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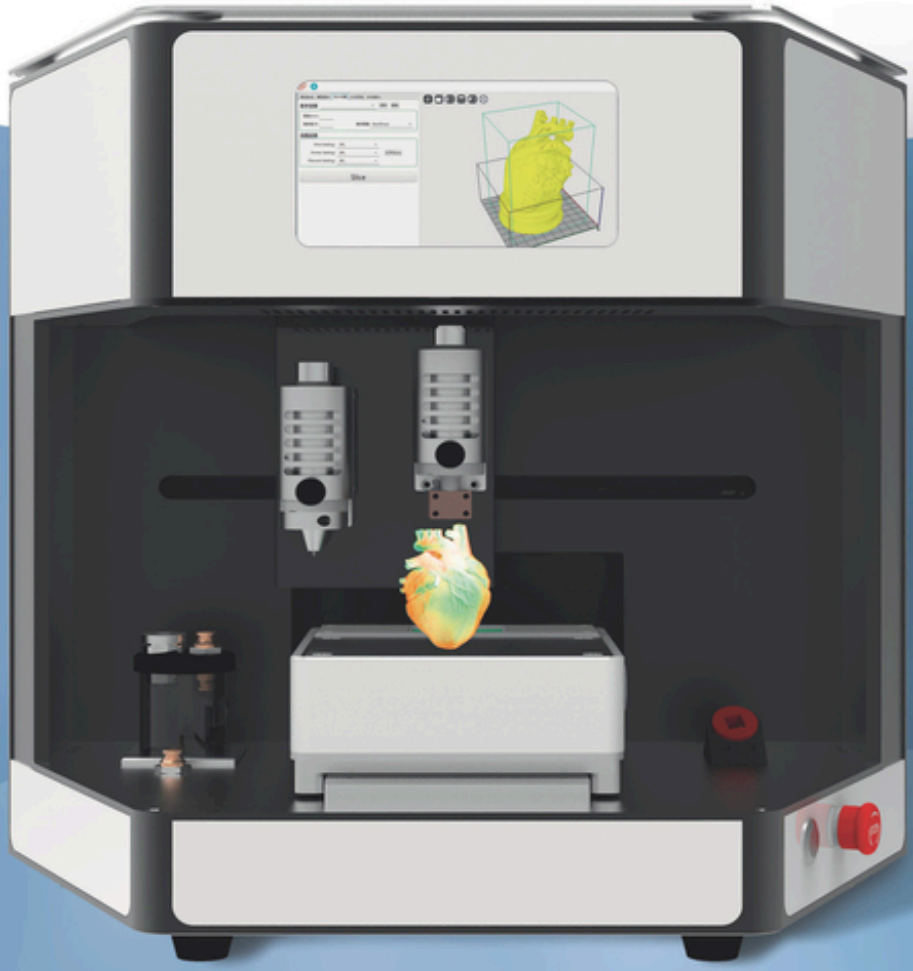
Cell Assembly with 3D Bioprinting



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Cover Image: Yong He

Library of Congress Card No.: applied for

British Library Cataloguing-in-Publication Data
A catalogue record for this book is available from the British Library.

Bibliographic information published by the Deutsche Nationalbibliothek
The Deutsche Nationalbibliothek lists this publication in the Deutsche Nationalbibliografie; detailed bibliographic data are available on the Internet at <http://dnb.d-nb.de>.

Print ISBN: 978-3-527-34796-4
ePDF ISBN: 978-3-527-82857-9
ePub ISBN: 978-3-527-82858-6
oBook ISBN: 978-3-527-82859-3

Cover Design Adam-Design, Weinheim, Germany
Typesetting Straive, Chennai, India

Printed on acid-free paper

10 9 8 7 6 5 4 3 2 1

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Preface

3D bioprinting has opened up a frontier in biomedical research, aiming at additive manufacturing or assembling living structures with cells, which provides the possibility to generate significant breakthroughs, yielding new treatments, and change the foundation of regenerative medicine. It is truly an interdisciplinary field, cross-fertilized ranging from mechanical engineering, materials science, and computer science to biology, medicine, pharmaceutical science, and so on.

The potential applications for 3D bioprinting include: (i) in vitro 3D tissue/organ models for drug screening, organ development, toxicological and cosmetic research, etc.; (ii) 3D biofabrication of living structures for clinical transplantation or tissue repair.

Compared to conventional additive manufacturing, 3D bioprinting possesses three remarkable characteristics: (i) **bioprinting usually utilizes cell-laden hydrogel** (called bioink) in terms of material use; (ii) **bioprinted structures have to go through hydrogel crosslinking process** (thermal, chemical, or enzymatic) during formation for manufacturing desired tissue structures; (iii) **cross-talking and functionalization of cells to acquire some tissue properties after printing is the goal**. In these regards, bioprinting faces two major challenges: (i) structural controlled manufacturing, which requires a stable printing process to ensure cell-laden hydrogels being accurately assembled. As the bioink is something like a soft tofu, precision manufacturing is difficult; (ii) functional controlled postprocessing, which needs to provide biomimetic microenvironment with physical and chemical stimulation for living constructs realizing functionalization.

Combined with our research experiences in 3D bioprinting over years, this book is outlined in 14 chapters: commonly used 3D bioprinting methods are summarized first; then the design of bioink is put forward; several bioprinting approaches are elaborated afterward including coaxial bioprinting, digital light projection, direct ink writing, and liquid support bath-assisted 3D printing; in the following parts, microgel-based, microfiber-based, and microfluidics-based biofabrication approaches and their applications are meticulously illustrated; and a protocol of 3D bioprinting is well represented in the end to show several examples of complete bioprinting process.

We wish to thank the valuable support from everyone who contributed to this book. This book would never have been published without your effort. Thanks to

Dr. Zeming Gu for help in writing Chapter 1; thanks to Prof. Changxue Xu for help in writing Chapter 2; thanks to Mr. Peng Zhang for help in writing Chapter 3; thanks to Dr. Chaoqi Xie for help in writing Chapter 4; thanks to Dr. Yuan Sun for help in writing Chapter 5; thanks to Mr. Cheng Zhang for help in writing Chapter 6; thanks to Dr. Weijian Hua for help in writing Chapter 7; thanks to Dr. Mingjun Xie for help in writing Chapters 8, 9, and 14; thanks to Prof. Lei Shao for help in writing Chapters 10 and 11; thanks to Dr. Jing Nie for help in writing Chapters 12 and 13. Thanks to Zhengyi Zhang, Danyang Zhao, Lily Raymond, Heqi Xu, Matthew Warner, and Beatriz Godina for their help in completing this book. Thanks to Ms. Katherine Wong for editing the manuscript.

