others are still under development. The 3D bioprinters currently on the market can cost around $100–200 K, depending on their unique capabilities, while 3D homemade bioprinters can cost less than $20 K. Examples of those commercially available are the NovoGen MMX Bioprinter™, the 3D Bioplotter® and Sciperio/nScrypt (see Fig. 7).

The NovoGen MMX (see Fig. 7(*a*)) is loaded with bioink spheroids, each of which contains an aggregate of thousands of cells. The NovoGen MMX first lays down a layer of a biopaper made of hydrogels such as collagen and gelatin. Bioink spheroids are then injected into this biopaper. Then, more layers are added one by one to build up the final object. Finally, nature takes over, and the bioink spheroids slowly fuse together. Another example of a commercial bioprinter is the 3D Bioplotter, which was created by a Rolf Muelhaupt's group at Freiburg Materials Research Center (see Fig. 7(*b*)) [150,155]. First, hydrogel scaffolds with a designed external shape and a well-defined internal pore structure were prepared by this technology. Recently, this bioprinter has been outputting bioink "tissue spheroids" and using supportive scaffold materials. The 3D Bioplotter can print solutions within temperatures ranging from −50 °C to 150 °C [149] and also print a wider range of biomaterials, including but not limited to ceramics and biodegradable polymers, which may support tissue constructs. In Sciperio/nScrypt (see Fig. 7(*c*)), a movable x–y stage and three z-directional printing heads work together to perform 3D printing. The first two heads are used to print the bioink particles, where the biopaper substrate is printed through the third head.

5 Limitations and Future Directions

Despite the great progress and many breakthroughs of the last decade, bioprinting technology is still in its infancy and has met several challenges and limitations associated with:

- resolution, repeatability, cell viability, practicality, and biocompatibility of bioprinting processes
- cell density, cytotoxicity, bioprintability, solidificability and solidification speed, mechanical and chemical properties, affordability and abundancy, and cell viability and long-term cell functionality of bioinks
- compactness, resolution, accuracy, high-degree-of-freedom motion capability and motion speed, commercial availability, full-automation capability, user-friendliness, sterilibility, affordability, and versatility of the bioprinters

This emerging technology appears to be more promising for advancing TE toward functional tissue and organ fabrication for transplantation, ultimately mitigating organ shortage and saving lives; however, some important aspects should be addressed in the near future. For instance, developing a standardized scalable fabrication method for the robotic delivery of cells is still a challenge. Moreover, further progress is needed in (1) improving nozzle and

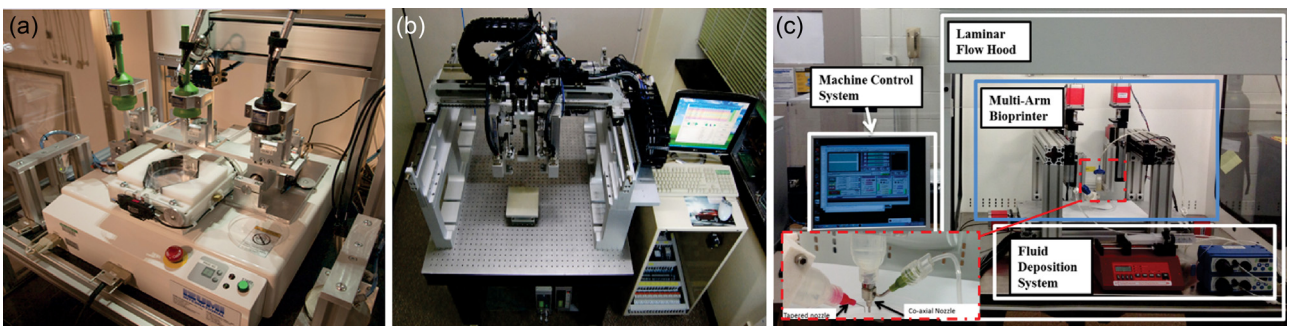

Fig. 6 Bioprinters: (*a*) Palmetto Bioprinter (courtesy of Michael J. Yost), (*b*) MtoBS (courtesy of Jin-Hyung Shim), and (*c*) the MABP

