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3D Bioprinting Technology – One Step Closer Towards Cardiac Tissue Regeneration

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Cardiovascular diseases are one of the leading causes of death across the globe. Heart transplantation has been used for end stage heart failure patients. However, due to the lack of donors, this treatment option usually depends on multiple variables and the result varies due to immunological issues. 3D bioprinting is an emerging approach for *in vitro* generation of functional cardiac tissues for drug screening and cardiac regenerative therapy. There are different techniques such as extrusion, inkjet, or laser-based 3D printing that integrate multiple cell lines with different scaffolds for the construction of complex 3D structures. In this review, we discussed the recent progress and challenges in 3D bioprinting strategies for cardiac tissue engineering, including cardiac patches, *in vitro* cardiac models, valves, and blood vessels.

Keywords: 3D bioprinting, scaffolds-based 3D bioprinting, 3D cardiac patch, cardiac regeneration, cardiac repair

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INTRODUCTION

Cardiovascular diseases (CVD) are common, enervating and fatal malady that devours thousands of life every year (Huang et al., 2021; Mei et al., 2021). Multiple conditions like congenital heart disease, ischemic heart disease and inflammation contribute to the loss of cardiac functions (Liu N. et al., 2021). Due to the limited regenerative capacity of cardiomyocytes (CMs), adult myocardium lacks ability to self-renew after injury (CMs) (Li et al., 2018). The current treatments for myocardial infraction (MI) relieves the symptoms and modestly prolong life rather than restore the lost cardiac tissue. Heart failure (HF), the common outcome of CVD, may require a heart transplant at the end stage (Wang Q. et al., 2021). However, the number of available donors lags far behind the number required by the patients (Liu N. et al., 2021). Allogenic organ transplantation is coupled with the high risk of immune rejections and surgical complications, where the desire for long term recovery of heart function is still unfulfilled.

In regenerative treatment approach, stem cell therapies have been considerably studied for cardiac repair (Berry et al., 2019; Oh et al., 2016; Zhu and Cheng, 2021; Tang J. N. et al., 2018; Tang J. et al., 2018). Mounting evidence suggested that stem cell therapies demonstrate treatment benefit through cardiac function enhancement, infract size reduction, and angiogenesis improvement (Su et al., 2018; Huang et al., 2018; Su et al., 2019; Huang K. et al., 2020; Cheng et al., 2012b). However, they are hampered by the intrinsic limitations after transplantation, such as low cell retention, low survival rate of the engrafted cells and lack of host tissue integration (Wang and Guan, 2010; Ong et al., 2017). Hence, applying contemporary technology to build functional artificial cardiac tissue *in vitro* has been a Frontier direction in tissue engineering (Tomov et al., 2019).

Cardiovascular tissue engineering encompasses cell biology, material science and biofabrication that facilitates the generation therapeutics for CVD (Noh et al., 2018; Zhu and Cheng, 2021). Within tissue

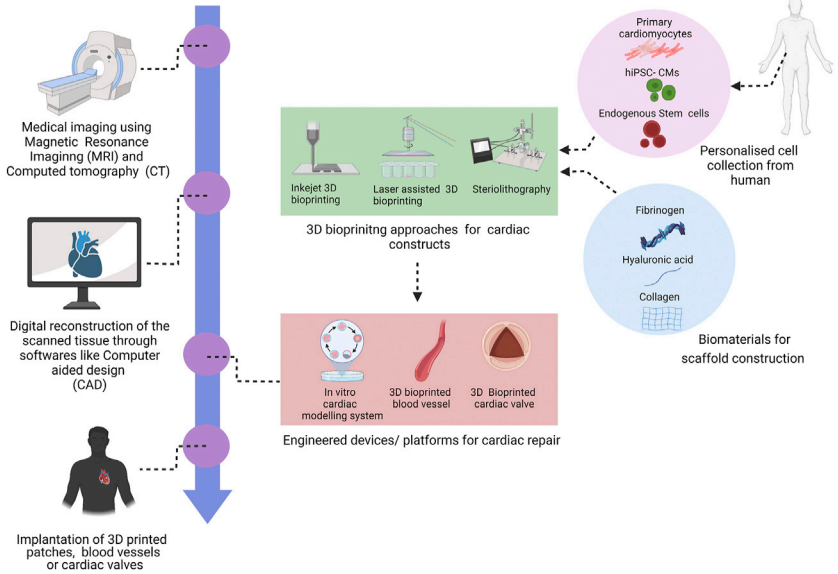

FIGURE 1 | 3D bioprinting technology for cardiac repair and regeneration.

TABLE 1 | Comparison Between different scaffold-based 3D printing techniques for cardiovascular applications.

