3D Printing for Tissue Engineering Applications

Derleme Makalesi / Review Article

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ABSTRACT

The goal of tissue engineering is to create functional tissues and organs for regenerative therapies, and total organ transplantation. Bioprinting tissues are one of the most attractive approaches for tissue engineering and regenerative medicine fields. Fabrication of a complex structure via bioprinting requires layer-by-layer fabrication strategy. Bioprinting is mainly based on three processes; imaging and computer aided the design of the tissue that we wanted to print, the production of bio-ink with the selection of proper substances, the choice of a proper bioprinter depending on the product that we want, for fabrication of scaffold and/or tissues. In recent years the 3D bioprinting technology has been developed and several approaches appear by the researchers. The approaches are biomimicry, autonomous self-assembly and mini-tissue building blocks. In this study, current and future potential applications of 3D printing for the tissue engineering and regenerative medicine will be discussed.

Keywords: Bioprinter, bioink, self-assembly, biomimicry, tissue engineering

1. INTRODUCTION

Creating functional tissues and organs and total organ replacement are the ultimate targets of tissue engineering for regenerative therapies. The most common tissue engineering strategy is to seed cells onto scaffolds, which can then direct cell proliferation and differentiation into three-dimensional (3D) functioning tissues. Synthetic and natural polymers have been used to produce various tissues. To be successful in this challenge, these materials must be biocompatible and biodegradable. Also, such materials having the proper mechanical strength have been used to support cell attachment, proliferation, and direct cell differentiation. Even though the research and clinical applications achieved significant success in the past years, it is obvious that due to their complex 3D structure, the organs require more precise multi-cellular structures with vascular network integration, which cannot be generated by conventional methods [1]. One of the most promising techniques to develop the field of tissue engineering and regenerative medicine is the usage of 3D bioprinters to produce artificial tissues and organs.

In 3D bioprinting, the layer-by-layer accurate positioning of biological materials, chemical materials, and living cells, with positional control of functional components, is used to fabricate 3D structures. There are several approaches to 3D bioprinting; biomimicry, autonomous self-assembly and mini-tissue building blocks. Researchers are developing these approaches and using them co-operatively to fabricate 3D functional living human constructs with proper biological and mechanical properties for the clinical restoration of tissue and organ function [2].

Biomimicry is the solution for complex human problems with the imitation of the models, systems, and elements of nature. Identical reproductions of the cellular and extracellular components of a tissue or organ are the application of biomimicry to the 3D bioprinting.

Autonomous self-assembly is the approach which is using embryonic organ development as a guide to copy a tissue. The early cellular components of a developing tissue produce their own extracellular matrix (ECM) components, proper cell signaling and autonomous organization and patterning to produce the desired biological micro-architecture and function [3].

The concept of mini-tissues is relevant to biomimicry and self-assembly strategies for 3D bioprinting. The mini-tissues by design and/or self-assembly can build larger constructs can be fabricated and assembled.

2. IMAGING & DESIGNING TISSUES

For printing out complex functional tissues, organs, it is very important to model these in an accurate way. The visualization techniques have been developed in time, and medical 3D visualization methods reached the accuracy of isualization an organ in details in a realistic scale. Medical imaging technologies provide the data on 3D structure and function at the cellular, tissue, organ and organism levels. Computer-aided design (CAD) and computer-aided manufacturing tools are providing collection and digitization with the complex architectural information for tissues and organs. It can also be modified or designed, with the benefits of the usage of computer-aided design tools, on demand. Much current imaging and diagnostic technologies, such as magnetic resonance imaging (MRI) and computer tomography (CT), have been explored to acquire information about the targeting tissues and achieve the CAD data of the grafts.

The CT imaging is based on the variable absorption of X-rays by different tissues. MRI also can provide high

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spatial resolution in soft tissue via using nuclear magnetic resonance. The contrast of biological structures can be strongly increased with the use of contrast agents. As contrast agents, while the barium or iodine are used for CT scans; the iron oxide, gadolinium or metalloproteins are used for MRI scans; [4].

