Enabling Microscale and Nanoscale Approaches for Bioengineered Cardiac Tissue

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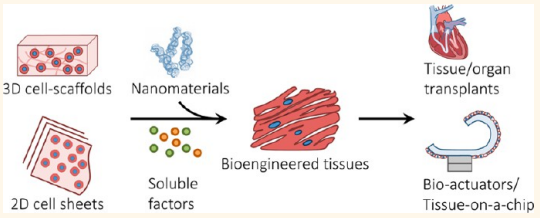
ABSTRACT In this issue of *ACS Nano*, Shin *et al.* present their finding that the addition of carbon nanotubes (CNT) in gelatin methacrylate (GelMA) results in improved functionality of bioengineered cardiac tissue. These CNT—GelMA hybrid materials demonstrate cardiac tissue with enhanced electrophysiological performance; improved mechanical integrity; better cell adhesion, viability, uniformity, and organization; increased beating rate and lowered excitation threshold; and protective effects against cardio-inhibitory and cardio-toxic drugs. In this Perspective, we outline recent progress in cardiac tissue engineering and prospects for future development. Bioengineered cardiac tissues can be used to build "heart-on-a-chip" devices for drug safety and efficacy testing, fabricate bioactuators for biointegrated robotics and reverse-engineered life forms, treat abnormal cardiac rhythms, and perhaps one day cure heart disease with tissue and organ transplants.



Cardiovascular disease is the leading cause of death in the United States. An estimated 82.6 million American adults (>1 in 3) have one or more types of the disease, resulting in over 2.4 million deaths with an estimated direct and indirect cost of $190.3 billion in 2008.[1] The disease often manifests as a heart attack, which is the sudden blockage of blood in the coronary artery caused by atherosclerotic buildup of plaque on the walls of the blood vessels. Blockages in the coronary arteries of the heart can reduce the blood supply to the heart. Without blood supply, the portion of the heart fed by the artery is starved for oxygen and begins to die within a few minutes. This is known as a myocardial infarction. Although the affected region may be small at first, the remainder of the heart works harder to compensate for the loss of pumping power. Over time, the entire organ may enlarge and start to fail.[2]

Tissue engineering is a promising strategy that could one day provide a cure for heart failure patients and relieve heart donor shortages by providing replacements for damaged tissue. This discipline involves seeding living cells in highly porous three-dimensional (3D) polymer scaffolds that provide biomechanical support for the cells to facilitate formation of structural and functional tissue.[3] The scaffolds can be used as a cardiac patch to replace the missing or damaged regions of a myocardial infarct and to provide temporary support for cells. These scaffolds can also be used to control the size, shape, strength, and composition of the bioengineered cardiac tissue. In addition, tissue engineering can be used to replace or to reconstruct defective heart valves or vessels normally associated with congenital or acquired heart defects.[4] Finally, bioengineered cardiac tissue can be used to build "heart-on-a-chip" devices for drug screening or bioactuators for biointegrated robotics and reverse-engineered life forms.

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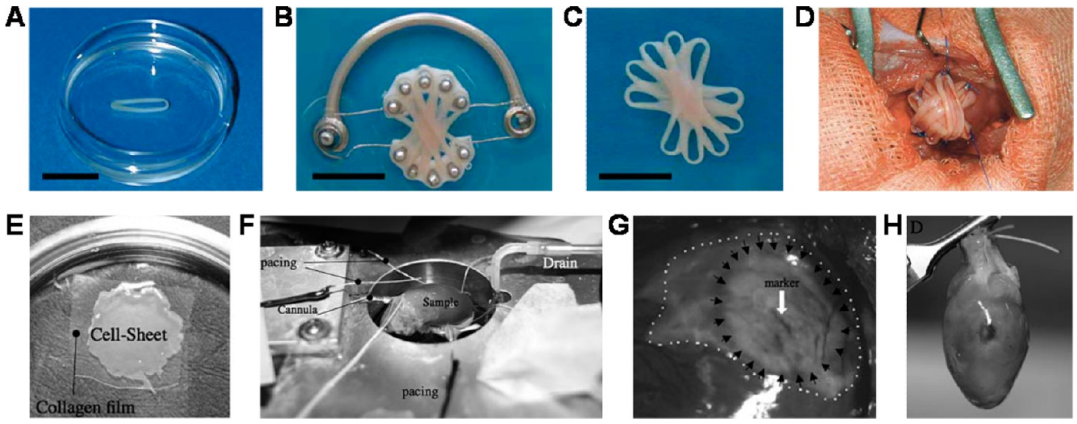

