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Electrical coupling of isolated cardiomyocyte clusters grown on aligned conductive nanofibrous meshes for their synchronized beating

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ABSTRACT

Myocardial infarction is often associated with abnormalities in electrical function due to a massive loss of functioning cardiomyocytes. This work develops a mesh, consisting of aligned composite nanofibers of polyaniline (PANI) and poly(lactic-co-glycolic acid) (PLGA), as an electrically active scaffold for coordinating the beatings of the cultured cardiomyocytes synchronously. Following doping by HCl, the electrospun fibers could be transformed into a conductive form carrying positive charges, which could then attract negatively charged adhesive proteins (i.e. fibronectin and laminin) and enhance cell adhesion. During incubation, the adhered cardiomyocytes became associated with each other and formed isolated cell clusters; the cells within each cluster elongated and aligned their morphology along the major axis of the fibrous mesh. After culture, expression of the gap-junction protein connexin 43 was clearly observed intercellularly in isolated clusters. All of the cardiomyocytes within each cluster beat synchronously, implying that the coupling between the cells was fully developed. Additionally, the beating rates among these isolated cell clusters could be synchronized via an electrical stimulation designed to imitate that generated in a native heart. Importantly, improving the impaired heart function depends on electrical coupling between the engrafted cells and the host myocardium to ensure their synchronized beating.

1. Introduction

Myocardial infarction is often associated with abnormalities in the electrical function of the cardiovascular system known as arrhythmias, subsequently impairing cardiac performance severely. As is well known, massive loss of functioning cardiomyocytes is responsible for triggering cardiac arrhythmias [1,2]. Despite the effectiveness of cell-based therapies in repairing infarcted myocardial tissues, the lack of functional coupling of donor cells with the viable host tissues can significantly impede their electrical communications. To reestablish the contractile function of an infarcted heart, we hypothesize on the ability to use electrically active scaffolds in order to integrate implanted cells with the host myocardium in a synchronized manner. The materials used to fabricate electrically active scaffolds require matched excitability of grafted and host cells, as well as support propagation of the electrical wavefront.

This work develops an aligned nanofibrous mesh with electrically conducting properties as a tissue-engineered scaffold to provide structural support for neonatal rat cardiomyocytes. The mesh, consisting of composite nanofibers of polyaniline (PANI)/poly(lactic-co-glycolic acid) (PLGA), is fabricated by an electrospinning technique. Electrospinning offers ultrafine fibers, provides high exposure to a cell environment, and allows nutrient transport. Additionally, the aligned electrospun nanofibers have structures that resemble those of the natural extracellular matrices (ECMs) of cardiac tissues [3,4]. This aligned fibrous mesh can thus serve as a functional unit by providing the topographic cue for cell alignment. In the native heart tissue, alignment of cardiomyocytes contributes to the anisotropic (i.e. directionally dependent) tissue structure of the heart, thereby facilitating its coordinated electrical propagation and mechanical contraction [3,5,6].

PANI is one of the best-characterized conducting polymers, due to its ease of synthesis, diversity of structural forms and highly environmental stability [7]. A π-conjugated backbone within PANI

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allows it to conduct electrons, providing PANI with the opportunity to couple grafted cells with the host tissue electrically. The PANI polymer has been used for biomedical applications both *in vitro* and *in vivo* [8–11].

The solubility of PANI in most common organic solvents is unsatisfactory, making it difficult to fabricate a uniform structure of electrospun nanofibers with PANI independently. Therefore, the aligned fibrous mesh is fabricated using a blend solution of PANI/PLGA for electrospinning. Owing to its excellent biodegradability, PLGA has received considerable attention as scaffold materials for tissue engineering [12–14].

This work elucidates the fundamental material properties of the fabricated fibrous meshes, including their morphology, surface characteristics, electrical conductivity, and cell compatibility. Neonatal rat cardiomyocytes on test fibrous meshes are also cultivated, along with their beating behaviors examined before and after electrical stimulation (Fig. 1). Moreover, synchronous contractions of the cultivated cardiomyocytes are induced by applying electrical signals designed to mimic those in the native heart.

