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Strategies for directing cells into building functional hearts and parts

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The increasing population of patients with heart disease and the limited availability of organs for transplantation have encouraged multiple strategies to fabricate healthy implantable cardiac tissues. One of the main challenges in cardiac tissue engineering is to direct cell behaviors to form functional three-dimensional (3D) biomimetic constructs. This article provides a brief review on various cell sources used in cardiac tissue engineering and highlights the effect of scaffold-based signals such as topographical and biochemical cues and stiffness. Then, conventional and novel micro-engineered bioreactors for the development of functional cardiac tissues will be explained. Bioreactor-based signals including mechanical and electrical cues to control cardiac cell behavior will also be elaborated in detail. Finally, the application of computational fluid dynamics to design suitable bioreactors will be discussed. This review presents the current state-of-the-art, emerging directions and future trends that critically appraise the concepts involved in various approaches to direct cells for building functional hearts and heart parts.

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1. Introduction

As one of the main causes of death and disability in the world, cardiovascular disease usually evokes the obstruction of blood flow and create oxygen deficiency in the heart muscles.[1–3] After myocardial infarction, also known as heart attack, the cardiac tissue undergoes a series of changes in its structures and function (called destructive cardiac remodeling), which are indicated by ventricular wall thinning and chamber dilatation.[4] Cardiac remodeling is a multifactorial procedure persisting for many years, influencing the structure of the whole heart, including infarct, border, and non-infarct areas.[5] From the other side, heart is not able to prevent the progress of cardiac remodeling and regenerate itself fully, especially in adulthood.[6,7]

For patients with mild symptoms of heart failure, pharmacological therapies are always applied to reduce work load and protect the heart from toxic humoral factors.[8,9] For patients with marked symptoms, implantations of pacing devices and controlled electrical/mechanical asynchronous strategies have been conventionally used. However, the implantation of these devices cannot adequately limit the disease progression towards the final stages.[10,11] As the only curative treatment for end-stage heart failure, heart transplantation also has some problems such as the limited number of organ donors and immune system rejection.[12–14]

As another strategy, cell delivery into the patient's heart muscle *via* direct injection of cardiac cells was recently applied.[15–17] However, it was reported that the success of this approach is limited because of a high rate of cell death as well as lack of control over the cell accumulation site after the injection.[18,19] Thus, it is necessary to develop new treatment strategies to overcome the problems associated with pharmacological and surgical treatments for heart diseases.[20]

Tissue engineering, as an interdisciplinary field and a fast growing area of research, provides new techniques to create

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functional constructs for the replacement of damaged tissues.[21] Developing a fully functional cardiac tissue requires the cooperative presence of biomaterials, biologically active molecules, and signals to assemble a 3D biological construct.[22] An engineered functional cardiac tissue requires well-aligned cardiac cells with regular contractions, and supportive extracellular matrix (ECM) with adequate mechanical strength.[23,24] Therefore, to grow such well-designed tissue constructs, the cellular microenvironment should be precisely regulated.

The development of biomimetic constructs for cardiac regeneration is an attractive field of research to deeply understand the effects of signals on cardiac cell behavior.[25] For example, the use of bioactive molecules (ECM proteins like collagen, fibrin, and laminin and short peptide sequences like arginine–glycine–aspartic acid (Arg-Gly-Asp, (RGD)) as biochemical signals could significantly improve cell attachment, adhesion and myoblast phenotype for cardiac tissue engineering.[26] Furthermore, it has been reported that native ECM proteins regulate cardiac cell behaviours such as attachment,[27] alignment,[28] migration,[29] and differentiation.[30] Thus, biocompatible scaffolds with various porous structures have been developed to actively interact with the cells, improve nutrient transport and ultimately enhance cell viability.[31] Recently, new strategies like micro-patterning of both topographical and biochemical cues have also been established to direct cells at the micro- and nanoscales.[32,33]

The native cardiac tissue has an anisotropic structure, which is directed by the transportation of electrical signals inducing synchronous mechanical contractions. It is known that collagen not only provides a structural support for the heart but also transduces the mechanical forces in systole, affords passive stiffness through diastole, and moderates the cell phenotype.[34] Not surprisingly, the biomimicry engineered constructs, as a key component of the cardiac tissue engineering field, should provide the mechanical integrity to support mechano-transduction among the cells and their environment similar to the native conditions. At the same time, the biomaterials should include adequate mechanical properties to allow easy handling in cell culture for the period of implantation.[35,36]

It is known that cardiac tissue is one of the major bioelectrical sources in the body. Thus, a successfully applied cardiac tissue engineered scaffold should be electro-conductive to facilitate electrical communications among the cells similar to that of the native tissue. Electrical stimulus improves contractile features and increases cell alignment and synchronous contractions.[37] For example, Ruan *et al.*[38] used cardiomyocytes

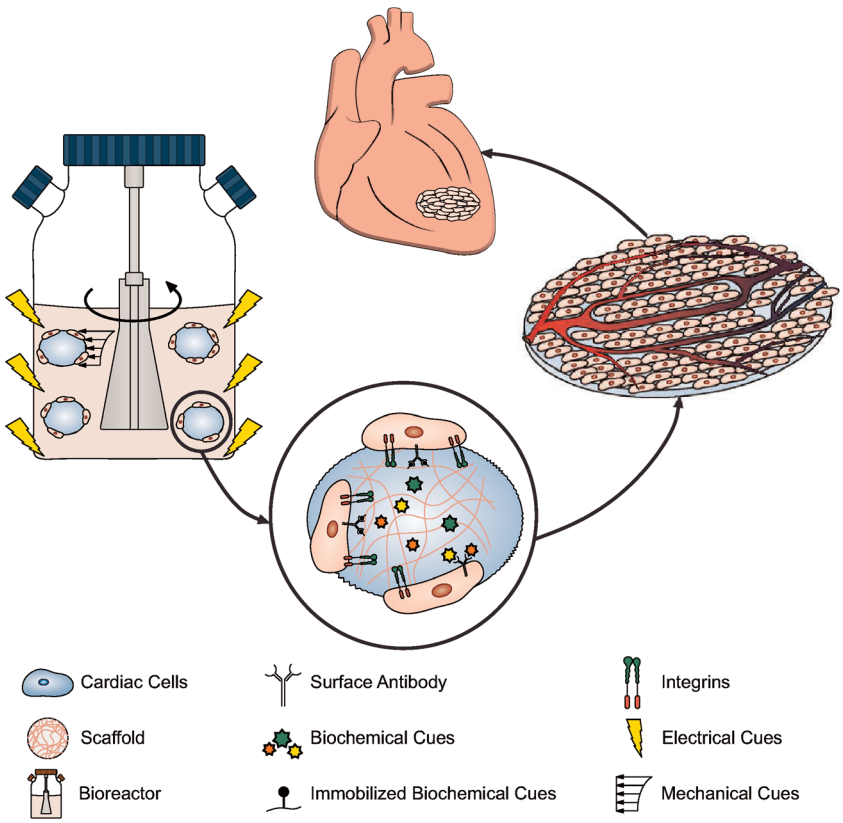

Fig. 1 A schematic illustration of the main effective signals such as topographical, mechanical and electrical cues on cardiac cell behaviors. The interaction between cardiac cells and scaffold surfaces is governed by the surface biomolecules of the scaffold biomaterial and morphological features. Mechanical and electrical signals are introduced to the cells *via* a bioreactor to regulate cell behaviors.

(CMs) to generate a bioengineered construct of collagen. These tissue constructs were exposed to electrical stimulations and static stress. The results showed a significant increase in the expression of Ryanodine receptor 2 (RYR2) and sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA2) which confirmed the maturation of excitation–contraction coupling.

This review first provides a general insight for different cell sources used in cardiac tissue engineering. Then, as shown in Fig. 1, we describe the most crucial cues such as topographical and biochemical signals to be incorporated into scaffolds aiming in directing cardiac cells to form an appropriate tissue for myocardial regeneration. Furthermore, pro-vasculogenic signals as one of the main challenges of tissue engineering is described in detail. Following that, different kinds of bioreactors to offer physiological conditions such as mechanical and electrical stimulation during cultivation will be explained. Finally, an overview of the numerical modeling strategies will be presented, which discusses the role of mathematical modeling in defining the bioreactor's conditions and establishing computed relations between the bioreactor's conditions and the subsequent engineered cardiac tissue properties.

2. Cells for cardiac tissue engineering

A suitable cell source to create a functional myocardial patch should be facile to harvest, expandable *in vitro* in large scales, non-immunogenic and capable of engrafting with damaged tissues. For stem cells, differentiation into mature, functional CMs should be possible. Although autologous cells have no immunologic problems, they are difficult to obtain and to expand. Allogenic cells are not only relatively difficult to obtain but they also evoke immune response in the host tissue. Because of the limited access to primary CMs and their minimal ability to proliferate *in vitro*, various stem cell sources have attracted research attention. So far, different cell sources have been applied in numerous studies. They could be classified into three groups: (I) somatic muscle cells including neonatal CMs[39–41] and skeletal myoblasts,[42–45] (II) stem cell-derived myocytes, such as embryonic stem cells, bone marrow-derived stem cells, adipose-derived stem cells and cardiac progenitor cells, and induced pluripotent stem cells (iPSCs),[46–48] and (III) angiogenic cells such as fibroblasts and endothelial progenitor cells.[49,50] Table 1 presents a summary of different cells and their advantages and disadvantages.

3. Let signals guide the heart

Abundant signals in the ECM microenvironment create different forces and interactions, which are transduced into intercellular cues and change gene expression and cell function. To imitate the native cardiac tissue, the major signals, including topographical, biochemical, mechanical and electrical, should be delivered to the cells to form a functional tissue *in vitro*. Some of these signals (*e.g.* topographical and biochemical) are delivered *via* the scaffolds and the others (*e.g.* electrical and mechanical) are sent by the bioreactor's environment. In the following sections, we will summarize the recent studies which have been developed to investigate the effect of each signal on cell responses.

