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Review

Recent advances in bioprinting technologies for engineering cardiac tissue

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ABSTRACT

Annually increasing incidence of cardiac-related disorders and cardiac tissue's minimal regenerative capacity have motivated the researchers to explore effective therapeutic strategies. In the recent years, bioprinting technologies have witnessed a great wave of enthusiasm and have undergone steady advancements over a short period, opening the possibilities for recreating engineered functional cardiac tissue models for regenerative and diagnostic applications. With this perspective, the current review delineates recent developments in the sphere of engineered cardiac tissue fabrication, using traditional and advanced bioprinting strategies. The review also highlights different printing ink formulations, available cellular opportunities, and aspects of personalized medicines in the context of cardiac tissue engineering and bioprinting. On a concluding note, current challenges and prospects for further advancements are also discussed.

1. Introduction

Cardiac-associated diseases are one of the most common causes of mortality globally (around 17.5 million deaths every year) and are expected to increase up to 23.6 million deaths by 2030 [1]. The main reason for these numbers is that the heart is one of the least regenerative organs of the human body due to cardiomyocytes' (CMs) limited renewal potential [2]. When heart attack or cardiac arrest occurs (due to loss of electrical impulse or lack of nutrients/oxygen supply from arteries), a billion of CMs are lost. Since there is no auto-regeneration or repair for these cells, the heart forms a scar tissue that cannot transmit the electrical signal and contractile activity, thus increasing the risk of heart failure [3,4].

Current treatments for cardiovascular diseases include cell therapy, autografts (*e.g.* coronary artery bypass graft with autologous vein), allografts (*e.g.* donor valve or heart transplantations), xenografts (*e.g.*, bovine or porcine heart valves, arteries) and artificial prostheses (*e.g.* biopolymer vascular grafts, mechanical valves, cardiac assist devices)

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[5]. However, these approaches suffer from several limitations, such as lack of donors, immune rejection, coagulants therapy, and limited durability. Nevertheless, direct injection of cells has shown some promising results in regaining muscle functionality, but low viability and lack of ability to develop extracellular matrix (ECM) and vascularization post-injection are the downsides [6].

In this regard, a promising solution is the emerging field of tissue engineering, a multidisciplinary approach that combines material engineering, life sciences, and computer modeling to fabricate functional scaffolds and artificial tissue constructs for biomedical applications and regenerative medicine [7]. As regard to cardiac tissue engineering (CTE), both the fabrication of artificial substitutes for damaged areas (*i.e.* cardiac scaffolds and/or patches) and *in vitro* models for drug testing and high-fidelity modeling of cardiac development and disease, are the main focus [8]. Each of these approaches is based on the choice of an appropriate cell source, biomaterials, and devising strategies for optimal oxygenation, media perfusion, and exposure to physiologically relevant stimulus [9–11].

Indeed, an ideal scaffold for CTE should mimic the native ECM in terms of morphology, electro-mechanical and biological properties [6]. The fabrication approaches for this 3D architecture spans from the simple use of hydrogels, to electrospuns nanofibers and decellularized tissues/organs [12], but with limited success due to poor control on scaffold architecture, an incomplete vascularization and few cell types to intervene in damaged tissue repair.

The introduction of Additive Manufacturing (AM) technologies, frequently referred to as 3D printing, has allowed overcoming these limitations. This approach facilitates the fabrication of complex 3D architecture through the layer-by-layer deposition of a wide variety of materials [13]. The application of AM technologies for creating functional living construct, using biological materials, including cells, biomaterials and growth factors, has been referred as 3D bioprinting (or, simply as Bioprinting) [14]. In the last decade, the applications of 3D bioprinting in medicine and bioengineering have advanced rapidly to fabricate tissues, organs, prosthetics and drug delivery systems. Moreover, bioprinting can create customized and patient-specific devices, thus enhancing their efficiency, durability and cost-effectiveness [15].

In this review, we present current opportunities and challenges in the field of CTE. We focus on the implications of 3D bioprinting in the treatment of cardiac diseases, presenting some of the most innovative and promising approaches classified according to the different fabrication techniques. In the last part, a brief overview of available cellular opportunities for CTE would be discussed.

2. Cardiac tissue: macro and micro-anatomical and histological features

The myocardium consists of four chambers, two atria and two ventricles, and is responsible for pumping blood to the body (Fig. 1A) [16,17].

Histologically, the heart is divided into three layers, namely the pericardium, myocardium, and endocardium (Fig. 1B) [16]. Each layer performs its own distinctive functions. The pericardium covers the heart and includes the root of the vessels together with the heart and is made up of two layers as the fibrous pericardium and serous pericardium. The fibrous pericardium is the pericardium's outer layer, protecting the heart from external factors and trauma. The serous pericardium is attached to this layer and directly contacts the pericardial fluid. The serous pericardium is further divided into two layers - visceral pericardium (inside) and the parietal pericardium (outside). The pericardial space exists between the two leaves [18].

Myocardium, on the other hand, is the muscular layer of the heart. It functions involuntarily and is innervated by sympathetic nerve fibers. Moreover, this layer is in the structure of intertwined fascicles of the fibers which form the cardiac muscle and is usually examined in three main components - ventricular, atrial, and conduction systems. Muscle fibers that form the ventricles are in two or three layers, and these layers follow each other. Atrial muscle fibers are in two layers - superficial and deep layers. While the fibers in the superficial layer wrap the atria together, the deep layer's fibers cover the atria separately. The muscle fibers belonging to the conduction system are specialized cardiac muscle fibers extending from the atrium to the ventricles [19].

In addition, the endocardium is the innermost layer of the heart composing of endothelial trabeculae and is mainly responsible for controlling myocardial contractility [16,20]. The structure and thickness of the endocardium are different in the auricle and ventricle. Together, these three layers (pericardium, myocardium, and endocardium) play distinctive and essential roles in maintaining myocardial function and health.

The heart is an organ which functions involuntarily due to the stimulation of the autonomic nervous system. The myocardium's stimulation system comprises the sinoatrial node, atrioventricular node, and atrioventricular bundle. The sinoatrial node is the structure consisting of atrial muscle cells and myofibrils. The stimuli is initiated at the sinoatrial node in normal adults where the heart rate is around 60–90 beats per minute (bpm), and is influenced by age. For example, the heart rate is between 90 and 140 bpm in children, whereas it is between 70 and 80 bpm in the elderly. From the sinoatrial node, the impulse reaches the atrioventricular node with normal muscle fibers in the atria. After that, the impulse is conducted to the ventricles *via* the Purkinje fibers [21]. The action generated by the CM surface leads to the contractile process in the cardiac muscle; the Ca^{2+} ion plays a crucial role in this event.

The cardiac muscle is a striated muscle like a skeletal muscle [22]. Typical myofibrils of the cardiac muscle contain actin and myosin filaments which are almost the same as those in the skeletal muscle. These filaments are intertwined and slide over each other during contraction, as in the skeletal muscle. However, there are substantial differences between the cardiac muscle and the skeletal muscle. All the cardiac muscle cells are interconnected with intercalated discs, thus creating functional integrity. The cardiac muscle contains a single type of fiber (slow-twitch fiber type) [23]. It has a unique conduction system (pacemaker), not observed in the skeletal muscles, which generates a stimulus on its own and spread it to all the heart cells. Another critical difference is that the heart can generate stimulation without any neural connection [24].

The cells forming the heart's shape are of mesodermal origin and consist of CMs, fibroblasts, and endothelial cells (ECs). CMs are the contractile cells that are responsible for the pumping and filling functions of the myocardium. When the histology of the heart is considered, CMs comprise 80% of it by volume. However, they only constitute 25% to 35% of the total cell population. In the connective tissue, CMs are surrounded by rich blood vessels and capillaries. The fibroblasts support CMs with ECM production [25]. Notably, CMs differ both structurally and functionally in the left and right chambers. The distribution of fibroblast cells in the heart is also not homogeneous, and it differs from layer to layer [26,27]. For instance, in the sinoatrial node, the number of fibroblast cells is higher than the CM cells [28]. In summary, the macro-/micro-scale organization of the heart is well-organized to support efficient pumping capacity.

3. Implications of 3D bioprinting for cardiac regeneration

Bioprinting pose remarkable promises for engineering functional scaffolds with complex 3D architecture by providing outstanding advantages, such as high-repeatability, reproducibility, controllability, and throughput [29–31]. By traditional classification, technological advancement for cellular bioprinting in CTE includes inkjet, extrusion, and laser-assisted bioprinting [6,29–33]. These technologies have also gained attention for bioprinting of cardiac patches without scaffolds or biomaterials [34–36]. With these approaches and systems, developed for bioprinting applications, conventional AM processes have also proven successful in fabricating scaffolds for CTE.

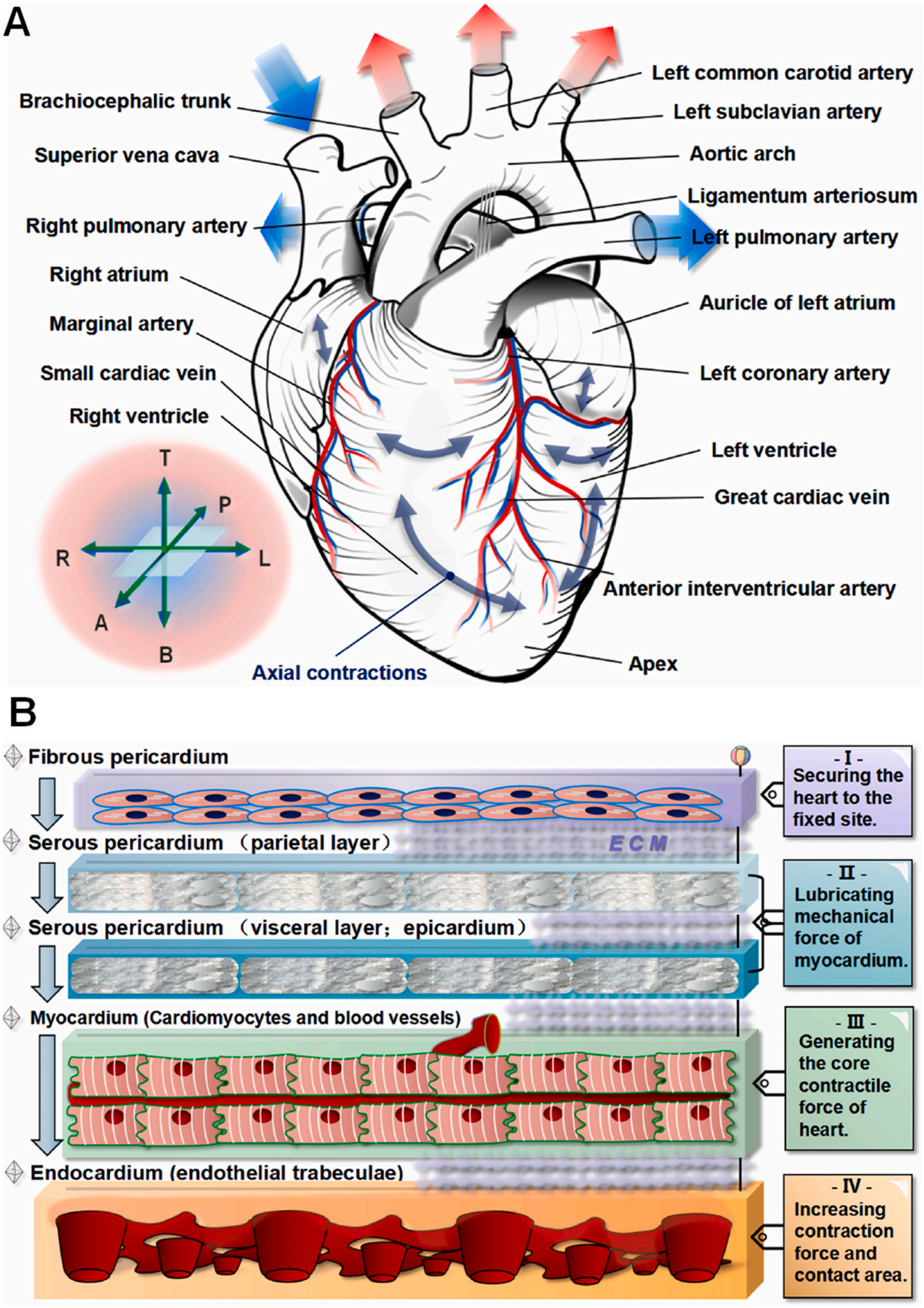

Fig. 1. Cardiac anatomy. (A) Structure of the heart. (B) Layers of the myocardium. Reproduced from [16] with permission from Elsevier.

The characteristics of each technique and printing materials for CTE are discussed in the next paragraphs. An overview of the open challenges towards personalized medicine (PM) is provided at the end of the section.

