Electrospun PLGA Fibers Incorporated with Functionalized Biomolecules for Cardiac Tissue Engineering

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Structural similarity of electrospun fibers (ESFs) to the native extracellular matrix provides great potential for the application of biofunctional ESFs in tissue engineering. This study aimed to synthesize biofunctionalized poly (L-lactide-co-glycolide) (PLGA) ESFs for investigating the potential for cardiac tissue engineering application. We developed a simple but novel strategy to incorporate adhesive peptides in PLGA ESFs. Two adhesive peptides derived from laminin, YIGSR, and RGD, were covalently conjugated to poly-L-lysine, and then mingled with PLGA solution for electrospinning. Peptides were uniformly distributed on the surface and in the interior of ESFs. PLGA ESFs incorporated with YIGSR or RGD or adsorbed with laminin significantly enhanced the adhesion of cardiomyocytes isolated from neonatal rats. Furthermore, the cells were found to adhere better on ESFs compared with flat substrates after 7 days of culture. Immunofluorescent staining of F-actin, vinculin, α -actinin, and N-cadherin indicated that cardiomyocytes adhered and formed striated α -actinin better on the laminin-coated ESFs and the YIGSR-incorporated ESFs compared with the RGD-incorporated ESFs. The expression of α -myosin heavy chain and β -tubulin on the YIGSR-incorporated ESFs was significantly higher compared with the expression level on PLGA and RGD-incorporated samples. Furthermore, the contraction of cardiomyocytes was faster and lasted longer on the laminin-coated ESFs and YIGSR-incorporated ESFs. The results suggest that aligned YIGSR-incorporated PLGA ESFs is a better candidate for the formation of cardiac patches. This study demonstrated the potential of using peptide-incorporated ESFs as designable-scaffold platform for tissue engineering.

Introduction

MYOCARDIAL INFARCTION OFTEN leads to necrosis or apoptosis of cardiomyocytes and left ventricular dilation, accompanied with reduced cardiac performance with high mortality of patients. Restoration of the damaged myocardium is desirable for the treatment of heart failure. Transplantation of tissue-engineered cardiac grafts is an emerging strategy to treat diseased myocardium or cardiac malfunction. Cardiac tissue engineering aims to repair damaged myocardium by combining cell biology, material science, and engineering principles. Engineered cardiac constructs composed of scaffolds alone or in combination with cells or growth factors have been widely investigated.^{1–5} The types of cardiac patches include acellular xenogeneic extracellular matrix (ECM),^{6–8} scaffolds^{5,9–12}, and cell sheets.^{13–15}

Morphological similarity between the native ECM and electrospun fibers (ESFs) provided great potential for the application of biologically modified ESFs in cardiac tissue engineering. Fibers fabricated by an electrically driven jet have

been employed in various biomedical applications such as tissue engineered constructs and wound healing patches.¹⁶ One of the advantages of ESFs is that a wide variety of macromolecules can be used for fabrication from biologically derived polymers such as gelatin, collagen, silk fibroin, chitosan, and cellulose^{17–21} to synthetic polymers such as polyglycolide, poly (L-lactide) (PLLA), poly(ϵ -caprolactone) (PCL), polyurethane, and poly(vinyl alcohol).²² ESFs made of natural polymers usually possess inferior mechanical properties, while those made of synthetic polymers often lack suitable biological activities.^{23,24} One solution is to biofunctionalize synthetic polymer-based ESFs to create desirable bioactivity for targeted tissues.²²

Biofunctionalization of ESFs made of biodegradable synthetic polymers is usually achieved by two strategies. First, polymers are co-electrospun with biomacromolecules such as ECM adhesive proteins to form composite electrospun scaffolds to enhance cell adhesion, for example, PCL/collagen²⁵ and PLLA/laminin²⁶ composite ESFs. Nevertheless, it is sometimes difficult to find a suitable solvent to

simultaneously dissolve hydrophobic synthetic polymer and hydrophilic proteins for electrospinning. Furthermore, native proteins are liable to denaturation when dissolved in an organic solvent. An alternative strategy is to physically or chemically immobilize proteins, adhesive peptides such as Arg-Gly-Asp (RGD), or ligands of cell surface receptors on the surfaces of ESFs,^{27–30} which significantly enhance cell attachment, proliferation, and functions. Nevertheless, surface-bound biosignals are only presented on the exterior and would disappear after surface erosion of the biodegradable ESFs, which thus no longer support cell activities.

