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# Cardiovascular 3D bioprinting: A review on cardiac tissue development

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ABSTRACT

Cardiovascular diseases such as myocardial infarction account for millions of worldwide deaths annually. Cardiovascular tissues constitute a highly organized and complex three-dimensional (3D) structure that makes them hard to fabricate in a biomimetic manner by conventional scaffold fabrication methods. 3D bioprinting has been introduced as a novel cell-based method in the last two decades due to its ability to recapitulate cell density, multicellular architecture, physiochemical environment, and vascularization of biological constructs with accurate designs. This review article aims to provide a comprehensive outlook to obtain cardiovascular functional tissues from the engineering of bioinks comprising cells, hydrogels, and biofactors to bioprinting techniques and relevant biophysical stimulations responsible for maturation and tissue-level functions. Also, cardiac tissue 3D bioprinting investigations and further discussion over its challenges and perspectives are highlighted in this review article.

## 1. Introduction

Cardiovascular diseases (CVDs) are the leading cause of death globally, involving one-third of the world's total death, and accounting for more than 19 million deaths each year [1]. CVDs are cardiac and vessel tissues' chronic pathological conditions, including coronary heart disease, rheumatic heart disease, and cerebrovascular disease among which, myocardial infarction is accounted as the primary cause of CVDs characterized by hypoxia-induced cardiomyocyte death and severe inflammation, leading to tissue degeneration and scar formation [2–4]. Clinically available methods for end-stage CVDs are majorly coronary artery bypass grafts (CABGs), prosthetic devices, and heart transplantation among which, cardiac transplantation methods are clinically restricted because of the shortage of donor organs and post-transplantation immunogenic rejection [5,6]. Although there have been many efforts to improve cell transplantation methods during the last decade, the low local cell retainability due to the blood flow and immunogenic inconsistency has remained as main challenges resulting in low integration with the host tissue [7,8]. Engineering a transplantable three-dimensional (3D) microenvironment for better cell-cell and cell-matrix interaction has the potential to replace the native tissue properties [9]. Tissue engineering is an arising alternative for end-stage CVDs, aiming to alleviate the current therapeutic complications and providing a potential platform for improving the efficiency of cell-based therapies. Owing to the vast advantages tissue engineering suggests, many challenges such as the organization of cardiomyocytes, limited self-renewal, electrical, and mechanical functions have been addressed [2]. Aside from the extensive research in tissue engineering, several challenges have majorly restricted its clinical application, including the lack of functional blood supply for cells, non-homogenous distribution of cells through the construct, and poor control over the 3D architecture precise structure of the construct, ultimately resulting in lack of integration. To address these complications, novel bioengineering methods are under investigation to improve the efficacy of cardiac tissue engineering strategies [10–12].

Among these novel methods, 3D bioprinting, as a powerful biofabrication technology stemming from additive manufacturing, has an exclusive potential in the fabrication of complex 3D architectures taking advantage of layer-by-layer deposition of biological components (cells, signaling molecules, etc.) and biomaterials, namely hydrogels known as bioinks [13]. Bioprinting enables precise control over the spatial distribution of cells, architectural organization, and compositional

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adjustment of the construct [4,10]. In fact, there have been various studies employing different kinds of cells, hydrogels, and architectures in biomimetic approaches to produce cardiac patches or organoids due to the capability of this method to fabricate geometrically complex structures in three dimensions and maintain cell proliferation, maturation, and long-term functions. However, due to the intrinsic structural and compositional complexity of the cardiac tissue, several significant elements need to be considered in cardiac biofabrication, including i) suitable manipulation of cells sources having high regenerative capacity, ii) appropriate biomaterials capable of replicating the native cardiac microenvironment, stimulating cell fate, providing mechanical stabilization, and non-immunogenicity, iii) well-defined structural organization, and iv) establishment of relevant physiological stimulations (mechanical and electrical) for efficient cellular maturity and functionality. Many bioprinting techniques have been introduced in the last decade based on extrusion, inkjet, and laser techniques, which provide a wide range of cell-friendly processes, adjustable precision, and compatible crosslinking methods. These techniques have advantages and disadvantages for any specific bioinks and crosslinking procedures, making the selection of the method extremely important [4,10,14–21].

There are many significant characteristics and functionalities in genuine cardiac tissue that makes the heart pump blood continuously. These specific features have already been attained in 3D bioprinted constructs designed by implementing biomimetic approaches. Today, bioprinted constructs with different organizations of endothelial cells allow vascularization and stimulate cardiomyocytes growth; anisotropic grid designs allow orientation, contractility; and other kinds of mechanical (e.g., external stretching and the microenvironment adjusting), electrical (e.g., direct and pulsatile impulses) and hemodynamic stimulations to bring maturation and conductivity [22–24]. In this review, we discuss several cell sources and hydrogels that have been used to prepare bioinks. We also review different bioprinting techniques, architectural designs, and stimulation methods that increase biological constructs' functionalities. Finally, we conclude with a discussion of cardiovascular bioprinting challenges and perspectives based on the last decade's studies.

## 2. Cell sources

3D bioprinting, aside from other cell-based technologies, aims to control the spatial distribution of cells in high resolutions and achieve a better mimicry of the natural cell microenvironment, leading to enhanced cell activity and higher tissue-level biological responses [25,26]. The introduction of bioprinted functional cells in nonfunctional defect sites is considered to trigger regeneration (mainly by differentiation of either transplanted stem cells or site-specific progenitors) and stimulate vascularization [27,28]. Hence, appropriate cell organization and tissue-level cell performance are two essential factors that should be strictly regulated for further tissue regeneration. To this end, cell sources should meet several characteristics, including i) facile and efficient differentiation of stem cells (increased number of both differentiated cells and tissue-specific gene expression), and ii) no pathogenic transference from the source to the host [27–30].

In contrast to non-parenchymal heart cells, parenchymal cardiomyocytes show minimal proliferative capacity, resulting in a low number of governing cells impairing tissue formation [31]. Therefore, stem cells, including autologous/allogeneic cardiac progenitor cells, skeletal myoblasts, mesenchymal stem cells (MSCs), embryonic stem cells (ESCs), and induced pluripotent stem cells (iPSCs), are potential sources for both experimental and clinical regenerative applications [29].

### 2.1. Cardiac progenitor cells

Based on earlier investigations, neonatal and fetal cardiomyocytes have suggested a favorable progression rate required for tissue regeneration. However, due to ethical issues, immune-rejection responses related to the allogeneic sources, and short cell survivability, their clinical application is limited [29,32,33]. Since immature early-stage cardiomyocytes possess high survivability, they have been exploited extensively in regenerative medicine. Cardiac progenitor cells are tissue-resident multipotent stem cells that can be harvested by minimally invasive biopsies and directed toward different myocardium lineages, including cardiomyocytes, smooth muscle cells, and endothelial cells [4,34]. Cardiac progenitor cells are harvested from two origins; leftover from the embryonic morphogenesis process or recruited from bone marrow by the circulating system, namely autochthonous and allochthonous [35]. Compared to other potent stem cells, progenitor cells are closer to the fully differentiated cardiomyocytes in the cardiac lineage. Therefore, they require fewer considerations for differentiation toward specific cells in 3D constructs. For instance, bioprinting of human cardiac-derived cardiomyocyte progenitor cells (hCMPCs) in various hydrogels has indicated a higher tendency to differentiate toward cardiomyocytes in 3D constructs [36,37]. However, the limited availability of these cells has restricted their clinical implementation. To obtain a cardiogenic construct, Gaetani et al. [38] evaluated extrusion-based bioprinting of modified alginate laden with hCMPCs. The results showed that the fetal cardiomyocytes appropriately migrated, formed tubular structure, and could also express early cardiac transcription factors [38].

In a study conducted by Bejleri, Donald, et al. [36], *in vitro* assessments of Human c-kit$^{+}$ progenitor cells (hCPCs) in bioprinted cardiac patches were accomplished, which showed an enhanced cell differentiation, angiogenic potential, and myocardial-matching mechanical properties. In another recent study by the same group [33], hCPCs were used to investigate *in vivo* outcomes of developed cardiac patches in a rat model of right ventricle failure. The results of *in vivo* analysis indicated that neonatal hCPC-laden cECM (cardiac extracellular matrix)-gelatin methacrylate (GelMA) patches could significantly enhance the right ventricle function and tissue remodeling compared to other groups [33,36].

### 2.2. Skeletal myoblasts

Skeletal myoblasts are among the most commonly used cell sources in cardiac regenerative medicine, which can be easily harvested by patients' skeletal tissue biopsies obviating further immunogenic responses [39]. Several specific characteristics are attributed to the increased interest in the use of skeletal myoblasts, which majorly include the high rates of cell proliferation, resistance to hypoxia, and the high potency of these cells in the establishment of myotubule structures, which can significantly improve cardiac functions [34,40,41]. These particular characteristics are also reported to be accompanied by a decrease in both tissue fibrosis and cardiomyocyte-hypertrophy [42]. However, after the formation of myotubules, the electrophysiological coupling was not well-established. This lack of electrophysiological coupling was due to the lack of the expression of gap junction-associated genes, including connexin-43 (Cx-43) and N-cadherin, which causes other arrhythmic behaviors [9,43,44].

### 2.3. Mesenchymal stem cells (MSCs)

MSCs, also known more recently as medicinal signaling cells, are multipotent stem cells isolated from different sources, majorly from bone marrow, adipose tissue, and umbilical cord tissue [45]. They can be signaled to differentiate toward mesenchyme-derived cells, including adipose, osteogenic, and chondrogenic lineages. Various studies have also suggested a different potency of MSCs to transdifferentiate towards other lineages, including cardiomyocytes [45–47].

However, the differentiation capacity of MSCs toward cardiomyocytes has been reported to be limited, producing cardiomyocyte-like cells with low functionality, which makes them less efficient cells

from a therapeutic perspective [48]. Due to this limited differentiation capacity of MSCs into cardiomyocytes, rather than being exploited as single regenerative cells, they are mostly implemented in co-culture systems. Several other transplant investigations reported enhanced survival and regenerative capacity of the MSC-Cardiomyocytes heterogeneous co-culture system [49,50]. Aside from the transdifferentiation capacity of MSCs, three other primary mechanisms were proposed for the enhanced regenerative capacity of MSC-cardiomyocytes: i) inducing the latent regenerative capacity by reprogramming the fully differentiated cardiomyocytes back to the therapeutically potent cardiac progenitor cells [51]; ii) a transient improvement in cardiomyocytes functions in response to the release of several paracrine factors, especially angiogenic factors [52,53]; iii) an electrophysiological coupling between MSCs and cardiomyocytes has been attributed to the formation of gap junctions between adjacent MSCs and cardiomyocytes [54].

### 2.4. Human embryonic stem cells (hESCs)

Cardiomyocytes derived from hESCs are accounted as another important cell source suggesting a strong potential for cardiac tissue engineering, which have been shown to form spherical cell aggregates of about 200 cells and up to 300 μm in diameter [55–57]. More specifically, hESCs are pluripotent stem cells harvested from the blastocyst's inner cell mass during embryogenesis with high regenerative potentials. They possess high self-renewal potential and can be differentiated toward an extensive choice of tissues with remarkable plasticity [4]. Besides the advantages hESCs exploitation suggests, several issues need to be mentioned. First, the differentiation of these pluripotent stem cells can occur in two different ways, spontaneously and through direct differentiation, due to the considerable heterogeneity of obtained cells within spontaneous differentiation. The direct differentiation of cells toward the myocardium cell lineage is more implemented [58]. However, co-cultured hESCs with murine stromal cells have resulted in a heterogeneous mixture of non-parenchymal and parenchymal cells [34,59]. In addition, the ethical dilemma concerning the exploitation of embryonic-derived stem cells is accounted as a strong limiting factor. Since research on these hESCs entails the elimination of human embryos, it is ethically and politically prohibited in a wide range of communities. Overall, the lack of controllability of cell direction, issues related to the immunogenic responses, and the ethical dilemma associated with their use have restricted these cells' extensive use in clinical applications [60,61].

### 2.5. Induced pluripotent stem cells (iPSCs)

iPSCs are generated directly by reprogramming the patient's fully differentiated cells to harbor pluripotency by introducing several stemness transcription factors, mainly through the exploitation of retroviruses or more recently-deployed, stimulating their expression through incorporating chemical factors [62]. Like ESCs, iPSCs have a high self-renewal capacity and high differentiation plasticity toward a wide variety of cell types, including cardiomyocytes [4]. Although it can be derived from heterogeneous healthy donors, the autologous sources of iPSCs can substantially ensure patient-specific immunogenic compatibility in four levels of genomics, transcriptomics, proteomics, and metabolomics [63,64]. Also, the high differentiation plasticity of iPSCs provides the potential for spontaneous generation and direction of different cell lineages, including parenchymal and non-parenchymal cells in cardiac tissue. However, in spontaneous differentiation, precise protocols are required for the direction of iPSCs toward intended cell types and suppress non-cardiac cell lineages generation [58].

There are several complications associated with iPSCs implementation in bioprinting for regenerative medicine. One of the significant concerns about the iPSCs is the possibility of teratoma formation and immaturity of governing cells. In this regard, electromechanical stimulations, introducing differentiative agents (e.g., Activin A) into the culture media or co-culturing them with endodermal cells, which is responsible for cell-controlled signaling molecules release, can control further maturation and cellular maintenance [59,64,65]. Also, *in vivo* transplantation of iPSCs has been reported to result in more mature cells attributed to the more compatible directing signals and microenvironment. Another significant complication is associated with the iPSCs generation, where significant genomic instability, mainly caused by unusual mutations during the reprogramming procedure, can be resulted [66].