We sincerely hope that our readers will find the book professionally written, richly illustrated, accessible, and most importantly, intriguing. We would be flattered if this book attracts new researchers from different disciplines into the field of 3D bioprinting. Due to limited time and scholars with different backgrounds involved in compilation, there may be some unsatisfactory points in the writing style or content of this book. Therefore, readers are welcome to put forward criticism or suggestions for our further improvement.

4 May 2021

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1

3D Bioprinting, A Powerful Tool for 3D Cells Assembling

1.1 What Is 3D Bioprinting?

3D printing, also known as additive manufacturing, is a layer-by-layer manufacturing approach, and it has been applied in many industrial applications and research fields. It could be thought of as an inverse process of potato cutting, assembling the chips or slices into integrity by certain rules. When 3D printing met biomedical engineering, 3D bioprinting was born. 3D bioprinting is an interdisciplinary science closely related to medicine biology, mechanical engineering, and material science. It can be divided into two concepts. Broadly speaking, 3D bioprinting refers to the use of 3D printing technology to achieve biomedical applications, such as the printing of medical aids, polymers, ceramics, or metal scaffolds [1–3]; in a narrow sense, this concept simply means 3D cells assembling through printing, therefore it can also be identified as cell printing or organ printing [4–6]. Here, this book is mainly focusing on the narrow viewpoint. A cartoon introduction of 3D bioprinting is illustrated in Figure 1.1.

In vitro bio-manufacturing of tissues/organs has always been a great dream pursued by mankind, driven by two needs: organ transplantation and accurate tissue models. First, there is a huge shortage of organs for transplantation. In 2016, there were 160 000 organ transplant recipients, but only 16 000 organ donors in the United States [8]. The complexity of human organs is not only reflected in the mechanism of organ growth that has not been revealed by biology, but also in the reproduction of fine structure manufacturing. The use of 3D bioprinting technology to solve the shortage of organ transplants is far too optimistic at the present stage. Second, traditional methods utilizing 2D cell culture were applied for drug screening and medical mechanism studies. However, microenvironment in vivo is far more complex than the 2D cell culture, and in some cases, 2D models may lead to opposite results. 3D bioprinting technology can realize spatiotemporal directional manipulation of various cells and has become the most ideal method to construct a 3D cell-laden structure in vitro.

In vitro models have undergone a meaningful revolution both in forms and functions: mini-tissue, organ-on-a-chip, and tissue/organ construct. Based on common bioprinting techniques, 3D mini-tissue in forms of spheres, fibers, or other geometric shapes could be fabricated [9, 10]. These models contribute to the simulation

Cell Assembly with 3D Bioprinting, First Edition. Yong He, Qing Gao, and Yifei Jin.

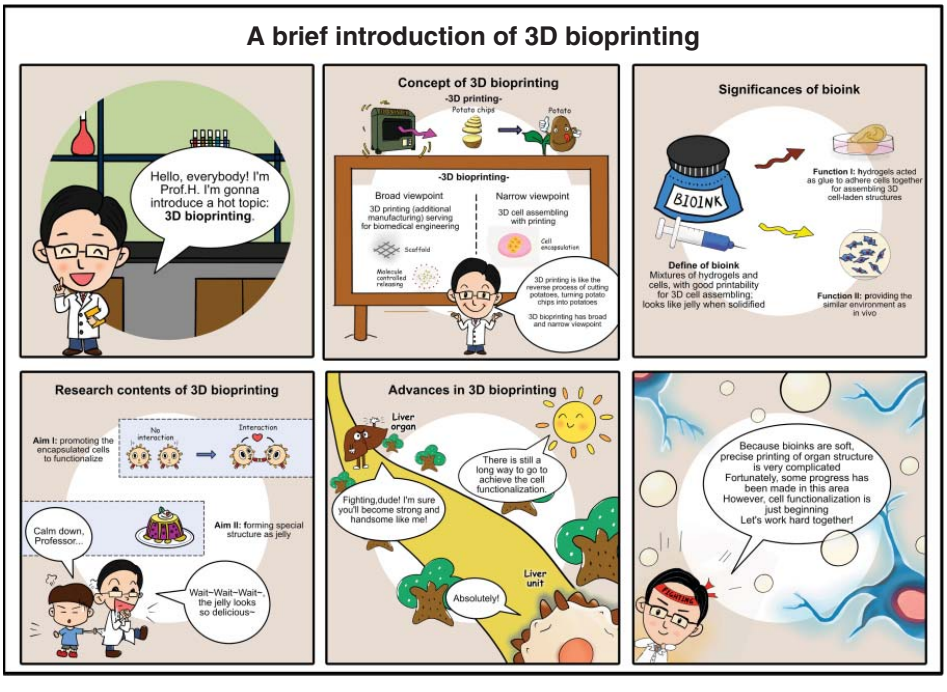

Figure 1.1 Introduction of 3D bioprinting. Source: He et al. [7]. Reproduced with permission of Springer Nature.

of functional units with simple composition and independent operation, which can be applied in high-throughput testing with a low dose. Besides, 3D bioprinting has been gradually involved in the setting up of organ-on-a-chip devices because of its excellent customizability and cell compatibility [11]. Modified microfluidic systems could be constructed with biomaterials through 3D bioprinting, on which specified cells are loaded and routine reactions were carried out. And the interactions and cross-talking between multiple organs can be well simulated by connecting different modules by means of microfluidic methods. Furthermore, 3D bioprinting has been further facilitated in the biofabrication of tissue/organ constructs with an inner channel network. A large number of 3D bioprinting strategies have been adopted in building 3D tissue/organ constructs with a vascular network, including coaxial bioprinting, projection-based bioprinting, as well as the integration of 3D bioprinting and sacrificial templates.

1.2 Evolution of 3D Bioprinting

As mentioned above, it is not practical to realize 3D bioprinting for full-function organ transplantation at present. However, it is an undeniable fact that bioprinting techniques have come a long way. Decades ago, pioneers such as Vladimir Mironov, Gabor Forgacs, and Thomas Boland saw the natural combination of technologies including cell patterning and others, such as commercial inkjet printing, to build living structures that might one day be used for human organ transplantation [6, 12, 13]. A timeline for the evolution of bioprinting technology up to state-of-the-art is illustrated in Table 1.1.

