Functional 3-D Cardiac Co-Culture Model Using Bioactive Chitosan Nanofiber Scaffolds

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ABSTRACT: The in vitro generation of a three-dimensional (3-D) myocardial tissue-like construct employing cells, biomaterials, and biomolecules is a promising strategy in cardiac tissue regeneration, drug testing, and tissue engineering applications. Despite significant progress in this field, current cardiac tissue models are not yet able to stably maintain functional characteristics of cardiomyocytes for long-term culture and therapeutic purposes. The objective of this study was to fabricate bioactive 3-D chitosan nanofiber scaffolds using an electrospinning technique and exploring its potential for long-term cardiac function in the 3-D co-culture model. Chitosan is a natural polysaccharide biomaterial that is biocompatible, biodegradable, non-toxic, and cost effective. Electrospun chitosan was utilized to provide structural scaffolding characterized by scale and architectural resemblance to the extracellular matrix (ECM) in vivo. The chitosan fibers were coated with fibronectin via adsorption in order to enhance cellular adhesion to the fibers and migration into the interfibrous milieu. Ventricular cardiomyocytes were harvested from neonatal rats and studied in various culture conditions (i.e., mono- and co-cultures) for their viability and function. Cellular morphology and functionality were examined using immunofluorescent staining for alpha-sarcomeric actin (SM-actin) and gap junction protein, Connexin-43 (Cx43). Scanning electron microscopy (SEM) and light microscopy were used to investigate cellular morphology, spatial organization, and contractions. Calcium indicator was used to monitor calcium ion flux of beating cardiomyocytes. The results demonstrate that the chitosan nanofibers retained their cylindrical morphology in long-term cell cultures and exhibited good cellular attachment and spreading in the presence of adhesion molecule, fibronectin. Cardiomyocyte mono-cultures resulted in loss of cardiomyocyte polarity and islands of non-coherent contractions. However, the cardiomyocyte-fibroblast co-cultures resulted in polarized cardiomyocyte morphology and retained their morphology and function for long-term culture. The Cx43 expression in the fibroblast co-culture was higher than the cardiomyocytes mono-culture and endothelial cell co-culture. In addition, fibroblast co-cultures demonstrated synchronized contractions involving large tissue-like cellular networks. To our knowledge, this is the first attempt to test chitosan nanofiber scaffolds as a 3-D cardiac co-culture model. Our results demonstrate that chitosan nanofibers can serve as a potential scaffold that can retain cardiac structure and function. These studies will provide useful information to develop a strategy that allows us to generate engineered 3-D cardiac tissue constructs using biocompatible and biodegradable chitosan nanofiber scaffolds for many tissue engineering applications.


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KEYWORDS: electrospinning; chitosan nanofibers; fibronectin adsorption; cardiomyocytes; co-cultures

Introduction

Heart disease is a preeminent cause of death in the world, responsible for about 40% of human mortality (Chen et al., 2008). Ischemic heart disease followed by an episode of acute myocardial infarction is the most frequent cause of left-sided heart disease. Myocardial infarction can result in myocardial fibrosis and failure, the replacement of the damaged myocardium with a non-contractile fibrous scar tissue compromises heart contractile efficiency. The damage to the heart wall is permanent because, following extensive cell loss, the myocardium does not possess intrinsic regenerative capability (Baig et al., 1998). As a result, the heart fails to pump sufficient amounts of blood to compensate the ventricles undergoing remodeling and hypertrophy; predisposing the heart to congestive heart failure. Pharmacological therapeutics can reduce the heart’s work load via diuretics, nitrates, and humoral factor blockers such as ACE inhibitors or B-blockers (Doughty et al., 1997; Sharpe et al., 1991). Interventional therapy, such as implanting pacing devices can be performed to control electrical/mechanical asynchrony (Martinez et al., 2007). Nonetheless, pharmaceutics and surgery fall short from preventing progression to end stage heart failure (Packer, 2002). Ultimately, heart transplantation is the final option...
for end-stage heart failure. The current survival rate of heart transplants at 1 year after surgery is ~85% and at 5 years it is 75% (Stehlik et al., 2011). However, due to the scarcity of heart donors and the complications related to immune suppression and rejection, researchers are turning towards cardiac tissue engineering to develop alternative solutions to revive the distressed heart.