Property	Extrusion 3D printing	Inkjet 3D bioprinting	Laser-assisted 3D bioprinting	References
Printing speed	0.1 mm/s -5 cm/s	1–10,000 droplets	1,600 mm/s	(Xu et al., 2009), (Hopp et al., 2012), (Guillotin et al., 2010), (Kim et al., 2010), (Cui et al., 2012a), Smith et al. (2007)
Viscosity	Medium to high	Low	Low to medium	
Gelation methods	Chemical Photo crosslinking	Chemical		
Material Integrity	High	Low	Medium	
Scaffolds	Fibrinogen Extracellular matrix (dECM) Collagen Methacrylate (GelMA) Gelatin	Gelatin alginate	Culture medium Geltrex Alginate	
Resolution	0.1–2 mm	Up to 100 µm	20–50 µm droplet	
Cell Viability	40–80%%	>85%	>95%	
Cell density (cells/m)	1×10^6 to 1×10^8	2×10^6 to $\times10^6$	2–100 $\times10^6$	
Cost	Medium to high	Low	Very high	
Status in cardiovascular tissue printing	Complete heart, arteries, valves, cardiac patches	Pseudocardiac tissues	Cardiac patches	

engineering, bioprinting both 2-dimensional (2D) and 3-dimensional (3D) has emerged as a modern fabrication method that utilizes live cells, bioactive molecules, and biomaterials for both *in vitro* and *in vivo* applications. Mimetic heart tissues are can fabricated using 3D bioprinting, which successfully capture the intricacy of the native cellular composition and matrix structure. (Bejleri et al., 2018; Ong et al., 2017; Liu et al., 2019; Chingale et al., 2021). These fabricated constructs have been further used for drug screening and regenerative studies (Ong et al., 2017; Liu et al., 2019). For the treatment of aortic valve disease and bypass procedures, 3D bioprinted aortic valves and conduits have been used. (Hockaday et al., 2012; Kang et al., 2016; Pountos et al., 2019). In ischemic events, the functionality of the cardiac tissue can be preserved using 3D cardiac patches (Gaetani et al., 2015; Jang et al., 2017; Izadifar et al., 2018). Also, 3D printed vascular grafts have enhanced structural stability and biocompatibility (Marga et al., 2012; Kucukgul et al., 2015). Using 3D bioprinted cardiac patch, fabricated by human coronary artery endothelial cells, methacrylate collagen micropatterning, and an alginate matrix (Izadifar et al., 2018) showed high cell proliferation, migration and differentiation. This review provides an overview of cardiovascular 3D bioprinting techniques, their potential applications, limitations and also the future prospects of this new technology (**Figure 1**).

3D Bioprinting Approaches for Cardiovascular Tissues

3D bioprinting is a layer-by-layer additive technology that precisely deposits biomaterials and active cells in accordance

TABLE 2 | Advantages and disadvantages of scaffold biomaterials used for 3D bioprinting of cardiovascular tissues.

Scaffold	Advantage	Disadvantage	References
Alginate	• High compatibility with many cells • Short crosslinking time • $CaCl_2$ don't not have significant effect on cell viability	• Unstable if used for prolonged culture • Low rate of degradation • Does not promote cell proliferation	Xu et al. (2009) Attalla et al. (2016) Kundu et al. (2015)
Collagen	• Good cell carrier • Easy to modify and add other scaffold materials	• Cannot be used alone • Long time for crosslinking	(Park et al., 2014) Skardal et al. (2012)
Hyaluronic acid	• Main component of ECM • Good cell carrier • Increases the mechanical stability and cell viability	• Low mechanical stability	Pinto et al. (2016)
Gelatin	• Has good thermoresponsive property • Good cell viability • Can be used as fugitive scaffold	• Not stable at physiological temperature	

with a certain spatial pattern that has high resolution stimulation of the heart (Mir & Nakamura, 2017). High resolution stimulation of is a method of evaluation of the functional attributes of the cardiac tissue and means of induction of various arrhythmia (Issa et al., 2019). Briefly, the 3D bioprinting procedure begins with the gathering of the patient's data through imaging tools like computed tomography (CT), magnetic resonance imaging (MRI) to design a computer-aided design (CAD) model. This model has a well-defined structure, architecture and porosity (Hockaday et al., 2012; Burke et al., 2017). 3D bioprinting techniques is further bifurcated scaffold-based printing and scaffold free printing (**Table 1**). The predominant approaches of scaffold based 3D bioprinting are extrusion, inkjet, laser assisted printing and stereolithography (Pountos et al., 2019). Using the various 3D bioprinting approaches, a number of tissue constructs like cartilage (Wang et al., 2016a; Luo et al., 2012; Shi et al., 2017; Cui et al., 2012a), neural tissue (Radulescu et al., 2007; England et al., 2017) heart valve (Hockaday et al., 2012; Duan, et al., 2013) have been successfully constructed.

Scaffold-Based 3D Printing

Scaffold based 3D bioprinting is used to generate tissue constructs using cells, biological macromolecules, and structural moieties. The scaffold-based materials have facile surface functionalization and superior physicochemical properties (Sarkhosh-Inanlou et al., 2021). These biomaterials facilitate the reconstruction and repair of various anatomical defects that may occur in the cardiac tissues. The porosity of the scaffolds allows the movement of the biological fluids which in turn amplifies the cell adhesion, proliferation, migration and differentiation (O'Brien, 2011). Furthermore, many scaffolds based 3D printing techniques have been under investigation that exhibit low inflammation and toxicity, and low rejection of scaffold through autoimmunity (O'Brien, 2011; Ng et al., 2012; Tariverdian et al., 2019). Extrusion-based bioprinting, inkjet-based bioprinting and laser-based bioprinting are the three major scaffold-based bioprinting modalities for cardiovascular tissue fabrication (Qasim et al., 2019).