After raw imaging data have been obtained from these imaging methods, the data must be processed to produce 2D cross-sectional images [5]. 3D anatomical representations can be produced for further analysis or modifica¬tion. The 3D CAD models can be separated into 2D horizontal slices to provide instructions to the bioprinter and direct the layer-by-layer depositions of the biological elements [2]. Modeling tissue for 3D printing is shown in Fig. 1.

A custom nozzle motion program is used for fabrication to adjust XYZ movement of nozzles, scan speed, temperature, air pressure and material information [6].

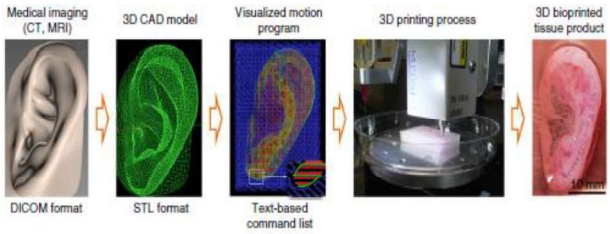


Figure 1. Modelling tissue for 3D printing [6].

3. BIOPRINTERS

There are mainly 3 strategies to produce a 3D tissue by bioprinting:

3.1. Ink-Jet Bioprinters

Inkjet printing takes a digital data from a computer representing an image or a character and reproduces it onto a substrate using ink drops as a non-contact technique (Fig. 2). They're also known as drop-on-demand printers. The controlled volumes of the ink materials are delivered to predefined locations by such ink bioprinters. The first inkjet printers used for the bioprinting applications were modified versions of the ink-based 2D printers [7]. There are three major types of drop-on-demand bioprinters: thermal, piezoelectric, and mechanical. Their basic structure has a cartridge, which is filled with the material to be printed, called as bio-ink. Bioink material is then forced through a microfluidic reservoir to an output nozzle.

Thermal inkjet printers use electrical heating of the print head to produce pulses of pressure that force droplets from the nozzle. In literature, it has been observed that there is no significant bad effect on the biological molecules because of this localized heating, ranging from 200 °C to 300 °C. Mammalian cells are affected by the viability or post-printing function in a negative way at these temperatures [8]. The thermal inkjet printers offer benefits such as high print speed, low cost, and wide availability. Therefore, exposing the cells and materials to a thermal and mechanical stress is the biggest disadvantage of this method. Moreover, low resolution, changes in the droplet size, and possible blockage of the nozzle tip are the other significant disadvantages for the use of these printers in bioprinting process [2].

In piezoelectric inkjet bioprinters, applying a voltage to a piezoelectric material induces a rapid change in shape and create acoustic waves to interrupt the liquid into droplets at regular intervals. Such acoustic waves generate the pressure inside bioink materials to eject droplets from the nozzle [9]. The piezoelectric printers lead to generate and control a uniform droplet size and ejection directionality as well as to prevent exposure of cells to heat and pressure stressors. That reduces the potential loss of cell viability and function and avoids the problem of nozzle blockage. Therefore, there are some concerns that the piezoelectric inkjet bioprinters with 15–25 kHz frequencies induce damage on the cell membrane and lysis [10]. In addition, such bioprinters also have limitations because of the material viscosity, which the excessive force required to eject drops using solutions at higher viscosities [11].

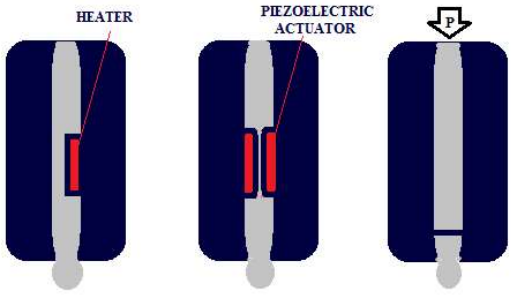


Figure 2. Schematic representation of inkjet printer methods. The figure on the left shows thermal inkjet bioprinter. The figure in the middle shows piezoelectric bioprinter. The figure on the right shows mechanical inkjet bioprinter

Mechanical inkjet bioprinters use pressure through the nozzle to flow of bioink material. The pressure source can be pneumatic or a pump based system. This types of inkjet bioprinters have a valve on the nozzle tip. The valve opens to allow bioink to flow through the tip, drop by drop [28]. The main disadvantages of these bioprinters are the cell damage caused by the mechanical pressure and nozzle clogging problems. It's an old technique compared to the others.