ESFs have not yet been widely applied to cardiac tissue engineering. Previously, primary cardiomyocytes were cultured on electrospun PLGA/PLLA scaffolds to form tissue-like constructs.³¹ Cardiomyocytes developed mature contractile machinery (sarcomeres) on electrospun PLLA scaffolds, and functionality (excitability) of the engineered constructs was confirmed. To the best of our knowledge, the biofunctionalized ESFs on cardiac tissue engineering have not been reported. This study was aimed to synthesize biofunctionalized PLGA ESFs for potential cardiac tissue engineering application. Laminin signaling was shown to be relevant to changes in autonomic regulation that occur during cardiac development or disease.⁹ Due to the advantages of adhesive peptides including cost effectiveness and less vulnerability to denaturation in comparison with intact adhesion proteins, laminin-containing adhesive peptides such as RGD and YIGSR³² were applied for biofunctionalization of ESFs. A simple but novel strategy to incorporate peptides onto PLGA ESFs was developed in this study. Both peptides were first covalently conjugated to poly-L-lysine (PLL), and then mixed with PLGA solution for electrospinning. In order to examine the effects that may due to the topographical structure (i.e., fiber orientation) of the ESFs, the two peptide-incorporated ESFs were fabricated into both random and aligned formats to investigate the effects of surface morphology and biofunctionality on cardiac cell culture.

Materials and Methods

Materials

Two types of peptides were synthesized by Kelowna International Scientific, Inc.: N-acetyl-GRGDSPGYG (abbreviated as RGD) and N-acetyl-GYIGSRGYG (abbreviated as YIGSR). Peptide concentration was determined according to UV absorbance at 275 nm from tyrosine residues (Y, molar adsorption coefficient $1420 \text{ M}^{-1} \text{ cm}^{-1}$). Poly (L-lactide-co-glycolide) (PLGA 85/15) was purchased from Bioinvigor. The other reagents were purchased from Sigma-Aldrich unless specified otherwise.

Cardiomyocyte culture medium consisted of 90% (v/v) Dulbecco's modified Eagle's medium high-glucose (DMEM-HG; HyClone) containing 1.5 g/L sodium bicarbonate, 1% (v/v) fungizone (Gibco), 0.5% (v/v) gentamycin (Gibco), and 10% (v/v) heat-inactivated fetal bovine serum (JRH). To avoid overgrowth of cardiac fibroblasts, the culture medium was supplemented with $2 \mu\text{M}$ of cytosine β -D-arabino-furanoside after 2 days of culture.

Preparation of PLL-g-peptides

RGD and YIGSR peptides were conjugated on PLL (MW 70–150 kDa) via a carbodiimide reaction, according to a

previous procedure.³³ Briefly, 12 mg ($13.2 \mu\text{mol}$) of RGD or 12.8 mg ($13.2 \mu\text{mol}$) of YIGSR was dissolved in 2 mL of deionized water and then mixed with 2 mL of a PLL solution (4.6 mg/mL in deionized water). N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride ($8.4 \text{ mg}/42.0 \mu\text{mol}$) and N-hydroxysuccinimide ($5.0 \text{ mg}/42.0 \mu\text{mol}$) were added in the mixture and allowed to react at room temperature for 24 h. The mixture was dialyzed against deionized water to remove the unreacted small molecules (MWCO 12,400 Da), followed by freeze-drying. The graft ratios of RGD and YIGSR to PLL were estimated as 15.0 and 13.9 mol%, respectively, with respect to the total moles of the amino groups of PLL, according to a previous procedure.¹⁵

Preparation of PLGA ESFs

PLGA solution (4–8 w/v% in 1.1.1.3.3.3-hexafluoro-2-propanol, HFP) with or without PLL-g-peptides was loaded into a 5 mL syringe fitted with a 21G blunted needle, which was connected to a high voltage (10–22 kV) power supplier for electrospinning. The polymer solution was fed at a rate of 4 mL/h using a syringe pump (Model 100 series; KDS). ESFs were collected on an aluminum foil or a rotating drum (6 cm in diameter and 1 cm in width) that was horizontally located at 10 cm from the needle tip. The collected ESFs were then placed in a 40°C oven to remove any residual solvent. Random ESFs were collected at a low rotating rate at 100 rpm, while aligned ESFs at high rotating rates of 2000 or 6000 rpm.

For preparation of peptide-incorporated ESFs, PLL-g-peptides and PLGA were dissolved in HFP to a final concentration of 6% (w/v) PLGA solution and 4.5 mM RGD, YIGSR, or RGD/YIGSR (1/1 ratio). Although peptide-conjugated PLL dissolves in HFP, PLL does not dissolve in HFP; so, PLGA/PLL solution could not be electrospun in HFP. Therefore, PLGA ESFs adsorbed with PLL or laminin were served as control groups. PLL or laminin-coated ESFs were used as controls. The pure PLGA fibers were covered with $100 \mu\text{L}$ of 1 mg/mL of PLL or laminin solution, and then allowed to air-dry. After rinsing with deionized water, the samples were ready for cell seeding.