Extrusion-Based 3D Bioprinting

The extrusion-based 3D printing is a facile, less complex and affordable 3D printing approach (Mandrycky et al., 2016; Kato et al., 2021). The cell deposition density usually ranges from 10^8–10^9 cells per mL which is crucial for cardiac tissue engineering (Pati et al., 2014). Although extrusion based 3D bioprinting offers high cell densities, due to high stress and dispensing pressure ($>6 \times 10^7$ mPa s), the cell viability of only about 40–80% (Mandrycky et al., 2016). Promising results are seen in 3D bioprinting of myocardium constructs, heart valves, and blood vessels (Alonzo et al., 2019). In this printing technique, the cells are suspended in a prepolymer solution, loaded in syringes and printed in the form of cylindrical filament precisely into the target 3D cardiac tissue structure by using pneumatic or mechanical forces (Liu et al., 2017; Mistry et al., 2017; Byambaa et al., 2017). To construct durable 3D cardiac tissues, factors like viscosity, shape of the needle, pressure and gauge size should be accurately optimized (Hölzl et al., 2016). Here, the 3D constructs are constructs by sequentially depositing the hydrogel filaments that have the diameters in a range of 150–300 μm (Pereira and Bártolo, 2015). Recently, a 3D human chambered cardiac muscle was constructed using a photo-crosslinking native extracellular matrix (ECM) proteins and human induced pluripotent stem cells (hiPSC)- laden structures (Kupfer et al., 2020). This 3D chamber muscle possessed a continuous action-potential propagation along with macroscale beating. Using freeform reversible embedding of suspending hydrogels (FRESH v2.0), a modified extrusion-based 3D bioprinting technique was developed to bioprint a model of the left ventricle of human heart left ventricle of human heart. Here,

TABLE 3 | Advantages of scaffold based and scaffold free 3D bioprinting.

	Advantages	Disadvantages
Scaffold-based	Consistent size and quality Better stiffness Robust Pre-vascularized	Immunogenic Lower cell density Probable toxic biodegradation
Scaffold-free	Non- immunogenic Less prone to infection 3D cell density	Not easy manipulate Need huge number of cells

collagen type I and human embryonic stem cell–derived cardiac fibroblasts (hESC-CFs) were used (Lee et al., 2019a). Using gelatin methacryloyl (GelMA) as a cell carrier, a combination of cardiac myocytes and cardiac fibroblasts was used to generate a self-contracting cardiac muscle, called engineered heart tissues (EHT) (Koti et al., 2019). Furthermore, GATA binding protein 4 (GATA4) -transfected Mesenchymal Stem Cells (MSCs) that were derived from human umbilical cord-derived (hUC-MSCs) expressed cardiac marker proteins and differentiated further into cells similar to cardiomyocyte. These naked plasmids when encapsulated in polyurethane (PU) hydrogel can upregulate specific cardiac marker genes and indicates as a new approach for *in situ* cardiac therapeutics (Huang N. C. et al., 2020). A tissue of cardiogenic potential was printed using human cardiac-derived cardiomyocyte progenitor cells (hCMPCs) in alginate scaffold that increase of the early cardiac transcription factor Nkx, Mef-2c, GATA4 also, late cardiac marker Troponin T (TnT) was also highly expressed (Gaetani et al., 2012).

Inkjet-Based 3D Bioprinting

Inkjet 3D based bioprinter allows the precise positioning of cells and biomaterials in the targeted cardiac tissue construct using droplets (1–100 pL) with cell densities as high as 10,000–30,000 cells/drop (Calvert, 2007), ejected via thermal (Cui et al., 2012b) or acoustic forces (Xu et al., 2008). In piezoelectric inkjet printer the piezoelectric crystals produce acoustic waves and force the liquid through the nozzle (Pereira and Bártolo, 2015). Whereas, in the thermal inkjet bioprinting, the droplets are expelled out from the nozzle by the pressure generated by the vaporizing the bioink around the heating element (Cui et al., 2012b). The inkjet 3D bioprinter is compatible with most of the biomaterials and has a cell viability of up to 90% (Wang Z. et al., 2021). The inkjet 3D bioprinter is compatible with biomaterials having specific viscosity of 3.5–12 mPa/s where specific viscosity is the increase in the viscosity of the scaffold beyond that of the solvent due to polymer additive (Murphy and Atala, 2014; Chawla et al., 2018). Inkjet printer was used to construct a layer-by-layer 3D bioprinted aortic tissue construct, using Mouse Embryonic Fibroblast (MEF) cell aggregates with hydrogel support. This tissue construct was a hierarchical design of functional cardiac pseudo tissue with balanced porosity, structural support and a beating cell response (Alonzo et al., 2019). In another study, 3D rectangular sheet was fabricated with alternating layers of alginate hydrogels and primary feline adult and H1 cardiomyocytes (Xu et al., 2009). However, due to the noncontact nature of inkjet printers and its dispensing mechanisms, the 3D constructs usually have weak mechanical properties (Lee et al., 2009). Therefore, the inkjet aided bioprinting of the cardiac tissue specifically is still in its infancy stage (Cui and Boland, 2009).

Laser-Assisted 3D Bioprinting

Laser-assisted bioprinting (LAB) facilitates the formation of *in vitro* tissue constructs. Based on the principle of Laser-Induced Forward Transfer (LIFT), LAB is being progressively used for tissue- and organ-engineering (Barron et al., 2004). LAB exhibits high precision and resolution which can print high cell density (10^8cells/ml) and viscosity (10–100) μm scaffolds. Using a laser pulse repetition rate of 5 kHz, and speed of 1,600 mm/s. LAB can print bioinks with negligible effect on cell viability and function (Ventura, 2021; Barron et al., 2004; Murphy and Atala, 2014). LIFT has been used to construct a defined patterned cardiac patch seeded with human umbilical vein endothelial cells (huvec) and hMSC on a Polyester urethane urea (PEUU) for cardiac regeneration (Gaebel et al., 2011). Also, laser bioprinting of undifferentiated hiPSCs in combination with different biomaterials like collagen, alginate, hyaluronic acid, fibrinogen, fibrin proved that hiPSCs are sensitive to the biomaterials, not to laser printing. Biomaterials, such as the hyaluronic acid, matrigel support hiPSCs differentiation and can be laser printed without losing their pluripotency (Koch et al., 2018).