Figure 1. Conventional methods for bioengineering cardiac tissue: (A) engineered EHT rings of prestretched cardiac muscle; (B) five single EHT rings on a custom-made device facilitated fusion; (C) fusion resulted in synchronous contraction of multiloop EHTs; (D) sutures fixed to multiloop EHTs were sewn on the recipients' hearts. Reprinted with permission from ref 8. Copyright 2006 Nature Publishing Group. (E) Macroscopic view of an engineered bilayer cell sheet with cardiomyocytes; (F) experimental chamber used to implant the cell sheet on infarct region of rat myocardium; (G) closer view of the grafted surface with cell sheet (dotted white line) covering infarcted region (black arrows); (H) seven days after transplantation, cell sheet completely merged with host heart. Reprinted with permission from ref 10. Copyright 2006 American Heart Association.

Conventional Approaches of Engineering Cardiac Tissue Constructs. In one of the earliest studies of cardiac tissue engineering, Leor *et al*. implanted fetal and human myocardial tissue (1−3 mm) in infarcted rat hearts and showed 2-month survival in the damaged myocardium. This raised hope for growing bioengineered cardiac tissues *in vitro* for myocardial repair.[5] Several groups followed by demonstrating that cardiomyocytes from neonatal rats and embryonic chickens could be reconstituted to 3D cardiac constructs using collagen gels, collagen fibers, collagen sponges, polyglycolic acid, and alginate. Two pioneering approaches emerged several years later to generate engineered heart tissue (EHT): (1) the classical cell-scaffold approach and (2) the cell sheet engineering approach.

Classical Cell-Scaffold Approach. Eschenhagen *et al*. engineered rings of cardiac muscle by mixing neonatal rat cardiomyocytes in collagen gel and casting them in a ring template to grow EHTs with the classical cell-scaffold approach (Figure 1A−D). A custom-designed stretching device was used to simulate heart contractions. Compared to former systems, stretched EHTs exhibited better cardiac tissue/matrix ratio, improved contractile function, and a high degree of cardiac myocyte differentiation. Additionally, action potential recordings revealed electrophysiological properties typical of cardiac tissue.[6,7] However, EHTs only exerted maximal forces of ~2 mN/mm^2, which is 10-fold less than the native myocardium because of the less dense and compacted EHTs. To create thicker tissue that could be implanted, five stretched rings were stacked together and fused using a custom-designed device. Implantation into rat myocardial infarction models indicated electrical coupling with native tissue, improved diastolic and systolic functions, and overall functional improvement.[8]

Cell Sheet Engineering Approach. Shimizu *et al*. successfully grew engineered heart tissue without the use of scaffolds, a technique known as cell sheet engineering (Figure 1E−H). Neonatal rat cardiomyocytes were cultured on poly(*N*-isopropylacrylamide) (PIPAAm), a temperature-responsive polymer that is slightly hydrophobic and cell adhesive at 37 °C. The PIPAAm undergoes a reversible phase transition below 32 °C, switching to a hydrophilic and nonadhesive state due to rapid hydration and swelling. This change releases the cell monolayers without the need for enzymatic digestion, which disrupts cell−cell junctions and adhesive proteins. The cell sheets were stacked on top of one another up to four layers until they fused together to form functional tissue. The thickness of the tissue was limited by oxygen and nutrient diffusion through the layers. It was observed through electrical stimulation that all the sheets beat in synchrony.[9] Two layers of cardiac cell sheets were overlaid and transplanted onto the infarcted region of a rat heart. The grafted sheets integrated with the host heart and contracted simultaneously. After one week, the measured conductance velocity of the infarcted myocardium was reduced by almost 50% in the fiber orientation compared to the normal myocardium. By four weeks, the infarcted myocardium with grafted sheets had recovered to its initial conductance velocity of ~100 cm/s.[10] Because of these and other successes, there are now ongoing human clinical trials to treat dilated cardiomyopathy using sheets of skeletal myoblasts.

