Nanobiomaterial Advances in Cardiovascular Tissue Engineering

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Introduction

Heart failure remains one of the deadliest, most expensive, and medically challenging problems in the modern world [1]. Occlusion of coronary arteries (e.g., as a result of atherosclerosis) leads to ischemic myocardial infarction (MI) which in turn can cause an irreversible substantial epicardial and myocardial tissue damage and loss of function [2]. The irreversibility of the loss of tissue is mainly due to the innately low self-renewal of adult cardiomyocytes (CMs), as human radioisotope studies show that less than half of the myocardium is renewed during the normal adult lifespan [3].

Various drugs (e.g., β-blockers and aldosterone), bare-metal stents, and drug eluting stents have been used to induce regeneration of CMs damaged by MI [4, 5]. Additionally, left ventricular polymeric restraints have been developed to prevent

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the negative remodeling events post-MI [6]. However, long-term use of drugs can have side effects and excessive costs. Surgical interventions can be also dangerous [7–9]. Cell therapy is an alternative approach that consists of injecting a bolus of cells intravenously, so they can home to the site of injury and become engrafted to replace damaged cells or exert therapeutic paracrine effects to retrieve the functions of stunned or hibernated CMs in the pre-infarcted area of myocardium [10–13]. This approach can be efficacious with cell types such as human umbilical vein endothelial cells (HUVECs) or bone marrow derived mesenchymal stem cells (MSCs), since these cells can be obtained from patients, have limited but clinically relevant expansion capacity in vitro, and have been shown to engraft at the injury site [14, 15]. Cell therapy has a high potential for regeneration of post-MI ischemic damage to the myocardium. However, due to the notoriously low renewal capacity of adult CMs, producing clinically relevant numbers of mature adult CMs is difficult. Thus, conventional cell therapy approaches have been thwarted, because of the inability of adult CMs to remain mature in vitro while undergoing expansion [16]. Under culture conditions where CMs remain mature, they remain viable only a few days, and under conditions where they proliferate and expand, they become immature [17, 18].

Because of the senescent nature of adult human CMs, researchers have turned to utilizing various stem cell types which can be expanded and then differentiated into CMs. The main stem cell types proposed for use in regenerative medicine are human embryonic stem cells (hESCs), human induced pluripotent stem cells (hiPSCs), and MSCs [19, 20]. HESCs once held a great promise as a source of species-specific biological cells which could be differentiated into adult cell types for regenerative therapy. The failures of this technology have since been evidenced due to ethical controversies, risk of immune rejection, immature phenotype resembling CMs of the primary heart tube, and tumor formation [21–23]. Unlike hESCs, patient-specific cells such as iPSCs and MSCs can partially avoid immune rejection and ethical controversies, as we can extract the cells from the patients and create patient-specific CMs [24–26]. Although these patient-specific CMs provided better opportunities to reduce the cardiac scar size, one of their main issues is the immature nature of the differentiated cells. Maturation of patient-specific CMs to a particular lineage cannot be achieved without providing stem cell or early stage differentiated CM cues on multiple spatial scales, from the molecular and nanoscale to the microscale [27–29]. Therefore, the field of tissue engineering (TE) has sought sources of patient-specific cells which can be engineered with spatiotemporal cues on the nanoscale. The nanoscale is the spatial domain of biological information and molecular recognition, thus giving the cells the ability to be expanded in a coordinated manner, and finally to mature into functional adult cells [30].

Two of the major candidate cell types currently carrying the most promise for cardiac TE are patient-specific human iPSCs and MSCs. In recent years it has become evident that each cell source carries drawbacks and advantages. Adult multipotent stem cells, such as MSCs, and progenitor cells have a capacity to differentiate into mature cardiac cell types; however limited self-renewal and potential for expansion remain a major hurdle [31]. iPSCs, obtained from patient cells such as skin or immune cells, can be reprogrammed to the pluripotent state and exhibit unlimited self-renewal. The reprogrammed cells display the capacity for in vitro expansion to

therapeutically relevant cell numbers and the ability to differentiate into immune-compatible CMs [32, 33]. The major drawbacks of iPSC-derived CMs include the immaturity of the resultant CMs, batch-to-batch variations, and lack of reproducibility [16, 34, 35]. The immaturity can cause low functionality, teratoma formation, arrhythmia, and low cardiac regenerative capacity [16, 34, 35].

The immature phenotype of iPSC-derived CMs produced from various biochemical cocktails (typically modulating the Wnt and TGF-β signaling pathways), using traditional culture materials, include microscopic details such as pleomorphic morphology, nanoscopic details such as disorganized, smaller sarcomeres, and clustered mitochondria, as well as molecular level Ca^{++} transport isotropy. This is in contrast to the cylindrical adult CMs with aligned, larger sarcomeres, distributed mitochondria, and directional Ca^{++} signaling [16, 36–38]. In addition, current methods of injecting a CM bolus for heart regeneration result in low retention and engraftment rates, lower than 10% in mice models [39]. Such a low survival percentage is due not solely to low engraftment but also to the immune reaction after the integration of the therapeutic cells into the tissue [40]. The idea that nanoenvironmental scaffolds which mimic the adult cardiac milieu can enhance iPSCs differentiation and maturation and serve as delivery platforms or enhancers for engraftment has been a major factor in the development of nanobiomaterials. In addition to iPSCs, human adult MSCs and CMs derived from animal models are being studied for their interactions with nanobiomaterials. As nanotechnology has advanced over the past few decades, with discoveries of new materials and methodologies, applications to TE have followed close behind.

Advances in Nanobiotechnology

The nanotechnology paradigm began in the 1960s, and the name was coined in the 1970s in connection with processing materials with nanometer accuracy [41]. The definition of nanotechnology is utilizing materials with one or more geometric dimensions smaller than 100 nm [42]. An alternative definition is, the building of materials from their atoms or molecules to suit an application (bottom-up nanotechnology), rather than devising applications based on a material's macroscopic property [43]. Since then, many nanomaterials have been discovered (e.g., buckyballs, carbon nanotubes, and graphene) and planned to be implemented in the field of TE beginning in the 2000s and cardiac TE within the past few years [44–47]. Aside from the discovery of new materials, in the past few decades, nanotechnological advances such as the manipulation of single molecules with atomic force microscopy (AFM), the deposition of nanoliter volumes of ink using nano three-dimensional (3D) printing, and improved methods for producing nanotopography (e.g., electrospinning, polymer-demixing, and colloidal lithography) have also led to a widespread interest and growth in nanobiotechnology for TE applications [48–50]. The main promise is to mimic the explosive growth in the electronics industry by controlling biological molecules on the smallest scales, a currently urgent initiative for cardiac TE considering the growing epidemic of heart disease in the younger populations of western countries [51, 52].

Utilization of biomaterials with nanoscale features and organized structures that can mimic natural biological structures on the smallest scales results in unique material properties unobtainable by mimicking gross morphological features of tissue or organs, alone. This is because incrementally changing the structure on a lower spatial scale can have nonlinear outcomes on material and biological properties, providing the possibility of mimicking diseased rather than healthy tissue nanostructure [53]. Therefore, the ubiquity of nanosurfaces in biological systems, such as the nanoscale domains of proteins responsible for molecular recognition, makes utilizing nanoscale approaches a necessity [30, 54]. In general, nanobiomaterials can create tissues with structures that mimic many physiological tissue characteristics including cell orientation, cell morphology, diffusion of gases/nutrients, extracellular matrix (ECM) topological structure, and mechanical/electrical properties [55–58].

