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REVIEW ARTICLE

3D Bioprinting: Recent Trends and Challenges

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Abstract | 3D bioprinting is an additive biomanufacturing technology having potential to fast-forward the translational research, as it has the capability to fabricate artificial tissues and organs that closely mimic biological tissues or organs. As an emerging area of research in the field of tissue engineering, 3D bioprinting has scope in the development of implantable tissues and organs, construction of tissue/organ models and high-throughput diseased/cancer models for pharmaceutical and toxicological studies. Further, this area has diversified with the continuous upgradation of 3D bioprinters and biomaterials, which play major roles in the architectural quality and functionality of bioprinted construct. Addressing these technological complexities requires an integrated approach involving expertise from different areas of science and engineering with lateral thinking. In this review, we highlight the recent trends in 3D bioprinting of tissues and organs including recent developments in usage of material, printers and printing technologies. In addition, importance has been given to various target tissues printed using this technology with an emphasis on bioprinted tissue/cancer models.

1 Introduction

Replication of biological tissue on the microscale ensures successful structural generation of tissue mimic[1]. Cells in the tissue mimics synthesize and remodel extracellular matrix, which in turn regulates cellular movement, growth and differentiation[2]. Extracellular matrix (ECM) also facilitates the microenvironment by harboring soluble factors, chemokines and growth factors[3]. It also provides physical cohesiveness and anchorage for cells through ligands[4]. Bioengineering approaches focus on reproducing these cellular and extracellular components present within a tissue to develop tissue replicates that can be used for clinical restoration of tissue or organ function[1]. One of the major challenges in this field is to reproduce the complex microarchitecture of the ECM, biochemical factors, their gradients and presence of multiple cell types in a particular tissue[1]. 3D bioprinting, a technological progress in the field of additive manufacturing technology, has displayed its potential to solve these issues, thus transforming tissue engineering and bioengineering approaches. 3D bioprinting allows precise positioning of biological materials such as cells, matrix materials and biochemical factors with spatial control over placement of functional components[1]. To develop tissues that have complex shapes and sizes with a single bioprinting technology remains challenging and hence determining the ideal technology becomes crucial[5]. The major bioprinting approaches that have been explored for bioprinting applications include inkjet-based[6], laser-assisted[7], and extrusion-based[8] bioprinting, each having its own specific strengths, weaknesses and limitations.

In addition to the printing technology, materials for printing also play a crucial role in recapitulating the overall properties of ECM. Hydrogels from various synthetic and natural polymers including decellularized ECM[9] and ceramics have been used for soft and hard tissue engineering,

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respectively. These printable materials, so-called "bio-glue", are deposited layer by layer to mimic the entire physical structure during bioprinting. The bioprinted tissues can actually recapitulate relevant attributes of in vivo biology, including cell–cell and cell–matrix interactions and deposition of native tissue extracellular matrix. Therefore, the tissues remain viable for extended periods in vitro, allowing the examination of low-dose drug treatments, various dose and regimen of drugs' action and possess architectural and functional features similar to that of native tissue environment. Furthermore, various end point analyses such as biochemical, genomic, proteomic and histological evaluation can be assessed over time in drug discovery research. Beyond toxicity, the human tissue models are also helpful for studying the development and progression of disease and provide an enormous opportunity to hasten drug discovery and screening. In this way; the cost and time can be reduced and saved[10]. Bioprinted cancer tissue models could also be used to study the primary and secondary effects of potential drug candidates. The advances in creating iPSCs from a specific patient and their redifferentiation into tissues of choice open up new possibilities for personalized tissue/tumor model development and in turn development of personalized therapy[11]. However, the main technological barrier is non-availability of suitable bio-glue or bioinks with good biocompatibility and mechanical strength that can be used to achieve biological function. In this review, we focus on different bioprinting strategies, emerging trends and applications of 3D bioprinting and describe the recent advances in terms of tissue bioprinting and their use in therapeutics.

2 Historical Evolution of 3D Bioprinting

3D printing was first demonstrated by Charles W. Hull in 1986, which was named solid image processing or 'stereolithography', in which layers of materials were printed sequentially to form a 3D structure. Later, this principle was applied to prepare biological scaffolds, with or without cells. In 1988, Klebe reported that cells can be positioned precisely in a predetermined design by using a technique named "cytoscribing", where collagen and fibronectin are deposited using HP thermal drop-on-demand (DOD) inkjet printer[12]. Further upgraded technology was introduced by Thomas Boland and his team in 2003[13], in which they used a customized thermal inkjet printer to print cells in a viable condition. Since then, scientists have been trying different biological materials, from growth factors[14] to decellularized extracellular matrix[15], to develop viable tissues. Most of the bioprinting works are hydrogel based; however, it is challenging to print tissues with both high resolution and high throughput with an affordable low-cost printer[16]. Hence, bioprinters are being modified to make it more advanced and accessible to everyone.

2.1 Inkjet-Based Bioprinting

Inkjet-based bioprinting technology was employed for initial bioprinting applications[17]. Inkjet-based bioprinting allows precise positioning of droplets containing cells and biomaterial in a desired pattern by employing either thermal or piezoelectric technologies as shown in Fig. 1. A bubble that is created due to heating of an element is responsible for pushing the material out of the nozzle in the thermal system, while the piezoelectric system uses acoustic waves to eject the material[18]. Confluent monolayer and cell–cell junctions were evident in microvessels that were fabricated by embedding endothelial cells in alginate printed using inkjet technology. It was also shown that the bioprinted vessels were similar to the native sample in terms of robustness. There are also reports showing the use of this technology for demonstrating the differentiation potential of primary muscle-derived stem cells to osteogenic and myogenic cells[19, 20]. An attempt to print full-thickness skin equivalents was done using the same technology, where cells showed high viability even after printing[21]. Apart from these works, inkjet bioprinting technology was also used to understand the basic parameters for printing, such as the optimum cell concentration required[6], viscosity of bioink[22] and thermal effects of cell viability[23]. Although there are advantages such as high speed, availability and low cost; there exist some disadvantages that include limited precision on droplet size and placement[24], requirement of low cell concentration and low viscosity bioinks[22] due to the current microelectromechanical system-based print heads. Hence in the recent past, researchers started modifying commercially available HP printers[25, 26] and there are also reports wherein efforts are made to increase the print resolution by using screw-based servos stages[27, 28].

2.2 Extrusion-Based Bioprinting

Extrusion-based printing is a widely used bioprinting technology employed for developing a 3D bioconstruct. Pneumatic and mechanical forces are responsible to drive material out of the

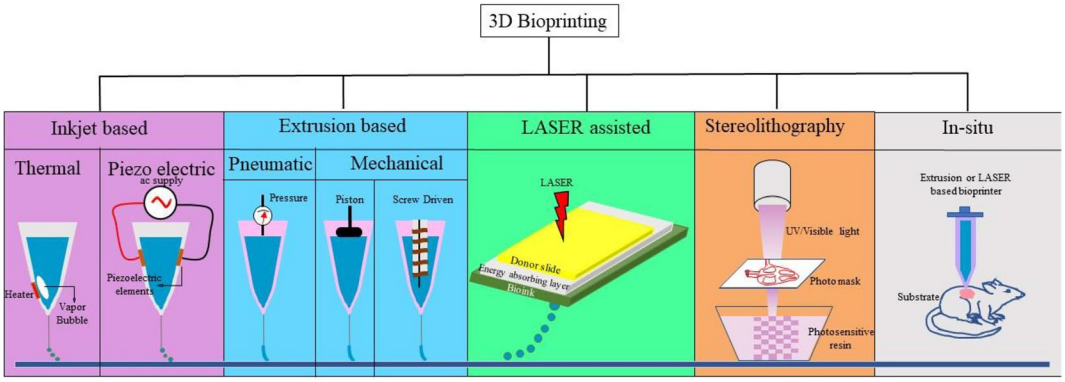

Figure 1: Schematic represention of various additive manufacturing technologies that are used for 3D bioprinting applications with their mechanisms.

nozzle in extrusion-based 3D bioprinting (Fig. 1). Unlike discrete droplets from inkjet printer, continuous strands of material can be deposited in extrusion-based 3D printing technology[24]. A typical extrusion printer design one can think of is a single print head that moves in *X* and *Y* directions. Technological advancements to this design included multihead tissue building system[29], thermal plates attached to print head and substrate[30, 31], co-axial nozzle[32], and employment of co-printing technique[32]. Osteochondral tissue equivalents fabricated using pressure-driven extrusion-based printing demonstrated the possibility of fabricating heterogeneous construct in centimeter scale[33]. In another study, heterogeneous printing of osteo and endothelial progenitor cells retained the diverse cell organization along with heterogeneous extracellular matrix formation after implanting in immune-deficient mice[34]. Besides printing hard tissues, pressure-driven extrusion-based printing technology has also been used to fabricate soft tissues such as liver[35], cardiovascular tissue[15, 36], heart valves[37], blood vessels and microchannels[38, 39], skin[40] and sweat glands[41]. Extrusion-based printing technology has the ability to print a high density of cells in higher viscosity bioinks in contrast to inkjet printers. Post-printing, due to applied pressure, decrement in cell viability and functionality, is a major limitation for this technology[1, 42].

2.3 Laser-Assisted Bioprinting

The other additive manufacturing technology, which was initially used for metal fabrication, but is modified for bioprinting applications, is laser-assisted bioprinting. This non-contact technique uses an energy-absorbing ribbon that can also transfer the energy to a ribbon containing a layer of bioink. This energy in turn pushes the bioink onto the substrate (Fig. 1)[24]. The scaffolds that were fabricated using this technology had a potential to direct embryoid bodies formation, allowing cardiogenesis[43], promote angiogenesis and enhance cardiac functionality, allowing printing of human stem cells for cardiac regeneration[44]. Printed grafts facilitated human adipose-derived stem cells to maintain their lineage and fabrication of functional skin tissue[45]. Being a non-contact technique, cells printed using laser-assisted bioprinting suffer less mechanical stress, unlike extrusion-based technique[42]. Also, higher viscous materials can be printed with ease, while inkjet technology does not allow printing high viscous materials[42]. With such promising features of laser-assisted bioprinting, researchers could achieve fabrication of capillary patterns, which would pave ways for developing macroscale tissues[7]. Nevertheless, there are limited number of printers that are based on this technology due to inadequate understanding of the effects of laser on different types of cells, their functionality and expensive machinery[42].

