Engineering of oriented myocardium on three-dimensional micropatterned collagen-chitosan hydrogel

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ABSTRACT
Introduction: Surface topography and electrical field stimulation are important guidance cues that aid the organization and contractility of cardiomyocytes in vivo. We report here on the use of these biomimetic cues in vitro to engineer an implantable contractile cardiac tissue.

Methods: Photocrosslinkable collagen-chitosan hydrogels with microgrooves of 10 µm, 20 µm and 100 µm in width were fabricated using polydimethylsiloxane (PDMS) molds. The hydrogels were seeded with cardiomyocytes, placed into a bioreactor array with the microgrooves aligned with the electrical field lines, and stimulated with biphasic square pulses at 1 Hz and 2.5 V/cm.

Results: At Day 6, cardiomyocytes were aligned in the direction of the microgrooves. When cultivated without electrical stimulation, the excitation threshold of engineered cardiac tissues using micropatterned hydrogels was significantly lower than using smooth hydrogels, thus showing the importance of cell alignment to cardiac function. The success rate of achieving beating constructs was higher with the application of electrical stimulation. In addition, formation of dense contractile cardiac organoids was observed in groups with both biomimetic cues. The cultivation of cardiomyocytes on hydrogels with 10 µm grooves yielded 100% beating tissues with or without electrical stimulation, thus suggesting a smaller groove width is necessary for cells to communicate and form proper gap junctions. However, electrical field stimulation further increased cell density and enhanced tissue morphology which may be essential for the integration of the tissue construct to the native heart tissue upon implantation.

Conclusions: The biodegradability of the hydrogel substrate allows for the rapid translation of the engineered, oriented cardiac tissue to clinical applications.

KEY WORDS: Cardiac tissue engineering, Collagen, Chitosan, Hydrogel, Topographical cues, Electrical field stimulation

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INTRODUCTION

The native myocardium is composed of a complex arrangement of cells. This organization is essential for the proper functioning of the cardiac tissue. Cellular arrangement is dependent on the microenvironment that provides various guidance cues to the cells. Neonatal rat cardiomyocytes (CMs), the cell type used in this study, have been shown to be responsive to guidance cues (1-4) as well as electrical field stimulation (2, 3, 5, 6).

Au et al (2, 3) previously studied the combined effects of surface topography and electrical field stimulation on the culture of cardiomyocytes. Polyvinyl surfaces were abraded with lapping paper to create V-shaped grooves of 13
In brief, 4-azidobenzoic acid (ABA, 80 mg/mL in DMSO; TCI America, Portland, OR, USA), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC, 140 mg/mL in distilled water) and N,N,N',N'-Tetramethylethylenediamine (TEMED, 300 µL; Sigma-Aldrich, St Louis, MO, USA) were mixed into a solution. Dissolved chitosan (75% to 90% deacetylated chitin, 400 mg dissolved in 15 mL distilled water; Novamatrix, Sandvika, Norway) was added to the solution in a drop-wise manner, after which the solution was left for 12 hours. The solution was put through numerous acid (pH 6) and base (pH 10) washes to remove the unreacted ABA. The final product was lyophilized for 3 days and stored at -20°C.

Fabrication of PDMS Stamps

Polyvinyl carbonate cover slips (22x22 mm²) were abraded using aluminum oxide lapping paper of various grain sizes. Silicone elastomer and curing agent (Sylgard 184, Essex Chemical, Newark, NJ, USA) were mixed in a 10:1 ratio and the mixture was poured onto cleaned, abraded polyvinyl surfaces or SU-8 master molds to be cured in the oven at 50°C overnight. The cover slips or molds were then removed, leaving patterned PDMS stamps with 10 µm, 20 µm, 25 µm, 50 µm and 100 µm wide grooves (Fig. 1). Stamps were cleaned by first removing particulate debris through sonication (Ultrasound cleaner, 1510R-MT; Misonix, Farmingdale, NY, USA) and then washing in soap and rinsing in distilled water. They were sterilized in 70% ethanol and dried under UV for 12 hours.