3.1. Bioinks and printing materials

Printing inks refers to the material formulations that could be printed in a layer-by-layer fashion to construct 3D structures. These printing inks may or may not contain viable cells; those containing them are precisely termed 'bioinks' [37]. As regard to cardiac TE, secondary cell lines, primary cardiac cells, and stem cell-derived cardiac cells are the major cellular components of bioinks. An ideal printing ink must possess certain properties such as printability, mechanical stability, biodegradability, tunability, and should promote post-printing maturation while ensuring viability and functionality maintenance of the cells for a prolonged period under culture conditions. Apart from these properties, various other parameters need to be considered during selection of the printing inks, including gelation properties/crosslinking ability, cost, printing time, industrial scalability, and permeability to gases, nutrients and wastes [38].

As far as materials are concerned, a wide range of materials, individual or in combination could be employed for developing printing inks; however, in context to cardiac TE, both natural and synthetic biomaterials have gained most popularity. Each of the material types has its pros and cons. Natural biomaterials, being naturally derived, are highly biocompatible and have intrinsic bioactivity that mimics cell's ECM. These may either be polysaccharide-based (agarose, alginate, chitosan), protein-based (collagen, gelatin, fibrin), glycosaminoglycan-based (hyaluronic acid, heparin) or even decellularized extracellular matrices (dECM) [39–45]. Although these materials are often the preferred choice, their inadequate mechanical properties, batch to batch variability, immunogenicity, and low tunability aspects often limit their applicability [46].

Synthetic materials, instead, are more defined and possess lower immunogenicity. Moreover, they are compatible with a wide range of physical and chemical modification, allowing fine-tuning of their physical, mechanical properties by pH, temperature, and light-based crosslinking, as per experimental requirements [178]. Polyethylene glycol (PEG) and polycaprolactone (PCL) are the most applied synthetic materials for cardiac tissue printing [47–51]. Interestingly, the derivatized form of natural/synthetic materials has experienced more comprehensive utility for 3D printing. Most evident examples of this are diacrylate/methacrylate derivatives of gelatin or PEG, unlike their pristine form, are photocurable and thus, could be used for stereolithography (SLA), a 3D printing strategy involving the use of light and photoinitiators for crosslinking (discussed in detail in the later sections) [43,52–55].

Besides, printing inks/bioinks for CTE may further be incorporated with other additives to improve their functional properties. For instance, metal/carbon-based materials (examples, such as graphene, graphene oxide, carbon or gold or transition metal dichalcogenides (TMDC)-based nanomaterials) or electroactive polymers (such as, Polypyrrole (PPy), polyaniline (PANi), and poly(3,4-ethylenedioxythiophene) (PEDOT)) have been added in the inks to impart much-needed conductive properties to the tissue construct [56–58]. Polymeric micro- or nanoparticles could also be incorporated to regulate the release of cells and other bioactive agents in a spatiotemporal manner. In fact, these additives, particularly nanomaterials, have also been shown to modulate the ink's mechanical and rheological properties, making them more printing- and/or cell-friendly [57,59]. Moreover, the crosslinkers' selection and concentration in the bioink formulation is also critical for effectively printing complex structures with required mechanical and degradation properties, without compromising cell viability.

In summary, the bioinks for CTE could be a composition of materials and other additives that could recreate a cardio-inductive microenvironment, both in terms of physicochemical and mechanical characteristics, post-printing. For this, one must understand the impact of these cues on cellular phenotype. Though a detailed discussion of these aspects is out of this review's scope, for the reader's understanding, we have provided their brief overview in the Appendix section S1.

3.2. Bioprinting strategies

To date, several strategies have been employed for 3D bioprinting in the sphere of CTE, focusing on both scaffold-based as well as scaffold-free approaches. Their basic working principle, advantages, limitations, along with representative examples of the technologies are discussed in the following subsections. Table 1 summarizes studies employing bioprinting for CTE, while Table 2 provides a brief overview of different bioprinting techniques.

3.2.1. Inkjet-based bioprinting

Inkjet-based bioprinting (IBB) is considered one of the most common 3D printing technologies for biological applications. It is based on the automated delivery of a controlled volume of bioinks, usually containing cells in a droplet fashion to predefined locations *via* a thermal or piezoelectric-driven mechanism. To release the bioink, thermal and piezoelectric methods employ a heating pulse from the micro-heater, and a piezoelectric actuator, respectively; both embedded in the printhead. In thermal inkjet printers, small air bubbles, generated by the heater, collapse, thereby providing pressure pulses to eject ink drops out of the nozzle. In piezoelectric inkjet printers, instead, the actuator of polycrystalline piezoelectric ceramic in each nozzle provides the transient pressure to eject the ink drops onto the substrate. Since piezoelectric actuators operate in the frequency range between 15–25 kHz that may lead to cellular damage, the thermal inkjet models are often preferred while working with cells [60].

IBB is relatively rapid and compatible with many biomaterials. One of the main advantages of this technique is its high resolution (<100 μm), attributed to the generation of tiny droplets (1–100 pl). Another advantage is the possibility to create concentration gradients of biomaterials, cells or growth factors, by merely varying drop density or size during the printing process [61,62]. However, IBB requires bioinks with lower viscosity (3.5–20 mPa·s); consequently, the fabricated constructs often lack structural integrity and mechanical strength [63]. Moreover, other disadvantages associated with IBB include non-uniform droplet size, low droplet directionality, mechanical, and shear stresses to the cells and frequent nozzle clogging.

As regard to CTE, one of the most exciting studies about IBB was reported by Xu et al. [64]. Here, the authors fabricated a 'half heart' structure by layer-by-layer inkjet printing of $CaCl_2$ cross-linker onto alginate formulation containing CMs. They showed how a controlled micro-structure and porosity could be obtained. Both microscopic and macroscopic contractile cellular activity were notable features of the developed constructs, highlighting IBB's potential to fabricate functional cardiac pseudo-tissues.

3.2.2. Laser-assisted bioprinting

Laser-assisted bioprinting (LAB) involves a high-intensity laser that propels the bioink droplets in a non-contact mode. A LAB bioprinter consists of three main components: a pulsed laser beam, a ribbon containing the bioink to print and a receiving substrate. The laser beam is transmitted through a transparent ribbon to the substrate. When the laser interacts with this intermediate layer, a droplet of bioink (usually containing cells) is ejected to the substrate (often coated with hydrogels that reduce the impact of previously placed droplets). Laser frequency, intensity, and motion control can be programmed. Since LAB is a nozzle-free technology, nozzle clogging is prevented, and high cell viability could be obtained due to low mechanical stress on cells during the printing phase. Moreover, LAB technique allows the deposition of high cell densities ($\sim 10^8$ cells/ml) with good resolution. Furthermore, a wide

Table 1
Other representative studies employing 3D bioprinting for cardiac regeneration.

S. no.	Bioprinting strategy	Bioink polymer(s)	Crosslinking	Cells	Construct	Therapeutic activity (evaluated *in vivo*)	Proposed/ highlighted application	References
1	μSLA	PEGDA	Photocrosslinking (Irgacure 819)	Human Lin-Sca-1+ CPCs	3D scaffold-in-scaffold system (Woodpile scaffold embedded in hydrogel)	–	–	[110]
2	DLP	PEGDA	Photocrosslinking (LAP)	Neonatal mouse VCM and human iPSC-CMs	Microscale force gauge arrays	–	Drug screening	[49]
3	Scaffold free	–	–	human iPSC-CMs, human AVCFs, and HUVEC	Scaffold free multi-cellular patch	Myocardial infarcted rat models	Tissue regeneration	[111]
4	Scaffold free	–	–	Rat neonatal VCMs, Human CMECs, Human NDFs	Scaffold free multi-cellular patch	Normal rat models	Tissue regeneration	[112]
5	Scaffold free	–	–	human iPSC-CMs, human NDFs, and HUVEC	Scaffold free multi-cellular construct	–	Drug screening	[113]
6	Extrusion	Porcine heart-specific dECM	Thermal crosslinking	Neonatal rat CMs	Cardiac gel patch	–	–	[44]
7	Extrusion	Fibrinogen, gelatin, aprotinin, HA	Enzymatic crosslinking (thrombin)	Neonatal rat VCMs	Cardiac gel patch	–	Tissue regeneration and pharmaceutical purposes	[114]
8	Extrusion	GelMA, PEGDMA, silk fibroin	Enzymatic crosslinking (HRP/H_2O_2) and photocrosslinking (Irgacure 2959)	Neonatal rat CMs and iPSC-CMs	Cardiac gel patch with perfusable vascular channels	–	Tissue regeneration, organ-on-a-chip, and drug screening	[50]
9	Extrusion	Gelatin	Enzymatic crosslinking (microbial transglutaminase)	Human bone marrow-derived MSCs and neonatal rat CMs	3D scaffolds	–	Tissue regeneration and pharmaceutical purposes	[115]
10	Extrusion	Furfuryl−gelatin and fibrinogen	Photocrosslinking (Rose Bengal photosensitizer) and enzymatic crosslinking (thrombin)	Human iPSC-CMs/ AC16 cells and human adult fibroblasts	Cardiac gel patch	–	Drug screening	[54]
11	Extrusion	PCL and CNT	–	H9C2 cardiomyoblast	3D scaffolds	–	Tissue regeneration	[51]
12	Extrusion	Alginate	Ionic crosslinking (calcium chloride)	Human fetal CMPCs	Cardiac gel patch	–	Tissue regeneration	[116]
13	Extrusion	Porcine heart-specific dECM	Photocrosslinking (vitamin B12) and thermal crosslinking	Human CPCs	3D construct	–	–	[117]
14	Direct-ink writing	Acetylated nanocellulose	–	H9C2 cardiomyoblast	3D scaffolds	–	–	[118]
15	FDM + extrusion	Alginate, low-melting agarose, platelet rich plasma	Enzymatic crosslinking (thrombin) and ionic crosslinking (calcium chloride)	H9C2 cardiomyoblast and HUVEC	Cardiac gel patch	–	Tissue regeneration	[39]
16	Multiphoton-excited 3D printing	GelMA	Photocrosslinking (MBS)	Human iPSC-CMs, iPSC-SMCs, and iPSC-ECs	Cardiac gel patch	Myocardial infarcted mouse models	Tissue regeneration	[119]

PEGDMA: poly(ethylene glycol) dimethacrylate, PEGDA: polyethylene glycol diacrylate, CPCs: cardiac progenitor cells, VCMs: ventricular cardiomyocytes, AVCFs: adult ventricular cardiac fibroblasts, CMECs: cardiac microvascular endothelial cells, NDFs: normal dermal fibroblasts, PEVA: poly(ethylene/vinyl acetate), CMCMA: carboxymethyl cellulose methacrylate, AlgMA: alginate methacrylate, CNT: carbon nanotube, CMPCs: cardiomyocyte progenitor cells, iPSC-SMCs: iPSC-derived smooth muscle cells, iPSC-ECs: iPSC-derived endothelial cells.

range of biomaterials can be used (1–300 mPa·s) in the printing process. Despite these advantages, some drawbacks can be highlighted. For instance, this technique is mostly used for 2D bioprinting (monolayer) and is often not suitable for depositing multiple cell types. It is time-consuming, costly and usually works with small size structures, and its clinical applications are minimal [5,15,33,65,66].

Previously, Gaebel et al. fabricated polyester urethane urea (PEUU)-based cardiac patch containing human mesenchymal stem cells (MSCs) and human umbilical cord vein-derived endothelial cells (HUVEC) in a defined grid pattern through LAB (Fig. 2) [67]. These patches, upon implantation in rat's left anterior descending (LAD) ligation model, demonstrated an improved cardiac repair along with reduced fibrosis. Groups implanted with untreated patches or those containing random cell distribution were associated with lower therapeutic outcomes.

3.2.3. *Extrusion-based bioprinting*

Extrusion-based bioprinting (EBB) is one of the most widely employed technologies for biological and non-biological printing. A computer-controlled system is used to extrude a continuous strand of bioinks onto a substrate to form a 3D construct in a layer-by-layer format [15]. The constant pressure to extrude the material, from the nozzle, can be generated by either pneumatic or mechanical (piston or screw-driven) system [68].

EBB can be classified as a direct and indirect extrusion method. The

Table 2
Summary of different bioprinting strategies in the sphere of CTE.