2. Materials and methods

2.1. Materials

PLGA (LA/GA = 75/25, MW = 66–107 kDa), 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP), PANI emeraldine base (PANI-EB) of MW 65 kDa, and hydrochloric acid (HCl) were purchased from Sigma–Aldrich (St Louis, MO, USA). Cell culture reagents were obtained from Invitrogen (Carlsbad, CA, USA).

2.2. Preparation and characterization of aligned nanofibrous meshes

PANI-EB was dissolved in HFIP (0.5% by w/v), and stirring was continued overnight. This PANI-EB solution was filtered through a 0.45 μm filter unit before adding PLGA (6%, 8%, 10%, or 12% by w/v). Both PANI and PLGA are soluble to some extent in HFIP [15,16]. HFIP is known to be cytotoxic. However, its boiling point is only 59 °C [17] and can be easily evaporated; therefore, HFIP has been commonly used as a solvent for electrospinning [3,16–18].

The mixed solution was loaded into a 1-mL syringe and delivered at a constant flow rate (1 mL/h) through a stainless steel needle (23 gauge) connected to a high-voltage power supply. Once a high voltage (12.3–13.6 kV) was applied, a thin polymer solution jet was ejected from the needle and deposited on a thick aluminum foil wrapped around a custom-made high-speed rotating mandrel (Fig. 1). After HFIP was evaporated at room temperature overnight, an aligned PANI/PLGA mesh was obtained. 1N HCl was used as a dopant to increase the conductivity of the as-prepared PANI/PLGA mesh, by converting the insulating PANI-EB into a conductive form (PANI emeraldine salt, PANI-ES) through protonation of the polymer overnight [19].

Morphology of the electrospun nanofibrous meshes was examined using a scanning electron microscope (SEM, Model JSM-5600, JEOL, Tokyo, Japan). Based on the obtained SEM images, the diameters and angle distributions of their constituted nanofibers were then evaluated using an analysis software (Image-Pro Plus, Media Cybernetics, Silver Spring, MD, USA) [16]. Next, the PANI/PLGA meshes were characterized by Fourier-transform infrared (FT-IR) spectroscopy (Perkin–Elmer, Buckinghamshire, UK) and X-ray photoelectron spectroscopy (XPS, ESCA PHI 1600, Physical Electronics, Chanhassen, MN, USA). Additionally, their conductivity was measured by using a 4-probe technique (Quatek 5601Y/QT-50, Taiwan). The mechanical properties of test meshes were measured using an Instron 5543 mechanical testing instrument (Instron Corp., Norwood, MA, USA).

Their protein adhesive properties were then investigated by soaking the undoped and doped PANI/PLGA meshes in the horse serum (Gibco, Grand Island, NY, USA) for 1 h and then washing them 3 times with deionized (DI) water. Subsequently, the meshes were stained with rabbit anti-laminin and mouse anti-fibronectin (Abcam, Cambridge, MA, USA). Additionally, fluorescent colors were obtained using different Alexa-Fluor secondary antibodies (Invitrogen). Finally, the stained meshes were examined using an inverted confocal laser-scanning microscope (CLSM, TCS SL, Leica, Germany).

2.3. Cardiomyocyte isolation and cell culture

Neonatal cardiomyocytes were isolated from the hearts of 1–2-day-old Lewis rats using a Worthington Neonatal Cardiomyocyte Isolation System (Worthington Biochemical, Lakewood, NJ, USA). Prior to cell seeding, test PANI/PLGA fibrous meshes were cut into small squares with an area of 1.5 × 1.5 cm^2, sterilized with UV light overnight, and pre-incubated for 1 h in the horse serum at 37 °C. The isolated cardiomyocytes were seeded onto test meshes at a density of 5 × 10^4 cells/cm^2 and then cultured in the growth medium (Dulbecco's modified Eagle's medium, Gibco) supplemented with 5% chick embryo extract (Sera Laboratories, West Sussex, United Kingdom), 10% horse serum, 100 U/mL penicillin, and 100 μg/mL streptomycin (Invitrogen).