The Heart at the Nanoscale

The heart is a complex three-dimensional organ with a multiscale structure consisting of CMs and supporting cell types and ECM. Cardiac muscle ECM has a role as both scaffold for the CMs to physically bind them into an organ unit and an intrinsic role in force transmission, CM mechanotransduction to control gene expression, propagation of electrical signals via inducing alignment of CM gap junctions, etc. [59, 60]. The collagenous matrix of the heart consists of the endomysium, a network weave-like structure wrapped around CMs, collagen struts connecting CMs, perimysium consisting of larger collagen fibrils, and the epimysium consisting of the largest and most ordered fibers [61]. This morphological multiscale structure is preserved among species and mechanical strength differences are mainly due to the concentration of collagen in the structure.

The importance of the cardiac ECM is highlighted by the finding that reversible ischemic CM injury results in structural changes to the collagenous network including uncoiling of fibers and discontinuities in the structure [62]. Such nano- and microscale differences between healthy and diseased ECM are important considerations in TE when the goal is either to model diseased tissue or engineer tissue replacements. For instance, the fractal dimension of liver tissue ECM as assessed by imaging software has been used to distinguish healthy from fibrotic tissue [63]. In addition to CMs, another important cell type are the Purkinje fibers and other cells which compose the conduction system of the heart and are also integrated with the nanoscale ECM into fractal-like networks [64]. These cells are responsible for propagating the electrical impulse of the Sino-atrial (SA) node and synchronizing CM contraction in a spatiotemporal manner [65]. With the discovery of conductive nanomaterials with various geometries similar to native cardiac ECM, this conduction system can also be mimicked with nanobiomaterial scaffolds that act as “artificial” Purkinje fibers to help synchronize CM electrical communication [66, 67]. Recent advances in TE have exploited the discovery of such materials to better mimic the properties of native heart tissue [56, 68].

While the actual arrangement of CMs, their connectivity, and the macroscopic structure of the heart have remained controversial, it is evident that the properties of functional heart tissue result from a unique microscopic structure that is ordered spatially down to the nanoscale. Therefore, a more physiologically functional tissue will result by taking this order into account [69, 70]. Little is known about how the nanoscale features of the heart relate to the structure and function at higher spatial scales, which is critically important for mimicking the natural structure of the heart, making the task of the cardiac tissue engineer even more difficult [71]. Despite this impediment, considerable progress has been made in the field of nanobiomaterials over the past few decades [72–74]. The increased understanding of cell signal transduction has integrated the nanoscale role of ECM and the physical nanoenvironment with biochemical signaling [75]. The central task of the cardiac tissue engineer is therefore to provide a nanoenvironment which induces proper biochemical/physical signal integration for producing a functionally mature cardiac tissue. This integration of signals will determine whether a stem cell becomes a mature CM and help the cardiac function.

The three main emerged strategies for nanoscale TE of cardiac tissue are the following: (i) creating nanoscale topography or patterns that guide cells into more mature morphological structures; (ii) using nanomaterials or composite scaffolds with conductive nanoenvironments; and (iii) utilizing nanoparticulate systems to engineer or deliver payloads to CMs.

Engineering Nanotopography of Cardiac Tissue Scaffolds

Perfectly smooth solid surfaces are rarely found in nature and are difficult to produce using current engineering techniques. Most surfaces even with fabrication techniques designed to reduce surface heterogeneities will have some degree of nano- or atomic scale heterogeneity, and on most engineered topographies, there will be multiscale features (Fig. 5.1d) [76, 77]. The biological influences of such surface nanoscale topographies are relevant to TE and can be utilized to mimic the natural ECM and control cell behavior through contact guidance in cardiac TE applications. Creating nanofibrous surfaces (Fig. 5.1a) and nanoscale surface structures such as tubes and pillars (Fig. 5.1b) and grooves (Fig. 5.1c) are some of the methods for engineering nanotopography of surfaces and systematically studying its effect on cardiac tissue regeneration.

The field of contact guidance probably first began with the culture of cells on spider webs during the early twentieth century where it was noted that cells aligned along the length of the fibers [78]. From the 1960s forward, the realization that surface topography and gradients control cell behavior gradually developed [79–81]. The cell cytoskeleton must be specifically oriented in space for proper signaling and maturation to ensue and cells will tend to align along structures of minimal curvature to prevent cytoskeletal deformation [82]. The creation of nanotopographical cues on a biomaterial surface is the extension of contact guidance down to the nanoscale motivated by biomimicry of the natural ECM. In

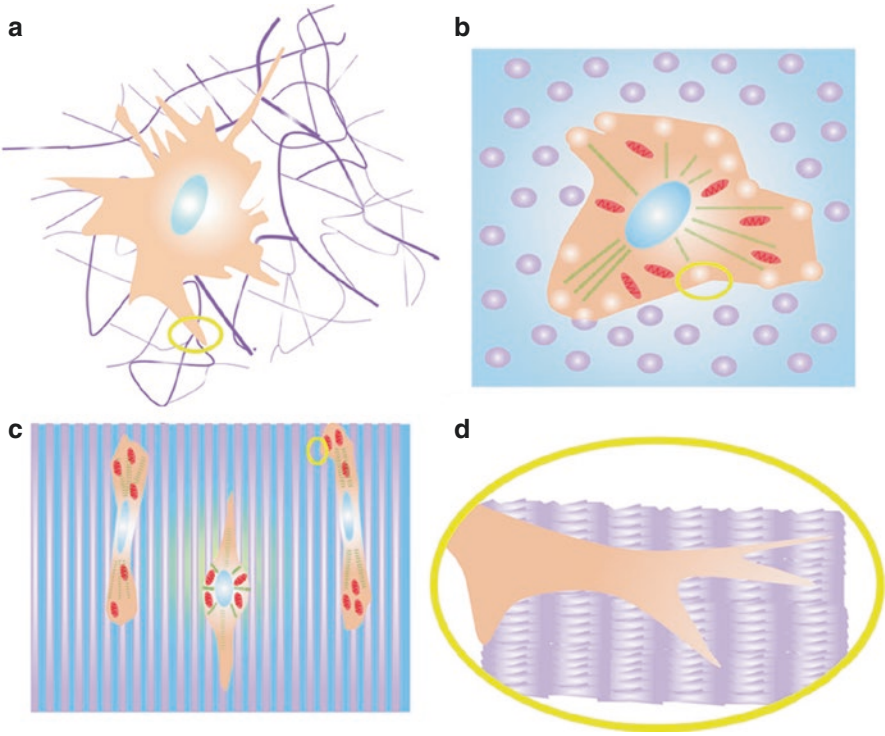


Fig. 5.1 (**a**) Schematic representation of CM cultured on nanofibrous scaffold where cell pseudopods are aligning along the fibers. (**b**) Schematic representation of CM cultured on nanostructure, showing alignment of cytoskeletal structures (sarcomeres are green mitochondria are dark red) with the topography. (**c**) Schematic representation of CM alignment on nanogrooves showing parallel alignment and spreading of CMs and cytoskeletal structures. (**d**) Magnified view (from yellow circles in **a**–**c**) of cells on nanostructures showing the features of topography interacting with cell membrane. Feature sizes are not to scale

this section, recent advances in nanotopographical strategies for biomimicry of heart ECM and cardiac TE will be discussed.

Nanofibrous Scaffolds

One of the most widely used strategies for mimicking natural cardiac ECM topography on the nanoscale is the creation of nanofibrous scaffolds using electrospinning of polymers or other fabrication techniques [83]. Such scaffolds can be fabricated with varying degrees of fiber orientation/alignment, porosity or space-filling character, and surface properties/hydrophilicity [84, 85]. The motivation for using this strategy is to mimic the complex multiscale fibrous network of the cardiac ECM. The main objective is to create nanotopographic cues on the scaffold surface to use contact guidance to control CM maturation. This includes focal adhesion

formation, CM aspect ratio, ion channel gene expression, and cytoskeletal arrangement [86–88]. In addition, stem cell differentiation can be directly influenced by topography via mechanotransduction mediated by focal adhesion kinase [89].