Printing method	Working principle	Strengths	Limitations	Applications	Remarks
IBB	- Ejection of droplets from a cartridge due to a thermal or piezoelectric actuator	- High resolution - High speed - Concentration gradients of materials/cells	- Mechanical and thermal stresses to cells - Low droplet directionality - Non-uniform droplet size - Nozzle clogging	- Functional cardiac pseudo tissues	- Cost-effective tool for hierarchical design
LAB	- A laser pulse is focused on bio-ink layer, generating a drop is ejected on a receiving substrate	- High resolution - High cell density - No nozzle clogging - Wide range of biomaterials	- Expensive technique - Mostly used for 2D bioprinting - Does not work properly with multiple cell types	- Cardiac patch	- Improved vessel formation and cardiac activity
EBB	- Extrusion of cell-laden hydrogel by a pneumatical/piston/screw-driven syringe pump	- Fast and controllable process - Possibility to print structures with clinically relevant size - Use of multiple biomaterials and cells	- Low resolution - Does not allow precise cell patterning and organization - Cell viability affected by hydrogel solidification	- Cardiac patch - Heart-on-chip	- Improved vascularization - Improved contractile activity - Cardiovascular toxicity evaluation
Coaxial nozzle-assisted EBB	- Use of coaxial nozzles to extrude biomaterials (core-shell structures) simultaneously	- Efficient for cell encapsulation - Easy fabrication of perfusable tube constructs	- Resolution of fabricated constructs are limited by the nozzle diameter	- Cardiac tissue repair - Vascularization	- Especially useful for vascularized tissues
FDM	- Extrusion of a polymeric thermoplastic material through and heated nozzle	- High repeatability - Low cost - Possibility to mix different materials	- Lower resolution - Not suitable for cell printing	- Cardiac scaffolds	- Improved scaffold properties mixing different materials
SLA and DLP	- Polymerization of a resin through a light source (often UV)	- High resolution - High printing speed using a digital mask	- Complex geometries need supports - Limited biocompatible photo-initiator	- Cardiac *in vitro* models	- High cell alignment achieved through printing pattern
Scaffold-free bioprinting	- Direct deposition of living cells (spheroids/organoids)	- No immune response due to the absence of other biomaterials	- Poor mechanical properties	- Cardiac models	- Improved cell proliferation
Suspension bath assisted bioprinting	- Use of moving extruder to locally fluidize suspension medium	- Reinforced cell survival - Fabrication without the presence of supporting materials	- Limited repeatability and precision	- Cardiac patch fabrication - Cellurized heart fabrication	- Especially suitable for bioinks with low viscosity - Suitable for the fabrication of tissues with complex shape
In situ bioprinting	- Direct printing on designated tissues or organs onsite	- Specialized in patterning on non-flat surfaces - Instant printing during a clinical process	- Potential negative immune response for non-autologous bioprinting	- Potentially cardiac patch fabrication	- Two main technical approaches, handheld and robotic arm

first method is based on the extrusion of bioinks into a cell-friendly environment. After extrusion, this hydrogel solidifies to form the 3D structure while cells can proliferate and undergo tissue remodeling. On the other hand, indirect extrusion is based on the fabrication of a sacrificial structure, which would act as a template/mold. The sacrificial structure can be printed directly in a bulk hydrogel, or this can be casted later. When the bulk hydrogel solidifies, the sacrificial material (usually made of gelatin, alginate, or agarose) can be removed thermally or chemically, thereby creating hollow structures.

The dispensed volume can be adjusted by varying bioprinting speed, bioprinting pressure, and nozzle diameter [66]. The advantage of using EBB is that the scaffold's biodegradability properties can be tuned (by modulating biomaterial composition) to match the extracellular matrix regeneration rate [33]. Moreover, multiple biomaterials and different cell types can be used simultaneously, due to the availability of multi-nozzle bioprinters allowing the fabrication of more complex structures. The major disadvantages of EBB are its lower resolution (>100 μm) compared to other techniques and the impossibility to obtain a precise cell patterning and organization. Besides, cell viability is also affected during gelation/solidification processes post-printing [5].

Many interesting applications of EBB can be found in the CTE domain. Bejleri et al. fabricated 3D bioprinted constructs with CPCs-laden gelatin methacrylate (GelMA)-heart-specific dECM composite bioinks. The study revealed that composite bioink exhibited better printability and supported better cardiomyogenic behavior and endothelialization than pure GelMA bioinks [43]. In another study, as an alternative strategy, angiogenic factors or dECM could also be employed for improving cardiac functionality and vasculogenesis. In this regard, Jang et al. bioprinted a vasculo-inductive patch to enhance the therapeutic efficacy of cardiac repair (Fig. 3) [69]. The construct was developed using heart-specific dECM bioinks with spatially patterned cells; hydrogel strands of bioink I (containing human cardiac progenitor cells (CPCs)) and bioink II (containing MSCs and vascular endothelial growth factor (VEGF)) were placed alternatively. When implanted *in vivo* in the mouse model, the fabricated cardiac patch significantly promoted a strong vascularization and tissue matrix formation, enhancing cardiac functionality while reducing hypertrophy and fibrosis. In contrast, constructs containing either only CPCs or a mixture of CPCs-MSCs-VEGF exhibited a lower therapeutic efficacy.

An advanced version of the EBB technology is coaxial nozzle-assisted bioprinting system. In a typical configuration, encapsulated cells are extruded through the central needle while a crosslinking solution stays in the outer portion of the needle (or *vice versa*) throughout the printing process [70–73]. Exploiting this strategy, Zhang et al. reported the fabrication of an engineered endothelized myocardial construct with potential applicability in regenerative medicines, drug screening, and disease modeling [74]. Firstly, the base microfibrous construct was bioprinted using ECs-laden alginate/GelMA composite bioinks; formation of endothelium and the vascular bed was evident during the post-culture *in vitro*. The seeding of CMs gave the contraction to the whole structure. In another interesting work, Maiullari et al. encapsulated HUVEC and induced-pluripotent stem cell-derived CMs (iPSC-CMs) into

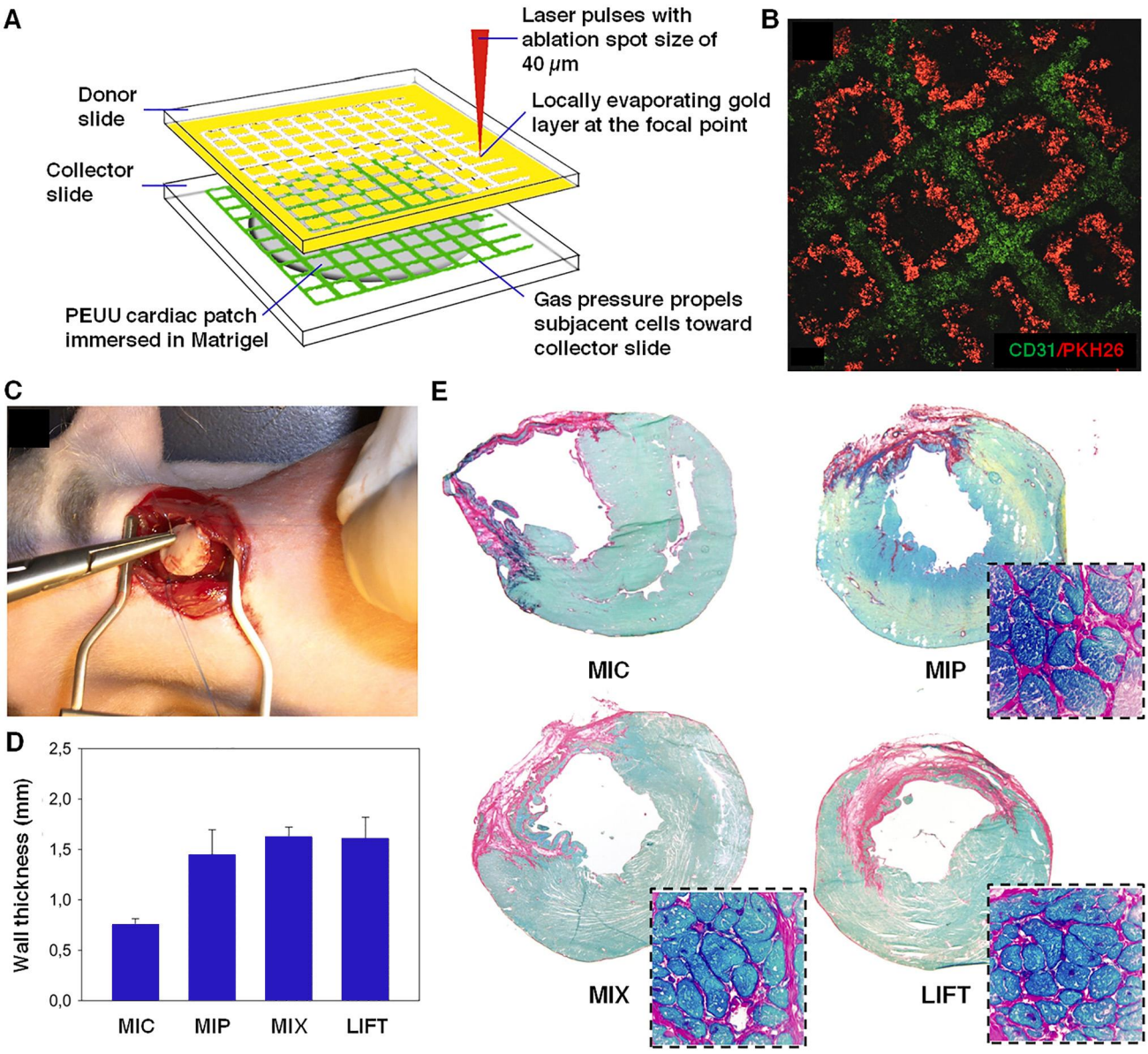

Fig. 2. (A) Schematic illustration of the LAB setup and the process of transferring cells on a PEUU cardiac patch. (B) Spatially organized human MSCs (PKH26+, red) and HUVEC (CD31+, green) were observed within grid patterns of cardiac patches after 24 h of printing. (C) *In vivo* implantation of bioprinted cardiac patch in rat's left anterior descending (LAD) ligation model. (D) Quantitative measurements of inner wall thickness in different groups: MIC (untreated LAD-ligation controls), MIP (LAD-ligation combined with untreated patch), MIX (patch with randomly seeded cell-co-culture), and LIFT (LAB-based cardiac patch with grid pattern). The inner wall thickness measurements showed no significant increase in the values for LIFT compared to MIP after 8 weeks of implantation. (E) Representative ventricular cross-sections from major infarcted regions in different groups. The sections were stained with Fast Green FCF and Sirius red for identifying the myocytes and collagen deposition respectively. The images highlighted in dotted square grids represent the Fast Green FCF and Sirius red-stained sections in border zone in different groups, indicating the extent of fibrosis. Reproduced with permission from [67]. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

an alginate-PEG-fibrinogen bioinks to construct cardiac-tissue structure through a customized bioprinter [53]. Three different geometries were employed by varying spatial organization of the two cell types: i) in the first type, both inks were extruded simultaneously with the same flow rate; ii) in the second, four layers of iPSC-CMs were alternated between two layers of HUVECs; iii) in the third, two layers of each cell type were alternated. Thanks to the printed geometry's high orientation, a well-structured cardiac tissue with blood-vessel-like structures were obtained (due to HUVECs) that enhanced integration with the host vascular system.

Liang et al. fabricated a living microscale 3D cell-laden structure, in which H9C2 rat myocardial cells exhibited uniform cellular distribution and high viability [75]. Their strategy incorporated an electrohydrodynamic printing platform, which had the advantage of high-resolution printing architectures by inducing electrohydrodynamic material flows. In another study, Colosi et al. developed a dispensing technique with a coaxial extrusion needle using a low-viscosity bioink to create highly organized 3D tissue constructs [76]. The strategy involved crosslinking of GelMA and alginate, encapsulation of HUVECs, simultaneous extrusion of $CaCl_2$ solution for the shell formation, followed by seeding of CMs. The resultant constructs yielded sufficient cell migration and synchronic beating of CMs.

Moreover, electroactive constructs have also been fabricated using EBB technologies. Ajdari et al. reported about conductive cardiac patches fabricated using nanocellulose, poly(glycerol sebacate) (PGS) and Ppy. The materials showed biocompatibility and cell proliferation

