



AN OVERVIEW: 3D BIOPRINTING TECHNOLOGY

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ABSTRACT

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

KEYWORDS: Bioprinting, bioinks, cells, hydrogels, scaffolds, organ transplantation.

INTRODUCTION

Recent advances in bioprinting technology have opened up new and exciting opportunities for the development of patient-specific medical treatments. The fabrication or printing of biomimetic tissue structures is a prerequisite for the advancement of emerging technologies such as drug testing, tissue engineering, biomimetic sensors and 3D tissue models. Due to the rejection problems associated with allo-geneic organ transplantation and scarcity of donors, ex vivo methods are being explored for tissue/organ transplantation. These methods involve the expansion of patient-derived autologous cells and their use as the primary cell source to develop tissues/organs for transplantation. These 3D tissue analogs can be achieved by incorporating native cells with suitable biocompatible materials using a precise and well-controlled fabrication process.^[1] Bioprinted 3D constructs are aimed to mimic the cell density, arrangement, niche and anatomical geometry of the native tissue and hence can be a promising solution for different regenerative medicine applications.^[2] A 3D object can be designed and fabricated using 3D printing techniques. In 3D bioprinting, a layer-by-layer assembly of inks is printed using computer-aided instructions to develop biological constructs.^[3] Bio-printing can be

defined as the use of materials science and fabrication techniques to build biological constructs containing tissues, cells and biomolecules with a particular organization and biological function.^[4]

Bioprinting techniques have been recently explored for different biological applications due to their potential to overcome most of the problems associated with the classical tissue engineering methods.^[5] Classical tissue engineering involves the combination of scaffolds, cells and compounds, such as growth factors.^[5,6] Scaffolds are seeded with the cells and compounds that promote tissue regeneration. Tissue engineering strategies have been utilized for the regeneration of various organs such as skin, trachea, bone, esophagus and myocardium.^[5] Though tissue engineering approaches have been shown to be clinically effective, all scaffolds up-to-date lack complex and intricate structures of the native tissue.^[7] In addition, the tissue engineered scaffolds do not mimic the native architecture of the tissues.^[8,9] The key requirements of a tissue engineered scaffold are (1) biocompatibility; (2) biodegradability; (3) adequate porosity; (4) mechanical strength; (5) biomimetic structure and (6) therapeutic activity.^[6] Various fabrication methods such as electrospinning, freeze-

drying, phase separation, gas foaming, particulate leaching and solvent casting have been developed to produce tissue scaffolds.^[10] However, tissue engineer-ed scaffolds do not completely mimic the native architecture of the tissues, have difficulties to support the growth of cells in 3D and have problems to deposit different cell types in the scaffolds at specified locations.^[8] Besides, many of these fabrication methods involve the use of organic solvents which impair the cellular growth.^[9] Further, tissue engineered scaffolds do not completely fulfill all the ideal requirements needed for tissue regeneration as discussed above. On the other hand, bioprinting offers an alternative approach solving most of the problems associated with the current tissue engineering methods. Tissue engineering strategies are mainly involved in the development of scaffolds to promote regeneration/ repair of tissue defects. While 3D bioprinting methods can also be used to develop whole or parts of organs, the main advantage is its potential to print whole organs for transplantation purposes.

Methods for Bioprinting Tissue/Organs

Bioprinting of a tissue or an organ is a complex process which depends on the inherent properties of the bioinks, printing techniques and cellular systems used for printing. Furthermore, the resolution of the printed structure is controlled by the parameters such as needle orifice size, surface tension and viscosity of the bioink, temperature, and humidity. A typical bioprinting system can dispense bioinks onto a suitable substrate of choice using a cartridge or a syringe. More advanced bioprinting systems contain multiple print heads, and each one can be loaded with the same or different bioinks.^[10-17] Printing patterns can be generated, modified and printed using computer-aided software such as CAD (Computer Aided Design). The turnaround time taken for making modifications in the CAD files is just seconds to minutes making this process easy and user-friendly.^[18] This is advantageous to bioprint custom made structures such as tissues and organs for transplantation. The prerequisites to develop a bioprinting process comprise characteristics, such as CAD, high resolution to obtain the micro/nanoarchitecture and high-precision to localize cells in a 3D environment. With these design strategies in mind, bioprinting is using biomimicry and 3D tissue generation. The biomimicry approach enables the fabrication of constructs with features that mimic the native architecture of the tissue as close as possible.^[19]

Key Requirements of Bioprinted Tissue/Organs

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

stability. Hence, structural features and their resulting properties are key requirements to develop 3D constructs for regenerative medicine applications.