In the electrical stimulation experiment, cells were labeled with a lipophilic dye (1,1′-dioctadecyl-3,3,3′,3′-tetra-methylindo-carbocyanine perchlorate, DiI) before seeding. Briefly, the isolated cardiomyocytes were incubated in a 5 μM DiI solution at 37 °C for 15 min. After the dye diffused laterally within the cellular plasma membranes, the supernatant was removed via centrifugation at 1000 rpm, and the cells were gently resuspended in a pre-warmed (37 °C) growth medium and then seeded on test meshes.

2.4. Cell viability tests

The viability of cardiomyocytes on test meshes was evaluated using a Live/Dead Viability Kit (Invitrogen) and photographed by a fluorescence microscope (Axio Observer Z1, Zeiss, Göttingen, Germany). As an additional test, the leakage of lactate dehydrogenase (LDH) in the culture media was measured. After cell culturing for distinct periods, media were collected and centrifuged; in addition, the activity of LDH released from the cytosol of damaged cells was assessed using the LDH Cytotoxicity Assay Kit (Sigma–Aldrich). The maximal LDH release of cells was determined by adding a lysis solution (0.8% Triton X-100), followed by incubation at 37 °C for 45 min [20]. Finally, the optical density at a wavelength of 490 nm was determined using a multiwell scanning spectrophotometer (Dynatech Laboratories, Chantilly, VA, USA).

2.5. Immunostaining of cardiomyocytes

After 60 h of culture, the cells on test meshes were fixed for immunofluorescent staining. The antibodies used were mouse anti-cardiac Troponin I and rabbit anti-connexin 43 (Abcam). Colored fluorescence was then generated using various Alexa-Fluor secondary antibodies. Finally, cells were costained to visualize the nuclei by propidium iodide (Sigma–Aldrich) and then examined using fluorescence microscopy.

2.6. Electrical stimulation

In this work, test PANI/PLGA meshes were placed on a glass slide. Two silver wires were placed under two ends of the mesh with the direction perpendicular to the major axis of the fibers (Fig. 1) [18]. The product was subsequently covered by a glass well (1 cm × 1 cm inner well dimension) that acted as a sealant and prevented the wires from coming into direct contact with the medium. This assembly was tightly sealed with a silicone paste (Dow Corning Corp., Midland, MI, USA) and sterilized by exposure to UV light overnight.

The DiI-labeled cardiomyocytes were then seeded on test meshes; following culturing, trains of electrical pulses (1.25 Hz, 5 V/cm) were applied, which is characteristic of native myocardium [21]. The beating behaviors of the cardiomyocytes (before and after electrical stimulation) were then observed and recorded using a fluorescence microscope with a CCD camera. The video was recorded at 30 fps and in a resolution of 640 × 480 pixels. The images were digitized frame by frame with Image-Pro Plus software. Finally, the beating rate of the culture was analyzed by selecting and tracking a small area of the video frame sequence in a cell cluster where brightness clearly oscillated due to the cell beating [22].

2.7. Statistical analysis

Two groups were compared by the one-tailed Student's *t*-test using statistical software (SPSS, Chicago, IL, USA). Data are presented as mean ± SD. A difference of $P < 0.05$ was considered statistically significant.

3. Results and discussion

After myocardial infarction, electrical integrity of the heart is often compromised because of massive loss of functioning cardiomyocytes. By combining cells and scaffolds, cardiac tissue engineering is a promising means of treating patients suffering from myocardial infarction [23–25]. One of the challenges for cardiac tissue engineering is that the implanted donor cells should be coupled electrically to the local host tissue [26,27]. The lack of electrical coupling of donor cells with the viable host myocardium significantly impedes cell-to-cell signaling, leading to unsynchronized beating between the tissue-engineered cardiac patch and the host tissue [26,28,29]. This work demonstrates the feasibility of using an aligned PANI/PLGA nanofibrous mesh as an electrically active scaffold to coordinate synchronous beating of grown isolated cardiomyocyte clusters.