



3D bioprinting for biomedical devices and tissue engineering: A review of recent trends and advances

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ABSTRACT

3D printing, an additive manufacturing based technology for precise 3D construction, is currently widely employed to enhance applicability and function of cell laden scaffolds. Research on novel compatible biomaterials for bioprinting exhibiting fast crosslinking properties is an essential prerequisite toward advancing 3D printing applications in tissue engineering. Printability to improve fabrication process and cell encapsulation are two of the main factors to be considered in development of 3D bioprinting. Other important factors include but are not limited to printing fidelity, stability, crosslinking time, biocompatibility, cell encapsulation and proliferation, shear-thinning properties, and mechanical properties such as mechanical strength and elasticity. In this review, we recite recent promising advances in bioink development as well as bioprinting methods. Also, an effort has been made to include studies with diverse types of crosslinking methods such as photo, chemical and ultraviolet (UV). We also propose the challenges and future outlook of 3D bioprinting application in medical sciences and discuss the high performance bioinks.

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1. Introduction

As the main process involved in cell growth and reconstruction of organs, tissue regeneration is currently under extensive study. Organ transplantation, replacement and repair are the options for patients with damaged organs depending on the situation and intensity of the damage. Extensively long waiting lists for organ transplantation exist all around the world. According to U.S. Department of Health & Human Services, as of June 2017, around 120000 patients are in need of lifesaving organ transplant in the United States while only about 5200 donors are available. Also, while the number of transplants performed every year since 2003 has been somehow constant, the number of patients waiting at the year-end has been growing (<https://optn.transplant.hrsa.gov>). Under these circumstances, scientists are eager to find alternative ways to compensate for this shortage of organ. Tissue engineering,

on the other hand, has been considered as an effective method to help save lives and improve the quality of life. Since proposed in 1993 [1], tissue engineering has been intended to develop practical replacements for damaged tissue by means of applying biology and engineering principles. Scaffolds have found their place in tissue engineering as templates for cell interaction, providing physical support to the afresh developed tissue [2]. Also, scaffolds can function as delivery vehicles to incorporate essential growth factors to control and enhance tissue growth [3]. A combination of cells and biomaterials is often employed as the printing precursor in 3D bioprinting of scaffolds. 3D Bioprinting is an actively studied method in tissue engineering since it shows effective control over scaffold fabrication and cell distribution. Printing resolution of 3D bioprinting techniques is 10–10000 μm which is a wide range showing flexibility of bioprinting compared to other assembly methods such as molding and porous scaffolds [4,5].

As an additive manufacturing technique, 3D bioprinting is based on deposition of biomaterials, either encapsulating cells or loaded with cells later on, in micrometer scale to form subtle structures comparable to tissue. In most cases, a three-axis mechanical platform controls the movements of extruders printing the bioink in

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the required algorithm and shape. This platform's movement is governed by coordinates created by the designer and saved in a file format such as g-code that could be easily followed by the printer. Due to vantages such as precise deposition, cost-effectiveness, simplicity, and cell distribution controllability, 3D bioprinting development and application has been increasing constantly over the past few years. As a result, need for new bioinks providing required properties for successful printing, such as printability, printing fidelity, and mechanical properties has been rising leading to extensive work to develop new materials. In the present review, an account of the most recent and functional research studies on bioinks and bioprinting developments is presented. To this end, first outstanding works in major bioprinting methods, including extrusion-based, inkjet, stereolithography-based, and laser-assisted bioprinting methods, are reviewed. Also, a brief review of the above mentioned bioprinting techniques is presented in Table 1 and a short summary of recent outstanding bioprinting studies is tabularized in Table 2. Next, the most fundamental recent studies in bioink development and applications are cited in "High performance bioink" section. Later on, challenges in bioink development and bioprinting, as well as applications and future perspective of bioprinting is discussed. Finally, a short summary of the present article is presented.

2. Extrusion-based bioprinting

Extrusion-based methods have been widely employed in recent years to provide researchers with alternative methods for scaffold fabrication. The extensive popularity of extrusion-based methods mostly relies on clear-cut processing method leading to simplicity, diversity and predictability of this technique. Bioinks having viscosity in the range of $30\text{--}6 \times 10^7$ mPa s are reported to be printable via extrusion printing [13]. In comparison with inkjet bioprinting, extrusion-based bioprinting offers higher cell densities but lower speed and resolution [13]. Wide range of printable biomaterials and inexpensive equipment are among extrusion bioprinting advantages. Many researchers have simply modified conventional commercial 3D printers to print biomaterials or developed their printing machines in-house to reduce the costs [2,24,29,31,33–35,38,41–43,49,55,56]. On the other hand, due to the need for development of bioprinters, commercial bioprinters have become widely available and employed by researchers [5,23,27,37,44–46,51–54], focusing on enhancing the printing quality and suitability for printing wider range of biomaterials. A review of the outstanding research works using extrusion-based techniques is presented in this section. Moreover, Fig. 1 illustrates

common extrusion-based printing methods categorized into pneumatic, piston-driven, and screw-driven dispensing. In pneumatic dispensing, air pressure provides the required driving force, while in piston and screw-driven dispensing, vertical and rotational mechanical forces initiate printing, respectively.

There are three main factors to take into account toward printability via extrusion bioprinting: 1) adjustability of the viscosity, 2) bioink phase prior to extrusion, and 3) material-specific biofabrication window [11]. Viscosity can be a function of temperature or shear thinning and therefore, needs to be adjusted for different printing methods. Also, bioink needs to be in liquid phase to avoid nozzle clogging. Finally, not all biomaterials are printable and those which are printable may not be printable in a wide range of processing parameters. To illustrate the current state of the art, the most recent extrusion bioprinting studies are cited in the following paragraphs.

To begin with, Rees et al. considered two types of oxidized nanocellulose 3D printed structures as wound dressings [23]. First type was prepared by (2,2,6,6-tetramethylpiperidin-1-yl) oxidanyl (TEMPO) mediated oxidation and the second type was prepared by carboxymethylation and periodate oxidation combined. The produced nanocellulose bioink was then used to print 3D porous structures, studied for bacterial growth support, and shown to have the potential to carry and release antimicrobial components while not supporting bacterial growth. Yu and Ozbolat utilized a coaxial nozzle system to print tissue strands as a bioink for organ printing [24]. Alginate-based bioink developed in this work showed mouse TC3 cell viability close to 90%. Also, human umbilical vein smooth muscle cells were incorporated in the bioink to fabricate structures similar to pancreatic tissue to further demonstrate the applicability of their method. In another study, a hydrogel based on gelatin, alginate, and collagen was used for cell-laden 3D printed tissue constructs [2]. One integral part of this work was to control the degradation rate of the hydrogel by changing the mole ratio of sodium citrate present in the medium to the sodium alginate present in the hydrogel. High cell proliferation rate indicated the possibility to improve the alginate bioink by utilizing the method used in this work.

Although bioprinting has been developing extensively in recent years, but the current technologies implemented in bioprinting are mostly incapable of printing functional solid organs. Researches have approached this issue by developing templates that could be used in vivo to support the development of vascularized solid organs such as bones [4]. Stem cells were encapsulated in a gamma-irradiated alginate-based bioink that was further reinforced by adding PCL fibers. RGD peptides were also incorporated to improve

Table 1
A brief review of common bioprinting techniques.