Several studies published in the early 1990s depicted long-term survival and differentiated phenotype of transplanted skeletal myoblasts in the ventricular myocardium (Koh et al., 1993a,b; Soonpaa et al., 1994). These studies paved the way for cardiac tissue engineering and shed light on the possibility of replacing the injured myocardium with healthy contractile cells. The ex vivo generation of an implantable myocardial contractile tissue-like unit is one of the most intensely researched topics in cardiac tissue engineering. The complexity of the myocardial tissue architecture demands the intricate design and integration of an extracellular matrix-like scaffold, cells, and biological factors. Cells sense the spatio-mechanical features of their microenvironment through integrin and mechanoreceptor mediated links between the external scaffold and the cell cytoskeleton (Inger, 1997, 2003; Stupack, 2005). This allows the cells to sense the scaffold’s chemistry, dimension, orientation, and mechanical properties that influence their survival and function (Schussler et al., 2010). Furthermore, the cellular composition of the engineered unit must resemble the native myocardium’s ratio of fibroblasts to myocytes (70:30) in order to closely replicate its physiological function (Banerjee et al., 2006; Vliegen et al., 1991). Fibroblasts have been shown to propagate electrical signals over distances of 100 μm via gap junctions and help synchronize cardiomyocyte contractions (Gaudesius et al., 2003). The intercellular dynamism channeled through gap junctions (connexin-43) and paracrine signaling between fibroblasts and myocytes affect myocyte structure and function (Laframboise et al., 2007). Hence, the biomaterial used for generating the scaffold, the cell species populating the scaffold, and the biological factors enhancing the cells’ performance and survival within the scaffold contribute to the successful fabrication of a myocardial contractile tissue-like unit.

Electrospinning is a fabrication technique by which submicron to nanometer fibers are produced as a non-woven mat from an electrostatically driven jet of polymer solution. Electrospinning is being extensively studied for tissue engineering applications because of its ability to produce nano-structures with a very high surface area to mass ratio (40–100 m²/g; Desai et al., 2008; Pham et al., 2006; Teo and Ramakrishna, 2006). In addition, the fibrous structure forms a network of interconnected voids that provides an environment that is similar to the in vivo ECM. Several synthetic or natural biomaterials have been used to fabricate nanofiber scaffolds using the electrospinning technique for cardiac tissue engineering applications (Rockwood et al., 2008; Shin et al., 2004; Zong et al., 2005). Although some advances have been made in the area, synthetic polymer-based scaffolds are not ideal for in vivo applications due to the toxicity of degradation products. Collagen is a natural polymer that has been commonly used in cardiac tissue engineering but its various applications have been limited by weak mechanical properties as a supportive scaffold and rapid in vivo degradation (Haugh et al., 2011; Venugopal et al., 2012).

Chitosan, a natural polysaccharide, is widely used in tissue engineering because of its biocompatibility, biodegradability, non-toxicity, and its pH dependent solubility facilitating its processing into micro- and nano-scaffolds (Cho et al., 2008b; Kim et al., 2008; Madhally and Matthew, 1999). The chemical structure of chitosan is similar to the glycosaminoglycans, such as hyaluronic acid, in the extracellular matrix, and its hydrophilicity enhances its interaction with growth factors, cellular receptors, and adhesion proteins (Kumar and Majeti, 2000; Matthew et al., 2003). Although electrospun nanofiber scaffolds using chitosan have been recently studied in many tissue engineering applications (Jayakumar et al., 2010; Ohkawa et al., 2004), there have been no reports on chitosan nanofiber scaffolds for cardiac tissue engineering applications. Furthermore, current cardiac tissue models are not yet able to stably maintain functional characteristics of cardiomyocytes in long-term culture.

