Video Article

Electrospun Nanofiber Scaffolds with Gradations in Fiber Organization

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Abstract

The goal of this protocol is to report a simple method for generating nanofiber scaffolds with gradations in fiber organization and test their possible applications in controlling cell morphology/orientation. Nanofiber organization is controlled with a new fabrication apparatus that enables the gradual decrease of fiber organization in a scaffold. Changing the alignment of fibers is achieved through decreasing deposition time of random electrospun fibers on a uniaxially aligned fiber mat. By covering the collector with a moving barrier/mask, along the same axis as fiber deposition, the organizational structure is easily controlled. For tissue engineering purposes, adipose-derived stem cells can be seeded to these scaffolds. Stem cells undergo morphological changes as a result of their position on the varied organizational structure, and can potentially differentiate into different cell types depending on their locations. Additionally, the graded organization of fibers enhances the biomimicry of nanofiber scaffolds so they more closely resemble the natural orientations of collagen nanofibers at tendon-to-bone insertion site compared to traditional scaffolds. Through nanoencapsulation, the gradated fibers also afford the possibility to construct chemical gradients in fiber scaffolds, and thereby further strengthen their potential applications in fast screening of cell-materials interaction and interfacial tissue regeneration. This technique enables the production of continuous gradient scaffolds, but it also can potentially produce fibers in discrete steps by controlling the movement of the moving barrier/mask in a discrete fashion.

Video Link

The video component of this article can be found at https://www.jove.com/video/52626/

Introduction

Nanofibers are a popular utility for tissue engineering because of their ability to mimic the extracellular matrix in its structure and relative size[1]. However, some native tissue interfaces, such as the tendon-to-bone insertion site, contain collagen fibers, which exhibit a variable organizational structure that increases in alignment towards the tendon and decreases at the bone site[2-5]. So, for effective tissue regeneration there is a need to fabricate a scaffold that could effectively mimic this structural gradient.

Previously, there has been research conducted on gradual changes in fiber composition, specifically, mineral content[6]. However, recreating the structural component of connective tissues remains largely unexplored. An earlier study examined morphological gradients by studying the effect of surface silica particle density on the proliferation of rat calvarial osteoblasts and found an inverse relationship between silica particle density and cell proliferation[7]. But the morphological changes that mediated cell proliferation in previous work were mostly related to surface roughness lacking the capability in mimicking fiber organizational changes[7,8]. One recent study attempted to fabricate a scaffold that mimicked the unique collagen fiber orientations by using a novel collector for electrospinning[9]. While this study succeeded in producing a scaffold with both aligned and random fibers, it failed to mimic the gradual changes exhibited in the native tissues. Also, in producing separate components, with an immediate change from aligned to random orientation, the biomechanical properties of this scaffold decreased significantly. No previous work has been able to produce applicable nanofiber scaffolds with continuous gradations in fiber orientations from aligned and random. Our recent study has shown successful recreation of nanofiber scaffolds with gradations in fiber organization that can potentially mimic the native collagen organization at tendon-to-bone insertion[10]. This work aims to present the protocols used for the production of nanofiber scaffolds with a structure that closely resembles that of fiber organization in the native tendon-to-bone tissue interface.

Gradient nanofiber structures have potentially far-reaching applications across a variety of fields. We focused on the applications to tissue engineering of the tendon-to-bone insertion site by combining our scaffolds with adipose-derived stem cells (ADSCs) which are already utilized for tissue regeneration on various substrates[11-14]. In addition, ADSCs are very similar in nature to bone marrow stem cells in terms of multipotency and their resource is abundant which can be harvested using a simple liposuction procedure[15,16]. Seeding these cells to gradated nanofiber scaffolds further enhances their tissue engineering applications by allowing for the controlled distribution of the cells that can potentially differentiate into various tissues. In addition to seeding stem cells, nanofibers can be encapsulated with signaling molecules for regulation of cellular response. Coupling nanoencapsulation with the organizational gradient of these scaffolds allows for the study of cellular behavior or possible implant designs and coatings. Encapsulation of functional molecules like bone morphogenetic protein 2 (BMP2), which has been shown to induce osteoblast differentiation[15,16], could further enhance the tissue engineering applications of these scaffolds[10].