2.4 Stereolithography-Based Bioprinting

Stereolithography (SLA) is a technique that was reported to print tissues with viable cells and tissues having high resolution than any other bioprinting technology. A photosensitive material that polymerizes upon exposure to light is the principle behind SLA. Exposure of bioink in a particular pattern cross-links the material, thereby forming the desired structure (Fig. 1)[24]. This printing strategy allowed researchers to produce complex tissues with control over porosity, scaffold architecture along with cell–cell interactions. Initially, UV light was used as a source to cure the bioinks[46, 47] which was quite successful, but the viability of cells was totally dependent

on the curing time and intensity of UV light, whereas insufficient curing would result in lack of mechanical strength. This was overcome by introducing visible light source replacing UV source and new biocompatible materials that would get cross-linked upon exposure to visible light[48, 49]. The limited availability of cytocompatible materials that can be cross-linked with light makes this technology a little less accessible for development of tissues.

2.5 In Situ Bioprinting

In situ bioprinting is a recent emerging indication of bioprinting technology for tissue regeneration. This technology allows researchers to directly dispense cell-laden bioinks onto the required site in vivo (Fig. 1). This being a method to directly write or deliver cells, only tissues such as skin, bone and cartilage have been explored using either inkjet or laser-assisted bioprinting technologies. There are few reports from literature showing in situ printing of skin cells to accelerate wound healing[50] and to treat skin burns[51, 52]. In situ bioprinted constructs also demonstrated that the regenerated skin was similar to native skin[51] with high level of re-epithelialization[40] and vascularization[53]. Despite the promising results, the newly formed tissue was reported to be anatomically dissimilar and printed cells could not integrate with the existing tissue. Apart from in situ skin printing, there are reports of bone[54, 55] and cartilage[56] in situ printing. However, with the current state-of-art of in vivo printing, the tissues that can be engineered or the engineered tissue regeneration is still limited. Apart from biological limitations, portable bioprinters with real-time imaging and modeling will be very useful for advancements in this technology[57]. In this budding stage, the above-mentioned technologies have their own sets of merits and demerits. Based on the application, the material and printing technology will vary and hence it is required to determine the best technology to accomplish a task.

3 Emerging Indications

3D bioprinting combines the concepts from various fields such as mechanical, electrical, electronics, instrumentation and biology, due to which it opens up new avenues for researchers pan world to explore the area. Many researchers have tossed themselves into this field and have achieved notable accomplishments, yet there are various points to explore to enhance the applicability of this technology. Nonetheless, noticing the collective developments in fields such as tissue engineering, regenerative medicine and rapid prototyping technologies, regenerating damaged tissues and production of functional organs do not seem very far. When it comes to 3D bioprinting, development of bioinks and printing technology turns out to be the key areas to work on for tissue engineering researchers.

3.1 Materials for 3D Bioprinting

The emerging bioprinting research works are mainly focussed on synthesizing various biomaterial formulations that possess properties such as biocompatibility, bioprintability, mechanical stability and shape fidelity. A wide variety of biomaterials including both natural and synthetic are available for 3D bioprinted tissue engineering applications. Naturally occurring materials such as gelatin[58, 59], alginate[60–62], collagen[63, 64] and silk protein[65–67] have been used as a bioink. These materials are best suited in terms of biocompatibility; yet, the necessity of using harsh cross-linkers to maintain structural shape[15], exhibiting inability to allow cell proliferation and differentiation[15, 68] and low mechanical integrity[69] have attracted researchers to explore more on the biomaterials. To increase the mechanical integrity of the printed construct and to control the biodegradation, synthetic materials such as poly(ethylene glycol) (PEG), poly(ethylene glycol) diacrylate (PEGDA) and gelatin methacrylate (GelMA) were used for bioprinting applications[49, 70]. With success in this, research groups came up with a technique called hybrid printing that involved simultaneous printing of both natural and synthetic materials[71]. This emerged as a potential technique that could overcome the disadvantages posed by employing either natural materials or synthetic materials as standalone bioinks[72]. There exist few reports which exhibited the use of various combinations of only natural materials as bioink. A combination of alginate, gelatin and collagen was used as a bioink to investigate the degradation kinetics of the printed constructs. Although results showed that the printed constructs had the advantages of the trio, the applicability of this material in 3D bioprinting does not seem promising due to the limited flexibility to modulate degradation[68]. Alginate being a bioinert material with limited biodegradability is modified as oxidized alginate to improve its degradation in vivo. Experiments were conducted by varying the concentration of the alginate solution with varied percentages of oxidation. Results demonstrated that with an optimized oxidation

and an optimized alginate concentration, this modified bioprintable alginate was degraded by day 8[73].

In another study, novel bioink that was developed based on polysaccharides, namely gellan and alginate, along with cartilage extracellular matrix particles, was used to print cartilaginous structures and showed promising cell proliferation and functionality[74]. In another interesting study, a multilayered cell-laden structure was developed using an innovative cell printing approach. Collagen was used in the core region to hold the cell and alginate as a sheath covering the collagen to protect the cells while printing and cross-linking alginate to stabilize the structure. This multilayered cell-laden structure exhibited excellent cell viability and functionality in contrast to the control[75]. Neural stem cells cultured in 3D-printed construct using novel bioink by blending three polysaccharides, viz, alginate, agarose as structural support materials and carboxymethyl chitosan as cell growth-supportive material, showed differentiation and other functional characteristics. These fabricated mini tissues can further be applied to understand the biology behind human neural development and neural disease progression studies[76]. A bioink composed of a blend of silk, gelatin and glycerol was reported to increase the print resolution for soft tissue fabrication[67].

In bioprinting, maintaining the optimal homogenous pH of the material and optimum temperature while printing is of utmost priority, since the viability of cells drastically varies if there is any deviation from the optimal conditions. Hence, the pH of natural materials such as alginate, gelatin and collagen is adjusted to physiological pH ~ 7.4[59–61]. The primary reason behind optimizing temperature for bioinks is to modulate their rheological parameters. While printing, the ideal temperature to be maintained depends upon the material's storage and loss modulus. Example, the storage modulus of collagen starts increasing after a certain temperature beyond 15 °C[15], meaning that, after that temperature, its viscosity increases thereby clogging the nozzle. Another example is gelatin whose storage modulus decreases after a certain temperature, after which its viscosity decreases and easily flows out from the nozzle. Though there is no drastic reduction in cellular viability for sudden change in temperature, structural stability depends on the printing temperature and hence materials are printed at a temperature lower than room temperature[59, 60]. To fabricate a mechanically strong, cell-compatible construct, it is necessary to develop a scaffold that blends harder and softer materials. There are reports from literature wherein a 12-week in vivo cultured bioprinted vertebra made of polycaprolactone (PCL) as load-bearing material along with RGD γ-alginate as bioink exhibited extensive vascularization and mineralization[77]. Multilayered scaffold developed by printing alternate layers of PCL/alginate composite and alginate bioink showed significantly higher osteogenic activity when compared to control scaffolds[78]. In an attempt to engineer a muscle tendon unit, researchers used a combination of both synthetic and natural materials. Based on the comparable mechanical properties of polyurethane (PU) and PCL, those materials were chosen for supporting myoblasts and fibroblasts, respectively, and a mixture of ECM components served as a bioink to hold cells. Although the developed construct requires longer culture durations, this heterogenous muscle tendon unit expressed good cellular viability, aligned morphology and increased MTJ gene expression on exposure to mechanical simulation similar to natural development[79]. Vascular biology plays a key role for enhancing the rejuvenation and functionality of the 3D tissue[80]. Due to lack of angiogenesis or vascularization, the developed 3D bioprinted constructs are not maintained in in vitro culture conditions for a long time[81]. The concept of hybrid printing was employed to print cell-laden collagen in a scaffold made of PCL to maintain the mechanical integrity of the construct. In the same study, hepatocytes were co-cultured with human umbilical vein endothelial cells (HUVEC) and human fibroblasts (HF) cells, showed significant urea and albumin secretion and there were preliminary signs of capillary network formation in the scaffold by day 14[82]. Another reason for using this technique is that it enables the development of structures with low viscous bioinks. The advantage with constructs that are printed with low viscous bioinks is that it has higher resolution due to use of lesser nozzle diameters, better cell alignment and increasing dispensing speed, thereby reducing print fabrication time. Although bioink prepared by blending alginate with GelMA had a lower viscosity, immediate cross-linking allowed a structure thickness of 1 mm that endorsed cell migration and orientation[83]. The above literature thus highlights the use of a wide variety of materials that are both printable and biocompatible which plays a key role in 3D bioprinting research and has been summarized in Table 1. It also helps us understand how critical it is to develop a material that best suits the application (Fig. 2).

Table 1: Summary of frequently used bioinks in the field of bioprinting.