Recently, there have been several studies on extracellular vesicles derived from stem cells and their potential effects on tissue restoration and cell viability. Extracellular vesicles derived from iPSCs have been shown to contain a wide range of proteins and non-coding RNAs (e.g., miRNAs), strongly influencing cell viability and cell cycle [65–68].

### 2.6. Non-parenchymal cells

In contrast to the parenchymal cardiomyocytes, non-parenchymal cells, including endothelial cells, fibroblasts, and smooth muscle cells, exhibit relatively high proliferative capacity [69]. These cells' phenotype and the source may present important properties affecting their functionality and parenchymal cardiomyocytes' survival and contractility. As an important case, both autologous and allogeneic sources of endothelial cells are utilized experimentally to induce vascular structures in bioprinted tissue, which plays substantial roles in barrier functions between cardiomyocytes and blood [70,71]. However, the utilization of primary endothelial cells from the aorta and human umbilical vein endothelial cells (HUVECs) has been found to suggest superior functionality and establishment of cardiomyocytes through paracrine signaling, specifically supporting high maturity and development (e.g., neuregulin and Neurofibromatosis type 1), survivability (e.g., neuregulin and Platelet-derived growth factor subunit B), and contractility (e.g., Endothelin-1) [55].

Fibroblasts are also supportive cells that mainly occupy the space between cardiomyocytes, regulate ECM synthesis, paracrine signaling, and remodel specific factors, including collagen, fibronectin, and other glycoproteins. Also, the fibroblast-cardiomyocyte coupling has effectively promoted the cardiac tissue mechanoelectrical behaviors, suggesting their utilization in heterogenic co-culture systems [4,18,70,71].

## 3. Hydrogels

Various bioinks have been developed for tissue bioprinting. They are mainly categorized into two groups of scaffold-based (e.g., polymer-cell suspension, microcarriers) and scaffold-free (e.g., cell/tissue spheroids) bioinks [72]. Scaffold-based bioinks are more common in cardiac bioprinting since incorporating biopolymers structurally supports tissue elasticity and myotubules formation, and bioactively can improve cellular fate by maintaining biochemical and bioelectrical signals [4,73]. Bioinks are classified as naturally-derived and synthetic materials. Naturally-derived materials are more commonly used due to their intrinsic biocompatibility and close ECM-resemblance. Here, we summarize the essential aspects regarding the majorly used bioinks in cardiac bioprinting comprising naturally-derived polymers, synthetic polymers, and also decellularized ECM.

### 3.1. Naturally-derived polymers

#### 3.1.1. Collagen

The commonly used naturally-derived hydrogels in cardiac bioprinting majorly include alginate, gelatin-based hydrogels, collagen, fibrin, and hyaluronic acid-based compounds [18,74,75]. Among them, collagen is highly favorable since it is one of the main ECM components. It largely contributes to cellular growth and organization. The collagen's elastic nature has provided smooth deposition of cells leading to an appropriate cell niche for further myocardium formation [76,77]. For

instance, Lee et al. [74] used collagen in a novel strategy named free-form reversible embedding of suspended hydrogels (FRESH) to bioprint human heart components at specific scales. pH-driven gelation enabled a 20-μm resolution that allowed rapid micro-vascularization, cellular infiltration, and optimized mechanical properties for multiscale vasculature perfusion. This approach of using collagen to fabricate cardiac tissue resulted in synchronous contractility and directional propagation of action potential due to the aligned organization of micro-vessels and cardiomyocytes and showed that the use of materials with inherent fibril structure is of more importance [74,78].

Also, collagen possesses poor immunogenic characteristics, which is majorly attributed to a similarity between different species [79]. However, the lack of mechanical stability after printing remains a significant complication that requires well-established crosslinking strategies. Solidification of collagen can be established through either permanent covalent bonds or physically crosslinked through temperature or pH changes [80].

Crosslinking can also influence the antigenic behavior of collagen. The non-helical telopeptide regions are majorly known as antibody-recognizing sites (epitopes). Crosslinking has been found to modify epitopes causing less interaction with antibodies and less antigenic responses [79,81]. However, along with collagen's high cell-friendly properties, its implementation possesses several complications, including low adjustability, fast degradation, and increased costs that have limited its wide applications [79].

### 3.1.2. Gelatin

Gelatin, the collagen partial hydrolysis product, has an uprising biological application as an alternative for collagen. Besides the high biocompatibility resulting from its peptidyl similarity to biochemical components of ECM, several significant advantages, including lower immunogenicity rather than collagen, low costs, and high availability, have attributed to its increasing application as bioinks [77]. Gelatin can be solidified through either chemical or physical crosslinking. Physical pH- or temperature-dependent crosslinking is relatively time-consuming, which decreases the printability of the bioink [77,82]. Also, it suffers from low rheological properties required for printing. To improve stability and adjustability, blending gelatin with a readily cross-linkable polymer (e.g., alginate) or implementing chemical modifications is proposed. As a widely used gelatin-modified bioink, gelatin methacrylate (GelMA), the product of gelatin methacrylation, has often been used. Besides improved shape fidelity and rheological characteristics, photo-crosslinking of gelatin through permanent interaction of methacrylamide groups indicates better biocompatibility and stability in the biological medium. These characteristics have made it a widely used hydrogel without a need for any further modifications [77,83].

### 3.1.3. Alginate

Alginate is a water-soluble polysaccharide composed of glucuronic (G blocks) and mannuronic (M blocks) residues, which can be readily crosslinked upon exposure to divalent cations (especially $Ca^{2+}$) through the establishment of electrostatic bonds between cations and G block residues [84].

Hence, G blocks' varying densities through the polymer chain can result in different crosslinking densities and mechanical strengths [84,85]. However, the solidification time should be optimized to prevent further cell mortality [86]. In a study conducted by Gao, extrusion-based bioprinting and organ weaving were combined to bioprint vascular conduits containing multilevel fluidic channels based on alginate. Multilevel micro-channels, with two fibroblasts and smooth muscle cells levels, were fabricated through coaxial extruding hollow cell-laden alginate filaments forming a tubular conduit concentric to the main macro-channel. Endothelial cells were subsequently seeded into the inner wall of the main channel [87]. Although, larger densities of G blocks, known as hard segments, have been shown to possess less biocompatibility.

For this reason, higher M/G ratios are favorable in biological applications [86]. As a significant drawback, alginate is a biologically inert material and supports low cell adhesion. Nevertheless, rapid solidification and its ability to maintain structural and mechanical stability have made it an indispensable component in various bioinks [84–86]. However, as a post-printing procedure, the post-crosslinking time of alginate should be strictly taken into consideration since it can significantly affect the viability of cells. To overcome the low cell-friendliness of alginate, chemical modifications and also functionalizing alginate with specific polypeptides supporting cell adhesion (such as RGD polypeptide) have been majorly investigated [88,89]. In this regard, alginate is mainly accompanied by gelatin to guarantee appropriate rheological, thermo-responsive, and cell-friendliness characteristics. However, in most bioengineered alginate-based bioinks, alginate serves as a sacrificial template component of the bioink, allowing the well-controlled deposition of hydrogel fibers. This is generally due to the presence of chelating EDTA in the medium, which uncrosslinks the alginate hydrogel by releasing the $Ca^{2+}$ cations. Also, to enhance this sacrificial characteristic, alginate polymer chains have been oxidized to increase the degradation rate of the polymer thanks to the presence of more reactive groups [18,90].

### 3.1.4. Fibrin

Fibrin, a biodegradable protein that naturally exists in the blood, suggests significant potentials for cardiac regeneration. It is noteworthy to mention the mimicry potential of fibrin's incorporation with various peptides, which acts as a local reservoir for specific growth factors. This reservoir behavior provides an appropriate microenvironment for instigating cellular fate [80,91]. This intrinsic bioactivity, along with fibrin fibers' structural strength and physical characteristics, has made it a potential matrix for endothelial cell adherence and angiogenesis [92]. Fibrin fibers undergo degradation and backbone cleavage through the activity of protease enzymes. Aprotinin is a protease inhibitor typically incorporated with varying concentrations in the medium of fibrin-based constructs to control the degradation rate [80]. However, diffusion of aprotinin in the medium leads to the loss of fibrinolysis protection. It was found that the aprotinin-conjugated fibrinogen was able to prevent the plasmin-mediated fibrinolysis, and its functionality was found to be as significant as the non-conjugated soluble form [93]. Also, the potential of autologous isolation of fibrin from patients is vital in eradicating the immunogenic responses. Nevertheless, weak mechanical stability, structural shrinkage, and the possibility of disintegration are the main complications associated with fibrin fibers, which can be adjusted to a great extent by varying concentrations of $Ca^{2+}$, buffers, crosslinking agents, or combining them with other supportive materials [80,91].

### 3.1.5. Decellularized ECM

ECM decellularization is currently being investigated as another method to prepare bioinspired bioinks. Potentially, decellularized ECM is an appropriate scaffold for regenerative medicine applications since it removes cells from the tissue's ultrastructure while preserving its biological properties encompassing non-living structural and signaling molecules (e.g., collagen, glycoproteins, and glycosaminoglycans) coupled with maintaining its mechanical properties. Using 3D bioprinting technology, cell-laden structures representing the intrinsic cues of natural ECM can be fabricated by layering ECM and autologous cells as a reproducible and accurate method [28,94,95].

Decellularization specifically aims to detach cells from their ECM to remove potential nuclear and antigenic components that may cause inflammation or further immune reactions. Hence, ECM's ultrastructure induces tissue repair, whereas the host tissue does not develop antigenicity, inflammation, or immunological response, increasing implantation success [94,95]. Furthermore, the intact decellularized ECM structure, along with preserving the natural cell binding sites, is a potential reservoir for various biomolecules found in native tissue,

including proteins and growth factors [94,95]. However, the perseverance of these characteristics in the final bioprinted construct is highly dependent on the implementation of decellularized ECM. Generally, in tissue Engineering, the decellularized ECM can be implemented as a whole organ/biopsy scaffold or as processed compartments including thermal hydrogels. In bioprinting application, the latter is the associated implementation method through which the decellularized ECM is first solubilized through enzymatic treatment and subsequently used as the bioink, which can form a gel upon increasing temperature up to physiologic temperature. Accordingly, in this case, the effects of architecture are no longer maintained in the final construct [28,41].

According to different studies conducted on heart-derived decellularized ECM, one of the main challenges within this field is the mismatch between the mechanical properties of the decellularized ECM and native cardiac tissue. However, there have been studies aimed to improve the mechanical properties through different methods of two-step crosslinking or the inclusion of methacrylated natural polymers [96,97]. More specifically, Jang et al. [96], in an attempt to improve the mechanical properties of heart bio-constructs, employed Vitamin B2 (VB2) into pepsin digested decellularized-ECM as a photoinitiator. This construct was further exposed to ultraviolet (UV) light to pursue photo-crosslinking. This first crosslinking step was followed by a thermal crosslinking, ensuring the stabilization of the final construct. The results indicated appropriate printability, comparable mechanical properties, and significantly higher cardiogenic differentiation [96]. In another study conducted by Yu et al. [97], GelMA was introduced into the decellularized-ECM with which the mechanical properties could be adjusted with the post-printing exposure time of UV light [97].

### 3.2. Synthetic polymers

Although naturally-derived materials possess more cell compatibility and relatively improved bioactivity, several associated disadvantages, including low mechanical stability, immunogenicity, and less reproducibility due to the batch-to-batch variations, have led to the uprising incorporation of synthetic polymers in bioinks [4]. Generally, synthetic materials such as polyethylene glycol (PEG) and polyethylene glycol-diacrylate (PEG-DA) suggest adjustable molecular and physicochemical properties, better physical integrity, and enhanced printability. However, they do not inherently support well-established cell-matrix interactions, do not closely mimic cardiac ECM, and possess less bioactivity [4]. The utilization of blends or composites of synthetic and naturally-derived polymers such as PEGylated gelatin methacrylate (PEGgelMA) is mostly under investigation in which synthetic polymers majorly contribute to the physical support of biologically advanced bioinks. Also, there are various thermoplastic polymers under investigation which are designed to be utilized in frameworks (e.g., polycaprolactone (PCL)) or as sacrificial polymers (e.g., polyvinyl alcohol (PVA)) [10].

Although PEG-based bioinks and other highly reproducible synthetic materials have contributed to promising results in bioprinting applications, another class of synthetic materials, synthetic self-assembling peptide (SAP) hydrogels, have indicated potential characteristics, which makes it one of the candidates for bioinks [14]. SAPs are sequences of particular peptides mostly self-assembled into nanofibrillar highly hydrated hydrogel via supramolecular interactions. The concentration of peptide nanofibers and their corresponding length must both reach a critical quantity to form a nanofibrillar hydrogel. Accordingly, different rheological behavior can be obtained by adjusting the length and concentration of peptides in different ways [14,15]. However, the lack of extensively discussed printability parameters (e.g., viscosity, shear-thinning, and filament analysis) has restricted the wide application of SAPs. This is why to increase the printability and shape fidelity of these peptides, a combination with other molecular structures as hybrid bioinks are used and the formation of complementary interaction in between is mostly responsible for the improved rheological behavior [14,17].

Despite having some intrinsic challenges in their current state, the potential properties that this class of materials suggests have made a great incentive toward increasing study over their translation into bioinks. Among the ranges of advantages they exhibit, the mimicking of native ECM structure and function coupled with the bioactivity can be highlighted [14,17].