3.1. Scaffold-based signals

An ideal engineered cardiac tissue needs to have some important features including cardiac cell alignment, extensive and dense microvasculature, synchronous electro-mechanical connections, and suitable mechanical and biochemical properties. Therefore, to engineer a functional cardiac tissue, a deep understanding of the whole cell–cell and cell–biomaterial and precise recapitulation are necessary.[61] Many certain and complex reactions occur between the cardiac cells and the biomaterial surface. There are important properties of the substrate such as topography, chemical composition, and stiffness, which regulate the reactions.[62] Therefore, it is essential to control substrate roughness, geometry, and biochemical and mechanical properties.

3.1.1. Topographical signals. It was reported that the structure of cardiac ECM influences cell behavior at the multiscale, including macro-, micro-, and nanoscale, in connection with morphology, certain gene and protein expression, cytoskeletal construction, and functionality.[63] For example, at the macroscale, cardiac tissue contains an aligned fibrous structure, which supports synchronised ventricle contraction and blood expulsion. Adult CMs (microscale), which are comprised of sarcomeres (nanoscale), allow the contraction of the cells and sarcomeres.[63]

Therefore, to engineer an ideal cardiac tissue, it is necessary to create a proper structure–function relation at different scales. The biomimetic constructs for cardiac tissue replacement should be fabricated with precise control over the micro- and nano-scale qualities of scaffold topography to be similar to those *in vivo* conditions. Many studies reported that the architecture and structure of the cardiac ECM environment influence cell behaviors such as adhesion,[64] differentiation,[65] orientation[66] and migration.[67] Topographical signals can affect CM attachment, ion channel activation, cytokine distribution, mechanical stress and the structure of CM remodeling.[68,69]

An important feature of CMs is cellular alignment, which enhances contraction, impulse spreading along the long axis of the cells, expression of cardiac genes, CM markers, and gap junctional proteins like Connexin43.[70,71] Thus, to induce cell alignment, many studies patterned the biocompatible constructs with micro- or nano-scale grooves to mimic the interactions between cells and the substrate surface.[72–75]

The methods of patterning can be classified into two main groups: surface patterning or indirect patterning including micro-molding[76] and photolithography[77] and biomolecular surface patterning (or direct patterning such as bioprinting,[78] micro-contact printing,[79] magnetic patterning,[80] and dielectrophoresis[81]). Each one has its own advantages and weaknesses. Collectively, bioprinting methods provide many benefits such

Table 1 Different cell types for cardiac tissue engineering

Cell	Advantages	Disadvantages	Clinical trial	Ref.
Neonatal CMs	Having natural electrophysiological, structural, and contractile properties	Allogenic Difficult to obtain, not expandable, cardiac myogenesis Risk of arrhythmia Poor coupling with the host tissue	No	51 and 52
Skeletal myoblasts	Form a muscle tissue Highly resistant to ischemia Available in a large volume Autologous	Risk of arrhythmia Poor electro-physiological coupling with the host tissue *via* gap junction proteins such as Connexin43 Low capacity to differentiate into cardiac cells	Yes	53–55
Embryonic stem cells	Pluripotency High plasticity The only pluripotent stem cell source with a complete phenotype	The possibility of tumor formation Ethical issues Having limited progress in clinical application Require immunosuppression, hESC-CMs do not reach full maturity, only 30–70% differentiated cells are ventricular CMs	No	46, 56 and 57]
Bone marrow-derived stem cells or crude bone marrow-derived/ circulating progenitor cells (BMPCs)	Autologous Available in a large quantity Easy to access High expansion potential Stable phenotype Plasticity (is able to alter the phenotype in response to form the target organ) Compatibility with different delivery methods and formulations	Low differentiation capacity *in vivo* Unable to explain the therapeutic effect provided to the infarcted heart	No	58
Mesenchymal stem cell (MSCs)	The ability of differentiation to CMs and ECs Autologous Available in a large quantity Easy to access Strong expansion capacity immuno-privileged Secrete large amounts of angiogenic and antiapoptotic factors *in vitro* and insulin-like growth factor 1 (IGF-1)	(MSCs) ability to differentiate into functional CMs was not examined clearly Low retention rate	Yes	55, 59 and 60
Adipose-derived stem cells	Having strong *in vitro* differentiation proliferation potential Can be easily isolated with less invasive procedures		No	40
Hematopoietic stem cells (HSCs)	Feasibility	The real effect of HSC on myocardial regeneration is still unclear	No	41
Cardiac progenitor cells	Multipotent Self- renewal differentiate to matured CMs and ECs No tumor formation reported Autologous	Small quantity Need cardiac biopsy or surgical sampling Heterogeneous population	No	45
Induced pluripotent stem cells (iPSCs)	Pluripotent Can be differentiated into CMs Autologous Available in a large quantity	Unpredictable genetic dysfunction Malignant formation CM differentiation: 55% Immuno-rejection	No	44
Endothelial progenitor cells	Stimulate revascularization	Having reduced capacity of re-endothelialization EPCs in patients due to carrying cardiovascular risk factors Heterogeneous population	No	43

as the possibility to pattern complex cellular constructs, and control and mimic the micro-environment of the cell with high accuracy based on natural conditions. Furthermore, bioprinting has high reproducibility and provides the possibility to print different types of cells. However, limited resolution, low speed of deposition and difficulty in storing cells within the bioink during printing time are major challenges in the efficacy of bioprinting. Furthermore, the application of most of the indirect patterning techniques such as bioprinting techniques is limited due to the choice of appropriate materials. The ideal biomaterials should provide many important features including bioprintability, proper functionality, mechanical strength, and availability.[82]

In surface patterning, the guidance cues are provided by the surfaces of biomimetic constructs to the cardiac cells. For example, Kim *et al.*[83] developed the patterned hydrogels with nano-scale grooves and exhibited that the CMs were aligned along the direction of the topographical cue due to the organization of focal adhesions and subsequently cytoskeletal proteins. The results also showed that cell alignment promoted Connexin43 expression and conduction velocity, which caused the cultured cells to exhibit characteristics similar to those in the native heart. Moreover, Annabi *et al.*[84] synthesized micropatterned, elastic methacrylated tropoelastin (MeTro) hydrogels by using recombinant human tropoelastin. They reported that the micro-patterned structure of these hydrogels enhanced cardiac cell alignment, considerably improved cell maturation and supported 3D modular assembly.

To evaluate the effect of topographic size, we can compare two separate studies,[85,86] which fabricated polyethylene glycol hydrogel at the micro- and nanoscale without any other difference in experimental conditions such as surface treatment and the groove ridges. Although they observed cell alignments in both studies, nano-topographic patterns could mimic the interaction between cells and ECM at the nanoscale. The comparison of cell penetration to the groove bottom between two different sizes of the grooves (400 and 800 nm) showed that the cells on the groove size of 800 nm were able to penetrate deeper than the cells on the groove size of 400 nm. The cells on the larger grooves experienced stronger contraction forces, which caused the enhancement in expression and improved conductivity.

Although these approaches (in direct patterning) provide a precise control for cell positioning, they are typically costly and have a long processing time. Moreover, they are not able to control the cell density properly.[87]

Biomolecular surface patterning (direct patterning) relies on printing cells encapsulated inside the appropriate biomaterial supplemented with different forces or inkjet or laser.[88] Bioprinting, as one of the most common direct patterning techniques, uses mechanical procedures and consistent biomaterials to fabricate 3D constructs according to the specific computer-aided designs.[88]

As another kind of direct patterning method, Birket *et al.*[79] used microcontact printing to pattern acrylamide gel and cultured cardiac progenitor cells (CPCs) on them and showed that CPCs can be patterned with good alignment. The results demonstrated that the differentiation of CPCs was controlled successfully, resulting in a proper re-creation of the heart. Microcontact printing methods do not need special equipment and do not damage the biomolecules. However, the possibility of stamp deformation due to the application of high pressure and the biomolecule diffusion process during printing limit its resolution.[89] In this method, different types of cells, biomolecules including collagen, laminin and fibronectin, or peptides containing cell-binding domains such as arginine–glycine–aspartate (RGD) are patterned at microscale or nanoscale to regulate cellular functions.[90] The cellular responses are a result of the mechano-transduction pathways mediated in part by integrin–ligand binding.[90]

Although these novel and advanced approaches allow the fabrication of 3D complex structures with exactly controlled constructions and numerous cell types, they need a relatively long time to correctly position cells inside the hydrogels. In addition, they need a surface for seeding cells, and their applications are limited because of the issues related to the incompatibility of high temperature and laser energy with the combination of different types of cells and biomolecules inside the hydrogels.[91]

We know that the cardiac ECM has a multiscale fibrous structure with changing morphologies and orientations. To mimic the cardiac structure, electrospinning has been developed which is a promising, easy, and cost-efficient method receiving increasing popular attention and with market growth.[92] Fibrous scaffolds have a high ratio of surface to volume, suitable porosity and the capacity to control the distribution of interconnected pores. Additionally, nanofibers provide a continuous release of growth factors and high loading efficiency.[93]

Recently, Zhang *et al.*[78] used bioprinting to create microfibers of alginate gel encapsulating endothelial cells and after the formation of an endothelialized bed (15 days), seeded neonatal rat CMs to obtain an engineered endothelialized myocardium with aligned CMs. The results of the immunofluorescence staining of Connexin43, sarcomeric α-actinin and CMs showed a well-aligned organization of the CMs.