In this study, we demonstrate for the first time the cytocompatibility of three-dimensional (3-D) chitosan nanofiber scaffolds for cardiac tissue engineering applications and improved cardiac function in the 3-D co-culture models. Chitosan nanofibers were fabricated using an electrospinning technique and their potential as a scaffold was studied for the development of 3-D cardiac tissue constructs. In order to enhance cell attachment and growth, fibronectin was incorporated onto the chitosan nanofibers by adsorption. Neonatal rat cardiomyocytes were cultured on the chitosan nanofibers and their viability, morphology, and function was assessed. For long-term survival and function, cardiomyocytes were co-cultured with either fibroblasts or endothelial cells on the chitosan nanofibers and examined by phenotypic and functional analysis.

Materials and Methods

Electrospinning of Chitosan

An 8% (w/v) chitosan solution was prepared by dissolving chitosan (medium molecular weight ~200 K, 75–85% deacetylation; Sigma, St. Louis, MO) in Trifluoroacetic acid (TFA; Fisher Chemicals, Pittsburgh, PA). The solution was stirred overnight at 40°C. Methylene Chloride (MeCl; Fisher Chemicals) was added to form a final volume to volume ratio of 80:20 (TFA:MeCl). The chitosan solution was fed into a 10 mL disposable syringe fitted with an 18 gauge needle. A DC voltage of 30 kV was applied to the needle and the planar collector was placed 30 cm from the needle. The polymer solution was pumped at a rate of 2 mL/h and the process was performed at room temperature.
and atmospheric humidity of 40–50%. Following vacuum drying at room temperature, the chitosan nanofibers were cut into pieces to fit into 35-mm dish and neutralized with 15N ammonium hydroxide: 100% ethanol (1:1 v/v ratio) for 30 min. The chitosan nanofibers were then washed with distilled water three times for 15 min each time. The chitosan nanofibers were then sterilized under a UV lamp for 20 min.

**Fibronectin Adsorption on Chitosan Nanofibers**

In order to evaluate fibronectin adsorption on chitosan, 24-well tissue culture dishes were coated with 1% chitosan and dried for 1 h before neutralization with 0.2 M ammonium hydroxide. Fibronectin (Sigma) solutions of different concentrations (0, 1.2, 5, 10, and 20 μg/mL in deionized water) were added into the dishes and incubated for 1 h at room temperature. The amount of adsorbed fibronectin was characterized by fluorescent staining using anti-fibronectin antibody (Sigma).

**Cell Culture**

Primary ventricular cardiomyocytes were isolated from 1-day-old neonatal Wistar rats (Charles River Laboratories, Wilmington, MA) using a collagenase procedure as described previously (Aoki et al., 1998) and cultured on 0.1% gelatin-coated dishes. Cardiomyocyte culture medium consists of DMEM (Gibco, Gaithersburgh, MD) with 10% FBS, 2 mM L-glutamine (Gibco), and 0.1% gelatin-coated dishes. Cardiomyocyte culture medium described previously (Aoki et al., 1998) and cultured on 1-day-old neonatal Wistar rats (Charles River Laboratories, Wilmington, MA) using a collagenase procedure as described previously (Aoki et al., 1998) and cultured on 0.1% gelatin-coated dishes. Cardiomyocyte culture medium consists of DMEM (Gibco, Gaithersburgh, MD) with 10% FBS, 2 mM L-glutamine (Gibco), and 0.1% gelatin-coated dishes. 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glutaraldehyde in PBS for 24 h at 4°C. The samples were then washed with PBS and serially dehydrated with 50%, 70%, and 100% ethanol for 15 min each. This was done to allow gradual dehydration of the cells preventing loss of cellular structural integrity. The samples were then vacuum-dried for about 6 h. The samples were coated with carbon and observed with SEM.