Characterization of ESFs

The morphology of ESFs after sputter-coating with gold was observed by a scanning electron microscopy (SEM; JSM-5310) at an accelerating voltage of 10 kV. Diameters of ESFs were analyzed from at least three SEM images with at least 25 fibers/image using Image J software (NIH). The orientation of ESFs was analyzed by the fast Fourier transform using Image J.³⁴

Incorporation of PLL-g-RGD to the ESFs was verified by conjugation of fluorescamine to the ESFs via a reaction with surface amines. A fluorescamine solution (1 mg/mL in acetone) was mixed with 50 mM borate buffer (pH 9.0) at a ratio of 10% (v/v). ESFs were rinsed with deionized water and then incubated with the fluorescamine solution for 15 min. The fibers were rinsed with methanol and then observed by a spectral confocal microscope imaging system (Leica TCS SP2). For quantification of surface amines of ESFs, the fluorescamine-conjugated PLGA fibers were dissolved in a mixture of HFP/DMSO (1/9 v/v), and then the fluorescence of the solution was read at emission wavelength of 475 nm with

excitation wavelength of 390 nm by a fluorometer. The amount of surface amino group was calculated from a calibration curve constructed by using PEG diamine and PLGA dissolved in DMSO.²⁷ The quantification of the density of amino groups embedded in the ESFs was carried out by the same method after the PLGA fibers were eroded in 0.1 N NaOH solution for 10 min.

Isolation and culture of cardiomyocytes

Primary cardiomyocytes were harvested from neonatal Wistar rats according to a previous procedure.³⁵ The procedure of animal experiments followed the ethical guidelines of Care and Use of Laboratory Animals (National Taiwan University, National Institutes of Health Publication No. 85-23, revised 1985) and was approved by the Animal Center Committee of National Taiwan University. The number and viability of isolated cardiomyocytes were determined by using a hemocytometer with trypan blue staining.

Isolated cells were plated onto the samples (1×10^5 cells/cm² for cell adhesion experiments and 5×10^5 cells/cm² for observing cell morphology and long-term cell culture), and cultured in a humid 37°C/5% CO₂ incubator. Cell numbers were determined by a lactate dehydrogenase assay, modified from a previous procedure.^{12,36} Cell morphology on ESFs was observed from SEM images. The preparation of SEM samples followed a previous procedure.¹³

Immunofluorescent staining

α -actinin, a protein locating at the Z-disk of the sarcomeric assembly and providing the cross-links that hold actin filament in muscle cells, was selected to examine the contractile characteristic of the cardiomyocytes on the surfaces.³⁷ In addition, α -actinin also served as a marker for identification of cardiomyocytes and cardiofibroblasts. On the other hand, F-actin, the major constituent of the cell cytoskeleton, was stained for observation of cytoskeletal network inside the cultured cells. N-cadherin, a transmembrane glycoprotein that belongs to the Ca²⁺-dependent cell adhesion family and is important in cell-cell connections among cardiomyocytes in intercalated disc of the heart

muscle, was also examined.³⁸ The expression of the above three proteins in the cardiomyocytes were visualized by immunofluorescent staining.

After culture for different time periods, the samples were rinsed with PBS, fixed with 4% paraformaldehyde for 20 min, and then permeated with glycine/triton solution (PBS/0.1% Triton/10 mM glycine). The samples were incubated with goat anti-N-cadherin IgG (1:300 dilution) (cat# sc-31030; Santa Cruz Biotech) and mouse monoclonal anti- α -actinin (1:300 dilution) (cat# A7811; Sigma) at 4°C overnight. After rinses, the samples were then incubated with anti-goat IgG-Alexa Fluor 633 (1:300 dilution) (cat# A21082; Invitrogen) and anti-mouse IgG-FITC (1:300 dilution) (cat# F2012; Sigma) for 60 min at 37°C. F-actin and nuclei was counter-stained with 500 nM Phalloidin-TRITC and 100 nM DAPI (Invitrogen), respectively, for 30 min followed by rinses with PBS. Fluorescent images were taken with a spectral confocal microscope imaging system (Leica TCS SP2).

Reverse transcription-polymerase chain reaction for analysis of the expression of cardiomyocyte genes

The expression of cardiac alpha-myosin heavy chain (α -MHC), atrial natriuretic factor (ANF), and β -tubulin genes was analyzed by reverse transcription-polymerase chain reaction (RT-PCR). The extraction of mRNA and the preparation of complementary DNA (cDNA) were performed according to a previous procedure.³⁹ PCR was performed in a thermocycler (PS320; ASTEC) by using Taq polymerase (M1865; PROMEGA).

The sequences of PCR primers (forward and backward, 5' to 3') were as follows:

GAPDH: sense 5'-GGAAAGCTGTGGCGTGATGG-3';
anti-sense 5'-GTAGGCCATGAGGTCCACCA-3';
 α -MHC: sense 5'-ACCGTGGACTACAACAT-3';
anti-sense 5'-CTTTCGCTCGTTGGGA-3';
ANF: sense 5'-GGGGGTAGGATTGACAGGAT-3';
anti-sense 5'-CAGAGTGGGAGAGGCAAGAC-3';
 β -tubulin: sense 5'-TCACTGTGCCTGAACCTACC-3';
anti-sense 5'-GGAACATAGCCGTAAACTGC-3'

FIG. 1. Six percent PLGA solution was electrospun at 18 kV and collected on a rotating drum at 100, 2000, and 6000 rpm. (A) The SEM images of PLGA electrospun fibers (ESFs). Magnification: 1500 $\times$; scale bar = 6 μ m. (B) The average diameters and alignment of PLGA ESFs at different rotation rates. The error bars represent the standard error of the mean from 100 fibers for each sample. * and ** represent $p < 0.01$ and 0.001 versus 100 rpm, respectively. PLGA; poly (L-lactide-co-glycolide); SEM, scanning electron microscopy.

