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Micro- and nanotechnology in cardiovascular tissue engineering

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Abstract
While in nature the formation of complex tissues is gradually shaped by the long journey of development, in tissue engineering constructing complex tissues relies heavily on our ability to directly manipulate and control the micro-cellular environment *in vitro*. Not surprisingly, advancements in both microfabrication and nanofabrication have powered the field of tissue engineering in many aspects. Focusing on cardiac tissue engineering, this paper highlights the applications of fabrication techniques in various aspects of tissue engineering research: (1) cell responses to micro- and nanopatterned topographical cues, (2) cell responses to patterned biochemical cues, (3) controlled 3D scaffolds, (4) patterned tissue vascularization and (5) electromechanical regulation of tissue assembly and function.

(Some figures in this article are in colour only in the electronic version)

1. Introduction

Tissue engineering aims to control the cellular environment to guide cell assembly and build a functional tissue from the bottom up with an aim of providing replacements for diseased or damaged native tissues. Additionally, engineered tissues may serve as models for studies in physiology, pathophysiology and drug testing. Heart failure in particular is a leading cause of death in the United States [1]. One in five people will develop heart failure in their life time. Such high risk is fuelled by the intrinsic inability of the heart to regenerate itself after injury. Upon myocardial infarction, the heart goes through an extensive period of remodelling that includes neurohumoral activation, cardiomyocytes death and fibrosis. As a result, the damaged tissue will lose contractile function. Reduction of contractile functions will initially be compensated by ventricle wall thinning and remodelling, thus resulting in maladaptive structure that may ultimately lead to heart failure [1].

The ultimate goal of cardiac tissue engineering is to heal the heart muscle by replacing the damaged tissue with engineered tissue patches capable of re-establishing normal contractile function and preventing pathological remodelling. However, growing such functional tissue constructs requires extensive control over the cellular environment. A functional cardiac tissue requires [1] well aligned myocytes with synchronized contraction [2], supportive extracellular matrix and scaffold structure to mimic the mechanical properties of the native tissue [3] and functional microvasculature to provide adequate nutrient and oxygen delivery within a tissue of clinically relevant thickness (∼1 cm). This engineering task will require collaborative efforts from multiple disciplines such as biomaterial science, microfabrication, stem cell biology etc [2].

Current advancements in microfabrication and nanofabrication techniques have touched many aspects in tissue engineering [3]. Micropatterning of both biochemical and topographical cues is well established to guide cell morphology and functionality. Micro-engineered scaffolds have shown remarkable mechanical properties that resemble native tissue. Microfabricated channel networks have demonstrated great potential for building microvasculature ready for perfusion.

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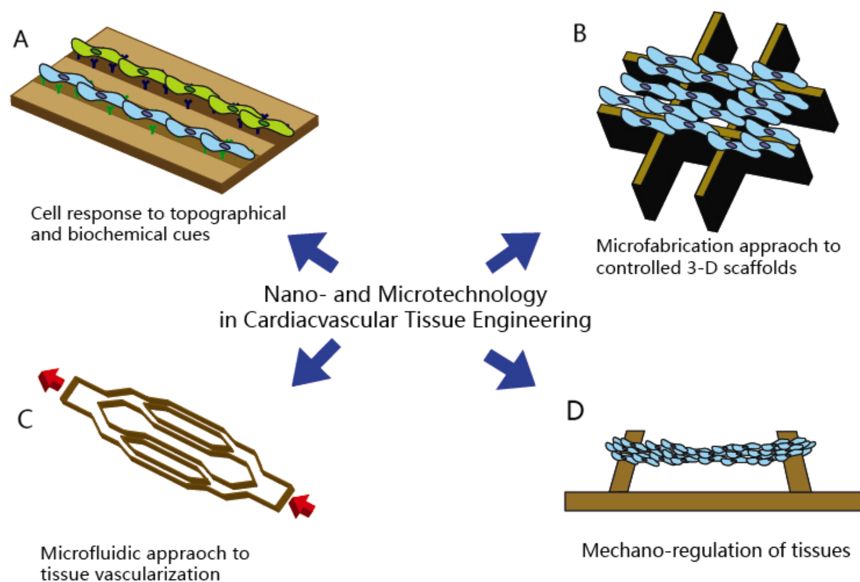

Figure 1. Micro-and nanotechnology in cardiovascular tissue engineering. (A) Cell response to topographical cues such as micro-and nanofabricated grooves and biochemical cues such as growth factors. (B) A microfabrication approach to controlled 3D scaffold generates structures that can assist cell assembly and alignment while mimicking the mechanical properties of the native tissue. (C) Microfluidic approach to tissue vascularization with micropatterned vasculature networks. (D) Mechano-regulation of cardiac tissue function can be examined by self-assembly of organoids around micro-posts.