	Extrusion	Inkjet	Stereolithography	Laser-assisted
Advantages	Simple, capable of printing various biomaterials, ability to print high cell densities	Ability to print low viscosity biomaterials, fast fabrication speed, low cost, high resolution	Nozzle-free technique, printing time independent of complexity [6,7], high accuracy and cell viability	High resolution, deposition of biomaterials in solid or liquid phase
Drawbacks	Only applicable for viscous liquids	Inherent inability to provide a continuous flow [8], poor functionality for vertical structures, low cell densities	UV light source and near-UV blue light's toxicity to cells [9,10], lack of printing multi-cells, and damage to cells during photo curing [11]	High cost, thermal damage due to nanosecond/femtosecond laser irradiation [12]
Speed	Slow [13,14]	Fast [13,14]	Fast [14]	Medium [14]
Cost	Moderate [8,15]	Low [8,15]	Low [8,15]	High [8,15]
Vertical printing ability	Good [6]	Poor [6]	Good [6]	Medium [6]
Cell viability	$89.46 \pm 2.51\%$ [16]	80–95% [17,18]	>90% [19,20]	<85% [12]
Cell density	High [21]	Low [21]	Medium [21]	Medium [21]
Resolution	100 μm [8]	50 μm [8]	100 μm [19,20]	10 μm [22]
Viscosity	$30\text{--}6 \times 10^7$ mPa s [13]	<10 mPa s [13]	No limitation [7]	1–300 mPa s [13]

Table 2

A short summary of outstanding recent bioprinting studies.

Ref	Material	Method	Commercial printer	Application	Research summary
[23]	Nanocellulose	Extrusion	Y	Wound dressing	Development of 3D porous structures
[24]	Alginate	Extrusion	N	Bioprinting of tissue/organ	New micro-fabrication technique to create tissue strands as a “bioink”
[2]	Collagen/gelatin/alginate hydrogel	Extrusion	N	Tissue engineering (general)	Printing cell-laden hydrogel to study cell proliferation
[4]	Gamma-irradiated alginate, poly(ϵ -caprolactone) (PCL) fibers	Extrusion	Y	Whole bone organ engineering	Biofabrication and in vitro and in vivo analysis of mechanically reinforced cartilaginous template
[5]	Gellan, alginate, cartilage extracellular matrix particles	Extrusion	Y	Tissue-specific and bioactive scaffolds	Bioprinting and cell proliferation study of grafts
[25]	M13 phages and alginate	Extrusion	N	Regeneration of various tissues	Printing 3D cell-laden matrices using genetically engineered M13 phage
[26]	Collagen, alginate, human adipose stem cells (hASCs)	Extrusion	N	Tissue regeneration and cell therapy	Fabrication and study of cell-laden 3D printed core-sheath structure
[27]	Alginate, carboxymethyl-chitosan, and agarose	Extrusion	Y	Neural tissue	Direct-write printing of cell-laden bioink to engineer a novel functional 3D neural mini-tissue construct
[28]	Commercial polyethylene glycol (PEG)-based bioink	Droplet-based	Y	Soft tissue models	Report of an integrative bioprinting strategy for industrial routine application
[29]	Gelatin-based bioinks	Extrusion	N	A referable template for designing new bioinks	Study of printing parameters effect on cell survival rate and printability
[30]	Poly(ethylene glycol) diacrylate (PEGDA), gelatin methacrylate (GelMA), eosin Y based photoinitiator	Stereolithography	N	Microscale cell patterning	Development of a low-cost printing system for visible light
[31]	Alginate, PCL/alginate mesh	Extrusion	N	Regeneration of hard tissue	stereolithography solution
[32]	Methacrylated gelatin (GM) and mature adipocytes	—	—	Adipose tissue engineering	Fabrication and in vitro study of mechanically reinforced cell-laden scaffolds
[33]	Hyaluronic acid	Extrusion	Y	Tissue engineering (general)	Evaluation of photo-crosslinkable (GM) and mature adipocytes as for 3D fatty tissue constructs
[34]	Polyurethane (PU), c2c12 cells, NIH/3T3 cells, hyaluronic acid, gelatin, fibrinogen	Extrusion	N	Muscle–tendon unit	Development of a dual-crosslinking hyaluronic acid hydrogel as a bioink
[35]	PCL, collagen, and three different types of cells	Extrusion	N	Liver tissue engineering	Development of a complex tissue construct for use in muscle–tendon tissue
[36]	Gelatin, polyethylene oxide (PEO), HEK293 cells, human umbilical vein endothelial cells (HUVECs)	Extrusion	Y	Tissue engineering (general)	Development and evaluation of 3D printed constructs for liver tissue engineering
[37]	Acrylated, pluronic F127	Extrusion	Y	Tissue engineering (general)	Development of bioinks suitable for freeform fabrication
[38]	Alginate in phosphate-buffered saline (PBS), hASCs	Extrusion	N	Hepatogenic differentiation of hASCs -embedded mesh structures	Finding a way to use pluronic as a biocompatible ink for 3D printing
[39]	Collagen/extracellular matrix (ECM) and alginate, hASCs	Extrusion	—	Tissue engineering (general)	Introduction of a new cell dispensing method using a core-shell nozzle
[40]	Hyaluronic acid and gelatin	Extrusion	N	Primary liver constructs with high viability	Introduction of a strategy for obtaining highly bioactive alginate-based ink
[41]	Type I collagen and chitosan–agarose blends, human bone marrow derived mesenchymal stem cells (hMSCs)	Extrusion	N	3D printed mesenchymal tissues	Development of stable printable bioink
[42]	Decellularized adipose tissue (DAT) matrix bioink, hASCs	Extrusion	N	Soft tissue regeneration	Study of purpose-driven printing and the parameters affecting printing quality
[43]	Alginate, GelMA, HUVECs	Extrusion	N	Tissue engineering (general)	Devising a biomimetic approach for printing adipose tissue constructs
[44]	Spider silk protein, human fibroblasts	Extrusion	Y	Tissue engineering (general)	employing decellularized adipose tissue
[45]	Poly(N-isopropylacrylamide), poly(N-isopropylacrylamide) grafted hyaluronan (HA-pNIPAAm), methacrylated hyaluronan (HAMA)	Extrusion	Y	3D printing at physiological temperature of a range of biopolymer solutions	Development of a versatile 3D bioprinting technique and a novel low viscosity alginate-based bioink
[46]	Sodium alginate, sodium periodate, Arginylglycylaspartic acid (RGD) peptides	Extrusion	Y	Controlled degradation of oxidized alginates in bioprinting	Development of a novel bioink without the need for post processing and better shear thinning properties compared to alginate
[47]	Fibroblasts, sodium alginate, polystyrene microbeads and 3T3 cells	Droplet-based	Y	Tissue engineering (general)	Improving glycosaminoglycan-based hydrogels' printing by blending
[48]	Gelatin, methacrylic anhydride	Droplet-based	Y	Tissue engineering (general)	Evaluation of alginate hydrogels with varied oxidation percentages and concentrations as bioinks

Table 2 (continued)