The PCR operation profile included initial denaturation at 94°C for 10 min, denaturation at 94°C for 30 s, annealing at 56.5°C for 30 s, and extension at 72°C for 40 s, for 35 cycles, and final extension at 72°C for 7 min. PCR products were analyzed by separating in a 1.5% agarose gel, followed by staining with ethidium bromide. The images of electrophoresis gels were taken under UV light, and the band intensity of every PCR product was quantified by NIH Image J. The intensities of the bands for α -MHC, ANF, and β -tubulin were normalized to that of GAPDH from the same sample.

Statistical analysis

Statistical analysis was performed using GraphPad Instat 3.0 program (GraphPad Software). The analysis between each group was determined with One-Way ANOVA and Student–Newman–Keuls multiple comparisons test. $p < 0.05$ was considered as significant difference.

Results

Fabrication and characterization of PLGA ESFs

The concentrations of PLGA solutions from 1% to 9% were first tested for electrospinning at a fixed voltage of 16 kV. At low PLGA concentrations (1% or 2%), only beads but not fibrous structures were formed. A 3% PLGA solution was fabricated into fibers that were decorated with beads. PLGA ESFs without beads were fabricated from 4% to 8% PLGA solutions (Supplementary Fig. S1 in the Supplementary materials; Supplementary Data are available online at www.liebertpub.com/tea). The diameters of PLGA ESFs increased with PLGA concentrations from ~ 412 nm for 4%, ~ 677 nm for 6% to ~ 1248 nm for 8%. Nevertheless, a 9% PLGA solution was too viscous to be electrojected from a needle.

Next, electrospinning of 6% PLGA solution at various voltages from 10 to 22 kV was evaluated. The diameters of PLGA fibers were decreased with increasing voltage (Supplementary Fig. S2 in the Supplementary data). Most of the fibers electrospun at 10 kV were wider than $1 \mu\text{m}$ (1145 ± 521 nm), while those electrospun at 18 kV mostly ranged from 500 to 800 nm (647 ± 235 nm). The average diameter of the fibers electrospun at 22 kV was 509 ± 204 nm. The uniformity of fiber diameters was enhanced at a higher voltage.

Since aligned fibers were desired in the application of cardiac tissue engineering, a rotating-drum collector was used for producing fiber alignment. The extent of alignment depends on the rotating speed of the rotating drum. At 100 rpm, no apparent orientation of PLGA ESFs was found (Fig. 1A). As the rotating speed was increased to 2000 and 6000 rpm, the alignment of the fibers was enhanced. Analyzed by the fast Fourier transform, only 20.7% of the fibers electrospun at 100 rpm were within $\pm 30^\circ$ from the rotating direction, while 58.6% and 67.1% of those electrospun at 2000 and 6000 rpm, respectively (Fig. 1B). In this study the fibers fabricated at 100 rpm were defined as random, while those at 6000 rpm were considered aligned. On the other hand, the diameters of ESFs were also affected by the rotating speeds. The average diameter of ESFs was ~ 750 nm at 100 rpm, and decreased to ~ 664 and ~ 521 nm at 2000 and 6000 rpm, respectively (Fig. 1B).

Fabrication and characterization of peptide-incorporated PLGA ESFs and laminin-coated ESFs

Although pure PLGA solutions at 4% and 5% could be electrospun as fibers at the given conditions, PLGA/PLL-g-peptide solutions at both concentrations were electrospun as bead structure at the same conditions. On the other hand, PLGA/PLL-g-peptide solutions at 6% could be electrospun at 18 kV as fibrous structure. Therefore, such conditions were used in the subsequent experiments. With the addition of PLL-g-peptide in PLGA solution, smaller-diameter ESFs were mingled with large-diameter fibers (larger than 400 nm), especially in the random ESFs (Fig. 2A). The average diameters of peptide-incorporated PLGA ESFs were smaller compared with pure PLGA fibers (Fig. 2B). The diameter for random pure PLGA ESFs (675 ± 257 nm) was

FIG. 2. Six percent PLGA solution containing RGD, YIGSR, or their mixture was electrospun at 18 kV and collected on a rotating drum at 100 (random) and 6000 rpm (aligned). (A) The SEM images of the ESFs. Magnification: $3500\times$; scale bar = $1 \mu\text{m}$. (B) The average diameters of the ESFs. The error bars represent the standard error of the mean from 100 fibers for each sample. *Represents $p < 0.001$ versus the corresponding random fibers, while #represents $p < 0.001$ versus the corresponding peptide-incorporated fibers.