Key Challenges to Address. While these two approaches have proven effective in some contexts, there are still many challenges in developing functional bioengineered cardiac tissue for replacement therapy. Simply seeding cells in porous 3D scaffolds often does not recapitulate sufficient tissue function characteristic of normal myocardial tissue. Experimental studies show that cardiac

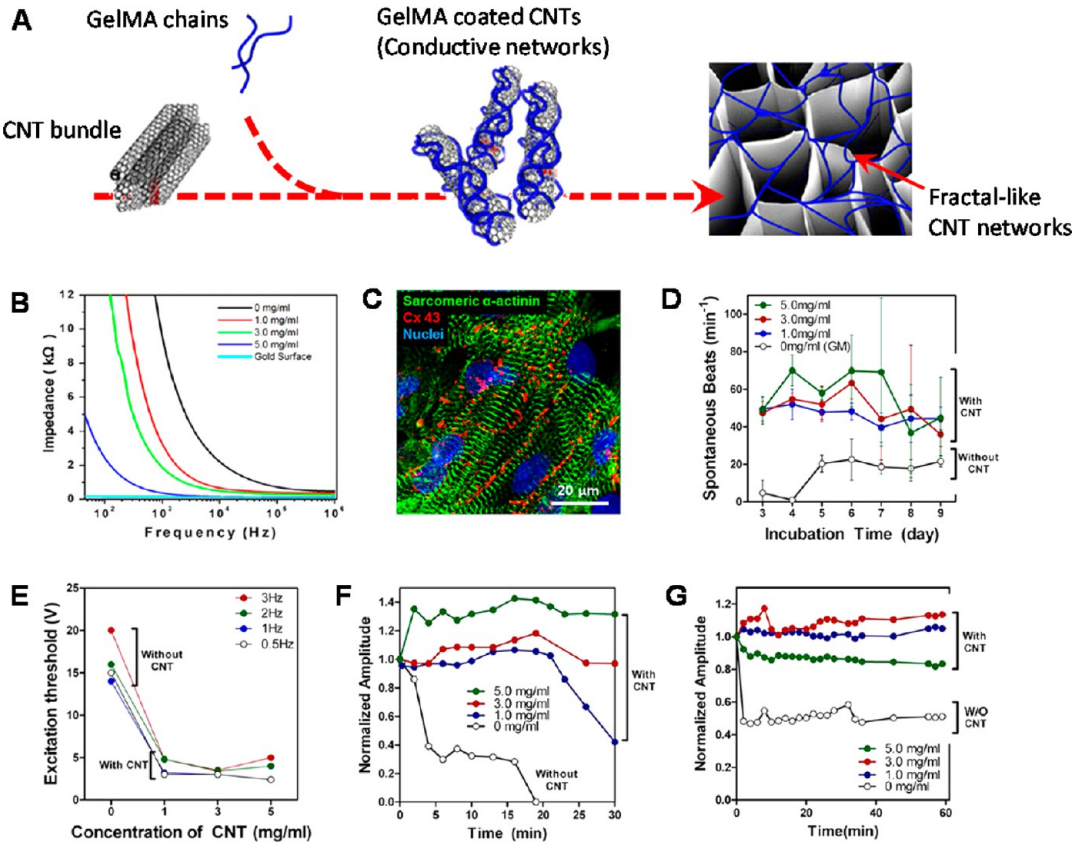

Figure 2. Carbon nanotube (CNT)-incorporated photocross-linkable GelMA hydrogels: (A) schematic diagram illustrating the preparation of fractal-like CNT networks embedded in GelMA hydrogels; (B) bulk impedance of a 50-μm-thick hydrogel thin film decreased with increasing CNT concentration; (C) partial uniaxial sarcomere alignment and connected intercellular electrically coupled gap junctions; (D) spontaneous beating rates of cardiac tissues showed more stable behavior with CNTs than without CNTs; (E) excitation thresholds were drastically reduced by 85% with CNTs; (F) protective effect of CNTs prevents heptanol from interrupting cardiac tissue beating propagation; (G) protective effect of CNTs minimizes the damage that doxorubicin causes on cardiac tissue. Reprinted with permission from ref 18. Copyright 2013 American Chemical Society.

tissue developed using current methods has weak cellular integrity, short duration of contractility, and inhomogeneous cell seeding.[11] While scaffolds are adequate base materials for cell growth, they are usually very insulating and prevent the propagation of electrical signals. Furthermore, there are subtypes of myocytes such as pacemaking, atrial, ventricular, and Purkinje cells that have different functional characteristics. To mimic the function of native tissue, contractions of engineered cardiac tissue should generate forces >25 mN/mm^2.[12,13] In addition, the propagation of electrical signals in atrial and ventricular cells has conductance velocities between 25 and 45 cm/s, while that of Purkinje cells has much higher conductance velocities between 200 and 400 cm/s.[14,15]

Implantation of thin bioengineered cardiac tissue has little or no effect on improving cell viability or tissue function in a damaged myocardium. To be effective, the engineered tissues need to be thick and to have high densities of myocytes and supporting cells. However, it is difficult to grow 3D structures that comprise more than a few layers of muscle cells. Most bioreactors simply cannot supply enough nutrients and oxygen to the growing tissue. Whereas human heart muscle is up to 2 cm thick,[16] growth in a bioreactor typically stops once the tissue is about 100−200 μm,[11,17] or 4−7 cell layers, thick. Beyond this thickness, the innermost cells are too far from a fresh supply of growth media to survive. Therefore, there needs to be a strategy to develop a vascular network in bioengineering cardiac tissue that can anastomose to the host myocardium.