In 1984, Charles Hull invented stereolithography (SLA) for printing 3D objects from digital data, symbolizing the birth of 3D printing. Bioprinting was first demonstrated in 1988 while Klebe using a standard Hewlett–Packard (HP) inkjet printer to deposit cells by cytoscribing technology [14]. In 1996, Forgacs and coworkers drew a conclusion that apparent tissue surface tension was the macroscopic manifestation of molecular adhesion between cells and provided a quantitative measure for tissue cohesion [15]. In 1999, Odde and Renn first utilized laser-assisted bioprinting to deposit living cells for developing analogs with complex anatomy [16]. In 2001, direct printing of a scaffold in the shape of a bladder and seeding of human cells took place [17]. In 2002, the first extrusion-based bioprinting technology was reported by Landers et al., which was later commercialized as "3D-Bioplotter" [18]. Wilson and Boland developed the first inkjet bioprinter in 2003 by modifying an HP standard inkjet printer [19]. Their team implemented cell-loaded bioprinting with a commercial SLA printer a year after [20]. Also in 2004, 3D tissue with only cells (no scaffold) was developed. In 2006, electrohydrodynamic jetting was applied to deposit living cells [21]. Scaffold-free vascular tissue was engineered through bioprinting by Norotte et al. in 2009 [22]. In 2012, in situ bioprinting was attempted by Skardal et al. on mouse models [23]. The following years saw the introduction of many new bioprinting products, such as articular cartilage and artificial liver in 2012, tissue integration with the circulatory system in 2014, and so on [24, 25]. In 2015, coaxial

Table 1.1 Timeline for bioprinting evolution.

Year	Development
1984	Stereo lithography was invented, representing the birth of 3D printing
1988	Bioprinting was first demonstrated by 2D micro-positioning of cells
1996	Cells sticking together during embryonic development was observed
1999	First use of laser technology demonstrating 2D patterning of living cells
2001	3D printed synthetic scaffold for human ladder
2002	First extrusion-based bioprinter was achieved
2003	First inkjet bioprinter was developed
2004	3D tissue with only cells (no scaffold) was presented
2009	Scaffold-free vascular constructs were fabricated
2012	In situ bioprinting was realized on animals
2015	Tubular structure was printed by coaxial technology.
2016	Rapid continuous optical 3D printing based on projection (DLP) was applied
2016	Cartilage model was obtained by ITOP system
2019	Cardioid structure was first bioprinted
2019	Collagen human heart at various scales was built using FRESH technology

technology was adopted by Gao et al. for the fabrication of a tubular structure [4]. In 2016, Pyo et al. applied rapid continuous optical 3D printing based on digital light processing (DLP) [26]. In the same year, a cartilage model was manufactured by Anthony Atala's research group using an integrated tissue-organ printer (ITOP) [27]. In 2019, Noor et al. succeeded in manufacturing a perfusable scale-down heart [28], and a few months later, bioprinting of collagen human hearts at various scales based on the freeform reversible embedding of suspended hydrogels (FRESH) technology was achieved by Lee et al. [29].

1.3 Brief Classification of 3D Bioprinting

Based on different printing principles, cell-laden 3D bioprinting can be divided into three types: extrusion-based, droplet-based, and projection-based bioprinting. Extrusion-based bioprinting generates continuous fibers to set up the structures; droplet-based bioprinting produces droplets as the basic unit for biofabrication and projection-based bioprinting takes advantage of the properties of photosensitive materials by stacking 3D models layer-by-layer. Different approaches possess diverse characteristics aiming at various scenarios and have specific requirements for bioinks.

Extrusion-based bioprinting is the most widely used method, which is suitable for a wide range of biocompatible materials. According to different liquid dispensing

modes, pneumatic-driven, piston-driven, and screw-driven extrusion systems are applied to extrude cell-laden bioinks in the form of continuous filaments.

Droplet-based bioprinting which employs discrete droplets stacked into constructs can be roughly divided into inkjet bioprinting [30], electrohydrodynamic jetting (EHDJ) [31], and laser-assisted bioprinting (LAB) [32] based on different droplets forming principles. Thermal and piezoelectric-driven technologies are most commonly used in inkjet bioprinting. EHDJ uses a high voltage motivated electric field to pull droplets out of the nozzle orifice. Changes in voltage certainly affect the size of each droplet, where the higher voltage leads to smaller droplets [33, 34]. LAB is a non-contact, nozzle-free bioprinting strategy used precisely to deposit bioink droplets. LAB technique includes laser-guidance direct writing (LGDW) and laser-induced forward transfer (LIFT). LGDW employs a light trap to guild cells onto a substrate, while LIFT uses a focused pulsed laser to induce partial evaporation of bioink coating to propel the biomaterial toward the receiving layer.

Projection-based bioprinting solidifies light-sensitive biomaterials to form constructs under precisely controlled lighting with high printing precision and fast printing speed. The most common use of projection-based bioprinting is to print cell-free scaffolds, where cells would be seeded post-printing. Currently, however, cell-laden projection-based bioprinting has also been reported using DLP technology.

1.4 Evaluation of Bioinks

Generally speaking, 3D bioprinting has three steps: preparing bioinks, printing the soft live structures with multiple cells, and rebuilding the interaction among cells. And that is why developing appropriate bioinks has always been a significant part, as it affects every step that follows.

The performance of bioinks can be measured by three main factors: printability, biocompatibility, and mechanical property. Printability is to assess the formability of bioinks, where adjustable material viscosity, rapid transition from sol state to gel state, and a broad range of printing parameters are necessary. Biocompatibility is a measure of biomimicry that requires bioink and printed cells to be as similar as possible in the microenvironment in vivo. The mechanical property requires that the cured bioink be strong enough to hold subsequent culture and implantation. Perfusion and degradation might occur during bioprinted constructs culture in vitro, which requires considerable strength to support.

Therefore, the choice of bioink necessitates compromise among printability, biocompatibility, and mechanical property. Considering the requirements of the bioprinting process, cell growth and proliferation, and structural integrity, reasonable bioink design can be carried out according to the actual cell type and printing resolution requirements. But in fact, these three requirements of bioink are inherently contradictory in the mechanism. For example, the higher the viscosity of biological ink, the better the printability, and vice versa, the poorer the biocompatibility.

Hence, bioink selection to meet the specific needs of different applications is a key step in bioprinting.