Although some natural polymers like hyaluronic acid can be used as bioinks, their low printability of them necessitates further chemical modification. More specifically, the solution of hyaluronic acid as bioink has shown no shape retention upon printing which is particularly due to the viscous shear-thinning preparations. Therefore, it is mostly functionalized or rather than being printed alone, integrated with other biomaterials, whether synthetic or natural polymers [30]. The presence of primary hydroxyl groups in hyaluronic acid allows the easy methacrylation of the polymer which can be further conjugated with a photoinitiator. Methacrylated hyaluronic acid has been found to show greater resistance to degradation and greater rigidity in comparison to unmodified hyaluronic acid. Methacrylation increases the final mechanical strength along with maintaining the main characteristics of hyaluronic acid including cell-supporting characteristics through the extrusion process, post-printing shape fidelity, tissue hydrodynamics, supporting cell migration and proliferation, and contribution to a particular cell receptor interaction [30,67].

### 3.3. Conductive bioinks

Since cardiac tissue is an electrically active tissue, the replication of a similar bio-construct as the final goal of bioprinting should be accomplished in a way that can preserve and establish the required biophysical stimulations including electrical conductance. As will be discussed in the following sections, through activating intracellular signaling pathways and altering intracellular microenvironments, proper electrical signals have been found to induce a substantial impact on encouraging and regulating cell growth performance, specifically including cell alignment, cell proliferation, cell migration, and cell differentiation. Although gelatin, alginate, GelMA, and their modified form are among the most used and promising hydrogels being widely used in cardiac tissue bioprinting, they cannot sustain and transduce the electrical signal. This is why an increasing interest has been directed toward the incorporation of electrically conductive biomaterials in bioinks [57].

Generally, these biomaterials can be classified into three groups, conductive polymers, carbon-based compounds, and metallic nanoparticles. Conductive polymers possess varying levels of conductivity depending on (i) the dopants and the levels of doping of the polymer and (ii) chemical functionalization, crossing several orders of magnitude [57]. The three common conductive polymers used in bioprinting and tissue engineering are polypyrrole [68], polyaniline [73], and poly (ethylene dioxythiophene): polystyrene sulfonate [77] owing to their exceptional electrical conductivity, chemical stability and biocompatibility. In fact, through *in vitro* and *in vivo* analysis the conducting polymers have been found to promote cell adhesion, cell proliferation, cell migration, cell differentiation, and secretion of proteins at the interface. However, since the pure films of these polymers show different drawbacks including high brittleness, poor solubility, and non-degradability, they are mostly utilized in different forms of blends, composites, and nanofibers [57]. Ajdary et al. [68] developed a biomaterial system incorporating nanocellulose, poly (glycerol sebacate), and polypyrrole as a heart patch with the ability to release curcumin. The results clearly showed a significantly improved electrical conductivity ($34 \pm 2.7$ mS. $cm^{-1}$) due to the incorporation of polypyrrole in the construct, which was coupled with improved H9c2 cardiomyoblasts cytocompatibility [68].

However, among the studies conducted on the bioprinting of cardiac tissue, the induction of electrical conductance is greatly done through the incorporation of carbon-based compounds including carbon

nanotube-based composites and graphene derivatives [57]. Along with exhibiting excellent processability, these compounds show superior specific surface area, electrical, and mechanical properties in comparison to conducting polymers. Although oxidized forms of graphene show lower electrical and mechanical properties, the proper dispersion of graphene oxide and reduced graphene oxide make them potentially better components to be incorporated in the hydrogels [57]. The easy dispersibility of graphene oxide in water can be also accounted as another factor in the interest toward the incorporation of graphene oxide over conducting polymers. To provide a more instructive microenvironment for cell growth and tissue development, Tsui et al. [22], developed hybrid bioinks composed of decellularized porcine myocardial extracellular matrix and reduced graphene oxide (rGO). The results associated with the electrical characterization of bioprinted constructs demonstrated a significantly higher electrical conduction in decellularized ECM-rGO compared to their decellularized-ECM counterparts which showed a maximum amount of $35.4 \pm 2.3$ cm/s being very close to the mean conduction velocity of isolated human left ventricular myocardium. The protein analysis on connexin gap junctions which is found to be responsible for the electrical transmission between cardiomyocytes also supported the same difference between the rGO incorporated and unincorporated constructs [22,57].

Along with the incorporation of organic compounds in the bioink, metal nanoparticles can be also incorporated into the bioinks to facilitate electrical conductance. Silver and gold are among the most investigated nanoparticles in the literature and each suggests particular advantages favoring their incorporation in bio-constructs [57]. The major challenge that should be considered when using metal nanoparticles is the cytotoxicity, which is highly dependent on the nanoparticle dimensions and concentration [57]. There would be a tradeoff between the resultant conductance and the cytotoxicity, which has shown to exhibit similar electrical conductance as the ventricles while preserving an acceptable cytocompatibility. In a study conducted by Zhu et al. [85], a bioink composed of GelMA incorporated with gold nanorods was developed for cardiac tissue bioprinting. Higher beating rate, elongation, and contraction rate were reported compared to alginate-GelMA bioprinted constructs. Accordingly, the incorporation of gold nanoparticles could increase electrical conductivity and therefore produce a more developed cardiac tissue capable of performing repetitive and concurrent beatings [85].

### 3.4. Considerations in bioink composition

To achieve an appropriate cell-cell and cell-matrix interaction required for tissue regeneration, the bioink should mainly support cell adhesion, cell alignment, oxygen transport, and, specifically for cardiac tissue, electromechanical synchronization through the successful establishment of gap junctions [98]. To this end, the bioink should possess several significant characteristics, including appropriate viscosity and shear thinning for enhanced printability, well-established solidification kinetics, high hydration ability, viscoelastic behavior close to the native cardiac ECM, and enough diffusivity to oxygen and cellular bioactivity to increase physiological synchronization (Fig. 1) [98,99]. In this regard, researchers are highly inclined to incorporate blended bioinks. Composite bioinks reported to be successful in biological characterizations are mainly alginate-GelMA, GelMA-cardiac

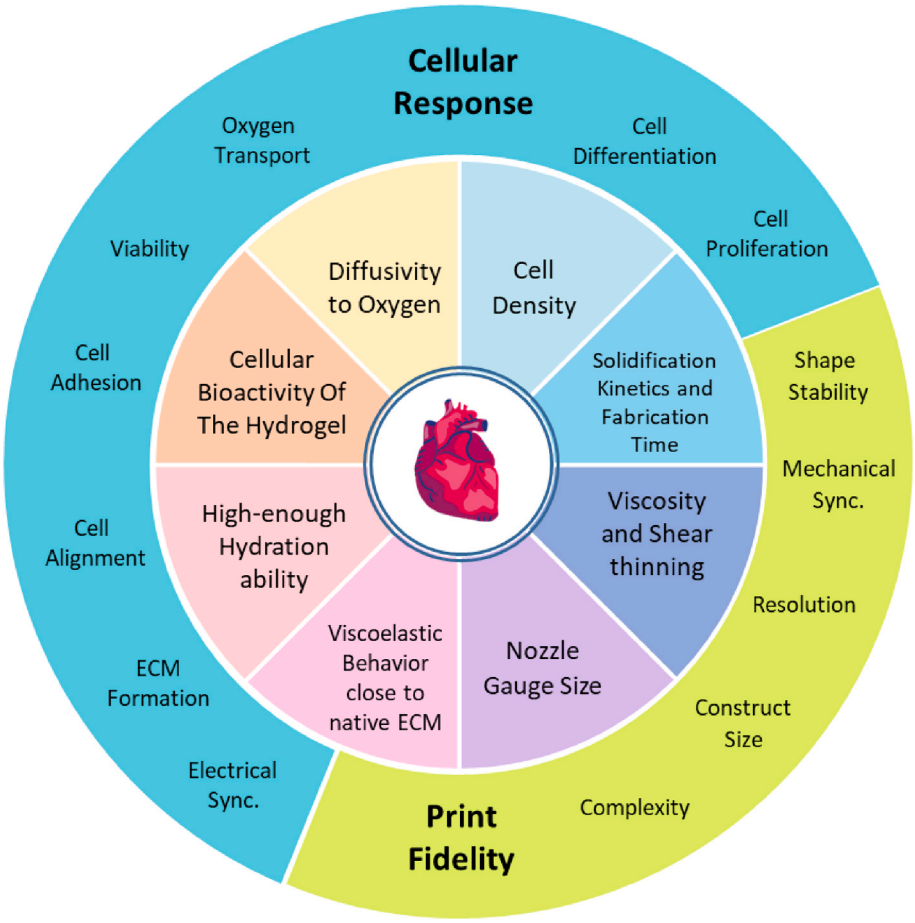


**Fig. 1.** Engineering considerations in Bioink compositions and correlated cellular and print fidelity challenges. Cardiovascular bioprinting is mostly correlated with several specific challenges subcategorizing in cellular challenges and those associated with print fidelity. To overcome these challenges, particular engineering considerations come into the role of establishing a well-organized bioink able to satisfy correlated challenges.

extracellular matrix, alginate-PEG-fibrinogen, and collagen-fibrin compositions. However, aside from bioink intrinsic properties and architectural considerations, adjusting composition and rheology can significantly influence the printability and biological characteristics of bioinks (Fig. 1) [90].

One of the deterministic factors influencing the rheology of bioinks is the concentration of the components. Although higher concentration may result in better mechanical stability and shape fidelity, it can significantly affect oxygen transportation [100,101]. An important parameter impacting the transport kinetics is the ratio of applied concentration (C) to the critical overlapping concentration (C*) of the polymer [102]. C* is the concentration in which the polymer chains start to overlap regarding their radius of gyration. Hence, the more C/C* ratio, the more densified solution results, which causes higher compaction and less porosity. This can significantly affect the transportation of biomolecules, especially oxygen [86,102,103], thus resulting in impaired maintenance of cell viability.

Another parameter that can be influenced by concentration is the shear-thinning behavior of the bioink [104]. Typically, shear stress is experienced by cells during bioprinting, while the biomaterial compartment of the bioink acts as a shield to mitigate the exerted shear stress on the cells. The shear-thinning characteristic is responsible for the mentioned behavior by which the exerted shear stress is damped by lowering the hydrogel viscosity. Therefore, a scientifically reasonable trade-off between printability and transport phenomena regarding the concentration of components is required [105,106].

Along with scaffold-dependent parameters, cell density can considerably influence the further regeneration potential of bioinks since key elements in regeneration, such as gene regulation, differentiation, and cell progression, are highly density-dependent elements [107]. Hence, optimizing the cell density is a crucial step toward developing a clinically applicable bioink. This can be discussed by accounting for two primary biological and mechanical considerations. Regarding the former, the cell type and associated proliferative capacity are determining factors in optimizing initial cell density [108–110]. The initial low density of highly proliferating cells may be reasonable, while the initial low densities can inhibit the cell population regarding the other cells with no or moderate proliferative capacity. This is due to the lack of required cell-cell interactions and the release of directing agents [110]. However, considerably high densities can also contribute to the formation of hypoxic regions due to an imbalance between uptake and intake rates of oxygen and nutrients. Inharmonious degradation of the hydrogels may also result in higher ECM production due to the utilization of high densities [100,110,111].

Regarding the mechanical considerations, higher cell densities increase the bioink viscosity requiring higher loads for printing. This can negatively influence cell viability to a great extent. Also, cells act as disintegration sites in the hydrogel in high densities, lowering deposited tissue's mechanical stability, and influencing long-term maintenance [100,111–113].

Modifying the internal structure of hydrogels through crosslinking is almost a post-printing process, which is determined with respect to the chemical composition of the hydrogel. The reaction can be generally established through physical and chemical routes. In each crosslinking method, specific considerations should be taken into account to obtain appropriate mechanobiological properties. Obviously, in chemical crosslinking, along with effective structural hardening, the crosslinker should elicit no cytotoxicity; in this regard, agents like genipin and EDC/NHS have suggested an appropriate biological response. Generally, in thermally and pH-dependent cross-linkable hydrogels like gelatin, the ideal plasticizing temperature is a point that has the minimum difference with the physiologic condition (37°C and pH = 7.4) accompanied by appropriate mechanical properties. Hence, the cells are less likely to undergo thermal or pH shocks [77].

In irradiation crosslinking, three particular parameters should be strictly controlled for enhanced and appropriate mechanobiological characteristics: the photoinitiator used for physical crosslinking, exposure time, and the irradiation wavelength. The commonly used photoinitiators are Irgacure 2959, Lithium phenyl-2,4,6-trimethylbenzoylphosphinate (LAP), and ruthenium-sodium persulfate. However, a study on cardiac patch bioprinting found that the Eosin Y system can more effectively act as a crosslinking agent in methacrylated collagen (MeCol) hydrogel accompanied by higher viable biological constituents. Regarding exposure, it is critically important to adjust the composition to minimize the exposure time [114]. Accordingly, as reported in a study conducted by Izadifar et al. [115], cell viability was characterized by varying UV exposure times of 45s, 120s, and 270s. Quantified results indicated an inverse relationship between exposure time and cell viability [115]. These results were consistent with the alterations in cellular morphology in a way that HUVECs were found to be able to preserve their stretchable morphology in MeCol hydrogel at 45s and 120s exposures. In comparison, in the 270s exposure time, the cells mainly stayed round in shape. The utilized irradiation wavelengths mainly were in the range of 300–500 nm. Nevertheless, in the same study that used the Eosin Y system, white light irradiation was used over UV exposure to decrease the induced cell death [77,114].

Although there is a great range of materials being investigated in the literature to be translated as bioink, there would always be a tradeoff between the above-mentioned factors in developing a tissue-specific or a universal bioink. However, there are numerous studies under investigation by both academic and industrialized groups attempting to narrow down this tradeoff region. Accordingly, there are increasing attempts to commercialize functional bioinks for a wide range of applications, which has shown to be successful to some points. In this regard, commercial products that have been studied on cardiac tissue mostly include Lifeink® 240 [106], Cellink GelMA-LAP, and AlloECM® [109] which are mostly based on collagen, gelatin, methacrylated alginate, and laminin. With respect to the necessity of electrical conductance in cardiac bioinks, the Cellink company has also developed a new bioink named Bio-Conductink [113] derived from the GelMA ink.