Carbon Nanotubes and Their Applications in Engineering Cardiac Tissue Constructs. Conventional methods of engineering cardiac tissue have not been able to recapitulate the organizational structure and functionality of the native myocardium. Accomplishing this complex task will require input from many researchers, each providing specific tools or methodologies that can be used to supplement conventional methods. The key to addressing the challenges facing cardiac tissue engineering is thus not only to develop new approaches, but to supplement existing methodologies with tools of physical, chemical, and electrical technologies that improve the physiology and function of the bioengineered cardiac tissue.

One example of a novel approach presented by Shin *et al.* in this issue of *ACS Nano* suggests that the addition of carbon nanotubes (CNT) to gelatin methacrylate (GelMA), a bulk scaffolding material, results in improved functionality of the engineered cardiac tissue (Figure 2A).[18] The resulting hybrid CNT–GelMA materials demonstrate cardiac tissue with (i) enhanced electrophysiological performance; (ii) improved mechanical integrity; (ii) better cell adhesion, viability, uniformity, and organization; (iv) increased beating rate and lowered excitation threshold; and (v) protective effects against cardio-inhibitory and cardio-toxic drugs.

A novel approach presented by Shin *et al.* in this issue of *ACS Nano* suggests that the addition of carbon nanotubes to gelatin methacrylate, a bulk scaffolding material, results in improved functionality of the engineered cardiac tissue

These CNT-embedded GelMA displayed enhanced electrophysiological performance as compared to pristine GelMA. The authors showed that the cross-linking between CNTs in the hydrogel matrix resulted in the formation of continuous fractal-like nanofibrous networks that were homogeneously distributed throughout the macroporous hydrogel. The electrically conducting CNT fibrous networks, which mimicked the submicrometer scale architecture of native ECM, bridged the insulating pore walls of the hydrogel and created additional pathways for current flow across the substrate. This acted to reduce the impedance between cells for charge redistribution and action potential propagation, much like the electrically conductive Purkinje fibers found in native heart muscle, thus improving the electrical conductivity of CNT–GelMA as compared to pristine GelMA hydrogels (Figure 2B).