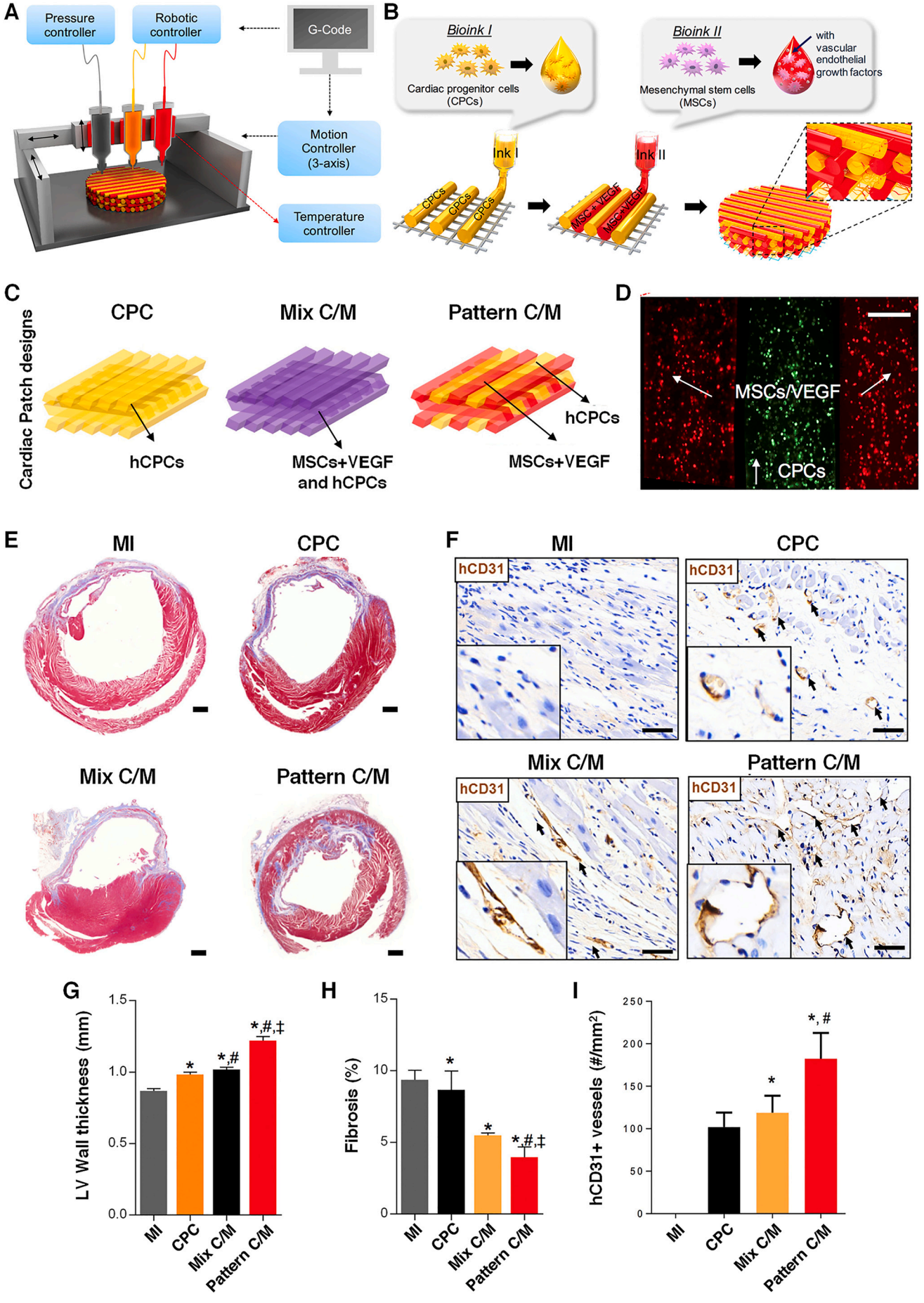

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Fig. 3. (A) Visual representation of the bioprinting process. (B) Demonstration of bioprinting process for a cardiac patch by using 2 different bioinks, deposited as alternate fiber strands on PCL based 3D printed support. (C) Illustration of various experimental variations included in the study: CPC (only CPCs), Mix C/M (randomly mixed CPCs, MSCs, and VEGFs) and Pattern C/M (patterning of CPCs-containing (bioink 1) and MSCs and VEGF-containing (bioink 2) strands, alternatively). (D) Alternate pattern design of CPCs and MSCs in the bioprinted cardiac patch fabricated on PCL support structures (Scale 200 μm). (E) Masson's Trichrome (MTC)-stained section of untreated MI group and those implanted with CPC, Mix C/M, and Pattern C/M patches (Scale 1 mm). (F) Immunohistochemistry-staining of infarcted regions in different groups against CD31. Assessment of (G) LV wall thickness and (H) fibrosis in various experimental groups after 8 weeks of implantation. (I) Quantification of vascularization in different groups, represented as number/mm^2 area. Reproduced with permission from [69].

promotion, as well as promising drug (3i-1000 and curcumin) release capability for long-term therapy in MI [56]. In a different work, Zhu et al. developed 3D constructs with CM-laden gold nanorods (GNR)-GelMA nanocomposite bioink using coaxial extrusion needle system [57]. The bioink (alginate + GNR-GelMA pre-polymers) was extruded through the internal needle and cross-linked with calcium chloride ($CaCl_2$), obtaining hydrogel microfibers. After layer-by-layer deposition of the microfibers, UV exposure was carried out to cross-link GelMA chains. The printed nanocomposite construct improved cell adhesion and synchronized contraction with respect to pure GelMA ones, thanks to the presence of GNR that enhanced electrical propagation between cells and promoted their functional phenotype.

Indirect EBB was also successfully utilized for bioprinting of vascular models [77]. As regard to CTE, Skylar-Scott et al. developed an interesting freeform printing method called Sacrificial Writing Into Functional Tissue (SWIFT) (Fig. 4) [78]. After creating spheroids from iPSC-CMs and fibroblasts, these were compacted (along with collagen-Matrigel solution) in a mold to create a living organoids matrix. Sacrificial ink was then printed inside this matrix, which allowed the formation of perfusable channels after its removal. This approach was further used to fabricate a cardiac tissue model, replicating the left anterior descending artery.

3.2.4. *Fused Deposition Modeling*

Fused Deposition Modeling (FDM) is a technique that allows the fabrication of an object using layer-by-layer extrusion of polymeric thermoplastic material (usually in the form of filament) through a heated nozzle [79]. FDM technology holds great potential for tissue engineering applications, although it delivers construct with a limited resolution, compared to previously cited techniques. Particularly, considering the fabrication of cardiac scaffolds, FDM allows obtaining highly customizable morphology and tunable mechanical properties that match the injured tissue. In a recent study, Yang et al. used PCL in combination with PGS to print 3D scaffolds [47]. PGS and PCL allowed tailoring of viscoelastic and mechanical properties, respectively, of the cardiac patch. Following an *in vivo* evaluation in the rat models with MI, PCL-PGS scaffolds showed improved cardiac functionality with positive impact on the left ventricle's remodeling. The authors also highlighted the versatility of these 3D printed structures and their great potential for treating cardiovascular diseases.

3.2.5. *Stereolithography (SLA) and digital light processing (DLP) technology*

SLA and DLP technologies are based on the polymerization of photo-cross-linkable materials (resins) using a light source (generally in the spectrum of UV light). The light source can be a laser or a projector that projects geometry to polymerize the resin and build the structure layer-by-layer selectively.

In the case of SLA (inverted setup), a build platform is lowered into the resin tank, leaving a thin layer of liquid between the platform and the bottom of the tank. A laser is directed by galvanometers through a transparent window at the bottom of the resin tank, drawing a cross-section of the 3D model and selectively polymerizing the material. Layer thickness can range from 25 μm to 100 μm. When a layer is complete, the part is peeled from the bottom of the tank, letting fresh resin flow beneath, and the platform is lowered once again. This process is repeated until the final structure is fabricated. Melhem et al. proposed an innovative cardiac patch based on MSCs-laden hydrogels with microchannels [48]. These channels with a controlled diameter (500–1000 μm) were obtained by a selective cross-linking of the solution using an SLA system. The introduction of microchannels reduced the number of cells required for cardiac regeneration and preventing fibrosis.

DLP is based, instead, on the projection of a mask. In this case, an entire layer (whose section is represented by the mask) is fabricated in a single exposure phase, and thus, printing time is reduced significantly. The image is generated through a digital micromirror device (DMD) where the user-defined patterns are consecutively loaded by turning on (UV light) and off mirrors [80]. Yu et al. used DLP to fabricate patient-specific tissue construct based on a photo-cross-linkable bioink from dECM (Fig. 5) [55]. Particularly, the authors showed that iPSC-CMs in structures bioprinted with dECM exhibited higher differentiated phenotype than those having collagen I as base matrix.

Among other DLP technology, Liu et al. employed the microscale continuous optical printing (μCOP), for developing biological structures for CTE applications in a layer-less fashion [52,81]. The μCOP is based on the continuous modulation, through the DMD, of 2D optical patterns and the simultaneous movements of the sample stage, containing a volume of the pre-polymer solution. This fabrication technology is well suited for processing cell-laden bio-inks, allowing controlled cell patterning, alignment, and concentration in the printed structure. Nevertheless, the use of a wide variety of photo-cross-linkable biomaterials is limited as there are a limited number of biocompatible photo-initiators [82]. There are two interesting applications of μCOP for CTE. The first study fabricated an *in vitro* model that mimicked the microarchitecture of the ventricular myocardium and its functionality [52]. In a second report, the researchers improved their model by adding a calcium monitor, which would benefit drug screening and cardiac tissue maturation analysis [81]. Moreover, different printing patterns were investigated by varying the design of the exposure mask: i) a simple slab, ii) lines, iii) grids, iv) dispersion and v) random. They showed that CMs aligned preferentially with the 3D printed architecture (lines pattern), and such bioprinted constructs produced higher contraction force than in case of other patterns.

Notably, due to the cytotoxic effects caused by currently available photoinitiators and UV light exposure, have recently motivated researchers to explore visible light-based photoinitiators or photoinitiator-free photopolymerization [83,84].

3.2.6. *Printing in suspension/support bath*

Another innovative form of EBB in trend employs suspension baths. The presence of suspension media possesses unique features that enable complete encapsulation and halt of fabricated material [85]. This specific technique allows reinforcing cell survival throughout the entire printing process and fabricating solid products without the existence of supporting materials for scaffolding [85]. Another advantage of the suspension bath extrusion system is that the rheological properties of bioinks have a reduced influence [86], which makes this technique more compatible with low-viscosity bioinks. Despite the advantage above, this approach holds a few weaknesses that restrain its repeatability and precision bioprinting, such as issues with the adhesion of continuous vertical layers, a blockage of the nozzle caused by the diffusion of the suspension media [86].

A widely known study that pioneered this technique was performed by Hinton et al. A 3D bioprinting technique named freeform reversible embedding of suspended hydrogel (FRESH) was developed to create

complex structures using consecutive hydrogel deposition in a thermo-reversible gelatin slurry support bath with the post-bioprinting temperature of 37 °C to ensure cell survival (Fig. 6) [87]. The developed system allowed bioprinting of most of the tissues and organs with convoluted shapes, including an embryonic heart. In another study, Lee et al. used the same method to accurately reproduce both a tree-leaflet valve and a heart miniature anatomical structure [42]. They showed how to 3D-bioprint collagen, controlling pH-driven gelation and obtaining a resolution up to 20 μm. The given porous microstructure supported rapid cellular infiltration and micro-vascularization, as well as mechanical strength for fabrication and perfusion of multiscale vasculature. Moreover, the cardiac structure seeded with CMs showed promising contractile activity. In another study, the FRESH method was also used to bioprint a full-size adult human heart using an alginate-based biomaterial [88]. The great potential of their research was proven by the high fidelity of the model obtained using a low-cost system, and the possibility to tune the mechanical properties and create a suturable tissue.

3.2.7. *Scaffold-free bioprinting*

All the techniques described above find a great number of applications for scaffold-based tissue engineering since there is always a hydrogel/biomaterial that gives structural support to cells for their maturation, migration, and proliferation. A scaffold-free approach, on the other hand, aims at fabricating artificial tissue or organs by directly printing living cells or spheroids into a pre-defined pattern. The advantage of not using a support biomaterial is that any inflammatory response due to the scaffold could be avoided and cells are able to differentiate immediately when introduced to a 3D environment [33]. Aspiration-assisted bioprinting [176], extrusion-based spheroid bioprinting [177], and the *Kenzan* [89] methods have been used to bioprint tissue spheroids, organoids and pre-defined building blocks in a scaffold-free manner.

Among different scaffold-free techniques, the *Kenzan* method is one of the most promising solutions to provide spheroids a spatial organization and the possibility to interact. After cells have been pre-assembled into spheroids, these are robotically inserted onto micro-needles (named "*Kenzan*") acting as a temporary support. *Kenzans* are