However, the electrospinning of pure natural polymers without using synthetic polymers, especially a mixture of different proteins, or whole ECM, is relatively difficult and so has been investigated rarely.[94,95] To overcome this challenge, Schoen *et al.*[96] used an electrospinning technique to form microfibers of decellularized porcine cardiac tissue without any variation in the ECM molecular structure and mechanical properties and seeded neonatal rat CMs on them. Upon seeding, they observed a normal alignment morphology of human mesenchymal stem cells (hMSCs) which penetrated into the scaffolds.

Moreover, Kharaziha *et al.*[97] applied an electrospinning approach to fabricate nano-fibrous scaffolds of poly(glycerol sebacate) gelatin that mimic the left ventricular myocardium architecture. Their results indicated that the aligned nanofibrous scaffolds promoted cellular alignment, induced

optimal synchronous contractions of CMs and supported cardiac cell organization, phenotype, and contraction. They found that the CMs on the aligned scaffolds increased organized and aligned sarcomeric α-actinin, Connexin43, and troponin I (calcium receptive component associated with cytoskeletal organization and maturation of CMs) in comparison with non-aligned scaffolds.

Nevertheless, the low thickness of the electrospun scaffolds creates difficulties in their handling and limits their clinical applications. To deal with this subject, Fleischer *et al.*[98] applied the layer-by-layer assembly method with different layers of fibrous albumin scaffolds. To induce cardiac cell alignment, they assembled the various patterned layers with different structures and functions (*e.g.* vascularization and releasing growth factors) which had been grown separately under the most appropriate conditions. Then they integrated the layers using a biological glue to produce a 3D engineered cardiac tissue and lastly transplanted the patches into the rat body. They predicted that this method could be considered as an applicable approach in the future to build thick and functional tissues due to its simplicity.

Scaffold topography also affects CM contractility by the enhancement of cytoskeleton alignment and nuclei elongation. For instance, in one study by Kim *et al.*[99] a 3D environment of grooved cantilevers was established and CM contractility was measured and compared with that observed in 2D flat cantilevers. Their results showed that the CM contractility on grooved cantilevers was 65–85% higher than those on flat ones.

Furthermore, to mimic the native tissue, it is necessary to control the cell alignment in 3D constructs since extending topographical cues from 2D to 3D will enable greater contraction forces that provides sufficient functionality. For example, Badrossamay *et al.*[100] produced nano-fibers (ranging from 50 to 3500 nm in diameter) by the electro-spinning technique and seeded neonatal rat CMs on the 3D fibrous scaffolds. They induced CM alignment into a beating tissue construct by highly aligned fibers and controlled the fiber morphology and diameter *via* changing polymer properties, nozzle geometry and rotation speed. Their results demonstrated that the CMs aligned into a beating tissue construct, which resembled closely the *in vivo* conditions.

Topography also has a key role in the creation of entirely synchronous engineered cardiac tissue through gap junction and cell–cell connection formations. For example, Patel *et al.*[101] grew CMs on a dimethylsiloxane (PDMS) micropeg with different topographies in size and spacing. Their results indicated that patterned topographies improved CM adhesion and proliferation and created a regular frequency of contractions through all micropegs.

Topographical cues can also direct stem cell differentiation to cardiac cells. For instance, Morez *et al.*[102] reported that the use of topographical cues could enhance the differentiation of cardiac progenitors into cardiomyocyte-like cells efficiently. In another study, human embryonic stem cells (hESCs) were differentiated to the cardiac cells by controlling the topographical properties of the substrates and subsequently the size of embryoid bodies (EBs). In this work, size-specified hESC colonies were formed by plating single-cell suspensions onto micropatterned extracellular matrix islands.

3.1.2. Biochemical signals. It is well known that the biochemical features of scaffolds can regulate cell growth, migration,[103] differentiation,[103] synthesis of ECM components and tissue morphogenesis. Therefore, different approaches have been developed to modify the biochemical properties of the scaffolds so as to regulate the reactions between cells and the biomaterial.[104] Bioactive molecules such as ECM proteins including collagen and laminin have been used to direct cardiac cell behaviors in many studies.[105] Thus, novel biomimetic constructs have been developed by immobilizing the bioactive molecules to provide specific inductive biological cues for the cardiac cell. Typical bioactive molecules which are used for the surface modification of biomaterials including ECM proteins and peptides and growth factors are presented in Table 2.

It has been shown that the proteins present in the basement membrane of the myocardium, such as laminin, enhance cardiac cell adhesion.[110] LaNasa *et al.*[111] estimated the effects of immobilized cell adhesion moieties on controlling the cellular attachment and phenotype of skeletal myoblast cells for cardiac muscle tissue engineering. In this study, they covalently attached collagen I, laminin and RGD to flexible hydrogels and found myoblast attachment and the development of an intracellular contractile network on these modified hydrogels. Finally, they suggested collagen and laminin as effective bioactive proteins for improving CM interaction with hydrogels.

Attaching ECM-derived peptides is a promising strategy to improve the surfaces of biomaterials.[105] For example, the synthetic arginine–glycine–aspartic–phe–lys–(RGDfK) peptide was covalently linked to the alginate scaffold. They reported that the modified alginate scaffolds, which were seeded with human mesenchymal precursor cells (hMPCs) and were implanted to the epicardial surface of the infarcted myocardium induced myocardial neoangiogenesis and significantly improved cardiac function without an overt immune response.[112] Shachar *et al.*[113] has covalently bound the RGD peptide to alginate to study its influence within the 3D constructs for cardiac tissue engineering. The results demonstrated that the RGD peptide modification promoted the relative expression levels of contractile, cell–cell adhesion proteins and prevented cell apoptosis.

Furthermore, Rosellini *et al.*[114] functionalized new synthetic biomaterials of polycaprolactone–poly(ethylene oxide)–polycaprolactone with H-Gly-Arg-Gly-Asp-Ser-OH (GRGDS) from fibronectin and H-Tyr-Ile-Gly-Ser-Arg-OH (YIGSR) from laminin for myocardial tissue engineering. They reported that these bioactive molecules distributed homogeneously and promoted cell adhesion and improved myoblast growth.

By using emerging microfabrication technologies, scientists could design the geometry of the attached cells and surface chemistry pattern, density and bioactive molecule specificity. For instance, Feinberg *et al.*[115] fabricated PDMS thin films

Table 2 Typical bioactive molecules and their impacts in cardiac tissue engineering

Bioactive molecules		Impacts on cell behaviors	Challenges	Ref.
ECM proteins	Collagen (different types)	Improve cell growth, provide structural support, connecting contractile elements of neighbouring myocytes together	Immunogenic problems	61 and 106
	Fibronectin	Improve cell adhesion, spreading and migration, improve healing and remodeling	Insufficient mechanical properties	
	Laminin	Improve cell migration, adhesion, growth and differentiation, improve healing and remodeling	Fast degradation procedure	
	Nephronectin	Improve cell adhesion, matured contractile apparatus with alignment of sarcomeres	The dependence on the source and extraction process	
	Gelatin	Improves cell adhesion, growth and differentiation Provides structural support	The possibility of contamination High production costs (except gelatin)	107
Short peptide sequences	RGD	Improves cell attachment Enhances CM contractility and cell viability Prevents cell apoptosis Direct interaction with cell surface receptors	High extraction costs	61 and 106
Growth factors	VEGF	Improves cell proliferation, migration and survival of endothelial cells Induces the formation of endothelial capillary structures	Quick degradation process Increases vascular leakage	61 and 108
	bFGF	Improves endothelial cell proliferation Induces the formation of endothelial capillary structures	Quick degradation process Uncontrolled release; mitogen for many different cell types	61 and 76
	Insulin-like growth factor	Improves cardiac contractility Promotes cardiac growth Facilitates glucose metabolism Increases cardiac DNA and protein synthesis	High extraction costs	109

which were patterned by ECM proteins and then neonatal rat CMs were cultured on them. These substrates were released from poly(*N*-isopropylacrylamide) (PNIPAAm), which is a temperature responsive polymer, and promoted myogenesis and indicated biomimetic functions effectively. Khademhosseini *et al.*[116] developed a microfluidic substrate of hyaluronic acid (HA) and sealed it on a glass slide. Then the fluid flow was transferred to selected areas of the substrate and indicated that CMs adhered successfully and aligned along the pattern direction of fibronectin and between HA patterns and the glass substrate. Another outcome of using biochemical signals is to induce vascularization in the functional constructs.