Stereolithography

Stereolithography (STL) is a nozzle-free technology based on photo-sensitive polymer formulation. It utilizes ultraviolet (UV) or visible light to cure photosensitive polymers in a layer-by-layer manner (Budharaju et al., 2021). This technique is fast and provides accurate fabrication, that has resolutions ranging from 5to 300 μm (Raman et al., 2016). Photoinitiators molecules are sensitive to different ranges of wavelength and initiate the polymer chains which in turn affects the stiffness and density of the cured resins (Pereira and Bártolo, 2015). Due to the toxic nature of some of the photoinitiators, commonly used least cytotoxic photoinitators are Irgacure 2,959 for UV cross-linkage and eosin Y for visible light (Noshadi et al., 2017). Since, UV light induce mutations, visible light-based photocross-linkage have been used more often in SLA (Ikehata and Ono, 2011). Using STL, a MSCs hydrogel patch was constructed with cross linked poly (ethylene glycol) dimethacrylate (PEGDMA) with desired diameter of microchannel suitable for sustained release of cytokines from cells resulted in enhance cardiac function and minimized cardiac remodeling (Melhem et al., 2017).

Digital Light Processing

Digital light processing (DLP) uses a digital micromirroe device (DMV) chip that is composed of approximately millions of micromirrors that reflects light and project an optical pattern that is dictated by CAD model on the photopolymer solution (Lu et al., 2006). The resolution of this printer controlled by the focal size of the light beam from each micromirror. In DLP printer prints parallely by projecting the entire plane of optical pattern onto the photopolyer solution, thus reducing the time required for the fabrication (Zhang et al., 2012). Compared to the other 3D bioprinting approaches, DLP has significant advantages in prining resolution, efficiency, and working condition (Zhang et al., 2019). Human embryonic stem cell derived cardiomyocytes (hECMs- CMs) and hydrogel were used to fabricate a cardiac tissue using DLP based 3D printing. The DLP facilitated the 3D bioprinting of hECMs- CMs mimicing the multilayed alignment of the myocardium. This tissue construct was furthre used to detect simultaneously both calcium transients and mechanical force that is essential for *in vitro* disease modeling (Zhang et al., 2012). Recently, using rapid light based 3D printing (DLP), a cardiac tissue construt

made of Human induced pluripotent stem cell - derived cardiomyocytes (iPSC-CMs) was used to detect the expression of mature cardiac marker genes (Ma et al., 2019).

Scaffolds for 3D Bioprinting

Cardiac tissue engineering aims to regenerate the injured cardiac tissue by combining cells and highly porous scaffold biomaterials to act as templates for tissue regeneration (O'Brien, 2011). It is of paramount importance that the scaffold possesses native ECM like texture and morphology for precise 3D cardiac construct fabrication (Zhu K. et al., 2017). The 3D scaffolds must have patterned channels that mimic the function of the endothelial network (Usprech et al., 2017). These patterned 3D channels promote the cell migration, physiology, morphology, and phenotype. They also play an important role in stem cell differentiation into cardiomyogenic phenotype (Zhang et al., 2016). In Tissue engineering, scaffold (hydrogel) materials should support cell adhering, cell proliferation along possessing key attributes like printability, degradation kinetics, biocompatible and material biomimicry (**Table 2**) (Murphy and Atala, 2014; Sahai and Gogoi, 2019). Hydrogels that are currently used in the cardiac regenerative medicine are either naturally derived polymers (like alginate, gelatin, collagen, chitosan, fibrin and hyaluronic acid) or synthetic molecules (polyethylene glycol, PEG) (Murphy and Atala, 2014; Sun et al., 2012; Spiller et al., 2011). Natural polymers provide the advantage in 3D bioprinting as they mimic the ECM both in terms of morphology and bioactivity. The synthetic polymers like hydrogels are also a preferred choice due to the ease of manipulating and tailoring and physical properties as per the target tissue. The challenges with the synthetic polymers include toxic degradation, loss of mechanical properties and poor biocompatibility (Li and Kawashita, 2011; Prasad, 2021).

For precise 3D printing of cardiac constructs, proper selection of scaffold based on the type of 3D bioprinting modality used is very crucial. Hydrogels used for extrusion based 3D bioprinting are usually non-Newtonian fluids, where the viscosity and sheer rate are corelated (Jungst et al., 2016). In addition, the scaffold should possess low surface tension and low adhesion properties along with rapid gelation characteristics (Jia et al., 2016). In inkjet-based 3D printing, the scaffold must have low viscosity and non-fibrous in nature to ensure easy flow through the tubing system. Also, the scaffold must have rheopectic behavior where the viscosity increases with applied shear (Mandrycky et al., 2016). A scaffold with sufficient adhesion and low surface tension characteristics can spread uniformly and adhere is best suited for laser assisted 3D bioprinting. Also, the scaffold chosen should exhibit viscoelasticity as it can enhance cell viability. The viscoelasticity properties of the scaffolds can be altered to achieve high cell densities (Nooranidoost et al., 2019). Also the scaffold should possesses rapid gelation capability, solidifying speed up to 10 s as it helps to maintain structural fidelity. (Wang et al., 2016b). The following functional scaffolds have been used in cardiac 3D printing.