In this section, numerous types of hydrogels, bioinks, and their characteristics have been discussed, which shows the wide range of biomaterials and the ability of the bioprinting approach that can be utilized to fabricate cardiac constructs. Besides, it shows that different kinds of functionalities necessitate the application of diverse hydrogels and nanoparticles.

## 4. Bioprinting techniques

Cells, biological macromolecules, and structural moieties are three main components that aim to provide a native environment to repair heart tissue injuries. However, fabrication and mimicking the complex 3D architecture of normal tissue is a challenging issue. Novel bioprinting systems have been developed to overcome this issue to a great extent. Aside from scaffold-free methods of bioprinting, other methods such as extrusion-based bioprinting (EBB), inkjet-based bioprinting, and light processing-based bioprinting (including laser-assisted bioprinting, stereolithography, and laser-Induced forward transfer (LIFT)) are three genuine scaffold-based techniques that exhibit high potentials in fabricating complex architectures (Fig. 2) [116–118]. However, among these various strategies, the extrusion-based bioprinting method, due to the specific characteristics suggested, is the most considerably applied method in cardiac bioprinting application, while to a great extent, no studies have reported the utilization of other approaches in cardiac bioprinting. Accordingly, here in this section, the focus has been established on extrusion-based and scaffold-free methods.

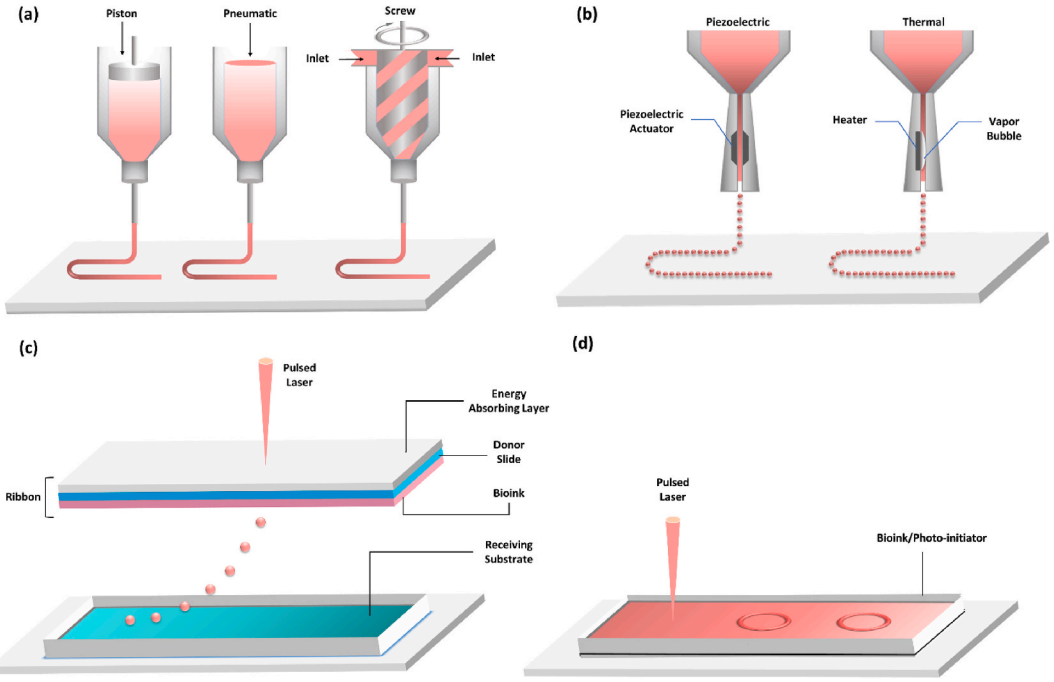


**Fig. 2.** Schematic illustration of 3D bioprinting technologies: a) Piston-based, pneumatic-based, and screw-based micro-extrusion technique b) Thermal and piezoelectric inkjet printing c) Pulsed-laser-assisted bioprinting d) Stereolithography.

### *4.1. Extrusion-based bioprinting*

EBB is a widely-applied method used to fabricate cardiovascular tissues by simultaneously dispensing cells and materials matrix. In this method, complex structures are biofabricated with various cells precisely. Mechanistically, EBB technology is very similar to the fused deposition modeling among 3D printing systems. It is based on extruding viscoelastic polymeric bioinks via an automatic robotic system to form 3D constructs layer by layer. An automated mechanical system can move in x, y, and z directions controlled and adjusted perfectly by a computer [72,117,119]. The dispensing system, which is pneumatic or mechanical (piston or screw-based), extrudes bioinks through nozzles and dispenses them on build-bed. Hence, a 2D pattern is primarily printed. After printing this 2D pattern, different curing methods solidify the bioink, and subsequently, the second layer is printed on the first layer to fabricate the 3D pattern layer by layer. Cell viability and cell density are critical factors in bioprinting. In EBB techniques, the effect of shear stress on cell viability is one of the parameters that should be controlled besides thermal stresses, which both can cause cell death. On the other hand, printing bioinks with high cell densities require higher shear stress. So, the printing pressure should be adjusted due to these two factors [72,116,118,120–126].

### *4.2. Scaffold-free bioprinting*

No biomaterials are used to print cells and generate a 3D structure in scaffold-free bioprinting. Cell pellets are fused in a 3D printed mold to secrete the extracellular matrix and to be held together. Through this method, the cells should go under a series of cell recapitulations to be further employed in bioprinting tissues. Since many cell expansions would be required to obtain tissue-level regeneration, this method may be slower than the scaffold-based methods. Despite cell proliferation inside the scaffold, many cells are poured into the scaffold to form the ECM in the shape of a cylinder, torus, spheroids, and honeycomb. Various developed methods are used to biofabricate scaffold-free structures, including hanging drop, pellet (re-aggregation) culture or conical tube, micro-molding, microfluidics (hydrodynamic cell trapping), liquid overlay, spinner flask, and rotating wall vessel techniques [116,127,128].

Scaffold-free bioprinting is a method that fabricates structures through bioprinting of living cells with designed patterns. Cells are deposited on a substrate or a spheroid mold to derive them in a specific module and are bioprinted layer by layer to form the final 3D structure. Using this method, many kinds of cells can be implemented to biofabricate tissue-like structures [116,127]. Compared to scaffold-based bioprinting methods, scaffold-free practices suggest a higher range of efficiencies, primarily attributed to the cells' self-assembly and high-speed bioprinting process. Furthermore, in this strategy, cell retention is high. Post-printing maturation time is comparable with other methods. One of the acute adverse effects of using these constructs is the possibility of immunogenic rejection unless the patient-derived cells would be employed [116,124,127,128].

### *4.3. Bioassembly*

Self-assembly bioprinting is a subclass of a scaffold-free method to bioprint desired microstructures. Several processes such as self-assembly, robotic assembly, Faraday acoustic assembly, bio-acoustic levitational assembly, magnetic assembly enable the user to organize, reorganize and regenerate basic units to form the 3D tissue architecture used in scaffold-free based bioprinting [120,129]. In this method, the micro-tissue bioinks are deposited in a closed area, fused, and further structured layer by layer to biofabricate the final construct. Flexibility, high scalability of desired architecture, reducing cardiac hypertrophy and fibrosis, high cell densities, and paracrine signaling are promising properties that have made this method more and more desirable.

Nevertheless, low architectural resolution and lack of control are two main disadvantages of using the self-assembly technique [126,128,129].

## 5. Architectural designs

The biological construct's architectural design is one factor that can be considered to provide cell-signaling features and affects cell-biomaterial interaction such as adhesion, cell growth, and especially migration, which directly leads to higher cell functions. Specifically, cell alignment, conductivity, and contractility are the functions that can be controlled under the influence of the design of the bioprinted construct [24,130]. Several architectural designs such as honeycombs, grids, strings, and even anatomical models have been investigated to induce higher cell viability and functions. In a study conducted by Zhang and colleagues, a biological construct was printed in an anisotropic honeycomb design, which provided higher connexin-43 expression and cell alignment in the direction of anisotropy compared to the isotropic design [24]. In another study, Noor et al. [131] used decellularized omentum tissue-based bioink to bioprint a vascularized cardiac patch in an anatomical design. The study indicated that the cardiac patch is contractile, bioprinted cardiomyocytes are aligned and elongated, and endothelial cells formed lumens, which shows that biomimetic structural design can influence the morphology and functions of cells [131].

Vascularization is a significant challenge that should be overcome in 3D bioprinting due to delivering nutrients and oxygen to the constructs' inner space [3]. Different designs are considered for endothelial cells and cardiomyocytes co-culture, mainly grids and layer by layer co-culture. In another study, Maiullari et al. used a bioink consisting of cardiomyocytes and endothelial cells in a grid design. In this design, a cardiomyocyte layer and endothelial layer were bioprinted respectively through a microfluidic printing head. Higher cell viability and tissue-level functions were provided by this design compared to other bioinks containing only cardiomyocytes [18].

Regarding converted 2D images of the human heart to 3D models, designed models could be produced and reproduced safely and in a patient-specific manner with complex architectures. In most designed structures, X-ray computed tomography, or MRI (magnetic resonance imaging), is used to capture the human heart's complex anatomy, and subsequently, 2D images are converted to a 3D image. The 3D image will be processed in Biomimics software to optimize the desired architecture. Thus, the final design will be converted to a G.code file to bioprint designed human heart model [132–134].

## 6. Biophysical stimulations

The heart is a highly active organ in which, in both developing and adult cardiac tissue, cardiomyocytes are subjected to various kinds of stimulations, including contractile mechanical forces, electrical forces, and hemodynamic stresses [16,135]. Numerous studies have revealed that these intracellular and extracellular stresses can have deterministic effects on cardiomyocyte maturation, mechanoelectrical coupling, and maintaining the differentiated phenotype of cells. Traditional bioengineering systems have shown immaturity in cardiomyocytes derived from stem cells such as iPSCs and skeletal myoblasts [136,137]. This immature characteristic of cardiomyocytes results in impaired synchronization and poor mechanoelectrical integration with the host tissue [2,137]. Hence, novel bioreactors and progressive methods are developed to provide relevant physiological states and deliver guiding stimuli to the bioengineered tissue to direct the cardiomyocytes' maturation through the construct. Here, we discuss major contributing stimuli in cardiac regeneration (Table 1).

**Table 1**
Different stimulations used in cardiac tissue development: types of stimulations, cells, biomaterials used to fabricate biological constructs or substrates, time, and conditions of stimulations.

| No. | Stimulations | Cells | Biomaterials | Time | Conditions | References |
|---|---|---|---|---|---|---|
| 1 | Passive mechanical stimulation (stiffness) | hESC-CMs | PDMS substrate | 9 days | Stiffness range: (5–101 kPa)<br>Pattern widths:12–24 μm | [139] |
| 2 | Passive mechanical stimulation (topography) | NRVM | PDMS substrate | 28 days | Microgrooves with 40, 60, 80 and 100 μm spacings | [140] |
| 3 | Active mechanical stimulation (static stretch) | hiPSC-CM | Collagen | 21 days | Progressive stretch rate: 0–0.32 mm/day | [141] |
| 4 | Active mechanical stimulation (cyclic stretch) | Neonatal rat cardiomyocyte | Fibrin hydrogel | 5 days | Sinusoidal waveform,<br>10% strain,<br>Frequency: 1 Hz | [142] |
| 5 | Pulsatile electrical stimulation | Neonatal rat cardiomyocyte | Decellularized pig's omenta tissue and synthetic graphite mixed in PDMS | 12 days | Voltage:7 V,<br>Pulse period: 50 ms,<br>Frequency: 1 and 2 Hz | [151] |
| 6 | Pulsatile electrical stimulation | hiPSC-CM | Collagen based | 7 days | Voltage:5 V/cm,<br>Pulse period: 5 ms,<br>Frequency: 2 Hz | [136] |
| 7 | Pulsatile electrical stimulation | hADMSC | PCL | 1 h | Voltage: 100 mV,<br>Pulse period: 0.2 ms,<br>Frequency: 3 Hz | [152] |
| 8 | Pulsatile electrical stimulation | pHCM | Collagen | 3 h | Voltage: 5 V,<br>Pulse period: 2 ms,<br>Frequency: 1 Hz | [153] |
| 9 | Hemodynamic stimulation (pulsatile perfusion) | Neonatal rat cardiomyocyte | Collagen and Matrigel | 5 days | Frequency: 1 Hz,<br>Flow rate: 1.50 or 0.32 mL/min | [156] |
| 10 | Hemodynamic stimulation (pulsatile perfusion) | NRVM | Alginate | 10 min | Pulsatile flow rate: 60, 120, and 180 pulses/min,<br>Shear stress: 0.6, 2.4, and 5.4 dyn/cm2 | [157] |

### 6.1. Mechanical stimulation

There is an increasing body of evidence that mechanical stimulations have profound impacts on cell physiology modulation and may, as a result, promote biosynthetic activities in cells residing in bioartificial matrices, thereby facilitating or speeding up tissue regeneration *in vitro*. Mechanical forces experienced by cardiomyocytes *in vivo* are exerted mainly by contractile forces, blood flow, shear stress, and pulsatile blood pressure [16]. These mechanical stimulations have been helpful in the anisotropic alignment of cells and directing functional and structural maturity of cells by regulating the genes and proteins expression [138]. *In vitro* mechanical stimulations can be established by adjusting the mechanical properties of the biomaterial compartment like altering stiffness or changing surface topography (passive stimulation) or exerting external stretching forces on the construct (active stimulation) [2,16,138,139].