These modification methods can be applied for both the surface (*e.g.* chemical,[117] photo-based methods[118] and plasma treatment[119]) and bulk (*e.g.* direct blending[120] and coaxial electrospinning[121]) of the biomimetic constructs. Generally, bulk modification approaches are not only quicker and simpler than surface modification methods but they also allow more bioactive molecule coupling and in consequence provide more guidance cues for cell–construct interactions.[61]

Chemical modification can be performed by grafting functional groups using different processes such as acetylation,[122] aminolysis,[123] hydrolysis,[124] silanization,[125] and modifications of the present functional groups *via* oxidation and reduction reactions.[126] For example, sodium hydroxide treatment was used to increase the hydrophilicity and roughness of the scaffolds and improve cell adhesion.[127]

However, some chemical reactions induce major issues which should be considered during use in cardiac tissue engineering. For instance, hydrolysis reactions are pH dependent and may result in undesirable reactions and productions. In aminolysis, a process temperature higher than 200 °C is needed to avoid salt formation.[61]

Photo-based approaches are cost efficient which need mild condition and only affect the biomaterial surface.[61] In one study, the poly(L-lactic acid) PLLA surface was modified to provide more hydrophilicity by attaching an acrylamide group *via* UV treatment.[118]

The use of plasma to graft functional groups is an effective method, which provides modifications of the biomaterial surface *via* alteration in neutral molecules, ions, radicals, and electrons and improves cell attachment and growth. Plasma treatment provides durable functionalization and the possibility of binding different monomers, which gain the desired features. However, the limited depth (only up to 10 nm) of its impact restricts its applications.[128] It was reported that the plasma treatment of PLGA by fibronectin coupling allowed better dispersion of neonatal rat CMs.[129]

3.1.3. Vascularization. It is well known that cardiac tissue is a dense and complex tissue that contains different cell types including CMs (30–40%) and endothelial cells, fibroblasts and vascular smooth muscle cells. The transport of nutrients and oxygen to this highly dense tissue is not feasible without an extensive vascular network.[130]

The engineering of thick and functional constructs for heart replacement demands a high-level density of the vascular networks. In the native cardiac tissue, a highly dense vascular network surrounds the aligned CMs, and the average distance among these micro-vessels is about 20 μm. Basically, this extensive and well-organized vascular system supports the high level of cardiac metabolic activity by the rapid transportation of nutrients, oxygen and metabolites to and from the cells efficiently.[131] Therefore, vascularization has a key role in developing practical engineered complex tissues such as myocardium. Unfortunately, the formation of a vascular network needs a long time (3–5 days at a minimum) and during this time, cell survival cannot be guaranteed. In addition, the resulting vascular network is discontinuous and randomly distributed which limits nutrient perfusion and oxygen transport.[132] Typical scaffolds have critical challenges such as the lack of control on the size of pores and their distribution. Therefore, bio-mimicking of native tissues is still a challenge in tissue engineering and micro-fabrication techniques have been developed to offer more appropriate scaffolds.[133]

By using the novel microfabrication approaches microfluidic channels are embedded into the constructs to provide oxygen, facilitate nutrient transport and remove metabolic waste products from the cells. These constructs are able to direct 3D cell behaviors, and improve vascularization and subsequently cell viability. Therefore, numerous techniques have been used to overcome these limitations by using microfabrication strategies to induce topographical signals and biochemical cues-based techniques to provide proper angiogenic biomolecular cues in the cell microenvironment.[134–138]

Numerous techniques such as direct patterning (*e.g.* bioprinting[139] and cell sheet technology[140]) and indirect patterning[141] (including PDMS molding,[84] sacrificial molding[142] and electrospinning[143]) provide the opportunity to organize the vascular cells and control their behaviors. In indirect patterning methods, tubule structures with a controlled geometry are prepared and then endothelial cells are seeded on them.[144]

Annabi *et al.*[145] designed a heart-in-a-channel platform to study cell–matrix interactions. They coated microfluidic channels with MeTro to improve cell attachment on the poly-PDMS surface. They observed improved adhesion, alignment and spontaneous beating of CMs compared to those covered by gelatin-based materials. This study proved the great potential of MeTro hydrogel to cover micro-channels in heart-on-a-chip systems.

All of these methods provide suitable control over endothelial cell organization in the tubule network and the channel diameter. However, the use of this indirect patterning mostly allows two-dimensional (2D) constructs which can be converted into 3D structures by stacking these 2D scaffolds. Another drawback of these methods is the presence of an impenetrable biomaterial surrounding the microchannels, which limits the extra remodeling of the vasculature network and nutrient transport. Furthermore, the inadequate resolution of these approaches limits the creation of a more complex network of capillary organization.[144]

By using direct patterning, particularly bioprinting, 3D constructs with more complex vascular networks can be attained, which results in additional remodeling and formation of more separate and interconnected tubule structures. Bioprinting offers the patterning of cell laden biomaterials or cellular aggregates at a particular location with a high level of accuracy.[146] However, the use of biomaterials has raised questions related to their degradation process and their toxic products. Moreover, the challenges include immunogenicity, host inflammatory responses, and fibrous tissue formation that affect the long-term function of the engineered tissue construct.[147]

Therefore, Ong *et al.*[148] have recently developed a 3D bioprinted cardiac construct without using any biomaterial (Fig. 2A). They created cardiac patches by aggregating different ratios of three cell types including human induced pluripotent stem cell-derived CMs (hiPSC-CMs), fibroblasts and endothelial cells in spheroid structures and then printing the spheroids. They reported that all cardiac patches of different ratios beat automatically and showed uniform electrical conductivity. The results of immunohistochemistry confirmed the presence of CD31 (related to neovasculogenesis) and Connexin43[148] (Fig. 2B).

A recent promising method for the synthesis of micro-channels in the constructs is to print living cells and biomaterials in 3D biological scaffolds called the bio-printing technique. Zhang *et al.*[149] proposed a novel hybrid strategy of 3D bioprinting and heart-on-a-chip systems. In this study, the endothelial cells were encapsulated within the bio-printed micro-fibrous scaffolds to resemble a blood vessel structure. The endothelial cells slowly migrated towards the scaffold fibers to create a layer of dense endothelium. Then the printed endothelium bed was seeded with CMs to form an engineered tissue with aligned CMs and synchronous contraction. Afterwards, the organoids were inserted into a certain microfluidic perfusion bioreactor to fabricate an endothelialized-myocardium-on-a-chip system (Fig. 3).

The biochemical based techniques can be classified into two types: using the gradient of angiogenic molecules in the microenvironment[150,151] and using the co-culture of different types of cells.[152–154] It was reported that by use of angiogenic biomolecular cues, vascularization is enhanced because it would be more similar to the *in vivo* microenvironment and angiogenetic factors would also be able to act more effectively for a longer period of time.[155] Lin *et al.*[156] studied the influence of self-assembling peptide nanofiber injection in combination with vascular endothelial growth factor (VEGF) on the improvement of post-infarct neovascularization in rats. Their result showed that nanofiber/VEGF injection enhanced angiogenesis, arteriogenesis, and cardiac performance. It also significantly reduced the side effects of systemic edema and proteinuria. In addition to the controlled local delivery of factors, it provided a proper microenvironment to recruit endogenous myofibroblasts and enhanced revascularization. It was demonstrated that the engineered vascular niche also attracted CM-like cells to the injected sites, which depicted the progression

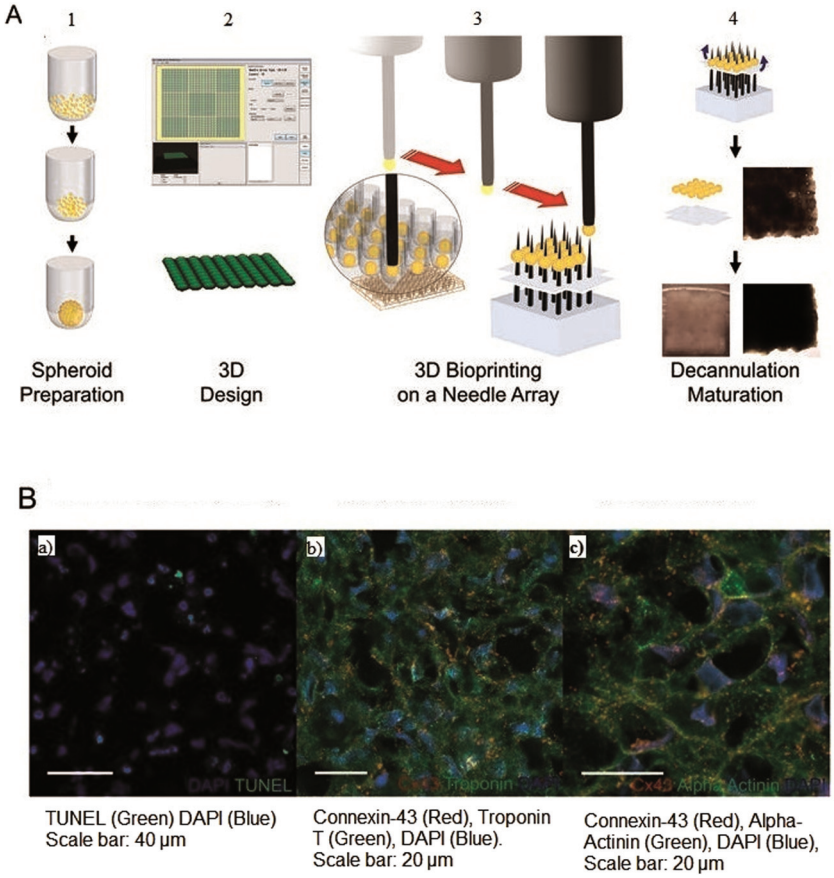

Fig. 2 (A) An illustration of biomaterial-free cardiac 3D construct formation: (1) Different types of cells including CMs, fibroblasts and endothelials were accumulated in 96-well plates to fabricate mixed cellular spheres. (2) Computer software was used to design the favourite 3D structure. (3) The bioprinter lifts the cellular spheres *via* vacuum suction and puts them on a needle array. (4) The cellular spheres were fuse to each other. (B) Confocal microscopic images of 3D bioprinted biomaterial-free cardiac constructs. Images reproduced with permission from ref. 148.

of CM regeneration. Another example of this approach is the study of Marsano *et al.*[157] on the combination systems of providing intrinsic vascularization stimulus and supplying adequate oxygen in an engineered cardiac patch. The results showed that the viability, vascularization, and functionality of the cardiac patch were enhanced. The *in vivo* study confirmed improved engraftment, survival and contractility of CMs. Chung *et al.*[158] have recently designed an epicardial delivery system of VEGF and cardiac stem cells (CSCs). They loaded VEGF in a series of PLLA mats and seeded the fibrous constructs with CSCs. This mat released VEGF for 4 weeks and promoted the migration and proliferation of both endothelial cells and CSCs. The sustained release of VEGF and the 3D microenvironment of the electrospun mat resulted in enhancing the *in vitro* capillary-like network formation of endothelial cells and increasing the expression of proangiogenic mRNAs of CSCs.