Micro-cantilever based systems have been used to either measure the contractility of microtissues or induce cell-extracellular matrix (ECM) remodelling guided by matrix tension.

This paper covers the use of micro-and nanotechnologies to control the structure and function of cardiovascular tissues. Specifically, we will focus on the cardiac muscle, myocardium and methods for obtaining tissue engineered microvascular beds. We will discuss microfabrication approaches to topography and biochemistry mediated cell assembly, precisely engineered scaffolds, functional vascularization and electromechanical cell coupling (figure 1).

2. Cell responses to micro- and nanopatterned topographical cues

It is well known that the architecture of the extracellular environment influences cell behaviour with respect to morphology, cytoskeletal structure and functionality [4]. Thus, substrates with different architectures can serve as platforms to study the effects of cell–substrate interaction on cell adhesion, orientation, migration and differentiation. It is also necessary to replicate, as closely as possible, the *in vivo* environment in terms of dimensional, configurational and compositional properties when designing tissue replacements [5]. Furthermore, to study the tissue, cellular and subcellular responses to different architecture features, the substrates should be fabricated with a high level of control over micro- and nano-scale features. Microfabrication, an emerging technique in tissue engineering, provides excellent control of the substrate microarchitectures and a platform to study the mechanisms of cell–substrate interactions (figure 1(A)) [6, 7].

The alignment of cardiomyocytes is important for the contraction and impulse propagation along the long axis of the cells [1]. It has been demonstrated that microfabricated substrates with micro-scale grooves can significantly enhance cell alignment [8, 9]. Moreover, the alignment of cardiomyocytes resulted in a greater expression of cardiac genes and cardiomyocyte markers, as well as gap junctional proteins such as connexin43 (Cx43) [10, 11]. Many studies have focused on reproducing this alignment with micropatterned substrates [8, 10, 12].

Due to the difficulty of separating cardiomyocytes from contaminating nonmyocytes, such as fibroblasts, from the primary cell source it would be beneficial to limit the proliferation of the nonmyocytes in the resulting heart cell cultures. Boateng *et al* found that the micropatterned substrates with arrays of micropegs (10 μm tall extruded cylinder providing a vertical surface for cell attachment) significantly inhibited fibroblast proliferation compared to the smooth flat substrates [13]. It was subsequently determined [14] that this effect was a result of the elongation in fibroblasts and the change in their nuclear shape.

The topographical cues regulate the structure and function of cardiac tissue constructs in the nano-scale as well. Fabricating on the nano-scale, although similar to the micro-scale in general procedure, has more stringent requirements on the properties of the master substrate used in order to resolve features on the nano-scale. Poly(dimethyl siloxane) (PDMS) has low elastic modulus ($\sim$1.8 MPa); thus it is difficult to obtain high aspect ratio features below 100 nm [15]. In addition, its high compressibility ($\sim$2.0 N mm^{-2}) tends to deform, buckle or collapse the relief features [15]. Lastly, due to the high surface tension relief features often have rounded corners. An alternative used to replace PDMS for nano-

scale fabrication is ultraviolet (UV) curable moulds made from poly(urethane acrylate) (PUA). PUA can be used to mould features as small as 50 nm on biomaterials such as polyethylene (PEG) hydrogels [16].

Kim *et al* [17] constructed an anisotropically nanofabricated substratum with PEG hydrogel patterned with arrays with groove sizes ranging from 150 to 800 nm to closely reproduce a nano-scale structure of the myocardial ECM composed of aligned fibrils approximately 100 nm in diameter. Although a single cardiomyocyte's width allows it to span more than ten nanoridges, the cell is still aligned along the direction of the topographical cue. Analysis showed that this alignment was due to the organization of focal adhesions, which followed the nano-grooves resulting in the organization of the cytoskeletal proteins and subsequent cell elongation. There were considerable differences between the non-aligned and aligned cell cultures in features important for cardiac tissue function such as: cell geometry, conduction velocity, and Cx43 expression, with aligned structures exhibiting properties similar to the native heart. This study also suggested that there was an optimal topography for cell–cell coupling, which maximized the area of cell–substrate contact.

Even though topographical cues on both the micro- and nano-scale can induce cell response, it is important to note that the mechanism might be different between these two approaches. Micro-scale topographical cues generally include features with sizes similar to the size of a single cell, thus might induce cell response through geometrical constriction or guidance. On the other hand, nano-scale topographical cues generally have features that are much smaller than single cells, thus might induce cell response through a more fundamental means such as stimulation or signalling of the cell surface receptors. In addition, nano-scale patterning resembles the size of the extra-cellular-matrix (ECM) in the native environment while micro-scale patterning often creates artificial constraints that may not be present *in vivo*.