Passive stimulations such as altering surface stiffness or topography have been used in many studies to maturate primary or stem cell-derived cardiomyocytes and activate cell alignment. Rodriguez et al. [139] fabricated five substrates from different mixtures of Sylgard 184 and Sylgard 257 (0, 5, 10, 15, and 20% Sylgard 184) to create a 5–101 kPa range of stiffness and compare the impact of stiffness and other conditions like cell densities and laminin printing on hESC-CMs maturation. It was shown that hESC-CMs have the highest degree of binucleation, calcium intensity, and the lowest amount of nuclear to cellular volume on the substrate with 21 kPa stiffness, which indicates the response of cardiomyocytes to stiffness [139]. In another study by Lind et al. [140], they used shear-thinning soft PDMS to bioprint microgrooves on an Organ-on-a-Chip system with different spacings (40, 60, 80, and 100 μm) and it was called cardiopatch. It was demonstrated that cardiopatches with spacings of 60 μm between grooves resulted in more aligned and organized neonatal rat ventricular myocyte (NRVM) compared to other spacings [140].

Although various studies indicate the potential impact of mechanical active stimulation on cells' functional and morphological organization, these mechanical stimulations have not been utilized with bioprinted constructs. Also, there is still little knowledge regarding particular specifications of mechanical stimulations' regimes of application (i.e., magnitude, continuous or sporadic, frequency). The mainly applied mechanical stimulations studied in the literature are static and cyclic forces. Suspending engineered cardiac tissue between fixed holders is the most straightforward approach to load static or cyclic mechanical forces; furthermore, static loading protocols can also be performed for extended periods without causing tissue rupture to apply progressive stretches. For instance, Lu et al. [141] developed a custom-made biomimetic tissue culture system that could provide progressive stretch at four different increments and used this system to investigate the impact of different amounts of stretches alongside electrical stimulations on tissue expansion and cellular functions. The results showed that a stretch rate of 0.32 mm/day resulted in the highest sarcomere length ($2.19 \pm 0.1$ μm), cellular volume, and cell alignment in comparison with the control sample. Cyclic stretching has been effective while only feasible for a limited period (7–10 days) without premature rupture [141]. In this approach, the period can be adjusted in two specific ways of (i) introducing advanced materials through which the viscoelastic properties of engineered tissue are changed and (ii) by adjusting the stretching algorithm to the intrinsic characteristic of engineered tissue [139]. It is required to mention that in the second approach, in optimizing the cycle length, it should be in harmony with the preexisting pacemaker cells within the bioengineered cardiac tissue. Ultimately, the tissue is suspended between resilient mounts to optimize auxotonic contractions of engineered cardiac tissue. This appears to mimic the physiologic contraction cycle substantially, but it is also the most challenging to generate in a way that corresponds to the constantly evolving contractile characteristics [139]. Massai et al. [142] developed an automated bioreactor to provide cyclic stretches to a 3D annular cardiac tissue seeded with neonatal rat cardiac cells and to investigate the impact of uniaxial cyclic stretches (sinusoidal waveform, 10% strain, 1 Hz) on the maturation of the construct in comparison with static culture. After 5 days of static culture, the construct was exposed to cyclic stretches for 4 days. Immunofluorescence assays demonstrated maturation and alignment of the cardiac cells compared to static culture and only the dynamic cultured construct was responsive to external electrical pulses, which showed the effectiveness of cyclic stretches [142].

In these mechanical stimulations, the extracellular mechanical stresses can be transmitted by transmembrane integrins binding, such as the tyrosine kinase receptor, which further activates particular intracellular pathways including Rho/ROCK, MAPK/ERK, FAK, and AKT [138,141–143]. These pathways and cellular fate largely contribute to regulating specific cellular activities, including hypertrophy, mitochondrial oxidative stress, calcium handling, and remodeling, causing anisotropic cell alignment and both phenotypical and functional maturation [144]. Hence, the bioink should be well-optimized to harbor dynamic mechanical stretch-relaxation stresses for long periods, while architectural and compositional considerations can establish phenotypical and functional maturation.

### 6.2. Electrical stimulation

The heart's electrical activity is attributed to the well-organized patterns of voltage-gated ion channels responsible for action potential formation and gap junction proteins contributing to the propagation of electrical signals through the cardiac syncytium. The electrical signals are converted to the contractile force by the excitation-contraction coupling (ECC) [2,145]. Hence, cardiac tissue's coordinated contraction primarily depends on gap junction proteins' presence and expression patterns, specifically Connexins (Cxs) [138,146,147].

Both direct and pulsatile electrical impulses are crucial in developing hearts regulating the expression of connexins and voltage-gated channel ions [138]. This is consistent with the finding that the conventional culturing of iPSC-derived cardiomyocytes has shown to lack voltage-gated ion channel expression, which leads to immature nonfunctional cardiomyocytes [148]. The bioengineered tissues may also possess electrically and mechanically active but occur at varying rates and less spontaneous behavior. Therefore, cells' electrophysiological activity in bioengineered tissues can indicate cardiac maturity [138,149]. Numerous studies have developed biomimetic systems to deliver electrical impulses to bioengineered cardiac tissue to harbor control over the tissue function and to develop mechanoelectrical properties, especially cell alignment, increased electrical coupling through up-regulation of junction proteins, amplified contractions concurrently, less arrhythmia, and highly organized ultrastructural organization [138,144,150]. For instance, through a study conducted by Asulin et al. [151] on one-step 3D bioprinting of cardiac patches with built-in soft and stretchable electronic systems, an electrical stimulation (7 V, 50 ms, 1 and 2 Hz) was applied to check the reaction to external stimulation and the construct contracted synchronously. It is demonstrated that electrodes built into the engineered tissue enable the monitoring of extracellular potentials, which gives a clearer picture of the tissue's function and more controllability [151]. In another investigation, Ruan et al. [136] studied the effect of electrical stimulation coupled with mechanical conditioning on the force maturation and contractility of iPSC-Derived human cardiac tissue. They indicated that the electric pacing (5 V/cm, 40 ms, 2, 2.5, and 3 Hz) cooperated with static stress conditioning has resulted in increased force production ($1.34 \pm 0.19$ mN/mm2), increased RYR2 (Ryanodine Receptor 2), and SERCA2 expression and hence, promoted maturation of excitation-contraction coupling [136]. In another study, Tracy et al. [152] used type I collagen and human adipogenic mesenchymal stem cells (hADMSCs) to prepare a bioink and to bioprint a Purkinje networks based on anatomical structure. In this study, connexin-40 (Cx-40) expression in manual pipetted, bioprinted, and stimulated-bioprinted

constructs (100 mV, 0.2 ms, 3 Hz) were compared and an equal Cx-40 to DAPI mean fluorescence intensity (MFI) was seen, which is believed to be downregulated by Purkinje cells upon pacing and shows the ability of Purkinje networks to adapt to increased conduction velocity to heart rate [152].

Besides stem cells, electrical stimulations have been used to enhance cell functions. Adams et al. [153] prepared a bioink from PCL and pHCM and bioprinted a construct out of this bioink to investigate the impact of electrical stimulation on the bioprinted construct. A low-cost electrical stimulation device (5 V, 2 ms pulses, 1 Hz) was developed and connected to a 6-well chamber to stimulate cells for 3 h. Immunofluorescence assays showed that 72.49% of cardiomyocytes were elongated and actin fibers were also seen in the stimulated construct, which showed that the electrical stimulation has enhanced cellular functions successfully compared to a control sample [153].

### 6.3. Hemodynamic stimulation

Aside from the conventional strategies of applying mechanical stresses, hemodynamic-induced stress, as an epigenetic factor, can significantly influence cardiomyocytes' maturation and functionality. The native shear stress experienced by cells due to the dynamic blood flow contributes to a highly organized gene expression modulation, resulting in particular morphological and functional maturity [4]. A significant result is the increased viability of cells attributed to the highly improved transport of nutrients, oxygen, and regulatory molecules. Static conditions are majorly involved with the diffusion exchange of biomolecules, a short-term phenomenon that does not efficiently contribute to macroscale constructs. Perfusion allows the more homogeneous spatial distribution of vital biomolecules such as oxygen through the dynamic convection of mass, crucial in large-scale constructs [4,138,154]. It has also been shown that the shear stress induced by perfusion results in improved compliance to the burst pressures, cellular polarization, angiogenic responses, and more organized ECM production [4,155].

Perfusion can be exerted in two ways; steady-state flow and pulsatile flow [138]. Based on the results from a study by Brown et al. [156], it has been shown that the pulsatile perfusion in high flow rates leads to a higher contraction strength and lower excitation thresholds required for coordinated stimulations. However, in low flow rates, the morphological changes are accompanied by hypertrophy of the biological construct [156]. In another study by Dvir et al. [157] about the perfusion impact, compared to the static conditions, a six-fold increase in the ERK1/2 signaling pathway has been reported, highlighting the crucial role of ERK1/2 contributing to the high expression of contractile and cellular junction proteins in cardiomyocytes. These modulations in the protein expression induced by pulsatile perfusion showed enhanced viability, cellularity, and ultrastructural organizations [157,158].

## 7. 3D bioprinting of cardiac tissue

In the last decade, the focus on cardiac tissue development has been increasing and many studies have been conducted to treat CVDs. Several approaches have been investigated to fabricate types of cardiac constructs such as patches, organoids, and other scaffolds to study cardiac tissue behaviors, drug tests, and the differentiation of several stem cells. These approaches alongside types of bioinks, cells, methods, and architectural designs are discussed in this section [159] (Table 2).

**Table 2**
Cardiac 3D bioprinting: bioink composition, bioprinting technique, and the architectural design used in cardiac 3D bioprinting case studies.

| No. | Hydrogels | Cells (density) | Bioprinting Techniques | Architectural Designs | References |
|---|---|---|---|---|---|
| 1 | - GelMA (low and high molecular weight)<br>- Alginate | - Human umbilical vein endothelial cell (HUVEC) ($1 \times 10^7$ cells/ml)<br>- Neonatal rat cardiomyocytes ($1 \times 10^6$ cells/ml) | Extrusion based bioprinting (pneumatic) | Anisotropic accordion-like honeycomb | [24] |
| 2 | Fibrin-based hydrogel, gelatin, glycerol and, hyaluronic acid | Neonatal ventricular cardiomyocytes ($10 \times 10^6$ cells/ml) | Extrusion based bioprinting (pneumatic) | String and patch form | [162] |
| 3 | Gelatin (crosslinked with mTgase) | - Neonatal rat cardiomyocytes ($2 \times 10^5$ cells/cm$^2$)<br>- Human mesenchymal stem cell (h-MSC) | Extrusion based bioprinting (pneumatic) | Micro-channeled sheet | [23] |
| 4 | - cECM<br>- GelMA | Human cardiac progenitor cell (hCPC) ($3 \times 10^6$ cells/ml) | Extrusion based bioprinting (pneumatic) | Grid | [36] |
| 5 | Alginate | Human coronary artery endothelial cells HCAEC ($0.6 \times 10^6$ cells/mL) | Extrusion based bioprinting (pneumatic) | Grid with different angles (15/165°, 0/90°, and 0/45/90/135°) | [163] |
| 6 | - CNT incorporated MeCol<br>- CNT incorporated alginate | HCAEC ($0.8–1 \times 10^6$ cells/ml) | Extrusion based bioprinting (pneumatic) | Accordion-like honeycomb | [115] |
| 7 | - GelMA<br>- Alginate<br>- Gold nanorods | Neonatal rat cardiomyocytes ($7.5 \times 10^5$ cells/well) | Extrusion based bioprinting (co-axial and pneumatic) | Grid | [164] |
| 8 | - Collagen type I<br>- Gelatin<br>- Fibrinogen<br>- Alginate | - h-MSC derived cardiomyocytes<br>- HUVEC<br>- Cardiac fibroblasts | Extrusion based bioprinting | Grid | [74] |
| 9 | - Alginate<br>- Polyethylene glycol monoacrylate-fibrinogen | - iPSC-derived cardiomyocytes ($8 \times 10^6$ cells/ml)<br>- HUVEC ($6 \times 10^6$ cells/ml) | Extrusion based bioprinting (co-axial microfluidic printing head) | Grid | [18] |
| 10 | - | - iPSC-derived cardiomyocytes<br>- Human cardiac fibroblasts<br>- HUVECs ($5–60 \times 10^3$ cells/Cardiosphere) | Extrusion based bioprinting | Sphere | [159] |
| 11 | | - iPSC-derived | Extrusion based bioprinting | - Crisscross | [131] |

(continued on next page)

**Table 2** (*continued*)

| No. | Hydrogels | Cells (density) | Bioprinting Techniques | Architectural Designs | References |
|---|---|---|---|---|---|
| | - Decellularized humans/pigs omenta tissue<br>- Gelatin as a sacrificial layer | Cardiomyocytes ($1 \times 10^8$ cells/ml)<br>- iPSC-derived endothelial cells ($2 \times 10^7$ cells/ml)<br>- Neonatal cardiomyocytes ($1 \times 10^8$ cells/ml) (Whole heart study)<br>- HUVEC ($1.5 \times 10^7$ cells/ml) (whole heart study) | | - Patient's heart vasculature (anatomical model)<br>- Whole rat's heart (anatomical model) | |
| 12 | - Decellularized pigs omenta tissue<br>- Synthetic graphite mixed in PDMS | - Neonatal rat cardiomyocyte | Extrusion based bioprinting | - spiral | [151] |