Given the goal of creating as realistic and natural a scaffold as possible, one natural choice for a fibrous TE cardiac scaffold is an injectable form of the decellularized cardiac ECM which can reassemble in vivo after injecting [90]. Such a scaffold presents not only native nanofibrous topography but also the solid phase protein molecular recognition sites (typically 50 nm^2 or less) of the native cardiac milieu [30]. This strategy was used to compare fetal and adult bovine cardiac ECM as a 3D scaffold for CMs which naturally have organized, periodic nanoscale cell-recognition sites in the gel molecular backbone [88]. HiPSC derived CMs were seeded onto two-dimensional (2D) layers or in 3D gels composed of the decellularized and digested scaffold proteins. Initial studies prior to digestion showed that adult ECM had more organized bundles of ECM proteins whereas fetal ECM was more disorganized. The 3D gels led to mature CM gene expression relative to 2D layers as determined via quantitative polymerase chain reaction (qPCR) with multifold enhanced expression of mature cytoskeletal, cell adhesion, and Ca^{++} handling and other ion channel genes, including Kir2.1, an ion channel with higher expression in mature CM, thought to prevent arrhythmia [91]. In addition, adult ECM had greater positive influence than fetal ECM on CM maturity.

While reconstituted natural ECM intrinsically carries the molecular recognition sites of the native myocardium, the methods for decellularization and processing create variability in mechanical properties and biocompatibility. To gain more engineering control over TE scaffold creation, synthetic polymers can also be used to fabricate biomimetic nanofibrous scaffolds [92]. Poly (lactic-glycolic acid) (PLGA) is one of the most common biodegradable TE materials which can also be used for nanofibrous scaffold creation via electrospinning [93]. Aligned PLGA nanofibrous scaffolds coated with gelatin were used for the maturation of hiPSC derived CMs. Resultant CMs were aligned with PLGA nanofibers via contact guidance relative to flat, smooth culture plates which was observed in scanning electron microscopic images (Fig. 5.2a). Sarcomeres were larger on the PLGA scaffold, closer to the size of native sarcomeres (~ 2 μm) than on flat surfaces and mitochondria were also aligned with the fibers showing more dense cristae. The rate of beating of CMs on the aligned structures was also approximately 50% higher than on a traditional, flat culture surface.

Electrospun polyurethane (PU) scaffolds have also been fabricated as scaffolds for mouse ESCs differentiation to CMs [96]. PU is a copolymer consisting of polyol, diisocyanate, and chain extender which self-organize into micro- and nanoscopic hard (diisocyanate and chain extender) and soft segment (polyol) [97]. Polycaprolactone diol (PCL) soft segment with a lysine-based diisocyanate and phenyl-alanine-based chain extender was used to create biodegradable electrospun PU. Such scaffolds could be spun with diameters ranging from hundreds of nm to μm; however, the multiscale hard/soft segment morphology can add another layer of nano- and microscopic complexity with incompletely known effects on protein adsorption and cell adhesion [98, 99]. Mouse ESCs could be differentiated to

CMs on these scaffolds with improved maturation characteristics on scaffolds with aligned fibers relative to those with more random orientation.

Surface Nanostructures

A second form of nanotopography which can give spatial control over the cues presented to CMs is creating nanogrooves or nanopillars on the substrate surface which can have varying diameter and separation [100, 101]. These controlled nanotopographies can be used to study fundamental mechanisms of cell interaction

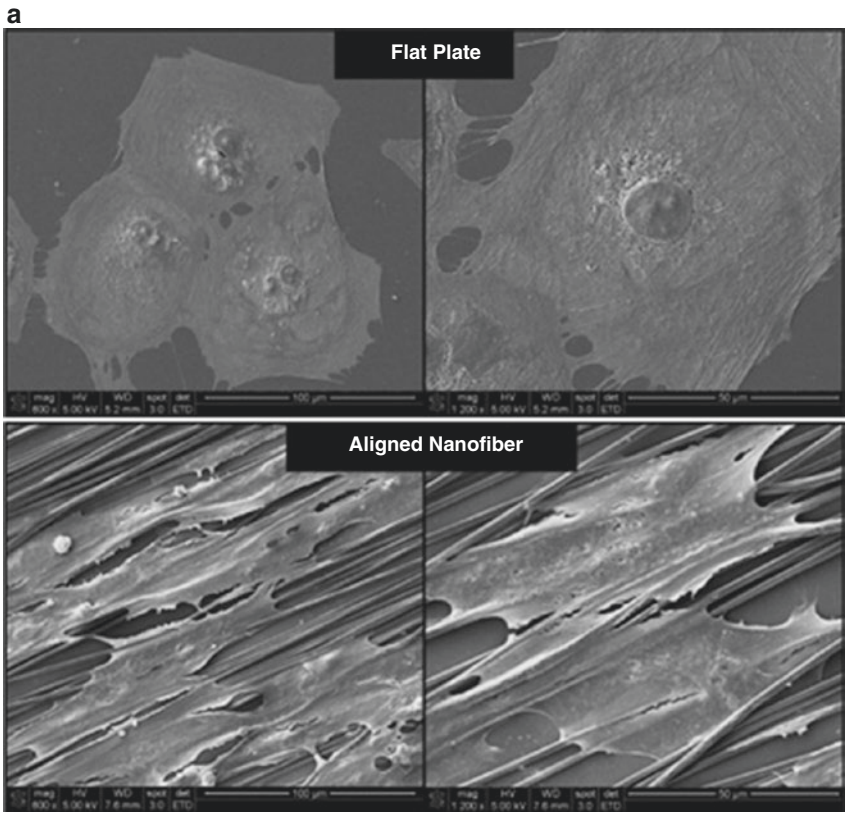


Fig. 5.2 Engineering nanotopography for Cardiac TE scaffolds: (**a**) SEM images of CMs on flat surfaces (top) and nanofibrous PLGA scaffolds (bottom), showing distinct alignment of CMs on nanofibers by contact guidance (Reprinted with permission from [93]). (**b**) CMs cultured on nanogrooves of varying geometry stained for α-actinin and F-actin (Reprinted with permission from [94]. Copyright 2016 American Chemical Society). (**c**) iPSC-CM on flat, 2D cell-imprinted, and multiscale cell-imprinted PDMS surfaces showing beating analysis in the insets. (**d**) qPCR analysis of CMs on various patterned PDMS substrates where multiscale imprinted PDMS had the highest expression of cardiac markers (**c** and **d** reprinted with permission from [95]. Copyright 2018 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim)