The nanofibrous architecture of CNTs embedded in GelMA improved the mechanical integrity of engineered cardiac tissue by providing a mechanical reinforcement to the hydrogels and improving the macroscale mechanical strength. The resultant 3-fold increase in the compressive modulus of the engineered tissue from 10–32 kPa is comparable to the compressive modulus of adult rat right ventricular myocardium, which ranges from 20 ± 4 to 54 ± 8 kPa.[19] As conventionally used scaffolds for cardiac tissue engineering are typically mechanically weaker than native tissue, the enhanced mechanical properties of CNT–GelMA demonstrate an advantage of this hybrid material over materials without a reinforcing CNT nanofibrous network.

The CNT networks in cell scaffolds resulted in increases in cell viability, adhesion, uniformity, and organization of engineered cardiac tissue. Cardiomyocytes cultured on CNT–GelMA substrates showed nearly 40% higher cell viability and adhesion as compared to pristine GelMA, with no cytotoxic effects of CNTs observed over a seven-day culture period. In addition, uniform sheets of cardiac tissue were observed on CNT–GelMA, but not pristine GelMA after one day of culture. Fast Fourier transform (FFT)-based image analysis of alignment showed a local alignment index 1.75 times greater in CNT–GelMA than in pristine GelMA. The improved viability, adhesion, and organization are attributed to the ECM-mimicking architecture of the CNT nanofibrous networks (Figure 2C).

The cardiac tissue cultured on CNT–GelMA demonstrated functional benefits such as increased beating rate and lowered excitation threshold (Figure 2D). Cardiac tissue cultured on both CNT–GelMA and pristine GelMA substrates showed that the spontaneous synchronous beating of the cell sheet could be electrically paced after one day of culture. However, cardiac tissue formed on CNT–GelMA displayed greater stability of beating and a significantly higher beating rate (69.8 ± 19.1 BPM) than on pristine GelMA (22.6 ± 11.1 BPM), bringing the engineered tissue beating rate in the range of native tissue (72 BPM). In addition, the excitation thresholds of the engineered tissue were reduced by approximately 85% in CNT–GelMA (Figure 2E). Shin *et al.* suggest that this decrease in excitation threshold and increase in beating rate could prove advantageous by preserving the engineered tissue from damage caused by high electric potential. They explain this improvement in functionality by the enhanced electrophysiological behavior and mechanical integrity observed in CNT–GelMA.

CNT–GelMA demonstrates protective effects against cardio-inhibitory and cardio-toxic drugs when used as a scaffold material for cardiac tissue. In response to heptanol, which inhibits the gap-junctional permeability of calcium ions and thereby interrupts the propagation of action potentials between cells, the cell sheets cultured on pristine GelMA started sporadic beating within 10 min and stopped synchronous beating within 20 min of drug introduction (Figure 2F). By contrast, the cell sheets on the CNT–GelMA took significantly longer to start sporadic beating (26–40 min) and to stop beating altogether (40–65 min). Shin *et al.* propose this result as an indication of the benefits of the conductive CNT nanofibrous network that allows propagation of electrical signaling between cells even when cell–cell gap junctional coupling is inhibited. The introduction

of doxorubicin, a cytotoxic compound used in cancer chemotherapy, to cardiac tissues grown on pristine GelMA demonstrated an immediate decrease in beating amplitude and rate followed by tissue detachment and debris formation after 6 h (Figure 2G). The same concentration of doxorubicin, when introduced to tissue grown on CNT–GelMA, did not significantly affect the beating amplitude and rate of the cell sheet. Shin *et al*. propose an explanation to this interesting phenomenon by postulating mechanisms through which CNTs act as scavengers of the free oxygen radicals generated by doxorubicin. The protective effect of CNTs against such oxidative stress, as well as the maintenance of electrical coupling between cells even after inhibition of gap junctional coupling, demonstrate two important advantages of the CNT–GelMA.