Alginate

Alginate is a common hydrogel used in 3D bioprinting due to its, easy of handling, price, crosslinking, bio-inertness, printability and biocompatibility (Paques et al., 2013; Selcan Gungor-Ozkerim et al., 2018). Alginate is chemical modified to promote desirable cell function and mechanical support (Agarwal et al., 2021). The viscosity of alginate depends on its concentration hence alginate at lower concentrations leads to shape distortion of the 3D constructs which in turn affects cell adhesion, proliferation and distribution of the differentiated cells (Paques et al., 2013). Modifying the alginate surface using peptides sequence (arginine–glycine–aspartic acid) RGD, results in enhanced cell attachment on alginate (Paques et al., 2013). Using chelating agents like ethylenediaminetetraacetic acid (EDTA), alginate can be eliminated after printing the 3D constructs. In clinical trials for patients affected by myocardial infraction, Algisyl-LVR™ has been approved for treatment (Liberski et al., 2016). The human cardiac-derived cardiomyocyte progenitor cells (hCMPCs) printed in alginate scaffold in *vitro* culture demonstrated increased cardiac commitment of hCMPCs without altering the cell viability and proliferation. A significant increase cardiac marker TnT and cardiac transcription factor Nkx2.5, GATA-4, Mef-2c was observed (Maiullari et al., 2018).

Hyaluronic Acid

Hyaluronic acid (HA) and its derivatives are biocompatible, not toxic and plays a central role in cell support. HA is modified to readily crosslink as it lacks spontaneous ionic/enzymatic gelation and used in 3D bioprinting due to this high viscosity (Duan, et al., 2013). It is a glycosaminoglycan (GAG) found in morphogenesis (Rosines et al., 2007), wound repair (Kikuchi et al., 2005), cell migration and signaling (Lokeshwar and Selzer, 2000). HA is a preferred choice in tissue engineering as its important role during early embryonic development its biocompatible nature and also the ability to control its architecture and degradation (Hölzl et al., 2016). HA displays a slow gelation rate, along easy mechanical and chemical modifications are required to enhance the rheological properties (Hospodiuk et al., 2017). Human cardiosphere-derived cells (CDCs) when delivered with FDA-approved Hyaluronan based scaffold *Hystem-C*™ hydrogel, showed high cell engraftment and therapeutic efficacy (Cheng et al., 2012a); . A photocrosslinked hydrogel constructed from oxidized and methacrylated HA which enabled the cell-hybrid hydrogel interaction stimulated cell migration, proliferation, GAG secretion. This study provides a proof that the hybrid hydrogels mimic layer specific valve ECM and are a good choice for heart valve tissue engineering (Duan, et al., 2013).

Fibrinogen

Fibrinogen is a fibrous plasma protein that is actively involved in wound thrombosis, wound healing, angiogenesis. Fibrinogen is also involved in cell adhesion, migration, proliferation and differentiation (Budharaju et al., 2021). Fibrinogen is often used in 3D printing of cardiac tissues as it undergoes spontaneous gelation in the presence of thrombin and has docking sites for many proteins like VEGF, IL-1, albumin, Von Willebrand factor (Brown and Barker, 2014). By adding FDA approved chemicals like aminocaproic acid, aprotinin during the gel preparation, plasminogen mediated proteolytic

degradation of fibrin can be avoided (Wang et al., 2018). Since fibrinogen is less viscous and poses weak rheological properties, fibrinogen is used with other biomaterials like Matrigel, collagen during the printing process (Hölzl et al., 2016). A advanced model of cardiac tissue constructed using HUVECs and iPSC-CMs encapsulated in alginate and PEG-Fibrinogen hydrogel strands demonstrated functional integration of the host's vasculature into the 3D bioprinted constructs, supplying blood to the implant and preventing necrosis and endorsing heart-like engineered tissue (Maiullari et al., 2018).

Decellularized ECM

Cardiac ECM contains is madeup of fibronectin collagen I, fibrillin 1, and laminin and other tissue specific growth factors (Williams et al., 2014). Decellularized ECM (dECM) has gained attention due to its high biocompatibility, printability and thermoresponsive nature (Garreta et al., 2017). Cardiac dECM can be prepared from porcine sheep by decellularization followed by lyophilization (He and Callanan, 2013). The dECM mimics the architecture of the cardiac ECM and facilitates the process of cell adhesion, proliferation and differentiation and migration (Ott et al., 2008). When myocardial ECM hydrogel was injected into the infract region of rat hearts, the RNA transcriptome analysis revealed the upregulation of neovascularization, cardiac transcription factors and the pathways associated. The factors associated with apoptosis, fibrosis were downregulated (Seif-Naraghi et al., 2013; Spang and Christman, 2018). Using dECM scaffold, a bioconstruct that mimicked native cardiac ECM microenvironment was fabricated that show high cell viability and proliferation of cardiac progenitor cells, that resulted in the increased cardiomyogenic differentiation (Jang et al., 2016).