**Calcium Transient Ion Staining**

Calcium indicator, fluo-4 AM (Invitrogen), was used to observe calcium ion flux of beating cardiomyocytes. The cells were incubated in 4 μM calcium indicator in cardiomyocyte medium for 45 min at 37°C. The cells were then washed with cardiomyocyte medium twice, followed by incubation in cardiomyocyte medium for another 30 min at 37°C. The stained cardiomyocytes were visualized by fluorescence microscopy. Videos of the calcium ion staining were captured using Nikon NIS-Elements image software. Pseudo-color images of the calcium ion fluorescence intensity were captured every 150 ms. The intensity of the calcium ion flux was determined by taking the average intensity from 12 pixel image points from each frame and plotting the average intensity over time.

**Quantitative Image and Statistical Analysis**

Nikon Imaging Solutions (NIS)-elements imaging software program (v.3-448) was used to measure the diameter distribution of the chitosan fibers. The measurements were done from five different SEM images on different regions of each sample. A total of ~150 measurements were analyzed for the fiber diameter. To quantify the amount of adsorbed fibronectin on chitosan surfaces, average fluorescence intensity from three to four random fields of images were measured and analyzed by the image software after staining with anti-fibronectin, alexa fluor 488. In order to analyze the cell attachment and spreading on chitosan and fibronectin-coated chitosan surfaces, the surface area of cultured cells was measured and quantified by the image software. Three to four random fields of image per sample were acquired after staining with actin/DAPI and quantified by the image analysis. Data from representative experiments are expressed as the mean ± standard deviation (SD) of duplicate or triplicate samples. All experiments were repeated at least 2–3 times and similar results were reproduced. Statistical

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**Figure 1.** SEM images of electrospun chitosan nanofiber scaffolds at (A) 5,000× original magnification and (B) 50,000× original magnification. C: Fiber diameter distribution of the chitosan nanofibers. D: Photography of the chitosan nanofiber mat.
significance was determined by a two-tailed student’s t-test (p < 0.01).

Results

Characterization of Electrospun Chitosan Nanofiber Scaffolds

Figure 1A and B shows SEM images of the nanofibrous chitosan non-woven mats fabricated using the electrospinning technique at 5,000x and 50,000x magnifications, respectively. The chitosan mats demonstrated homogeneous cylindrical morphology and well-formed fibers with a fiber diameter ranging from 50 to 450 nm, and an average of 188 nm, as illustrated in Figure 1C. The thickness of the mats used in this study was ~150 μm. The random orientation of the fibers produces many interconnected spaces. The fibers did not dissolve and maintained their cylindrical morphology after neutralization with ammonium hydroxide.

Fibronectin Adsorption on the Chitosan Nanofibers

Cellular attachment to the fibers and infiltration into the interfibrous spaces were enhanced by immobilizing fibronectin onto the chitosan nanofibers by adsorption. To establish the concentration at which chitosan is saturated by fibronectin adsorption, immunofluorescent staining of adsorbed fibronectin using varying concentrations (0–20 μg/mL) was performed. Figure 2A illustrates an increase in observed fluorescence intensity with fibronectin concentration, which suggests that fibronectin adsorption on chitosan coated wells is dependent on the concentration of the fibronectin solution. There was a steady increase in the amount of adsorbed fibronectin as the concentration increases and the adsorption plateaus beyond 10 μg/mL. As a result, the 10 μg/mL solution was used to adsorb fibronectin on the chitosan nanofibers for improved cell adhesion and migration.