Controlling cell assembly and alignment in 2D provides insights on how cardiac cells respond to these topographical cues and the extent of these responses. However, building a functional cardiac tissue that is relevant for clinical applications requires expanding topographical cues to 3D. For a clinical application, a single sheet of cardiomyocytes may not be sufficient. Aligning cardiomyocytes in 3D will enable much greater contraction forces that can match the native tissue and provide adequate functionality to replace damaged tissues. Micro-and nanofabrication techniques provide a method to control geometrical patterns in 2D on a plane surface; however, these techniques may not directly translate to 3D. Therefore, assembling well organized and defined structures in 3D has to be achieved by combining these techniques with other means.

Controlling cardiomyocyte alignment in a 3D construct was often achieved by generating well aligned and defined nano-fibres through spinning techniques. Mohammad *et al* [18] produced nano-fibres (ranging from 50 to 3500 nm in diameter) embedded with neonatal rat cardiomyocytes by rotary jet-spinning replacing the conventional electrospinning technique which requires high-voltage electric fields that are detrimental to cells [19]. Fibre morphology and diameter as well as porosity of the resulting scaffold were controlled by controlling the nozzle geometry, rotation speed and polymer properties. Highly aligned fibres induced cardiomyocyte self-organization and alignment into a beating tissue construct mimicking the laminar, anisotropic, architecture of the heart muscle. Culturing cardiomyocytes in a topographically controlled 3D environment resembles more closely the *in vivo* conditions. This is a significant advantage over 2D culture where cell–cell communication and assembly are artificially constrained to a plane.

Recently, it has been reported that the cardiogenic differentiation of human embryonic stem cells (hESCs) can be regulated by controlling the size of embryoid bodies (EBs). The size of the EBs can be controlled by the topographical features of the substrates [20–23]. By plating single-cell suspensions onto micropatterned extracellular matrix islands, size-specified hESC colonies formed under physical and extracellular matrix patterning constraints and maintained undifferentiated proliferation. For instance, by allowing suspended cells to settle into microfabricated micro-wells these wells can physically constrain the aggregation of the cells and thus control the size of the EBs based on the diameter and depth of the micro-wells. Hwang *et al* have fabricated arrays of wells with sizes ranging from 150 to 450 μm on poly(ethylene glycol) (PEG) hydrogel. These colonies grew into monodisperse EBs after transferring into suspension culture [23]. Gene and protein expression analysis of these micropatterned hESC populations revealed that different differentiation trajectories were affected by the size of the EBs [20]. Specifically, cardiogenesis was enhanced in larger EBs. Subsequent study that combined this technology and a controlled bioreactor system for EB cultivation demonstrated improved cell growth and cardiogenic differentiation of hESCs [21]. Hwang *et al* also demonstrated that the EB-size mediated differentiation was driven by differential expression of WNTs [22]. Other than topographical control, the size of the EBs can also be controlled through microcontact printing techniques. Similar effects can be achieved controlling the size of each cell clusters by limiting the size of patterned ECM. Niebruegge *et al* [21] have demonstrated large scale production of EBs with uniform sizes with microcontact printing. In addition, they have shown improved cardiac differentiation due to the better control over the size of the EBs.

3. Cell responses to patterned biochemical cues

Not only is the ability to precisely control the geometry of the attached cells important, advancements in microfabrication technologies including microcontact printing, microfluidic patterning and photolithography, offer additional opportunities to control surface chemistry pattern geometry, density and bioactive molecule specificity.

Cardiac tissue engineering applying microcontact printing (μCP) and microfluidic patterning (μFLP) will be reviewed here. μCP is the most widely used soft lithographic technique due to its simplicity, cost effectiveness and flexibility. An elastomeric stamp casting over a microstructured master is