Zhang et al. [24] fabricated a biological construct emphasizing the impact of anisotropic design using an extrusion-based 3D bioprinter. Different types of bioinks were prepared so as to contain different GelMA and alginate concentrations, and dual crosslinking was employed for alginate and GelMA. In this research, prepared bioinks were extruded via coaxial nozzles, which have been used to crosslink alginate during the printing process and provide higher shape fidelity before the second step of GelMA crosslinking by UV exposure. HUVECs with a cell density of $1 \times 10^7$ cells/ml were encapsulated in alginate. Bioinks were further bioprinted in a grid design with different aspect ratios of unit grids ($2 \times 2$, $2 \times 3$, $2 \times 4$, and $2 \times 5$) to form an accordion-like honeycomb structure, to mimic genuine endothelium, and stimulate cell orientation in the direction of bioprinting. The anisotropic design and surface-to-volume ratio of the construct were found to affect the migration of HUVECs forming vascularized structures [24,160]. After 15 days, neonatal rat cardiomyocytes were cultured on the confluent layer of endothelial cells, which was prepared from the same bioink without encapsulated cells to investigate the impact of endothelium layer on cardiomyocytes viability and functions for three days. Cardiomyocytes were matured and expressed proteins necessary for conductivity, contractility, and synchronous beating rate. It was also demonstrated that the biological constructs with anisotropy design ($2 \times 5$ sample) expressed Cx-43 with a coverage area of about $8.02 \pm 0.54\%$, which provided a higher synchronous beating rate and a higher percentage of aligned cardiomyocytes. Zhang et al. [24] employed the CD31 surface marker and GFP-HUVECs to demonstrate the formation of lumen-like endothelial layers on the surface of microfibers and the migration of endothelial cells from the core of microfibers to the surface of them respectively. The results indicated that the endothelial cells migrated through microfibers to form the endothelium-like layer because of the high HUVECs density ($10 \times 10^6$ cells/ml). To evaluate cardiac tissue functions, Cx-43 and sarcomeric actinin were used to compare cardiomyocyte contractility, orientation, and beating in anisotropic constructions with other $2 \times 2$ and $3 \times 3$ isotropic samples. Eventually, the organization of cardiomyocytes was evaluated after culturing them on the endothelium-like bioprinted construct. It was illustrated that the existence of VGEF, which was secreted from endothelial cells, enhanced the tissue-specific functions of cardiomyocytes [24,160,161]. Preparing GelMA-based bioink and using alginate as a sacrificial material is one of the excellent methods for bioprinting; however, using photoinitiation in the UV range is still harmful to cells. Besides, preparing a construct with anisotropic structure helps cardiomyocytes elongate significantly in the long term and encourages contractility.

Wang et al. [162] have studied another composite bioink containing neonatal ventricular cardiomyocytes with a density of $10 \times 10^6$ cells/ml and fibrin-based hydrogel, which was prepared out of fibrinogen, gelatin, glycerol, aprotinin and, hyaluronic acid with different ratios to fabricate a contractile and functional cardiac tissue. The bioink incorporated a sacrificial hydrogel, which contained the same bioink without fibrinogen and aprotinin, to a reinforced cell-laden hydrogel. They used an extrusion-based tri-nozzle bioprinter to deposit two types of constructs, including string and patch form out of the bioink and the sacrificial hydrogel, surrounded by a PCL frame. First, the frame was printed to anchor the biological construct from two sides to enable intrinsic forces. After 30 min of resting in the chamber, the sacrificial hydrogel and cell-laden hydrogel were bioprinted at 18 °C. Subsequently, functional characterization of contractility, cell alignment and, electromechanical coupling of the construct was investigated for three weeks [162]. The construct's contractility was localized and limited after three days; however, the synchronous and spontaneous beating was detected after four weeks. By detecting α-actinin and connexin, elongation of cardiomyocytes was observed, without appreciable differences in both designs. A contractility and maturation positive feedback loops were observed, allowing the maturation of the constructs. Cardiomyocytes from single cells to a dense tissue-like structure were assessed with the same immunofluorescence markers to evaluate string and patch forms in 3 weeks. The results showed that both designs supported cardiomyocytes' functions properly. After four days, cardiomyocytes were just aggregated in the bioprinted construct; however, there were more elongated and denser populations of cells after three weeks in cardiac patch form. This study showed that design plays a significant role in cardiac tissue development and contractility. After three weeks, the patch sample provided higher concurrent contractility, and cardiomyocytes proliferated more than the string sample [162].

One of the bioprinting applications is to support 3D culture and the differentiation of stem cells. Tijore et al. [23] utilized this capability to differentiate human mesenchymal stem cells (hMSCs) toward cardiomyocytes and further investigated the impact of micro-channeled hydrogel design on differentiation. In this study, neonatal rat cardiomyocytes with a density of $2 \times 10^5$ cells/cm$^2$ were seeded on similar micro-channeled scaffolds to investigate the impact of micro-channeled design on elongation, contraction, and alignment of cardiomyocytes. The gelatin-based hydrogel was further crosslinked with microbial transglutaminase (mTgase) overnight at 37 °C to form a rectangular hydrogel sheet with a microchannel structure. After the crosslinking process, the micro-channeled construct was soaked in culture media for 24 h, hMSCs with cardiomyocytes were seeded on the plain and microchannels of printed hydrogels. Cell viability, cardiomyogenic lineage commitment, and cell alignment were evaluated over nine days. The difference between the impact of plains, micro-channels, and spacings between them was discussed after evaluation. The results showed that the micro-channels facilitated elongation of cellular morphology, well-established F-actin anisotropy, and a significant increase in mature cardiac markers. Also, seeded cardiomyocytes exhibited synchronized beating and more alignment. Tijore et al. [23] used β-myosin heavy chain antibody (β-mhc) along with DAPI and Phalloidin (Ph) to compare the influence of spacing between microchannels and plains on the hMSC morphology and demonstrated that stem cells cultured on microchannels with 500 μm spacings were stretched in the direction of gelatin pattern more than samples with 1000 μm spacings. The morphology of cultured stem cells on micro-channels and plains was assessed with Ph and DAPI. The assessments showed that 40% of hMSCs cultured on microchannels were orientated 0–10°; however, about 7% of plain cultured cells were oriented in the same degree interval [23].

Izadifar et al. [163] prepared an alginate-based bioink mixed with Human coronary artery endothelial cells (HCAECs). The constructs were

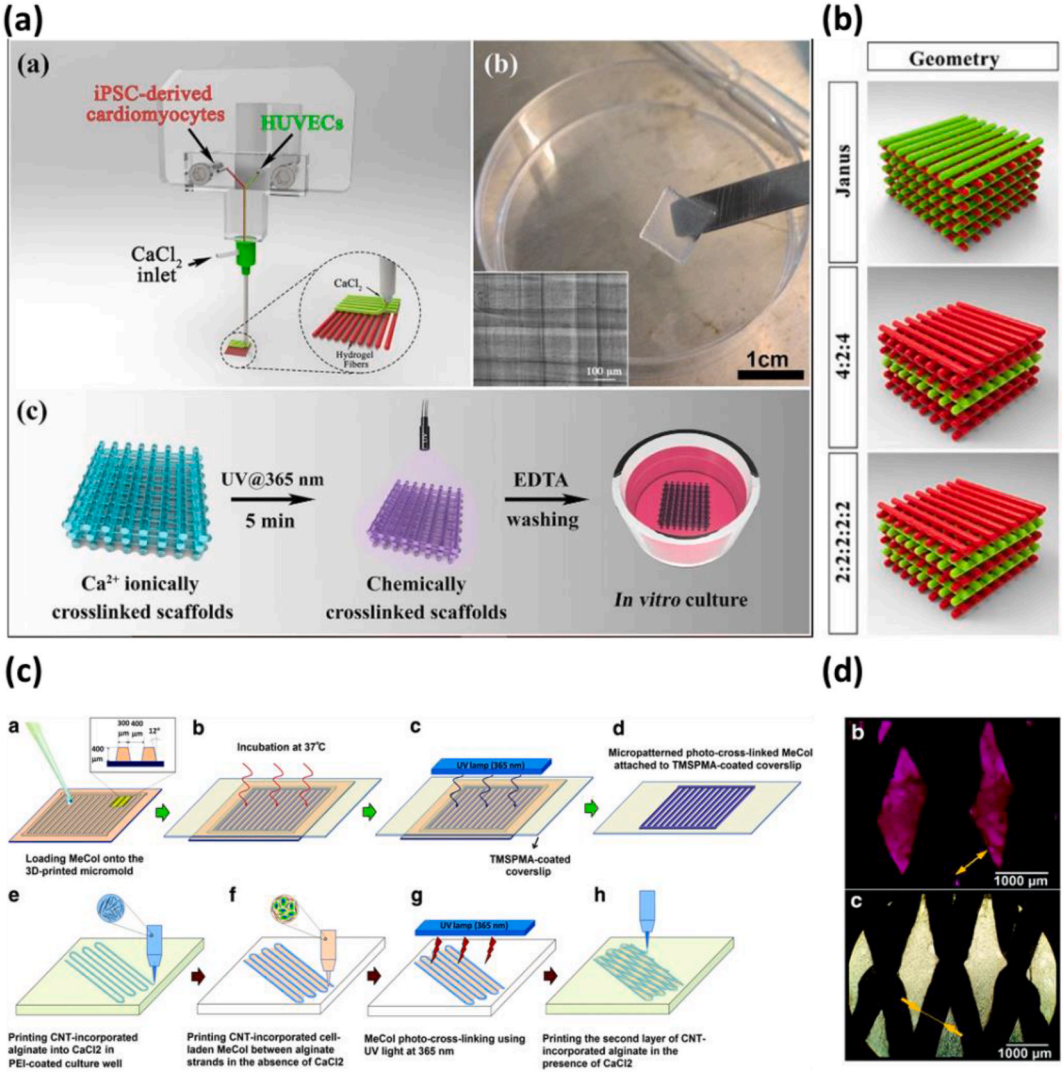


**Fig. 3.** Illustration of methods and designs used in cardiac 3D bioprinting: a) cell-laden CNT-incorporated MeCol and alginate bioink 3D bioprinting and crosslinking with $Ca^{2+}$ and UV exposure [115] b) architectural design of cell-laden CNT-incorporated MeCol and alginate [115] c) preparation of bioprinted construct with grid design out of cardiomyocyte- and endothelial-laden alginate, polyethylene glycol monoacrylate-fibrinogen through microfluidic printing head and processes of crosslinking with $Ca^{2+}$ and UV exposure [18] d) different architectural designs of bioprinted constructs (Janus, 4:2:4, 2:2:2:2:2) [18].

printed in grid designs with different strand alignment angles (15/165°, 0/90°, and 0/45/90/135°) to demonstrate the effect of mechanical properties and design on viability and functions of endothelial cells. It was indicated that the construct with 0/45/90/135° strand alignment angle provided higher compressive modulus, stiffness, and conductivity, leading to higher cell viability and cell functions [163].

In another study, Izadifar et al. [115] introduced a new hydrogel mixture prepared out of MeCol, alginate, which was reinforced with functionalized carbon nanotubes (CNTs) to improve mechanical, electrical, and biological performance properties. CNTs were functionalized with carboxylic groups and were incorporated into crosslinked alginate and MeCol. A photopolymer (verowhite fullcure 835) was printed in parallel strands micro-mold. Before printing the bioink, HCAECs were encapsulated in MeCol and mixed with alginate hydrogel with a density of 0.8–1 $\times$ $10^6$ cells/ml. The cell-laden hydrogel mixture was bioprinted on the micro-mold, and alginate was crosslinked through $Ca^{2+}$. Subsequently, the MeCol was photo-crosslinked with methacrylic anhydride chemistry through UV exposure. The micro mold was removed, and a micro-patterned bioink after crosslinking was left. Patches were soaked in calcium-free DMEM (Dulbecco's modified eagle media), and the impact of the CNT nanotubes density on mechanical, electrical, and biological behavior was evaluated in several days. The results displayed that CNT-incorporated alginate enabled a highly interconnected meshwork with robust viscoelastic and electrical conductivity of photo-crosslinked MeCol and alginate (Fig. 3 a-b) [115]. Izadifar et al. [115] assessed cardiac patches' cell viability and compared the influence of UV exposure on cell viability. Cells were colored with Calcein-AM and Hoechst dyes, and it was shown that with an increase of time of the UV exposure, cell death would be higher after three days. Izadifar et al. [115] demonstrated the impact of CNTs with the biomimetic view from Purkinje fibers on the morphology and orientation of cardiomyocytes. The results showed that HCAECs entrapped in the CNT-incorporated hybrid were more elongated and aligned in the direction of CNTs than the alginate-HCAEC bioprinted construct (Fig. 4 a) [115].