Bioprinting technique	Bioink material	Mechanism of cross-linking	Bioprinting characteristic	References
Extrusion-based bioprinting (supports wide range of bioinks)	Matrigel	Thermal	Natural biomaterial Mimics ECM	34
	Gelatin	Thermal	Natural biomaterial High cell viability	221
	Chitosan	pH assisted	Natural biomaterial High cell proliferation rate	222
	Alginate	Calcium chloride-assisted reaction	Natural biomaterial Sustained growth factor release	223
	Agarose	Thermal	Natural biomaterial Good printability High cell viability	224
	Fibrin	Fibrinogen–thrombin assisted reaction	Natural biomaterial Cell layer sheets can be made Complex cellular architecture can be made	225
	GelMA	Photopolymerization (UV assisted)	Synthetic biomaterial Good cytocompatibility Good mechanical properties	226
	PEG	Photopolymerization	Synthetic biomaterial Biocompatible material Good mechanical properties	227
	Decellularized extracellular matrix	Thermal	Natural biocompatible material, Biological and chemical cues, Provides tissue specific microenvironment	15
Laser-based bioprinting (supports only bioinks that can be irradiated)	Poly(DL-lactide co-glycolide)	Photopolymerization	Synthetic biomaterial High resolution structure can be constructed Good cytocompatibility	228
Droplet-based bioprinting (supports only bioink with lower viscosities)	Collagen/gelatin	Thermal	Natural biomaterial Good cytocompatibility	229

Biomolecules can be mixed with bioink to stimulate cell differentiation, proliferation, survival and tissue regeneration. Growth factors are basically proteins or steroids produced by specific cell types, tissue or gland. Addition of specific growth factor into the bioink depends on the specific cell types encapsulated with it, such as bone morphogenic protein (BMP) family[84], which stimulates bone-related differentiation, whereas vascular endothelial growth factor (VEGF), angiopoietin-1[85], stimulates the vascularization process and transforming growth factor beta 1(TGF-β1) heps in differentiation of mesenchymal stem cell to chondrocyte[86]. Blood plasma also can be mixed with bioink, as it has an all-natural mixture of vital protein. Gruene at el. mixed blood plasma with alginate to encapsulate adipose-derived stem cells (ASCs) for adipogenic differentiation[87] and skin tissue engineering[45].

It is quite difficult to fabricate organs that have different shape and size complexities with a single bioprinting technology. Hence, it becomes crucial to determine which printing technology must be used that can print the bioink while providing other advantages such as high resolution and high cell viability during printing.

4 Bioprinted Tissues: An Overview

3D bioprinting is an emerging trend in the field of therapeutics, which can translate the word therapeutics into personalized treatment in medical industry. 3D bioprinting emerged as a powerful tool with which development of customized, personalized solutions became a reality[88]. Considerable progress has been made in designing tissues, innovative biologically superior biomaterials and printing technologies. Here, a number of bioprinted tissues targeted by various researchers globally is enlisted.

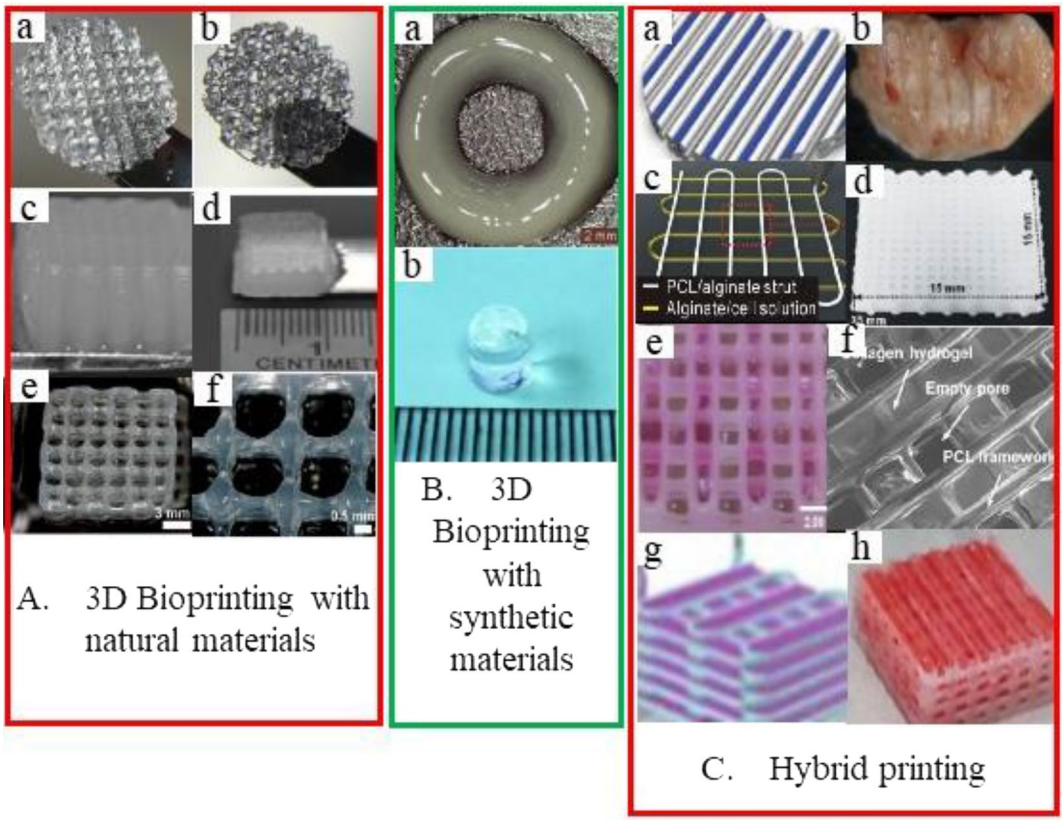

Figure 2: Various natural (A) and synthetic (B) materials as bioink for 3D bioprinting. (C) Structures printed simultaneously with synthetic and natural material—hybrid printing. (A) 3D printed gelatin scaffold (a, b) (58), alginate (c, d) (61), silk (e, f) (64), (B) structures fabricated using synthetic materials: (a) PEG (69), (b) PEGDA + peptide (135), (C) mechanically robust structures developed with hybrid printing technique using PCL/alginate (a, b, c, d) ((76), (77)), PCL/collagen (e, f) (81), PCL/adECM (g, h) (15).

4.1 Muscle

Recent advancements in 3D bioprinting technology have accelerated the growth in the field of tissue engineering and regenerative medicine. There are many different organs that are 3D bioprinted and are reported functional at the microscale. One such tissue that researchers have started to develop in vitro in the recent past via 3D bioprinting technology is the skeletal muscle. Laboratory engineered skeletal muscle could serve as an alternative for tissue flaps, muscle reconstruction surgeries[89], drug discovery/testing model[90] and biological actuators[91], also assisting in understanding the effects of mechanical stimulation[92]. A construct developed by 3D extrusion-based bioprinting of a hydrogel composite containing gelatin, fibrinogen, hyaluronic acid (HA) and glycerol with cell type mouse myoblasts along with PCL and pluronic F-127 as supporting materials exhibited higher viability and muscle-like structure with aligned myotubes. Further, in vivo studies showed well-organized muscle fiber structures and mechanical integrity, and their response to electrical stimulation and immunofluorescent staining confirmed the presence of myosin heavy chain (MHC)[93]. The 3D-printed muscle construct developed using muscle progenitor cells for functional muscle reconstruction could successfully integrate with the native tissue and restore muscle functionality in vivo. The functionality of the mature scaffold was confirmed by the expression of the MHC marker[89]. A muscle construct was printed using a blend of thermosensitive and natural material, viz., pluronics/alginate with C2C12 cells. The expression of markers such as myogenin, α-sarcomeric actin and myoblast differentiation protein proved that this 3D bioprinted construct possessed viability and differentiated multinucleated myotubes[94]. To explore the effects of topographical cues on cells for muscle regeneration, a hierarchical construct was developed by printing C_2C_{12} cell-laden alginate on micro/nanofiber assembly printed using PCL. This study revealed that fiber alignment

has influence on the morphology and alignment of the formed myotubes, also causing significant changes in the levels of myogenic genes[95]. Another similar study revealed the fabrication of a 3D microfibrous bundle structure with collagen as a biomaterial. Due to the aligned topology and high biocompatibility of collagen, the expression of myogenic genes such as *MYF5, MYH2, MYOD* and *MYOG* was enhanced implying the formation of myotubes[96]. Bioengineered muscle also finds its importance in the field of biosensors and actuators and is being explored in recent time. A blend of hyaluronic acid, gelatin and fibrinogen along with myoblasts was 3D bioprinted to develop bioactuators. This printed construct exhibited viability, differentiation and contractile properties upon electrical stimulation. The expression of two transcription factors, *MYOD* and myogenin, marked early stage differentiation, while there was presence of myosin heavy chain I and II when the tissue started to mature. It was also found out that the force that is generated could be modulated with mechanical stiffness and frequencies, demonstrating printed tissue adaptability to applied stimulus[94]. In a similar study, cells along with bioink were printed onto a cantilever using thermal inkjet technology for biosensors application. The developed bioprinted conjugated construct expressed strong proliferation, differentiation and functional properties in a very short culture period of 4 days in contrast to a culture period of 14 days without printing[97]. Instead of using a blend of ECM components or a mixture of natural materials as bioink, hydrogel prepared from decellularized tissue has proven to have much more potential for improved cell structure and functionality[15]. Skeletal muscle tissue thus printed using decellularized porcine skeletal muscle as bioink provided adequate microenvironment for C_2C_{12} cells to show enhanced proliferation and differentiation capacity that was represented by higher myogenic gene (*MYF5, MYOG, MYOD* and *MHC*) expression[98]. Biologically printed muscle tissue integrated with MEMS technology has its applications in various areas and has a capacity to offer solutions for biosensors, heart pumps and actuators. Moreover, standalone bioengineered muscle tissues could provide a route to improve the treatment strategies for musculoskeletal disorders in the field of medicine. Nevertheless, the development of full-thickness, mechanically robust, vascularized muscle tissue grafts with neuromuscular junctions remains challenging[99] (Fig. 3).

4.2 Liver

Liver diseases are one of the major causes of death worldwide. Tissue engineering provides an alternative strategy to restore the hepatic functions by developing artificial or natural whole organ substitutes[100, 101]. Apart from their uses in transplantation, tissue engineering can also aid in the development of in vitro models that can be used for preclinical drug toxicity testing and as disease models[102, 103]. The importance of 3D bioprinting can be well understood while developing complex organs like liver. It is because of its complex geometry, assorted cellular populations and sophisticated microenvironment, the liver is able to function very efficiently. In the recent past, researchers have developed different techniques to develop 3D models of liver using the basic technologies available in 3D printing.