Protocol

1. Preparation of the Solution

1. Prepare a solution of Poly (ε-caprolactone) (PCL) (M_w= 80,000 g/mol) at an approximate concentration of 100 mg/ml. Dissolve PCL in a mixture of dichloromethane (DCM) and N, N-dimethlyformamide (DMF) at a ratio of 4:1 (v/v) with a concentration of 10% (w/v).
2. Place the solution in a 20 ml glass tube for mixing. Place glass tube into ultrasonic cleaner for 30 minutes, or until solution is translucent.

2. Apparatus Preparation

1. Add the prepared PCL solution into a 5 ml syringe with a 21 gauge blunt needle attached.
2. Place syringe pump in a vertical electrospinning position, according to **Figure 1**.
3. Use a stainless steel-gap collector with an open space of 2 cm x 5 cm for the aligned fiber substrate. Place the collector 12 cm from the needle tip.
4. Connect the Direct Current (DC) High Voltage power supply to the needle and ground the collector. Ensure that the collector is individually grounded with no contact to the other lab equipment.

3. Electrospinning

1. Set syringe pump to 1.50 ml/hr, until the droplets forming at the needle tip are immediately replaced when removed. Then, set the flow rate to 0.50 ml/hr.
2. Turn the voltage operator to 12 kV.
3. Electrospin until the uniaxially aligned fibers completely cover the gap on the collector.
4. Apply glue to the edges of a small glass plate and transfer the fibers from the gap collector to the glass plate. Place the glass plate on the top of a piece of aluminum foil that has the same size as the glass plate and is grounded.
5. Position the second syringe collector according to **Figure 3**.
6. Attach the plastic mask to the second syringe pump and position it 2 mm above the collector.
7. Add Coumarin 6 at 1% (w/w) to the PCL solution—if nanoencapsulation is desired— and mix until the solution is translucent.
8. Load the PCL or PCL/Coumarin 6 solution into a 5 ml needle with a blunt 21 gauge needle. Set vertical pump to 0.50 ml/hr and horizontal to pull at 9 ml/hr or at an approximate speed of 1 mm/min.
9. Electrospin until the mask has moved almost completely off the collector, but with the edge area still under the mask.

4. Fiber Characterization

1. Place samples with double-sided conductive tape to the metallic stud and coat with platinum for 40 sec using a sputter coater at 40 mA.
    1. Examine fibers through scanning electron microscope (SEM) according to our previous studies[17,18].
    2. Collect images at an accelerating voltage of 15 kV.

2. Perform fast Fourier transform (FFT) analysis on a separate fiber sample to measure fiber alignment. The detailed information on measuring fiber alignment by FFT can refer to previous studies[19,20].

5. Seeding Stem Cells.

1. Sterilize the fiber samples by soaking in a 70% ethanol solution for 2 hr. Then wash the fibers with distilled water to remove any contaminants.
2. Obtain human ADSCs and culture cells in a 25 cm^2 flask at 37 °C in an atmosphere of 95% air/5% CO_2. Change the cell culture medium every other day[10].
3. Trypsinize the cells and count the number of cells. Specifically, remove all culture medium from the cell culture flask and wash the cells with phosphate buffered saline (PBS) twice. Then add 1 m^l of a 0.25% trypsin-EDTA solution (pre-warm in water bath to 37 °C) to cover the cell monolayer and incubate the flask in the cell culture incubator for 2-3 minutes. Add 4 ml culture medium and wash out all the cells from the surface by pipetting the medium all over the surface. Centrifuge the cell suspension and re-disperse the cell pellet in the culture medium. Count cells via hemacytometer. Seed around 1 × 10^4 cells to the nanofiber scaffold placed in a 35 mm -culture dish and incubate for 3 and 7 days.
4. Stain cells using fluorescein diacetate (5 mg/ml in acetone) (FDA) then image the samples using a fluorescent microscope. Specifically, add 100 µl of FDA solution to the culture dish and incubate for 30 min. Wash cells with PBS three times before fluorescent imaging. Analyze cell orientation by a customized mat-lab program based on our previous studies[10].