It is reported that cardiac fibroblasts affect the maturation and function of cardiomyocytes. However, because of the high proliferation of fibroblasts compared to cardiomyocytes, conductivity and contractility of cardiomyocytes could be impaired if fibroblast proliferation is not properly controlled. To overcome this challenge, CNTs, gold-based nanomaterials such as gold nanorods, nanowires, and nanospheres can be used to induce higher electrical conductivity. Zhu et al. [164]

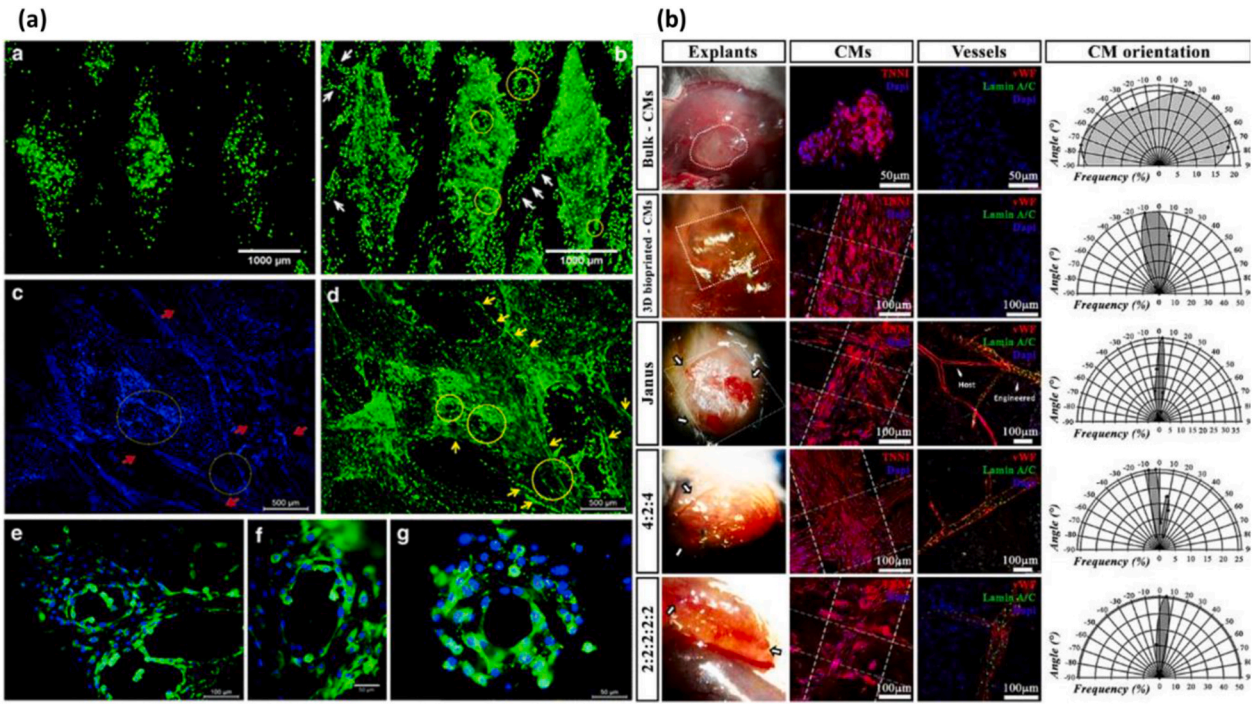


**Fig. 4.** Immunofluorescence and immunocytochemistry images of cardiomyocytes and endothelial cells: a) Investigation of cardiomyocytes and endothelial cells behaviors (orientation, alignment, and migration) in CNT-incorporated MeCol and alginate 3D bioprinted constructs [115] b) Images of explants, orientation of cardiomyocytes and vascularization of the 3D bioprinted constructs prepared out of cardiomyocyte- and endothelial-laden alginate, polyethylene glycol monoacrylate-fibrinogen bioink [18].

prepared gold nanorods and coated them with GelMA to fabricate a cardiac patch with higher conductivity and contractility. In this study, neonatal rat ventricular fibroblasts and cardiomyocytes were suspended in Alginate and gold nanorod (G-GNR)-incorporated GelMA hydrogel and printed in a grid pattern. Higher beating rate, elongation, and contraction rate were reported compared to Alginate-GelMA bioprinted constructs [164]. To this end, it was shown that a bioink consisting of CNTs or GNR could provide higher electrical conductivity, leading to a more developed cardiac tissue that can perform a repetitive and concurrent beating.

The cardiac patch is a proper substitute for the cell therapy method due to the limited retention and low efficiency of cell-based approaches. Bejleri et al. [36] fabricated a cardiac patch using extrusion bioprinting. Human cardiac progenitor cells (hCPCs) have been suspended in the cECM to prepare a novel bioink. However, due to the low mechanical properties of cECM, GelMA was added to the bioink composition. The biological construct was bioprinted in a grid design, and white light was used to crosslink GelMA instead of UV radiation to decrease cell death. The cECM-GelMA bioink provided a higher mechanical storage modulus and cell viability [36]. Mechanical compressive or tensile strength of a construct is one characteristic that usually has not been considered in cardiac 3D bioprinting; however, it was demonstrated that it could impact cardiomyocytes' viability and functions. Investigation of mechanical properties of the cardiac patches alongside electrical properties can lead to a better understanding of cardiac patch development.

Printing hydrogel-free bioink is another method that has been used to fabricate cardiac patches. This method was called the cardiosphere by Ong et al. [159]. It was made out of 3300 primary cells in total, including human cardiac fibroblasts, human iPSC-derived cardiomyocytes, and HUVECs, which were co-cultured with different ratios (70:15:15, 70:0:30, 45:40:15) to produce mixed-cell aggregate. They were placed on a needle array by an extrusion-based bioprinter. It was then put on a shaker in an incubator for 72h to allow the fusing of spheroids before removing the needle array. Printing spheroids with different cell densities in precise positions is the critical factor of this method, enabling the fabrication of a whole cardiac patch. In another study, Yeung et al. [165] used the 70:15:15 sample and showed higher contractility, ejection fraction, and vessel count, provided by the higher concentration of fibroblasts and endothelial cells [159,165].

Lee et al. [74] have introduced a novel reversible freeform embedding of suspended hydrogels (FRESH v2.0), a buffer system consisting of a thermo-reversible bath of gelatin microparticles that unmodified collagen can be extruded in it. This method enables collagen with high concentration to self-assemble in a gelatin bath by rapid pH change of the gelatin bath and allows fabricating structures with high resolution. Gelatin baths act as a support to the biological construct and can be uncrosslinked at 37°C. Another improvement of FRESH v2.0 is using morphological uniform microparticles with a diameter of 20 μm, which provides a support with higher mechanical properties. In this study, different advantages of this method to bioprint components of the cardiovascular system were evaluated. Collagen type I was bioprinted with this method, and gelatin microparticles were purposely incorporated in the construct and melted away to provide a porous structure. The porous and non-porous constructs were implanted *in vivo* to evaluate cell infiltration. This comparison showed that porous uniform structure provides higher cell-infiltration because of uniform 25 μm pores prepared by the FRESH v2.0 method. Both constructs were later incorporated with vascular endothelial growth factor (VEGF) and fibronectin to provide a microenvironment for angiogenesis and were compared again after subcutaneous implantation. FRESH-printed construct provided enhanced vascularization after ten days. The same method was used to print the left ventricle model with two nozzles. One of them printed collagen bioink in two shells, and another bioprinted a bioink consisting of human embryonic stem cell-derived cardiomyocytes and cardiac fibroblast in the spaces between two shells. After four days, the construct contracted, and after seven days, the contraction was synchronous throughout the entire construct. Eventually, a structural model of the human heart was printed out of a bioink consisting of neonatal rat cardiomyocytes and collagen hydrogel to show the capability of this approach to bioprint micro-scale structure [74]. Mirdamadi et al. [166]

repeated this method using the same bath, but GelMA was substituted by alginate [166]. Collagen is one of the functional proteins of cardiac tissue ECM; However, fabricating a construct from only one type of protein might not lead to a complex structure with tissue-level functions. Due to the different microenvironments of cell types, different types of proteins and polysaccharides should be considered in preparing a bioink to bioprint a complex structure from a biomimetic point of view.

Co-cultured cardiomyocytes and endothelial cells have been commonly used in cardiac tissue engineering; Maliauri et al. [18] researched the same topic by combining microfluidic and bioprinting approaches to print more precise structures. They introduced a microfluidic printing head made of polycarbonate with a Y-junction microchannel structure connected to a coaxial syringe. Alginate and polyethylene glycol monoacrylate-fibrinogen (PF) bioink containing iPSC-derived cardiomyocytes with an initial concentration of $8 \times 10^6$ cells/ml and HUVECs with the density of $6 \times 10^6$ cells/ml were bioprinted in a grid design. Alginate hydrogel was crosslinked ionically while the bioink was printed through a coaxial microfluidic nozzle. After bioprinting the construct, PF was crosslinked by photochemical cross-linkers (Fig. 3 c). Cell viability, several functional gene expressions, including cardiac early and late genes, and different tissue evaluations like orientation and contractility were studied in 14 days. It showed

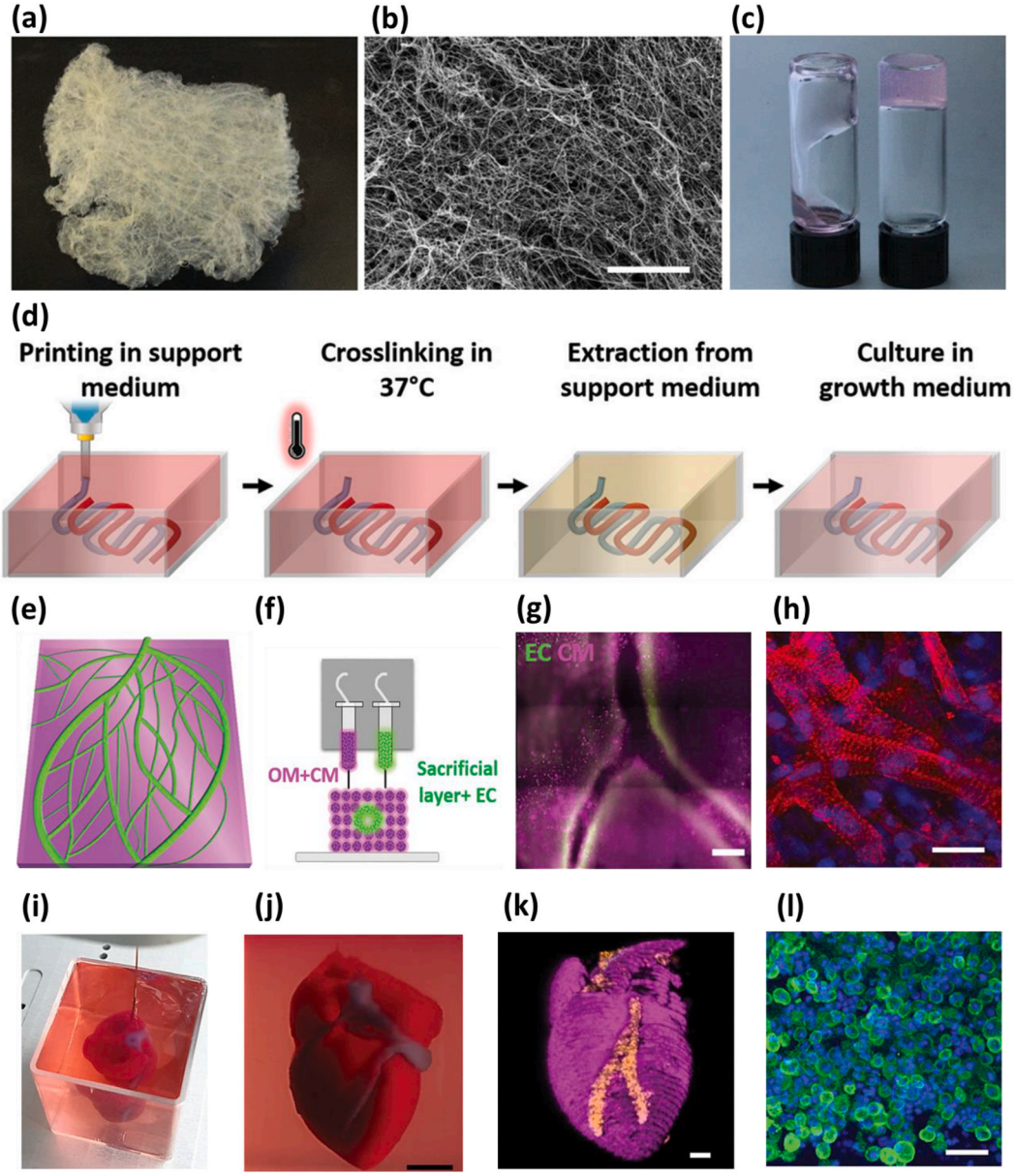


**Fig. 5.** Biofabrication of cardiac patch and whole heart anatomical model: a) decellularized human omentum tissue b) SEM image of a personalized hydrogel structure based on decellularized omentum c) A personalized hydrogel before gelation at room temperature (left) and after gelation at 37 °C (right) d) Schematic steps of free-form 3D bioprinting of the personalized hydrogel in the support material, crosslinking at 37 °C, extraction of the biological construct by an enzymatic or chemical degradation process of the support material, and transferring into culture medium e) A 3D model of a vascularized cardiac patch f) The concept which was used to bioprint the patch g) A bioprinted iPSCs-derived cardiac patch where the blood vessels are marked by CD31 (green) and cardiomyocytes are marked by actinin (pink) h) Sarcomeric actinin (red) and nuclei (blue) staining of sections from the explanted patch i-j) biofabricated anatomical model of a human heart k) 3D confocal image of the bioprinted heart (Cardiomyocytes in pink, endothelial cells in orange) l) Cross-sections of the anatomical model of human heart immunostained against sarcomeric actinin (green) nuclei (blue). Scale bars: (b) = 10 μm, (g) = 500 μm, (h) = 25 μm, (j) = 0.5 cm, (k) = 1 mm (l) = 50 μm [131]. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

printed cardiomyocytes' orientation and organization is significantly similar to genuine myocardium compared to the casted construct.

On the other hand, HUVECs were stained with von Willebrand factor (vWF) and DAPI to indicate the influence of vascularization in different geometries. The orientation, organization, and microenvironment of cardiomyocytes affect the functionality of these cells extensively. Hence, cardiomyocytes' orientation was compared in three mentioned designs, bulk hydrogel, and 3d bioprinted hydrogel. Cardiomyocytes demonstrated more alignments in the direction of printing and more maturation in the Janus geometry (one layer of cardiomyocytes: one layer of HUVECs) compared to two other constructs, which were 4:2:4 (four layers of cardiomyocytes: two layers of HUVECs: four layers of cardiomyocytes) and 2:2:2:2:2 (two layers of cardiomyocytes: two layers of HUVECs: two layers of cardiomyocytes: two layers of HUVECs: two layers of cardiomyocytes) (Fig. 3 d and Fig. 4 b). Besides, it is believed that the maximum oxygen and nutrients diffusion distance to obtain the highest cell viability and functionality without vascularization is approximately 100–200 μm. Hence, more layers of endothelial cells in the Janus design compared to two other biological constructs helped cardiomyocytes be more aligned and organized (Fig. 4 b) [18].