used. In direct printing, the stamp prints a selected cell responsive protein, whereas, in indirect patterning, a secondary bioconjugate modifies the areas of interest which in turn can be functionalized with counter-conjugated protein (e.g. Biotin–avidin). Cimetta *et al* have used μCP to pattern laminin onto soft polyacrylamide-based hydrogels photopolymerized onto glass and showed patterns of cardiomyocytes forming myofibres [24]. Furthermore, to investigate the role of microfibrillar structure and organization on cardiomyocyte contractile function, Parker *et al* patterned square ECM islands and showed that cardiomyocytes reoriented their stress fibres and focal adhesions to concentrate their tractional forces in the corner regions, in contrast to circular patterns, where edges and corners were absent, and myofibrils assembled in a random manner [25]. The μCP enabled geometrical cues provided a platform to study mechanical interactions between cardiomyocytes and ECM that modulate cytoskeleton tension. In addition to the basic geometry patterning, to simulate the tissue's natural structure, high-resolution diffusion tensor magnetic resonance imaging (DTMRI) has been combined with μCP to fabricate a 2D replica of heart tissue. The orientation of the myofibers was measured by DTMRI and the images were converted to soft-lithography photomasks used to make silicon masters and polydimethylsiloxane (PDMS) stamps. The PDMS stamps were used to create fibronectin lanes in the same orientation as the cardiac myofibres in the native heart, followed by the seeding and cultivation of cardiomyocytes on the transferred fibronectin patterns. With optical mapping, it was shown that microscopic changes in cell orientation and ventricular tissue boundaries independently and synergistically increase the spatial dispersion of conduction velocity propagation [26, 27]. The ability to create cardiomyocyte micropatterns mimicking cell orientation in the native heart allows structure–function studies bridging to 3D studies.

To extend to 3D, Feinberg *et al* took a unique approach by culturing neonatal rat ventricular cardiomyocytes on ECM protein micropatterned polydimethylsiloxane thin films that could be released from a thermally sensitive polymer substrate and constructed to create functional three-dimensional conformations [28]. By varying tissue architecture, thin-film shape, and electrical-pacing protocol, with fine spatial and temporal control, the constructs can perform tasks such as gripping, pumping, walking and swimming. Another study by Domian *et al* showed seeding of progenitor cells, isolated by expression of Isl1-dependent enhancer of the Mef2c gene or cardiac-specific Nkx2.5 enhancer [29]. The cells aligned on micropatterned surfaces and formed a beating muscular film *in vitro*.

In our laboratory, to promote site-specific vascular differentiation for vascularized cardiac tissue engineering, Chiang *et al* applied μCP to pattern vascular endothelial growth factor-A immobilized onto collagen IV onto a non-cell adhesive chitosan layer. It was shown that Flk1 + progenitor cells derived from mouse embryonic stem cells preferentially differentiated to endothelial cells on an immobilized vascular endothelial growth factor (VEGF) surface, and to smooth muscle-like cells on surfaces without VEGF [30].

Some limitations to μCP include the range of mechanical strength of the stamp material that must be soft enough to enable conformal contact with the substrates, yet rigid enough to create precisely defined micropatterns. The resolution and possible geometries are thus constrained. Creating multicomponent surfaces with three or more molecular species requires applying different inks onto the chemically patterned stamp or micro-aligning and repeating the patterning procedure. Stencils can be used for this type of multicomponent surface functionalization and cell co-culture [31].

In μFLP, microfluidic channels created by the stamp are used to deliver fluids to selected areas of a substrate. Khademhosseini *et al* reversibly sealed PDMS microfluidic mould on a glass slide and flowed through cell adhesive molecules. Excess fluid and mould were removed from the surface leaving physisorbed patterned molecules. In this study, contractile cardiac organoids were formed with preferential adhesion of neonatal rat cardiomyocytes to fibronectin over hyaluronic acid [12].

Photolithography utilizes geometric features drawn on a mask transferred by UV illumination onto a substrate, a spin-coated photoresist or a photo-crosslinkable biodegradable polymer. After photoresist development, a thin layer of bioactive molecules such as peptides, proteins or bioactive polymers, is sometimes deposited. Using glass coverslips coated with positive resist with arrays of circular patterns 100–400 μm in diameter, Sasaki *et al* showed mouse ESC cultured in 200 μm diameter domains to be optimal for the cardiac differentiation [32]. Other photo-crosslinkable materials can also be used with no chemical crosslinker required. Karp *et al* used azidobenzoic acid modified chitosan spin-coated onto glass cover slips or tissue culture polystyrene. UV illumination through a patterned design mask resulted in cell-repellent chitosan patterns and cell adhesive glass area suitable for cultivation of cardiomyocytes [33]. Crosslinkable substrate modified with cell adhesion molecules can also be used directly in photolithography.

Overall, μCP induces various cell responses by stimulating the cell surface receptors and activating biochemical pathways. This approach is similar to nano-topography where cell alignment is induced also through surface receptor interaction. However, cell alignment induced through μCP by constraining the cells to the patterned area might be similar to constraining the cells through micro-topography. The exact mechanisms of these interactions and the activation of particular biochemical pathways still need to be further investigated.