3.1. Microextrusion Bioprinters

The extrusion based bioprinting technique has a fluid-dispensing system and an automatic robotic system in order to extrude the gel-form bioink and bioprint a model [12]. The bioink is located using a positioning system, with computer-aided design tools. The printed products are desired 3D custom-shaped structures that are formed with cylindrical filaments. These cylindrical filaments may contain cells and other biological materials. Such a better integrity on the structure can be provided by a rapid

manufacturing method due to the continuous deposition of filaments.

The fluid polymers can be dispensed using systems of the pneumatic, screw-driven, piston or solenoid-based system; however, the solenoid-based dispensers are not convenient to use for bioprinting [13].

The pneumatic system uses pressured air with/without a valve configuration. The valve-free system has been widely used because of its simplicity. Therefore, the preferable configuration is the valve-based system can be because it has higher resolution than the other system due to its pressure control and pulse frequency control [13].

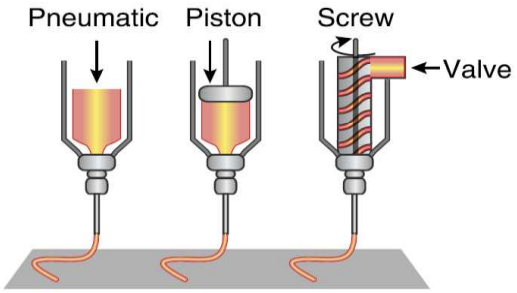


Figure 3. Microextrusion bioprinting systems [2].

Mechanical microextrusion systems use a piston or a screw-driven configuration; working principle is shown in Fig. 3. The piston-driven dispenser maintains more direct control over the bioink flow through the nozzle [14]. On the other hand, the screw-driven dispenser may give more positional control and is more useful for processing the high viscous bioinks [14].

However, the screw-driven dispenser can damage the loaded cells, because its mechanism exposes bigger pressure drops along the nozzle. Thus, the rotating screw gear of the dispenser must be carefully designed as an extrusion-based bioprinter. Both piston and screw types of mechanical microextrusion can work synergistically. For example; the screw-driven dispenser melts polycaprolactone (PCL) before deposition while the piston-driven dispenser, having syringe pumping, extrudes hydrogel [15].

Extrusion based bioprinters are simple to construct and affordable. High viscous biomaterials can be used as bioink for producing a tissue-specific scaffold or small tissues. Also, large constructs can be created. Cell spheroids, cell aggregates can be used as bioink and them self-assembled into complex tissues by this printing method.

The drawback of these methods is that only high-viscous materials can be extruded. Low-viscous materials need high pressure for extrusion and this cause high shear stress, which tends to kill cells. Cell survival rates decrease with increasing pressure.

3.1. Laser-Assisted Bioprinting

Laser-assisted bioprinting (LAB) is based on the principles of laser-induced forward transfer. The laser-induced forward transfer is a printing process allowing the deposition of a small amount of material in solid or liquid phase with high resolution [16]. A typical LAB device consists of a pulsed laser beam, a focusing system, a ribbon that has a donor transport support usually made from glass that is covered with a laser-energy-absorbing layer and a layer of biological material prepared in a liquid solution, and a receiving substrate facing the ribbon. LAB functions using focused laser pulses on the absorbing layer of the ribbon to generate a high-pressure bubble that moves cell-containing materials toward the collector substrate [17]. The LAB setup is shown in Fig. 4.

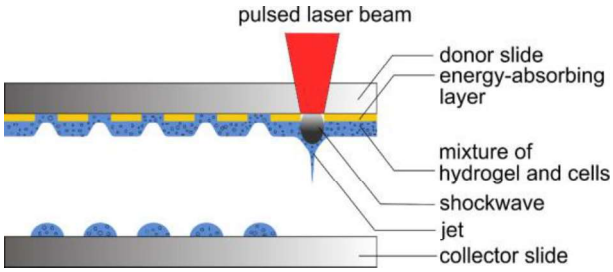


Figure 4. Schematic laser-assisted bioprinting setup [17].