Shin *et al*. demonstrated that the continuous and branching nanofibrous networks of CNTs embedded within GelMA hydrogels can improve electrophysiological performance, enhance mechanical integrity, result in better cell viability, adhesion, uniformity, and organization, improve contractile behavior, and form protective effects against cardio-inhibitory and cardio-toxic drugs. They propose that this multifunctionality is preserved even after the degradation of the GelMA matrix since the CNT networks remain embedded in the cardiac tissue. This study incorporated CNTs into GelMA hydrogel substrates, but the approach is applicable to other biomaterial scaffolds as well. The findings of this study thus introduce a new tool for improving engineered cardiac tissue functionality that supplements conventional methods.

Other Notable Enabling Tools and Approaches. Many groups have introduced novel strategies to engineer cardiac tissue that more closely mimics native myocardium. A few pioneering approaches that specifically address the challenges of scaling tissue into geometries and length scales relevant for tissue engineering and tissue-wide organization of 3D muscle architecture are presented below. These methodologies provide tools to supplement conventional approaches, and approaches that combine the advantages of some or all of these methodologies would promise to advance the field of cardiac tissue engineering significantly.

Decellularized Matrices. The high oxygen and energy demands of cardiomyocytes in engineered tissue, which cannot be satisfied by mere diffusion past a thickness of 100 μm, require a complete vascular architecture to overcome mass transfer limitations. Ott *et al*. have proposed a method of engineering bioartificial hearts with intact 3D geometry and vasculature by repopulating decellularized whole rat hearts with neonatal cardiac cells (Figure 3A,B).[20] These recellularized hearts were mounted into a bioreactor that provided pulsatile coronary perfusion to the entire construct over a period of 8–28 days. Eight days after initial cell seeding, the whole heart constructs demonstrated contractile behavior in response to electrical stimulation. This contractile behavior led to a pump function equivalent to 2% of native adult heart and 25% of 16-week fetal heart function, suggesting the need to improve the mechanical and electrophysiological performance of these whole-heart constructs. The decellularized scaffolds were also separately repopulated with rat aortic endothelial cells. This resulted in the re-endothelialization of some coronary vessels and ventricular cavities. This method of engineering bioartificial hearts proposes promising solutions to three grand challenges in this field: creating a complex 3D scaffold with complete vascular architecture, populating the scaffold with an appropriate composition of cells, and maturation of this construct within a controllable system until the development of contractile function. However, this approach requires some modifications in order to achieve the functionality demonstrated by native heart and does not address the combined challenge of both recreating the native vasculature using endothelial cells as well as repopulating the whole heart with cardiomyocytes.

Engineered Vascular Architecture. A major roadblock in the engineering of functional cardiac tissue is thus the incorporation of a mature and stable vascular architecture into the engineered whole heart system. Koike *et al*. have demonstrated a method of constructing stable networks of blood vessels by coimplantation of vascular endothelial cells and mesenchymal precursor cells in a 3D collagen gel (Figure 3C,D).[21] These engineered 3D vascular networks were implanted into mice and observed over a period of several months through transparent "windows" incorporated into the mice. Over time, the implanted constructs integrated with the existing circulatory system and became perfused. The engineered arterial vessels showed constriction behavior in response to local administration of a vasoconstrictor, indicating that the engineered vessels could demonstrate functionality similar to native tissue. Integrating these engineered functional vascular systems within large-scale bioartificial hearts, such as those demonstrated by Ott *et al*., would be a significant step forward in the engineering of functional cardiac tissue with engineered vascular architectures.