Fabrication of photocrosslinkable collagen-chitosan gels

The lyophilized Az-chitosan (described above) was reconstituted in 0.9% saline (NaCl solution in water) to a concentration of 10 mg/mL. Collagen I (3.67 mg/mL; BD Biosciences, Franklin Lakes, NJ, USA) was mixed with Az-chitosan at different collagen-chitosan mass ratios (0:1, 1:200, 1:120 and 1:50) for studies to determine the optimal gel composition. For further studies, the 1:50 collagen-chitosan ratio was used to give concentrations of 0.19 mg/mL collagen I and 9.5 mg/mL chitosan. The gel solution, 20 µL, was pipetted onto the sterilized PDMS mold (either abraded or smooth) as a droplet. The droplets were exposed to UV irradiation at a distance of 1.5 cm for 30 minutes at room temperature (UVP, 365 nm, 1 mW/cm² at 3° distance, 64 µm in width and 700 nm in depth (2). These surfaces were seeded with fibroblasts and cardiomyocytes and placed between two electrodes. The topographical cues and electrical field stimulation both led to cell elongation, with topographical cues also having significant effect on cell alignment. Further, Au et al (3) incorporated topographical cues and electrical field stimulation onto cell culture chips consisting of microgrooves created by hot embossing of polystyrene surface and two gold electrodes electrodeposited at the ends of the chip at 1 cm apart. As expected, cardiomyocytes seeded onto the micropatterned surfaces were elongated and aligned along the microgrooves. Since the cells were organized by surface topography, the application of electrical field stimulation led to the formation of gap junctions confined to the cell-cell end junctions and significantly enhanced cardiac function. It is clearly evident that the combination of two biomimetic cues produces a synergistic effect for cardiac tissue engineering. While this interactive effect has been shown for cardiomyocytes cultured on non-degradable, two-dimensional (2D) hard substrates such as fibronectin-coated polyvinyl and polystyrene surfaces, there is an incentive to apply the same principle to achieve functional engineered cardiac tissues on implantable soft substrates.

Here we describe the engineering of a beating cardiac tissue on a biodegradable, collagen-chitosan hydrogel with microgrooves, with the application of electrical field stimulation during cultivation (Fig. 1). The advantage of using a biodegradable hydrogel substrate is the ease of translation of the engineered tissue to a clinical setting. Moreover, the hydrogel substrate provides a three-dimensional (3D) environment that is similar to the in vivo extracellular matrix in its components and its ability to remodel according to the beating of the cultivated cells. Fibroblasts and cardiomyocytes were able to align along the grooves on the hydrogel substrates. Moreover, the topographical cues led to improved contractile properties. The use of electrical field stimulation further improved functionality of the engineered cardiac tissues on the micropatterned hydrogel substrates and aided the formation of dense contractile cardiac organoids.

MATERIALS AND METHODS

Preparation of Az-chitosan

Az-chitosan was synthesized by chemically modifying chitosan with azidobenzoic acid, as described previously (7). In brief, 4-azidobenzoic acid (ABA, 80 mg/mL in DMSO; TCI America, Portland, OR, USA), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC, 140 mg/mL in distilled water) and N,N,N',N'-Tetramethylethylenediamine (TEMED, 300 µL; Sigma-Aldrich, St Louis, MO, USA) were mixed into a solution. Dissolved chitosan (75% to 90% deacetylated chitin, 400 mg dissolved in 15 mL distilled water; Novamatrix, Sandvika, Norway) was added to the solution in a drop-wise manner, after which the solution was left for 12 hours. The solution was put through numerous acid (pH 6) and base (pH 10) washes to remove the unreacted ABA. The final product was lyophilized for 3 days and stored at -20°C.

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Chiu et al

The bioreactor array (Fig. 1A) was milled from a polycarbonate sheet and consisted of five sample wells situated between two parallel carbon electrodes (1/4 in diameter; Ladd Research Industries, Williston, VT, USA) that were 1 cm apart and connected through platinum wires to a stimulator (Grass S88X; Grass Technologies, West Warwick, RI, USA). The bioreactor array was placed in a plastic 100 mm diameter x15 mm height Petri dish. Cells (3T3 fibroblasts or neonatal cardiomyocytes), 1000000, were seeded onto each hydrogel in 10 µL medium, consisting of high glucose Dulbecco’s Modified Eagle Medium (DMEM) with 4.5 g/L glucose, 4 mM L-glutamine, 10% fetal bovine serum (FBS), 10 mM HEPES Buffer, 100 units/mL penicillin and 100 µg/mL streptomycin. The samples were incubated for 40 minutes at 37°C before 25 mL culture medium was added to each Petri dish. The cells were cultured for 6 days with medium exchange every other day. For the stimulated groups, electrical stimulation was applied as biphasic square pulses at 2.5 V/cm, 1 Hz and 1 ms (per phase), unless otherwise stated for studies involving different electrical stimulation regimes (2.5-8 V/cm and 1-2 Hz). A supplementation of 10 µM ascorbic acid (Sigma A4544; Sigma-Aldrich, St Louis, MO, USA) was added to the medium at every culture medium exchange.