An ideal bioink would certainly be close to the natural extracellular matrix (ECM), and it would need to be adapted to match different types of cells. Therefore, it could not be better to add specific substances in bioinks that cells possibly need during proliferation and functionalization. For example, when bioprinting chondrocytes, the addition of HA, a common component of cartilage, can significantly promote later culture and functionalization.

Typical bioinks applied in bioprinting may include hydrogels, decellularized matrix components, microcarriers, tissue spheroids and strands, cell pellet, and/or some advanced bioinks such as multi-material, interpenetrating network, nanocomposite, and supramolecular bioink, etc. [35, 36]. Among them, hydrogels are considered to be one of the most important biomaterials in bioinks, because of their outstanding capability of providing a viable microenvironment for cell adhesion, growth, and proliferation. Natural/synthetic hydrogels including alginate, fibrinogen, gelatin, collagen, silk fibroin, chitosan, agarose, pluronics, HA, GelMA, PEG, PEO, etc., have been found in countless applications in bioprinting. They are either ion-sensitive, photosensitive, thermosensitive, enzyme-sensitive, or pH-responsive, so they can be easily gelated to form constructs before, during, and/or after bioprinting [37].

1.5 Outlook and Discussion

3D bioprinting technologies still need further improvement. The complexity of tissues and organs has brought great difficulties to accurate bioprinting. One of the major disadvantages of current bioprinting technologies is the low accuracy of bioprinting compared to natural tissues/organs. Most tissues/organs are more delicate than current bioprinting devices. Another common drawback of bioprinting is the slow speed of bioprinting of complex scale-up structures, especially when it comes to multi-material alternate biofabrication.

Vascularization is the basis of bioprinted structures. Same as the challenge of tissue engineering and regenerative medicine, ensuring adequate vascularization in bio-manufactured structures is a key factor in 3D bioprinting. The effective construction of a multi-scale perfusion vascular network and the promotion of its vascularization by mechanical or chemical stimulation are the basis of the biological fabrication of scale-up constructs.

Functionalization is the primary goal for 3D bioprinting. Most of the current research is still focused on the manufacturing idea-oriented printing process and mechanism, while functionalization is the core factor leading 3D bioprinting from basic research to practical application. In order to be functional, bioink needs to have excellent biocompatibility and mechanical properties to meet the requirements of nutrient perfusion and implantation. In addition, the construction of microenvironments that mimic in vivo scenarios, including mechanical and

chemical stimuli such as perfusion culture and growth factors, is also critical for the functionalization of bioprinted structures.

Combined with the outlook of 3D bioprinting, there are several printing methods that are quite promising: DLP, coaxial bioprinting, and embedded bioprinting. Due to its intrinsic principle, DLP has a much higher printing resolution and speed than other bioprinting approaches. As a key application of 3D bioprinting technology, in vitro tissue models need to be standardized not only in sizes, but also in biological and mechanical properties, while DLP owns excellent uniformity and reproducibility compared to other methods. Additionally, coaxial bioprinting has become an increasingly popular extrusion-based bioprinting method since it was introduced into the field of tissue engineering in 2015 [4], especially in the area of blood vessel biofabrication/vascularization. The biggest advantage of coaxial bioprinting is its ability to construct hierarchical tubular structures with tunable biological/mechanical properties. It is well known that hydrogels with good biocompatibility tend to have insufficient mechanical strength. Coaxial bioprinting can partly solve the problem with its core-shell structure: core materials guarantee biocompatibility, while shell materials provide mechanical strength and vice versa. The use of sacrificial materials as the core material would also contribute to the convenient bioprinting of hollow tubular structures. Besides, embedded bioprinting allows anti-gravity writing of 3D freeform constructs within yield stress and gel-based supporting bath, which would be further removed post-printing to retrieve models with desired shapes or channels. Other than traditional bioprinting approaches, it can achieve the fabrication of discrete patterns, which are not mechanically supported [38–40].

In addition to the challenges including bioinks design, bioprinting techniques, vascularization, and functionalization, issues such as cell sources, bioreactor construction, and even ethical problems also require considerable attention. 3D bioprinted fully clinical translation could take a long time until bio-artificial tissues such as cartilage or skin, to be applied in transplantation. We all hope that 3D bioprinting can find its way from structural similarity into functional realization.

This book is organized into 14 chapters. This chapter "3D Bioprinting, A Powerful Tool for 3D Cells Assembling," covers the definition, evolution, and classification of 3D bioprinting. Chapter 2 "Representative 3D Bioprinting Approaches" and Chapter 3 "Bioink Design" demonstrates a variety of commonly used 3D bioprinting methods in detail, and introduces the principle of bioink design. In Chapter 4 "Coaxial 3D Bioprinting," Chapter 5 "Digital Light Projection-Based 3D Bioprinting," Chapter 6 "Direct Ink Writing for 3D Bioprinting Applications," and Chapter 7 "Liquid Support Bath-Assisted 3D Bioprinting," four types of promising 3D bioprinting technologies and their applications are highlighted respectively. Chapter 8 "Bioprinting Approaches of Hydrogel Microgel," and Chapter 9 "Biomedical Applications of Microgels" provides the manufacturing process and medical use of microgels. Chapter 10 "Microfiber-Based Organoids Bioprinting for in vitro Model" and Chapter 11 "Large Scale Tissues Bioprinting" are mainly concerned with biofabricated organoids and scale-up tissues. In Chapter 12 "3D Printing of Vascular Chips" and Chapter 13 "3D Printing of in vitro Models," vascular chips and

in vitro models by 3D printing approaches are well presented. Finally, Chapter 14 "Protocol of Typical 3D Bioprinting," comes up with an integrated blueprint for 3D bioprinting.

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2

Representative 3D Bioprinting Approaches

2.1 Introduction

Three-dimensional (3D) bioprinting, defined as the spatial patterning of living cells, biomaterials, drugs, growth factors, and genes in a layer-by-layer manner, has been rapidly developed in recent years and widely used for fabricating living tissue and organ constructs for various biomedical applications [1–3]. It is envisioned as the first step toward the fabrication of functional replacement human organs in the future by bridging the gap between numerous biologics and integrated 3D living constructs.

In 3D bioprinting, building blocks are the fundamental units to construct living tissue and organ structures. Based on the methodology to form basic building blocks, current 3D bioprinting techniques can be divided into two categories: orifice-based and orifice-free [4]. In orifice-based bioprinting, nozzles with microscale orifices are used as the tools to form either cell-laden spheroids or strands as the building blocks. Inkjet bioprinting and extrusion bioprinting are two representative orifice-based 3D bioprinting techniques. In orifice-free bioprinting, different light sources are used as tools to form cellular layers instead of microscale orifices. Thus, the orifice-free bioprinting is also known as light-based bioprinting, as shown in Figure 2.1.