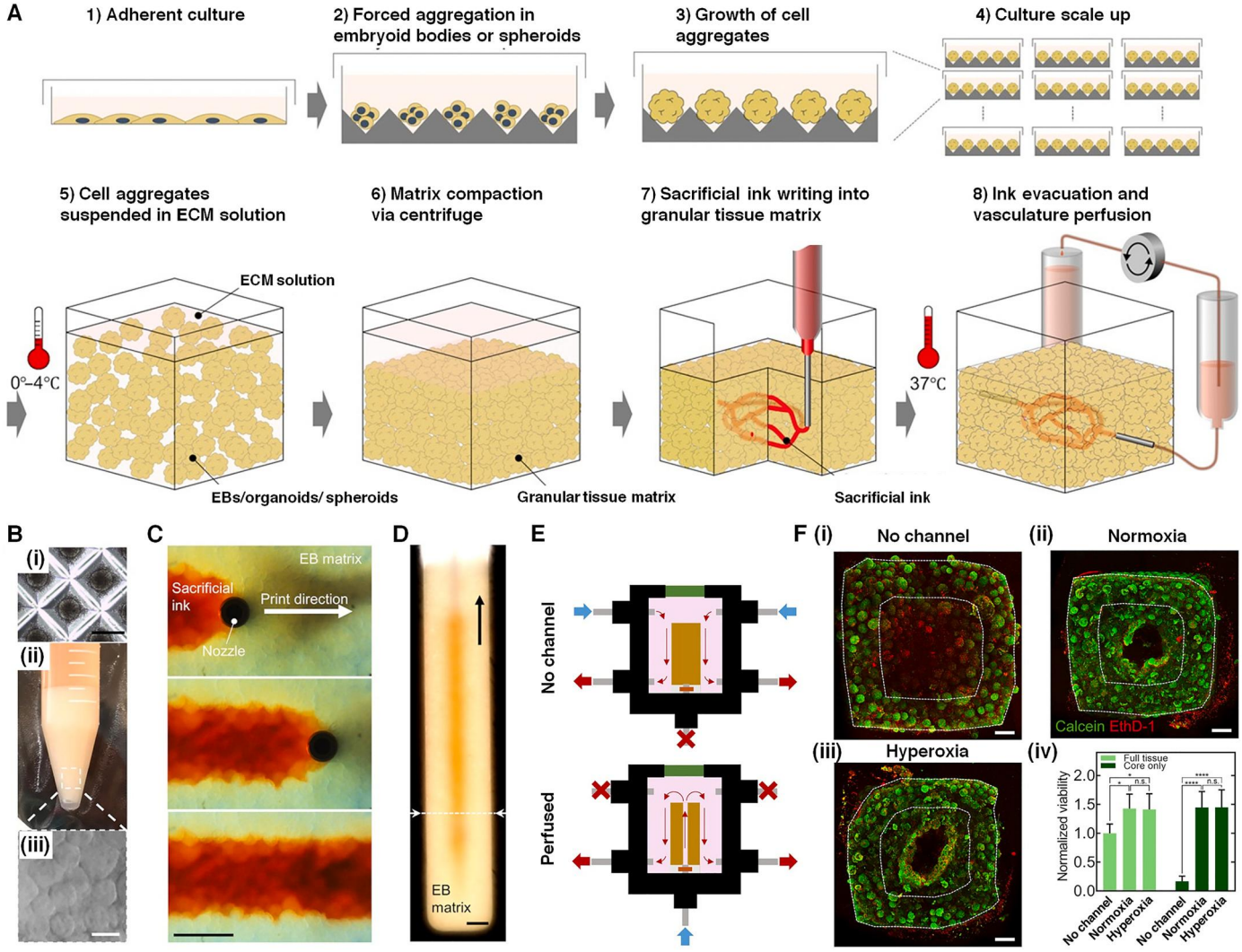


Fig. 4. (A) Schematic representation of the SWIFT process. (B) (i) Large-scale microwell culture of iPSCs (scale 300 μm), (ii) harvest of approximately 2.5 ml volume of embryoid bodies (EBs), (iii) and an image of the compacted form of EBs to from organ building block (OBB) tissue matrix (Scale 200 μm). (C) Time-lapse images of 3D printing of a sacrificial writing ink (orange) in an EB matrix (Scale 1 mm). (D) Sacrificial ink embedded by 3D printing in an EB matrix (Scale 1 mm). (E) Perfusion system employed to assess tissue viability of EB tissue matrix after SWIFT process. (F) Live/dead staining analysis carried out for the EB tissue matrix after 12 h of culture with: (i) no channel configuration (ii) normoxic (21% O_2) (iii) hyper-oxygenated (95% O_2) media (iv) quantification of normalized viability under different perfusion strategies in the full tissue (sea green) and core region (dark green), as indicated by grid lined rectangles in (i) (ii) and (iii) (scale 500 μm). Reproduced with permission from [78]. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

160 μm thick needles made of stainless steel, placed at 500 μm distance. Since spheroids have to come in contact, their diameter has to be in a precise range (400–600 μm). The efficiency of Kenzan bioprinting method is therefore related to the quality of spheroids assembly, and also requires a complex 3D bioprinter and vision system for an accurate manipulation [89,90]. The main disadvantage of this strategy is the need of spheroids within a specific diameter range to fit into the needle array, which is challenging and needs extensive optimization studies of cell type, densities, and culture duration [89,91].

In this regard, Arai et al. created a cardiac model by printing multicellular spheroids, consisting of iPSC-CMs, HUVEC, and dermal fibroblasts, onto a needle array to form a tubular construct [92]. Their

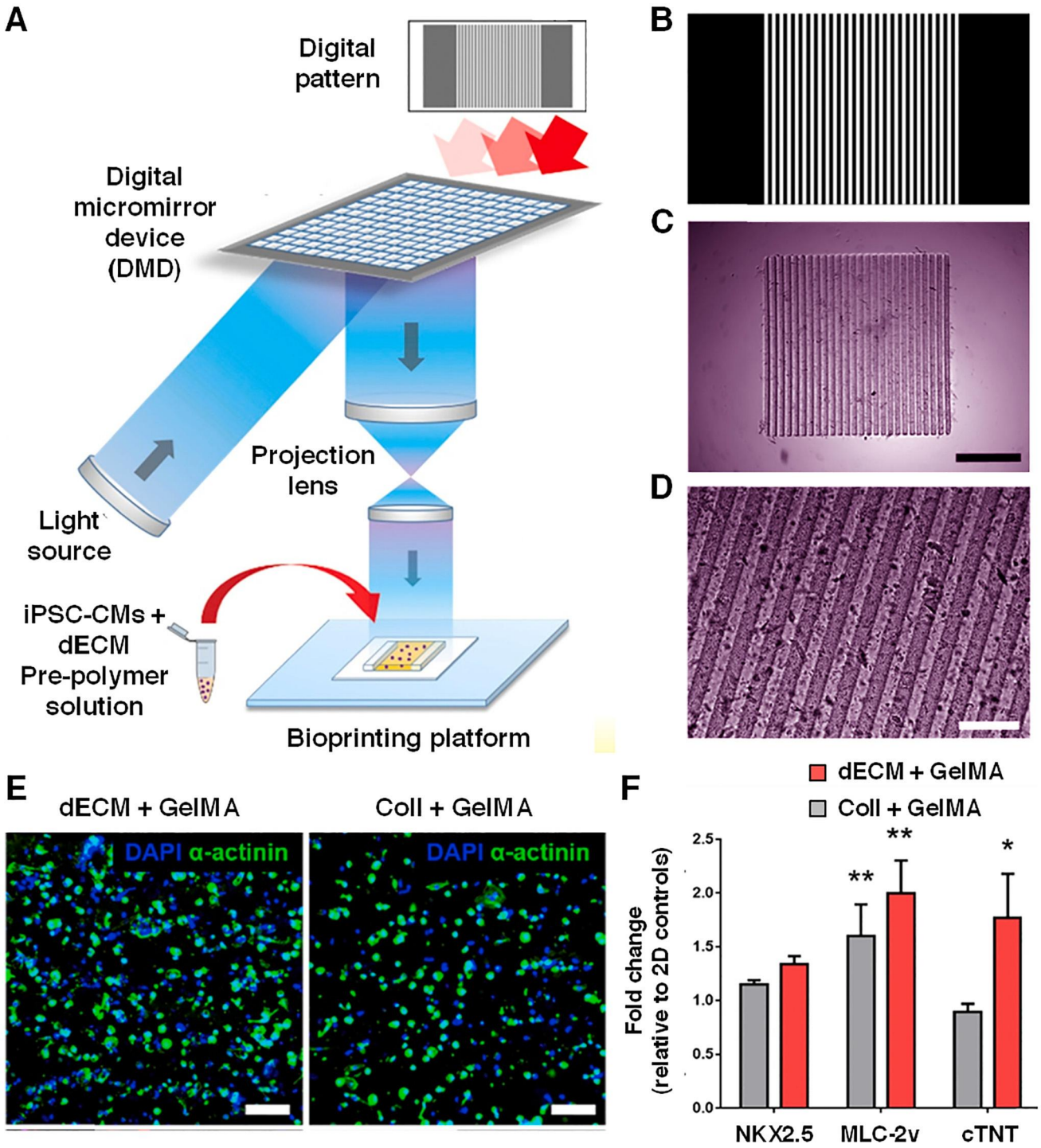


Fig. 5. (A) A schematic illustration of the overall bioprinting process. First step involved differentiation of iPSCs into iPSC-CMs, which are further formulated with cardiac tissue-specific photocrosslinkable dECM-based bioink. Digital files for 3D constructs to be printed are designed and given as input to a DLP-based bioprinters to print the microarchitecture constructs within seconds. (B) Pattern present in the digital file as input to the DLP-based bioprinter. (C) Gross morphology (scale 1 mm) and (D) microarchitecture (scale 200 μm) of the acellular bioprinted cardiac tissue construct by using DLP-based 3D bioprinter. (E) Immunohistochemical staining (α-actinin, actin) of cells in the constructs after 7 days of culture (scale 50 μm). Nucleus was stained with DAPI. (F) Gene expression profile cardiac specific markers (NK2 homeobox 5 (NKX2.5), myosin regulatory light chain 2 (MLC-2v), cardiac Troponin T (cTNT)) in the bioprinted constructs. Bioprinted collagen constructs were used as control for the study. Reproduced with permission from [55].

model was used to evaluate the drug response, in terms of contractile force and beating rate of the cardiac construct. On the same note, Ong et al. also bioprinted a functional cardiac patch using spheroids formed from iPSC-CMs, HUVEC, and adult ventricular cardiac fibroblasts with different aggregation ratios (Fig. 7) [35]. The fabricated patch exhibited good mechanical properties and spontaneous beating activity. Besides, engraftment and vascularization of the patch post-implantation in the rat native myocardium were the noticeable results of the study.

3.3. Stepping into the era of personalized medicines for cardiac therapy with bioprinting

PM aims at developing patient-specific therapies that, eventually, could allow the shift from a "one-size-fits-all" medicine to individual treatment [93]. Crucially, PM is the paradigm switch from the "reductionist" theory towards a more "holistic" (or integrative) view. The progress in "omics" field and bioinformatics is at the core of PM advancements. However, the latest discoveries in the biomedical field are opening an avenue to several new opportunities to improve PM [94]. So far, different materials and cell engineering strategies have been achieved. In particular, biosensors for monitoring the health status that performs fast diagnosis and precise drug release; reprogrammed immune-cells and iPSCs for cell-therapy respectively; bio-scaffolds or organs-on-a-chips suitable for drug discovery and testing are, at present, available and represent the newest healthcare tools to be applied to PM [95].

Patient-specific 3D printed models have also been introduced in cardiology and cardiac surgery, showing a pivotal role in the domain of heart disease and a valid option to overcome the problem of cardiac organ shortage. However, the generation of constructs that accurately mimic the anatomical vascularized structures remains the main CTE challenge. So far, cardiac stem cell therapy – in which stem cells are injected directly into the myocardium – has been developed to treat heart diseases and favor tissue regeneration [96,97]. However, several issues remain unaddressed, such as containment of stem cells in a specific area, the integration with the host tissues, and maintaining their survival.

In a recent study, Tal Dvir's group at Tel Aviv University 3D bioprinted fully personalized cardiac patches and hearts (Fig. 8) [45]. In particular, iPSCs were reprogrammed from patient biopsy and differentiated into CMs and ECs. Both cell lines were mixed separately with personalized bio-ink and 3D printed in a support bath to obtain cardiac patches. Interestingly, the authors demonstrated the capability to fabricate volumetric, freestanding, cellularized structures, including whole hearts and blood vessels. The authors have reported a method to 3D-print thick, vascularized, and perfusable cardiac patches compatible with the patient's immunologic, cellular, and anatomical features.

In the same milieu, scaffold-free bioprinting approaches also offer tremendous opportunities as patient-specific cells could directly be employed to develop multicellular spheroids and print them into a personalized patch/tissue using the Kenzan method – as discussed above. However, none of the studies, reported to date, have developed completely personalized cardiac constructs.

Nevertheless, several challenges remain to be addressed. In particular, efficient expansion protocol of stem cells still needs to be improved to fulfill all the stringent requirements for translation into the clinics. Moreover, *in vitro* long-term culturing protocols for the engineered tissue/organ are still missing. Finally, new strategies and advanced technologies to recapitulate the entire blood vessel network – particularly the small capillaries – remain an unmet issue that, until will remain unsolved, will limit the scalability of all tissue engineering strategies.