Reliable in vitro liver models which can be maintained for long is an area of continuous research interest due to this biggest organ's role in the detoxification process and metabolism and also in its application in the screening of new drugs[104]. Bioprinted human liver tissues are one of the first ever bioprinted human tissues, which found commercial application in toxicology and disease modeling[105]. However, this is a rapidly evolving field with many academic and industrial R&D laboratories that are simultaneously pursuing research toward the fabrication of liver tissues by various bioprinting approaches[105].

In a study by Nguyen et al.[106], a novel bioprinted human liver tissue was developed from patient-derived hepatocytes and non-parenchymal cells in a defined architecture. A dose-dependent toxicity was observed at clinically relevant doses (<4 μM), demonstrating 3D bioprinted liver microtissue can be used as a model for drug-induced liver injury (DILI). In a different approach, HepG2 and human umbilical vein endothelial cells (HUVEC) were encapsulated in collagen type I and gelatin, respectively, and printed on a PCL scaffold. The functionality and viability of HepG2 cells were found to be increased in 3D culture compared to 2D[107].

Aggregates of cells can be printed onto removable support matrices, after which the spheroids fuse together to produce a material-free scaffold[108]. This technology has been licensed to Organovo's as NovoGen MMX bioprinter™[109, 110]. Using this method of printing, Organovo created stable liver tissue composed of endothelial cells, stellate cells and primary hepatocytes or hepatocytes derived from induced pluripotent

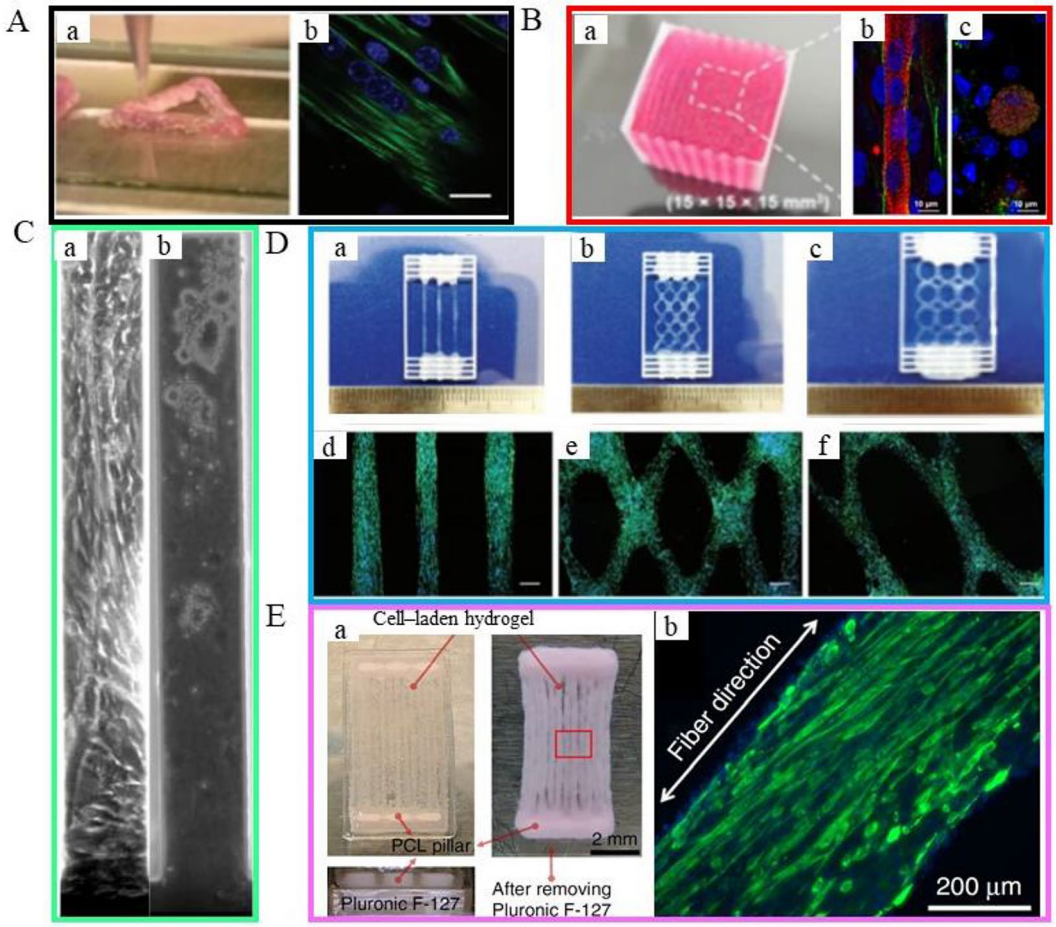

Figure 3: 3D bioprinted skeletal muscle tissue. (A) A simple design showing 3D bioprinted bioactuator (a) along with cell expressing MYHCII and nuclei stains (b) (90). (B) Multilayered bioprinted skeletal muscle (a) composing myofiber bundles, cells expressing contractile proteins when printed (b), no contractility when not printed (c) (88). (C) Image showing formed myofibers due to bioprinting of cells, (a) cells that are non-printed are randomly distributed (b) (96). (D) 3D cell printed in various designs (a, b, c) with corresponding fluorescent images stained with f-actin and DAPI (d, e, f) (97). (E) 3D printed muscle scaffold with PCL and pluronic F-127 as supportive materials (a), aligned myoblasts expressing myosin heavy chain (b) (92).

stem cells (iPSCs). When evaluated histologically, it was found that hepatocytes and endothelial cells formed sinusoidal-like microvascular structures. The viability and the phenotypic stability increased when smaller spheroids were cultured[111]. However, the microvascular and ductal system of the liver presents a major challenge to engineering of liver tissue. A scaffold-free 3D bioprinted model was developed by Kizawa et al.[112], using spheroids which were skewered onto a needle array and cultured on perfusion for 4 days. A microarray analysis was done for metabolic and functional genes involved in gluconeogenesis (*GCGR, G6GP*), glycolysis (*INSR, AKT1*), tricarboxylic acid (TCA cycle) (*IDH1*), glycogen synthesis (*GYS2, PYGL*), fatty acid synthesis (*SREBF1, ACACA*), cholesterol synthesis (*SREBF2, HMGCR*) and urea cycle (*OTC, ARG1*). The comparison showed expression of the functional genes on levels similar to native levels, which was also maintained for an extended period of 2 weeks. The bioprinted liver tissue also showed glucose production, which could be suppressed with insulin and bile acid secretion that accumulated in the culture medium over time.

4.3 Kidney

Kidney, one of the important organs to remove waste from the body, is also one of the major organs that is in huge demand for transplantation. The treatment options that are available for an end-stage renal disease patient is either

kidney transplantation or dialysis. The rise in need of donor organs is pushing researchers to discover ways to fight organ crisis. Printing complex organs such as kidney is quite difficult in one attempt. Therefore, printing it with its small constructs such as nephron in kidney and then fusing it together to make a whole organ is a better way for organ bioprinting. Tissue engineering company Organovo Inc. and Murdoch Children's Research Institute developed kidney tissue constructs by their proprietary 3D bioprinting platform for disease modeling and drug screening. A human in vitro proximal tubule was developed using a thermoresponsive bioink along with renal fibroblasts and HUVEC[113, 114].

Currently, large-scale 3D kidney complex tissue fabrication is not yet achieved and hence researchers are trying to fabricate small-scale level tissue constructs on mini tissue building blocks. Researchers from Harvard's Jennifer Lewis laboratory have been trying to develop the small-scale functional renal tissue structure and nephron and recreated the proximal tubule segment of a nephron[113, 115].

Just like, Organovo's NovoGen Bioprinter, the Fabion 3D bioprinter is used to develop functional and implantable human organs in the near future. To bioprint mini kidney using kidney organoids, they are collaborating with the University of Oulu and 3DTech Oy. Fabion is equipped with five deposition nozzles to dispense bioink having cell suspension, spheroids and other materials. The other two nozzles are for dispensing biopaper, which can be polymerized by UV radiation[113]. The kidney model can be used to study drug secretion, drug–drug interaction, kidney injury by drugs, etc., and the challenges to bioprint kidney include its size and variety of cells. Major challenges include variability of cells, and vascularization of kidney and ductal system to drain out urine is a most difficult task to maintain the functionality of bioprinted kidney.

4.4 Spinal Cord

Spinal cord injury (SCI), a neurological disorder that interrupts communication within the central nervous system (CNS), can lead to diminished function[116]. Spinal cord tissues contain various cell types organized in high order of spatial distribution. One has to take it into account while engineering this structurally heterogeneous tissue construct[117]. Aspect Biosystems' novel RX1 bioprinter was used[116] to bioprint hiPSC-derived neural progenitor cells (NPCs) along with the unique fibrin-based bioink, which conserved high cell viability and proved to be functional by expressing spinal cord motor neurons (MNs)-associated markers. Joung et al. used a variety of hydrogels, namely Matrigel, gelatin/fibrin (GEL/FIB) and GelMa, alginate (AG) with methylcellulose (MC) to 3D bioprint-induced pluripotent stem cell (iPSC)-derived spinal neuronal progenitor cells (sNPCs) and oligodendrocyte progenitor cells (OPCs) in a layer-by-layer manner to create multiple channels. The axons were extended via the bioprinted sNPCs and the activity of these neuronal networks was confirmed by the calcium flux studies[117]. Alginate gelatin hydrogel was printed in an extrusion-based bioprinter by Li et al.[118] along with Schwann cells which demonstrated good cell viability and functionality even after 14 days. Lee et al.[119] 3D bioprinted rat embryonic neurons and astrocytes were printed on a planar collagen scaffold by inkjet printing method, which has undergone gelation when reacted with sodium bicarbonate solution. However, reduced cell viability was observed due to decreased level of perfusion; inhomogeneity during cell printing as well as altered mechanical property including porosity.