Ref	Material	Method	Commercial printer	Application	Research summary
[49]	Gelatin, methacrylamide, gellan gum	Extrusion	N	Tissue engineering (general)	Development of a versatile bioink for inkjet bioprinting allowing for addressing ECM-based hydrogel matrices with a broad range of physical properties Enhancement of rheological and mechanical properties of gelatin-methacrylamide by addition of gellan gum
[50]	MG63 cells, alginate, PCL electrospun scaffold,	Laser-assisted	N	Tissue engineering (general)	Study of layer-by-layer fabrication effect on cell proliferation in vitro and in vivo
[51]	Polylactic acid, gelatin methacrylamide-gellan gum, Mesenchymal stem cells (MSCs)	Extrusion	Y	Living tissues constructs	Development and study of cell-laden gelatin-based bioink
[52]	Alginate, gelatin, hydroxyapatite, hMSCs	Extrusion	Y	Tissue engineering (general)	Modified alginate-gelatin based hydrogel for stable 3D bioprinted constructs
[53]	Nanofibrillated cellulose (NFC), alginate	Extrusion	Y	Bioprinting of living tissues and organs	Development and in vitro analysis of NFC-alginate based bioinks
[54]	Various natural and synthetic materials such as PEG and gelatin	Extrusion	Y	Tissue engineering (general)	Study and characterization of various printable gel-phase bioinks
[55]	Silk fibroin, gelatin, Human turbinate mesenchymal stromal cells (hTSMCs)	Extrusion	N	Tissue engineering (general)	Development and in vivo study of silk fibroin-gelatin bioink
[56]	decellularized adipose (adECM), cartilage (cdECM), and heart (hdECM) tissue, PCL	Extrusion	N	Tissue engineering (general)	Development and in vitro study of cell-laden novel dECM bioink

osteogenesis for bone tissue engineering applications. In this work, a cartilaginous construct similar to vertebral body was fabricated and shown to support vascularized bone development in vivo. In most cases, researchers use a combination of multiple biomaterials to achieve the required properties by the application. For instance, in one study, alginate and gellan were used along with BioCartilage (clinical product) to prepare a new-fashioned bioink for printing cartilage grafts proved to support chondrocytes' proliferation [5]. Furthermore, a cation-loaded polymer was also utilized to stabilize overhanging structures in this strong but ductile bioink.

While alginate is a common biomaterial employed as bioink, most studies are based on native alginates with limited degradation. In a study, oxidized alginate hydrogels with various degrees of oxidization were studied as bioinks with controlled degradation

[46]. Effect of viscosity and density of the alginate solutions on their printability was studied. Furthermore, alginate solutions with various biodegradability were loaded with hASCs and were shown to provide the ability to control proliferation and spreading of the cells.

Also, alginate-based bioinks often exhibit low cell-activating properties. Lee et al. tried to overcome this weakness by printing porous 3D constructs with a novel bioink consisted of collagen/ECM and alginate [39]. Cell studies showed that the developed bioink in this study displays decent cell viability and higher osteogenic activity compared to conventional bioinks based on alginate.

The biomaterials chosen for the bioink development play pivotal role in the research. For this reason, researchers prefer to use biomaterials previously proven to be compatible with cells in

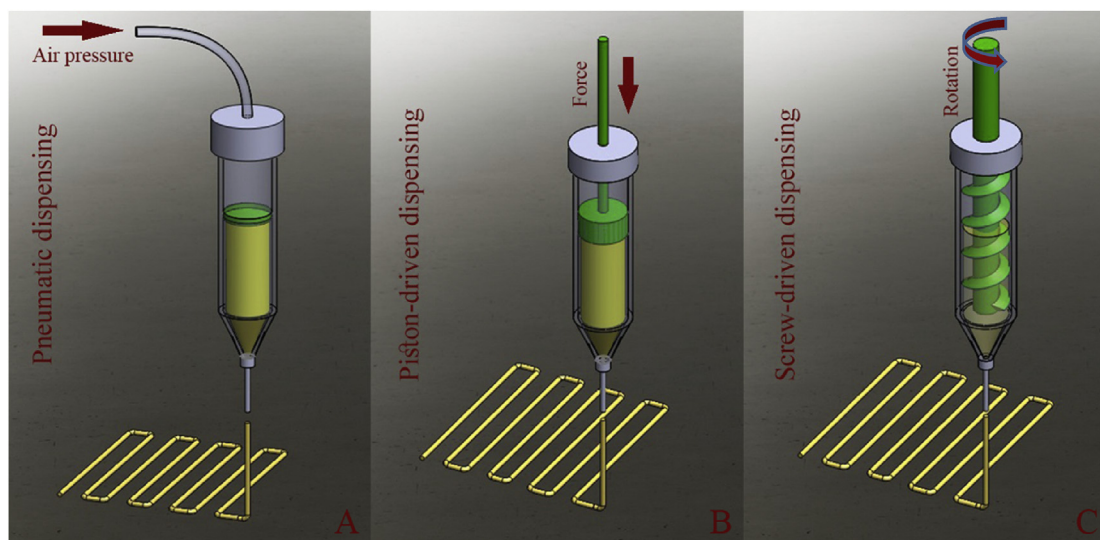


Fig. 1. Schematic diagram of common extrusion-based bioprinting methods: (A) pneumatic, (B) Piston-driven, and (C) screw-driven dispensing method. In pneumatic dispensing air pressure provides the driving force while in piston and screw-driven dispensing, mechanical displacement and rotation are utilized to drive a continuous flow of biomaterial through the nozzle.

commercial or non-commercial products or devices. Hence, the variables in the projects are decreased and the outcome is more likely to be predicted. As an example, RGD-phage solution was employed in one study to develop a versatile bioink with cell printing ability [25]. In particular, M13 phages were shown to provide good blending properties with alginate. Also, the proliferation of MC3T3-E1 cells were shown to improve proportionally with concentration of phages. In another study, Pati et al., devised a new method to print cell-encapsulating DAT bioink [42]. Porous dome-shaped structures were prepared and tested in vitro and in vivo for cell viability and differentiation of hASCs. The DAT 3D printed constructs were found to express more adipogenic lineages than that of non-printed DAT gel.

Integrated organ printing (IOP) is a technology focused on tissue-like structures which is especially useful in systems with local differences in cell types and mechanical properties. Merceron et al. employed an IOP system to fabricate a muscle-tendon unit construct composed of four different elements [34]. Thermoplastic PU and PCL were used to provide the structure with elasticity for muscle development and stiffness for tendon development, respectively. These constructs showed above 80% cell viability one week after printing.

3D printing is considered as a new concept in tissue engineering. Previously, cell studies took place using 2D structures, but with the introduction of 3D printing to the tissue engineering, it became possible for researchers to use 3D scaffolds instead of 2D ones. Lee et al., for instance, worked on the development of 3D structures with improved mechanical properties for liver tissue regeneration [35]. A multi-head tissue printing system was employed to print PCL as a framework material to provide proper mechanical properties. Also, three types of cells were printed in the PCL canals to study liver cells' proliferation. Results of this work suggested that the employed co-cultured microenvironment promoted heterotypic cellular interaction within a 3D construct.

In another study, gelatin was employed in free form fabrication of 2D and 3D cell encapsulating constructs [36]. PEO was also utilized with gelatin to enhance printing precision. Printed hydrogels showed support for cell proliferation and spreading. Skardal et al., in another study, considered a blend of hyaluronic acid and gelatin to prepare liver-specific bioink with the ability to be further exploited for other tissue types [40]. PEG crosslinkers with various molecular weights were utilized to facilitate bioprinting. A 2-crosslinker, 2 stage polymerization method was employed to improve bioink properties. This research outcome showed high cell viability for the proposed bioink. Kesti et al. also, employed a tandem gelation mechanism (thermally and photo-triggered) to crosslink a blend of poly(N-isopropylacrylamide) grafted hyaluronan (HA-pNIPAAm) with methacrylated hyaluronan (HAMA) [45]. The proposed bioink displayed good printing fidelity as well as fast gelation and proper mechanical stability. Although no direct toxicity was observed in cells cultured on the surface of the 3D constructs, encapsulating cells in the bioink led to high cell fatality. However, cell death decreased significantly by removing HA-pNIPAAm in a brief washing step.