Effect of Fibronectin Immobilization on Cellular Morphology, Focal Adhesion Expression, and Cytoskeletal Organization

In order to investigate the effect of fibronectin immobilization on chitosan material in cell cultures, the morphology, cytoskeletal protein distribution, and vinculin focal adhesion expression of various cell types were monitored on 2-D chitosan films with and without fibronectin adsorption. As shown in Figure 3, when the cells were cultured on chitosan film, they maintained a rounded morphology, minimal vinculin focal adhesion expression, and a diffused F-actin cytoskeletal organization. In contrast, the cells cultured on fibronectin coated chitosan films demonstrated enhanced cellular spreading, significant increase in vinculin expression and a well-organized fibrous F-actin cytoskeleton.

Characterization of Cellular Behavior in the Chitosan Nanofiber Scaffolds

Assessment of cellular viability and morphology was performed to evaluate electrospun chitosan nanofibrous mat potential as cellular scaffolds. Fibroblasts, endothelial cells, and cardiomyocytes were seeded onto fibronectin coated chitosan mats and cultured over 3 weeks. Figure 4A–C depicts the live–dead cell staining of fibroblasts cultured on the chitosan nanofiber scaffolds, indicating that the chitosan nanofibers do not adversely affect cell viability. Similar results were observed for other cell types, including cardiomyocytes and endothelial cells (data not shown). Some cells formed filopodia-like extensions to attach to the fibers, assisting them in spreading inside the chitosan nanofibrous scaffold (Fig. 4D–F). In addition, the SEM images exhibit the formation of a film-like material surrounding the densely seeded areas, indicating the

Figure 2. Fibronectin (FN) adsorption on chitosan surfaces (2-D film and 3-D nanofibers). A: Relative fluorescence intensity and images of fibronectin adsorbed on chitosan coated tissue culture dishes at various fibronectin concentrations by immunofluorescence staining. B,C: Phase and immunofluorescence staining for anti-fibronectin of chitosan nanofibers adsorbed by fibronectin solution (10 μg/mL) at 200 x original magnification. Data are expressed as means ± SD.
secretion and immobilization of cell secreted ECM components.

Cardiac Function in Mono- and Co-Cultures on the 2-D Chitosan Films and 3-D Nanofiber Scaffolds

Cardiomyocyte morphology and gap junction formation were monitored via alpha-sarcomeric actin (SM-actin) and connexin-43 (Cx43) staining, respectively. Cardiomyocytes’ SM-actin and Cx43 expression was examined on both fibronectin adsorbed chitosan films (2-D) and fibronectin adsorbed chitosan nanofibers (3-D). In each condition, cardiomyocytes were cultured in mono-cultures (cardiomyocytes only) and co-cultures (cardiomyocytes–fibroblasts or cardiomyocytes–endothelial cells).

In the 2-D systems, the cardiomyocyte mono-culture (Fig. 5A and D) exhibited low expression of SM-actin and the cardiomyocytes lost their structural polarity and acquired a rounded morphology. Gap junction protein Cx43 expression was minimal in the mono-culture system. In contrast, the cardiomyocytes co-cultured with 3T3-J2 fibroblasts maintained a highly polar morphology and the SM-actin was strongly expressed along the axis of morphological polarity (Fig. 5B and E). In addition, Cx43 expression was the highest in the fibroblast
co-culture which enabled the cardiomyocytes to contract in a tissue-like synchronized manner (Supplementary Video 1). The cardiomyocytes co-cultured with endothelial cells (Fig. 5C and F) demonstrated a spherical morphology with lower levels of SM-actin and Cx43 expression than those in the fibroblast co-culture as well as isolated contractions (Supplementary Video 2).

To assess the chitosan nanofibers’ potential as a cardiac tissue engineering scaffold, the same cardiomyocyte mono-culture and co-culture studies were performed on the 3-D chitosan nanofibers. The cardiomyocyte mono-culture (Fig. 6A and D) and cardiomyocytes–endothelial cell co-culture (Fig. 6C and F) did not have any visible SM-actin or Cx43 expression. The cardiomyocyte-fibroblast co-culture resulted in elongated networks of contracting cardiomyocytes (Supplementary Videos 3 and 4) with the highest expression of SM-actin and Cx43 (Fig. 6B and E).