4.5 Bone

Recently, 3D bioprinting of bone is in rise with a variety of combination of synthetic polymers, natural materials and modified natural materials. Most of the reported works used hydrogels along with cells and osteoconductive or osteoinductive elements. Although there are not much reported preclinical studies to rate the success, potential feasible in vitro studies are plenty including mineralized, vascularized networks[120]. Though a number of materials are under research, the challenge is to bioprint constructs with structural stability, sufficient mechanical properties and suitable function for the required time until complete healing occurs[120]. Though most combinations of thermoplastics such as polylactic acid (PLA) and PCL exhibit excellent mechanical property and biocompatibility, their higher processing temperature makes it unsuitable for 3D bioprinting. Therefore, hydrated polymers known as hydrogels are widely being tested for bioprinting[121]. Here, we discuss few latest studies on 3D bioprinting of bone tissue with different materials and cell types using different printing technologies.

Using thermal inkjet bioprinter, a thermoresponsive composite hydrogel made up of collagen type 1 and agarose was printed with human mesenchymal stem cells (hMSCs)[121]. The printed

construct supported cell proliferation (>98% cell viability after 3 weeks), cell spreading and branching. It was observed that MSCs showed osteogenic differentiation in the constructs with high collagen content and less mechanical stiffness[122].

Kang et al. modified the printing technologies of fused deposition method (FDM) along with inkjet-based bioprinting and used to create a large bone tissue construct. A cell-laden hydrogel comprising human amniotic fluid stem cells (hAFSCs) suspended in a solution (gelatin, fibrinogen, hyaluronic acid and glycerol mixed with DMEM) was printed along with synthetic biodegradable biomaterial PCL that acted as support material and pluronic F-127 sacrificial hydrogel. This was done using an integrated tissue organ printer (ITOP) system to attain mechanically stable constructs in the shape of the mandible and calvarial bone (hAFSCs), cartilage (rabbit ear chondrocytes) and skeletal muscle (mouse myoblasts). This study showed the printing mechanism for some of the largest bone constructs in the field of bone tissue engineering[93].

In another study, using a dual extrusion-based bioprinter, core–shell structures were bioprinted where the core was made up of alpha-tricalcium phosphate (α-TCP) with the shell made up of mouse bone/calvaria MC3T3-E1 preosteoblast cells that were suspended in alginate hydrogel. This construct was then assessed for cell viability and checked for mechanical strength and it was found that the compression strength (3.2 MPa) and Young's modulus (10.92 MPa) were maintained till 5 weeks in vitro with >90% cell viability[123]. Similarly, composite hydrogel core made up of collagen type 1 and alpha-tricalcium phosphate (α-TCP) using extrusion-based bioprinting was used. The core was later coated with collagen type 1 bioink containing mouse bone/calvaria MC3T3-E1 preosteoblast cells. In this study, the cross-linker used was tannic acid and the printed structure was mechanically stable with an elastic modulus of 0.10 MPa and showed good cell viability (>90%) with high metabolic activity[124].

The recent trend in bioprinting is in situ bioprinting with the aim of making it clinically friendly. In such a study, Biopen, a handy device, was developed that directly prints human amniotic mesenchymal stromal cells (hAMSCs) on the chondral wound sites. Biopen also facilitates in situ UV cross-linking while printing the matrix materials such as GelMA or methacrylated hyaluronic acid along with cells. The photoinitiator used was Irgacure 2959 and the cells showed cell viability as of day 7 in the printed construct[125]. In another study, UV in the presence of photoinitiator Irgacure 2959 was used to cross-link methacrylated hyaluronic acid (MeHa) hydrogel encapsulated with human bone marrow-derived mesenchymal stromal cells (MSCs) and printed using piezoelectric inkjet-based bioprinter. Post-printing showed increased storage and elastic moduli. The increased gel rigidity led to osteogenic differentiation of MSCs, and with the addition of BMP-2, this bioprinted bone construct showed further matrix mineralization[126].

The desired mechanical stability for a specific time duration is considered as one of the biggest challenges in bone tissue engineering. In an attempt to address this issue, hMSCs were suspended in PLA fibers functionalized with the growth factor BMP-2 and printed using extrusion-based FDM bioprinter. Similarly, HUVECs, as well as hMSCs, were co-encapsulated with methacrylated gelatin (GelMa) hydrogel functionalized with the growth factor VEGF and printed using SLA bioprinter in the shape of tubes. The entire construct was incubated in a custom-designed bioreactor. Post-printing of the biphasic bone construct and osteogenic differentiation as well as the formation of a complex vascular network were observed[127].

A mixture of alginate, polyvinyl alcohol (PVA) and hydroxyapatite (HA) was optimized for biomimetic rheological properties. Mouse calvarial 3T3-E1 (MC3T3) cells were suspended in this formulation, and extrusion-based bioprinter was used to 3D print the bone constructs. The alginate–PVA–HA biomaterial gave a bone-forming environment for 14 days; it was biodegradable and supported cell growth (77.5% cell viability post-printing and post calcium bath) with compressive moduli of 2.4 kPa at the end of 14-day culture[128].

With the aim of development of vascularized bone tissue construct, the team developed an extrusion-based bioprinting protocol that allows direct writing or surgical fabrication of bone tissue constructs on large defects. HMSCs and HUVECs were suspended in GelMA hydrogel with a low degree of methacrylation and printed to form the core of a fiber-like construct. To create perfusable vascular channels, the second bioink was prepared using GelMA hydrogel conjugated with VEGF and mixed with silicate nanoplatelets and hMSCs which also initiated osteogenesis, as differentiated osteoblasts were seen in the construct on maturation by day 7. Cell proliferation, capillary formation and stability of construct up to 21 days of culture were observed. Bone, as well as vascular tissue, was printed in one construct[129].

Using laser-assisted bioprinting in addressing tissue regeneration of bone, in situ bioprinting using mouse bone marrow mesenchymal stromal (BMSC) precursor D1 cells was done. The group directly printed cell-laden collagen mixed with nano-hydroxyapatite (nHA)-based hydrogel onto a calvaria defective mice model to show its impact on bone regeneration. Different cell geometries were tested for cell viability and metabolic activity, and it was found that there was significant healing 2 months post-operation (defect creation) when the cell-laden hydrogel was patterned in a disc shape on defect/wound site[55].

Development and characterization of an extrusion-based bioprinting protocol was done of mouse calvaria 3T3-E1 (MC3T3) cells suspended in a formulation of chitosan hydrogel, chitosan–nano-hydroxyapatite (nHA) hydrogel, alginate hydrogel and alginate–nano-hydroxyapatite (nHA) hydrogel for the purpose of bone tissue engineering. All the formulations were viscous, particularly chitosan-based hydrogels, and showed good printability, and cell viability and proliferation were observed. Osteogenic differentiation peaked in chitosan–nHA formulation up to 21 days of culture[130].

4.6 Cartilage

Cartilage, a highly heterogeneous tissue with complex microarchitecture, comprised different zones, namely the superficial zone, the middle zone and the deep zone[131]. Conventional clinical and tissue engineering technologies have failed to recapitulate this zonation, resulting in disorganized tissues with compromised mechanical properties. 3D bioprinting technology promises to overcome the challenge of zonation owing to its ability to make patterns and deposit cells layer by layer with high precision. Based on the approach, cartilage bioprinting is categorized into three sections, namely scaffold-based bioprinting, scaffold-free bioprinting and the technique with more translational value, in situ bioprinting[132].

Naturally available materials such as agarose, alginate, collagen and synthetic materials such as PCL[133], PEGDA[134], GelMA[56] and PEG[135] were used in engineering cartilage tissues in vitro. In a recent study, a blend of collagen or agarose with sodium alginate was used as a bioink. This in vitro developed scaffold housing chondrocytes was mechanically strong and facilitated in cell adhesion, which remarkably enhanced proliferation. Expression of cartilage-specific markers such as aggrecan and SOX9 further displayed the potential of this bioink when compared with the other control groups[136]. Fabricating cell-laden cartilage scaffold using GelMA and PEGDA as material components was reported by Zhu et al. Mesenchymal stem cells along with growth factor TGF-β1, the developed structure-maintained cell viability and growth factor bioactivity. Also, due to inclusion of TGF-β1 in the form of nanospheres, a sustained release was maintained in the construct up to 21 days, which supported differentiation of MSC to chondrogenic lineage[86]. Nguyen et al. showed that a bioink comprising nanofibrillar cellulose with alginate maintained the pluripotency of iPSC in the construct with COL-II being expressed, while iPSC in nanofibrillar cellulose with HA could not maintain their pluripotency[137]. In different studies, materials such as PCL/alginate[133], PEGDA[134] and GelMA[56] were used to fabricate cartilage scaffolds that were implanted into animal models to test their efficacy in in vivo conditions. PCL/alginate cartilage construct maintained viable cells and secreted cartilage matrix components after 21 days post-implantation[133]. In another study, after implantation, vascular tissue membrane formation was reported surrounding the cartilage tissue developed with PEGDA, as reported by Gao et al.[134]. As per Di bella et al. the cartilage construct produced by using GelMA showed higher amount of newly formed cartilage along with aligned chondrocytes in vivo[56]. Recently, Apelgren et al.[138] used 3D network of bacterial nanocellulose (BNC) fibrils for cartilage 3D bioprinting which exhibits a proliferation of chondrocytes in vivo from 32.8 ± 13.8 to 85.6 ± 30.0 cells per mm^2 from 30 to 60 days. The bacterial nanocellulose fibrils were disassembled using aqueous counter collision (ACC) method. The ACC method helped in enhanced printability, mechanical stability and structural integrity probably due to the disentanglement of BNC and the increased length of fibers makes it highly suitable for 3D bioprinting (Table 2).