Bioprinting Methods

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

Microextrusion

Microextrusion is a 3D printing method used for biological and mostly for non-biological purposes. Printers that use the microextrusion method normally utilize a thermo-regulated handling and dispensing system, a piezoelectric humidifier and a stage with provisions for movements along the x, y and z directions.^[24] The deposition area is illuminated with a light source that enables the activation of photoinitiators. A video camera is attached to the xyz stage to monitor and control the printing process.^[25] Microextrusion technique has been successfully used to print scaffolds for tissue engineering.^[26] The microextrusion head deposits the material onto the substrate as continuous beads based on the instructions from the CAD-CAM software. Initially, the beads are deposited in the x-y direction, then by moving the extrusion head (or) stage in the z-axis, complex 3D structures are fabricated. Biocompatible polymers, cell spheroids and many hydrogels have been shown to be compatible with microextrusion. Two main dispensing systems that are used to extrude biomaterials are mechanical and pneumatic.^[27] The bioink flow is better managed in mechanical dispensing rather than pneumatic dispensing method.^[28] The compressed gas volume in the pneumatic system can delay the ink flow. Pneumatically driven printer systems operate with only

air-pressure and are more suited for applying limited force during printing.^[29]

Inkjet Bioprinting

Inkjet printers are referred to as drop-on-demand printers since these printers can reproduce digital information by printing small bioink drops onto the pre-defined location in a suitable substrate.^[30] These printers are widely used for many biological and non-biological applications.^[31] The cartridges can be refilled with bioinks, and the substrate is controlled by an electronic stage to enable z-axis movements.^[32] Nowadays, custom-designed inkjet printers are available that can use different bioinks with enhanced speed, accuracy and resolution.^[33] Inkjet-based printers utilize acoustic and thermal forces to eject bioinks on the substrate.^[34] In the case of acoustic forces based printers, a piezoelectric material is fixed to the needle that generates an acoustic wave to break the ink into small droplets at pre-determined intervals.^[35-46] When a voltage is applied, the piezoelectric material rapidly undergoes shape transformations which produce adequate pressure to eject bioink from the needle orifice. Some inkjet printers use acoustic radiation coupled with an ultrasonic sound to pump out the ink.^[47] In this method, the parameters of ultrasound such as amplitude, time and pulse can be varied to control the rate and size of the ejected droplets.^[48] Further, the desired ink droplet size can be easily generated and monitored. In this method, cells containing bioinks are not subjected to pressure and heat, hence better cell viability.^[49] In addition to this, nozzle-less print heads can be used to avoid exposing cells to shear stresses which may also improve cell viability.^[50] However, an important problem involved in this type of printing is the use of 15-25 kHz frequencies to eject ink, which causes cell membrane damage.^[50] Also, it is hard to use bioinks with high viscosity.

Laser-assisted Bioprinting

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

pressure bubble from the bioink layer which transfers the bioink onto the substrate. The resolution of the laser-assisted bioprinting system depends on the laser energy, air gap between the absorbing layer and substrate, nature of the substrate surface, surface tension and viscosity of the bioink. It is a nozzle-free printing method, and hence clogging of bioink/cells can be completely avoided.

Integrated Tissue Organ Printer (ITOP)

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

Robotic Bioprinting of Organs

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

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

Bioinks for 3D printing

The 3D printing technology was initially developed for many non-biological applications that involve the use of high temperature and toxic organic solvents. These harsh conditions are not suitable for printing biological cells and other biomaterials. Hence, it is essential for printing to find suitable bioinks with desired functional and mechanical properties in order to come close to native tissue. Both natural polymers (such as collagen, gelatin, alginate, fibrin, hyaluronic acid and chitosan) and synthetic polymers (such as polyethylene glycol (PEG), poly(L-lactic acid) (PLA) and poly(ϵ -caprolactone)(PCL)) are predominantly used as bioinks. Ultrashort peptides that can self-assemble into nanofibrous structures have recently been proposed as novel bioinks and are attractive candidates for bioprinting due to biocompatibility and processability. This newly developed bioink contains helical fiber structures that strongly resemble collagen fibers in topography and diameter. Printability is an important feature of an ideal bioink. During printing, the bioink should be accurately deposited in the construct providing the desired temporal and spatial resolution. For example, thermal inkjet printers require bioinks of lesser thermal conductivity to improve the cell viability.