Any material has its own properties which may or may not be suitable for 3D printing of scaffolds. Pluronic, for example, is a thermo-sensitive polymer that has been in use for applications such as drug delivery [57,58] and wound dressing [59]. Block copolymer Pluronic is known to have good printing properties but weak cell-culture properties. However, Muller et al. proposed a method to improve the biocompatibility of Pluronic [37]. This goal was achieved through blending acrylated with unmodified Pluronic F127 followed by UV crosslinking.

Recently a new extrusion-based method has emerged. This method utilizes a core-shell nozzle and a crosslinking agent is being

printed at the same time the bioink is being dispensed from the core of the nozzle. In one study, core-shell printing was employed for rapid printing and gelation of cell-laden alginate 3D constructs [38]. The printed mesh structures, showed cell viabilities of 93% and 92% for preosteoblasts and hASCs. Yeo et al. also, employed this method to print cell-laden bioink based on alginate and collagen [26]. Cell encapsulating collagen bioink was loaded in the core barrel and alginate was loaded in the shell to improve cell viability during printing and crosslinking as well as enhancing overall printing fidelity. An aerosol crosslinking method was used to achieve multi-layered mesh constructs. 3D constructs prepared using core-shell method in this study, showed noticeable higher cell viability compared to regular alginate-based bioinks.

Silk protein is another material exploited in bioinks for 3D printing of tissue-like constructs. Its potential as a bioink has been evaluated alone and in blends with other biomaterials. Studies suggest that silk fibers are biocompatible, possess unique mechanical properties, and allow for decoration with growth and adhesion factors due to their diverse side chain chemistries [60]. In one study, Schacht et al. fabricated cell encapsulating three-dimensional spider silk structures [44]. Robotic dispensing was employed for printing the constructs without any crosslinking additives. Different cell lines were cultivated on the hydrogels and cell adhesion and proliferation were studied. Furthermore, unlike common biomaterial as a bioink, no post print crosslinking is not needed. Good cell viability and proliferation for at least one week was reported. Also, a blend of silk fibroin and gelatin was utilized to print 3D tissue constructs in another study [55]. Mushroom tyrosinase and physical crosslinking via sonication were employed as crosslinking mechanisms. In vitro studies were taken place on the bioink encapsulating human nasal inferior turbinate tissue-derived mesenchymal progenitor cells. The 3D printed structures were shown to support multilineage differentiation of encapsulated stem cells. Furthermore, a blend of silk, gelatin, and glycerol was employed to enhance the printing resolution to meet patient-specific needs for soft tissue regeneration [61]. In vitro and in vivo studies showed that the developed material is stable and biocompatible while supporting tissue integration.

3. Properties and parameters

Engineering bioinks directly affects mechanical and biological properties. Even by slightly changing the concentration of the components, the gelation, printability, and properties of the resulting 3D constructs can be affected significantly. Thus, it is of great importance to design the bioink based on the requirements of the application. As an example, Gu et al. developed a novel bioink for neural tissue construction based on alginate, carboxymethyl-chitosan, and agarose [27]. Fast gelation, stable crosslinking, and porous surface of the cell-encapsulating bioink was shown to be promising in human neural development and may also be applicable to other types of cells. Also, in another study, 3D printing technique was utilized to mimic liver tissue [62]. HepG2 cells were encapsulated in alginate bioink and multilayer three-dimensional constructs were fabricated. Stable cell proliferation and enhanced gene expression profiles were observed.

Printing parameters, such as printing speed and temperature, could also affect cell survival and printability. For this reason, some researchers focus on evaluation of these potential dependencies. To do so, Zhao et al. studied the effect of composition, concentration, temperature and holding time on printability and also, cell survival after extrusion printing [29]. In this study, cell survival rate was found to decrease when viscoelasticity of the gelatin-based bioinks were increased. Also, in a recent study, different compositions of alginate with low and high molecular weight were loaded with NIH

3T3 fibroblast cells and effects of alginate molecular weight on printability and cell viability were studied [63]. It was concluded that 3 wt% alginate composed of a blend of high and low molecular weight alginate with the ratio of 2:1 offers the optimum results with respect to printability and cell response.

To achieve required mechanical properties for any specific application, it's necessary to reinforce the scaffolds in some cases. Different methods have been used to accomplish this goal. In one study, for example, PCL/alginate struts were coated with alginate-based bioink to reinforce the structure [31]. The ratio of alginate crosslinking agent was also varied to find the optimum conditions for cell-coating. Using this method, multi-layered reinforced cell-laden scaffolds were constructed. Moreover, dual-crosslinking is another method employed to improve the quality and stability of printed constructs. Ouyang et al. utilized this method to prepare a printable hydrogel ink based on hyaluronic acid [33]. Guest-host assembly and covalent crosslinking were used to include self-healing and shear-thinning properties in the bioink. They were able to prepare structures with more than 16 layers which were stable over a month without loss of mechanical properties. The developed bioink was later functionalized to improve cell adhesion.

In an interesting study, effects of stiffness and 3D structure of the printed constructs on cell differentiation were studied [41]. Collagen type I, agarose, and chitosan blends were employed to study human mesenchymal stromal cells differentiation. Among the studied blends, osteogenesis was shown to be more likely in anisotropic soft collagen-rich substrates while adipogenesis was more likely in isotropic stiff agarose-rich matrices. Different ratios of collagen type I and agarose blend were concluded to suit wide range of mesenchyme-based applications.

Effective blend of alginate and GelMA has also been employed as a bioink in literature [43]. GelMA is used because of its ability to form stable hydrogels via UV crosslinking above alginate physical crosslinking. A coaxial extrusion printing system was used to print 3D constructs using low viscosity bioink with high cell viability in vitro. GelMA has also been shown to promote cell adhesion and migration [64]. In another work, a highly concentrated bioink consisting of alginate and polyvinyl alcohol was developed and studied for co-printing with bovine serum albumin (BSA) and bone morphogenetic 2 (BMP-2) [65]. It was shown that the release profiles of BSA and BMP-2 were strongly dependent on the micropores in the scaffolds which was related to the polyvinyl alcohol (PVA) sols.

4. Specific applications

Another application of bioprinting is in bone and cartilage tissue engineering. In one study, a bioink mainly consisted of alginate sulfate and nanocellulose encapsulating bovine chondrocytes was developed and printed via extrusion printing [66]. However, it was shown that printing cell laden bioink resulted in lower cell proliferation compared to non-printed samples. Heo et al., also, developed a new bioink consisting of alginate and bone formation peptide-1 to enhance bone regeneration [67]. In vitro and in vivo studies showed that the developed bioink provided a stable environment for the cells to proliferate. In another study, bioprinted calcium sulfate hydrate (CSH)/mesoporous bioactive glass (MBG) scaffolds were loaded with human bone marrow-derived mesenchymal stem cells (hBMSCs) and studied in vitro and in vivo [68]. Results revealed that CSH/MBG scaffolds promoted cell adhesion and proliferation, enhancing new bone formation. In a similar study, printed mesoporous silica/calcium phosphate cement porous scaffolds were fabricated and loaded with recombinant human bone morphogenetic protein-2 (rhBMP-2) and studied in vitro and in vivo [69]. It was concluded that this blend is able to

eliminate tissue necrosis issues during regeneration process. Yang et al., in one study, employed extrusion printing to print novel PCL scaffold with spiral struts encapsulating MG63 cells [70]. In vitro studies indicated that novel spiral-like struts did improve cell attachment, proliferation and differentiation with respect to normal struts. 3D bioprinting has also been used to print GelMA scaffolds on titanium implant surface, triggering mineral deposition of MG63 osteoblasts and human osteoblasts [71]. It was shown that while directly grafting on titanium alloy within a groove system, the hydrogel can survive from shear forces in a marrow implantation model.