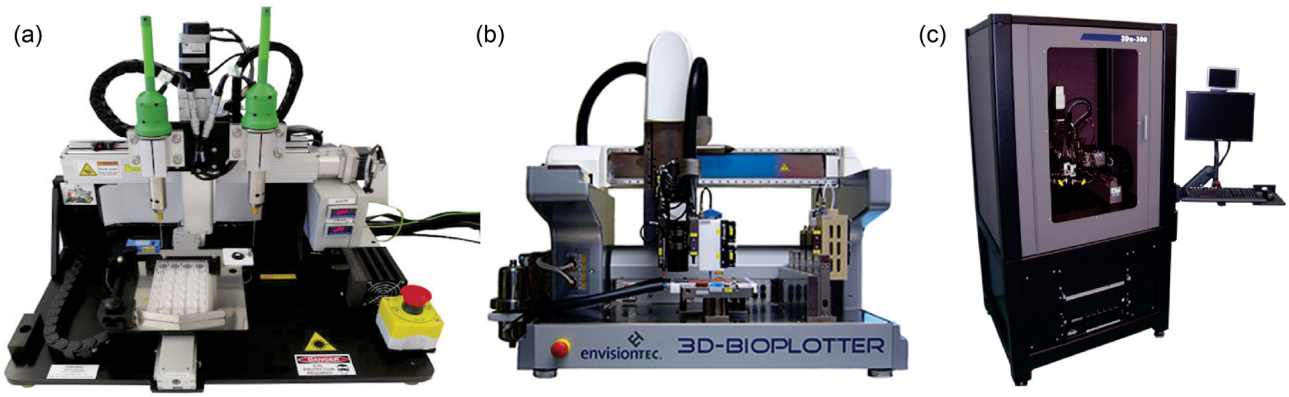

Fig. 7 Commercial bioprinters: (*a*) NovoGen MMX Bioprinter (courtesy of Organovo, San Diego, CA), (*b*) fourth generation 3D Bioplotter (courtesy of Envisiontec GmbH, Gladbeck, Germany), and (*c*) Sciperio/nScrypt (courtesy of BioAssembly Tool, Orlando, FL)

cartridge design, (2) increasing the diversity of bioprocessable and functional bioink with high cell density, (3) printing an intra-organ branched vascular tree for occlusion- or leak-free perfusion, (4) developing enabling technologies for multibioink multiscale hybrid bioprinting processes, and (5) developing accelerated tissue maturation technologies. Furthermore, to accurately capture and simulate dynamic tissue maturation, designing a bioprinter to consider postprocessing remodeling after printing is a challenging task. Consequently, 3D cell printing requires a controlled microenvironment around the printing stage for temperature, buffering, oxygenation, sterility, and delivery of trophic factors.

As far as future trends, 4D bioprinting can be considered a promising direction in the fabrication of living tissues in a shorter period of in vitro culture time. With the rapid fusion, folding and remodeling capabilities of cell-aggregate-based bioinks, tissues can be generated in shorter times, enabling bioprinting in the fourth dimension "time." In 4D bioprinting, printed cell aggregates fold and fuse to each other to create large-scale tissues within hours or a few days. This period depends on several factors, including cell phenotype, optimized media conditions for cocultured systems including medium and growth factors, culture conditions (i.e., static or dynamic) and applied cell aggregation methods. Besides, maturation of cell aggregates also plays a significant role in the folding and fusing capabilities of the bioink. The more the bioinks mature, the less the folding and fusion capabilities.

In general, cells encapsulated in hydrogels require a prolonged in vitro culture time for their proliferation in the order of months, which is relatively shorter in vivo as the nature can be considered as the best bioreactor. In this regard, phenotypic stability of cells becomes a problem after few weeks of in vitro culture and tissue generation attempts usually fail. A recent approach in bioprinting cell-laden hydrogel microcarriers can be a new direction while the microcarrier technology provides high cell concentration with preserved phenotypic stability [161]; however, printing cell-aggregate-based bioink overcomes several issues associated with scaffolding, where tissue remodeling and generation time is shortened remarkably, and the phenotypic stability of cells and tissue functionality can be preserved for longer periods of time. Although printing complex functional organs seems far-fetched, 4D bioprinting can enable the fabrication of mini-organs rapidly for pharmaceutical tissue models or cancer studies in the very near future.

Despite the great advantage of cell-aggregate-based bioinks, there is still a plethora of research that needs to be done to accelerate tissue maturation further. In this regard, the integration of well-connected capillaries inside the bioink will be another milestone toward functional tissue and organ printing. The bioink should be developed in a way that multicellular aggregates need to possess self-vascularization through biologically driven vasculogenesis or artificially developed vascular network in submicron scale. Otherwise, viability can be a concern for highly metabolic cells (i.e., beta cells, hepatocytes, cardiomyocytes, etc.) when the aggregates are stacked together. It will advance the tissue folding and remodeling process significantly and improve cell viability with preserved tissue functionality for a prolonged time. In addition to capillaries inside the bioink, the integration of macroscale vascular networks and the connection of these networks with capillaries is crucial to get into larger-scale models. Thus, a mold-free process design is needed for fully vascularized scale-up tissues and organs.

6 Conclusion

This review paper provides a general overview of the current state of the art of bioprinting technology and describes the broad range of bioprinters now being used to deliver cells and biomaterials in preclinical studies. Distinctions between laser-, extrusion-, and inkjet-based bioprinting technologies and appropriate and recommended bioinks are discussed. Commercially available bioprinters are demonstrated, and challenges and limitations associated with the current bioprinting technology are presented as well.

Although the "two-step" biofabrication of solid synthetic biodegradable scaffolds using a 3D bioprinter (first step) and sequential cell seeding of scaffold in an artificial or natural bioreactor (second step) has been preferred in TE due to its simplicity and speed, the "one-step" bioprinting approach based on simultaneous deposition of hydrogels mixed with living cells is more appropriate for biofabrication of highly metabolic 3D thick soft tissues [150,162–165]. The technology, however, is moving toward hydrogel-free bioprinting for its great advantage in the fabrication of functional tissue in a short period of time. Currently, there is a plethora of research being done on bioprinting technology and its potential as a future source for tissue grafts and full organ transplants. In particular, the development of a bioprinter has been one of the issues that attracts interest. Therefore, a number of bioprinting techniques have been developed in this regard, such as inkjet-, extrusion- and laser-based techniques, as discussed extensively in this paper.

Acknowledgment

This study was supported by NSF CMMI CAREER-1349716, a grant from the Diabetes Action Research, and Education Foundation and a grant from The State of Iowa through The University of Iowa Research Foundation.

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