Representative Results

Using this protocol, a fiber mat with an organizational gradient was formed. **Figure 3** shows the SEM images taken at various locations on the nanofiber scaffold. Qualitatively, it can be determined that there is a progression from the uniaxially aligned fibers at 0 mm (**Figure 3A**) to a random fiber assortment at 6 mm (**Figure 3D**). The FFT gives a quantitative value to the fiber alignment, specifics on the quantitative processes are detailed here[19]. Fibers at 0 mm exhibit an FFT that indicates fiber alignment, and at 6 mm the FFT pattern signifies a random orientation.

There is a clear progression in the SEM images (**Figure 3**) from an aligned fiber organization to an increasingly random fiber deposition (**Figure 3B**-**C**).

ADSCs underwent morphological changes based on their location in the nanofiber scaffold. **Figure 4** shows images taken with the fluorescent microscope (Zeiss) at 3 days (**Figure 4A**-**D**) and 7 days (**Figure 4E**-**H**). The distribution of the stem cell angle was quantitatively assessed by a customized MATLAB program and analyzed using the Kolmogorov-Smirnov test at various distances. **Figure 4I** shows the distribution of cell angle at different locations. At 0 mm, or the region of aligned fibers, 70% of the cells appeared within 20° of the axis of nanofiber fabrication. In contrast, the ADSCs seeded on the random portions of the fiber scaffolds lacked this organizational structure, with only 20% of the cells appearing within 20°. Finally, the formation of the chemical gradient using Coumarin 6 - loaded PCL fibers was studied using fluorescence microscopy. The chemical gradient was qualitatively confirmed using the microscopy image (**Figure 5A**). The image confirms the increasing chemical concentration across the scaffold, which is exhibited by the steadily increasing intensity of the fluorescent image. The graph of the fluorescent intensity (Image J) (**Figure 5B**) confirms the gradient of the chemical concentration by exhibiting a linear growth across the scaffold.