It is well known that cell migration is an important step in creating a tubular shape of endothelial cells. A density gradient of growth factors in cell laden hydrogels or even in a microfluidic device stimulates the cells to migrate from a region of low concentration of growth factors to a high concentration region.[159,160] For example, Wu *et al.*[160] fabricated a basic fibroblast growth factor (bFGF) gradient environment by the injection method. The density of bFGF gradually increased from about 130 to 300 ng cm^{-2} and was sensitive to the vascular smooth muscle cells (VSMCs). The results showed that more than 70% of the cells migrated to the area with a higher concentration of bFGF.

Furthermore, Wu *et al.*[159] showed the effect of the gradient density of the VEGF environment on cell migration. They calculated several cell mobility parameters such as total migration distance, net displacement, chemotactic index (CI) and the percentage of cells moving towards the gradient, which demonstrated that most of the cells migrated to the higher density region. Moreover, vascularization can be induced by seeding vascular cells with different types of cells, which can secrete angiogenic bio-molecules in a microenvironment. For example, in the co-culture of endothelial cells and adipose-derived stem cells (ASCs) in fibrin gel, the formation of tubule structures was successfully guided by ASCs.[161] Fibroblasts also have the role of supporting vascularization through improving endothelial cell survival and proliferation and increasing vessel size and density in the pres-

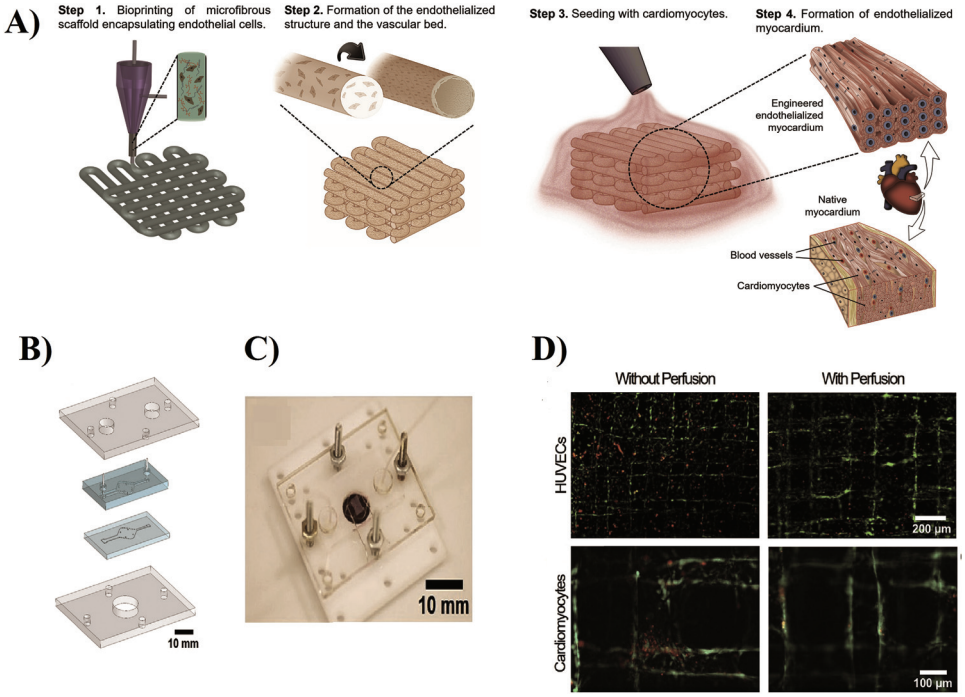

Fig. 3 (A) Schematics of the fabrication process of the endothelialized myocardium bioprinting method. (B) Schematics of the microfluidic bioreactor's components, which are surrounded by a pair of PMMA layers. (C) Photograph of the designed bioreactor containing a bioprinted scaffold. (D) Live/dead micrographs and quantified cell morbidity of bioprinted endothelial cells and CMs on the scaffolds without and with perfusion in the bioreactors; red: dead cells, green: live cells. Images reproduced with permission from ref. 149.

ence of growth factors such as VEGF, platelet-derived growth factor (PDGF), *etc*. In comparison with the monoculture systems, this co-culture environment better represents the *in vivo* microenvironment.[154]

Another example of taking advantage of co-culturing different cells in cardiac tissue engineering has been done by Sakaguchi *et al*.[153] They utilized a combination of single patterned cell sheets and co-culture of endothelial, cardiac, fibroblast and myoblast cells in order to control the shape of endothelial cell networks. Endothelial cells spontaneously formed vascular networks in co-culture with cardiac cells, fibroblasts, and myoblasts. Although the designed vascular networks were effectively connected to the host capillaries after transplantation, the thickness of the engineered tissue was limited. Therefore, new triple-layered cell sheets were placed on the first one repeatedly to obtain a thick and desirable cardiac implantation. A cardiac tissue with about 1 mm thickness could be produced by this method.[153]

Furthermore, Okano's group designed a vascular bed bioreactor to form perfused vessel networks within the engineered cardiac and skeletal muscle tissue sheets.[76,162] Upon implantation, these tissue patches were connected to the host vasculature, resulting in increased viability compared to non-vascularized controls.[162]

3.1.4. Scaffold stiffness. Cardiac tissue engineering allows the control of the scaffold properties to direct cell response to different specific cues. While the tissue regenerates, the cells apply forces to their supporting scaffold which gradually changes its structure at the microscale. The mechanical properties of the scaffold regulate the opposite reaction forces applied to the cells. By taking advantage of microfabrication techniques, many studies were developed to find out the cell–scaffold mechanical interaction.[84,215,216] For instance, Annabi *et al.*[84] fabricated a highly elastic and mechanically stable MeTro hydrogel from human tropoelastin. This study confirmed that the elasticity of the constructs is a major factor to maintain the proliferation of neonatal rat CMs. Compared to the gelatin methacryloyl (GelMA) gel which has extensibility less than 100%, MeTro gel can provide significantly higher extensibility (~400%) while maintaining a tensile modulus (15 kPa) close to those of the rat CMs (30 kPa). The results confirmed that high elasticity and resilience of functional biomimetic MeTro hydrogels provided the superior contractile performance of CMs.

To study the effect of scaffold stiffness on CM alignment individually, Sinha *et al.*[215] investigated the alignment of neonatal CMs on polyacrylamide gel coated with collagen type I with stiffness values of 1, 10, and 50 kPa. Cells that were cultured on the scaffolds with a stiffness of 10 kPa had the most defined and aligned striations, as in this case, the scaffold stiffness matched the native embryonic tissue. Unaligned cells on stiffer scaffolds showed the importance of scaffold stiffness in providing an *in vivo*-like niche. In another study, porous, elastomeric poly(glycerol sebacate) (PGS) scaffolds with an accordion-like honeycomb structure were developed with tunable tensile properties and microstructural anisotropy.[216]

The results showed that the alignment of CMs cultured on anisotropic scaffolds was significantly higher than that cultured on isotropic scaffolds. While these methods are able to control the structure and elasticity of the scaffold, they failed to mimic the fibrillary structure of cardiac ECM. To overcome this challenge, electrospinning has been used to create fibrous scaffolds with tunable micro-scale structures and chemical qualities.[217] The mechanical properties of the scaffold are not only a function of biomaterial formulation and concentration but also depend on the microstructure of the scaffold, which can be engineered in the electrospinning process.[218,219] Furthermore, studies have been performed to evaluate the influence of construct stiffness on the rate of CM contractility,[220] stress production,[221] and handling of intracellular calcium.[222] For example, Galie *et al.*[206] showed that scaffold stiffness affects both the stress and strain created inside the CMs. Moreover, there was a time-based change in two parameters which suggests that the CM response *in vitro* is an adaptive, time-related procedure.

3.2. Bioreactor-based signals

3.2.1. Bioreactors and cultivation systems. The influence of different signals on cell behavior is one of the most significant principles of cardiac tissue engineering. To mimic the native heart tissue closely, it is crucial to use the most suitable cell source, biomaterial scaffolds, and the development of a suitable environment to deliver appropriate signals to the cardiac cells.[163–165] In static cultivation, there is a large diffusional gradient between the cells and their surroundings. Therefore, the process of oxygen and nutrient delivery and waste removal does not perform sufficiently for the cells in the center of the construct and consequently the cells finally die.

Bioreactors have been developed to simulate culture conditions *in vitro* according to native tissues, such as efficient exchange of metabolites and transport of chemical, electrical and mechanical cues to or from cells and their environment. These signals can increase contractile potency,[166] improve electrical conductivity,[167] direct stem cell fate,[168–170] enhance vascularization[171–173] and encourage cellular responses to engineer specific tissues and organs.[48] The responsive cells in cardiac tissue engineering are highly influenced by the cues, which were discussed in section 3. It requires robust control for the effective regeneration of the heart. Generally, bioreactors can be classified into two groups: (1) macro-scale (or conventional) and (2) micro-engineered bioreactors (or heart-on-a chip systems), which help to control the microenvironment of cardiac cells.

3.2.2. Macro-scale bioreactors. Macro-bioreactors with different geometries and fluid patterns have been designed to allow the continuous flow of culture medium, improve mass transport, stabilize the pH and promote cell viability.[132] Generally, different kinds of flow patterns can be obtained based on the bioreactor's structure. Not surprisingly, the control of the cell environment and transport efficiency is performed by the bioreactor design and substrate material of biomimetic constructs, which directly influences cell viability, proliferation and differentiation *in vitro*. It was reported that perfusion bioreactors provide more uniform cell seeding, increase Connexin43 expression, support stronger contractions, and decrease the excitation threshold.[174,175]

One of the flow patterns in practice is the spinner flow, which has been used as a cell culture widely because of its easy setup and accessibility. It was reported that by changing the agitation rate, it is possible to apply these kinds of bioreactors for different cell tapes.[176] For example, Niebruegge *et al.*[177] and Zandstra *et al.*[178] demonstrated that CMs could be grown successfully at 60 RPM. Although these bioreactors provide a well-mixed and homogeneous environment around the cell constructs and minimize the stagnant layer at their surface, they do not present the best conditions for cardiac cells due to the harmful effects of eddies in the turbulent flow at the surface of the scaffolds.