**Intracellular Calcium Ion Flux Analysis**

The intracellular calcium ion staining was used to monitor calcium ion flux of beating cardiomyocytes. Figure 7 shows the pseudo color time-frame images of the 3-D cardiomyocyte co-culture system. The co-culture system with fibroblasts demonstrated elongated cardiomyocyte morphology and a rhythmic and frequent change in intracellular calcium ion concentrations (Supplementary Video 5). The image analysis of the calcium flux showed a frequency of $17 \pm 3$ contractions per minute. In contrast, the 2-D and 3-D mono-cultures of cardiomyocytes lost their functionality and their polarized morphology after 4 days of culture (data not shown).

**Discussion**

The aim of this study was to fabricate 3-D chitosan nanofiber scaffolds using an electrospinning technique and to test for the first time the feasibility of 3-D chitosan nanofibers as scaffolds for cardiac tissue engineering applications. The results demonstrate that the chitosan nanofibers retained their cylindrical morphology in long-term cell cultures and exhibited good cellular attachment and spreading in the presence of adhesion molecule, fibronectin. In both 2-D and 3-D cultures on the chitosan scaffolds, cardiomyocyte-fibroblast co-cultures resulted in polarized cardiomyocyte morphology with high levels of SM-actin and Cx43 expression over long-term culture.
periods. In addition, the fibroblast co-cultures demonstrated synchronized contractions involving large tissue-like cellular networks, indicating the maintenance of long-term and stable function of cardiomyocytes gap junctions. To our knowledge, the results of the present study provided the first evidence that 3-D chitosan nanofibers can be used as a potential scaffold that can retain cardiomyocyte morphology and function. Further details of the chitosan nanofiber scaffolds on their physical, chemical, and thermal properties will be reported separately.

Cardiac fibroblasts are the most abundant non-cardiomyocyte cells in the mature heart. Their functions include deposition of the extracellular matrix (ECM), paracrine signaling, and propagation of the electrical stimuli. In this study, we used murine 3T3-J2 fibroblasts cell line (derived from Swiss mouse embryo by Howard Green) for cardiac co-cultures because of their easy access, propagation, and high induction of parenchymal cell functions (e.g., primary hepatocytes; Bhatia et al., 1999; Khetani et al., 2004). It was reported that co-cultures of primary parenchymal cells (e.g., cardiomyocytes, hepatocytes) with non-parenchymal stromal cells are able to maintain their phenotype and function for a longer period of time, depending on co-culture cell type (Cho et al., 2008a; Driesen et al., 2005; Goulet et al., 1988; Narboneva et al., 2004), but the effects of co-cultures are not species-specific (Clement et al., 1984). Potential mechanisms include gap junctional communications on heterotypic cell–cell contacts (Gaudesius et al., 2003; Kizana et al., 2006; Rook et al., 1992) and production of the extracellular matrices (Guillouzo et al., 1984; Loreal et al., 1993). However, details of the regulatory mechanism on the role of cell–cell interactions in co-cultures are still unknown. The key feature of our approach is the co-culturing of cardiomyocytes with either fibroblasts or endothelial cells within a 3-D scaffold for long-term functionality of the cardiomyocytes. SM-actin expression was solely found in cardiomyocytes and was expressed the most in the fibroblast co-cultures. Cx43 is the gap junction protein that is mainly found in ventricular cardiomyocytes (van Veen et al., 2001). The Cx43 mediates fibroblast heterogeneous coupling, such as between cardiomyocytes and fibroblasts (Snider et al., 2009). These gap junctions with fibroblasts are known to propagate electrical stimuli for 100 μm (van Veen et al., 2001). The results from both the 2-D and 3-D cultures indicate that fibroblast co-cultures resulted in high levels of SM-actin and Cx43 expression, suggesting fibroblasts are