In orifice-based bioprinting technologies, the typical biofabrication process consists of two main steps: printing and cross-linking. The core function of the printing step is to form building blocks at a liquid state and then deposit them based on the designed trajectories. In inkjet bioprinting, different methods are used to form cell-laden spheroids, namely droplets, as the building blocks, while in extrusion bioprinting, cell-laden filaments are generated with continuous, cylindrical morphology to construct 3D structures. The main purpose of the subsequent cross-linking step is to solidify the deposited droplets and/or filaments rapidly. Thus, the solidified droplets and/or filaments cannot only keep their shapes as designed, but also have the mechanical stiffness to support the following deposited droplets and/or filaments. By repeating the printing and cross-linking steps, 3D structures can be fabricated by orifice-based bioprinting technologies. As a result, classic orifice-based bioprinting is performed in a "solidification-while-printing" fashion.

Orifice-based 3D bioprinting technologies have many advantages. First, the independent cross-linking step makes it possible to print biomaterials with different

Cell Assembly with 3D Bioprinting, First Edition. Yong He, Qing Gao, and Yifei Jin.

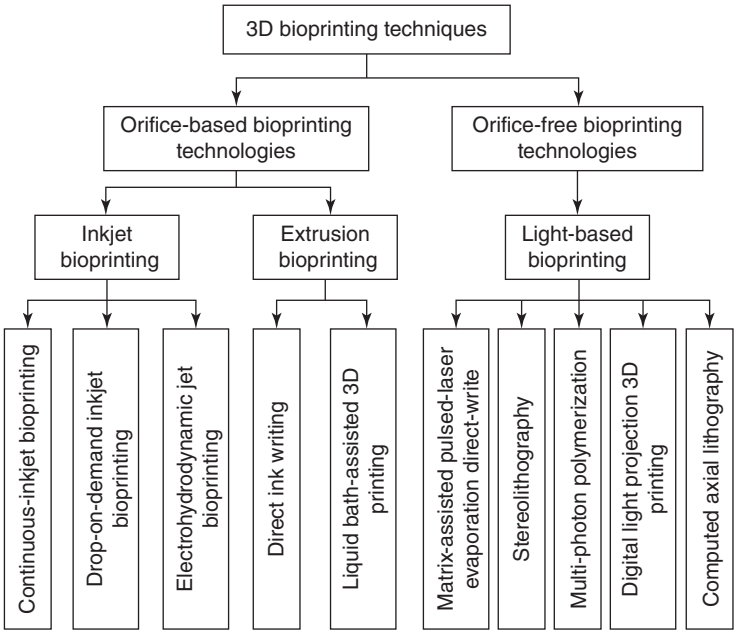

Figure 2.1 Classification of representative 3D bioprinting approaches. Source: Yifei Jin.

cross-linking mechanisms. Thus, orifice-based bioprinting technologies have a wider range of printable materials. Second, it is feasible to improve fabrication efficiency by simultaneously printing via numerous printheads with microscale nozzles. Third, orifice-based bioprinting technologies provide a technical solution to print cellular constructs with different cell types. In both inkjet and extrusion bioprinting, multiple nozzles can be integrated within one printing system to deposit different cells through corresponding nozzles, facilitating the fabrication of spatially heterocellular constructs. Finally, current inkjet bioprinters and extrusion bioprinters can be easily implemented at affordable prices. This is due to the fact that ink-jetting and extrusion are mature technologies and widely used in painting/printing and polymer/metal processing, respectively. However, orifice-based 3D bioprinting technologies have complications. The main challenge during orifice-based bioprinting is the high shear-stress-induced cell damage when cells flow through microscale nozzles. For inkjet bioprinting, the typical cell viability after printing is above 85% [1]. To form droplets with a well-defined shape, cell density in inkjet bioprinting is always controlled at a low level, less than 10^6 cells/ml. For extrusion bioprinting, living cells are propelled through nozzles with microscale diameters to form filaments. During this process, high shear stress may bring damages to cell membranes and kill cells. Thus, typical cell viability in extrusion bioprinting falls in the range of 40–80%, even lower than that in inkjet bioprinting [1].

Orifice-free 3D bioprinting technologies cover several light-based 3D printing approaches. Based on the different functionalities of a light source, they are divided into different subcategories. In matrix-assisted pulsed-laser evaporation (MAPLE)

direct-write, the laser beam is illuminated on biomaterial-coated quartz to repel the localized materials away from the quartz in the morphology of droplets. This approach is also known as laser-assisted 3D bioprinting. Since MAPLE uses droplets as the basic building blocks to construct 3D structures, the fabrication process is also composed of printing and cross-linking steps, similar to that of inkjet bioprinting. However, different from inkjet bioprinting, living cells do not experience orifice-induced high shear stress during droplet formation. As a result, cell viability in MAPLE is above 95%, much higher than those in both inkjet bioprinting and extrusion bioprinting. Despite the high cell viability, MAPLE is also constrained by some complications. On the one hand, preparation of coated quartz is time-consuming, which makes the fabrication efficiency relatively low. On the other hand, the high price of laser system increases the cost of MAPLE significantly, which is another concern for clinical tissue engineering applications.

Aside from MAPLE, other light-based 3D printing approaches combine the printing step with the cross-linking step. Thus, cellular biomaterials are loaded in a container before printing and then cross-linked in situ via different light sources. In particular, during stereolithography (SLA) and multi-photon polymerization (MPP), one or multiple light spots are used to selectively cross-link cellular biomaterials in a pixel-by-pixel or voxel-by-voxel manner, while during digital light projection (DLP) and computed axial lithography (CAL), each cross-section of the designed 3D structure is projected via light pattern to cause the solidification of biomaterials in a layer-by-layer manner. Since no orifice is used during printing, the cell viability of these light-based 3D printing approaches can be very high. In addition, the resolution of the printed 3D structures mainly depends on the size of the light spot and the accuracy of the light pattern, which can be easily controlled and improved. As a result, these 3D printing approaches are promising when creating 3D structures with high resolution. The disadvantage of the aforementioned light-based 3D printing approaches is the limited material selection. To facilitate the printing process, biomaterials must be photocurable, which severely constrains the applications of these 3D printing approaches to fabricate living tissue and organ constructs from various non-photocurable materials.