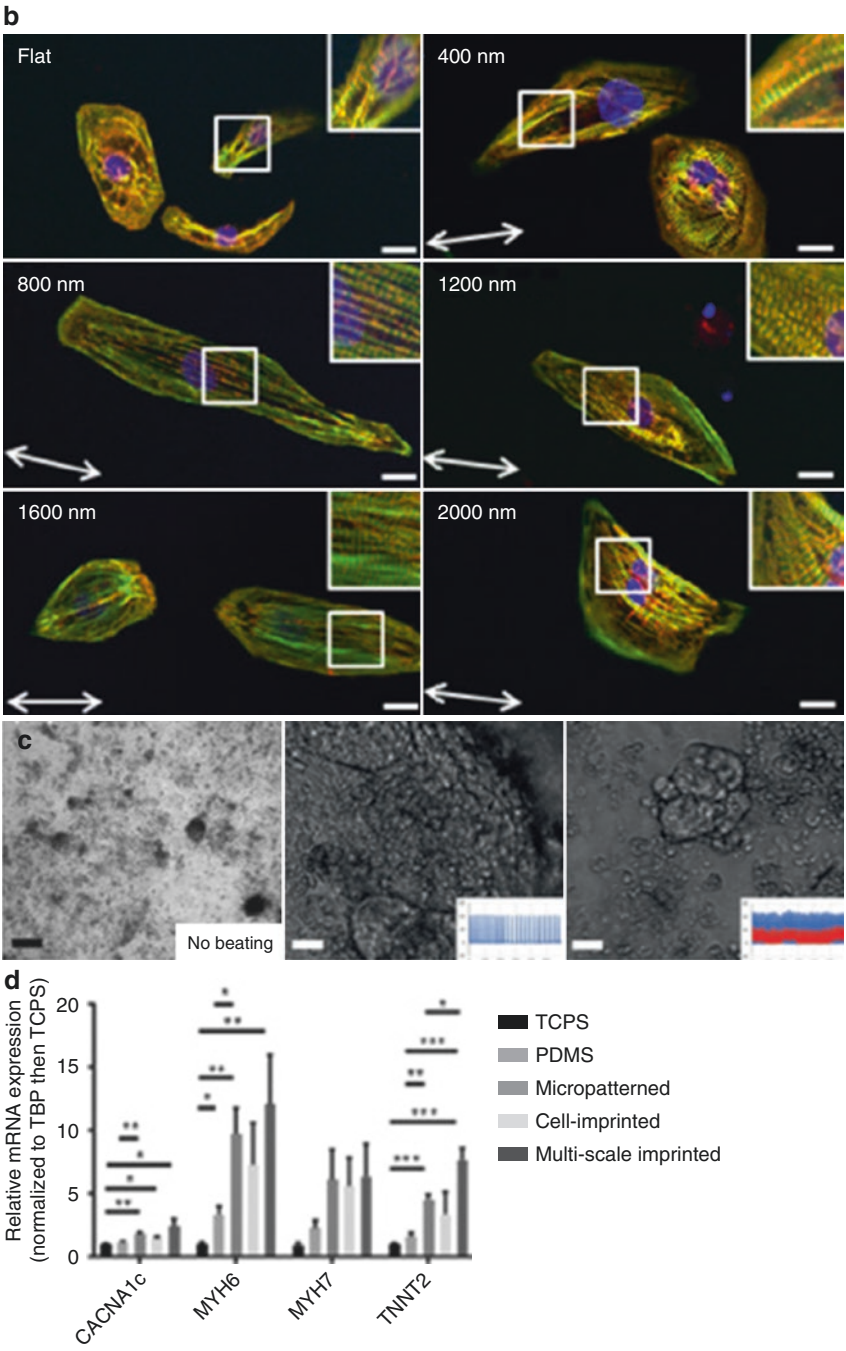


Fig. 5.2 (continued)

with topographies of different dimensions and geometries [86]. One such strategy utilized poly(ethylene glycol) (PEG) diacrylate-based aligned nanofibers formed with surface nanogrooves as scaffolds developed from a UV cross-linking capillary molding technique [27]. The groove/ridge width was 150/50–800/800 and the depth of grooves was 200–500 nm in height. The hydrophilic nature of PEG creates a high water content scaffold with aligned fibers similar in diameter to native collagen. Neonatal rat ventricular CMs were used to create highly aligned beating cardiac monolayers. Cell cytoskeletal proteins such as sarcomeric α-actinin and F-actin are also highly aligned on such nanotopographic surfaces relative to control unpatterned surfaces.

The creation of nanogrooved surfaces has also been combined with variation of substrate stiffness to examine the important influence of the deformability of the nanostructured surfaces using photolithography techniques [102]. PU and polystyrene nanogrooved surfaces were fabricated to compare nanotopographical surfaces with differential stiffness. Rat CMs were cultured on engineered substrates. The surface nanogrooves of 100 or 350 nm depth controlled the cell alignment, regardless of surface stiffness, with 350 nm giving greater alignment than 100 nm grooves. However, CM contractility differed between PU (softer, ~4 MPa) and polystyrene (stiffer, ~2 GPa) and synchronous contractility was longer maintained on the former, indicating that the stiffness of topographical features is also an important parameter. When human ESCs were differentiated on poly (acrylamide) gels of variable stiffness, expression of Brachyury (a marker of mesendodermal fate) on day 1 peaked on gels of intermediate stiffness, which also lead to the highest expression of Troponin T expressing CM [103]. However the impact was less significant if differentiation was initiated on TCP, to the point of cardiac progenitor stage, and subsequently transferred to substrates of variable stiffness.

Nanogrooves imprinted in PU-acrylate (PUA) with self-assembled (due to a PUA binding domain) chimeric peptides promoted cell adhesion [94]. Various groove/ridge widths were fabricated from a few hundred nm to 2 μm, all of which had uniform adhesion of hiPSC derived CMs after coating with the peptide. Each nanogroove substrate enhanced CM morphological anisotropy. The 800 nm grooves resulted in greatest cell-spread area as demonstrated by immunofluorescent staining of α-actinin and F-actin (Fig. 5.2b).

Creating shapes other than grooves, such as nanopillars, has also been recently achieved using a UV-curable PEG derivative [100]. The tapered pillars, created by capillary lithography were ~ 100 nm wide and a few hundred nm in height. Rat CMs were seeded on the nanopillar substrate and observed by environmental scanning electron microscopy (ESEM) to be migrating across PEG pillars, causing them to bend. Due to the non-fouling characteristics of PEG, cell adhesion was restricted and ephemeral. The posts, however, could be used to study the dynamics of cell focal adhesions on nanopillars. Immunofluorescence studies revealed that cytoskeletal filaments were aligned with the underlying PEG nanopillars.

Molecular and Cellular Imprinting

Molecular imprinting has also emerged as a rising strategy for TE including cardiac regenerative medicine. Molecular imprinting began in the 1930s, when it was discovered that silica gelled in the presence of a specific molecule will have increased selective adsorption of the molecule [104]. In general, the method usually consists of curing a cross-linkable polymer on a molecular template and subsequently removing the template to obtain geometrically defined cavities which mimic binding sites of the molecular template [105]. Importantly, the cavities will have complementary binding sights (lock-and-key type specificity) and chemical recognition of the template species. In this way, molecularly imprinted surfaces can act as "artificial antibodies." Molecular imprinting has been used for separation/sorting systems, bio sensing, drug delivery, and catalysis [105]. Recently, this methodology has been extended to the imprinting of bacterial and mammalian cells for selective adsorption or adhesion [106]. Cardiac applications for molecular imprinting include templated polymers to detect cardiac troponin T and myoglobin, while previous cell imprinting applications included the imprinting of mature CMs for iPSC differentiation as well as mature chondrocytes and keratinocytes for MSC differentiation [95, 106–109].

Cells in different 2D or 3D microenvironments can be imprinted to achieve different results. One photolithographic method created cell-imprinted surfaces with multiscale topography by seeding primary human CMs on a 3D semicylindrical micro-molded poly(dimethyl siloxane) (PDMS) surface with a pattern aspect ratio similar to in vivo CM morphology. PDMS was poured over adult primary human CMs, aligned within the 3D patterns, and cured to create a mold of the mature cells with topography at the μm and sub-μm level, which could be used as a substrate for the differentiation of iPSCs to CMs. In addition to the asymmetrical microscale mature cell-shaped pattern, the nanoscale topography of the adhesion molecules of primary human CM was used as complementary binding sights for the iPSCs throughout their differentiation to CMs. CMs produced on these substrates started beating earlier, showed enhanced expression of cardiac markers, and mature calcium handling characteristics relative to traditional culturing surfaces. Contractile function of CMs was analyzed at day 16 of differentiation on multiscale imprinted PDMS, and was compared with CMs generated on tissue culture plastic (TCP), showing no beating (Fig. 5.2c). QPCR data (Fig. 5.2d) showed highest expression of cardiac markers on multiscale CM imprinted PDMS compared to TCP, micropatterned PDMS, or PDMS imprinted by adult primary human CMs on 2D surfaces.