Cell Seeding and Cultivation for Cardiac Constructs

Enriched cardiomyocytes were isolated from 2-day-old neonatal Sprague-Dawley rat hearts by serial collagenase digestion and pre-plating, as described previously (8), in accordance with the protocol approved by the University of Toronto Committee on Animal Care. The hydrogels were placed, with abraded surfaces facing upward, in the bioreactor array. The bioreactor array (Fig. 1A) was milled from a polycarbonate sheet and consisted of five sample wells situated between two parallel carbon electrodes (1/4 in diameter; Ladd Research Industries, Williston, VT, USA) that were 1 cm apart and connected through platinum wires to a stimulator (Grass S88X; Grass Technologies, West Warwick, RI, USA). The bioreactor array was placed in a plastic 100 mm diameter x15 mm height Petri dish. Cells (3T3 fibroblasts or neonatal cardiomyocytes), 1000000, were seeded onto each hydrogel in 10 µL medium, consisting of high glucose Dulbecco’s Modified Eagle Medium (DMEM) with 4.5 g/L glucose, 4 mM L-glutamine, 10% fetal bovine serum (FBS), 10 mM HEPES Buffer, 100 units/mL penicillin and 100 µg/mL streptomycin. The samples were incubated for 40 minutes at 37°C before 25 mL culture medium was added to each Petri dish. The cells were cultured for 6 days with medium exchange every other day. For the stimulated groups, electrical stimulation was applied as biphasic square pulses at 2.5 V/cm, 1 Hz and 1 ms (per phase), unless otherwise stated for studies involving different electrical stimulation regimes (2.5-8 V/cm and 1-2 Hz). A supplementation of 10 µM ascorbic acid (Sigma A4544; Sigma-Aldrich, St Louis, MO, USA) was added to the medium at every culture medium exchange.

Supplementary Fig. 1 - Pattern fidelity of micropatterned collagen-chitosan hydrogels. (A) Cross sections of the PDMS molds with groove width of 25 µm, 50 µm and 100 µm, showing the height of the grooves to be around 220 µm. (B) The pattern is maintained throughout the copying and cultivation process, as shown by the pattern on (i) the PDMS mold with 100µm wide grooves, (ii) the hydrogel on Day 0 after rehydration and removal from the PDMS mold but before being immersed in culture medium, and (iii) the hydrogel after being immersed in culture medium for 6 days.
Engineering of oriented myocardium on micropatterned hydrogel

For studies to determine the best orientation of topographical cues with regards to the electrical field, the sample wells in the bioreactor array were abraded with lapping paper to achieve microgrooves parallel or perpendicular to the electrodes. The wells were coated with Matrigel and seeded with 100000 cardiomyocytes. Electrical stimulation was applied as biphasic square pulses at 2.5 V/cm, 1 Hz and 1 ms. The abraded surfaces were placed such that the grooves were oriented either parallel or perpendicular to the electrical field lines.

Assessments

Functional Testing

Functional properties of the constructs were determined at Day 6 by evaluating three parameters: excitation threshold (ET), maximum capture rate (MCR) and success rate, as described previously (5). ET is the minimum voltage, using monophasic square pulses at 1 Hz and 2 ms, required to induce synchronous contractions of at least 75% of the cells in field of view. MCR is the maximum pacing frequency for synchronous contractions at 200% of ET. It was measured by increasing the stimulation frequency until the contractions stopped or became asynchronous. Success rate is defined as the fraction of tissue constructs with attainable ET and MCR, using stimulation not exceeding 8 V/cm, out of all tissue constructs for the experimental group.

Live/dead staining

Live/dead staining was performed using carboxyfluorescein diacetate succinimidyl ester (CFDA, staining live cells green) and propidium iodide (staining dead cells red), at concentrations of 10 µM and 75 µg/mL, respectively, according to the manufacturer’s protocol (Molecular Probes, Eugene, OR, USA). Briefly, culture medium was removed from the Petri dish. Samples were then transferred, with the patterned side facing upward, to a 96-well plate. Live/dead staining solution, 200 µL, was added to each well and the plate was incubated at 37°C for 40 minutes. The samples were washed twice in PBS and imaged using fluorescence microscopy. Aspect ratio and orientation angle were evaluated from live/dead staining images as described previously (2).