In the following sections, the mechanism, printable biomaterials, and representative biomedical applications of each 3D bioprinting strategy will be introduced in detail. In particular, Section 2.2 will focus on inkjet bioprinting technology. Section 2.3 will cover basic information on extrusion bioprinting, which will further be discussed in detail in Chapters 6 and 7. Section 2.4 will introduce some well-developed light-based 3D printing techniques such as MAPLE, SLA, MPP, and several newly proposed printing strategies, such as DLP and CAL.

2.2 Inkjet Bioprinting

Inkjet bioprinting is a droplet-based 3D bioprinting technique in which cell-laden droplets are used as the basic building blocks to construct complex 3D structures. Since this technique makes it feasible to accurately control the volumes and locations

of the deposited bioink, it has been widely used in tissue engineering to fabricate spatially heterocellular constructs from various cellular inks. In this section, we will introduce this 3D bioprinting technique from three aspects: droplet formation mechanisms, materials, and representative biomedical applications.

2.2.1 Mechanisms of Droplet Formation

In inkjet bioprinting, a cellular bioink is squeezed through a microscale nozzle to generate cell-laden droplets. By leveraging multiple factors including gravity, surface tension, and the fluid mechanics of the bioinks, the droplets are ejected from the nozzle and land on a receiving substrate. Based on different mechanisms to form droplets, inkjet bioprinting can be classified into three subcategories: (i) continuous-inkjet (CIJ), (ii) drop-on-demand (DOD), and (iii) electrohydrodynamic (EHD) jet, as shown in Figure 2.2.

2.2.1.1 Continuous-Inkjet Bioprinting

In CIJ bioprinting, cellular bioink is pushed through a nozzle under constant pressure to form a continuous liquid jet. Due to Rayleigh–Plateau instability, this liquid jet rapidly breaks up into a stream of droplets before landing on a substrate, as shown in Figure 2.2a. During the droplet formation, Rayleigh–Plateau instability plays an important role in which the liquid jet is perturbed by several factors including potential energy (gravity), surface energy (surface tension), and kinetic energy [5, 6]. When the wavelength of the perturbed jet is larger than the initial radius by a

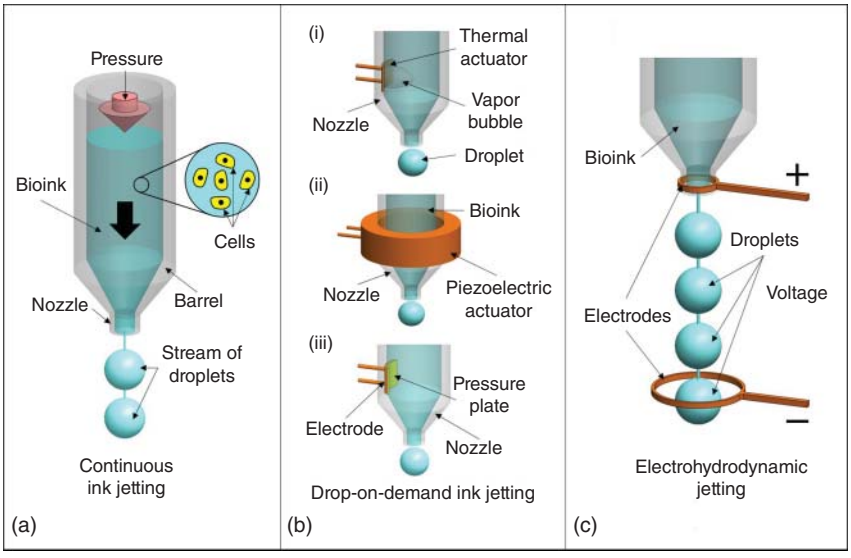

Figure 2.2 Mechanisms of droplet formation in inkjet bioprinting. (a) Continuous ink-jetting based on Rayleigh–Plateau instability. (b) Drop-on-demand ink-jetting including: (i) thermal ink-jetting, (ii) piezoelectric ink-jetting, and (iii) electrostatic ink-jetting. (c) Electrohydrodynamic jetting based on an electric field. Source: Yifei Jin.

certain limit, the perturbation grows rapidly, breaking the jet into a series of droplets to minimize its potential energy. The condition to form droplets in CIJ can be simply modeled as a function of the number of waves per unit length (k) on the perturbed jet and the initial jet radius (R_0). When kR_0 is less than 1, the jet can distort itself into continuous droplets. The detailed information regarding Rayleigh–Plateau instability can be found in other published reports [7].

2.2.1.2 Drop-on-Demand Inkjet Bioprinting

Different from CIJ, DOD inkjet bioprinting can generate droplets based on requirements. Thus, it is much easier to accurately control the locations of the deposited droplets and pattern cells and other biologics accordingly. As a result, DOD is the most popular inkjet bioprinting technique for cell bioprinting purposes. In DOD inkjet bioprinting, bioinks are transferred from fluid chambers to nozzles and squeezed out of the nozzles using different mechanisms. Since multiple nozzles can be used to print simultaneously, DOD inkjet bioprinting has the capacity of printing 3D cellular constructs with high efficiency. Currently, there are three main mechanisms to generate droplets during DOD ink-jetting including: (a) thermal inkjet, (b) piezoelectric inkjet, and (c) electrostatic inkjet.

A. *Thermal inkjet bioprinting*. In thermal inkjet bioprinting, a thermal actuator is integrated with a nozzle and heats bioink under a voltage pulse. This localized heating leads to the formation of a vapor bubble as shown in Figure 2.2b(i), which rapidly expands in the nozzle and generates pressure to the localized bioink, pushing it to the exit of the nozzle. Eventually, the bioink overcomes surface tension at the nozzle's exit and forms a droplet. Thermal inkjet bioprinting has been widely used to print living tissue constructs from various liquid bioinks such as polyethylene glycol dimethacrylate (PEGDMA), alginate, and cell suspensions. For example, Xu et al. [8] printed complex and heterogeneous 3D tissue constructs using a modified thermal inkjet printer. In their study, they mixed human amniotic fluid-derived stem cells (hAFSCs), canine smooth muscle cells (cSMCs), and bovine aortic endothelial cells (bECs) separately with calcium chloride ($CaCl_2$) as the cell-laden inks and then printed them layer-by-layer to the predetermined locations in a sodium alginate-collagen composite, as shown in Figure 2.3a. The ionic cross-linking between alginate and $CaCl_2$ led to the formation of a solid composite gel with encapsulated heterogeneous cells. The printed cellular constructs were able to survive and mature into functional tissues after incubation and generated adequate vascularization *in situ*, which validates the feasibility of inkjet bioprinting heterogeneous living tissue constructs with multiple cell types.
B. *Piezoelectric inkjet bioprinting*. In piezoelectric inkjet bioprinting, a piezoelectric actuator is used to generate a localized pressure pulse in the nozzle. The piezoelectric actuator can provide a radial deformation when a voltage pulse is applied, leading to the change of the bioink volume in the nozzle. Thus, a given volume of bioink is pushed toward the exit of the nozzle. After overcoming the surface tension at the exit, the bioink is ejected in the form of droplets, as