Another flow pattern in macro-bioreactors can be obtained by rotating bioreactors. These bioreactors provide a laminar flow without mixing, gas bubbles and gas/fluid interfaces and destructive shear stress. The mass transport between the scaffold and bioreactor media is governed by dynamic laminar convection, while the transport inside the scaffold is based on the molecular diffusion model. These kinds of bioreactors have a great advantage due to the absence of the agitator or mixing device. Because the links between the cells and microcarriers are maintained with no trouble. However, the microcarrier's motion relative to the fluid flow creates mixing and minor shear stress in the rotating bioreactors.[179] Carrier *et al.*[180] compared rotating and mixed bioreactors and indicated that the expression of specific cardiac markers was higher in rotating bioreactors. However, these bioreactors are not able to provide medium perfusion into the center of the constructs because of the lack of capillary network in the engineered tissue. Therefore, microfluidic bioreactors have been developed to optimize the transport of nutrients and metabolites, especially for limited distances by diffusional penetration depth for different molecules such as oxygen.

Furthermore, pulsatile flow is another kind of fluid pattern which improves the contractile properties and excitability of CMs.[181,182] However, high shear stresses raised during interstitial perfusion may deleteriously influence cell viability. Moreover, the removal of perfusion conditions at the time of implantation is likely to result in rapid tissue ischemia and death *in vivo*. In Table 3, the macroscale bioreactors with different stimulations to achieve more sophisticated and functional engineered cardiac constructs are summarized.

3.2.3. Micro-engineered bioreactors and heart-on-a-chip systems. The reduction of bioreactors size from macro- to micro-scales provides the opportunity to mimic the micro environment of the cells, control the effective factors, improve the vascularization through embedding microvessels in the engineered constructs, decrease the number of experiments, save time and reduce cost.[195]

On the other hand, we know that *in vivo* CMs are very close to each other and form a dense construct of the cells and have a high level of glucose uptake due to their functionality. To

Table 3 A summary of the developed bioreactors for cardiac tissue engineering with mechanical and electrical stimulation

Type of bioreactor	Scaffold	Cells	Target tissue	Physical stimulation	Flow	Ref.
Static flask	Fibrin	Neonatal rat CMs	Cardiac tissue	Cyclic stretch (5% with a 50% duty cycle at 1 Hz) and electrical field (rectangular, biphasic, 1 ms, 3 V cm^{-1} at 1 Hz)	Static	183
Static flask	Collagen type I	Neonatal rat CMs	Cardiac tissue	Static stretch (5%) and electrical field (rectangular, biphasic, 1 ms, 3–4 V cm^{-1} at 1 Hz)	Static	184
Static flask	Rat tail collagen Type I and Matrigel	Murine iPSCs/human ESCs/human iPSCs	Cardiac tissue	Cyclic stretch (10% at 1 Hz)	Static	185
Static flask	Rat tail collagen Type I and Matrigel	Neonatal rat CMs	Cardiac tissue	Cyclic stretch (10% sinusoidal stretch with a 100% duty cycle at 1 Hz)	Static	186
Static flask (petri-like culture chamber)	Porous alginate disks	Neonatal rat ventricular CMs	Cardiac tissue	Electrical field 1. Under static conditions (5 V, bipolar, 2 ms pulse, 1 Hz) 2. Under perfusion conditions (74.4 mA cm^{-2}, 1 Hz, bipolar, 2 ms, 80% duty cycle)	Static and perfusion	187
Static flask	Porous poly (glycerol sebacate) (PGS)	Neonatal rat CMs	Cardiac tissue	Hydrodynamic shear force (<1 dyne cm^{-2}) Electrical field (3 V cm^{-1}, 3 Hz, monophasic square wave)	Perfusion	188
Static flask (petri-like culture chamber)	Ultrafoam collagen sponges	Neonatal rat ventricular myocytes	Cardiac tissue	Electrical field (monophasic pulses of 0.2–10 ms duration, 1, 3, and 5 Hz, 1–6 V cm^{-1})	Static	189
Static flask (petri-like culture chamber)	Bovine fibrinogen + Matrigel	Rat ventricular cardiac cells	Cardiac tissue	Electrical field (2 ms symmetric biphasic square pulses, 4 V cm^{-1}, 1 Hz)	Static	190
Static flask (petri-like culture chamber)	2D cell culture on glass slide	Mouse adipose-derived stem cell line (m17.ASC line)	Cardiac tissue	Electrical field (monophasic, 8 V, 2 ms, 1 Hz) Electrical field (1 ms and 4 V, 1 ms; 1 Hz)	Static	191
Static flask	Ultrafoam collagen sponges	Rat cardiac cells	Cardiac tissue	Electrical field (monophasic pulses of 2 ms duration, 3 Hz, 3 V cm^{-1})	Perfusion	130
Static flask (modified Schott Duran square cut flask)	Rat acellular whole-heart scaffolds	Human umbilical vein endothelial cells (HUVEC) and neonatal CMs	Cardiac tissue	3D stretch	Perfusion	192
Spinner flask	Gelatin porous microbeads	Neonatal rat mixed cardiac cells	Cardiac tissue	Hydrodynamic shear force electrical field (0.5–1 Hz, 7–9 V cm^{-1})	Dynamic	193
Static flask	2D cell culture on gelatin coated glass slides	Human cardiac progenitor cells (hCPCs)	Cardiac tissue	Electrical field Monophasic (2 ms, 1 Hz, 5 V amplitude) Biphasic (2 ms, 1 Hz, ±2.5 V)	Static	194

deliver glucose to this large number of cells, medium perfusion has been used. However, the medium renewing process in the perfused macro-bioreactors not only could not precisely imitate the cell microenvironment but it also washed out the biomolecules secreted by the cells. Microfabrication technologies provide a precise control over the cellular microenvironment through the topography, size, and elastic properties of the 3D constructs and facilitate biochemical signal delivery to the cells *via* the continuous perfusion of the culture media.[195]

To mimic the circulation conditions in the human body, the newly established heart-on-a-chip system has been developed by using soft lithography to integrate tissue constructs in a microfluidic platform.[196,197] In a heart-on-a-chip system, there is a microfluidic device with continuous culture media flow to mimic the physiological conditions of the native tissue. By using heart-on-a-chip platforms, different kinds of signals can be applied to the cell microenvironment to direct them on a desired path. For example, electrical stimulation,[198] cyclic mechanical shear stresses,[199] and strain[200] can be used to guide cells to resemble the physiological conditions in the body. Therefore, this novel technology has excellent potential to advance the cardiac tissue engineering field.

Microfabrication techniques and heart-on-a-chip systems have been developed to set up hypoxia/normoxia conditions and cardiac cell behaviors *in vitro* for pharmacological and electrophysiological studies.[201] Novel micro-engineered systems precisely address the needs of the cardiac microenvironment

and in particular cell–ECM interactions *via* different entering signals (*e.g.* topographical and biochemical cues), which were explained in the previous sections for the specific signal in detail. Heart-on-a-chip systems such as the generation of micro-actuators of beating CMs[202] and heart-like pumps[203] have been used to mimic the native cardiac tissue environment to promote cardiac tissue maturation *in vitro*. The formation of micro-bioactuators is an approximately novel and progressing subdivision of microfabrication. This kind of system has a wide range of possible applications in drug delivery and testing drug in heart-on-a-chip systems. The development of micro-pumps is an emerging field of research in micro-bio-actuators, which have common properties such as high biocompatibility, absence of the need for any external power source, and creation of fluid flow using the contractile force of CMs to bend thin PDMS layers. However, the capacity of pumping of these systems is very low and needs to be improved in the future.

In the following sections, we will summarize the role of micro- and macro-scale bioreactors in the stimulation process to deliver mechanical and electrical cues to the cells for the formation of engineered functional cardiac tissues.

3.2.4. Mechanical signals. Cardiac tissue has an elastic nature and is exposed to continuous mechanical stimulation. Therefore, the synthesis of biomimetic materials for the replacement of myocardium tissue requires a mindful focus in the field of mechanical signals effects on cardiac cells behaviors. These mechanical cues influence the biochemical pathways of native CMs and changed cellular behaviors by extracellular stress and intracellular tension. Then, CMs respond to the mechanical signals *via* integrin binding followed by the activation of receptor tyrosine kinase and cell membrane GTPase.[204]

An ideal myocardium construct should imitate the anisotropic and complex hierarchical structure of cardiac ECM while resisting the native mechanical deformations present *in vivo*. Passive mechanical cues such as scaffold stiffness and active mechanical cues such as cyclic stretch have been previously shown to direct cells to form 3D applicable engineered tissue.[205] Either passive or active mechanical signals can increase cell alignment;[206] enhance sarcomere organization,[207] contractile properties,[208] and calcium handling;[209] and upregulate the expression of cardiac-specific genes and proteins.[210]

Scaffold stiffness and cyclic stretching forces are the main approaches of employing mechanical cue to the cardiac cells. However, in reality, mechanical signals are imported into cells *via* shear stress induced by blood flow, compressive forces such as hydrostatic pressure, and scaffold topography.[211] The effect of substrate stiffness on cardiac cell behavior was explained in the previous sections. In this section, we will discuss the bioreactor environment in which cyclic stretch is applied.