4. Microfabrication approaches to controlled 3D scaffolds

Never resting, cardiac tissue contracts repeatedly, resisting fatigue, over three billion cycles throughout an average human lifespan [34]. The synchronized periodic contraction of cardiac tissue is made possible by the highly packed and aligned muscle cell network of the native tissue. To engineer a myocardial patch that mimics the native tissue

would require precisely controlled scaffold properties such as porosity for cell seeding and nutrient delivery, anisotropic mechanical properties for tissue contraction and cell alignment, biodegradability, and biocompatibility. Conventional bulk scaffold construction techniques such as solvent casting, particulate leaching etc [35] are inherently limited and fail to deliver the desired scaffold properties. However, the advancements in both microfabrication techniques and biomaterial science have enabled patterning of biocompatible materials layer by layer and thus precise control of the intrinsic structure of the 3D scaffold (figure 1(B)).

Albert *et al* [36] demonstrated the moulding of polyurethane in a PDMS based microfluidic device and subsequent stacking of multiple polyurethane structures to form a scaffold with a controlled interconnected network. Although only fibroblasts were cultured in this system, it served as the basis for microfabrication based scaffolds. Scaffolds microfabricated with biodegradable materials such as poly(DL-lactide-*co*-glycolide) (PLGA) [37] and collagen gels [38] were demonstrated with controlled cavities or features as small as 10 μm.

Giovanni *et al* demonstrated the difference between three different moulding techniques for scaffold fabrication (micro-moulding method, microfluidic method and spin coating method) with PLGA [37]. The micro-moulding method yielded a good lateral resolution but poor vertical resolution while the spin coating method yielded a high vertical resolution but poor lateral resolution. Both the micro-moulding method and the spin coating method tended to generate uneven surfaces. In contrast, the microfluidic method could generate flat surfaces with high lateral and vertical resolution, but the patterned geometry choices were limited due to the high pressure drop during injection [37]. In addition, they demonstrated that conventional techniques such as particulate leaching can be combined with microfabrication techniques to create even smaller randomly distributed pores between the micropatterned networks.

PLGA has rigid mechanical properties and loses its integrity rapidly through bulk degradation. These problems were overcome by new materials such as poly(glycerol sebacate) PGS, which maintains integrity during the degradation process through surface erosion [34, 39–41]. Freed and colleagues microfabricated accordion-like honeycomb scaffolds from PGS (figure 2(A)) [42, 43]. These scaffolds yielded anisotropic mechanical properties similar to the native myocardium that were preserved even after weeks of *in vitro* cyclic fatigue testing [42]. Furthermore, the stiffness of the structure could be fine tuned by varying the curing time of PGS. By subsequently seeding neonatal rat heart cells, the alignment and elongation of these cardiac cells within the scaffold pores was observed after one week (figure 2(B)). Single-layered scaffolds were microfabricated, two-layered scaffolds with fully interconnected pore networks were produced by stacking and lamination, and these scaffolds were seeded with heart cells and cultured with perfusion to yield synchronously contractile engineered tissue as demonstrated with electrical field stimulation [42, 43]. The platform was also extended to include micromoulded gratings on the surface of the scaffolds,

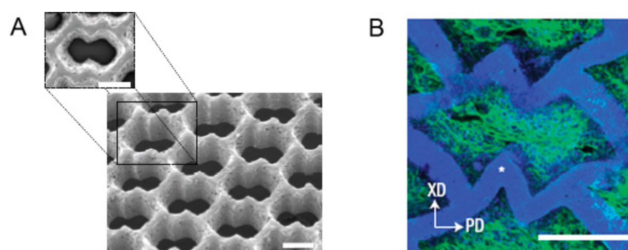

Figure 2. Accordion-like honeycomb scaffolds for tissue engineering of cardiac anisotropy. (A) Scanning electron micrographs show the structure of excimer laser micro-ablated accordion-like PGS scaffolds. (B) Neonatal rat heart cells, fluorescently labelled for F-actin (green) and counterstained for nuclear DNA (blue), cultured on the PGS scaffolds (blue) for one week. Scale bars (A–C) 200 μm. Adapted with permission from [42, 44] (Macmillan Publishers, Copyright 2008).

which further enhanced alignment of cultured muscle (C2C12) cells [44].

Other than 3D microfabrication, geometrically controlled scaffolds can also be generated through other nanofabrication methods such as electrospinning where biomaterials are spun into nano-fibres to create a 3D scaffold closely mimicking the ECM network *in vivo*. The advantage of using nano-fibres is that the created nano-scale mesh, although randomized, resembles more closely the native ECM than scaffolds created through microfabrication. Using an electrospinning technique, Chengyu *et al* [45] produced nano-fibres ranging from 400 to 800 nm in diameter with a biodegradable material, poly(L-lactide-*co*-ε-caprolactone)[P(LLA-CL)](75:25) copolymer. Human endothelial cells and smooth muscle cells were cultured on the scaffold for seven days. Both smooth muscle cells and endothelial cells were able to maintain their phenotype and integrate with the nano-fibre to form a three-dimensional cellular network.