The resolution of LAB is influenced by many factors, including the laser flow rate, the surface tension, the ability to get substrate being wet, the air gap between the ribbon and the substrate, and the thickness and viscosity of the biological layer [18]. LAB is a nozzle-free method; therefore the clogging problem with the bioprinting materials and cells is executed. It is compatible with a range of viscosities and can print mammalian cells with a low effect on cell viability and function [17].

Despite such advantages, the high resolution of LAB demands a rapid gelation rate to gain high shape suitability, which results in a relatively low overall flow rate. That disadvantage prevents the production of highly complex tissues by this technique. Another disadvantage of this method is the metallic absorbing layers which produce metallic residues on the product. Metallic contamination of tissues must be inhibited. Besides these drawbacks, LAB is a very expensive device [2]. To eliminate these disadvantages, there are many types of research are in progress. LAB may be an effective prospect for tissue engineering applications.

4. MATERIALS & BIOINK

Selection of proper biomaterials as the bioink is a vital step to gain a successful bioprinting product. Bioinks based on both naturally derived and synthetic biomaterials have been developed to provide a few key properties, such as biocompatibility and appropriate physical properties, to provide printability and long-term functionality following deposition [5].

The materials selection and their performance in order to use in bioprinting depend on printability, biocompatibility, structural, mechanical degradation, and biomimicry properties. Materials should be proper for

printing processes, gelation methods material viscosity are important. The materials also should be biocompatible for not creating an immune response in the host. Materials' degradation products should be non-toxic, and their degradation rate should be proper to tissue. Mechanical properties should be similar to the tissue that we constructed. The selected bioink materials should mimic the tissue-specific endogenous material compositions of the tissues [2].

Naturally-derived polymers, such as collagen, chitosan, and some of the synthetic polymers, such as polyethylene glycol (PEG), poly(lactic-co-glycolic) acid (PLGA), are widely used in regenerative medicine field and tissue engineering applications. Materials of bioinks must be biocompatible, with proper mechanical and structural properties. Moreover, bioinks should be compatible with 3D printing application. Because of these obligations generally, natural polymers are proper materials as a bioink material. Due to the application of bioprinting, bioinks may be composed of cells, cross-linked polymers, stem cells and cell signaling molecules.

In literature, the methods for bioink printing can be observed in two pathways; printing bioink as functional scaffolds with various polymers, or scaffold-free printing methods which only use cells as bioink material to print. In both cases, viable bioprints which have loaded with the cells, need a sufficient nutrient transport through vasculature mimics or pores. If the material is not adequately presented excessive swelling, the crosslinked porous and vascular structure can be lost, which ends up with the prevention of nutrients from supplying the cells in the constructs. The printed materials have to also physically support the desired structure, so mechanical properties must be suitable for the designated tissue or location.

4.1. Cells for Bioink

We can use any type of cells as a bioink material. The cells that we selected may be mature or pluripotent stem cells, due to the printing strategy and procedure, but they are not useful by themselves. Cells need a delivery medium for the printing process, and they need a growth media for a successful product at the end of printing. That indicates the importance of proper polymer material selection.

4.2. Polymers for Bioink

Materials must be selected properly for bioink success. When polymers selected as a bioink material, it's required to understand how polymer characteristics influence printing efficacy and cytocompatibility. Because using of these bioinks during the printing process, they are expected to maintain structural integrity for the printed product. Polymer materials using for 3D bioprinting is shown in Fig. 5. Table 1 shows the polymers which have been used as bioink materials and their properties, in detail.

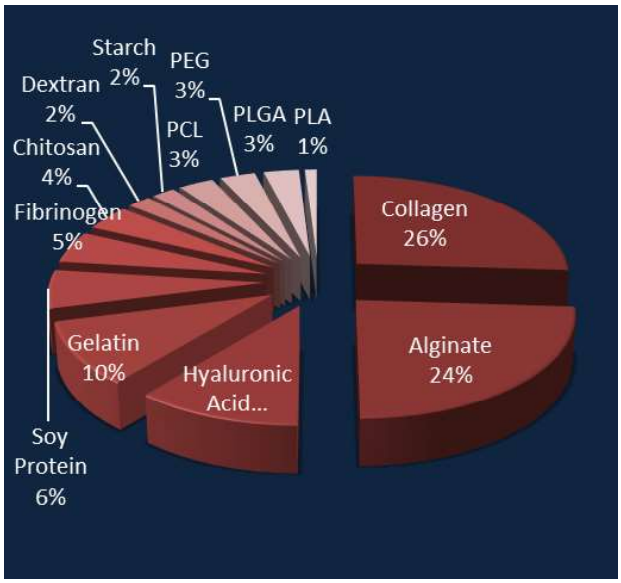


Figure 5. Chart diagramming natural and synthetic polymer distributions for use as bioinks compiled from relevant literature [19].