Natural Polymers

(1) Alginate Sodium alginate (alginate) is a raw material extracted from brown seaweed. Alginate is a polysaccharide and anionic in nature. It is a linear block copolymer having M (β -D mannuronic acid monomers) and G (α -L-guluronic acid blocks) domains. Alginate structure has a mixture of M and G domains. G-blocks can form ionic bonds when interacts with divalent cations and become gels in solutions. Biomimetic structure, suitable viscosity, gelation at ideal temperatures and high biocompatibility are some of the properties of alginate that makes it suitable for bioprinting. Cell-laden 3D alginate hydrogels were prepared using inkjet printing. Although this hydrogel

provides biocompatibility and mechanical strength, it lacks cell recognition motifs. Moreover, bioprinting alginate constructs of thick tissues with well interconnected pores is yet to be achieved.

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

(3) Hyaluronic acid Hyaluronic acid is a linear polysaccharide made of (β -1,3) β -1,4-linked D-glucuronic acid and N-acetyl- D-glucosamine disaccharides. It is a viscoelastic, bio-degradable and highly biocompatible polymer. Hyaluronic acid is an interesting candidate for bioprinting, but its high hydrophilicity limits its application. Chemical cross-linking methods and derivatization of hyaluronic acid with hydrophobic side chains have been attempted to reduce hydrophilicity but still not successful in bioprinting. Blending hyaluronic acid with some photocrosslinkable materials such as Dex- HEMA have been shown to improve the cell viability of chondrocytes.

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

Synthetic Polymers

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

Some of the synthetic polymers used for bioprinting are discussed as follows.

(1) Poly(lactide-co-glycolide) (PLGA) PLGA is a copolymer of lactide and glycolide, synthesized via ring opening polymerization mechanism. It can be synthesized with different copolymer ratios, and their degradation rates can be controlled. PLGA has been successfully used as bioink to create 3D vascular networks. Human umbilical vein endothelial cells (HUVECs) were deposited on the PLGA based biopaper by using biological laser printing method.

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

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

(4) Poly(ϵ -caprolactone) (PCL) PCL is a synthetic polyester which is semicrystalline, biocompatible and biodegradable. It is an easily processable bioink due to its excellent properties such as low melting point, thermoplastic behavior, hydrolytic degradation and excellent mechanical properties. Initially, PCL being a viscous solution had difficulties in printing because of the requirement of large diameter nozzle and high pressure. To overcome this problem, an electrohydrodynamic jet technique was used to print PCL bioinks. Applying electrohydrodynamic forces created a temperature gradient in the ink and high resolution (10 μ m) 3D constructs were formed.

Ultrashort Peptides

Hauser and coworkers have recently reported that distinct peptides selected from the earlier discovered class of self-assembling ultrashort peptides can be used as bioinks for bioprinting applications. These ultrashort peptides have an innate tendency to self-assemble into hydrogels with a nanofibrous topography that closely

resemble collagen and thus mimicking the native architecture of tissue ECM.

Applications of Bioprinting

Bioprinting makes use of novel bioinks and 3D printing techniques to fabricate closely resembling organs/ tissues for regenerative medicine applications. Bio-printing techniques make it possible to print cells in the constructs in specific locations which is important for mimicking native tissue architecture. The vasculature of 3D constructs is essential to improve nutrient delivery, tissue ingrowth, and regeneration. Cells in tissues are mostly found within 100-200 μ m away from adjacent blood vessels. Cells that are present within this limit of 100- 200 μ m receive nutrition and oxygen through diffusion from the nearby capillaries. Cell viability and vasculature are some of the important parameters that need to be considered to develop 3D constructs for regenerative medicine applications.

CONCLUSIONS

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

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