Gelatin

Gelatin is a derivative of collagen that contains RGD cell-binding motifs like collagen. Gelatin is less immunogenic and takes active part in cell adhesion, differentiation, migration, and proliferation (Davidenko et al., 2016). Gelatin is sensitive to temperature, concentration, solvent and crosslinking agents that in turn affects the thermoresponsive nature of gelatin (Zhu W. et al., 2017). Due to poor mechanical characteristics, gelatin is chemically cross linked with agents like glutaraldehyde before using for 3D bioprinting (Hellio and Djabourov, 2006). Using extrusion-based three-dimensional (3D) bioprinting (Yin et al., 2018) reported accurate deposition of cell-laden GelMA in microarchitectures that showed high cell viability. To build patient specific soft tissue, gelatin-methacrylate gelMA hydrogel using poly -actic-co-glycolic acid nanofiber fragments (PLGA-NF) was fabricated that enhanced the biomechanical properties and stability and also promoted fibroblast proliferation in gelMA/PLGA-NF hydrogel (Ko and Kwon, 2020).

Cell Sources for 3D Printing

For the accurate functioning of the fabricated constructs, the proper choice of cells for tissues is crucial. There are various cell types in tissues and organs that are endowed with specific functions, and these attributes must be recapitulated in the new 3D bioprinted construct. The cells present in the native organ are mainly involved in providing structural support and are actively involved in maintaining the stem cell niche (Murphy and Atala, 2014). For the 3D bioprinted cardiac construct to be functional in the long term, the cardiac tissue construe must maintain homeostasis, respond to tissue injury and self-renew (Budharaju et al., 2021).

Primary Cardiomyocytes

The adult primary cardiomyocytes are terminally differentiated and where the Ca^{+} regulates the functional characteristics, thus making them an excellent choice for tissue engineering (Foglia and Poss, 2016). By using primary cardiomyocytes and polycaprolactone support frame, a contractile cardiac construct was developed that was structurally organized and scalable The 3D bioprinted cardiac tissue construct was a dense, aligned cardiac muscle bundles that possessed synchronous contraction and responded to known cardiac medications. These medications acted by blocking which notch pathway and promoted improved cardiac tissue formation (Wang et al., 2018). A EHT constructed, contained aligned homologous cardiomyocytes and a synthetic supporting polymer polyethylene vinyl acetate (PEVA) that enhanced the expression of cadherins, integrin proteins, differentiation and maturation with increased functionality (Das et al., 2019).

Stem Cells

Stem cells like iPSCs, ESCs, MSCs (Li et al., 2022), cardiac progenitor cells have been cultured in 2D cell cultures and further differentiated to functional cardiomyocyte (Kattman et al., 2006; Wang X. et al., 2021). The growth factors and differentiation factors present in the matrix of scaffolds play an important role in the cell fate. Among the different proteins in the ECM, integrin is important as it activates transcription factors to future differentiate into cardiomyocyte (Lee, 2018). Mouse ESCs plated on polyglycolic-acid (PGA) material patches and transferred to ischemic and peri-ischemic mice myocardium 3 days after seeding. The survival rate, blood pressure along with ventricular function in the mice heart with ESCs patch was better than the sham operated and cell free patches mice hearts thus demonstrating that ESCs aided the cardiac repair of infracted myocardium (Ke et al., 2005).

hiPSC-Derived Cardiomyocytes

To design an accurate cardiac construct, researchers have integrated stem cells derived human cardiac cells in the construct (Liu et al., 2019). Human induced pluripotent stem cell-derived cardiomyocytes (hiPSC-CMs) are superior to primary cardiomyocytes as they can expand prior to differentiation to cardiomyocytes and also are easy to scale up (Zuppinger, 2019). GelMA-a photopolymerizable ECM has adhesion moieties and natural degradation making it a popular choice for printing encapsulated cells (Yu et al., 2020). To design hiPSC-CMs models, cells have been encapsulated in ECM, Fibrin or gelatin methacrylate (GelMA) (Veldhuizen et al., 2019) to facilitate the formation of connections in 3D space (Ma et al., 2018). The recently developed micro-continuous optical printing system (μCOP) is a perfect 3D bioprinting technique

that supports high 3D printing resolution, biocompatibility and speed (Ma et al., 2018; Liu et al., 2020). 3D cardiac tissue that is constructed using hiPSC-CM and human cardiac fibroblasts (HCF) that is coated with (ECM) and plates on the fabricated HBC gel frame, enhances the contractile function and vascular function (Tsukamoto et al., 2020). A study established that a 3D *in vitro* model of cardiac fibrosis made of TGF-β1 treated 3D cardiac microtissues promoted myofibroblast proliferation and differentiation (Lee et al., 2019b). However, hiPSC-CMs are not preferred for adult cardiac tissue constructs as the new differentiated hiPSC-CMs possess limited tissue alignment and have deficient calcium handling (Ronaldson-Bouchard et al., 2018).

Scaffold–Free Bioprinting

Though scaffolds have been successfully used in tissue engineering, the issues that still persist are low biodegradability and adverse immune response which are life threatening (Gao et al., 2017). Hence, scaffold free printing, has emerged as an alternative for artificial tissue/organ fabrication (Zhu W. et al., 2017). In scaffold-free 3D bioprinting, cell adhesion is crucial as it compensates for the scaffold support. For tissue repair and regeneration, several scaffold free printing methods have be developed like pallet culture (Estes and Guilak, 2011), hanging drop (Lee et al., 2012), hydrodynamic cell trapping (Fu et al., 2014) and spinner flask (Rodday et al., 2011).