Another application for 3D bioprinting is in fabrication of human bilayered skin. Cubo et al., for instance, utilized an extrusion-based technique to print bioinks containing human plasma as well as primary human fibroblasts and keratinocytes [72]. In vitro and in vivo studies revealed that the printed skin was very similar to human skin and was indistinguishable from handmade dermo-epidermal equivalents.

5. Inkjet bioprinting

Inkjet printing application in 3D bioprinting has been limited compared to extrusion-based studies. The main reason for that is the inherent inability of printing head to provide a continuous flow which limits its application in bioprinting [8]. Bioinks with viscosities lower than 10 mPa s have been reported printable via inkjet printing. In comparison with other methods, inkjet printing offers fast fabrication speed but low cell densities [13]. Inkjet printing methods could be classified into three groups: continuous-inkjet bioprinting, electro-hydrodynamic jet bioprinting, and drop-on-demand inkjet bioprinting. The latter category happens to be the largest and the most common one consisting of thermal, piezoelectric, and electrostatic inkjet bioprinting [73]. Thermal and piezoelectric inkjet printing are shown schematically in Fig. 2. A few outstanding studies in this area are reviewed in this section.

Although currently extensive research is being done on bioink development, not all of these works are likely to be commercialized. For a new bioink to become commercially available, it has to be cost effective and show the potential to be standardized according to industrial environments and requirements. To this end, Rimann et al. developed an all-in-one printing method for soft tissue construction [28]. In this work, a PEG-based bioink was developed and used along with a commercial 3D discovery inkjet bioprinter. Printing took place in sterile environment. To verify the applicability of their work, long-term culture of the printed structures was carried out. The results approved the human primary dermal fibroblasts viability and proliferation up to seven weeks.

In another study, droplet formation process during inkjet printing of cell-laden bioink consisting of fibroblasts and alginate with different cell concentrations was studied [47]. Breakup time, droplet size, droplet velocity, and satellite formation were among the parameters studied in this work. It was reported that increasing cell concentration, decreases velocity and droplet size while increasing breakup time. Also, the process was compared to polystyrene microbead-laden suspension inkjet printing to illustrate the effect of particle physical properties on the droplet formation. Furthermore, double chemical functionalization of gelatin was undertaken in another novel work to control its physical and chemical properties for bioprinting [48]. This was achieved by methacrylation and acetylation of free amino groups to gain control over viscosity and mechanical properties of the bioink. The resulting soft hydrogels were printed by drop-on-demand inkjet printing and shown to be cytocompatible and suitable to print viable mammalian cells.

It is worth mentioning that thermal inkjet printing is not

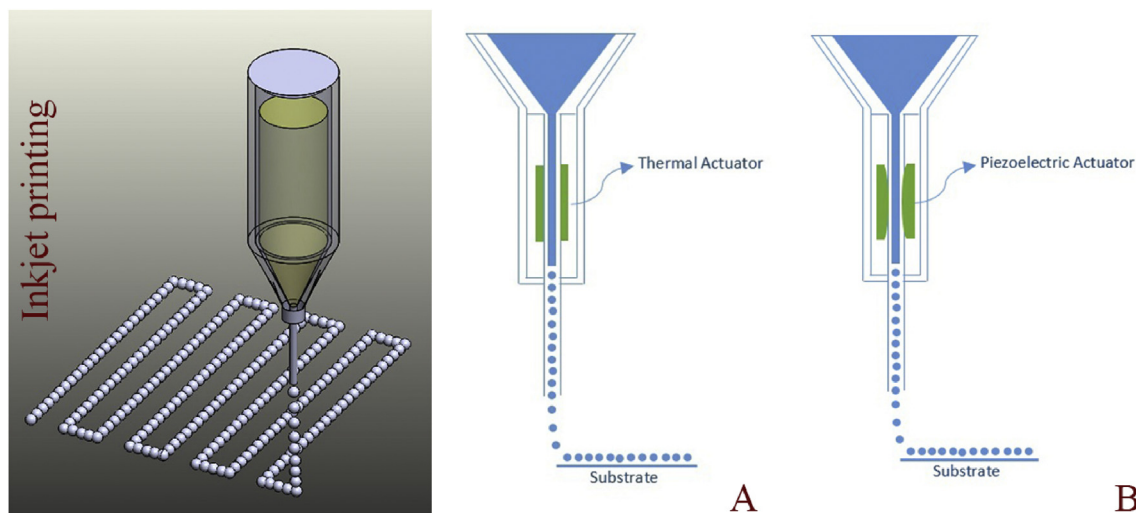


Fig. 2. Schematic diagram of drop-on-demand inkjet printing method using A) Thermal, and B) Piezoelectric actuators. A thermal printing head employs a heating element that raises the temperature locally and creates a bubble that drives droplets through the nozzle. A piezoelectric head is utilized with a material that changes shape upon voltage application and pushes droplets out.

common in tissue engineering due to activity loss resulting from very high temperatures which may reach above 200 °C. For instance, Setti et al. reported 15% activity loss while printing β -galactosidase (GAL) [74]. However, some studies do employ piezoelectric inkjet bioprinting in their research.

As an example, a piezoelectric inkjet printing system was utilized in one study to print breast cancer cell suspensions [75]. Preparing neutrally buoyant suspensions using Ficoll PM400, it was shown that nozzle clogging was eliminated and dispensing accuracy was enhanced. Through this work, improved dispensing by rheological manipulation was studied. Furthermore, Xu et al. proposed a novel 3D bioprinting system capable of scaffold-free printing of 3D cellular tubes [76]. Cell viability of constructed cell-based tubes was reported as high as 82% even after 3 days of incubation. Gudapati et al. have well expanded the droplet-based bioprinting methods including inkjet printing, common biomaterials, and cells employed [73].

6. Stereolithography-based bioprinting

Stereolithography printing is based on polymerization of light-sensitive polymers by precisely controlled light glinted from

digital micromirrors. In comparison with other methods, Stereolithography is a technique with high printing quality, speed, and cell viability. However, drawbacks have been reported resulting from using this method. For instance, UV light source which is the common polymerization method, has been reported to be harmful for DNA cells and even cause skin cancer [9,10]. To address this issue, visible light stereolithography bioprinting systems have gained attention. As an example, Wang et al. employed a bioprinting system consisted of a beam projector and blends of PEGDA, GelMA, and eriosin Y based photoinitiator as bioinks [30]. This work's results of NIH 3T3 cell bioprinting indicated that the proposed low cost system is capable of printing and visible-light curing of hydrogels with 50 μ m resolution and relatively high cell viability. Fig. 3 schematically shows stereolithography using a beam projector.