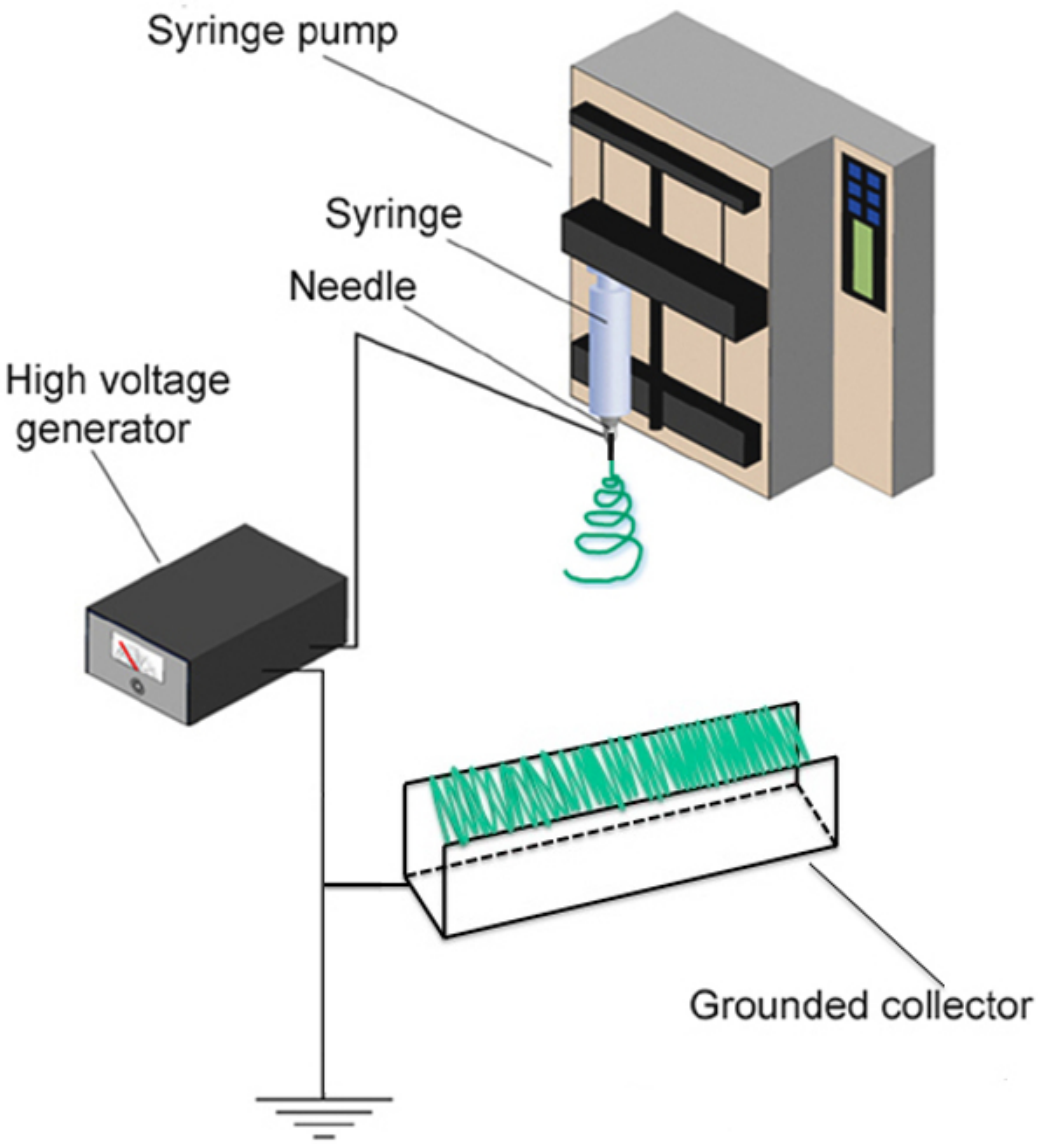

Figure 1: Shows the schematic of experimental setup for the preparation of the uniaxially aligned fiber substrate.