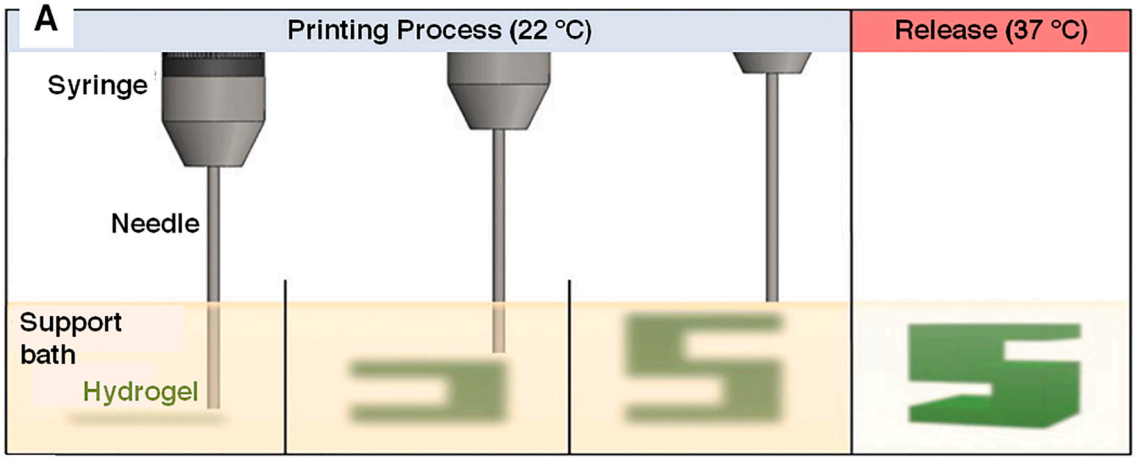
3.4. Exploring possibilities of cardiac regeneration with bioprinting at the defect site in vivo

Currently, *in vivo* application of bioprinted multiscale cardiac constructs is limited to surgical implantation. In this regard, recent developments aim to bioprint constructs directly at the defect tissues or organs onsite during a clinical process, a technology precisely termed as *in situ* bioprinting [98]. The first *in situ* bioprinting based on an inkjet bioprinting system was introduced approximately a decade ago [99]. This strategy is typically specialized in persistent patterning on intricate surfaces, while most of the traditional 3D bioprinting systems focused on the fabrication on a flat construct [100,101]. Based on its enormous potential in tissue and organ regeneration, various *in situ* bioprinting techniques have been developed for different tissue types. Current *in situ* bioprinting systems are mostly utilized for the repair of skin, cartilage, and bone defects with the two main technical approaches, hand-held and robotic arm [98,102–107,179]. In terms of biological perspective, *in situ* bioprinting of autologous cells has been considered a promising strategy as these cells can be directly obtained from the patients, thus avoiding negative immune responses [32]. For example, Hakimi et al. and Albanna et al. demonstrated the *in situ* bioprinting for skin repair with robotic arms and hand-held approach, respectively [102,103]. Especially in work done by Albanna et al., incorporating autologous skin cells with the *in situ* bioprinting technique resulted in significant time reduction for wound closure and repair [102]. Although these reported studies for *in situ* bioprinting using autologous cells were not directly associated with cardiac tissue regeneration, this approach possesses sufficient room to be suitable for the fabrication of cardiac patches. For instance, autologous cardiac cells can be substituted for the cells used in the studies above when performing CTE. More importantly, bioprinting of cardiac patches requires fabrication steps on intricate surfaces, for which this approach is specifically suitable. One of the major drawbacks of this strategy is the need to expose the application site. In this regard, advanced technologies of intravital bioprinting [108] and non-invasive *in vivo* bioprinting [109] have been introduced, recently. Both technologies allow the fabrication of tissue-like constructs using a non-invasive approach, based on the capability of Near-infrared (NIR) light to penetrate into deep tissues. In the first, a photosensitive polymer solution is injected in the target anatomical site and successively polymerized through a two-photon excitation ($700\ nm < \lambda < 850\ nm$), obtaining a 3D structure. Similarly, in the second, a photosensitive bio-ink is injected beneath the tissue and is cross-linked layer-by-layer using a NIR light ($\lambda = 980\ nm$) and a DMD chip.

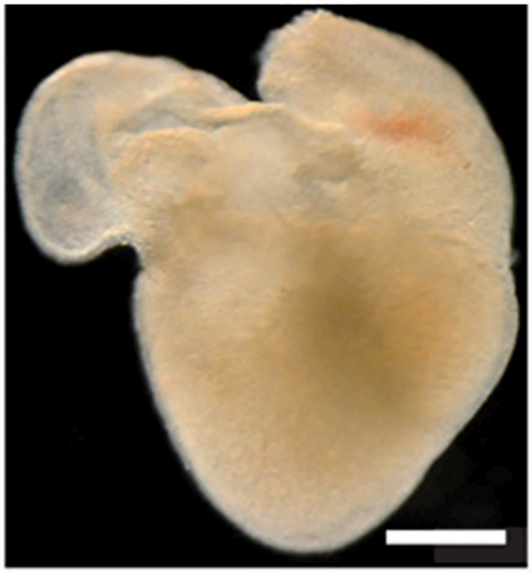
4. Advancing into four-dimensional (4D) printing for cardiac regeneration

4D printing, in which the fourth dimension of 'time' has been integrated with 3D printing, has recently emerged as a future solution in tissue engineering [120]. It presents the possibility of creating complex and functional architectures or replicating much-required dynamic physiological microenvironment of tissue remodeling and addressing the limitations of the static nature of 3D bioprinting [121,122]. 4D printed objects are made of stimuli-responsive materials (SRMs), or by integrating transformation information into the initial structural design. They are capable of dynamically change their shapes, physical properties, or functionalities with time, when subjected to different stimuli or when cellular self-assembly occurs [120,123–125].

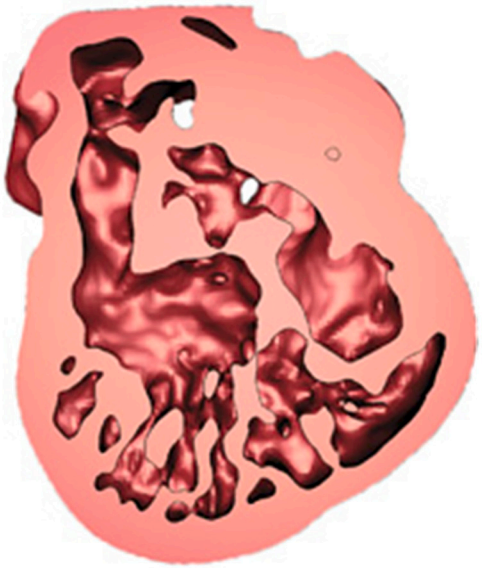
Many expertise are needed to be joined to fabricate a 4D printed structure: 1) adequate stimulus to change structure shape or functions, 2) printing technologies and optimally equipped facility, 3) mathematical and theoretical studies to predict physical or chemical behavior of the materials, and 4) integration of SRMs or anisotropic structural design [124,126,127]. SRMs employed in 4D printing can be sensitive to changes in external (e.g. magnetic and electric fields, temperature, acoustic waves, and light) or internal triggers (e.g. enzymes, glucose, microenvironment pH, ionic strength) [128]. Thus, the material has to be appropriately selected to ensure transformation over time once the stimulus is applied [125,129]. Currently, the most common SRMs for 4D printing are shape-memory materials (SMMs) due to their ability to



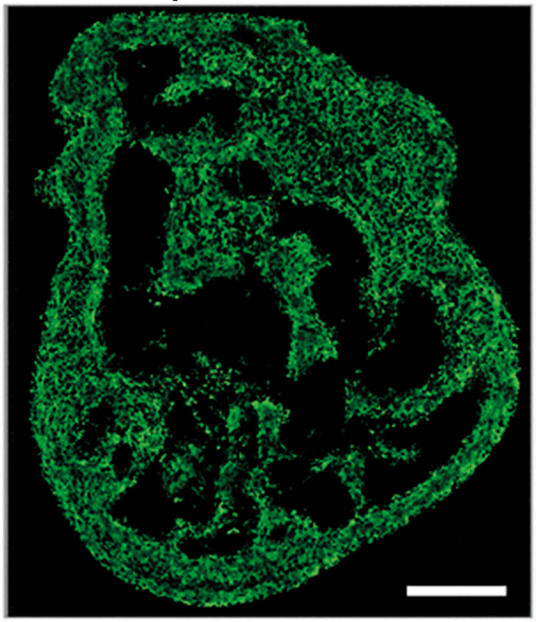
B Explanted embryonic chick heart



C 3D CAD model of embryonic chick heart



D Cross section of the 3D printed heart



E 3D printed heart with internal structure

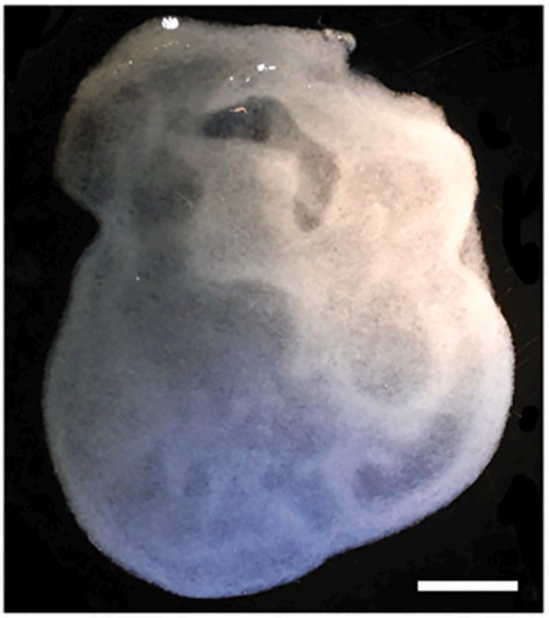


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Fig. 6. (A) A schematic illustration of FRESH process of bioprinting. Hydrogel (green) is deposited and crosslinked within thermo-reversible gelatin support bath (yellow) at 22 °C. Post-printing the temperature was raised to 37 °C to release the final structure and melt gelatin. (B) Representative dark-field image of an embryonic chick heart explant (scale 1 mm). (C) 3D CAD model of a cross section of the embryonic heart with internal trabecular features, generated based on confocal imaging data. (D) Representative image of cross section of the bioprinted heart with fluorescent alginate (green), with clear visualization of internal trabecular features of the 3D CAD model, the 3D CAD model of heart was scaled by factor of 10 to meet the printing resolution of the printer (scale 1 cm). (E) A dark-field image of the translucent bioprinted heart (scale 1 cm). Reproduced with permission from [87]. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

memorize and restore their original shape in response to specific internal or external triggers. SMMs could further be classified into shape memory polymers (SMPs), shape memory alloys (SMAs), shape memory composites (SMCs), and shape memory hybrids (SMHs) [124]. Among them, SMPs and SMAs are the most used in biomedical applications. In particular, SMPs exhibit advantageous properties such as biodegradability, biocompatibility, recoverable deformation and high strength to weight ratio [130].

As regard to 4D printing of the myocardium, it is still at the early stages, but there are published works that provide crucial insights. Miao et al. fabricated innovative 4D organized micropatterns by photolithographic-stereolithographic-tandem strategy (PSTS) with smart soybean oil epoxidized acrylate (SOEA) inks to obtain a 4D layered patch, supporting culture, and cardiomyogenic differentiation of human bone marrow MSCs. The authors observed that PSTS flat sheet scaffold changed its shape, autonomously bending or self-assembling upon immersion in ethanol, performing the 4D effect. By simply adjusting the thickness of the sheet, it was possible to precisely control their bending, thereby allowing them to obtain various curvatures to substitute damaged tissues or organs [131].

In a different study, Cui et al. developed a 4D physiologically adaptable cardiac patch using beam-scanning SLA with GelMA-PEGDA bioink [132]. After printing, the patch could switch from a 3D flat pattern (wavy microarchitecture) to 4D self-morphing curved (mesh-like microarchitecture) shape, following diastolic and systolic phases of the cardiac cycle. *In vitro* evaluation of constructs tricultured with iPSC-CMs, ECs, and MSCs revealed an evident variability in cellular phenotype as a function of microarchitectural wavy/mesh patterns (Fig. 9). Long-term studies in murine chronic MI model showed enhanced cellular engraftment and vascularization at the patch implantation site. This advanced 4D dynamic feature displays excellent potential for CTE and organ regeneration applications.

Another study, in this regard, was presented by Lind et al., who made significant progress in mimicking the cardiac microphysiological system using multimaterial 3D printed devices. They designed six functional inks, based on biocompatible soft materials having piezo-resistive and high-conductance characteristics, enabling the integration of non-invasive sensors within the microstructures that guided cardiac cells self-assembly. These sensors provided quantitative and continuous electronic readouts of cardiac micro-tissue contractions. The devices were even applied to study a series of drug responses and the contractile development of human cardiac tissues [133].

However, because of the early nature of 4D printing of tissues, new research and technological improvements need to be developed. Currently, there are only a few SMMs that are contemporarily highly biocompatible and suitable for 4D printing [130]. In particular, there is a need to develop new SRMs, offering multi-stimuli or a reversible response to replicate continuous contraction and elongation activities of the native heart. Another possible limitation in 4D printing is related to the presence of the internal or external stimuli, generating a response from the material and that may damage or kill living cells. Thus, the stimulus must be opportunely titrated to limit unintended tissue damages [134].

Recently, Cui et al. developed novel NIR-responsive 4D printed constructs [135]. The light-switch stimulation enabled a remote, meticulous, and dynamic spatial-time control. Especially, long-wavelength NIR is considered as a human benign energy form, which is capable of adequately passing through the targeted tissue with no biological damage, compared to other energy sources. Similarly, Wang et al. also fabricated NIR-responsive 4D cardiac structures with adjustable curvature design using the DLP-based printing technique. In order to mimic the native aligned architecture of the human myocardium, such as microgroove arrays and curved structure, they synthesized and utilized a NIR-sensitive bioink with graphene and PEGDA [136]. As a result, the fabricated 4D constructs exhibited an even distribution of seeded cells and myocardial lineage development without cellular or tissue damage.

5. Current cellular opportunities in CTE

For tissue engineering applications, cellular components are also a prerequisite [137]. A wide range of studies have already evidenced faster and efficient regenerative outcomes in the case of cell-laden tissue constructs than the acellular ones [138]. Selection of an ideal cell source is of particular importance to attain effective therapeutic outcomes and must fulfill several requirements: (1) the capacity of fast proliferation and easy differentiation/maturation into a target cell type, (2) easy accessibility to the cell source and autologous origin preferably, and (3) non-antigenicity [30]. In context of cardiac tissue, native CMs, progenitor, and stem cells are the most potent candidates.