Briefly in all these techniques, the cells are outspread in a 3D environment where their differentiation results into a mass due to cell-cell adhesion. After the cells grow in a definite shape, they are placed in a layered pattern with the aid of 3D printer and then cultured with other cells to form a tissue like structure (Tan et al., 2014). A biomaterial free cardiac patch was developed to deliver hiPSC-CMs, fibroblasts (FB) and endothelial cells (EC) as mixed cell spheroids. These 3D printed patches show uniform electrical conduction and also exhibit action potential waveforms as in the ventricle. Upon implantation, vascularization and engraftment with the myocardium was seen (Ong et al., 2017) thus, suggesting a next generation stem cell based treatment for heart failure. A scaffold free cardiac graft patch was developed that was made of spheroids containing CMs, FB and EC that promoted rapid self-organization and vascularization (Noguchi et al., 2016). Though a thicker myocardial graft could not be fabricated, this approach could potentially be developed to treat myocardium injury. In a scaffold free 3D cell sheet, MSCs are seeded and cultivated on temperature-responsive polymer-grafted cell culture dishes (TRCD) at 37°C. At confluency, the cells deposit ECM and form interactions along with forming channels with the neighboring cells. The cells are detached at confluency by reduction of temperature from 37 to 20°C to induce changes in the surface properties, that results in the change from hydrophobic to hydrophilic nature of the culture dished and releases the adherent cells. Spontaneous tissue contraction upon the release from the 2D monolayer resulted in the increase in the thickness of the cell sheet rendering an increase in the volume of the cardiac like tissue construct. This tissue has demonstrated an upregulation in the expression of vascular hepatocyte growth factor (HGF), interleukin -10(IL-10), endothelial growth factor (VEGF) (Bou-Ghannam et al., 2021). The advantage and disadvantages of scaffold-based or scaffold-free bioprinting were summarized in **Table 3**.

APPLICATION

Though the cardiac tissue is complex, 3D bioprinting has emerged as a next generation treatment approach for generating cardiovascular implants that have biomimetic qualities. These 3D implants recapitulate both morphological as well as biochemical attributes of the native cardiac tissue. Here, we discuss the 3D bioprinting strategies used to fabricated cardiac tissues such as myocardium, cardiac valves, and cardiac models for drug screening.

3D Bioprinted Myocardium

The myocardium is an intricately organized muscle layer in the heart wall that plays a crucial role in the contraction and relaxation of the heart. Myocardium is made of nearly 2-4 billon cardiomyocytes (Laflamme and Murry, 2011). During a cardiac injury, there is a loss of cardiomyocytes accompanied with modification in the cardiac ECM (Gaetani et al., 2012). Hence cardiac 3D bioprinted constructs/implants aim in repopulating the cardiomyocytes and ECM for cardiac repair (Mathur et al., 2016). An engineered human myocardium (EHM) that mimicked the postnatal heart both in terms of structural and functional properties was fabricated *in vitro* under well-defined serum free conditions using ESCs and iPSCs. These EHM exhibited attributes like cardiomyocytes with M bands, systolic twitch, positive force frequency response along with molecular advancement. This EHM can potentially be used for cardiac repair, drug screening and disease modeling (Tiburcy et al., 2017). 'VentriGel'- a first in man clinical research implant, fabricated from porcine heart-derived dECM-based hydrogel and transendocardially injected in early and late post MI patients with left ventricular dysfunction. Post injection, there was an increase in the vascularization, reduced cardiomyocyte dystrophy and fibrosis (Traverse et al., 2019). Cardiac patches composed of cECM, human cardiac progenitor cells (hCPCs) and gelatin methacrylate (GelMA) were implanted *in vivo* on rat hearts. After 14 days of implantation, an increase angiogenic potential (>2 fold) along with improved endothelial cell tube formation was observed which indicated that these patches could be used as potential treatment for repairing the damaged myocardium (Bejleri et al., 2018).

3D Bioprinted Cardiac Valves

Apart from myocardial damage, cardiac valve dysfunction represents another major reason for heart failure (Howell and Butcher, 2012). The cardiac valves are mainly made of valve interstitial cells (VICs), SMCs, and valvular endothelial cells (VECs) (Klebe, 1988). In cardiac valve diseases, the valves are either incapable to close or are too contracted to open entirely. In such conditions, valve replacement surgery is the only option where prosthetic valves are employed. Though the prosthetic

valves have high longevity, thrombogenicity is a major issue (Maxson et al., 2019). A 3D bioprinted aortic heart valve scaffold was fabricated using rat MSCs onto gelatin support gel. *In vivo* studies proved that this scaffold was capable of alteration and an increase in elastin, vimentin, alpha SMA, and CD31 was observed in all 12 weeks (Maxson et al., 2019). 3D bioprinted aortic valve conduit constructed directly by encapsulating aortic root sinus smooth muscle cells (SMC) and aortic valve leaflet interstitial cells (VIC) within alginate/gelatin hydrogel discs improved cell viability, migration and proliferation (Duan et al., 2013).