Fig. 2 - The use of electrical field stimulation at 1 Hz and 2.5V/cm aids functional properties of engineered cardiac tissues. Matrigel was used to coat the polycarbonate surfaces for culture. (A, B) Excitation threshold and maximum capture rate for cardiomyocytes cultured on Matrigel under electrical field stimulation of 1 Hz and different voltages. (C, D) Excitation threshold and maximum capture rate for cardiomyocytes cultured on Matrigel at 2 Hz and different voltages. * denotes statistical significant difference (p<0.05 one way ANOVA with post-hoc Tukey tests).
After cultivation, the tissue constructs were fixed in 10% formalin for 1 hour and then sent to the Pathology Research Program (PRP) laboratory for paraffin embedding and sectioning. The samples were stained to determine cellular expression of troponin T, as previously described. Briefly, the sample slides were incubated at 58°C for 30 minutes. Deparaffinization and antigen retrieval were performed by incubating the slides in reveal solution in a de-cloaking chamber for 20 minutes at 95°C. The slides were blocked with 10% normal horse serum (NHS) in PBS for 40 minutes at room temperature in a humidified chamber. Primary antibody (mouse troponin T, MS-295-P; Fisher Scientific, Waltham, MA, USA) was applied at a dilution factor of 1:50 with 1.5% NHS and 0.5% Tween-20 in PBS at 4°C overnight. Then, secondary antibody (rhodamine goat anti-mouse, 115-025-166; Jackson ImmunoResearch, West Grove, PA, USA) and DAPI were applied at dilution factors of 1:50 with 1.5% NHS and 0.5% Tween-20 in PBS for 30 minutes. The slides were mounted with Fluoromount and imaged using fluorescence microscopy.
Engineering of oriented myocardium on micropatterned hydrogel

Regime for electrical field stimulation

A bioreactor array equipped with electrical field stimulation was designed as in Figure 1A. The first aim of the study was to find the appropriate electrical stimulation regime for cultivating cardiomyocytes in the developed bioreactor array. Cardiomyocytes were cultivated under 0, 2.5, 5, and 8 V/cm and frequencies of 1 Hz and 2 Hz (Fig. 2). There was no significant difference in ET values between the 1 Hz and 2 Hz groups at the same voltage (Figs. 2A and C). However, the MCR was significantly higher when a frequency of 1 Hz was used compared to 2 Hz for both 5 V/cm and 8 V/cm groups (Figs. 2B and D; p=0.0041 for 5 V/cm; p=0.0097 for 8 V/cm). Thus, the use of 1 Hz is more appropriate for the cultivation of neonatal rat cardiomyocytes on these biodegradable substrates compared to 2 Hz. Moreover, the groups stimulated at 2.5 V/cm and 5 V/cm at 1 Hz had significantly lower ET values compared to the corresponding non-stimulated control group (Fig. 2A, one-way ANOVA, p=0.0075). The group stimulated at 2.5 V/cm showed slightly higher MCR value compared to the 5 V/cm group (Fig. 2B, one-way ANOVA, p=0.0431). Thus, 1 Hz and 2.5 V/cm were the stimulation parameters used for subsequent studies.

Regime for topographical cues

The second aim was to determine the optimal configuration of the topographical cues on the hydrogel with respect to the electrical field. The abrasions in the bioreactor array were oriented to be either parallel or perpendicular to the field lines (Fig. 1A) and coated with Matrigel. Cardiomyocytes were then cultivated on these Matrigel-coated surfaces (Fig. 3). Live/dead staining (Fig. 3A) showed alignment and elongation of cardiomyocytes along the direction of the field lines. Live/dead staining images of cardiomyocytes on (A) chitosan only, (B) 1:200 w/w collagen-chitosan (9.9 mg/mL chitosan and 0.05 mg/mL collagen), (C) 1:120 w/w collagen-chitosan (9.8 mg/mL chitosan and 0.08 mg/mL collagen), and (D) 1:50 w/w collagen-chitosan (9.5 mg/mL chitosan and 0.19 mg/mL collagen). CFDA stains live cells green, PI stains dead cells red.

Statistical analysis

Statistical analysis was performed using Graphpad Prism 5.0. T-tests were used for comparisons of two groups; one-way ANOVA with post-hoc Tukey’s tests or two-way ANOVA with Bonferroni post tests were used for comparison of multiple groups; p<0.05 was considered statistically significant for all tests. Data were represented as means ± standard deviations.