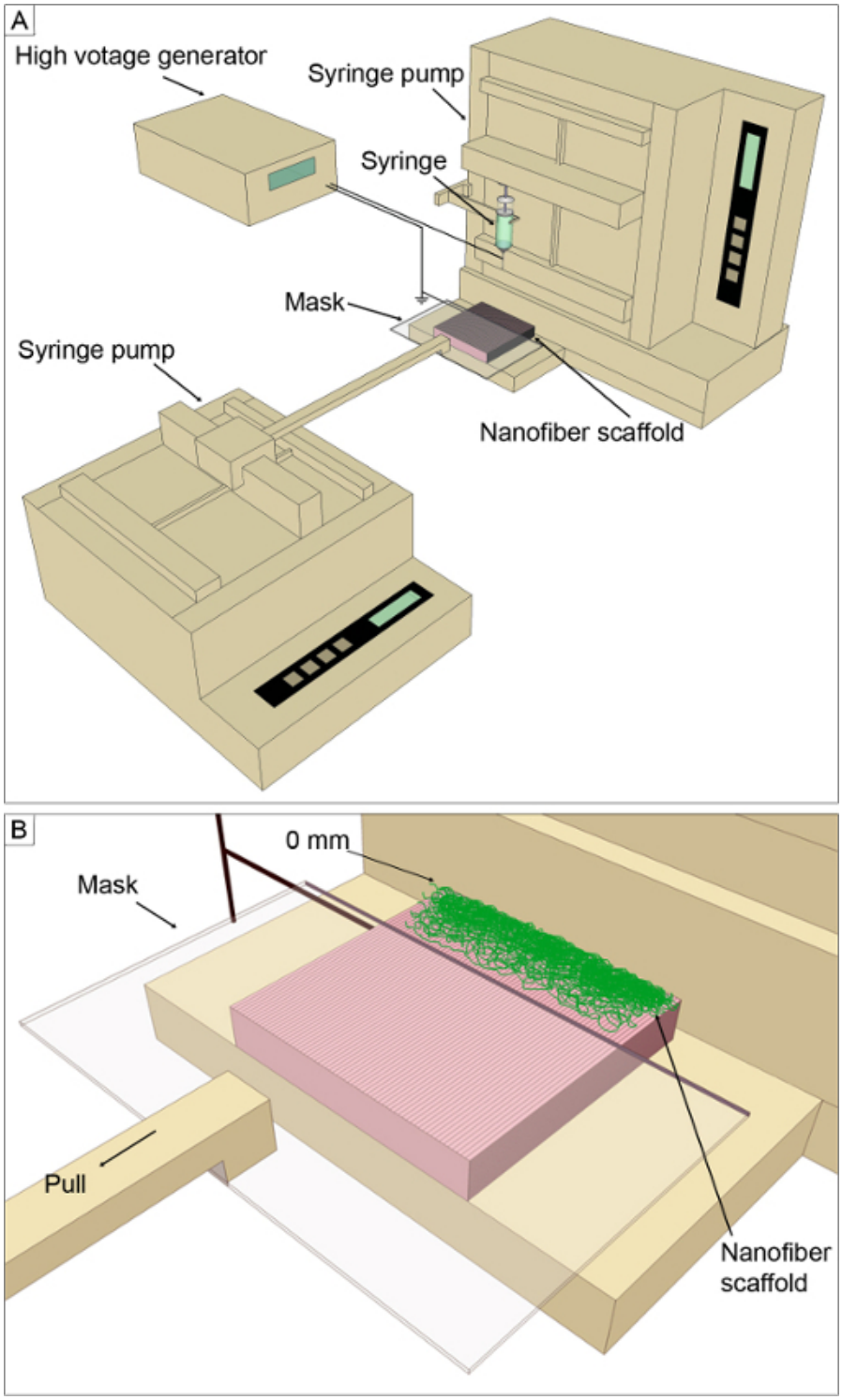

Figure 2: (A) Shows placement of the second syringe pump for the fabrication of the gradient scaffold. (B) Placement of the mask above the collector. This figure has been reprinted from [10] Macromol. Biosci., 12, Xie, J., Ma, B., Michael, P. L. & Shuler, F. D. Fabrication of Nanofiber Scaffolds With Gradations in Fiber Organization and Their Potential Applications. 1336–1341, Copyright 2012, with permission from Wiley-VCH.