Conductive Nanomaterials

Over the past few decades, the discovery of graphene and the earlier discovery of carbon nanotubes have led to an explosion in research in conductive materials that is predicted to revolutionize the electronics industry [110]. This excitement has fueled a parallel interest in the field of nanobiomaterials. The reason for this interest is the

concept of engineering a tissue on the nanoscale that can mimic the complex electrical activity and signal propagation of the native heart [65, 111]. Materials such as graphene, carbon nanotubes (CNTs), gold nanorods, and silver nanoparticles have become routinely used fillers or as pure materials in scaffold design [112–120]. Such materials have a niche in cardiac TE due to the need for CMs to propagate electrical signals to synchronize cell-cell communication (in the absence of a native conduction system). There is also need for conductive tissue elements to interface with a patient's heart tissue for TE cardiac grafts [121]. This strategy is becoming popular due to the high electrical impedance of most traditional cross-linkable materials used for TE purposes, such as hydrogels and various synthetic polymers which can interfere with the conduction velocity of engineered CM tissues.

Spherical Conductive Nanomaterials

Spherical conductive nanoparticles have been incorporated in biomaterials for different applications such as creating scaffolds with actuation, with antibiotic properties, or with reduced impedance to enhance CM cell-cell communication [122, 123]. They are typically incorporated into a fibrous matrix or a hydrogel material [115]. One study used gold nanoparticles (AuNPs) combined with an autologous ECM matrix to create conductive immune-compatible scaffolds which can be used with patient-specific cells. AuNPs were decorated on the scaffolds by e-beam evaporation. Cardiac patches were made by seeding neonatal rat CM on Au-coated ECM. These scaffolds could maintain the population of CMs, against being overpopulated by fibroblasts, relative to control non-conductive scaffolds. Additionally, the Au-coated ECM resulted in greater contractile forces and lower excitation threshold for induced contraction by external electrical stimuli. Ca^{++} transients on scaffolds decorated with 4 and 10 nm AuNPs were two to threefold greater than that on pristine ECM, demonstrating an overall more mature electrophysiological behavior. The cytoskeletal alignment and organization of CX-43 also increased with increasing AuNP concentration (Fig. 5.3a).

Silver (Ag) nanoparticles have attracted attention due to their antimicrobial and possible anti-inflammatory effects [126, 127]. One method of producing a natural conductive ECM is to use collagen protected with nano-Ag for electrospinning, which can produce electrical conductivity at low μmolar Ag concentration [128]. The Ag nanoparticles were added to the collagen solution prior to electrospinning followed by a glutaraldehyde cross-linking process [129]. Neonatal rat CMs were plated on the Ag containing scaffolds and controls with no Ag. After electrical pacing, the CMs on the Ag-containing scaffolds exhibited greater expression of gap junction protein CX-43 and protein Ki67 (a marker of proliferation) compared to control collagen fibers. The Ki67 expression increase may have been due to increased survival and growth of the neonatal CMs. In addition,

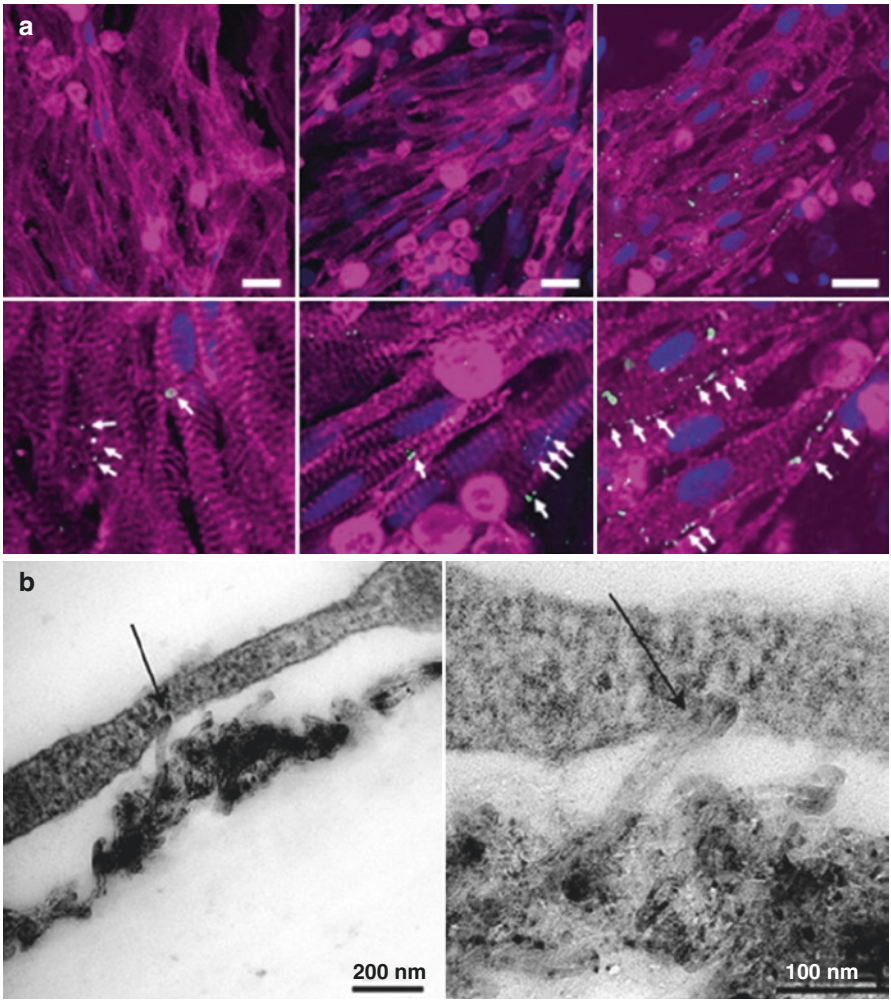
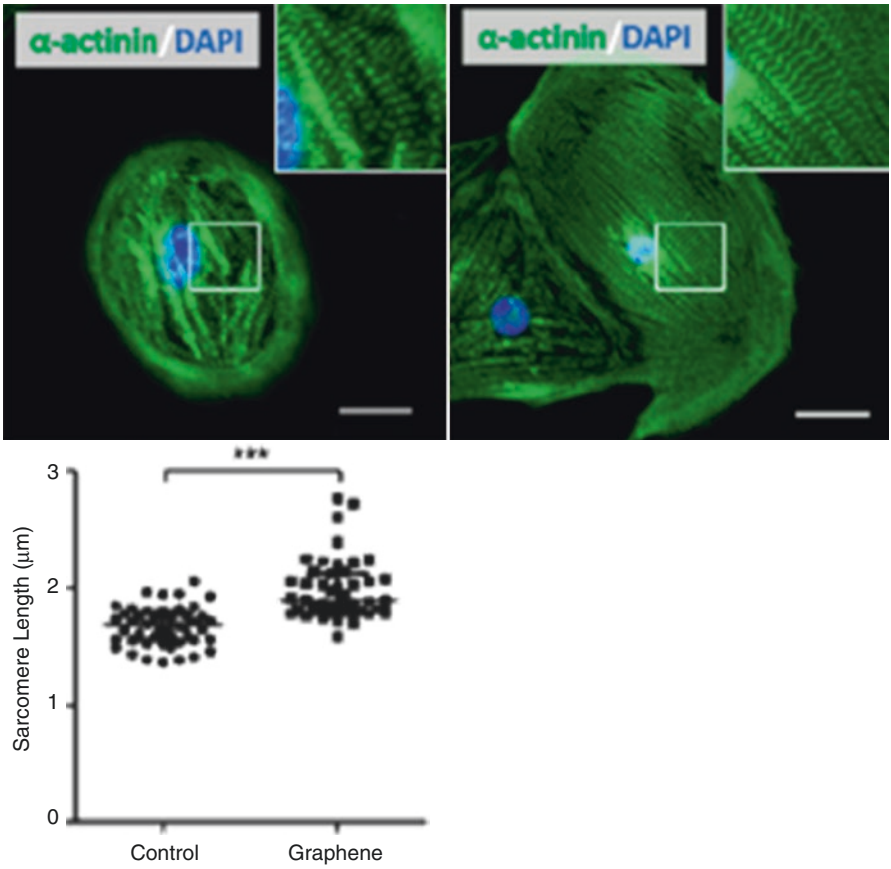