RESULTS

Regime for electrical field stimulation

Fig. 4 - The increase in collagen concentration in photocrosslinkable collagen-chitosan hydrogels improves survival of cardiomyocytes. Live/dead staining images of cardiomyocytes on (A) chitosan only, (B) 1:200 w/w collagen-chitosan (9.9 mg/mL chitosan and 0.05 mg/mL collagen), (C) 1:120 w/w collagen-chitosan (9.8 mg/mL chitosan and 0.08 mg/mL collagen), and (D) 1:50 w/w collagen-chitosan (9.5 mg/mL chitosan and 0.19 mg/mL collagen). CFDA stains live cells green, PI stains dead cells red.
of the grooves. There were significant differences between the ET values (Figs. 3B, D, and F) for non-stimulated and stimulated groups of cardiomyocytes cultivated on smooth surfaces (p=0.0058) and on surfaces with grooves perpendicular to the electric field (p=0.0166), but not on surfaces with grooves parallel to the electric field (p=0.3419). Thus, topographical cues had an effect on the functionality of cardiac tissues, but this effect diminished when electrical stimulation was applied as there was no significant difference between the smooth and parallel groups when stimulation was applied during cultivation.

When stimulated, both perpendicular and parallel groups had significantly higher aspect ratio compared to smooth group (Fig. 3H, p<0.001 for both perpendicular and parallel). Furthermore, the cells grown on grooves that are parallel to the electric field lines showed higher aspect ratio compared to the cells grown on grooves that are perpendicular to the electric field (p<0.001), consistent with our previous work (3). More importantly, the stimulated parallel group had significantly higher aspect ratio compared to
its corresponding non-stimulated group (t-test, p=0.0064), showing that electrical field stimulation improves cell elongation. Similarly, the parallel group had the lowest orientation angle amongst the three surfaces for both non-stimulated and stimulated conditions (Fig. 3I, p<0.0001). The stimulated parallel group also had significantly lower orientation angle compared to its corresponding non-stimulated control group (p=0.0072). Although topographical cues did not have an effect on the functional properties once electrical stimulation was applied, the cells showed enhanced elongation and orientation for the parallel group, which may be important for improving functional properties at higher cell densities and for integration of the engineered tissue upon implantation. As such, the constructs were placed with microgrooves parallel to the electric field lines in subsequent studies (Fig. 3).
in their ability to support the survival of cells and the engineering of cardiac tissues based on primary cardiac cells (Fig. 4).

Since Az-chitosan can be crosslinked when exposed to UV light and collagen I cannot, it is necessary to find optimal concentrations of both so that the mixture remains crosslinkable and the resulting gel supports cell survival. As expected, hydrogel solution made purely of collagen I was not able to gel after UV exposure since collagen was not rendered photocrosslinkable by chemical modification with azidobenzoic acid. Its inability to gel impedes pure collagen to be micromolded into patterned hydrogel substrates. On the other hand, chitosan-only gels composed of 20 mg/mL chitosan did not support cell survival (Fig. 4A). A composite gel (50:1 Az-chitosan:collagen ratio) with 0.19 mg/mL collagen and 9.5 mg/mL chitosan yielded high cell viability (Fig. 4D). Gel solutions that were made with a Az-chitosan:collagen ratio of less than 50:1 were not able to form stable gels for cell culture following UV radiation (data not shown).

Using the hydrogel composition of Az-chitosan:collagen 50:1, micropatterned hydrogels (Fig. 1B) were fabricated using soft lithography. Pattern fidelity was achieved throughout the fabrication process (Supplementary Fig. 1B). The hydrogels could be molded in PDMS stamps to fabricate hydrogel substrates with microgrooves of different widths, 25 µm, 50 µm and 100 µm (Fig. 5). The height of the grooves was ~220 µm on all PDMS stamps (Supplementary Fig. 1A). As expected, fibroblasts seeded onto the hydrogel attached, aligned and elongated along the microgrooves (Fig. 5). The cell viability was high on all surfaces.