FIG. 3. (A) RGD-incorporated aligned PLGA ESFs, before and after 10-min treatment of 0.1 N NaOH solution, were conjugated with a fluorescent dye, and then observed under a fluorescent microscope. The control was pure PLGA ESFs coated with poly-L-lysine (PLL). Scale bar = 25 μ m. (B) The average diameters of pure PLGA and RGD-incorporated PLGA ESFs before and after 10-min treatment of 0.1 N NaOH solution. *Represents $p < 0.001$ versus NaOH-treated RGD-incorporated fibers. Color images available online at www.liebertpub.com/tea

decreased to 480 ± 222 nm for peptide-incorporated fibers, while that for aligned PLGA fibers (521 ± 192 nm) was decreased to 391 ± 120 nm for peptide-incorporated ESFs.

The incorporation of PLL-g-RGD in PLGA fibers was visualized by surface conjugation of a fluorescent dye, fluorescamine, via a reaction with the amino groups of PLL and then observed under a fluorescent microscope. RGD-incorporated PLGA fibers displayed blue fluorescence after conjugation of fluorescamine (the upper-right panel in Fig. 3A), indicating appearance of amines on the surface of the ESFs. Similarly, PLL-adsorbed PLGA fibers were stained by fluorescamine (the lower-right panel in Fig. 3A). However, after 10-min NaOH treatment, the PLL-coated PLGA fibers whose diameter was slightly decreased from 521 to 509 nm (Fig. 3B) were not stained by fluorescamine anymore (the lower-left panel in Fig. 3A), indicating that surface-coated PLL was removed by NaOH. On the other hand, the PLGA/PLL-g-RGD fibers whose diameter was decreased from 391 to 285 nm by NaOH erosion (Fig. 3B) were still stained by fluorescamine (the upper-left panel in Fig. 3A), suggesting that PLL-g-RGD not only appears on the surfaces but also is buried inside the ESFs. The laminin coating was also demonstrated by fluorescamine staining on the laminin-coated ESFs, shown as Supplementary Figure S3 in the Supplementary data.

The surface contents of RGD were estimated by the conjugation of fluorescamine. The fluorescamine-labeled ESFs were dissolved in HFP/DMSO and the intensity of the solution fluorescence was used to estimate the amount of PLL-g-RGD. The amounts of amines on PLGA/PLL-g-RGD ESFs were 924.3 ± 96.4 and 749.5 ± 156.6 nmol before and after NaOH treatment, respectively. The densities of amines after normalization by the surface area of ESFs were 3.61 ± 0.38 and $3.23 \pm 0.0.68$ nmol/cm² before and after NaOH treatment, respectively, suggesting that PLL-g-RGD may be uniformly distributed inside the ESFs. The surface RGD densities could be estimated by using the grafting mole ratio of RGD to the amino groups of PLL (15.0 mol% indicated in preparation of PLL-g-peptides). The surface density of peptides was calculated within the range of 371.0–566.6 pmol/cm².

Attachment of cardiomyocytes to ESFs

The effectiveness of peptide incorporation in the enhancement of cardiomyocyte adhesion to PLGA ESFs was investigated under serum-containing or serum-free conditions. Laminin- or PLL-coated ESFs were used as controls to evaluate the effects of peptides. Tissue culture polystyrene (TCPS), a conventional tissue culture vehicle, was also used for a reference substrate for comparing cell adhesion. In the presence of serum, all samples except the PLL-coated one significantly increased cell adhesion when compared with the unmodified PLGA fibers (Fig. 4A). The ineptness of PLL in enhancing cell attachment indicates that the increase in cell adhesion by incorporation of PLL-g-peptide is mainly contributed by the adhesive peptides, but not PLL. Incorporation of peptides (RGD, YIGSR, or RGD/YIGSR) increased cell adhesion slightly better than the laminin-coated substrates, but TCPS remained the best vehicles in supporting cell adhesion. Since the adsorption of serum adhesion proteins mediates cell adhesion to a substrate, we also evaluate cell adhesion in the absence of serum to evaluate the efficacy of biomolecule conjugation alone in mediating cell adhesion alone. The increase in cell adhesion by laminin or peptides was more significant under serum-free condition, at least five-folds compared with pure PLGA ESFs ($p < 0.001$, Fig. 4A).

We next evaluated cell adhesion after surface degradation of ESFs by pretreatment of the fibers in 0.1 N NaOH for 10 min. Alkaline erosion of pure PLGA fibers did not affect cell adhesion (comparing the leftmost bars in Fig. 4A, B). However, the enhancement in cell adhesion by laminin-coating of PLGA fibers disappeared after alkaline treatment, suggesting that NaOH incubation removed surface-coated laminin (Fig. 4B). On the other hand, the peptide-incorporated PLGA fibers still maintained their ability in enhancing cell attachment after alkaline erosion, proving that cell adhesive peptides remain appearing on the surfaces of ESFs.

The culture of cardiomyocytes on flat and ESFs samples

The effects of ESF structure on the culture of cardiomyocytes compared with flat substrates were next evaluated.