3D Bioprinted Blood Vessels

Impaired blood circulation to heart underlies enervating conditions like ischemia, heart failure and stroke (Mozaffarian et al., 2015). Functional vascular network facilitates the adequate delivery of oxygen, nutrients, unwanted metabolite removal and constant circulation of immune cells which in turn plays a crucial role cardiovascular regeneration (Duan, 2017). 3D bioprinted viable cardiac tissue constructs require imbedded blood vessel networks for nutrient transport and cellular waste disposal (Jafarkhani et al., 2019). Using bone marrow MSCs and sodium alginate/gelatin, a cardiac patch was designed and implanted on the injured heart. 5-azacytidine (5-Aza) induced differentiation of MSCs leds to morphological changes and expression of α-striate muscle actine antibody (α-SCA) and desmin antibody (DES) and other proteins. This cardiac patch repairs the injured cardiomyocytes by generating new cardiomyocytes, rather, than increasing the blood supply and increasing the cardiac functionality by promoting vascular proliferation. Also, the implanted cardiac patch demonstrated improved epicardial activation that promoted angiogenesis and EMT through WT1 (Wilms tumor protein 1)-mediated Wnt/β-catenin signaling pathway (Liu X. et al., 2021). Using the *in vivo* priming strategy, bone marrow MSCs were primed *in vivo* by genetically induced hepatocyte growth factor -expressing MSCs (HGF-eMSCs). These MSCs and HGF-eMSCs were encapsulated within a 3D cardiac patch. On implantation on MI induced heart, an improvement in cardiac function, prevented apoptosis along with the enhancement of vessel formation was observed post MI (Park et al., 2020).

3D Bioprinted for Drug Screening *In Vitro*

3D printing offers a great platform in pharmaceutics for drug discovery and development (Sharma et al., 2021; Vijayavenkataraman et al., 2018). In cardiac regenerative medicine, efforts are mainly focus on recreating biomimetic microtissues of left ventricular myocardium as it is the primary pumping chamber and site for pathologies (Ma et al., 2018). Recently, an μCOP system was adopted to construct a scalable 3D model that mimicked the function and microarchitecture of the ventricular myocardium (Liu et al., 2020) that could be used in drug screening. A customizable force computing asymmetric system was designed that directly printed encapsulated cardiomyocyte. Here the NMVCMs formed a contracting tissue that possessed improved alignment physiological responsiveness to ionotropic stimulation (Liu et al., 2020). Using microfluid technology 3D endothelialized microfibrous scaffold with precisely controlled macroscale microfibers and seeded cardiomyocytes that induced the formatin of myocardium that possessed spontaneous and synchrous contraction (Zhang et al., 2016; Veldhuizen et al., 2019).

LIMITATIONS

The innovations in 3D printing in tissue engineering for cardiovascular repair and regenerative research has made tremendous stride in the recent years. The advantage of 3D bioprinting approach is its ability of accurately printing high resolution constructs of different biomaterials, cells, and therapeutics for the fabrication of highly complex 3D cardiac constructs. Despite of the significant progress and sophistication in 3D bioprinting in tissue engineering, the fabrication of a fully functional heart is yet to achieve. In 3D bioprinting, one of the major challenges is the printing resolution. For the fabricated new tissue to be closely mimetic to the native tissue, the ideal resolution much be comparable to the cell size. For the clinical application, a multilayered tissue is required. It is a challenge to generate controlled vascular network for the survival for the cells (Lovett et al., 2009). In the recent years, the 3D bioprinting technique has advanced in achieving structural complexity, but the 3D bioprinting of soft materials is still immature (Lee and Dai, 2017). Another limitation of the 3D bioprinting is that there is no standard and accepted method to access the accuracy and efficiency of the fabricated models which results in the variability. Some of current biomaterials used do not truly replicated the mechanical properties of the human heart limiting the evaluation of the tissue behavior (Harb et al., 2019). Another issue in 3D bioprinting is the determination of scaffold and optimizing the process parameters. Also, the present polymeric materials lack appropriate conductivity and weak mechanical strength as compared to native cardiomyocytes. For CTE engineers, degradability, and biocompatibility of scaffold along with cells interaction and migration in the scaffolds remains an important challenge. In the present times, 3D bioprinted of cardiac tissue is still in infancy, in the coming years, once the above issues are addressed, 3D bioprinting will enable the translation of technology to personalize therapeutic and pharmaceutical applications.

PROSPECTS

Cardiovascular constructs (vasculature constructs, heart valves, myocardium), have been successfully 3D bioprinted with discrete structure and function. However, all these techniques are still in infancy and face several challenge which hinder the accurate construction of cardiac analogs with full functionality and complex micro-architecture (Cui et al., 2018). The recent advancements on the field of 3D printing and tissue engineering have shown to provide a platform for studying and understanding the unknown mechanism of development

of diseases and its responsiveness to new developing drugs. This science continues to grow and evolve with the addition of new advanced technologies like bioelectronics, next generation sequencing etc. For instance, the convergence of bioelectronics and 3D printing helps to generate a functional live tissues like cardiac muscles with electrophysiological signals (Ershad et al., 2019). Also, the integration of biosensors and engineered tissue devices further helps in real-time monitoring of parameters which in turn can provide insight in morphogenesis, pathogenesis, and drug responsive remodeling processes in disease conditions (Ni et al., 2017). Using patient driven stem cells, 3D printed cardiac tissues can be used to study the *in vitro* responses of individual characteristics (Sun, 2020). This facilitates higher treatment efficacy by enabling personalized prescriptions and treatments.

CONCLUSION

The 3D bioprinting technology is novel and cutting-edge. it is one of the most popular tissue engineering methods as it facilitates the rapid creation of complex biostructures. Currently, significant efforts are being made to develop the technique to improve printing accuracy and speed, as well as the capacity to capture the tissue's complexity. Future advancements in the field of 3D bioprinting will offer new doors and speed up cardiac regenerative medicine research.

AUTHOR CONTRIBUTIONS

MC wrote the text of this review paper with guidance from KH and KC. All authors have reviewed the final version and approve of the content in this manuscript.

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