The effects of hydrogel surface topography and electrical stimulation on cell alignment and cardiac function were further evaluated on the cultivation of cardiomyocytes in vitro, using the parameters optimized in the previous aims. The micropatterned hydrogels were placed in the stimulation bioreactor array (Fig. 1A) such that the microgrooves were aligning parallel to the electric field lines. Electrical stimulation was applied at 1 Hz and 2.5 V/cm. After 6 days of cultivation, cardiomyocytes were aligned along the microgrooves in all groups with micropatterned hydrogels (Fig. 6). Interestingly, the cell density was visibly higher in the stimulated groups compared to the corresponding non-stimulated groups. This could be due to the ability of electrical stimulation to increase cell-cell coupling and contractile activity, in turn motivating a dense arrangement of connected cardiomyocytes. It may also be due to an

Effect of electrical stimulation and topographical cues on collagen-chitosan gels

The disadvantage of using Matrigel as a substrate is the inability to control concentrations of proteins present, as well as the stiffness of the biomaterial. The goal is to create an oriented substrate that is biodegradable to support cell alignment and elongation. To do so, different concentrations of collagen I and Az-chitosan were used to create photocrosslinkable hydrogels, which were then evaluated
increase in the density of fibroblasts or other cell types. Furthermore, cardiac organoids were formed on the micropatterned hydrogels with electrical stimulation. When no electrical field stimulation was applied, there was significantly decreased excitation threshold in micropatterned gels compared to the smooth gels (Fig. 7, p<0.05 two-way ANOVA). This can be explained by the improved end-to-end connection between the aligned cardiomyocytes on micropatterned gels compared to a random arrangement of cells on the smooth gels, thus easing the propagation of pacing signals and decreasing the voltage necessary to attain synchronous beating of the tissue. In addition, contact guidance of cardiomyocytes can lead to the organization of the contractile apparatus, in turn improving contractile properties. The success rate of achieving contractile cardiac tissues was increased with the application of electrical field stimulation during cultivation (Tab. I). In addition, there was a significant decrease in excitation threshold when electrical field stimulation was applied, as compared to no stimulation, for all gels except for the gels with 10 µm grooves (Fig. 7). The gels with 10 µm grooves yielded 100% success rate for both non-stimulated and stimulated groups (Tab. I). There were no significant differences in maximum capture rates among all groups (Fig. 7, p>0.05, two-way ANOVA).

**DISCUSSION**

The field of tissue engineering has been developed to fill the escalating gap between the need for organs and tissues and the lack of supply. Regeneration of functional cardiac tissue is complicated by the complex arrangement of cells in the native heart tissue. As such, there is much interest in research on engineering cardiac constructs in vitro to be implanted in vivo. Recent studies in cardiac tissue engineering have used various polymeric and naturally-derived fibrous or porous scaffolds (9-12), as well as decellularized native tissues and organs (13, 14), as engineering biomaterials. Cell sheet engineering was also used to generate cardiac tissues without scaffolds, by growing monolayers of cardiomyocytes on a temperature-sensitive polymer that allowed the detachment of cell sheets at a lowered temperature and then stacking the cell sheets together (15-17). The cardiac tissue is made of elongated cardiomyocytes that contract in synchrony with gap junctions or intercalated discs that form connections between adjacent cells, allowing action potentials to propagate throughout the tissue. For this reason, the coupling of cells and the organization of the myocardium are of utmost importance to cardiac function (2, 18).

Ventricular CMs beat in response to electrical impulses whose rapid propagation requires high cell density and formation of gap junction between adjoining CMs (5). Previous studies demonstrated that the use of suprathreshold monophasic pulses on enriched CMs cultured on scaffolds could improve the structural and functional properties of engineered myocardium (6). The application of electrical stimulation to cultured cardiac constructs induces hyperpolarization at the anode end of the cell and depolarization at the cathode end of the cell (6). The cells that align with the electric field lines are subjected to the largest voltage difference and they generate action potentials and contract. The processes forming at the ends of the cells promote the formation of intercellular connections, propagation of pacing signals and generation of action potentials that lead to synchronous macroscopic contractions and organization of sarcomeres (6). This improves the response of the construct to electrical pacing.