Versatility, controllability, and precision of stereolithography has been studied by Melchels and colleagues [77]. Porous scaffolds were designed and fabricated with either a poly(D,L-lactide)-based resin or a poly(D,L-lactide-co- ϵ -caprolactone)-based resin. It was shown that by varying the composition of the macromeres and the pore architecture, mechanical properties of the scaffolds can be controlled. Elomaa et al. also prepared scaffolds by employing a

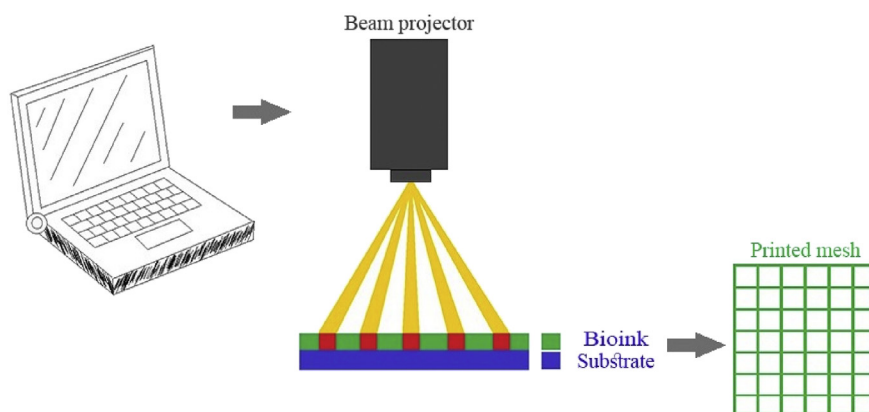


Fig. 3. Schematic diagram of stereolithography using beam projector. Focused light beams allow for precise photopolymerization of layers of light-sensitive polymer to apply any desired pattern to the bioink.

photocrosslinkable PCL-based resin with high gel content networks [78]. Porous scaffolds were prepared by stereolithography using the resin prepared by Irgacure 369 photoinitiator, inhibitor and dye. The fabricated scaffolds matched the design and proved the suitability of the prepared resin for fabrication of tissue engineering scaffolds.

In a novel study, a projection stereolithography (PSL) platform was proposed to design 3D tissue scaffolds based on computer aided design [19]. Various structures and concentrations of GelMA were employed to control the mechanical properties of the scaffolds. Complex porous constructs were seeded with HUVECs and were studied in vitro. Precisely fabricated scaffolds with interconnected pores were shown to support cell growth resulting in high cell densities.

Melchels et al., in another work, employed stereolithography to fabricate porous constructs from a resin based on a 2-armed poly(D,L-lactide), ethyl lactate, photoinitiator, inhibitor and dye [79]. Good pre-osteoblast adherence and comparable proliferation to high-molecular weight poly(D,L-lactide) and tissue culture polystyrene were reported. Shie et al. employed a commercial 3D printer using blue light digital stereolithography to prepare polyurethane with hyaluronic acid for cartilage repair [80]. The printable photosensitive material developed in this work was shown to be non-toxic, supporting high resolution printing, cytocompatible, and promote cell adhesion, proliferation, and differentiation. In another study, fumaric acid monoethyl ester-functionalized poly(D,L-lactide)/N-vinyl-2-pyrrolidone resins were prepared and used with stereolithography to fabricate scaffolds [81]. Mouse pre-osteoblasts were shown to adhere and spread well onto the material. Also, a resin composed of Poly(propylene fumarate) (PPF), diethyl fumarate (DEF), and bisacrylphosphine oxide (BAPO) has been utilized to fabricate scaffolds by stereolithography [82]. Fabrication of constructs with controlled microstructure by optimizing resin composition and laser parameters was studied in this work. To optimize the microstructure and achieve high porosity, sugar particles have also been used in a projection-based stereolithography [83]. This method was shown to increase the porosity of the scaffolds by two times in comparison with the current stereolithography method.

Application of PEG hydrogels with stereolithography have also been reported in literature [84]. Due to presence of the photo-reactive groups, UV light can crosslink PEG into a hydrogel in the presence of a photoinitiator. Complex multilayer 3D PEG hydrogel constructs were prepared by using stereolithography and two different molecular weight of PEG. Effects of factors such as photoinitiator, photopolymer concentration, and energy dose on the gel properties as well as effects of stereolithography parameters on in vitro cell studies were investigated. Use of Poly(trimethylene carbonate)-Based Resins in stereolithography has been also reported [85]. Results of this work approved attachment, diffusion, and differentiation of bovine chondrocytes, providing evidence for applicability of the proposed resin for cartilage tissue engineering.

Scaffolds made by stereolithography have been also used for heart valve tissue engineering [86]. A blend of a thermoplastic elastomer, a poly-4-hydroxybutyrate (P4HB) and a poly-hydroxyoctanoate (PHOH) was used to form the resin. Direct pressure measurements of the sample heart valves revealed synchronous opening and closing of the valves in a pulsatile flow bioreactor. Another application of stereolithography is to prepare sacrificial moulds for scaffold preparation. Chopra et al., for instance, were able to control the architecture of gel-cast glass-ceramic tissue scaffolds [87]. Similarly, Bian et al. employed ceramic stereolithography and gel casting to fabricate beta-tricalcium phosphate/collagen scaffolds for osteochondral tissue engineering [88]. Using this method, high resolution scaffolds ideal for bone

tissue engineering were developed. Furthermore, development and application of epoxy/hydroxyapatite in stereolithography made scaffolds has been reported [89]. Prepared scaffolds were kinetically characterized and importance of factors such as weight percentage of ceramic powder and viscosity of the suspensions for fabrication was studied. Green ceramic bars fabricated in this work were reported to offer good mechanical properties. Zheng et al. also used stereolithography to fabricate very precise and complex moulds using 3D models designed based on Computed tomography (CT) images of rat mandible [90]. A silicon tissue transformation mould was prepared using the prepared precise moulds by stereolithography and bone formation was observed by X-ray. The applicability of this method for in vivo tissue transformation for vascularized bone reconstruction was reported. For further reference, a comprehensive review of the materials processed using stereolithography has been presented by Skoog et al. in 2014 [91].

7. Laser-assisted bioprinting

Laser-induced forward transfer (LIFT) is technique presented more than 30 years ago by Bohandy et al. [92]. This technique allows high resolution deposition of material in solid or liquid phase. While several versions of this technique exists, a solid phase material printing version is illustrated schematically in Fig. 4. In one study, Matrix-assisted pulsed-laser evaporation direct-write, one variation of LIFT technique, was employed for cell printing [93]. Sodium alginate loaded with NIH 3T3 mouse fibroblast cells was used as the bioink along with calcium chloride as the crosslinking agent. Effects of alginate gelation and concentration, gelation time, and laser fluence on cell viability were studied. It was observed that longer gelation time decreases the cell viability after 24 h of incubation due to the reduced nutrition and oxygen transfer through the thick gel wall.

Although cell transfer using LIFT technique has been successful but, cell survival rate is often below 85% [12]. Thermal damage due to nanosecond laser irradiation was recognized as the main cause of cell death. To decrease the damage to the cells, femtosecond lasers were employed. Particularly, absorbing film-assisted LIFT (AFA-LIFT) method, which is an improved LIFT method, was studied by

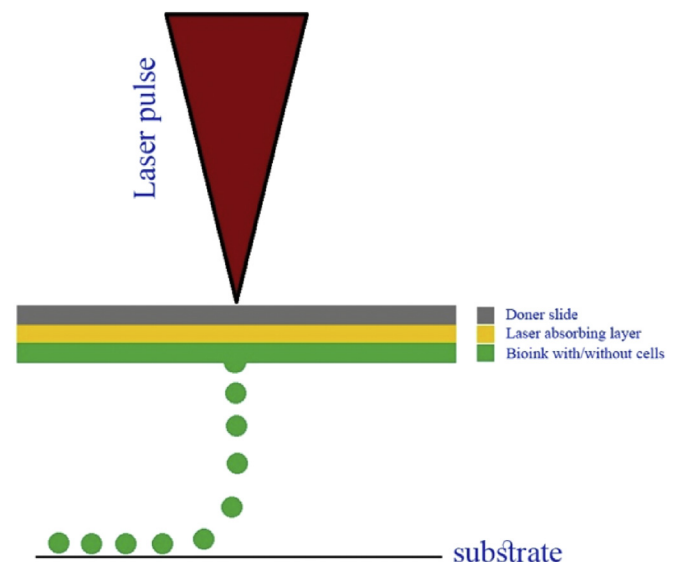


Fig. 4. Schematic diagram of laser-assisted bioprinting. A nozzle-free technique using pulsed laser source to deposit microdroplets of bioink with/without cells on a substrate.