4.7 Heart

The most challenging part of the cardiac tissue bioprinting is vascularization while achieving synchronous rhythm. A recent study illustrated fibrin-based bioengineered cardiac tissue constructs printed on gelatin-based sacrificial scaffold, and PCL as supportive framework with cardiomyocytes demonstrated calcium influx as well as synchronous contraction after 3 weeks[139]. Alginate as a material for 3D bioprinting of myocardium demonstrated high cardiogenic potential[140]. Human-derived cardiac-derived cardiomyocyte progenitor cells also migrated to

Table 2: Summary of classification of bioprinting modalities

Bioprinting modalities	Features	Advantages	Limitations	Application	Refernces
Inkjet/droplet based bioprinting	Material viscosities—3.5–12 mPa/s Gelation methods—chemical, photo-cross-linking Preparation time—low Print speed—fast (1–10,000 droplets per second) Resolution or droplet size—< 1 pl to > 300 pl droplets, 50 µm wide Cell viability—>85% Cell densities—low, < 106 cells/ml Printer cost—low	Bioinks with low viscosity can be printed easily Higher print resolution High throughput Printers commercially easily available	Nozzle clogging if cell density is high Drop size not uniform Possible cross-contamination if multiple bioinks used	Liver models	30, 230
Extrusion-based bioprinting	Material viscosities—30 mPa/s to > 6×10^7 mPa/s Gelation methods—chemical, photo-cross-linking, sheer thinning, temperature Preparation time—low to medium Print speed—slow (10–50 µm/s) Resolution or droplet size—5 µm to millimeters wide Cell viability—40–80% Cell densities—high, cell spheroids Printer cost—medium	Wide range of bioinks can be printed Allows for scaffold-free bioprinting Can be used to prepare organ-on-chips, cancer-on-chips for drug testing Commercially easily available	Extrusion causes shear stress that leads to lower cell viability Low resolution Difficulty in scaling up bioprinted models	Cornea, muscle, cardiac tissue, liver, bone, kidney models	106, 115, 139, 231_234
Laser-based bioprinting 1. Cell transfer 2. Photolithography	Material viscosities—1–300 mPa/s Gelation methods—chemical, photo-cross-linking Preparation time—Medium to high Print speed—medium–fast (200–1600 mm/s) Resolution or droplet size—micro-scale resolution Cell viability—>95% Cell densities—medium, 10^8 cells/ml Printer cost—high	Narrow range of printable bioinks Free of shear stress to bioink Highest resolution High cell viability	Bioink preparation requires precision and time Models with heterogeneous cell population are hard to print Printer cost and maintenance is very high	Skin	45, 235

form tubular structures while maintaining their functional properties, indicating their use in therapeutic cell delivery. Recently, Wang et al.[139] developed a contractile cardiac tissue with cellular organization, uniformity and scalability by using three-dimensional bioprinting strategy. They observed the alignment of cardiomyocyte and also the expression of α-actinin through the entire length of cardiomyocyte and connexin, expressed in between cardiomyocytes. The synchronous contractile nature could be visible by the calcium flux at the time of contraction demonstrated by Fluo-8 AM. Liu et al.[141] used human embryonic stem cell-derived cardiomyocyte that was printed by using light-based micro continuous optical printing. This printed model was reported to be used as a powerful tool for drug screening and helpful to analyze cardiac tissue maturation[142]. Although the bioprinting process promotes better differentiation and functional organization than scaffold-based strategies, there is a need to improve printing strategies and incorporation of other cell types for tissue-specific functionality[143]. In this regard, Maiullari et al.[144] developed a microfluidic-based printing head (MPH) for the co-axial needle system to extrude two biopolymers, alginate (ALG) and PEG monoacrylate–fibrinogen (PF) with $CaCl_2$ to cross-link alginate. In vitro developed cardiac tissue was matured in in vivo conditions along with developing vasculature in the transplanted tissue. Addition of multiple cell types such as fibroblasts and HUVEC along with cardiomyocytes aids in maturation of tissue rather than using cardiomyocytes alone[144, 145].

4.8 Skin

Autologous split-thickness skin graft (ASSG) remains the gold standard in the clinic for large wound healing[146]. In ASSG surgery, doctors remove a piece of skin by dermatome from a secondary surgical site of the patient, stretching the skin and reapplying the skin to the wound site. Its limitation with respect to wound size led to the development of skin scaffolds using natural and synthetic materials[147, 148]. Cells spraying in wound site can heal better and faster than scaffold-based therapy[149], but harvesting is effective only in smaller areas. The scenario of manually seeding cells or spraying cells is changed to layer-by-layer freeform fabrication techniques to fabricate complex tissues[150]. Bogaard et al.[151] demonstrated first 3D skin in vitro model for skin disease psoriasis by using different T cell population, where they studied migration of immune cells and secretion of proinflammatory cytokines. Bioprinting of skin tissue can be done by using either spheroids or collagen hydrogel[50]. Lee et al.[52] bioprinted a 13-layer tissue construct using collagen hydrogel by bioprinting of skin tissue using an eight-channel valve-based bioprinter.

In situ bioprinting has huge potential in the treatment of skin wounds that are patient and site specific at a faster pace[1]. The prevailing in situ skin bioprinting process is done using extrusion-based system with custom geometries, yet skin contraction, scar formation and vascularization in large wound remain unsolved[52]. Wound contraction in in situ skin bioprinting can be reduced by using autologous cells rather than using only matrix or allogenic cells[52]. Although, in situ bioprinted skin constructs demonstrated vascularity and low inflammation, limited cosmetic outcome, pigmentation and hair follicles within constructs are yet to be addressed. In vitro 3D printed skin construct with air–liquid interface (ALI) culture allows vascularization of mechanically robust tissues. It also aids to terminally differentiate keratinocyte to corneocyte, which is important for multilayer keratinocyte maturation[50, 152, 153].

In case of in situ bioprinting, an inkjet-based bioprinting is used generally due to its ability to print drop-on-demand of irregular shape of wound[50]. Although in situ bioprinting can save the treatment time and close the wound very rapidly, it has major limitation to treat large wounds. For proper vascularization and keratinocyte maturation, 3D bioprinted skin equivalents are being fabricated. Collagen-based scaffold printed using laser-assisted and extrusion-based technique demonstrated epidermis maturation similar to native tissue[45, 154, 155]. For full-thickness skin substitute, incorporation of skin appendages like hair follicles, sweat gland, melanocyte and sebaceous glands ise necessary, which is however extremely challenging.

4.9 Blood Vessel

Recreating the hierarchical architecture of the blood vessels is essential for engineering complex tissues and organs such as heart, liver, lungs and kidneys[156]. Apart from providing nutrients and oxygen, vascularization also influences tissue formation[157, 158]. Hence, integration of vasculature in tissue-engineered constructs would significantly improve clinical outcomes by improving innervation, as there are studies that show cross talk between vascular promoting factors, endothelial cells and nerve cells[159–162]. Vascular tissue engineering on the other hand refers to the

construction of independent vessels for cardiovascular diseases such as coronary heart disease, cerebrovascular disease, deep vein thrombosis and peripheral artery disease[163].

3D bioprinting techniques have opened avenues that enable us to fabricate an organ in a predefined size and shape. One major challenge limiting the bioprinting applications is the vascularization of the printed organ. Depending on the size of the construct, the cellular and matrix composition, the maximum distance from the blood flow that the cells can survive is around 1–2 mm[143, 164]. Two different strategies are used for printing the vascular tissue: the direct printing of hollow channels or indirect printing using a sacrificial material within the hydrogel. Gao et al.[165] used co-axial printing for delivering endothelial progenitor cells and atorvastatin, a proangiogenic drug, using vascular tissue-derived decellularized extracellular matrix and alginate. However, this method is unable to mimic the three-layer architecture of the blood vessels. Schöneberg et al.[166] printed blood vessels consisting of endothelium containing tunica intima, an elastic smooth muscle containing tunica media and fibroblasts encapsulated in collagen mimicking the tunica adventitia, but printing of small arteries or capillaries is difficult using this technique. To overcome these issues, co-axial printing using sacrificial materials is carried out to vascularize the construct. The commonly used sacrificial materials are agarose[167], carbohydrate glass[168], pluronic[164] and gelatin[143]. After printing, this material is selectively removed to create hollow channels through which media can flow. Zhu et al.[169] printed a prevascularized tissue using a rapid microscale continuous optical bioprinting (μCOB) method. In this technique, they first printed a honeycomb-like structure using GelMa and LAP (as a photo initiator), which was then cured by UV light and then printed with endothelial cell-encapsulated bioink. But the major problem is the resolution and large construct, because in vivo channel formation by endothelial cell is dependent on both cell number and shear stress. In this regard, Xu et al.[170] established a co-axial printing technique for both large blood vessels by direct method and small capillaries by an indirect method. They used cartilage-derived dECM and endothelial cell encapsulated within a sacrificial material like pluronic F127 for large vessels and silicone SE1700 as a support material for small blood vessel. In both cases, they used HUVEC, human aortic vascular smooth muscle and human dermal fibroblast for tunica intima, media and adventitia, respectively.

4.10 Lung

The main function of the lung is to support gas exchange, and any defect in the structure and function of lung can create fatal consequences. Most of the knowledge we have is gained from studying animal lung models. However, animal models are not always fully capable of recapitulating human lung development and disease. That is why in vitro lung model gives an opportunity to mimic human lung model to understand critical developmental and pathological mechanisms[171]. Ott et al.[172] and Petersen et al.[173] have successfully implanted engineered lungs that exchanged gas for several hours. Recently, a model has been developed with three different layers such as lung epithelial cell, endothelial cell and basement membrane, which can be fabricated by using extrusion-based bioprinter for the basement membrane layer with cells and Matrigel[174, 175].

4.11 Cornea

Cornea is the transparent outermost portion of the eye, which plays a pivotal role in eyesight, as visible light is transmitted and refracted when passing through the cornea. Therefore, irreversible damage to the cornea results in blindness[176]. Moreover, there is shortage in donor corneas mainly because of the increase in the number of laser-based treatments and surgery, which disqualify the donated cornea for transplantation. There are synthetic substitutes that are available including KPro (PMMA)[177] and AlphaCor™ (PHEMA)[178], but they have various side effects such as immune reactions because of difference in material properties with respect to native tissue. However, it offers a fertile ground for 3D printing strategies as they can easily mimic the tissue properties. Also, the cornea has a relatively homogeneous cell population, low metabolic requirements and is completely avascular, making it a targeted tissue in the field of bioprinting[179].

In an attempt to engineer a corneal model, stromal keratocytes were encapsulated in a mixture of agarose and collagen I and printed into a self-supporting dome-shaped structure. The cells were viable within the encapsulated gel post-printing and showed characteristics similar to that of native keratocytes. They were able to generate translucent corneal stromal equivalents with similar optical properties as well as their geometry[180].