Microfabrication Technologies. Shin *et al*. sought to improve the functionality of engineered cardiac tissue by developing a hybrid bulk material (CNT–GelMA) that enhanced the mechanical integrity and electrophysiological performance of cardiac cell sheets. Another approach to enhancing the functionality of engineered tissue, which can be used in conjunction with modified bulk materials, is that of developing techniques to align and to organize the tissue on local and tissue-wide scales. To this end, Bian *et al*. have demonstrated a method of engineering 3D muscle tissue architecture *in vitro* using

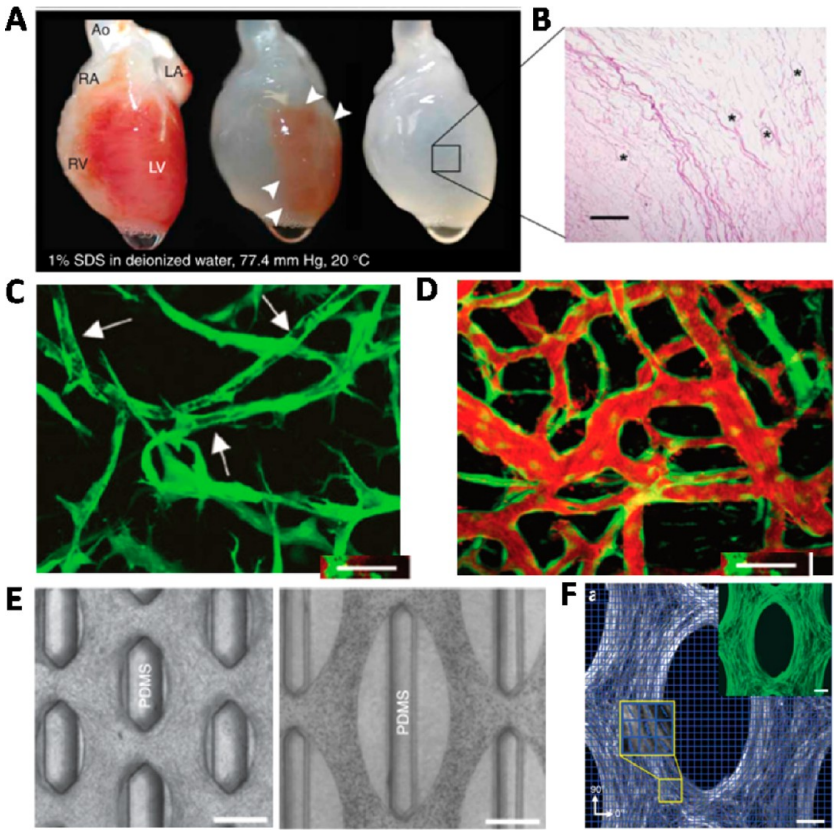

Figure 3. Enabling tools and approaches for bioengineered cardiac tissue: (A) perfusion decellularization of whole cadaveric rat hearts using SDS detergent; (B) hematoxylin and eosin (H&E) staining of decellularized heart showing no intact cells or nuclei with preserved vascular conduits. Scale bar, 50 μm. Reprinted with permission from ref 20. Copyright 2008 Nature Publishing Group. (C) HUVEC and 10T1/2 cells co-cultured in collagen gels shown four days after implantation into mouse model have long interconnected tubes but no evidence of perfusion; (D) HUVEC and 10T1/2 construct four months after implantation in mouse model with stable, perfused, and functional engineered vessels. Scale bar, 50 μm. Reprinted with permission from ref 21. Copyright 2004 Nature Publishing Group. (E) Cell-hydrogel compaction around microfabricated PDMS posts of different sizes and spacing between posts. Scale bars, 500 μm; (F) map of muscle cell alignment in cell-hydrogel tissue construct. Scale bar, 200 μm. Reprinted with permission from ref 22. Copyright 2009 Nature Publishing Group.

a microfabricated template as a mold for a cell/hydrogel composite material.[22] Cell/hydrogel mixtures are loaded into polydimethylsiloxane (PDMS) molds containing arrays of mesoscopic posts of precisely defined size and spacing (Figure 3E). These posts served as topographical constraints to cell-mediated compaction of the hydrogels, which in turn created a precise and reproducible spatial pattern of mechanical tension. This tension guided the local cell alignment in 3D and was used to engineer cardiac tissue with uniformly aligned and differentiated fibers (Figure 3F). Bian *et al.* envision that this method of engineering 3D muscle architecture can be used to differentiate and to align stem cell-derived muscle tissue, enabling this approach to be applied to the field of regenerative medicine and tissue engineering in the coming years.