Hopp et al. as a method of controlled living cell transfer onto various acceptor surfaces [12]. However, experimental results of this work revealed that femtosecond AFA-LIFT caused higher fatality rates in cells compared to nanosecond AFA-LIFT which was mainly attributed to the strong photomechanical influences of laser pulse. Laser-assisted bioprinting by LIFT technique was further investigated to print cell-laden three-dimensional structures [94]. Collagen encapsulating fibroblasts and keratinocytes was employed to print 3D skin tissue like structures. These lines of cells were previously proven to be resistant to damage during laser-assisted printing process [95]. Proliferation of cells over a period of 10 days was studied and the ability of 3D printed cells to form real tissue was demonstrated.

In general, cells could be either printed onto/in the depth of ECM layer or printed as encapsulated particles in an ECM-like printable biomaterial. It is important to know the effects of different printing parameters on cell viability. In one study, effects of laser pulse energy, ECM thickness, and viscosity of the bioink on the cell viability was studied [96]. Cell viability 24 h post-printing was measured to compare different printing settings. It was concluded that while higher laser energy leads to more cell fatality, increasing film thickness as well as bioink viscosity results in increased cell viability. Furthermore, effects of bioink viscosity, laser energy, and printing speed on printing resolution was studied by Guillotin et al. [97]. It was shown that microscale resolution and 5 kHz printing speed are within reach. This work is another proof for applicability of printing blends of cells and ECM via laser-assisted bioprinting to fabricate soft free form tissue able to host a high cell density *in vivo*.

8. High performance bioink

Among all the research works on bioinks, there are some studies that stand out by the benefits they offer. Specific applications, new methods, and spectacular properties are some of the reasons making these type of studies inspiring.

Application specific studies engineer the bioink based on the requirements of the application. In certain biomedical devices, for instance, conductivity can be of great importance while in scaffolds, cell support is essential. In a novel study, a special bioink was developed for cardiac tissue regeneration [98]. This bioink was developed to provide proper conductivity and avoid delayed electrical coupling in cardiac cells. This new gold nanorod-integrated gelatin methacryloyl-based bioink was shown to be accurately printable, cytocompatible, and enhance cardiac cells functionality. Nerve [99], kidney [100], and cartilage [5] regeneration and repair as well as bionic ear [101] are other specific applications studied for bioink development. Flexible electronics for bioelectronic interfaces are also under extensive research currently [102,103]. In one study, a new method for fabrication of inkjet-printed flexible gold electrodes was demonstrated [103]. Fabricated gold electrode arrays were shown to be mechanically and electrically promising for bioimpedance and biopotential measurements. Also, to increase the survival time of the bioprinted tissue, development of bioinks for vascularized bioprinted tissue has been studied [104,105]. Sensing applications such as tactile sensors are also focus of many studies. Guo et al., for example, employed a multifunctional bioprinting method for fabrication of stretchable tactile sensors [106]. Fabricated sensors in this work, were shown to be able to measure finger motions and pulse. Furthermore, inks have been developed to fabricate strain sensors within structures guiding the self-assembly of cardiac tissue [107]. This versatile fabrication approach was claimed to be applicable to a wide range of instrumented micro-physiological devices, further expanding *in vitro* tissue engineering. A typical image of a 3D printed hydrogel in the form of a mesh structure as well as a 3D printed conductive sensor

is displayed in Fig. 5.

Many research works could be found in the literature that focus on specific properties of the 3D bioprinted constructs, properties such as high strength structures. Zhu et al., for instance, employed polyion to prepare ultratough hydrogels by extrusion printing [109]. Also, Qin et al. combined 3D bioprinting and computational modelling to evaluate mechanical behavior of elastomeric webs mimicking spider webs [110]. This work's results suggest that loading pattern governs the material distribution in the spider web. Based on that and computational modelling, authors showed that mechanical functions of 3D printed Polydimethylsiloxane (PDMS) webs are controllable by material distribution.

Development of new methods is also vital for the expansion of bioprinting. Enhancing the printing resolution and versatility of the current methods as well as development of new ones is an ongoing research. As an example, self-healing hydrogels were shown to provide support for direct printing of high resolution 3D constructs by utilizing shear-thinning hydrogels, providing the ability to print in any direction [111]. In another study, a new approach to print nonviscous photo-crosslinkable bioinks was introduced [112]. In this method, light is introduced to the hydrogel via a photo-permeable capillary immediately before deposition, allowing for high resolution and uniform filaments with high cell viability. Also, Colosi et al. developed a low viscosity bioink based on mixing alginate and gelatin methacryl during the extrusion process and crosslinking of alginate just prior to the deposition [113]. Using this versatile approach, printing highly functional tissue-like structures with high resolution was demonstrated. Furthermore, significant enhancements in microdrop bioprinting have been reported by Pataky et al. [114]. Compatibility of this method with alginate and collagen printing as well as the ability to print resolutions comparable to industrial prototyping was demonstrated. In another study, Rutz et al., proposed a bioprinting method capable of extruding various natural and synthetic gel-phase bioinks [54]. Authors proved the versatility of this approach by designing and printing 35 formulations of bioinks. Overcoming vascularization in tissue-like structures by implementing fluidic channels is another method receiving a great deal of attention recently. Gao et al., for instance, employed extrusion bioprinting to fabricate multilayer macro-channel embedded alginate-based structures loaded with two type of cells [115]. These printed multilayer constructs with multilevel fluidic channels were reported to be biocompatible and have acceptable mechanical strength. In yet another interesting and recent study, programmable structures capable of changing to complex 3D morphologies were studied [116]. Inspired by botanical systems, Gladman et al. printed composite hydrogel constructs using four-dimensional printing pathways which are capable of changing shape upon localized swelling due to water absorption. This approach can potentially lead to new shape transforming structures and find applications in tissue engineering and biomedical devices.

9. Challenges, applications and future perspective

Despite all the progress over the years in tissue engineering, many challenges still remain unsolved. Challenges fall into two main categories: 1) biomanufacturing which involves 3D fabrication of the cells and biomaterials and 2) *in vivo* integration which involves post-implantation functionality and integration. One challenge in fabrication process is nozzle clogging in nozzle-based fabrication methods. Depending on the application, fabrication time can take several hours. To avoid nozzle clogging in these cases, printing precursor needs to be homogenous and have proper viscosity and shear thinning properties. Another challenge is that the 3D constructs need to be sufficiently stable and mechanically rigid