The orthogonal arrangement of collagen I or lamellae within the corneal stroma is one of the important factors necessary for maintaining the transparency. The collagen fibrils in the cornea have a diameter of about 25 nm. During

printing, parameters like feed rate, discharge rate and nozzle diameter are optimized, and the shear that is induced on the decellularized corneal stroma hydrogel enables the alignment of the encapsulated keratocytes along the printing path. These cells over the course of 28 days produced new ECM where the collagen fibrils had a diameter of about 40 nm and were perpendicularly stacked. This corneal construct that was developed showed better transparency when compared to the other groups and showed no in vivo degradation of collagen after transplantation[181]. This study reveals the superiority of bioprinting techniques that is not reported with the conventional tissue engineering technology.

Recently, attention has been focused on the development of 4D biomaterials or smart materials that can reversibly or irreversibly change shape, size, composition or texture in response to an internal or external stimulus[182]. Cornea-shaped curved stromal tissue equivalents were generated via controlled cell-driven curving of collagen-based hydrogels. This was achieved by the use of bioactuators in limited regions of the gels with the help of a contraction-inhibiting peptide amphiphile (PA). Tissue self-curvature was achieved by gel contraction of the cells, which was limited by the addition of PA. The gels retained the curvature even after extensive handling and there was no impact on transparency due to the curvature. After 5 days culture in gels, the cells assumed a more pronounced ellipsoid shape, with curvature angles of $20° \pm 1°$, and a ratio between diameter/curvature radius similar to that of the native cornea (i.e., ≈ 1.4)[182]. The expression of α-SMA was significantly low, indicating that the cells could retain their phenotype. The cells also displayed orthogonal arrangement after a few days in culture.

The corneal decellularized extracellular matrix bioink is an important step forward in the direction of stromal tissue engineering. It provides a cornea-mimicking environment that supports and maintains the characteristics of encapsulated cells. Apart from the biochemical environment, the mechanical properties of the gel are another important factor when considering using it as a scaffold as it can influence the phenotype of the cells. Moreover, the curvature of the tissue and the lamellar arrangement of the matrix need to be considered when mimicking the corneal tissue.

5 Bioprinted Cancer/Tumor Models

One of the major applications of bioprinting is to serve as a model tissue, with a wide range of applications in industries and in research as well (Fig. 4). 3D bioprinting can replicate the tumor microenvironment and tumor heterogeneity to a higher degree than compared to in vitro models developed conventionally. Advances in 3D printing technique allow the development of preclinical tumor models in the form of 3D in vitro cancer models that are physiologically relevant. Biomimetic 3D printed models can be constructed because bioinks for cancer models

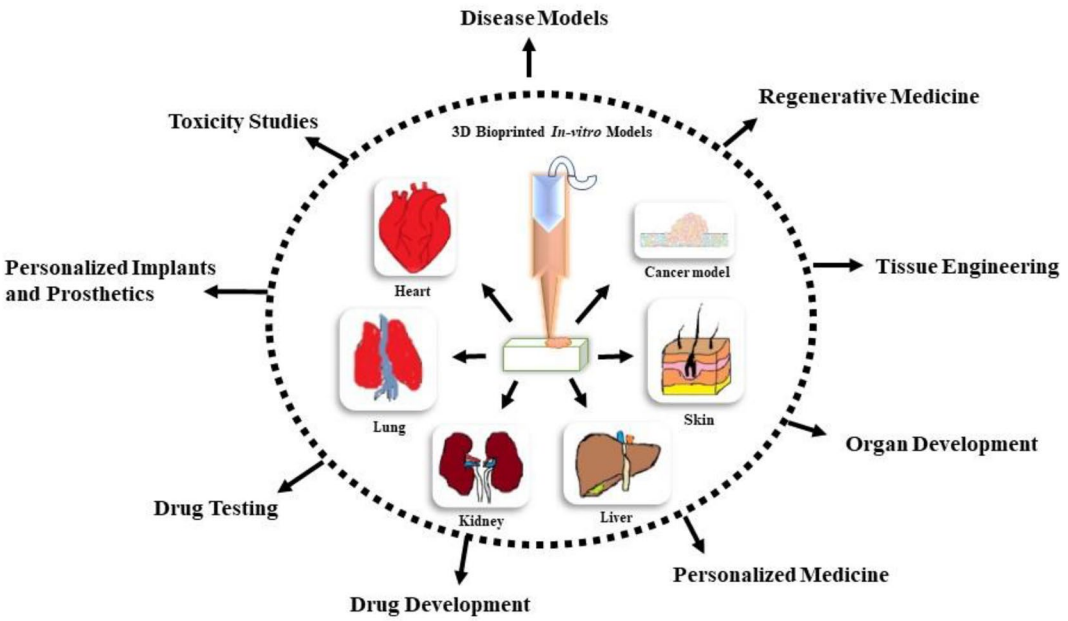

Figure 4: Various applications of 3D bioprinted in vitro models.

can carry various types of tumor-associated cells, ECM components such as collagen and gelatin, and other necessary proteins that can create the desired cancer niche for the bioprinted cancer model[183–185]. These 3D bioprinted cancer models serve as a bridge between in vitro conditions and in vivo models[186, 187]. 3D cancer spheroid models represent in vivo conditions in a tumor more realistically as compared to 2D planar models[188, 189]. 3D bioprinting helps in mimicking the complexity and heterogeneity of a tumor, making the results of preclinical studies more significant[190, 191] and also allowing for a superior degree study of cancer cell–cell signaling and cell–cell interactions with the tumor matrix[192].

Schwartz et al. discussed the importance of the addition of three dimensions to cell culture. Development of 3D bioprinted cancer models started with 3D culturing of cancer spheroids or tumoroids in vitro[193, 194]. These cancer spheroids mimicked in vivo conditions and served as a pedestal for the development of bioprinted models to provide a microenvironment similar to that of a tumor that can be further experimented on for biological studies[195, 196].

3D bioprinting also allows modulation of the architecture of scaffold position of pores, pore size as well as the number of layers of cells establishing a gradient similar to the tumor morphology[184, 197], therefore allowing a model with excellent biomechanical properties and controlled tissue organization to be fabricated to study cancer cell behavior in all its complexity and mimetic native tissue microenvironment[184]. There is a comparable difference in cancer cell behavior in 2D monolayer culture as compared to cells in 3D printed models including proliferation rate[198], gene expression and protein expression[199], as well as their resistance to anticancer drugs[200].

Zhao et al. showed higher viability of HeLa cells in a gelatin/alginate/fibrinogen (GAF) hydrogel-based 3D printed cervical tumor model with compared to cells in 2D monolayer, and they matured to form cellular spheroids by 8 days mimicking the tumor architecture. These cells also showed higher chemoresistance to the drug paclitaxel as compared to cells grown in 2D monolayer conditions[198].

Similarly, a study presented by Dai et al.[201] showed that glioma stem cells SU3 showed increased expression of nestin, a biomarker for cancer stem cell in 3D bioprinted glioma model as compared to SU3 cells grown in monolayer culture. The group also reported an increased expression of VEGF and higher chemoresistance to temozolomide in the cells within this 3D bioprinted model. Similar observations have been seen in case of 3D printed breast cancer model by Palomeras et al.[202], where an increased proliferation of cancer stem cells was seen when MCF-7 cells were seeded on 3D printed circular PCL scaffolds.

Using two-step biofabrication method, Wang et al.[203] fabricated a methacrylated gelatin (MeGel) scaffold and observed that hypoxia faced by cancer cells in cancer niche promotes invasion and migration by increased expression of genes such as VEGF, Snail and MMP-1 in patient-derived breast cancer cell line 21PT in 3D encapsulated hydrogel model as compared to 2D cultures.

One-step biofabrication strategy allows for a higher degree of spatial control over patterning of different cells found in a cancer niche along with native ECM component together to form a biomimetic 3D bioprinted cancer model, which leads to better and relevant results concerning cell-to-cell interactions, cellular signaling and drug screening[202–205].

A recent study by Puls et al.[185] showed a novel bioprinting technique wherein pancreatic cancer cells were suspended in an oligomer solution and then extruded out onto a prefabricated platform. The group fabricated this 3D bioprinted model to study the invasion and migration of highly metastatic pancreatic cancer cells PANC 10.05 in the presence of cancer-associated fibroblasts (CAFs) and, as an application, use this bioprinting technique for rapid drug screening based on patient-specific 3D pancreatic cancer models.