Outlook and Future Directions. There have been many encouraging advances in the field of cardiac tissue engineering and development of biohybrid actuators recently. This can be attributed to the increasingly interdisciplinary nature of the field, with collaborations being established between disciplines such as bioengineering, regenerative medicine, developmental biology, stem cell engineering, genetic engineering, polymer chemistry, biomaterials, and microfabrication. The study by Shin *et al.* addressed several challenges of engineering functional cardiac tissue: to enhance mechanical integrity of their hybrid CNT–GelMA scaffold material; to improve cell adhesion, viability, uniformity, and organization; and to enhance electrophysiological coupling between cells in the scaffold. These improvements led to an observable increase in functionality by increasing the beating frequency and decreasing the excitation threshold of cardiac tissue grown on the hybrid CNT–GelMA materials. The challenge that remains to be addressed is the scaling of these engineered tissues into thicknesses and geometries that enable their application in tissue engineering and regenerative medicine. This is a complex problem that is yet unsolved, but the work done by Shin *et al.* brings forward a promising new tool for researchers aiming to improve the mechanical and electrophysiological performance of their engineered cardiac tissues. This tool, when used in conjunction with

other approaches that specifically target scaling of cardiac tissue into length scales relevant for tissue engineering, may prove to address the grand challenges of engineering functional cardiac tissue.

The challenge that remains to be addressed is the scaling of these engineered tissues into thicknesses and geometries that enable their application in tissue engineering and regenerative medicine.

Additionally, many other emerging biological applications based on bioengineered cardiac tissue are currently being developed. Some of these applications may not require materials of the same hierarchical tissue complexity as a cardiac patch or organ transplant, making it easier to produce. One notable example is a "heart-on-a-chip", which can be used to help screen and to detect drugs that stimulate or inhibit heart tissue contraction. The contractile response of the heart tissue can be observed to study the effects of physiological factors or to test drugs for cardiotoxicity. Using muscular thin film (MTF) technology of two-dimensional (2D) engineered cardiac tissue on elastic substrates, Grosberg *et al.* developed contractility assays in fluidic chambers and multiwell plates for automated tracking and analysis.[23] Boudou *et al.* fabricated arrays of tissue microgauges (μTUG) to generate cardiac microtissues embedded in collagen/fibrin 3D matrices for high-throughput, low-volume drug screening, and tissue morphogenesis studies.[24] By replicating small segments of heart tissue, it is possible to measure contraction data at the tissue level rapidly, rather than just studying individual cells. Another notable example is the development of bioactuators for biointegrated robotics and reverse-engineered life forms. In addition to Shin *et al.*, several groups have shown autonomous locomotion of 2D engineered cardiac tissue on soft materials using micromolding[25,26] and 3D printers.[27] Sakar *et al.* showed that the contractions of engineered, light-activated muscle could be controlled spatiotemporally for multiple degrees of freedom (multi-DOF),[28] setting the stage for controlled locomotion. Nawroth *et al.* reverse-engineered a jellyfish with rat heart cells on well-organized elastic substrates.[29] Our group demonstrated biohybrid actuators driven by cardiac cells that can walk on a surface in fluid.[27] Thus, there are many emerging applications for bioengineered cardiac tissues: to build "heart-on-a-chip" devices for drug safety and efficacy testing, to fabricate bioactuators for biointegrated robotics and reverse-engineered life forms, to treat abnormal cardiac rhythms, and to cure heart disease one day with tissue and organ transplants.

Conflict of Interest: The authors declare no competing financial interest.

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