Fig. 5.3 Conductive nanomaterial strategies: (**a**) immunofluorescent staining of α-actinin and CX-43 on pristine (left), 4 nm AuNP (center), and 10 nm AuNP (right). Arrows point to CX-43 fluorescence (Reprinted with permission from [122]. Copyright 2014 American Chemical Society). (**b**) TEM images showing the close approximation between CM membrane and CNT substrate creating electrical shortcuts (Reprinted with permission from [124]. Copyright 2013 American Chemical Society). (**c**) α-actinin staining of CMs on control culture dish (left) and graphene substrates (right), showing sarcomere length is significantly greater on graphene substrates (Reprinted with permission from [125]. Copyright 2017 American Chemical Society). (**d**) SEM of CNT-Gel-MA networks showing fractal-like structure of CNTs (left) and CNT-Gel-MA supporting adherent CMs as free-floating bioactuator, capable of spontaneous movement in suspension (Reprinted with permission from [47]. Copyright 2013 American Chemical Society)



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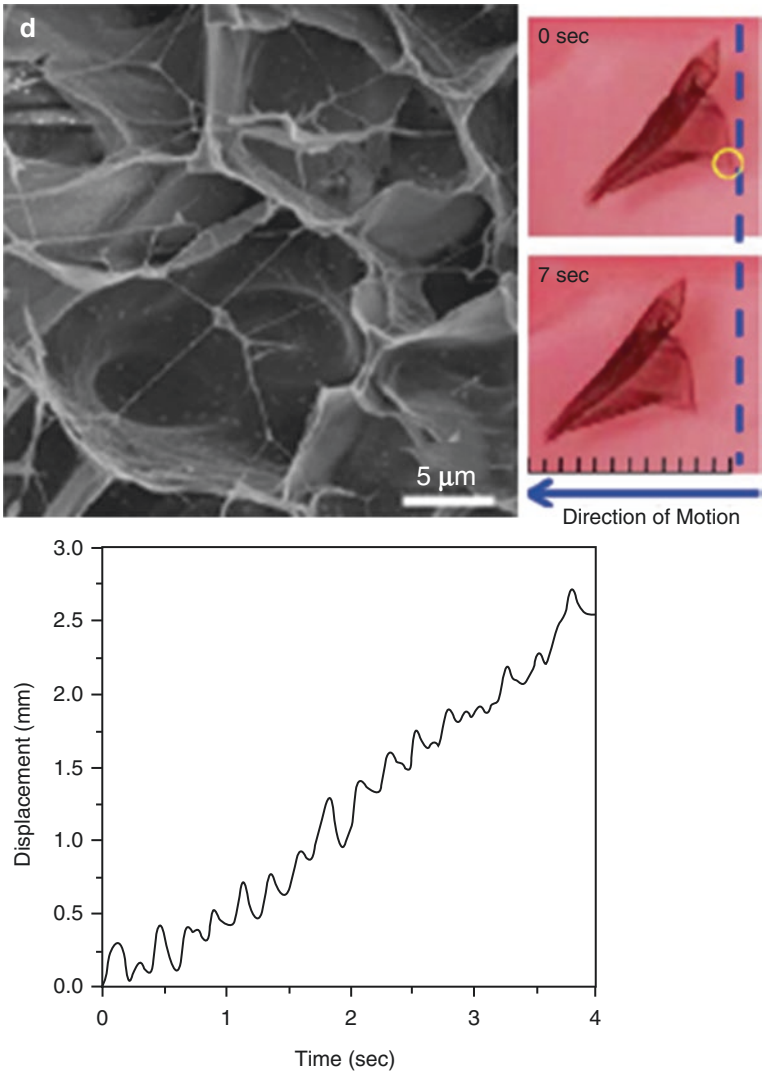

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antibacterial properties of Ag nanofiber scaffolds were evident by the ability to prevent *Pseudomonas aeruginosa* biofilm formation [130, 131].

High-Aspect Ratio Conductive Nanomaterials

Conductive one-dimensional (1D) or 2D nanomaterials with high aspect ratios have recently been used as substrates for CM maturation or iPSC differentiation to CM. For 1D materials, CMs were matured on CNT layers attached to glass [124,

132]. Since CNTs do not passively bind with glass, they were first functionalized using 1,3-dipolar cycloaddition of azomethine ylides [133]. They can then form 100 to 200 nm films on the glass and be defunctionalized by heating under N_2 resulting in a layer of pristine CNTs. Transmission electron microscopic (TEM) images showed that rat ventricular CM membranes had close approximation to CNT bundles that were periodically distributed over the basal surface (Fig. 5.3b). CMs also had greater proliferative capacity on CNTs than control gelatin films, while fibroblasts had no proliferative advantage on CNTs versus gelatin. Smooth conductive control surfaces such as amorphous carbon and indium tin oxide were also compared and did not demonstrate the enhanced proliferative effect on CMs; however fibroblast proliferation rate on those surfaces was slightly increased. In addition to this enhanced proliferative effect, CMs on CNTs exerted more negative action potentials and higher probability of firing an action potential relative to CMs on control surfaces.

To study 2D materials, both hESCs and hiPSCs were differentiated to CMs on 2D graphene substrates [125, 134]. HiPSCs were differentiated by small molecule modulators of Wnt signaling pathway while hESCs were differentiated by removing fibroblast growth factor from the medium and adding fetal bovine serum. Both cell types showed increased expression of cardiac markers and hiPSCs had increased alignment of sarcomeres as shown by α-actinin staining (Fig. 5.3c) and larger mitochondria that were aligned with myofibril bundles. The hESCs had higher early expression of cardiac mesodermal gene MESP1, higher SOX 17, similar ectodermal genes, and higher later expression of CX-43, NKX2.5, cardiac and troponin T, indicating a promotion of mesodermal and eventually cardiac mesodermal fate. However, beating CMs were not observed in the absence of small molecule inhibitors or cytokines typically used for CM differentiation, indicating that graphene alone, promoted cardiac differentiation but not complete maturation. Graphene was also shown to enhance BMP4 signaling in hiPSCs relative to oxidized graphene, showing a direct role of substrate conductivity in cardiac differentiation. This is despite the fact that CMs on smooth conductive surfaces (such as amorphous carbon) did not have enhanced maturation, demonstrating that scaffold conductivity and nanostructure can have diverse pathways in affecting cell function [124].