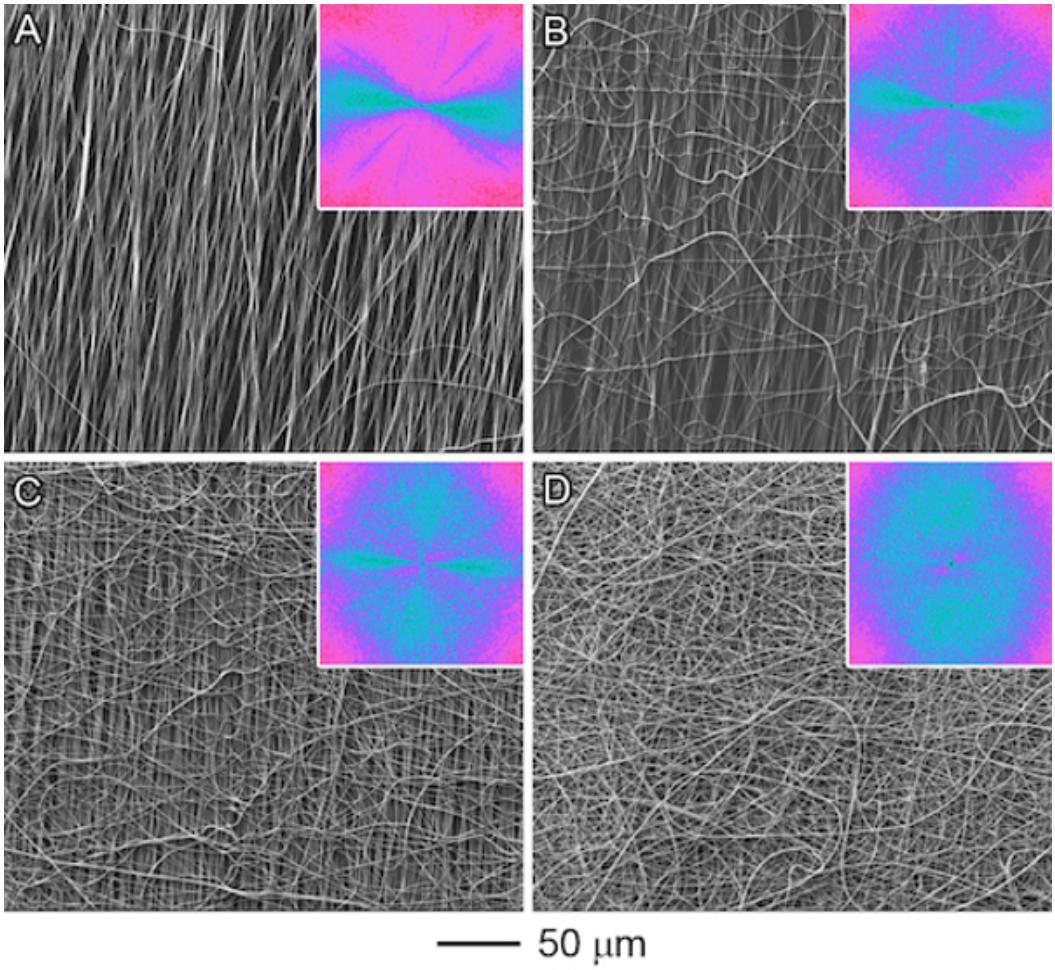

Figure 3: SEM images of the PCL gradated nanofiber scaffold at 0 mm (**A**), 2 mm (**B**), 4 mm (**C**), and 6 mm (**D**). The secondary images are Fourier fast transfer patterns (FFT). Pattern at (A) is that of aligned fibers, (D) suggests random fiber deposition. This figure has been reprinted from [10] Macromol. Biosci., 12, Xie, J., Ma, B., Michael, P. L. & Shuler, F. D. Fabrication of Nanofiber Scaffolds With Gradations in Fiber Organization and Their Potential Applications. 1336–1341, Copyright 2012, with permission from Wiley-VCH.