CMs, native cell population of cardiac tissue, have been used in various studies pertaining to cardiac regeneration. However, low availability of the cell source, particularly the autologous sources, and limited proliferative capability of the isolated CMs, highly limit their translational ability [139]. Though, there are growing evidence that the CMs may undergo dedifferentiation and recover their proliferative phenotype [140]. Besides, current research thrust is on trans-differentiation of the cardiac fibroblasts, direct or *via* CPCs intermediate state, to the CMs [141]. Various strategies (transduction/transfection-, microRNA-, and small molecule-mediated) have been reported for direct trans-differentiation to the CMs, as summarized recently by Monaghan et al. [141]. But still these aspects are in R&D phase and not yet established for clinical application.

Besides this, the stem cells also offer promising prospects for CTE. In that context, multiple types of stem cells, including embryonic stem cells (ESCs), iPSCs, and adult stem cells (ASCs), retaining flexible degrees of self-renewal and differentiation ability, have been recognized suitable [142,143]. Each of these cells has their own advantages and disadvantages.

ESCs and iPSCs, available pluripotent stem cells (PSCs), are highly proliferative and are capable of differentiating into CMs [144–146]. ESCs are derived from the embryos, while iPSCs are induced artificially from the somatic cells. Different strategies for directed differentiation of PSCs to CMs have recently been reviewed by Jiang et al. [147]. However, practical applicability of PSC-CMs is mainly limited by their immature phenotype that is characterized by short and less organized sarcomere, absence of transverse tubules (T-tubules), reduced contractibility, altered metabolic, and electrophysiological properties, as compared adult CMs [148]. Besides, these cell sources are still associated with prevailing ethical and other safety concerns.

MSCs, a type of ASCs, are relatively safer candidates than PSCs and have witnessed a wide applicability in cardiac regenerative therapies, either directly or post-differentiation into CMs [149–151]. MSCs have been shown to exert anti-apoptotic and anti-fibrotic effects, promote neovascularization, and regulate immune responses at the injured cardiac tissue post-implantation [152]. Moreover, there are evidence

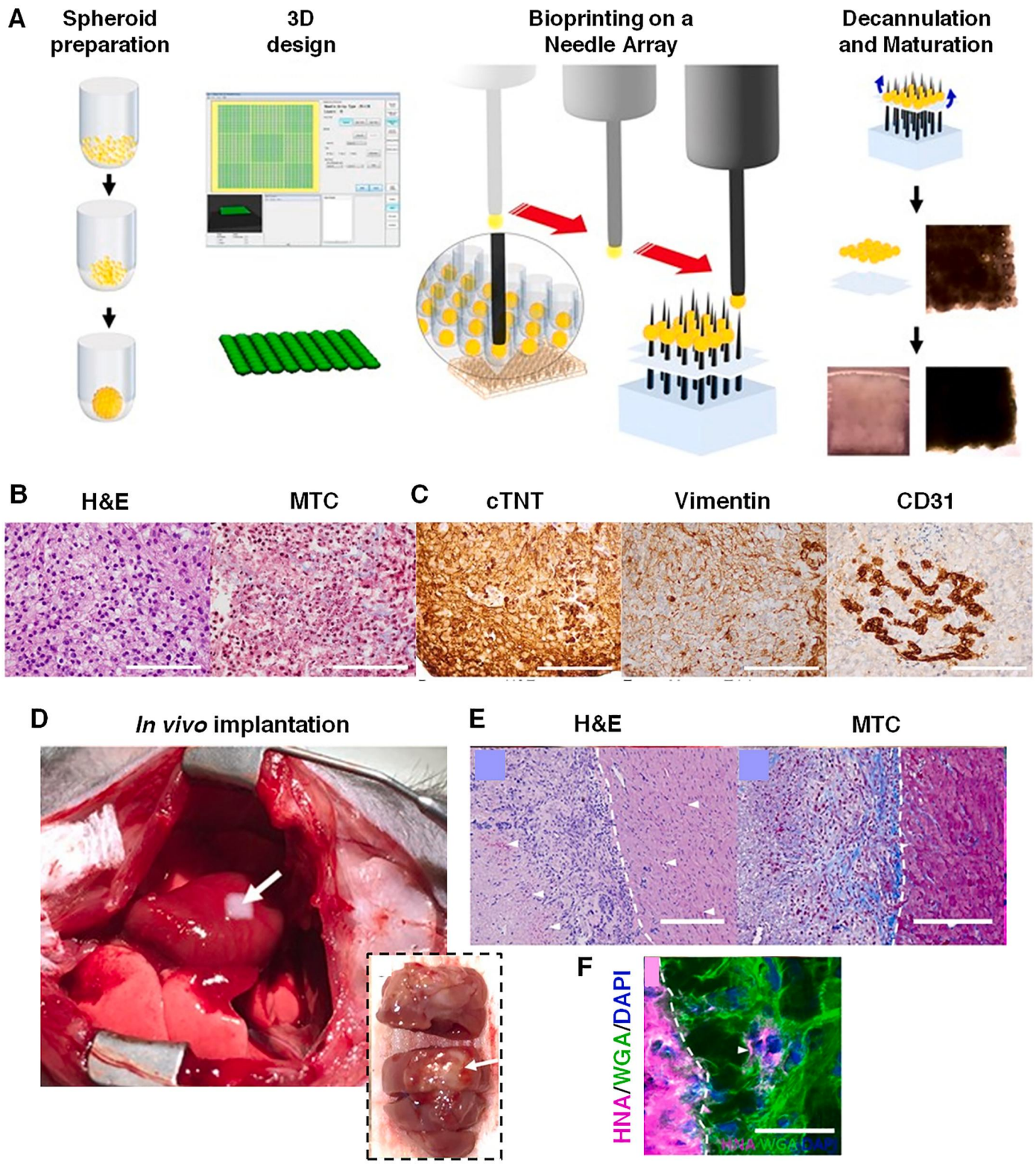

Fig. 7. (A) Schematic representation of Kenzan method of scaffold-free bioprinting for the fabrication of 3D cardiac patch. First step involved the formation of cardiac spheroids in ultra-low attachment 96 well plates. Next the design of the 3D construct is prepared and provided to a 3D bioprinter. The 3D bioprinter picks up a cardiac spheroid and places onto a needle array. The cardiac cell spheroids are allowed to fuse to form 3D bioprinted cardiac patch. The 3D cardiac patches are removed and cultured to promote maturation. (B) Histological analysis of 3D bioprinted cardiac constructs: Hematoxylin and eosin staining (H&E) and MTC staining (scale 100 μm). (C) Immunohistochemistry analysis of 3D bioprinted cardiac constructs cTNT, vimentin, and CD31 (scale 100 μm). (D) *In vivo* implantation of 3D bioprinted cardiac construct on anterior surface of rat heart. The highlighted image within dashed lines represents the anterior aspect of the explanted heart. (E) Histological analysis of the implanted 3D bioprinted cardiac construct by H&E and MTC staining (scale 400 μm). (F) Immunohistology analysis of implanted bioprinted constructs stained for human nuclear antigen (HNA), Wheat Germ Agglutinin (WGA), and DAPI (scale 40 μm). The dotted line indicates the border between native rat myocardium and 3D bioprinted cardiac construct. White arrow indicates the integration of the bioprinted cardiac construct with the native rat myocardium. Reproduced with permission from [35].

regarding trans-differentiation potency of MSCs into CMs *in vivo* [153] but there also exists other reports with contradictory results [154,155]. As regard to *in vitro* conditions, MSCs also hold high proliferative capacity and potent cardiogenic differentiation ability upon exposure to proper cardio-inductive conditions [151,156,157]. MSCs could even impart mature phenotype in iPSC-CMs, in terms of structural organization, cell-cell communication, and contractile behavior, upon direct/indirect coculture *via* secretion of soluble factors like cytokines and exosomes [158]. Though, MSCs have proven quite effective, but variability in their source of isolation (such as bone marrow, umbilical cord, amniotic fluid, and adipose tissue) and culture conditions, may largely impart differences in their therapeutic efficacy, differentiation potential, and immunoregulatory effects [156].

Off late, resident cardiac stem cells (CSCs), which are tissue-specific progenitor cells, have been traditionally used to preserve myocardial cell homeostasis [142,159]. Similar to the other stem cells used in CTE, CSCs have the capability to differentiate into myocytes, vascular ECs, and smooth muscle cells *in vitro*, *in vivo*, and *ex vivo* [30,159–161].

Besides the direct application of cells, exosomes (cell-derived extracellular vesicles) have also demonstrated promising therapeutic effects, post-cardiac injury [162]. Exosomes are endosomal in origin and are released by multitude of cells ranging from adult cardiac cells (*e.g.*, cardiac fibroblasts, CMs, cardiac ECs, epicardial adipocytes, and cardiac telocytes) to stem/progenitor cells (*e.g.*, CPCs, MSCs, iPSCs) [163–165]. These exosomes are involved in variety of cellular processes associated with cardiac tissue, including cell migration, proliferation, angiogenesis, apoptosis, hypertrophy, fibrosis, and even repair and regeneration [166]. Such an effect exerted by exosomes is primarily due to a myriad of biological molecules including nucleic acids (DNA, mRNA, miRNAs), proteins (heat shock proteins, enzymes, transcription factors), amino acids, and lipids, contained within [162,164]. Nevertheless, the type of biomolecules and their directed function is highly dependent upon the physiological and pathological states of the secreting and target cell type [167,168]. These exosomes could also be engineered to improve their functional aspects or improving their targeting capability [169–171].

As regard to delivering these exosomes to the injured tissue site, injection *via* intracoronary/intramyocardial/intravenous routes delivery is usually performed (for details refer to Appendix section S2) [162]. Another strategy is to encapsulate the exosomes in the engineered cardiac constructs [172,173]. In the milieu, exosomes could also be added to bioink formulations to fabricate spatially-defined bioactive cardiac constructs using 3D printing technologies. To date, none of the study has integrated 3D printing and exosomes in context of CTE but, a proof-of-concept was recently presented for osteochondral tissue regeneration [174] and neovascularization [175]. Both the studies confirmed that exosome-incorporated bioprinted constructs positively influenced regenerative outcomes and thus this strategy holds promising prospects for future development.

6. Conclusion and outlook

Although many anatomical in-depth studies have been carried out to unravel the architecture of the heart, explicitly long-term sustainable cardiac tissue regeneration approaches and development are still somewhat questionable. These drawbacks are due to the heart's complex structure. The techniques to regenerate it rely on many uncertain factors including the biocompatible scaffold material selection, scaffold fabrication method, transplantation of scaffold *in vivo*, cell selection, and cell cultivation *in vitro*. During the past decades, researchers from diverse fields have put much effort into the development of more feasible and sustainable methods of biophysical and biochemical CTE. Among the previously performed studies in the field, many of them have made a level of achievement to fabricate either partial cardiac tissues or an entire heart artificially. Currently, many researchers from diverse fields put their effort towards improving the bioprinting technique in CTE. As a result, the aforementioned advanced bioprinting systems have been invented and developed.

To date, we are still far from reaching an acceptable quality of engineered constructs, and some critical issues remain unmet. Providing a proper microenvironment for cell culture, effective cell differentiation/tissue maturation protocols, the integration of a functional blood vessels network, and obtaining a suitable overall mechanical stability represent the key challenges to be overcome. Future studies should also focus on enhancing the patient-specific compatibility and *in vivo* engraftment of the patches/artificial organs. The combination of better tissue/organ defect 3D reconstruction – using the modern non-invasive *in vivo* imaging techniques to generate CAD models – and novel differentiation protocols for multi-potent cells – to fully eliminate the risk of tumorogenesis and the development of arrhythmias – will be the roadmap to follow. Further, the development of innovative biomaterials with novel mechanical properties, high levels of biocompatibility, and dynamic behavior should be mandatory to sustain the printed architectures and to promote vascularization and innervation. Another important aspect that needs consideration is overcoming hypoxia related issues that may consequently affect overall therapeutic efficiencies of the cardiac construct. In this regard, recent research efforts have been directed towards incorporating oxygen-releasing materials, thereby presenting scope for further exploration [11].

As pointed out in the text, addressing the aforementioned challenges will undoubtedly require the use of advanced bioreactors which should be optimized to provide the needed multi-scale support to the fabricated engineered cardiac constructs. So far, the number of commercial bioreactors for CTE is still limited (refer to Appendix section S3), and thus today researchers have no choice than developing customized systems depending on their specific needs. Despite valuable proofs-of-concept, such systems are envisioned for lab-scale, pilot experiments lacking of standardization, advanced automation, and integration of useful sensors for live monitoring of key parameters.

Last but not least, it is worth mentioning that the whole process should be addressed in agreement to the existing and future legal regulations and ethical guidelines from international agencies/authorities. In fact, biofabrication techniques are not fully regulated, highlighting the necessity to start discussing, developing, and eventually adopting detailed guidelines for the fabrication of such advanced products.