To further enhance tissue organization, nano-fibres can be organized unidirectionally to promote cell alignment. Yuliya *et al* [46] produced aligned nano-fibres with prismatic cross sections (700–1000 nm in width and 300–500 nm in height) from polymethylglutarimide (PMGI) with electrospinning. The alignment of the nano-fibres was controlled by the collector which included a rectangular hole. The orientation and dimension of the rectangular holes controlled the orientation and length of the nano-fibres. The positioning density of the nano-fibres was controlled by varying the time of fibre deposition. Seeded cardiac cells proliferated and aligned into contractile tissue. The elongation and alignment of the cardiac cells were characterized by the orientation of the α-actin filaments. The significance of this work is the demonstration of both three-dimensionality of the scaffold and the resulting tissue as well as the structural anisotropy.

5. Microfluidic approaches to tissue vascularization

Other than achieving the desired structural and mechanical properties, to build a thicker tissue construct with clinically relevant dimensions (e.g. 1 cm in thickness) adequate vascularization is required to provide nutrient delivery and

oxygen transport. Methods to achieve this in scaffolds include control of porosity and pore size [47], controlled release of angiogenic growth factors [48, 49] or covalent immobilization of angiogenic growth factors to the scaffolds [50, 51]. Such methods are successful to only a certain extent, since they rely on promoting the in-growth of the host vasculature into the biomaterial. Overall, a vascular network takes a long time to develop (3–5 days at minimum) during which time cell survival cannot be guaranteed. In addition, the resulting vascular network is discontinuous and randomly distributed which limits nutrient perfusion and oxygen transport [52]. However, with microfabrication techniques, vascular tree-like organization can be embedded within biodegradable polymers and subsequently seeded with endothelial cells to form rudimentary vasculature (figure 1(C)). Such vascular networks will be continuous and may facilitate *in vivo* tissue integration through immediate anastomosis to the host vasculature [52].

Engineering vascular networks through microfabrication was first demonstrated on silicon and Pyrex wafers [53]. Silicon and Pyrex were single-level etched with branched vascular network composed mostly of densely spaced grooves (10–25 μm wide) mimicking the capillary bed. By coating the wafer with vitrogen, Matrigel or gelatin, seeded hepatocytes and endothelial cells could be lifted off as a monolayer sheet [53]. Biocompatible polymers such as PDMS, PLGA and PGS were also used to form enclosed channel networks where endothelial cells were seeded and allowed to grow to confluence in four weeks to form endothelialized microvasculature [54–56].

Several issues must be considered when designing the vascular network. Firstly, the pressure drop to provide adequate mass transfer across the network should not be greater than the one found under physiological conditions. This requires reducing the resistance across the network while maintaining tightly spaced capillary channels. Secondly, the flow pattern through the network should be uniform which requires the special consideration of the pressure drop distribution among the capillaries. Jeffrey *et al* had demonstrated a design that satisfies both criteria [54]. Thirdly, non-Newtonian fluids, such as blood that consist of two phases: plasma and cells, present additional complications with respect to the fluid dynamics of the vascular network. Fourthly, the interactions between the red blood cells and the walls will induce the migration of the red blood cells towards the centre of the channel which would reduce the transit time of the red blood cells (Fahraeus–Lindqvist effect). Finally, multiple cell types will be required to construct actual functional cardiac tissue with proper cell–cell interactions and alignment in addition to vascularization.

To move a step closer to vascularized functional tissue, cell seeded hydrogels (alginate, collagen and fibrin) moulded with a microvascular network were demonstrated [57]. Hydrogels were moulded with a vascular network by micropatterned meshes of gelatin that were subsequently melted to leave behind interconnected channels in the hydrogel. The microvascular channels were seeded with endothelial cells to form rudimentary endothelial networks, where culture medium was continuously perfused to feed the endothelial cells and cells within the hydrogel. Perfusion of propidium iodide to label dead cells showed a 96% survival rate which emphasized the critical role of nutrient delivery through vascularization. After culturing up to five days, cells deformed the sharp corners of the channel while the overall channel network was preserved to allow sustained perfusion of culture media.