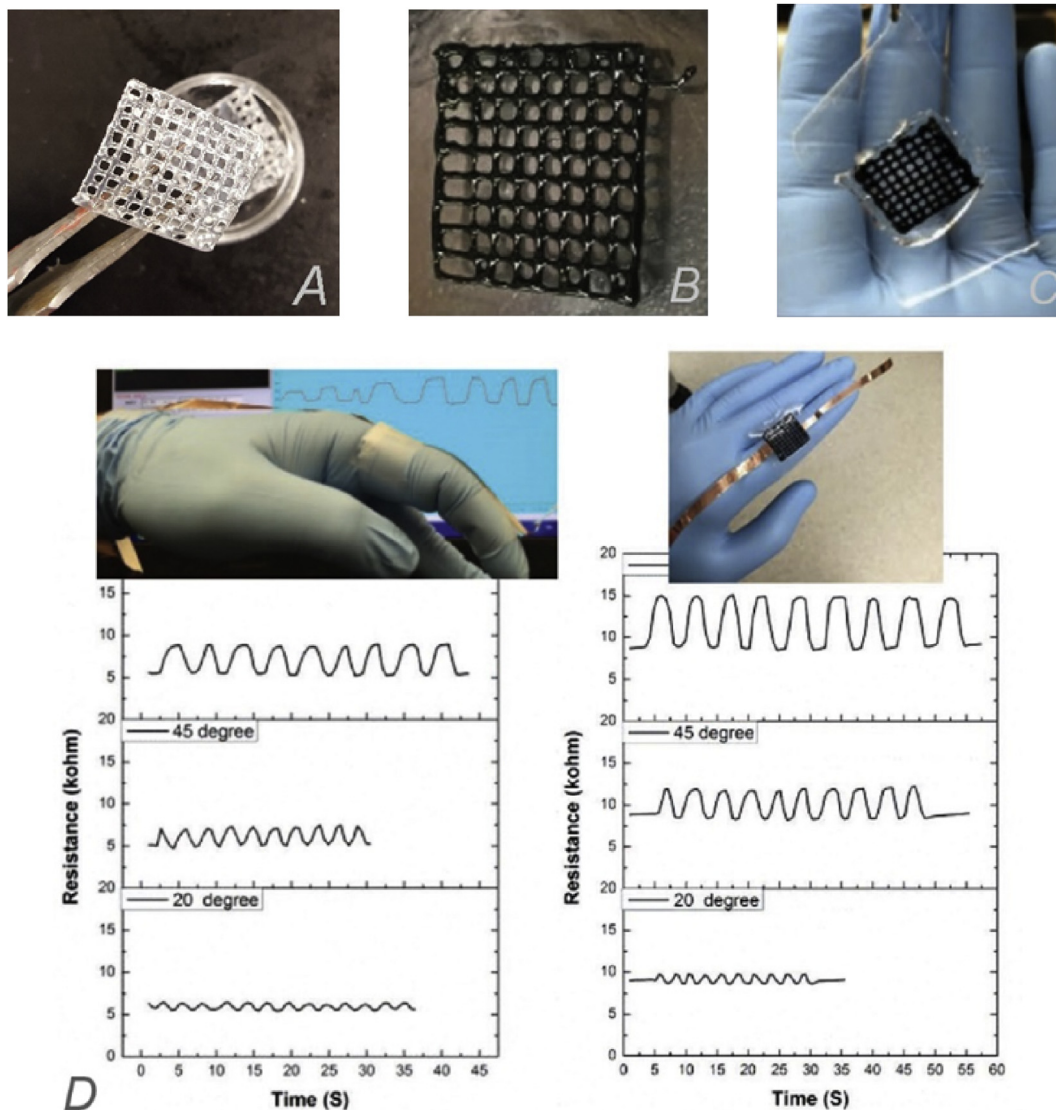


Fig. 5. 3D printed constructs of conductive and nonconductive bioinks. A) A typical chitosan-based extrusion bioprinted mesh structure, B-D) a conductive 3D printed sensor based on chitosan and acrylic acid, sealed in PDMS. The resistance response at various bending angles from testing the hydrogel as a sensor in strip form (left) and in 3D printed mesh form (right) is displayed. © 2017 Reprinted with permission of John Wiley and Sons [108].

to ensure successful transplantation. For example, in the case of hard tissue repair, elastic modulus of the scaffolds needs to be high enough to maintain its designed structure and porosity while implanted to support natural cell growth [117]. If the scaffold is not capable of maintaining its structure and provide mechanical support, any newly formed tissue will probably fail as a result of scaffold deformation [118].

Bioprinted constructs for tissue engineering, being ultimately implanted in body, need also to support vascularization *in vivo* to provide the cells with sufficient nutrition, growth factors, oxygen and remove waste. *In vivo*, capillaries are found within a distance of 100 μm from most cells so that there is sufficient diffusion for the cells to survive [119]. For distances more than that, such as thick tissues in printed organs, additional means for diffusion may be needed. To overcome this challenge, Hutmacher et al. suggested an artificial vascular system to enhance transportation of nutrients and removal of waste products [120].

3D bioprinting is currently expanding swiftly toward a large industry due to its diversity and potential applications. 3D printing

market size is predicted to reach \$10.8 billion in 2021 from \$2.2 billion in 2012 [121]. Currently, several companies are working on 3D bioprinting products for tissue engineering applications such as cartilage, liver tissue, breast, and bone [122]. Tissue Regeneration Systems is among the companies that have already produced commercially available bioprinted products. This company develops bioprinted PCL-based solutions customized for individual patients to repair skeletal defects [123]. This solution was approved by the food and drug administration in 2013 as the first implant for skeletal reconstruction and bone regeneration prepared by 3D bioprinting. Furthermore in 2014, Organovo introduced bioprinted human liver tissue, named exVive3D™ Liver, designed to evaluate drug toxicity [124]. While this product offers *in vitro* drug screening, a commercially available liver tissue has not been successfully developed yet. Generally, bioprinting applications can be categorized into two major groups: 1) tissue regeneration and regenerative medicine and 2) biomedical applications. The first group is about applications of bioprinted constructs such as vascular grafts, skin, neuron, bone, and liver while drug discovery

and biopreservation fall under the second category [122].

Needless to say, bioprinting has been constantly evolving over the past decade and this trend seems to be continuing. As more research is done on bioprinting techniques, printing resolution and quality will eventually improve, providing capability to print more complex 3D constructs. Natural organs are often very complex structures consisting of different types of tissue, ligaments, etc., each having their specific functions. By further advancement of bioprinting, biofabrication of complex constructs accurately mimicking natural organs becomes practical. Structural complexity of the bioprinted products can also be improved by precise fabrication of multi-material 3D constructs. Simultaneous deposition of materials with different physical and chemical properties is also a useful approach to fabricate organs with various properties in different regions. Multimaterial bioprinting provides the ability to adjust factors such as concentration of growth factors, cell adhesion, and degradation rate in different regions of the printed object. Capability to load different type of cells in different zones and on compatible biomaterials is another benefit of this approach allowing for closely mimicking of natural cellular diversity and activity.

Future development of bioprinting can also potentially overcome vascularization challenge which is among the most important factors limiting bioprinting applications in tissue engineering [125]. Biofabrication of microstructures within scaffolds by employing technologies such as microfluidic systems [126] and layer-by-layer assembly [127]. Furthermore, it is predictable that advances in biofabrication, will also benefit related fields such as imaging and diagnostic applications.

10. Summary and conclusions

In this text, recent research on development of bioinks, 3D bioprinting methods, as well as current state of art is discussed. Extensive research on 3D bioprinting over the past decade is a sign of its wide applications and promises in tissue engineering. However, to overcome challenges such as vascularization, bio-manufacturing issues, and unfit properties, more research on bioink development and 3D bioprinting techniques is required. Further expansion of multimaterial hydrogels, development of more accurate bioprinting methods, and combining different printing techniques are some of the most important areas that can help advance the applications of bioprinting in tissue engineering. A few bioprinting products have been already introduced and are commercially available in the market. Given the swift development of this industry over the past years, it is predictable that more bioprinting products will eventually become available in the market to help patients suffering from a wide range of diseases and 3D bioprinting will continue to be a strong fabrication tool for tissue engineering and development of biomedical systems.

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