4.3. Cell Aggregates

Scaffold-free bioprinting techniques are generally based on self-assembly approaches. With cell aggregate techniques, tissues can be engineered and modified with specific compositions and shapes. These tissues are printing with the advantage of cell-cell adhesion and they can grow and evolve in their own natural ECM. That reduces inflammatory responses and increases the biocompatibility of the engineered tissue [21].

For self-assembly applications, cell aggregates are the bioinks for bioprinting. These techniques are the closest endeavor to produce a whole complex organ. Some techniques have been developed and made a success of producing simply structured tissues such as skin, bone cartilage tissues without using a scaffold [22]. Self-assembly of cellular components perform the principles of embryonic development.

These techniques start with extremely high cell numbers. The aim of these techniques is triggering the cells to place to ECM in specific locations for inducing a proper growth of the tissue that we wanted. Schematic illustration of cell spheroid printing is shown in Fig. 5. The cell aggregates that we used for bioprinting can be found as cylinder, torus, spheroids, and honeycomb. Using cell spheroids for 3D bioprinting applications is a huge trend in the literature [14].

Table 1. Biocompatible polymers used as bioinks for stem cell, cell delivery, and scaffold materials are presented along with their crosslinking features and application in bioprinting stem cells [20].

BIOINK	PROPERTIES	CROSSLINKING FEATURES	EXAMPLES
ALGINATE	Inexpensive, natural polysaccharide derived from algae. Bioinert, which may lead to anoikis and is often modified with RGD or additives such as hydroxyapatite.Crosslinking occurs rapidly hence alginate is very popular as a bionk.	Instant gelation in Ca^{2+} solution.	Fabrication of osteochondral tissue equivalents.
AGAROSE	Bioinert. Forms cytocompatable and structurally stable hydrogels. Solidifies slowly, resulting in bioink spreading. Not biodegradable in mammals.	Thermal gelation, cells mixed at 40 °C and gelates at 32 °C. No other polymerizers needed.	Printing of bone marrow stromal cells in agarose has been assessed.
HYALURONIC-MA	A non-sulfated glycosaminoglycan, usually used for producing soft tissue like hydrogels rather than ones confering structural stability. Often mixed with gelatin, dextran or other polymers to overcome bioinertness and mechanical weakness.	UV triggered free radical polymerization.	Adipose stem cells printed in Gel Ma/HA Ma hydrogel, confering high cell viability detected after 1 week (97%).
FIBRIN	Natural protein comprised of cross-linked fibrinogen, has quick crosslinking rate and is glue like in form. The mechanical stiffness is low, so often used in conjunction with other polymers.	Crosslinks through the thrombin cleavage of fibrin.	Blended with collagen to deliver stem cells by inkjet with the application of skin regeneration.
SILK FIBROIN	Good biocompatability and mechanical properties. Mixed with gelatin to prevent nozzle clogging.	Crosslinked with tyrosinase or by sonification.	Silk fibroin-gelatin bioink used to print human nasal inferior turbinate tissue derived MSC that supports multi lineage differentiation.
GELATIN	Formed from partially hydrolysed collagen. More soluble than collagen. Melt/gelation temperature 30 °C–35 °C, requires secondary crosslinking for applications at physiological temperatures. Matrix can be remodelled by cells.	Crosslinked using gluteraldehyde, carbodimiide or transglutaminase. UV irradiation of the methycrylated form.	BMSCs printed in gelatin MA with BMP2 or osteogenic medium.
COLLAGEN-I	Rich in the intergrin binding RGD motif. The ionic or pH changes involved in gelation are usually not gentle enough to allow cell bioprinting, however water soluble forms do exist. Collagen hydrogels are formed at low concentration. That confer for low elastic modulus. Unfortunately a 100% collagen hydrogel may not be ideal as a cellularized construct due to water exclusion and contraction induced by hydrophobic peptide residues.	Gels through hydrophobic bonding with a slow rate of crosslinking, so can be blended with faster crosslinking polymers such as alginate or fibrin.	MSCs in collagen hydrogel differentiate towards chondrocytes, expressing cartilage proteins.
dECM	Supplies a natural like ECM niche for the stem cells. The stem cells seeded in dECM scaffold show greater degree of differentiaiton than cells seeded in collagen.	Can form a bioink that remains as a solution below 15 °C and gels after 30 min at 37 °C.	Adipose, cartilage, and heart dECM used as cell printing bioink for adipose derived SCs and human inferior turbinate tissue derived MSC.
MATRIGEL	ECM like hydrogel rich in laminin, collagen and heparan sulfated proteoglycan. Has been used extensively for 3D cell culture.	Thermal gelation.	Not widely employed for bioprinting, used for printing HepG2 cells by temperature comtrolled syringe.
METHYL CELLULOSE	Can be used to aid printing of another polymer and is then released. Enhances print viscosity and porosity following release.	Thermal gelation.	Blended with alginate to print MSCs into a low concentration alginate hydrogel.
PEG	Bioinert, variable molecular weight allows tunable properties, altering stiffness can aid stem cell differentiation. Can easily joined to other molecules. Requires modification to allow crosslinking.	UV initiated photocrosslinking of the PEGDMA.	Bone marrow derived MSCs printed for osteogenic and chronogentic differentiation.