With the system described above, the diffusion coefficient of relevant molecules and the rates of cell metabolism in the hydrogel can be measured and correlated with a quantitative convection–diffusion model. In fact, the diffusion of fluorescein and FITC-BSA within the micro-channel moulded calcium alginate hydrogel seeded with cells was studied extensively [58]. Nak *et al* examined their transient diffusion profiles under pulsed flow at different flow rates to determine their diffusivity in gel. This study indicated that the pore size of 4% (w/v) calcium alginate was too large to hinder the diffusive motion of both small and large molecules. Furthermore, the spacing between the channels was studied to determine the design rules for maintaining uniform metabolic environment within the bulk gel [58]. By combining microfluidic technology with biomaterial science, more realistic tissues can be created *in vitro* which is critical for further advancement in both clinical applications and basic biological studies.

6. Mechano-regulation of tissues on the micro-scale

The electromechanically dynamic properties of the heart are relatively complex and difficult to study at the macro level [59]. Microfabrication techniques have been employed to isolate many of these cell–cell and cell–ECM interactions to better understand how they affect the tissue remodelling capabilities of the various cell types found in the heart [60]. Both direct and indirect techniques have been advanced in the field which allow for exquisite control of mechanical stimulation and measurement parameters [61–65]. Development of high-fidelity microenvironments that accurately represent the *in vivo* niches and cells' native environment, such as substrate stiffness [66, 67], will enhance our understanding of the mechano-transduction biology in the heart.

Generating a physiologically accurate cardiac *in vitro* model is challenging. Much of the effort in interrogating and recapitulating the *in vivo* environment has been focused on the strains and stresses (post-diastolic expansion and post-systolic contraction respectively) encountered by cardiac myocytes and fibroblasts alike in the mechanically dynamic environment of the heart [68]. Two-dimensional monolayers have been largely used as a platform for studying the mechanical interactions of cells and their matrices [69, 70]. Although 2D models offer practicalities in experimental methods including the ease of isolation of different parameters, these models do not accurately mimic the physiology of the microenvironment of interest.

A primary obstacle with more realistic 3D models, however, is the diffusion limitation that is associated with thick tissues, both for oxygen and nutrient exchange [71] as well as for antibody-based immunofluorescence imaging.

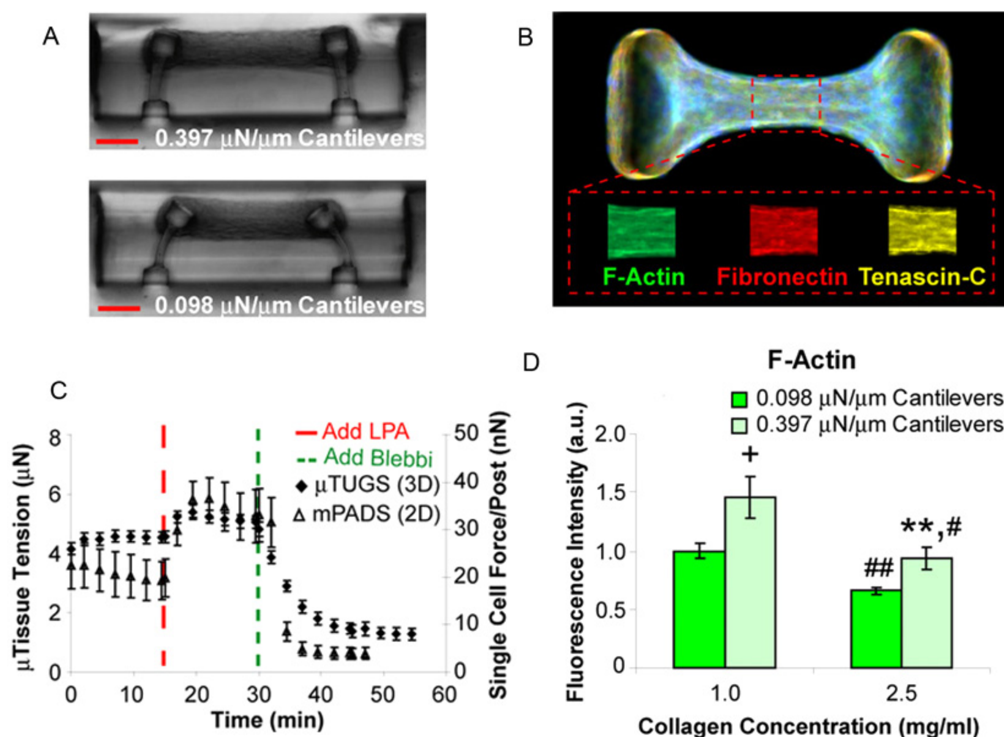

Figure 3. Microfabricated tissue gauges can be used to measure the temporal contractility response of microtissues. (A) Representative cross sections of fibroblast based microtissues tethered to rigid or flexible cantilevers. (B) Representative immunofluorescence overlay of cytoskeletal and ECM proteins within microtissues. Mean fluorophore intensity was measured over a 30 μm long segment at the tissue midsection using distinct fluorophores for each protein. Inset (C) the temporal response of 3D microtissues (closed diamonds) and single cells on 2D micro-needles (open triangles) in response to 10 g ml^{-1} LPA and 50 M blebbistatin. (D) Average relative fibrillar actin expression under each of the four combinations of collagen density and cantilever stiffness. Scale bar: 100 μm. Adapted with permission from [75].