FIG. 4. The attachment of cardiomyocytes to PLGA ESFs containing RGD, YIGSR, or both peptides after 24 h of incubation in serum-containing or serum-free media before (A) and after (B) 10-min treatment of 0.1 N NaOH solution. PLGA ESFs coated with laminin or poly-L-lysine (PLL) were used as controls. Error bars = standard deviation ($n=5$). * $p<0.01$ with serum and ** $p<0.001$ w/o serum for each sample compared with PLGA ESFs.

After 1 day of culture, the cell attachment to the flat samples was generally higher compared with their corresponding ESF samples (Fig. 5A). However, after 7 days of culture the cell numbers significantly decreased on all the flat samples ($p<0.001$ vs. day 1), while the cell numbers on the ESF groups remained at a similar level on day 1. The cell numbers on the laminin-coated or RGD-incorporated ESFs

were close to the value on TCPS. The cells on the ESF samples displayed an elongated morphology along the aspect of ESFs. On the other hand, the cells cultured on the flat samples showed an aggregated morphology. Figure 5B and C shows representative immunofluorescent staining images of vinculin in the cardiomyocytes on PLL-g-RGD-incorporated ESF and flat surfaces after 3 days of culture. Vinculin, a focal adhesion-associated protein, was more expressed by the cells cultured on the ESF samples compared with those on the flat surfaces.

The morphology of the cardiomyocytes cultured on the random and aligned ESFs was observed by SEM, and the representative SEM images after 5 days of culture are shown in Figure 6. Compared with the unmodified PLGA ESFs, the cardiomyocytes possessed a more spreading morphology on the laminin-coated or peptide-incorporated ESFs samples. Moreover, the cardiomyocytes cultured on the aligned ESFs extended in a specific direction that is close to the aspect of ESFs, while the cells on the random fibers showed an overall disorganized profile.

Immunofluorescent staining of cardiomyocyte-specific proteins

After 7 days of culture, the formation of α -actinin and F-actin filaments was visualized by immunofluorescent staining (Fig. 7). The cardiomyocytes attached poorly on the unmodified PLGA ESFs, and the formation of α -actinin and F-actin filaments was not significant compared with the laminin-coated and peptide-incorporated ESFs (Fig. 7A). The formation of α -actinin and F-actin filaments was greatly enhanced on the laminin-coated ESFs (Fig. 7B). Furthermore, α -actinin formed striated morphology broadly in the cells on the laminin-coated ESFs (e.g., the circled areas in Fig. 7B, and the enlarged images in Supplementary Fig. S4 of the Supplementary Data). The arrangement of striated α -actinin on the aligned laminin-coated ESFs generally complied with the aspect of the fibers, while the arrangement of striated α -actinin was disorientated on the random laminin-coated fibers. The cardiomyocytes on the RGD-incorporated ESFs did not spread as well as the cells on the laminin-coated fibers (Fig. 7C). The cells cultured on the aligned fibers were also in parallel with the direction of the fibers. The immunofluorescent staining of α -actinin on the RGD ESFs

FIG. 5. Culture of cardiomyocytes on flat surfaces and ESF. (A) Cell densities after 1 day (1D) or 7 days (7D) of culture. Error bars = standard deviation ($n=5$). *** $p<0.001$. (B) and (C) illustrate the immunofluorescent staining images of vinculin (green) in the cardiomyocytes on PLL-g-RGD incorporated ESF and flat surfaces after 3 days of culture. Cell nuclei were stained by DAPI (blue). Scale bar = 20 μ m. Color images available online at www.liebertpub.com/tea

FIG. 6. SEM images of cardiomyocytes on random or aligned PLGA ESFs after 5 days of culture. The double-headed arrows indicate the direction of the aligned fibers. Scale bar=100 μ m.

was not as intense as that on the laminin-coated fibers. In addition, the striated α -actinin structure was not obvious on the RGD-incorporated ESFs. The organization of α -actinin and F-actin on the YIGSR-incorporated ESFs was similar to that on the laminin-coated fibers (Fig. 7D). Striated α -actinin was broadly found in the cells (e.g., the circled areas in Fig. 7D, and the enlarged images in Supplementary Fig. S4 of the Supplementary Data). On the other hand, on the RGD/YIGSR-incorporated ESFs the extent of immunofluorescent staining of α -actinin and F-actin was similar to that on the YIGSR-incorporated samples, but few striated α -actinin was found

(Fig. 7E). The cells seeded on the flat laminin-coated surfaces formed layered cell clusters and did not organize distinguished F-actin or the α -actinin stripes of the cardiomyocyte characters (Fig. 7D). The positive yellow N-cadherin staining was found on the aligned laminin-coated or YIGSR-incorporated fibers but not the other types of ESFs (Fig. 8A, B). The results indicate that cell-cell contacts are enhanced on the aligned ESFs decorated with laminin or YIGSR. The homophilic cell-cell contact is probably important for the reformation of intercalated discs and for the attachment of the myofibrils within the intercalated discs.³⁸