shown in Figure 2.2b(ii). By depositing the resulting droplets layer by layer, 3D constructs can be fabricated. Different from thermal ink-etting in which the bioink is heated periodically, the pressure pulse generated in piezoelectric ink-jetting is caused by the deflection of the actuator. Thus, the entire droplet formation is considered as an isothermal process and the effect of temperature change on the cell viability can be ignored. As a result, piezoelectric inkjet bioprinting is popularly used in tissue engineering to fabricate 3D tissue constructs. For example, Xu et al. [9] used a piezo-based inkjet bioprinting system to successfully print overhang tubular constructs as shown in Figure 2.3b. In their study, they used sodium alginate with a low concentration (2% [w/v]) as the matrix and added fibroblasts (3T3 cells) to prepare the cell-laden bioink. Thus, droplets containing 3T3 cells were deposited based on the designed trajectory in a $CaCl_2$ bath to form solid cellular constructs. The post-printing cell viability was above 82%, proving the cytocompatibility of the piezoelectric inkjet bioprinting approach.

C. *Electrostatic bioprinting*. Similar to piezoelectric inkjet bioprinting, in electrostatic bioprinting, droplets are generated by the pressure pulse resulting from the deformation of a pressure plate, without heating the bioink as in thermal inkjet bioprinting. When a voltage pulse is applied between the pressure plate and an electrode, the pressure plate deflects to increase the volume of bioink in the nozzle. After removing the voltage pulse, the pressure plate rapidly regains its original shape and subsequently ejects droplets, as shown in Figure 2.2b(iii). This inkjet 3D bioprinting approach has also been used to fabricate 3D cellular and/or acellular constructs. For example, Nishiyama et al. [10] built up a homemade 3D printer based on an EPSON SEA-Jet™ inkjet nozzle head (static electricity-actuated inkjet). They used alginate as the main component of the bioink and printed different acellular and cellular constructs including gel lines, gel sheets, gel laminations, and gel tubes with HeLa cells (as shown in Figure 2.3c) to prove the feasibility of this bioprinting strategy.

2.2.1.3 Electrohydrodynamic Jet Bioprinting

In both CIJ and DOD bioprinting, bioink is propelled under different mechanisms through a nozzle to form droplets. However, when the nozzle diameter is extremely small, a high-level pressure must be required and the resulting high shear stress in the nozzle is fatal to living cells in the bioink. In particular, when the concentration of the bioink and/or cell concentration is high, the extremely high shear stress can tear cell membranes in the nozzle, leading to cell death during printing. As a result, these two bioprinting approaches cannot achieve microscale droplets with high cell viability. To overcome this challenge, a new inkjet bioprinting approach EHD jet has been developed. In EHD bioprinting, an electric field is used to pull the bioink droplets from the microscopic nozzle without the utilization of high pressure. The working mechanism of EHD bioprinting is illustrated in Figure 2.2c in which a back pressure is applied to the bioink in a metallic nozzle and forms a spherical meniscus at the exit of the nozzle. Then, an electric field with a high voltage range of 0.5–20 kV

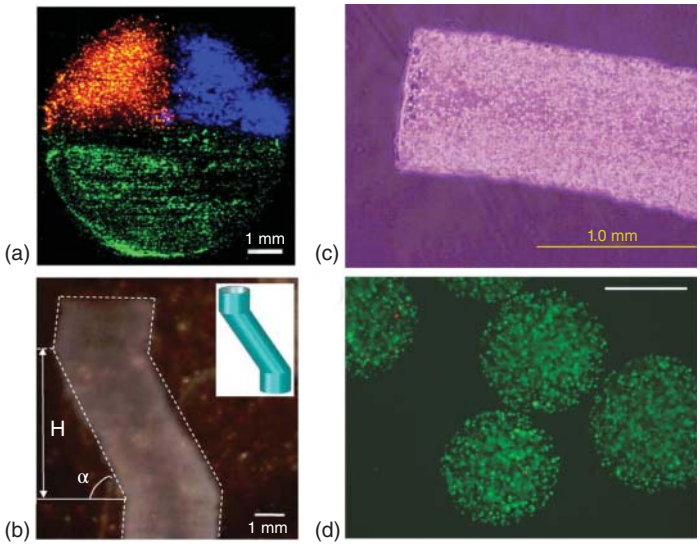

Figure 2.3 Representative inkjet bioprinting results based on different droplet formation mechanisms. (a) 3D multi-cell "pie" construct printed by thermal inkjet bioprinting. Source: Xu et al. [8]. Reproduced with permission of Elsevier. (b) Cell-laden zigzag tubes printed by piezoelectric inkjet bioprinting. Source: Xu et al. [9]. Reproduced with permission of John Wiley and Sons. (c) Gel tube including HeLa cells printed by electrostatic bioprinting. Source: Nishiyama et al. [10]. Reproduced with permission of Elsevier. (d) Cell encapsulations printed by electrohydrodynamic jet bioprinting. Source: Workman et al. [11]. Reproduced with permission of PMC.

is applied between the metallic nozzle tip and the receiving substrate, which accumulates mobile ions in the bioink near the surface of the bioink meniscus. Due to the electrostatic repulsions between ions, the bioink meniscus deforms into a conical shape and eventually forms a droplet when the electrostatic stress exceeds the surface tension at the orifice. By increasing the applied voltage, the jetting mode varies from dripping to cone-jet, and the droplet size decreases. EHD bioprinting is significant in the biomedical applications that require a nozzle diameter of less than 100 μm and bioink concentration higher than 20% (w/v). One of EHD bioprinting is illustrated in Figure 2.3d. In this study, bio-electrospraying was used to produce microspheres containing THP-1 cells, collagen, and alginate as a model cell line. It was found that bio-electrospraying technology did not affect cell viability and cell proliferation ability [11].