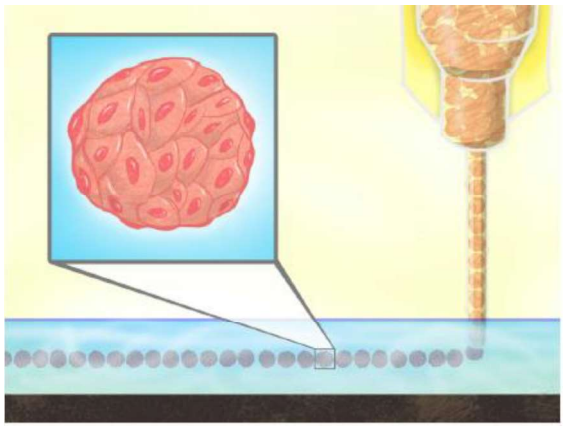


Figure 6. Spheroids are printed into ‘‘biopaper’’ which is a layer of hydrogel [23].

Tissue spheroid technique has some disadvantages due to its bioink properties. Tissue spheroids require a degradable carrying medium like thermo-sensitive hydrogel medium for extrusion through the nozzle tip. Such hydrogels are bioinert to cell adhesion. Moreover, tissue spheroids can fuse together very quick; so, they may start to accumulate in the nozzle and that affects their printability in a challenging way. In addition, for a complete fusion, tissue spheroids should be in contact tightly with each other, therefore, during the printing process, they may not be in contact tightly. That gives a leaky tissue as a product [14].

Instead of delivering cells in spheroid form with high density, delivering them in pellet form works more effectively [24]. In this technique, cells are printed into micro molds to form and aggregate in a strand shape. Molds are constructed with hydrogels, such as agarose or alginate, which are inert to cell adhesion [25]. The biggest obstruction with this approach is the necessity of a temporary molding material for making large-scale tissues. So, tissue strands can be considered as an alternative approach to overcome this challenge. Tissue strand printing uses long strands of tissues as bioink material. With tissue strand technique, the hard procedure of the spheroid preparation and loading, printing difficulties can be eliminated. The strands can be printed in coordination with the vasculature. That is the biggest advantage because that gives a convenience as a fabrication method to print out vascularized tissues. Tissue strand printing shows a great potential for fabricating large-scale tissues and organs in the future [26]. The concept of tissue printing is shown in Fig. 7.