FIG. 7. The immunofluorescent images of α -actinin (green) and F-actin (red) structure of cardiomyocytes on random and aligned ESFs after 7 days of culture: (A) pure PLGA ESFs; (B) laminin-coated ESFs; (C) RGD-incorporated ESFs; (D) YIGSR-incorporated ESFs; (E) RGD-incorporated ESFs. The double-headed arrows indicate the direction of the aligned ESFs. Circled areas indicate striated α -actinin. Color images available online at www.liebertpub.com/tea

RT-PCR analysis of the expression of cardiomyocyte-specific genes

The expression of three cardiomyocyte-specific genes, α -MHC, ANF, and β -tubulin, in the cells that were cultured on the ESFs was evaluated by semi-quantitative RT-PCR. The expression of the three genes was normalized to the expres-

sion of GAPDH on the same samples. We found that no significant difference in the expression of all genes between PLGA and RGD samples (Fig. 9). On the other hand, the expression of α -MHC and β -tubulin on YIGSR was significantly higher compared with the expression level on PLGA and RGD. Noticeably, the expression of α -MHC was only detected on YIGSR.

FIG. 8. The immunofluorescent stained images of N-cadherin (yellow) and F-actin (red) on (A) the aligned YIGSR-incorporated ESFs and (B) the aligned laminin-coated ESFs after 7 day-culture of cardiomyocytes. Cell nuclei were stained by DAPI (blue). Scale bar = 15 μ m. The double-headed arrows indicate the direction of ESFs. Color images available online at www.liebertpub.com/tea

Contraction of cardiomyocytes

The contractile properties of the cardiomyocytes on various surfaces were inspected under an optical microscope. The cells cultured on TCPS started beating on the first day of culture, in advance of the cells on the other substrates. On the second day, the cells cultured on the laminin-coated or peptide-incorporated random ESFs beat vigorously, while fewer cells on the aligned ESFs started beating. However, after 5 days of culture cardiomyocyte sheets with synchronized contraction were observed on all the laminin-coated and peptide-incorporated samples except the pure PLGA ESFs. Furthermore, the cardiomyocytes cultured on the aligned fibers of laminin-coated, YIGSR- or RGD/YIGSR-incorporated ESFs contracted along the direction of ESFs. The synchronization was observed in a higher degree in the aligned ESFs groups compared with the random ESFs groups. After 7 days of culture, the beating rates of the cardiomyocytes on the random ESFs of RGD, RGD/YIGSR decreased significantly, while those on the aligned ESFs of RGD/YIGSR, YIGSR, and laminin remained their original beating rates. After 2 weeks of culture, the contractile activities of the cell sheets on the laminin-coated ESFs (Supplementary Video S1) and the YIGSR-incorporated ESFs (Supplementary Video S2) remained much better compared with the other substrates such as RGD/YIGSR- and RGD-incorporated ESFs and TCPS (Supplementary Video S3). We found that the sizes of synchronously beating cell sheets were generally larger on the YIGSR-incorporated and laminin-coated ESFs compared with the other types of ESFs.

Discussion

In this study, RGD- or YIGSR-conjugated PLL were applied to fabrication biofunctional ESFs for tissue-engineered cardiac patches. Peptide-conjugated polyelectrolytes have been previously used for surface modification to enhance cell affinity of biomaterials.^{15,33,36,40} We suggest that “isolation” and reassociation” of bioactive domains of a protein in polyelectrolytes such as PLL construct a biofunctional “imitative-protein” that can perform specific functions of a protein. Such “imitative proteins” have some advantages over native proteins. First, peptides are more economic and less vulnerable to denaturation in comparison with intact proteins. Second, the conjugation densities of a biosignal in an imitative protein can be modulated at will to meet re-

FIG. 9. After culture for 7 days on PLGA, RGD-, and YIGSR-incorporated aligned ESF electrospun films, the expression of alpha-myosin heavy chain (α -MHC), atrial natriuretic factor (ANF), β -tubulin, and GADPH of mesenchymal stem cells (MSCs) was determined by reverse transcription-polymerase chain reaction (RT-PCR). (A) Electrophoresis images for the RT-PCR products of α -MHC, ANF, β -tubulin, and GADPH of MSCs. (B) Normalized band intensities for α -MHC, ANF, and β -tubulin relative to the densities of GADPH. (* $p < 0.05$ and ** $p < 0.001$)

quirement. Third, different biosignals can be associated in a single macromolecule without including multiple proteins. Such “imitative proteins” can be adsorbed, conjugated onto a surface, or incorporated into scaffolds to enhance bioactivity.

In this study, both the laminin-coated ESFs and the YIGSR-incorporated ESFs are superior to the other types of ESFs regarding the formation of striated α -actinin and cell contraction. However, we prefer using “imitative proteins” for biofunctionalization of ESFs owing to the following reasons. First, it is often difficult to find a suitable solvent for co-electrospinning of a water-soluble protein and a hydrophobic synthetic polymer due to their intrinsic difference in solubility. Since the physicochemical properties of peptide-conjugated polymers could be easily modulated by varying polymers and peptides, it is easier to find a suitable solvent for electrospinning by using peptide-conjugated polymers. Second, native proteins are liable to denaturation by organic solvents. Last, compared to surface immobilization of proteins or peptides, our method incorporates biosignals throughout ESFs. Therefore, after surface degradation of ESFs, the interior peptides would be exposed to support subsequent cell attachment and proliferation.