Furthermore, the added dimension in a 3D tissue brings with it complexity in the analysis of cell–cell and cell–ECM interactions. Miniaturizing 3D tissues to the sub-300 μm scale to eliminate diffusion barriers greatly simplifies matters. Microtissues can also be generated with low volume components, and can be engineered to be highly controlled and reproducible. Hydrogel-based microtissues that self-assemble under gel compaction are a simple-to-generate system that is widely used for multiple cell types and matrices (figure 1(D) [72–74].

A study by Legant *et al* makes use of this technique alongside a microfabricated device to both measure and manipulate forces exerted by micro-scale tissues [75]. The study employed rectangular micro-wells containing two micro-cantilevers which simultaneously constrained the remodelling of the collagen with embedded 3T3 fibroblasts and reported real-time dynamic forces exerted by the contained microtissue (figure 3). Similar cantilever-based force sensors have also been employed for studying the traction force of single cells [76]. The study goes on to show that forces exerted by the cells (contractility), increased with tissue matrix and boundary rigidity, whereas protein expression associated with ECM and cytoskeleton correlated with mechanical stress. These types of microsystems are geared towards drug screens; compared to typical 2D culture systems used for screening purposes, these systems provide *in vivo* like organoid morphology and can serve as a validation to preliminary hits.

Tissues for eventual organ repair can also be engineered on a larger scale still employing microfabrication methods to achieve physiological properties. Bian *et al* have developed a method to generate aligned collagen-based tissue using a bed of macropillars [77]. The technique takes advantage of gel compaction around an array of 2 mm tall pillars; the geometry and spacing of the pillars are strategically designed such that the surrounding collagen remodelling results in tension-mediated alignment.

Controlling cell assembly and tissue formation are not only achieved through the micro- and nanofabrication approach, instead methods such as mechanical [78, 79] and electrical stimulation [80] also play a critical role in building a functional cardiac tissue. Both mechanical and electrical stimulation have been shown to induce cell alignment in both 2D and 3D tissue constructs. In addition, both have been shown to induce cell maturation, ultrastructural organization and improve functional properties of the tissue such as contractile force. These methods are discussed in detail in other review articles [81]. Combination of electrical, mechanical stimulation and micro/nano control of substrate topography is expected to significantly improve functional assembly of cardiac tissues *in vitro*.

7. Summary and future directions

Micro and nanofabrication technologies provide important cues to control the microenvironment of cardiovascular cells

during a tissue engineering process. The concept of a heart on a chip has not yet been realized, however, there have been a few studies pushing in this direction [82, 83]. A critical component for recapitulating a simplified heart on a chip is achieving mechanical 'synchronicity' of an atrium and ventricle compartment respectively. Once the timing of these two primary heart compartments is set, pulsatile flow can be initiated around a closed loop powered by contracting cardiomyocytes. The ability to derive different sub-populations of cardiomyocytes from human embryonic stem cells or induced pluripotent stem cells will enable engineering of human models of healthy and diseased myocardium. These models will be indispensable in studies aimed at the discovery of new markers for heart disease and drug testing. In addition, these models will be valuable tools in studies aimed at discovering the side effects of environmental pollutants or viruses (such as e.g. HIV) on the cardiovascular system. Progress in this research field will inevitably be accelerated by the design of several new analytical techniques that allow real-time monitoring of cell and tissue function to gain insight into the complex mechanisms of cardiogenesis and myocardial repair. Additionally, novel scaffolds and improved bioreactors capable of simultaneous application of multiple biochemical (e.g. growth factors) and physical (e.g. electrical, mechanical, perfusion) stimuli will be required. Bioreactor systems can also be used for rigorous studies of cardiac development and function to evaluate parameters such as the relative importance of each stimulus, when it should be applied and at what level. For these studies, we anticipate that microfluidic and BioMEMS (Micro-Electro-Mechanical Systems) techniques will be invaluable. The ability to control the microenvironment of cardiac cells at the nano-scale will provide appropriate cues for functional assembly of these model tissues.

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