In the context of active mechanical signal influence, a static stretch is loaded to the scaffolds by either a time-dependent enhancement in stretch over time or an early-stretch subsequent plating that is continued by the time in culture. The recent studies not only proved the importance of mechanical cues for CM growth and proliferation but also facilitated the engineering of functional cardiac tissue for clinical applications.[199]

Inspired by the effects of mechanical cues in the native cardiac tissue, the creation of dynamic stretch in the bioreactors can imitate mechanical stimulations with enough accuracy. Most of the bioreactors have been designed to load cyclic stretch on the engineered cardiac scaffolds.[212–215] In one of the early bioreactor designs to confirm the key role of dynamic stretch for the fundamental improvement in cardiac tissue function, the isolated neonatal rat CM was used and encapsulated inside the combination of Matrigel and collagen I gel.[216]

Many attempts have been made to indicate the effect of dynamic stretch on intracellular organization,[217] intra- and extracellular tension, focal adhesion formation,[218] gene expression,[219] and protein expression.[220] Mechanical stretch has a different influence on cardiac cell behavior. For instance, Lux *et al.*[221] used a promising technique to investigate the effect of cyclic mechanical stretch and perfusion on cardiac cell behavior within the scaffolds with clinically relevant dimensions. They applied a bioreactor system to load cyclic stretch and perfusion simultaneously to observe the maturation process in the large-scale (2.5 × 4.5 cm) decellularized scaffolds based on Biological Vascularized Matrix (BioVaM) and 3D scaffolds containing neonatal rat CMs. Their results showed that the use of cyclic mechanical stretch not only improved the contractility, alignment and gene expression of CM markers but also enhanced the vascular network formation of endothelial cells within the cardiac scaffolds. They also studied the effect of cyclic mechanical stretch dimensions and perfusion of the starter matrix on cardiac cell maturation.

It is also well known that mechanical signals affect the stem cell differentiation and maturation process by the alteration in gene and protein expression. Li *et al.*[222] investigated whether the elastic modulus of highly flexible poly(*N*-isopropylamide) (PNIPAAm) hydrogels can stimulate the encapsulated cardiosphere derived cells (CDCs) to differentiate into cardiac cells under static and dynamic conditions. They developed hydrogels with a similar chemical structure, which their elastic modulus did not alter under static conditions and dynamic stretching for 14 days. Under dynamic conditions, a stretching force (12% strain and 1 Hz frequency) was applied to CDCs encapsulated into the hydrogels to mimic the native cardiac tissue conditions. The results showed that CDC growth was stimulated under the dynamic stretching conditions and the maximum differentiation of CDCs was observed in the hydrogels with a stiffness of 40 kPa.

3.2.5. Electrical signals. It was indicated that the design of bioreactors, which generate electrical signals, is simple. The carbon or platinum electrode pairs could be set in a certain device to mediate the electric pulse through the culture medium.[223] Monophasic or biphasic electrical cues can be delivered to the cardiac cells continuously or in a pulsatile manner.[224] However, effective, microscale accuracy with local

motivation cannot be obtained with these simple systems of electrode pairs. To overcome this limitation, multielectrode arrays (MEAs) have been developed using lithographic techniques.[225] The main advantage of MEAs is the generation of electrical fields in a micro-scale area for the stimulation of a single cell.[226] Besides MEAs, 2D metal electrodes with more limited applications were recently used which were deposited on glass substrates.[224]

It is well known that bioelectrical stimulation and transmembrane potentials have an important role in cardiac cell behaviors and the myocyte contractions are controlled by the electroconductive networks. For the high-level performance of the heart, appropriate electrical connection between cardiac cells is essential for proper signal communication.[227] Thus, electrical stimulation can induce synchronous contractions in the cardiac scaffolds and influence the rate, time and number of action potentials in CMs. It can enhance the percentage of naturally beating cells, improve synchronous contractions, increase tissue homogeneity and interconnectivity and finally result in higher contraction force production. Several studies indicated that electrical stimulation improves functionality[227] and induces the differentiation of stem cells toward cardiac lineage with different efficiencies and specific gene regulations.[228,229]

Natural hydrogels such as collagen and chitosan are not electrically conductive *in vitro*.[230] Therefore, it is necessary to use new biomaterials for obtaining regular contractions across a 3D engineered tissue. Thus, in some studies, electrically conductive scaffolds have been developed by the addition of conductive materials such as graphene oxide,[231,232] carbon nanotubes,[233] and Au nanorods.[234] For instance, Shin *et al.*[233] incorporated carbon nanotubes within GelMA hydrogels to enhance the electrical conductivity and seeded neonatal rat CMs on their surface. According to the authors, carbon nanotubes improved the mechanical properties and electrical conductivity of the hydrogels and consequently cell adhesion, alignment, organization, and maturation. In another study, a conductive hybrid hydrogel was fabricated from GelMA and reduced graphene oxide (rGO). The authors evaluated the hybrid hydrogel biocompatibility by culturing primary CMs onto rGO-GelMA samples and observed that the cellular DNA content did not change during 9 days for all samples proving that the hydrogels were not toxic. The results showed that the combination of rGO and the GelMA hydrogel considerably increases both the electrical conductivity and mechanical features and improves cell viability, proliferation, and maturation in comparison with ones seeded on the hydrogels without rGO. Furthermore, they examined the CM phenotype by immunostaining and found that the rGO-GelMA samples provided better construction with uniaxial and well-aligned sarcomeric structures than pristine GelMA scaffolds. They indicated a more homogeneous distribution of Connexin43, which is responsible for the fast transduction of beating signals and reported that CMs showed improved cell–cell coupling, improved contractile properties and faster spontaneous beating rate on the rGO-GelMA hydrogel than on pristine GelMA scaffolds.[231] Recently, carbon nanofibers were used to fabricate a conductive chitosan-based scaffold by the precipitation technique. The results showed that chitosan/carbon scaffolds were conductive and highly porous, with suitable connections between pores, and their elastic moduli were comparable to that of rat myocardium.[51] Navaei *et al.*[234] developed a conductive scaffold of the incorporation of Au nanorods and GelMA to increase the functionalities of cardiac cells. The results showed that the addition of GNR leads to excellent cell adhesion and cell viability and improved metabolic activity.

Although the addition of the mentioned materials resulted in a supporting scaffold for primary CM proliferation *in vitro*, there are two main problems related to finely tune the mechanical properties and conductivity of scaffolds and proper dispersion of the additive material in polymeric matrices.[230]

Except carbon nanotubes, graphene and a few metals, some synthetic polymers such as polyaniline[235] and polypyrrole[236] have electrical conductivity properties. However, these conductive polymers still show the main technical restrictions such as cytotoxicity, lack of cell binding sites, low solubility and biodegradability, variation of their physical features and high expression of pro-inflammatory cytokines.[230]

To overcome these challenges, Noshadi *et al.*[230] recently used bio-ionic liquid (Bio-IL) in combination with GelMA. Choline-based Bio-ILs are organic salts, which are water soluble, ionic conductive and stable electrochemically, biocompatible and non-toxic. The results showed that Bio-IL conjugated hydrogels had high electrical conductivity and biocompatibility *in vitro* and *in vivo* and support primary CM growth and function. Another advantage of these conductive hydrogels was related to the biodegradation process, which leads to low immunogenicity in rats during implantation. Therefore, it can be predicted that the application of Bio-ILs in biomedical and tissue engineering will increase due to their excellent properties.

The early combined electrical and mechanical stimulation was investigated in cardiac tissue constructs by Morgan *et al.*[199] They used pressurized air to induce cell stretching and carbon rods for electrical stimulation and then compared the effects of the mechanical or electrical stimulation separately and with simultaneous electrical and mechanical stimulations. They observed no significant difference between individual mechanical and electrical stimulations while the combined electromechanical stimulation upregulated the expression of SERCA2a and TnT proteins after two weeks of culture.

Barash *et al.*[187] developed a perfusion bioreactor to provide a homogeneous interstitial fluid flow throughout the cardiac cell construct and controlled electrical stimulation. The results confirmed the ability of medium perfusion to induce cardiac tissue formation and electrical stimulation to enhance cell elongation, striation, and upregulation of the gap junction protein, Connexin43, by 4 days after cell seeding. However, the effect of these cell stimulation modes is still limited in improving the contractile features for thicker patches. Radisic *et al.*[184] studied the effect of electrical stimulation in CMs growth by

using electrical pulses (rectangular, 3–4 V cm^{-1}, 1 Hz) to imitate the native myocardium. They reported that electrical stimulation improved the conductivity and contractile features of the cardiac scaffolds by increasing the levels of sarcomeric α-actinin, actin and Troponin I considerably.

Furthermore, the influence of electrical stimulation can also be assessed in blending with other biochemical signs, such as small changes in integrin binding and growth factor delivery. For example, insulin-like growth factor-1 (IGF-1) is recognised to maintain CMs from hypertrophic and oxidative tensions and increase survival subsequent injury. Park *et al.*[237] cultured cardiac cells on a biodegradable, micro-patterned PGS scaffold and added IGF-1 with electrical stimulation. According to their reports, after 8 days all scaffolds could be paced and expressed the cardiac protein troponin-T. Their results showed that Connexin43 expression and cell diameter increased in the samples with both IGF-1 and electrical stimulation more than the scaffolds with electrical stimulation and IGF-1 individually. They found that IGF-1 reduced apoptosis, enhanced cell-to-cell connectivity, and decreased the excitation threshold which is an index of electrophysiological activity.

4. Computational simulation strategies

Bioreactors have been designed in biotechnological applications to meet the cell culture environment necessities *via* controlling effective parameters like oxygen, temperature, pH, nutrients, metabolites and biologically active molecules. Since the design of a bioreactor is a major determinant of cell behavior, hydrodynamic conditions of bioreactors should be evaluated for the suitability to apply physiologically acceptable cues. However, the parameters enabling the success of this process are not clearly understood.[238] Therefore, more research needs to be done to develop and optimize bioreactors conditions which provide a suitable environment to direct the cell's behaviors. The complex correlation between the hydrodynamic conditions and neighboring space of the tissues directly impacts on the design of suitable bioreactors.