2.2.2 Hydrogel-Based Bioinks for Inkjet Bioprinting

Among various biocompatible materials, hydrogels have unique properties including tunable printability, excellent biocompatibility, suitable biodegradability, and enhanced cell adhesive properties. Thus, hydrogels have been widely used as the extracellular matrix (ECM) materials for preparing cellular and/or acellular bioinks. In this chapter, the main properties of hydrogel-based bioinks for inkjet bioprinting will be introduced and some commonly used hydrogels will be summarized.

2.2.2.1 Material Properties for Inkjet Bioprinting Applications

From the 3D bioprinting aspect, there are two main properties to evaluate the functionality of hydrogels for inkjet bioprinting applications: rheological and cross-linking properties. Rheological properties are used to determine printability, which is the ability that hydrogels can generate droplets. Cross-linking properties are adapted to solidify the deposited droplets as designed to form solid 3D constructs.

2.2.2.1.1 Rheological Property

Viscosity is one of the primary rheological properties of hydrogel-based bioinks during inkjet bioprinting, which is predominantly determined by the polymer concentration and molecular weight. Since surface tension is the main driven force to generate droplets in most inkjet bioprinting approaches, the viscosity of hydrogel-based bioink is required to be relatively low to enable a fluid jet to break up into droplet(s). The optimal viscosities of hydrogels for inkjet bioprinting fall in the range of 3.5–12 mPa·s [1].

Shear thinning is another important rheological property of hydrogel-based bioinks, which indicates the phenomenon of decreasing viscosity with increasing shear rate [12–14]. It has resulted from the reorganization of polymer chains to a stretched conformation under shear stress. Thus, the entanglement of polymer chains reduces from microscopic analysis and further leads to the decrease of viscosity from macroscopic behavior. After removing shear stress, polymer chains regain the original entangled status, and the viscosity recovers to the original value. This shear-thinning property effectively facilitates the squeezing of bioinks out of the nozzle or orifice to form droplet(s).

2.2.2.1.2 Cross-linking Property

Cross-linking property is defined as the capacity of gelation/curing/solidification of a bioink after printing, which is necessary to preserve the shape of a printed 3D construct [15]. This property is particularly important in inkjet bioprinting in which bioinks usually have low viscosities and cannot prevent the shape change of printed structures without cross-linking. Physical cross-linking and chemical cross-linking are two main categories of cross-linking mechanisms during 3D bioprinting.

Physical cross-linking depends on nonchemical interactions between molecular polymer chains such as entanglements, ionic interactions, and hydrogen bridges. These interactions are always reversible. Ionic and thermal cross-linking are two primary methods to design cross-linkable bioinks. In ionic cross-linking, di/trivalent cations are used as cross-linkers to bond different polymer chains together. Alginate is an exemplary ionic cross-linked hydrogel, which consists of mannuronic and glucuronic acid residues. After adding Ca^{2+} or Al^{3+}, these acid residues can bond with each other rapidly via cations, leading to the gelation of alginate [16]. In thermal cross-linking, hydrogels are composed of thermosensitive polymer chains, which can form stable 3D networks at one temperature, but collapse into independent polymer chains at another temperature. Gelatin is an example of the thermal cross-linkable hydrogel. At higher temperatures such as physiological temperature, the protein chains in gelatin are short and lack the ability to self-assemble into

well-organized fibers, leading to a liquid status of gelatin. However, upon cooling, some segments in gelatin return to the triple-helical conformation and form junctions between gelatin molecular chains. Thus, gelatin transitions from its liquid to solid states [17].

Chemical cross-linking mainly relies on the formation of covalent bonds between hydrogel polymer chains, which can provide cross-linked hydrogels with excellent mechanical strengths. Chemical cross-linking is always achieved by mixing monomers and initiators. Monomers provide hydrogel polymer chains, while initiators start the cross-linking reaction. Thus, when the gel point is reached, hydrogel polymer chains can rapidly develop into 3D networks, resulting in the increase of viscosity and transition of hydrogel from liquid to solid [18].

2.2.2.2 Commonly Used Hydrogels in Inkjet Bioprinting

Due to the severe requirement of viscosity, only a handful of hydrogels have been used in inkjet bioprinting including alginate, collagen, fibrin, gelatin-methacryloyl (GelMA), and polyethylene glycol (PEG).

Alginate, also called alginic acid, is naturally derived from the cell walls of brown algae. It consists of a family of unbranched binary copolymers of 1,4 linked β-D-mannuronic acid (M units) and α-L-guluronic acid (G units). It undergoes physical cross-linking when it interacts with divalent ions (such as Ca^{2+}) and/or trivalent ions (such as Al^{3+}). Gelation occurs as such cations form interchain ionic bonds between G units, forming a stable 3D network of calcium and/or aluminum alginate. Due to its tunable viscosities and mild cross-linking conditions, alginate has been used in inkjet bioprinting to make cellular tissue constructs. For example, Xu et al. [9] printed zigzag cell-laden tubular constructs using alginate-based bioink composed of 2.0% (w/v) sodium alginate solution and 3T3 fibroblast cells, as shown in Figure 2.3b.

Collagen is a triple-helical protein and also a primary component of ECM materials in the human body. Collagen is a thermal cross-linkable biomaterial, which is a liquid state at low temperature (~4 °C), but cross-links at room temperature or physiological temperature [19]. It has been extensively utilized for the fabrication of living tissue constructs due to its excellent biocompatibility and cell adhesive properties [20]. For example, Skardal et al. [21] mixed amniotic fluid-derived stem (AFS) cells and bone marrow-derived mesenchymal stem cells (MSCs) with fibrin-collagen gel and inkjet-printed over the wound site on a mouse model. It was found that cellular construct-driven wound closure and re-epithelialization were significantly better than closure and re-epithelialization in wounds treated by acellular fibrin-collagen gel only.

Fibrin is a hydrogel formed by the reaction of protease thrombin on fibrinogen. Since it can support cell growth and proliferation [21, 22], fibrin has been widely used to fabricate skin grafts in wound healing. For example, Cui et al. [22] printed human microvascular endothelial cells (HMVEC) and fibrin into micron-sized fibrin channels to investigate microvasculature construction. It was found that the printed cells aligned themselves inside the channels and proliferated to form confluent linings, which promoted HMVEC proliferation and microvasculature formation.