Comparing cell adhesion to 2D and fibrous substrates, we found that the cardiomyocytes initially attached better on the flat surfaces (Fig. 5A). However, cell numbers were greater on the fibrous substrates after 7 days of culture. Focal adhesions formed more significantly on the ESFs compared with the flat substrates (comparing Fig. 5B, C), suggesting that cardiomyocytes adhere more tightly on the fibrous structure compared with 2D surfaces. The cardiomyocytes may detach more easily from a 2D surface during contraction, which results in less amount of cells observed in longer culturing time. These findings also supported previously published researches. For instance, Zong *et al.* showed that cardiomyocytes interacted with the sub-microfibrous PLGA network and reorganized to follow the scaffold-prescribed direction.³¹ More recently, Kenar *et al.* seeded mesenchymal stem cells (MSCs) on a 3D micro-fibrous mat, where the cells aligned in a similar way to cell organization in native myocardium and formed a thick myocardium-like patch.⁴¹ These evidences imply that fibrous 3D architecture might be more suitable for cardiac cell culture compared with 2D flat surfaces.

We also found that aligned fibers benefit the organization of the cardiac cells into a physiological tissue *in vitro* compared with random fibers. The cardiomyocytes cultured on the peptide-incorporated aligned ESFs possessed better characterization of mature cardiomyocytes than their corresponding random ESFs. The observations were similar to our previous studies on MSCs and cardiomyocytes on the grooved topographical substrates such as polystyrene and polyurethane.^{35,42}

In this study, cardiomyocyte interactions with two types of cell adhesive peptides, which both exist in laminin, RGD, and YIGSR, were investigated. Laminin exists in the ECM in myocardial tissue and is critical for myocardium development. For instance, Matrigel[®], mainly composed of laminin, had been demonstrated to enhance myocardial repair after infarction.^{10,11} We found that although cell numbers were similar on both RGD- and YIGSR-incorporated ESFs, the behavior of cardiomyocytes were quite different. First, as shown in Figure 7, more intense staining of F-actin was found on the YIGSR samples than the RGD samples, suggesting that the organization of F-actin is more mature on the YIGSR-incorporated ESFs compared with RGD-incorporated ones. We also found that cardiomyocytes spread less on the RGD ESFs compared with the YIGSR samples, suggesting that the adhesion of cardiomyocytes to RGD-incorporated scaffolds was less tight. The distribution of intracellular structural proteins such as vinculin and F-actin on different biofunctionalized surface regulated the organization of the actin cytoskeleton and focal adhesion complexes, and consequently the contractile behavior of the cardiomyocytes.³⁵ Second, more striated α -actinin structure was found on the YIGSR ESFs than the RGD ESFs. Since the formation of striated α -actinin is a sign of maturation of cardiomyocytes,¹⁴ we suggest that the YIGSR-incorporated ESFs are more suitable for myocardium formation compared with the RGD substrates. Similarly, N-cadherin was also found on the YIGSR-incorporated aligned ESFs but not the RGD-conjugated scaffolds. Third, the expression of β -tubulin, a cytoskeleton protein that regulate the contractility of myocytes, and α -MHC, a myosin protein that is important for the movement and morphology of the cardiac

muscle cells, on YIGSR-incorporated aligned ESFs scaffolds was better compared with PLGA and RGD samples. Last, the contraction of cardiomyocytes was faster and lasted longer on the YIGSR ESFs than the RGD ones. The results highlight the important role of YIGSR sequences, indicating that YIGSR should be more important than RGD as biosignals for myocardium formation. The behavior of cardiomyocytes on the YIGSR-incorporated ESFs is similar to that on the laminin-coated ESFs. YIGSR is a cell-adhesive peptide derived from laminin, suggesting that the binding of cardiomyocytes to laminin is important for the physiologic activity of cardiomyocytes. Since laminin contains other biofunctional signals such as EGF-like domains that might benefit the growth and functions of cardiomyocytes, these peptides could be incorporated in ESFs in the future to improve the outcome of cardiac tissue engineering.

Conclusion

The uniqueness of our studies is the incorporation of bioactive peptides into electrospun fibrous meshes. The short peptides conjugation allows a precise and controllable manner for both biochemical and physical properties of the ESFs. Tuning the biochemical clues from protein to peptides levels can lead to a better understanding of the mechanism of integrin–ligand interaction of cardiac tissue. Our results indicated that the cardiomyocytes cultured on the YIGSR-incorporated and the laminin-coated aligned ESFs presented physiological-like morphology and contracted more than 14 days. The potential of using peptide-incorporated ESFs as designable-scaffold platform for cardiac repair was demonstrated.

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Disclosure Statement

No competing financial interests exist.

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