Although elongation on non-abraded surfaces can be modulated by electrical field stimulation, topographical cues are a stronger determinant for cardiomyocyte orien-

<table>
<thead>
<tr>
<th>Experimental Group</th>
<th>Non-stimulated</th>
<th>Stimulated</th>
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<tbody>
<tr>
<td>Smooth</td>
<td>2 out of 6 gels (33%)</td>
<td>3 out of 5 gels (60%)</td>
</tr>
<tr>
<td>10 µm grooves</td>
<td>5 out of 5 gels (100%)</td>
<td>5 out of 5 gels (100%)</td>
</tr>
<tr>
<td>20 µm grooves</td>
<td>2 out of 3 gels (67%)</td>
<td>10 out of 14 gels (71%)</td>
</tr>
<tr>
<td>100 µm grooves</td>
<td>5 out of 7 gels (71%)</td>
<td>5 out of 6 gels (83%)</td>
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Results obtained after 7-day cultivation of cardiomyocytes on smooth and micropatterned hydrogels with or without electrical field stimulation.
Cells and tissues are sensitive to local micro- and nanoscale topography (18). These topographic patterns are present in the in vivo environment due to the structure of the extracellular matrix (ECM) (18). Although in vivo ECM molecules form fibrillar structures on the order of 10 nm to 100 nm, topographical guidance cues in the microscale are physiologically relevant. In the native rat heart, myocytes are tightly spaced between capillaries that are approximately 20 µm apart. Not surprisingly, guidance cues of structures on the order of microns are well documented (2, 18, 19). Cardiomyocytes that were cultured on thin films with micropatterned ECM proteins formed thin muscular constructs that performed gripping and pumping functions (20). In another study, cardiomyocytes cultured on polyeurathane films that were patterned by microcontact printing of laminin lanes were spatially organized (19). Further seeding of cardiomyocytes on top of the initial layer of patterned cardiomyocytes led to the organization of cells to form thin organized tissue sheets of <1 mm in thickness (19). Badie and Bursac (21) generated angled parallel lines on PDMS stamps by performing a two-dimensional projection of three-dimensional maps of local cardiac fiber directions in mouse ventricles. This allowed the printing of fibronectin patterns so as to guide local cell alignment to generate realistic ventricular cross-sections.

Due to the ability for topographical cues to guide alignment of cardiomyocytes and electrical field stimulation to enhance cell-cell coupling and construct contraction, we used a combination of these two biomimetic cues in this study. The bioreactor array used to cultivate the cardiac constructs (Fig. 1A) was developed such that five samples could be cultured and stimulated in one array. In addition, the bioreactor was fabricated using transparent polycarbonate sheet, allowing the ease of observing the cultivated cells under the microscope. It was further determined here that the developed bioreactor could consistently produce beating cardiac tissues (Figs. 2, 3, 6, and 7). The optimal parameters for electrical field stimulation for this bioreactor array were found to be 1 Hz and 2.5 V/cm, with microgrooves aligned perpendicular to the electrodes. Biphasic pulses at 1 Hz and 2.5 V/cm were also previously used to create contractile cardiac tissues on poly(ethylene glycol)-diacylate discs based on cardiomyocytes, fibroblasts and endothelial cells (5). In addition, orienting the microgrooves perpendicular to the electrodes helps to align the cells with the electric field lines, thus allowing the electrical field stimulation to act maximally on cells for the generation of action potentials (6).

Previously, Au et al (2, 3) studied the interactive effects of surface topography and electrical field stimulation on the organization of cardiac cells. Cardiomyocytes cultured on abraded surfaces could orient and elongate, with additional improvement in contractility when the cells and the topographical cues were aligned in parallel with the electric field lines (2). However, cardiomyocytes were grown on fibronectin-coated polyvinyl and polystyrene surfaces in the studies by Au et al (2, 3). The use of these non-biodegradable substrates led to several disadvantages, including the inability of the cells to remodel the substrate during culture. Although it was reported that cardiomyocyte beating does not have a significant effect on cell orientation and elongation (3), the ability of cells to remodel the substrate may be important for the formation of functional cardiac organoids. This is evident in the three-dimensional tissue structures in the stimulated groups as shown in Fig. 6, as compared to the cardiac monolayers in previous studies (2, 3). Also, the cardiac monolayers grown on polyvinyl and polystyrene surfaces cannot be implanted in vivo due to the non-biodegradable nature of the substrates.