Until recent years, 3D bioprinted cancer models had a considerable lack of vasculature, which is otherwise seen in tumors in vivo. Co-printing of cancer cells and endothelial cells to form a 3D printed model complete with its vasculature allows for real-time observation of the progression of cancer metastasis[199]. Also, printing of template with sacrificial material has been proved to be advantageous up to an extent to mimic native vasculature in printed construct[206] (Fig. 5). By changing the composition of bioinks composed of different cell types, surrounding tissue components for each tissue type and establishing a biochemical gradient, a realistic 3D bioprinted model can be fabricated to study cancer invasion and metastasis mode l[202, 203, 207]. Cancerous tumors are known to undergo the process of angiogenesis and allow nutrients and oxygen to reach to tumor's hypoxic core via the formation of poorly organized blood vessels often known as 'leaky vessels'[208]. 3D bioprinting technology now allows the microenvironment to be modulated in

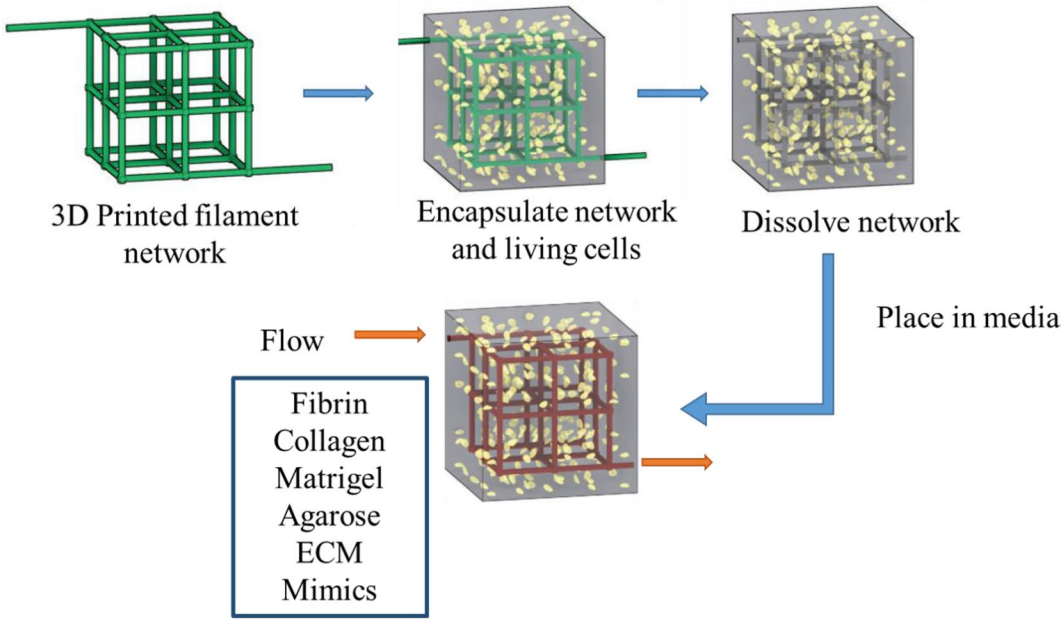

Figure 5: 3D bioprinting of vasculature mimics using sacrificial template technique. Figure illustrating sequence of steps that are followed for this technique[206].

such a way that leaky blood vessels can be introduced to these models. 3D bioprinting can recapitulate the formation of leaky vessels and help us study cell migration[209, 210] and angiogenesis in a heterogeneous tumor in a realistic manner[211]. Another important implication is that the fabrication of these 3D leaky vessels will allow for better drug screening, as it would help in optimizing the mode of nanomedicine delivery and efficient concentration[47, 212].

3D bioprinted cancer models are physiologically relevant and allow repeatability that can be explored for industrial applications such as large-scale screening and development of anticancer therapeutics[198, 213]. 3D bioprinted cancer models with tumor heterogeneity also allow for efficient drug screening.

3D bioprinted cancer model can lead to advancement in the field of personalized medicines. Patient-specific 3D bioprinted cancer models can be used to assess chemoresistance, pharmacogenetics as well as pharmacokinetics leading to efficient drug development[214].

6 Characterization of Bioprintability

A number of parameters have to be assessed before considering a particular bioink for printing. The bioink has to be printable and meet the criteria required for application. The bioink can be characterized for the printability in two steps[215]. Firstly, it is required to perform a screening test in advance to check the fiber formation during printing. This can be done by applying different pressure manually until the material is extruded in the form of a fiber rather than droplets. Analysis can be done by using a stereomicroscope or by taking an image for different parameters such as fiber thickness, shape fidelity and retention of multiple layers. Secondly, rheology, a well-known method for evaluating the flow behavior and reproducibility, can be performed to establish the optimum condition for printing of the bioink formulations. The printing temperature can be decided from the temperature where the viscosity begins to increase sharply. Before initiating the printing process, the developed hydrogel is subjected to various rheological analyses including steady shear sweep, i.e., viscosity v/s shear rate, amplitude sweep, temperature sweep, etc. Viscosity v/s shear rate at a constant temperature can give us a clear idea about the behavior of the gel; generally; all gels used for 3D bioprinting should exhibit shear thinning behavior. Temperature ramp of the bioink is used to study the gelation kinetics and to find out the complex process of gelation in a time- and temperature-dependent manner under shear. Generally, a range of temperature from 4 to 37 °C with an increment of 0.5–1 °C/min was kept for this study. Amplitude sweep gives us an idea about the viscoelastic property of the gel and also gives us information about shape retention. The loss

modulus and storage modulus are plotted with strain percentage on the *x*-axis. It is interesting to note that from temperature 4–26 °C (gelation point), the loss modulus is higher than the storage modulus exhibiting liquid behavior, while from 26 to 37 °C the storage modulus is greater than the loss modulus exhibiting cross-linked gel behavior in case of dECM bioink, which can retain its shape[15]. A stable storage modulus indicates the stable state of the printed gel and the storage modulus v/s time tells us the printable time duration.

7 Existing Challenges in 3D Bioprinting

In comparison to non-biological printing, 3D bioprinting has to encounter various challenges such as limitations in the material selection, cell viability, ethical issues and many more to make it clinically relevant or industrially attractive. For a better understanding, the challenges are classified into two categories: technical challenges and ethical or legal challenges.

7.1 Technical Challenges

One technical issue to be addressed is printability, which defines a precise and accurate deposition of the bioink. Higher resolution will enable better interaction and control in the 3D microenvironment. To reach up to a commercial level, faster printing and scaling up of the process is highly necessary[1]. Based on the native tissue, structural and mechanical properties of the 3D scaffold should be taken into consideration. Use of sacrificial material at the time of printing or alternatively incorporating into the construct is necessary for increasing the mechanical property[1]. However, maintaining adequate mechanical strength of the desired tissue remains a challenge, bearing the availability of the bioinks in use today. There are various trial and errors to solve the issue of vascularization, and one such approach is to build vasculature during bioprinting either with biodegradable or synthetic polymers that eventually lead to a vascularized tissue[24, 216]. Another approach is to mix angiogenic factors in the bioink that can attract the cells within the construct and a vasculature can be formed later[217]. There are also a few other technical limitations faced by researchers while 3D bioprinting any tissue or organ. Designing a tissue or organ blueprint due to complex architecture and heterogeneity of cells is a significant hurdle.

The next challenge is equalizing the mechanical properties of 3D printed organs such as bone, articular cartilage and meniscus to the physiologically relevant tissues by maintaining its biological activity. However, a material of such kind is yet to be introduced. This would involve using scaffolds or ECM components that mimic mechanical properties such as elastic moduli of human tissue. Small structures such as hair follicles and sweat glands as well as organs such as liver or brain or spinal cord with complex architecture remain to be printed in a fully functionalized manner, same as endocrine organs with heterogeneous population of cells.

Another challenge is the possible immunoreaction by recipient in response to scaffolding material or cells present in the 3D printed tissue construct. A possible solution is using native decellularized ECM-based bioink for printing such tissues and organs as well as introduce immunosuppressant molecules during the incubation period of printed tissue or organ in the bioreactor before implantation. Development of personalized patient-specific tissue or organ will remain an issue, as it would take not only more resources with respect to acquisition and isolation of stem cells and decellularized ECM, but also more time to print at a commercial level.

Bioprinting, like any other technology, needs constant updates to move toward the aim of printing a biomimetic structure and functional organ. Some of the significant elements to reach toward this Herculean aim are developments from the hardware of bioprinters, the bioink composition, as well as printing techniques.

7.2 Ethical Issues

The first concern that arises is whether there is anything that ought not to be printed. It is important to weigh the potential negative and positive consequences as well as mechanisms available for reducing the risk of the negative consequences. Even after obtaining positive results during the testing protocols before the human trials, sustainable results are not guaranteed due to each patient's unique genetic makeup. Due to such barriers, a new regulatory framework of clinical evaluation may need to be specifically developed for 3D bioprinting to answer the core issue associated with standardization and scaling up personalized medical treatments. Regulatory agencies across the globe, including the European Commission, have found 3D bioprinting challenging and are still undecided on how to address the potential and uncertain risk of harms associated with this technology[218]. According to the FDA's recent Technical Considerations for Additive Manufactured

Devices, biological, cellular or tissue-based products manufactured using 3D printing technology are excluded as they "may necessitate additional regulatory and manufacturing process considerations and/or different regulatory pathways" (FDA.gov 2016). From a worldwide perspective, to date, only South Korea (Ministry of Food and Drug Safety) and Japan (Pharmaceuticals and Medical Devices Agency) have provided some kind of specific regulatory guidance loosely applicable to 3D bioprinting. Even so, the guidance provided is quite broad (MFDS 2015).

8 Conclusion and Future Perspective

Bioprinting remains a promising solution for addressing the increasing organ shortage for transplantation globally. The ability to generate tissues for transplant with a lower immune response risk holds significant promise in the fabrication of artificial organs. The recent progress in hydrogel science, including the development of dynamic switchable hydrogels[219] and oxygen-producing hydrogels[220] provides more and more methods that enable control of cell microenvironments. However, the full potential of 3D bioprinting can be realized by improving upon the printing speed, capability to bioprint at various scales, availability of bioprintable materials and hydrogels, vascularization of tissues, innervation of tissues and on-demand scaffold fabrication and cell maturation mechanisms.

This review paper provides the current state of the art of bioprinting technology application in the field of tissue engineering and regenerative medicine. It highlights different interesting applications of bioprinting technology in organ printing and cancer models. 3D bioprinting involves printing cells and materials like ECM components together to form a viable tissue construct following a one-step biofabrication process. This is followed by incubation of the 3D printed tissue or organ in a bioreactor before any further experimental procedures are performed and then passed on for preclinical and clinical trials.

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Ashis Kumar Bera is a recipient of CSIR-UGC JRF fellowship for pursuing PhD. He holds a bachelor's degree and a master's degree in human physiology. In the field of regenerative medicine, 3D Bioprinting is the most promising technology to design and fabricate tissues to restore parts that are lost in trauma or diseases. In this regard, fabricating a vascularised, structural and functional 3D bio-printed tissue stands challenging. He is passionate to develop a highly vascularized 3D bioprinted tissue model for tissue repair.

Dr Sri Sai Ramya Bojedla She is an Oral physician and a Maxillofacial Radiologist by profession with a good clinical experience. She is currently a research fellow at IIT Hyderabad working with a great passion to provide the best health care and to improve the present-day treatment strategies. She is working on the development of customized implants for maxillofacial reconstruction.

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