The advantages of the bioinks having cell aggregate compounds shows are well cellular interactions, close biomimicry, quickly tissue forming and long-term stability of cells in the 3D architecture. Although, they have a few limitations. Preparation of an essential amount of aggregates needs a quite high number of cells can go up to a few hundred million cells. In general, enlarging cells with such numbers are expensive, requiring intense effort, and some cell types cannot proliferate fast; therefore, their applicability and availability are limited [14].

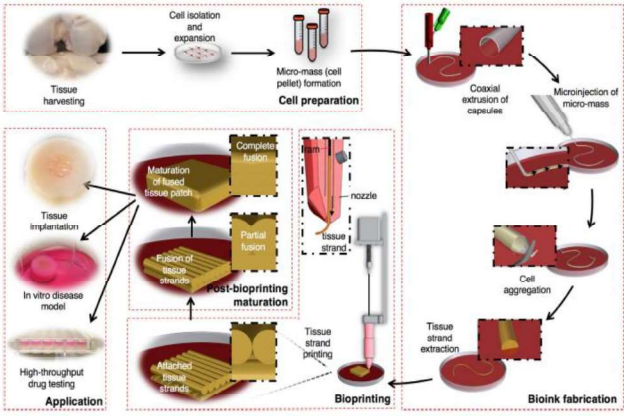


Figure 7. The concept of tissue printing using tissue strands as bioink [26].

4.4. Decellularized Extracellular Matrix

With the developments in hydrogel-free approaches, obtaining extracellular matrix from natural sources has been considered as a new bioink source for tissue and organ fabrication. After discovering the decellularization techniques of tissues, researchers aimed to use decellularized extracellular matrix (dECM) components in printing tissue analogues. In a recent study, they decellularized tissues and degraded them into smaller pieces, then loaded the mini-decellularized tissues with cells and printed with a polycaprolactone frame to support the mini tissues (Fig. 8). The natural differentiation of the cells observed with three different cell types loaded in their native dECM [27].

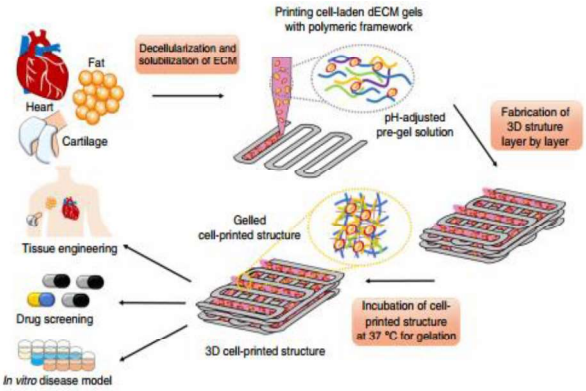


Figure 8. Schematic procedure for dECM bioink bioprinting [27].

With today's technology, decellularization of matrix components have some limitations which are related to their low affordability and abundance of the bioink, with the protocols which have been used. It's possible to have very small pieces of dECMs via the decellularization of tissues, therefore; a large volume of tissues is needed in order to produce tissues via bioprinting. Furthermore, the decellularization of ECM can cause losing mechanical and structural integrity. And, some toxic residuals can stay in the separated dECM components. Because of these problems, the printed bioink cannot allow forming cells while cells can absorb the matrix components or the matrix shrinks significantly. Using a hard material is more beneficial to support the dECM structure due to the weakness of the mechanical properties. Thus, printing a frame with dECM supports, the dECM structure prevents collapsing of the printed product [14].

5. CONCLUSIONS & FUTURE PERSPECTIVES

Bioprinting applications for tissue engineering is a very attractive subject for producing novel solutions to the many problems of the medical world. Present fabrication procedures are not sufficient to produce complex whole organs, but simple-structured organs and tissues can be produced with current approaches. In this study, the working principle of bioprinters and some significant approaches have been discussed. It has been observed that there is a great potential with current approaches, which can be classified as biomimicry, self-assembly and tissue building blocks. It seems like that, the key to success is the usage of these approaches co-operatively. The improvement of bioprinting applications also depending on the stem cell technology, materials science technology and high-scale production capabilities of the ingredients of bioink. In addition, post-printing treatments to the printed tissues may be considered as a solution to fabricate complex organs, with the principles of self-assembly, self-organization and embryonic development. When these problems solved, bioprinting will be one of the most important inventions of the world.

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