Here we developed a photocrosslinkable hydrogel composed of collagen I and Az-chitosan that contained enough collagen I to support cell survival and enough Az-chitosan to yield stable biomaterial for cell culture (Figs. 4, 5, and 6). Hydrogels are highly hydrated crosslinked polymer networks, making them applicable in the development of matrices for tissue engineering. They can act as replacements of the extracellular matrix to organize cells into a three-dimensional architecture that mimics their organization in the native tissue, thus helping to restore natural function (22). Collagen and chitosan are naturally derived polymers that are extensively studied and widely applied in tissue engineering. Collagen I is a major component of the extracellular matrix of the native heart wall (23). It is biocompatible and promotes cell attachment and proliferation (24). The disadvantage of collagen-only hydrogels is the fast biodegradation rate and low mechanical strength (24). In this study, we combined collagen with chitosan to improve the mechanical strength of the hydrogel (25). Chitosan is a naturally occurring polysaccharide that is commonly used to create a biodegradable biomaterial. The chitosan backbone consists of amino groups, thus allowing conjugation with azidobenzoic acid to render it photocrosslinkable. The chemically modified chitosan, Az-chitosan, can be gelled upon brief exposure (~5 min) to UV light (7). The gelation of
Engineered oriented myocardium on micropatterned hydrogel

Photocrosslinkable chitosan does not require photoinitiators (1). Thus, it is non-toxic and biocompatible (1). It has been shown previously that Az-chitosan does not support heart cell adhesion and survival well on its own (7). The increase in the proportion of chitosan can inhibit cell growth in collagen-chitosan hydrogels (25). Here we found an optimal collagen-chitosan ratio such that the gelation occurred so as to form a stable hydrogel and the growth of fibroblasts and cardiomyocytes was supported. This collagen-chitosan ratio was also important for the gel solution to cover and penetrate the grooves of the PDMS molds to achieve pattern fidelity in the hydrogel patterning process.

Importantly, both collagen and photocrosslinkable chitosan are biodegradable. It was previously shown that 85% of photocrosslinkable chitosan degraded after 14 days of subcutaneous implantation in mice (26). Similarly, a honeycomb scaffold made of collagen I was shown to degrade in vitro when seeded with human fibroblasts for 60 days (27). The biodegradability of the collagen-chitosan hydrogel described here is consistent with its envisioned ultimate application in vivo. Neonatal cardiomyocytes are committed, specialized cells with a characteristic phenotype and functional properties. These cells do not proliferate. However, they have been well-documented to respond to contact guidance (2, 28) as well as electrical field stimulation (2, 6, 29). Cardiac fibroblasts, another significant cell population of the heart, are able to proliferate. Cardiac fibroblasts normally regulate myocardial development via mechanical, chemical and electrical interactions, and are responsible for the synthesis of ECM proteins such as collagen type I.

Both cardiomyocytes and fibroblasts could be organized by the micropatterned collagen-chitosan hydrogel (Figs. 5 and 6). In the absence of electrical field stimulation, topographical cues improved the contractile function of the tissues engineered based on cardiomyocytes, according to success rates (Tab. I) and the measurements of ET (Fig. 7). Electrical stimulation further improved the functional properties of the engineered cardiac tissues (Fig. 7). Interestingly, cardiomyocytes grown on the gels with 10 µm grooves showed high success rate for formation of contractile force and low excitation threshold in both stimulated and non-stimulated cases. Moreover, the stimulated group of gels with 10 µm grooves showed a uniform coverage of the substrate by the aligned cardiomyocytes, rather than a formation of dense cardiac organoids. As such, cell morphology and functional properties suggest that smaller grooves are more desirable for providing contact guidance for cardiac tissue engineering. However, it was not possible to create hydrogels with grooves smaller than 10 µm using the current fabrication technique. Small patterns that are densely spaced in PDMS molds are known to collapse easily, leading to the failure in replicating the patterns in subsequent copying processes (30). To investigate gels with grooves narrower than 10 µm, techniques such as imprint lithography or ultraviolet curable molds could be used (30).

While there was success in cell alignment and improvement of cardiomyocyte functionality on two-dimensional patterned substrates (2, 18), this study is the first step in aligning cells on a three-dimensional hydrogel. The resulting oriented cardiac tissue can be used as an in vitro model for physiological studies, or implanted directly for cardiac repair.

CONCLUSIONS

In this study, we developed a 3D collagen-chitosan hydrogel with microgrooves to guide the alignment and elongation of fibroblasts and cardiomyocytes. Moreover, we applied electrical field stimulation to enhance the functional properties of the cardiac constructs. Cardiomyocytes cultured on the gels with 10 µm-wide grooves were well organized into a 3D beating tissue. Since the grooves were perpendicular to the electrodes, cells could align with the electric field lines and were exposed to the greatest voltage difference. In turn, electrical field stimulation significantly affected the ability of the cardiomyocytes to contract and couple. Since the micropatterned hydrogel substrate is biodegradable, the engineered cardiac construct can be directly used in clinical applications while it was not possible in previous studies involving micropatterns on non-biodegradable substrates.

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