Abbreviations

2D	two dimensional
3D	three dimensional
4D	four dimensional
CMs	cardiomyocytes
ECM	extracellular matrix
CTE	cardiac tissue engineering
AM	Additive Manufacturing
ECs	endothelial cells
PEG	polyethylene glycol
PCL	polycaprolactone
SLA	stereolithography
DLP	digital light processing
UV	ultraviolet
dECM	decellularized extracellular matrix
μCOP	microscale continuous optical printing
DMD	digital micromirror
iPSCs	induced pluripotent stem cells
iPSC-CMs	iPSC-derived CMs
GelMA	gelatin methacrylate
IBB	inkjet-based bioprinting
EBB	extrusion-based bioprinting
LAB	laser-assisted bioprinting
MI	myocardial infarction
PEUU	polyester urethane urea
CPCs	cardiac progenitor cells

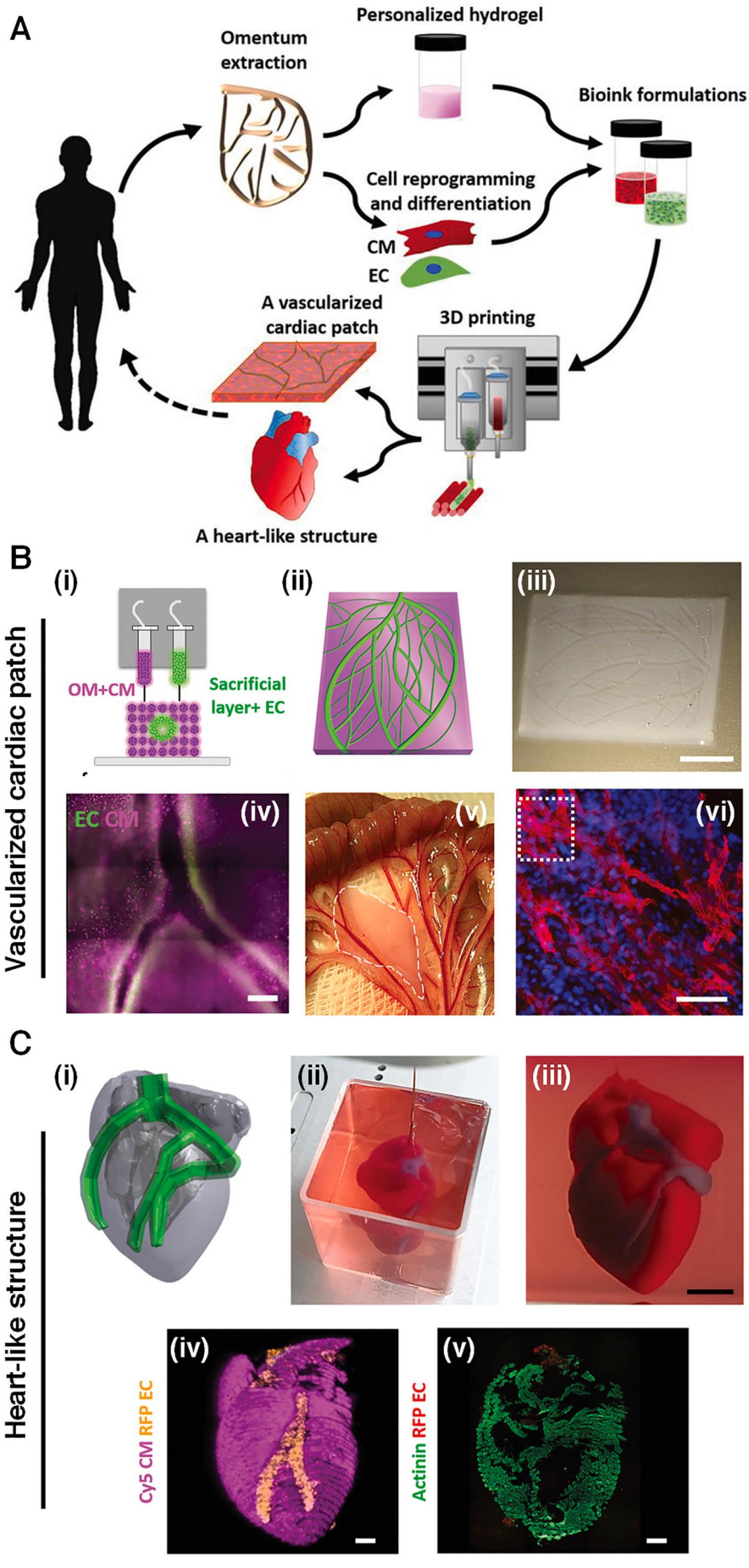

Fig. 8. (A) Schematic workflow applied by Noor and colleagues. Briefly, after biopsy, personalized hydrogels and iPSCs – differentiated into CMs and ECs – were used to produce bioinks. The bioinks were then 3D bioprinted to generate vascularized patches or complex scaffolds suitable to be transplanted back into the patient. (B) 3D personalized cardiac patches. (i) Schematic printing concept (ii) A digital model of the cardiac vascularized patch. (iii) 3D printed cardiac patch, where (iv) the blood vessels (CD31 in green) are closer to the cardiac tissue (actinin in pink). (v) *In vivo* cardiac implant. (vi) Sarcomeric actinin (red) and nuclei (blue) staining of sections from the explanted patch. (C) Representative image of the heart. (i) CAD model of the heart. (ii, iii) The printed heart within a support bath. (iv) The confocal image of the printed heart (CMs in magenta, ECs in orange) and (v) the cross-sections of the heart stained against sarcomeric actinin (green) and ECs (red). Reproduced with permission from reference [45]. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

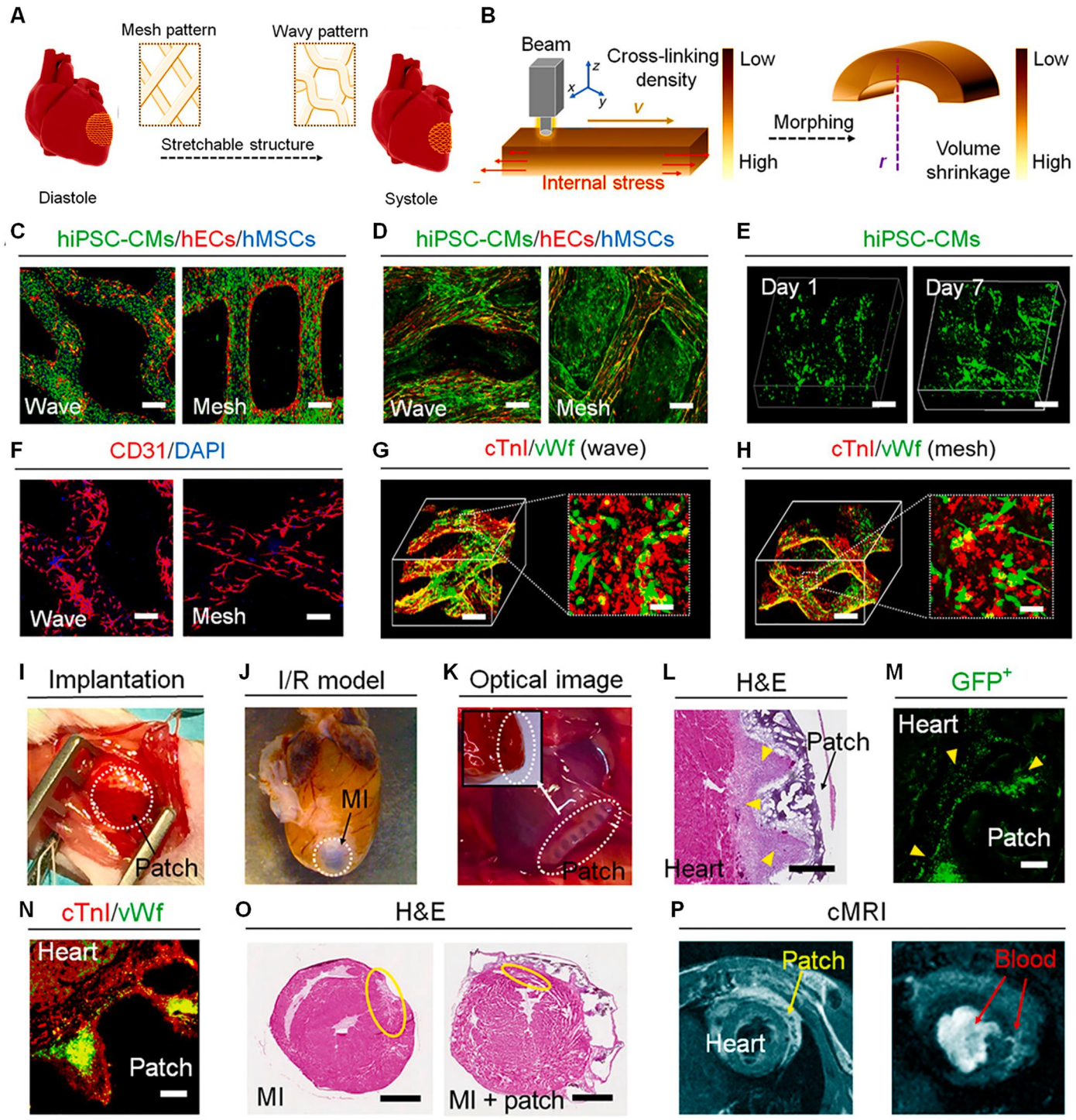

Fig. 9. *In vitro* characterization of the fabricated cardiac patches using coculture of iPSC-CMs, ECs, and MSCs with different patterns and *in vivo* long-term evaluation of the fabricated cardiac patches. (A) CAD model of 3D stretchable construct. (B) A schematic illustration of internal stress-induced morphing mechanism. (C) 1 day and (D) 7 days of cell culture (Scale: 200 μm). (E) Confocal microscope images of green fluorescent protein-transfected iPSC-CMs after 7 days (Scale: 100 μm). (F) Immunostaining images of EC distribution to indicate capillary formation using CD31 on the fabricated cardiac patches (Scale: 200 μm). Immunostaining images of cardiac Troponin I (cTnI, red) and von Willebrand factor (vWf, green) on (G) wave-patterned and (H) mesh-patterned cardiac patches. (I) Implantation of the cardiac patch into the mouse heart. (J) An optical image of a heart model with I/R MI after 4 months. (K) An optical image of the cellularized cardiac patch after 3 weeks of implantation. (L) H&E assessment image of the cellularized cardiac patch after 3 weeks, where yellow arrows indicate dense cell clusters (Scale 400 μm). (M) A confocal microscope image of green fluorescent protein-transfected iPSC-CMs after 3 weeks, where yellow arrows indicate high engraftment (Scale 100 μm). (N) Immunostaining image of cTnI (red) and vWf (green) of the cellularized cardiac patch after 3 weeks (Scale 100 μm). (O) H&E assessment image of the original MI heart (left) and the heart with the cardiac patch after 10 weeks (right). The yellow circles indicate infarction (Scale 800 μm). (P) Cardiac magnetic resonance images (cMRI) of the heart with the cardiac patch after 10 weeks. The locations of the heart and the patch are indicated (left), and the blood perfusion is highlighted (right). Reproduced with permission from [132]. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

MSCs	mesenchymal stem cells
VEGF	vascular endothelial growth factor
HUVECs	human umbilical cord-derived endothelial cells
PGS	poly(glycerol sebacate)
GNR	gold nanorods
SWIFT	Sacrificial Writing Into Functional Tissue
FDM	Fused Deposition Modeling
FRESH	freeform reversible embedding of suspended hydrogel
CAD	computer aided drawing
ADSCs	human adipose stem cells
PM	personalized medicine
SRMs	stimuli-responsive materials
SMPs	shape memory polymers
SMAs	shape memory alloys
SMCs	shape memory composites
SMHs	shape memory hybrids
PSTS	photolithographic-stereolithographic-tandem strategy
SOEA	soybean oil epoxidized acrylate
NIR	near-infrared light
ESCs	embryonic stem cells
ASCs	adult stem cells
PSCs	pluripotent stem cells
CSCs	cardiac stem cells
PPy	polypyrrole
PEDOT	poly(3,4-ethylenedioxythiophene)
PANi	polyaniline

CRediT authorship contribution statement

Tarun Agarwal: Conceptualization, Writing–Original draft, Writing–Reviewing and Editing; Gabriele Maria Fortunato: Writing–Original draft, Writing–Reviewing and Editing; Sung Yun Hann: Writing–Original draft, Writing–Reviewing and Editing; Kiran Yellappa Vajanthri: Writing–Original draft (supplementary file); Bugra Ayan: Writing–Original draft (supplementary file); Dario Presutti: Writing–Original draft; Haitao Cui: Writing–Original draft (supplementary file); Alex H-P Chan: Writing–Original draft (supplementary file); Marco Costantini: Writing–Reviewing and Editing; Valentina Onesto: Writing–Original draft; Concetta Di Natale: Writing–Original draft (supplementary file); Ngan F. Huang: Writing–Reviewing and Editing; Pooyan Makvandi: Writing–Reviewing and Editing; Majid Shabani: Writing–Reviewing and Editing; Lijie Grace Zhang: Writing–Reviewing and Editing; Carmelo De Maria: Writing–Reviewing and Editing; Tapas Kumar Maiti: Writing–Reviewing and Editing.

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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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.msec.2021.112057.

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