One of the most popular strategies for creating conductive TE substrates is mixing a natural hydrogel material with conductive nanomaterials. One of the major drawbacks using 3D nanomaterials such as spherical particles is the high filler concentration required to achieve percolation and highly reduced impedance [135]. The obvious drawbacks to such high nanofiller concentrations for biomedical applications are the high cost of nanomaterial as well as the release of nanomaterials which can interface with cells in vitro or tissues in vivo, in an uncontrolled manner. Substituting 1D or 2D conductive nanomaterials with high aspect ratios can substantially reduce the percolation threshold for nanomaterial concentration, allowing for conductive composites with reduced risk of particle accumulation in the system. One of the early trials utilized gold nanowires, mixed with alginate, to create electrical connectivity between CMs via mechanically and electrically connecting the individual pores containing CMs [121]. The resultant neonatal rat heart cells

demonstrated increased alignment and expression of cardiac markers. It was suggested that such engineered tissues could be used as a regenerative cardiac patch that will be degraded and replaced with natural ECM with the exception of the gold-nanowires.

This strategy was subsequently extended to other ECM molecules containing a variety of nanomaterials with high aspect ratios (1D or 2D nanomaterials), which are a preferred filler due to the lower concentration percolation threshold, minimizing the amount of nanomaterial in the system. For instance, CNTs were used with a gelatin methacrylate (Gel-MA)-based scaffold for maturation of rat neonatal CMs [47]. Gel-Ma is a UV-curable gelatin derivative that can be mixed with CNTs and cured to form a composite hydrogel. SEM images of dried Gel-MA laden with CNTs revealed nanotube networks had a morphology reminiscent of Purkinje fiber networks in the heart (Fig. 5.3d). Gel-MA-CNT scaffolds, fabricated at ~50 μm thickness, showed enhanced expression of cardiac proteins as well as enhanced alignment, lower excitation threshold, and more synchronous CM beating behavior. In addition, the scaffold exerted a protective effect against free radicals, possibly due to absorption via the CNTs. The engineered tissue could then be released from the substrate to form a spontaneously beating, floating bioactuator (Fig. 5.3d). This strategy was later extended to create biomimetic swimming bioactuators utilizing the Gel-MA-CNT-based CM sheets as the actuator in the shape of a batoid fish [136].

Conductive nanomaterial culture substrates can also be used for purposes other than contact guidance or maturation/differentiation of cells. In one case, a platinum nanopillar electrode array was fabricated using photolithographic methods for the purpose of culturing hiPSC and hESC-derived CMs and having the ability to measure action potentials in a more efficient, high throughput manner than traditional patch-clamp techniques [137]. The fabricated nanopillars were 200 nm in diameter and 1.5 μm in height and the surface of the substrate was electrically insulated to isolate the signal of the nanopillar electrodes. The nanopillars created nanoholes in the CMs via electroporation prior to measuring action potentials. The potential recording from the nanoelectrode array was compared with that obtained from traditional patch-clamp technique, showing that action potential shape was of different amplitude but identical shape. The array was also capable of determining CM subpopulation, determining what percentage of CMs were atrial, ventricular, or pacemaker types. This system was also able to differentiate between normal CMs versus those with long QT syndrome.

Engineering Nanocarriers for Cardiac Tissue Repair

Using nanoparticles to deliver molecules and engineer tissues is another nanobiotechnological strategy where rapid progress has been made. The basic concept involves encapsulating delivery agents inside colloidal scale matter with variable dimensions and geometry, which can be made to protect the payload until it reaches the desired location in the body. Such targeted delivery in which collateral damage

is completely avoided is called a "magic bullet" and is often considered the *holy-grail* of nanomedicine and has been increasingly used in cardiac TE [138, 139].

Therapeutic payloads such as interleukin 10 (IL-10) have recently been used inside blends of biodegradable poly(D-lactic acid) (PLA) and PLGA to target cell types that form coronary atherosclerotic plaques [140]. A targeting polymer consisting of PLGA, PEG, and collagen IV (PLGA-PEG-Col-IV) was mixed into the blends at a small percentage to impart the specificity of the nanoparticle. The basic concept is to target collagen IV basement membrane protein, which will be exposed due to injury/inflammation at the site [141]. These particles were capable of reducing reactive oxygen species of macrophages in vitro by ~30–60%. Additionally, in a mouse model of atherosclerosis, the fibrous cap thickness could be increased, while the necrotic area of the plaque was reduced (Fig. 5.4a).

Decorating cell surfaces with nanoparticles, which can then control cell binding behavior or be internalized by the cells stimulating signaling cascades, is another engineering strategy which has been developed [142, 144]. In one case, iron oxide nanoparticles were used for increasing the expression of CX-43 in cardiomyoblasts, with inherently low CX-43 expression, to enhance therapeutic efficacy and cell-cell communication [142]. Iron oxide nanocubes were the particle chosen for this study, due to their higher colloidal stability and magnetization. Particles are internalized by the cardiomyoblasts after exposure with endosomal localization, which partially ionizes them due to the lower pH. These iron ions caused JNK-mediated CX-43 expression. To examine the influence of this increased gap junction protein expression cardiomyoblasts were cultured with MSCs (cMSCs) after taking up two dyes, one of which can pass through gap junctions (Calcein-AM). After 48 hours, more MSCs had calcein AM when plated with iron oxide nanoparticle containing cardiomyoblasts than those plated with control cardiomyoblasts (Fig. 5.4b). The MSCs were then sorted from the cardiomyoblasts, and those which had communicated with iron oxide containing cardiomyoblasts had greater expression of cardiac genes. The results of the injection of iron oxide nanocube treated MSCs versus control groups in a model of rat ventricular infarction were then examined histologically (Fig. 5.4c). The extent of collagenous remodeling and blue-colored fibrotic scar tissue formation was reduced in the treatment groups versus control MSCs.

Magnetotactic bacteria (MB) are another naturally occurring nanobiomaterial that have recently gained attention. MB are bacteria that naturally synthesize magnetic nanoparticles at density sufficient to allow their alignment with the Earth's magnetic field. They were examined as a contrast agent for cardiac TE by infecting CMs with the non-pathologic bacteria (commercially available "Magnelles®") [143]. The nanoparticles inside the bacteria were visible in TEM images and by immunofluorescence (within CM cytoplasm, Fig. 5.4d). CMs could then be injected in a mouse model and their magnetic resonance imaging could be compared with that of cells injected after decoration with iron oxide particles. The iron oxide particles tend to remain in the tissue even after cell death while the MB are present only with living cells, giving a more objective determination of the engraftment efficiency.