Figure 5. Morphology and phenotypic characteristics of cardiomyocytes on 2D Chitosan-FN film. A,D: Cardiomyocytes cultured alone, (B,E) cardiomyocytes cocultured with 3T3-J2 fibroblasts, and (C,F) cardiomyocytes co-cultured with microvascular endothelial cells after 7 days of culture. Cardiomyocytes were immunostained for α-sarcomeric actin (SA-actin) and connexin-43 (Cx43) gap junction expression. Neonatal cardiomyocytes (CM), 3T3-J2 fibroblasts (FB), microvascular endothelial cells (EC). 200× original magnification.
essential in maintaining cardiomyocytes viability and function in vitro. Optimal cardiac tissue for therapeutic applications should involve a mixture of cardiac cells that more closely resemble native tissue but the optimal cell type to be used still remains uncertain. Thus, in order to mimic cardiac structure and function in vitro, a basic understanding of the role of each of these interactions will be essential to progress in the field.

Another advantage of our system is the use of electrospun chitosan to create nano to micro-sized fibers that reproduces the spatial dimensionality of the fibrous component of the ECM. The fact that cardiomyocytes are able to survive and contract on chitosan nanofibers mats has never been reported before, as per our knowledge. It is projected that these mats can be layered on top of each other to create a prevascularized thick tissue-like structure composed of cardiomyocytes, fibroblasts, and endothelial cells (Sasagawa et al., 2010). The fibroblasts will enhance the electrical synchronization of the cardiomyocytes, while the endothelial cells have the potential to facilitate vascularization into the graft to avoid diffusion limitation of oxygen and nutrients. Chitosan can interact electrostatically with cells since cells carry an overall slightly negative surface charge and chitosan’s free amine group can become protonated allowing ionic interactions (Chatelet et al., 2001; Prasitsilp et al., 2000). Our data suggests that cells cultured on chitosan surfaces maintained rounded morphology with poor cell adhesion. However, cells cultured on fibronectin coated chitosan surfaces exhibited a typical elongated shape with improved cell adhesion. Fibronectin is a large ECM glycoprotein which facilitates cell adhesion and spreading via α5β1 and αvβ3 integrin receptors in cells (Amaral et al., 2009). The integrins recognize and interact with RGD cell adhesion domains initiating cell signaling pathways that control cell survival, proliferation, differentiation, and remodeling of the ECM (García and Boettiger, 1999). The amine groups present in chitosan are engaged in fibronectin adsorption (Amaral et al., 2005). The functional activity of fibronectin is conserved because there is minimum protein unfolding conserving the cell adhesion sites (García and Boettiger, 1999).

In conclusion, results of this study demonstrate that chitosan nanofibers can be used as scaffolds for the development of 3-D cardiac tissue constructs that more closely resemble native heart tissue. Our results suggest that cardiac co-culture model is a promising system for the maintenance of long-term survival and function of cardiomyocytes. The engineered 3-D cardiac co-culture
and blue representing low calcium ion concentrations. Ion flux were pseudo-colored, with red representing high calcium ion concentrations intracellular calcium ion flux in the 3-D co-culture. The fluorescence images of calcium intervals with fluo-4, AM indicator. 200 images of calcium ion changes in fluorescence intensity, monitored at 0.7-s time culture of cardiomyocytes and fibroblasts after 7 days of culture.

Figure 7. Intracellular calcium ion flux of beating cardiomyocytes in 3-D co-culture of cardiomyocytes and fibroblasts after 7 days of culture. A: Pseudo-color images of calcium ion changes in fluorescence intensity, monitored at 0.7-s time intervals with fluo-4, AM indicator. 200× original magnification. B: Frequency of intracellular calcium ion flux in the 3-D co-culture. The fluorescence images of calcium ion flux were pseudo-colored, with red representing high calcium ion concentrations and blue representing low calcium ion concentrations.

model using chitosan nanofiber scaffolds will be useful for the design and improvement of engineered tissues for the repair of myocardial infarcts, tissue engineering applications, and drug testing.

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