Decellularized human tissues are one of the most promising resources to develop hydrogels as a component of a bioink. Noor et al. [131] used decellularized omenta from humans or pigs mixed with iPSC-derived cardiomyocytes for the main bioink to bioprint vascularized cardiac patches as a proof-of-concept for the patient-specific treatment (Fig. 5 a-c). Gelatin was mixed with iPSC-derived endothelial cells and bioprinted as a sacrificial bioink to induce vascularization. To this end, the orientation and 3D structure of a patient's heart vasculature were identified through a computational tomography image and Computer-aided design that can be attached to the patient's heart with the same vasculature structure (Fig. 5 d-f). Blood vessels and cardiomyocytes were identified by CD31 and actinin, respectively (Fig. 5 g), and the contraction of the cardiac patch was observed through transient calcium. Elongated and aligned cardiomyocytes were detected (Fig. 5 h). It was demonstrated that a contracting cardiac patch could be prepared by a patient's own cells with similar cell activity. To assess this method's ability to bioprint larger constructs with more complexity, Noor et al. [131] mixed neonatal cardiomyocytes with the same personalized bioink to bioprint an anatomical model of a whole human heart (Fig. 5 i-j). HUVECs were combined with gelatin sacrificial bioink like the previous step to print complex vasculatures. The whole heart tissue had close mechanical properties to the rat's heart. According to the bioprinting plan, a confocal image of the bioprinted heart showed that the same spatial organization of cardiomyocytes and endothelial cells was achieved (Fig. 5 k). Sarcomeric actinins were observed after one day, which showed an internal compartmental structure close to the rat's heart (Fig. 5 l) [131].

The limited electrical function is one of the most critical issues that cause the disability of cardiac patches to treat the diseased areas of the genuine cardiac tissue. Asulin et al. [151] studied the fabrication of stretchable and flexible planar electronic systems by lithography and integration of it in a bioprinted construct to produce controlled electrical function. Three types of bioink were prepared, a cell-laden bioink to encapsulate cardiac cells and other two PDMS bioinks to act as electrodes and dielectric, respectively. Cellular bioink was prepared by decellularization of pigs' omenta. Two other bioinks were prepared by mixing graphite synthetic powder and span 80 with PDMS to conduct and passivate electrical signals, respectively. First, the electrical and mechanical properties of the bioinks were investigated. To determine the optimum conductivity, bioinks with different concentrations of graphite flakes were prepared, and the conductivity was measured, and it was demonstrated that 45% (wt) showed the highest conductivity. Subsequently, stress-strain behavior of the ink was evaluated that showed robustness about 50% and elongation under 20%, which is close to the mechanical behavior of cardiac tissue. Change of resistance by degradation and mechanical evaluation were assessed for the printed passivation bioink that showed no significant change of resistance and 135% elongation. Next, the main construct was printed to assess the electrical and mechanical ability of the cardiac patch. Neonatal rat cardiomyocyte containing bioink was printed with eight electrodes, including six core electrodes which had a passivation layer around them; however, the end of each electrode was left without a passivation layer for stimulation and point sensing. Two outer electrodes were exposed for field stimulation. The cardiac patch showed high levels of actinin and synchronized contractions through four regions of the patch after 12 days [151]. To this end, bioprinting of hydrogels from decellularized tissues alongside conductive biomaterials in a denser hydrogel bath is one of the most accurate methods which can be used to biofabricate a complex micro- and macrostructure with specific tissue-level functions.

Human native cardiac tissue has vital abilities such as contractility, excitability, conductivity, and automaticity, which should be considered in cardiac tissue bioprinting. Preparing an appropriate microenvironment encourages cells to develop a tissue-like structure and operate tissue-level functions. Researchers working on cardiac tissue bioprinting have used many natural and synthetic hydrogels; however, hydrogels based on decellularized tissues have provided a more effective environment than other natural hydrogels. Proteins in cardiac tissue ECM such as collagen and fibrin alongside hydrogels based on decellularized tissue have been used extensively to meet the expectation of a genuine microenvironment from a biomimetic point of view. Crosslinking the hydrogel composition is one of the challenges that should be overcome in cardiac tissue bioprinting. UV crosslinking is one of the crosslinking methods that have been used repeatedly in studies. It has been reported that UV radiation cause cell death and decreases cell viability which is the first step to having functional tissue. Hence, photoinitiators that work in the visible range with higher length wave has been utilized to overcome this challenge. Using gold nanorods or CNTs is the next step in this field to provide higher tissue-level functions. These nanorods or nanotubes can stimulate contractions and alignment of the cardiomyocytes, leading to better contractile cardiac tissue development. Besides, investigating the differentiation and growth of MSCs toward cardiomyocytes during embryo development could be crucial in achieving and eventually developing more similar constructs to genuine cardiac tissue. The presence of endothelial cells or endothelium lumen-like structure, fibroblasts, and VEGF are significant factors alongside printing constructs with anisotropic architecture, hydrogel's mechanical, chemical and electrical properties that help cardiomyocytes cell growth and functions such as elongation and orientation; however, it might not be enough to obtain a tissue formation and tissue-level functions [167–170]. Another challenge that should be considered is the maturation of bioprinted constructs. Microfluidic bioreactors with a biomimetic point of view are one of the elegant designs proposed by Zhang et al. [24] to mature bioprinted constructs with perfusion of culture medium and showed that more mature tissue-like structures could be obtained compared to static cultures. Overall, to achieve similar bioprinted constructs with native cardiac tissue, many aspects should be studied to have a biomimetic point of view, from the embryo development in the early stages of seeding to different bioink compositions, designs, and maturation techniques [72,133,171] (Fig. 6).

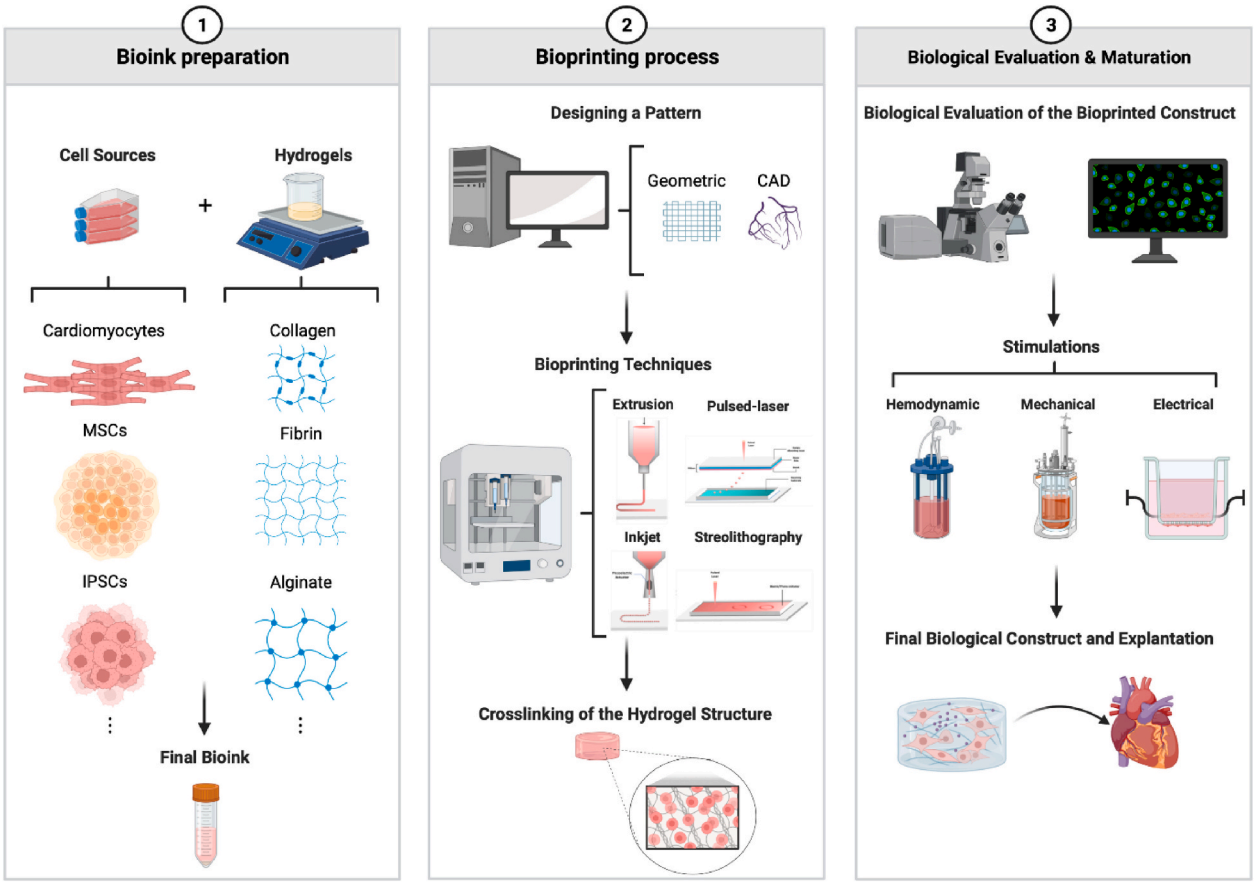


**Fig. 6.** Schematic representation of bioink preparation, bioprinting process, and maturation of the cardiac construct (Created with BioRender.com).

## 8. Conclusion and future outlook

Cardiovascular bioprinting has drawn remarkable attention during the last decade, providing precise control over fabricating 3D biological constructs. As pointed out in the text, several cell types, natural or synthetic hydrogels, and biochemical factors have been used to prepare bioinks and these compositions have been selected based on specified considerations related to cardiac tissue structure and functions. Also, the architectures of the bioprinted constructs are designed based on anatomical or geometric structures and then bioprinted by different bioprinting techniques, which are mostly extrusion-based. In the last step, cellular activities were assessed and several stimulations have been inducted to maturate the cardiac construct with functions such as conductivity and contractility.

The significant progress in cardiovascular bioprinting includes improved cell-cell integration, vasculature incorporation, well-organized cell alignment, better management of complex architectures, and better functions. These are majorly attributed to the development of bioprinting strategies and the incorporation of more biologically responsive bioinks. The latter has resulted from more diversified natural and synthetic hydrogels and the utilization of cells with regenerative potential. Despite the remarkable advances, specific challenges restrict the fabrication of functional large-scale cardiac tissue. Full maturation of cardiomyocytes has remained a significant challenge limiting the tissue-specific functions, which further impedes integrating the host tissue. There have been remarkable attempts toward employing physiologically relevant stimulations inducing tissue-specific gene expression. However, reaching an appropriate population of fully mature cardiomyocytes remains a substantial challenge. The lack of vasculature with high density in the bioprinted construct is another challenge that has significantly restricted the clinical application of bioprinted cardiac tissues. Efficient mass transfer in the construct is a critical issue that allows uniform distribution of heterogeneous cell types through the scaffold, improved cell viability, and better cellular activity. Although bioprinting allows precise control in fabricating complex architectures, supplying a vasculature network with the approximate density of 3000 cells/mm$^2$ requires novel bioprinting systems with higher resolutions and improved modeling properties. The novel systems should pave the way for improved mimicking of the cardiac tissue complex heterogeneous architecture.

Also, novel and closely tissue-mimicking bioink systems should be developed to improve the construct cellular functionality and mechanical stability. Mechanical characteristics of the construct could strongly influence both the integration with the host system and the direction of cells toward cardiomyocytes. Thus, there should be a trade-off between the mechanical properties and the processability of the bioink.

Bioprinting of bioinks with different architectures influences cardiomyocytes' organization in cardiac patches and leads to higher proliferation, biological functions such as notch signaling. On the other hand, bioprinting of bioinks made out of hydrogels with high conductivity like CNTs is another way to stimulate cardiomyocytes to higher cell growth and enhance functional gene expression leading to contractility. New techniques like bioprinting scaffold-free cells have been used to fabricate cardiac patches with determining cell densities of different cells along cardiomyocytes, which produce their own ECM similar to genuine tissue in composition structure after *in vivo* implantation. Fabricating vascularized biological constructs with different designs that lead to more tissue-like structures and organoids is another application of this technology. Bioprinting layers of endothelial cells between cardiomyocytes layers is one of the approaches that highlight endothelial cells' impact on cardiomyocytes' viability and function. Similar strategies like culturing cardiomyocytes after fabricating

endothelialized constructs have been employed to mimic cardiac tissue structure. It has been shown that anisotropic endothelialized structure leads to more oriented cardiomyocytes with higher frequencies of contractions. The combination of hydrogels with additives with high conductivity, the architecture of 3D bioprinted constructs, and the co-culture of cardiomyocytes and endothelial cells are the major strategies that have been used to improve cardiac tissue functions, which influence cell aggregation, proliferation, and differentiation to achieve tissue-like contractile structures with beating areas. Besides, there have been endeavors to fabricate chambered cardiac organoids with macro-scale beating and the ability to pump blood through *in situ* differentiation of hiPSCs to overcome the challenge of high cell density fabrication.

As an exciting perspective in cardiovascular bioprinting, the fabrication of bioprinted tissues under zero- or microgravity conditions can promise other abilities in the field. Contrary to the current situation on earth, the microgravity conditions suggest implementing lower viscosity bioinks and more satisfactory resolutions in printing at a single-cell scale. These conditions can remarkably improve the current mentioned challenges in cardiac bioprinting.

## Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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