Cardiac hypertrophy is another variant of heart disease caused by adaptation of the heart to pathological mechanical stress levels caused by various conditions. Its uncontrolled progression can lead to heart attack or death. An endogenous peptide, apelin, with a short plasma half-life and incompletely known function was recently encapsulated in liposomal nanocarriers with PEG surface decoration for use in a model of cardiac hypertrophy [145]. Previous studies had shown that loss

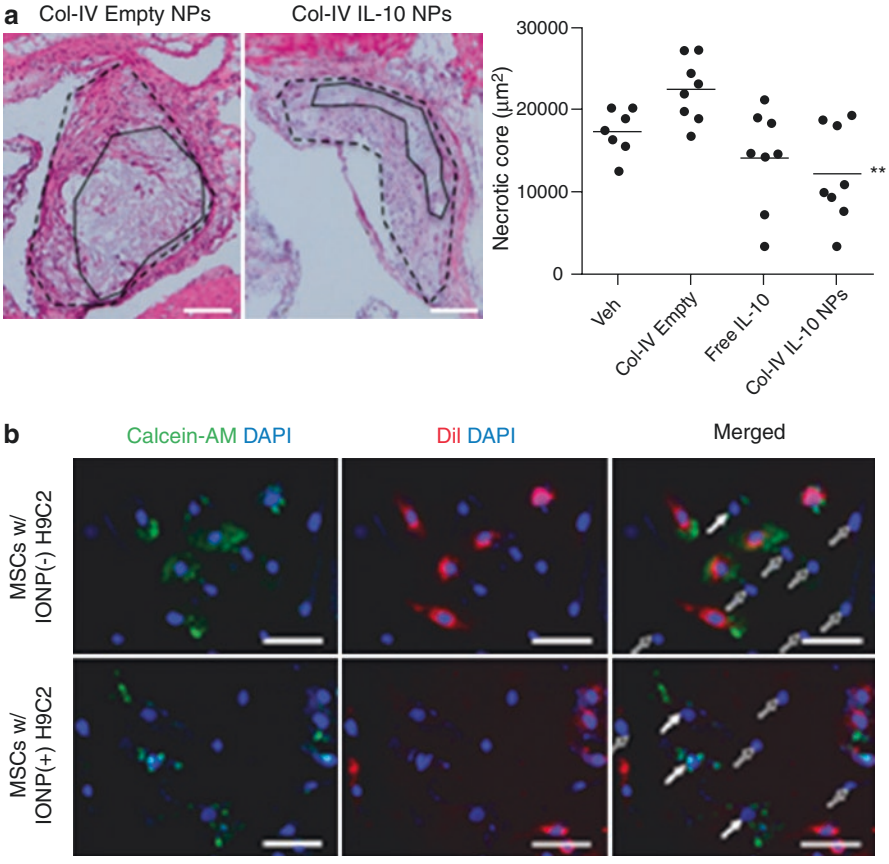

Fig. 5.4 Engineering Nanocarriers for Cardiac Tissue Repair: (**a**) Histological slices of sacrificed mouse heart with H&E staining comparing size of necrotic core between treatment and control groups where necrotic core was substantially reduced for targeted IL-10 containing NPs (Reprinted with permission from [140]. Copyright 2016 American Chemical Society). (**b**) 48 hours after staining cardiomyoblasts in coculture with MSCs, having green but no red dye denotes MSCs with gap junction crosstalk, while having DAPI but no stain denotes MSCs with no gap junction crosstalk. (**c**) Histological sections of rat heart 2 weeks after treatment with MSCs, stained with Masson's trichrome stain to assess the extent of collagenous fibrous tissue formation (**b** and **c** reprinted with permission from [142]. Copyright 2015 American Chemical Society). (**d**) TEM images of magnetotactic bacteria showing the synthesized magnetic nanoparticles (top) and immunofluorescence showing the Magnelles® (red) inside CMs (Reprinted with permission from [143])

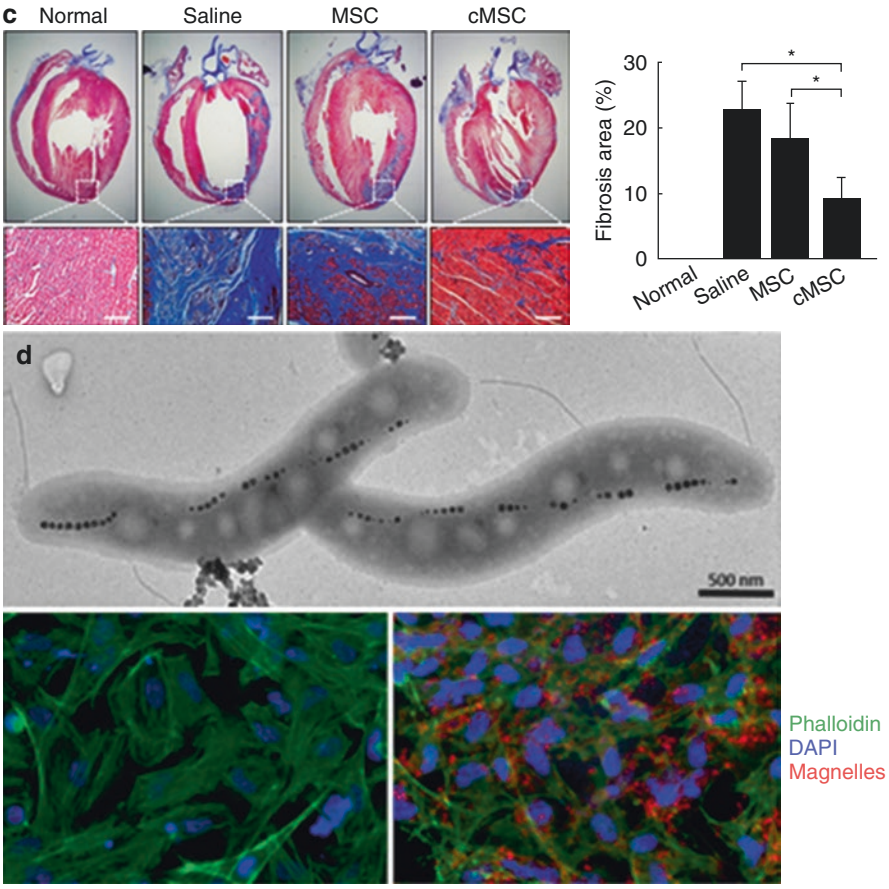

Fig. 5.4 (continued)

of function of the peptide or its receptor is associated with cardiovascular diseases [146, 147]. Specifically [Pyr 1]-apelin-13 was used in a transverse aortic constriction mouse model. The nanoencapsulated apelin (lipoPEG-PA13) was compared with direct administration of PA13 and saline injection. LipoPEG-PA13 attenuated the adaptive hypertrophic response compared to the two control groups and resulted in a sustainably elevated level of plasma PA13 up to 6 days post injection [148]. The extent of fibrotic scar tissue was also significantly less with the nanoencapsulated peptide relative to the free peptide, demonstrating the controlled and sustainable release which could overcome the short plasma half-life of the peptide.

Future Outlook

Top-down strategies for cardiac TE, such as decellularized heart tissue, are popular and plausible strategies to utilize the native architecture of the heart, in the absence of fundamental principles for engineering cardiac tissues [149]. However gaining

more engineering and manufacturing control and avoiding immunogenicity issues have led to the continuous search for bottom-up engineering strategies. A greater understanding of fundamental mechanisms of cell interactions with nanofeatures and assessment of in vivo performance of the nanomaterials will be required to advance the nanobiomaterial technologies. This knowledge could particularly accelerate the use of nanomaterials for obtaining sufficient quantities of patient-specific mature CMs. In vitro experiments typically use purified proteins to coat scaffold surfaces and defined cell culture medium. These strategies can work for producing CMs in vitro, but would not be able to predict the fate of CMs or nanostructured grafts in vivo, where they will be exposed to a complex mixture of thousands of plasma proteins and many cell types. Upon administration in vivo and exposure to the blood, nanostructured materials will be spontaneously coated with protein corona [150]. Recent studies suggest that the biological identity of this corona may change based on patient disease phenotype [151, 152]. An increased emphasis on realistic nanosystems with biomimetic structures that examine more faithful conditions will move the field of cardiac nanobiomaterials closer to a stage of viability and ubiquity.

Despite the significant progress in using various nanobiomaterials in cardiovascular TE applications, the clinical use of such material systems has not been achieved. To advance toward clinical applications, socioeconomic aspects of the technology as well as the time frame and speed of development should be considered. Furthermore, the specific needs of patients and physicians should be assessed as guiding factors in the design of the material, treatment, or implantable device.

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