One way to understand the hydrodynamic properties of a bioreactor is to use flow visualization methods for exploring fluid flow behavior before performing any cellular experiment. Particle image velocimetry (PIV),[239] tracer liquid image velocimetry (TLIV),[240] and laser-induced fluorescence (LIF)[241] techniques are some experimental visualization methods that have been used to understand the hydrodynamic conditions in the bioreactors. Although these experimental methods are reliable, they are time consuming and require extensive image processing. Thus, computational fluid dynamics (CFD), as a cost-efficient tool, has been applied to provide a better understanding of the role of the hydrodynamic environment and the factors that modulate it. Computational models are crucial for enabling the prediction and subsequent testing of a large number of parameters that influence cells, tissues, and organs in a tissue engineering context.

The equations governing a 3D fluid field are complicated partial differential equations and hence do not have an exact solution. By discretization of these equations and truncation of higher-order terms, unsolvable equations would transform into solvable equations, which represent an approximate solution for the problem. Indeed, CFD could be used for not only solving the fluid flow, but also for solving any equations describing the electrical field, or chemical concentration of species within the fluid domain. This feature distinguishes computational methods from other experimental visualization methods.

Another advantage of CFD is to enable the visualization of the flow phenomena when it is infeasible to position probes within the fluid domains to measure a couple of parameters such as pressure and velocity. Despite these important advantages, numerical methods have some limitations. For instance, an excessive error can be generated when they are used alone in bioreactor design. Indeed, computational methods are usually coupled with experimental analysis to ensure that the simulation results are trustworthy. This limitation of CFD is not in contrast with the idea of substituting experimental studies with computational simulations because by using mathematical models, the number of experimental tests would decrease dramatically and the experiments are only for the validation of the CFD-derived results. Sucosky *et al.*[242] were among the pioneers to study the experimental and numerical characterization of the flow field within a spinner bioreactor under operating conditions. They carried out experiments in a scaled-up model bioreactor using the PIV approach to investigate two parameters, the velocity and shear rate, in the vicinity of the construct. They also performed numerical computations by using software to simulate the flow pattern in a similar bioreactor under the same operating conditions.

Furthermore, Ramaswamy *et al.*[243] designed a new bioreactor for the tissue engineering of the heart which was able to induce mechanical signals including cyclic flexure, stretch, and shear stress. They performed 3D CFD simulations to examine the effects of flow rate and viscosity on specimens within the bioreactor. Shear stress distribution on the surface of the specimens was determined during each cycle of flexure and/or stretch to check its magnitude with the physiological level. They found that applying shear stress up to 9 dynes per cm^2 combined with cyclic deformation for two weeks increased the collagen content per DNA mass by ~35% compared to those specimens subjected to cyclic deformation and non-physiological shear stress. In another study, Pavesi *et al.*[191] have developed a simple and cost-effective bioreactor and provided controlled electrical stimulation to explore the impact of electric field in cardiomyogenesis. This bioreactor contained an electrical stimulator and 12 culture chambers (Fig. 4a–c). They also applied a 3D computational model to investigate the distribution and the intensity of the electric field in the vicinity of the cell culture volume. This numerical model enabled them to characterize the alignment and uniformity of the elec-

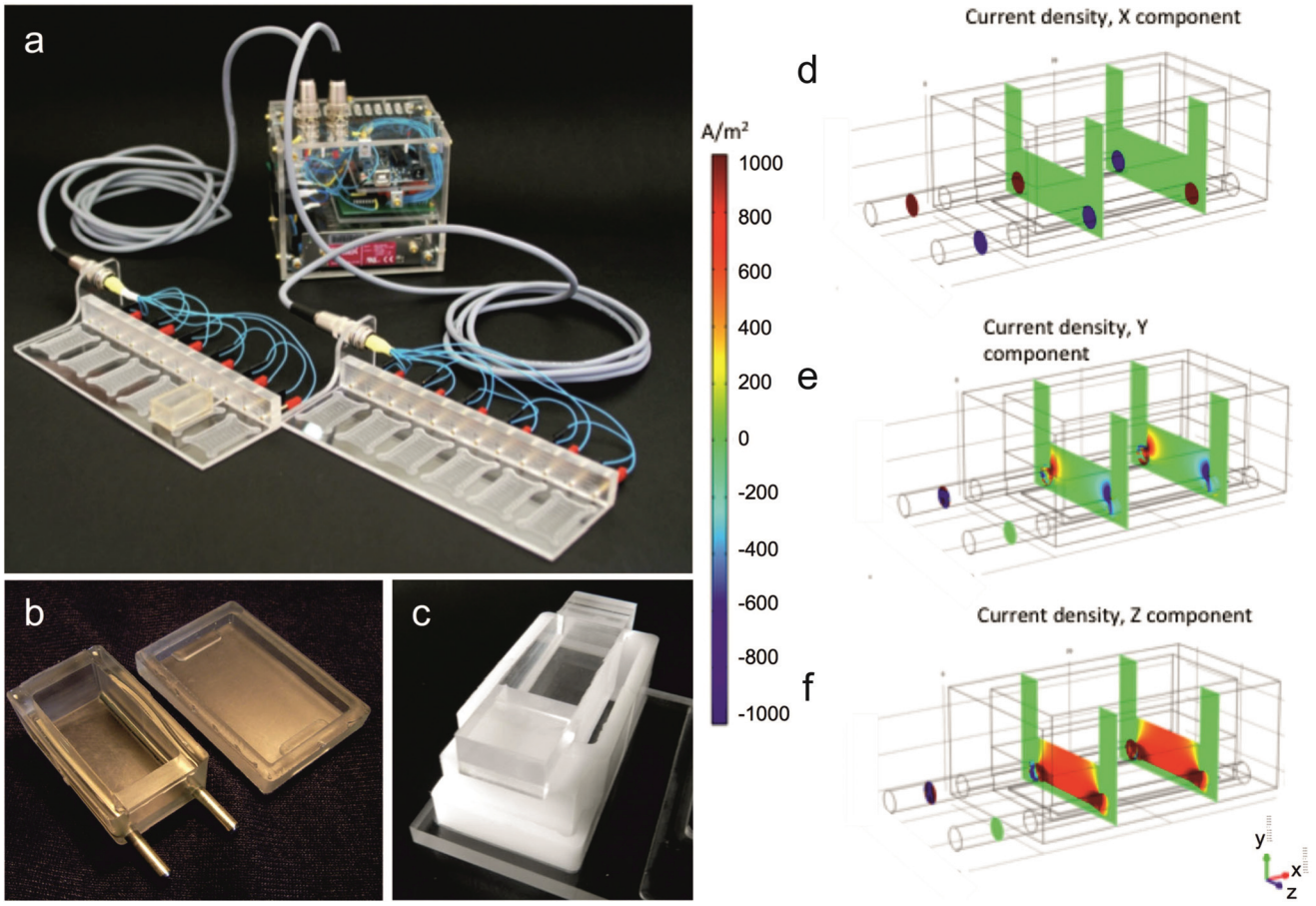

Fig. 4 (a) The bioreactor structure. (b) Rectangular-shaped PDMS culture chamber. (c) Manufactured mold to generate culture chambers. (d–f) 3D computational model results of the current density components on a cross-sectional plane of the bioreactor. Image reproduced with permission from ref. 191.

tric field and help them to avoid field distortions, practically (Fig. 4d–f). Consequently, they could find precise conditions to direct adult stem cells toward a myocardial phenotype by physical stimuli without using additives and expensive bioactive molecules. In addition, they found out that the electric field uniformity was dependent on the culture medium volume; thus the desired uniformity could be achieved by quantifying the culture medium volume.

5. Conclusion and future perspectives

Cardiac tissue engineering holds great promise in the field of regenerative medicine due to the increasing number of patients with heart disease and the limited availability of organ donors. Cardiac tissue engineering strategies employ various biomaterials and cell sources to fabricate biomimetic constructs, aiming to construct a functional tissue to restore the function of a diseased or defunct heart. An engineered functional cardiac tissue needs regular contractive well-aligned cardiac cells, which provide support for cell growth and a well-designed practical bioreactor to mimic the native tissue conditions. To synthesize functioning 3D constructs, the optimum topographical, biochemical, mechanical, and electrical cues should be delivered to the cells to control their behavior. Therefore, it is necessary to understand the role of different stimulus signals on pathway development that results in a fully functional cardiac tissue. In the generation of cardiac patches, which meet clinical demands, macro- and microscale bioreactors have been developed to optimize all of the needed signals in the cell microenvironment. In addition, it might be beneficial to develop perfused bioreactors, which use simultaneous electrical and mechanical signals. This direction is essential to meet the urgent need to treat numerous patients. Some challenges for the application of classical scaffolds for thick tissues such as the heart are the lack of vascularization and controllable pore size. Therefore, novel microfabrication techniques have been developed to induce the vascularization processes and consequently enhance cell viability. These innovative approaches are molding with microneedles or soft lithography and bio-printing. However, the other major challenges such as CM maturation, organization, and survival and synchronised beating should be considered to fabricate proper cardiac

constructs. Therefore, new technologies should be developed to control the cell organization precisely in 3D constructs. Both experimental and computational methods are capable of solving the fluid flow parameters to compare the results with the *in vivo* recorded data. CFD is a potent, time-consuming, cost-effective tool to expedite the process design of the bioreactors. As custom-made bioreactors for specific tissues are under development, the need for CFD contribution in attaining functional engineered cardiac constructs will increase.

Conflicts of interest

There are no conflicts to declare.

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