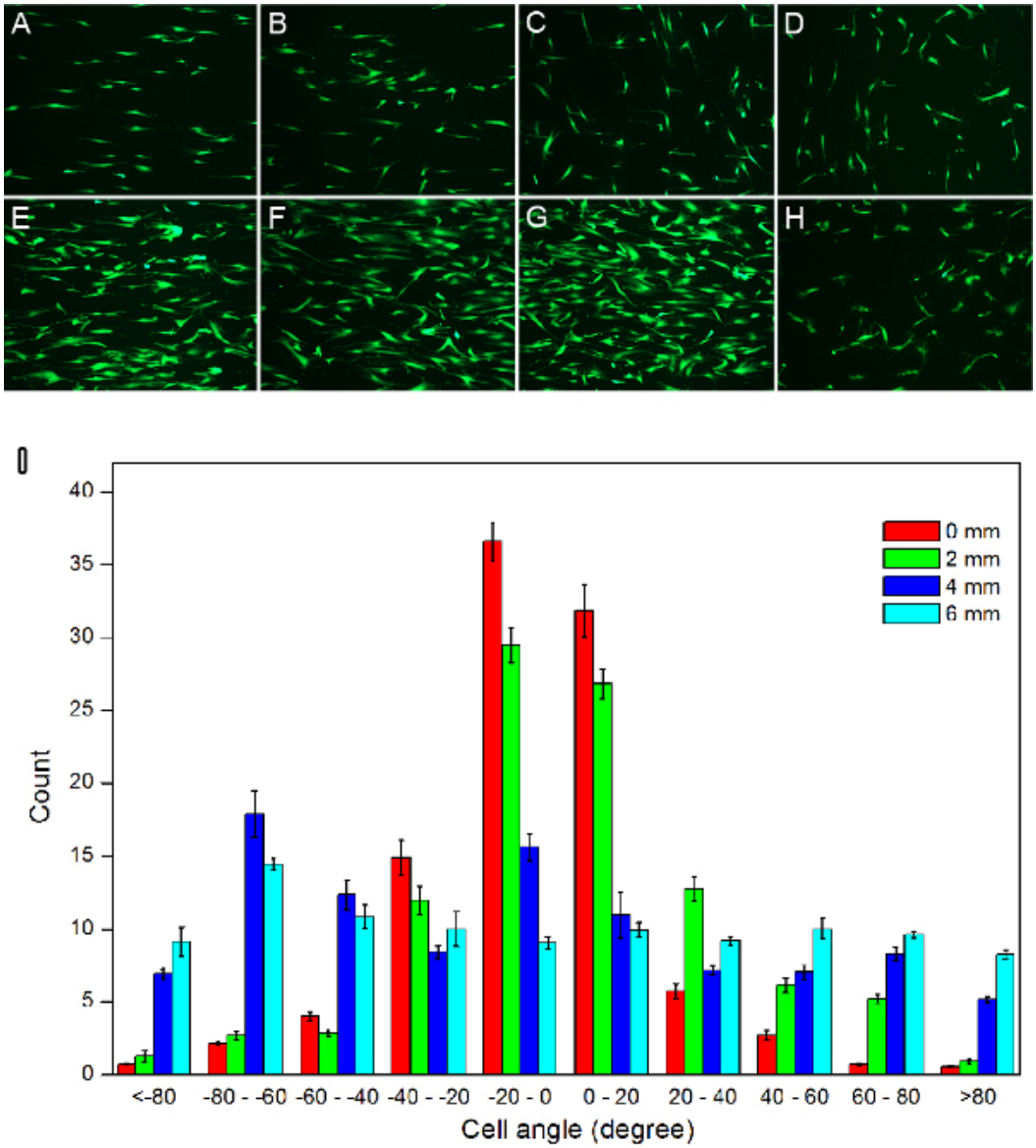

Figure 4: Fluorescence microscopy images showing ADSCs after incubation for 3 days (**A**-**D**) and 7 days (**E**-**H**). Images exhibit the various morphologies of ADSCs in different locations of the gradated scaffold. (**I**): The distribution of cell angles at different locations of scaffolds. Cells were much more concentrated between 20° of the axis of nanofiber alignment on aligned fibers (0 mm). This figure has been reprinted from [10] Macromol. Biosci., 12, Xie, J., Ma, B., Michael, P. L. & Shuler, F. D. Fabrication of Nanofiber Scaffolds With Gradations in Fiber Organization and Their Potential Applications. 1336–1341, Copyright 2012, with permission from Wiley-VCH.

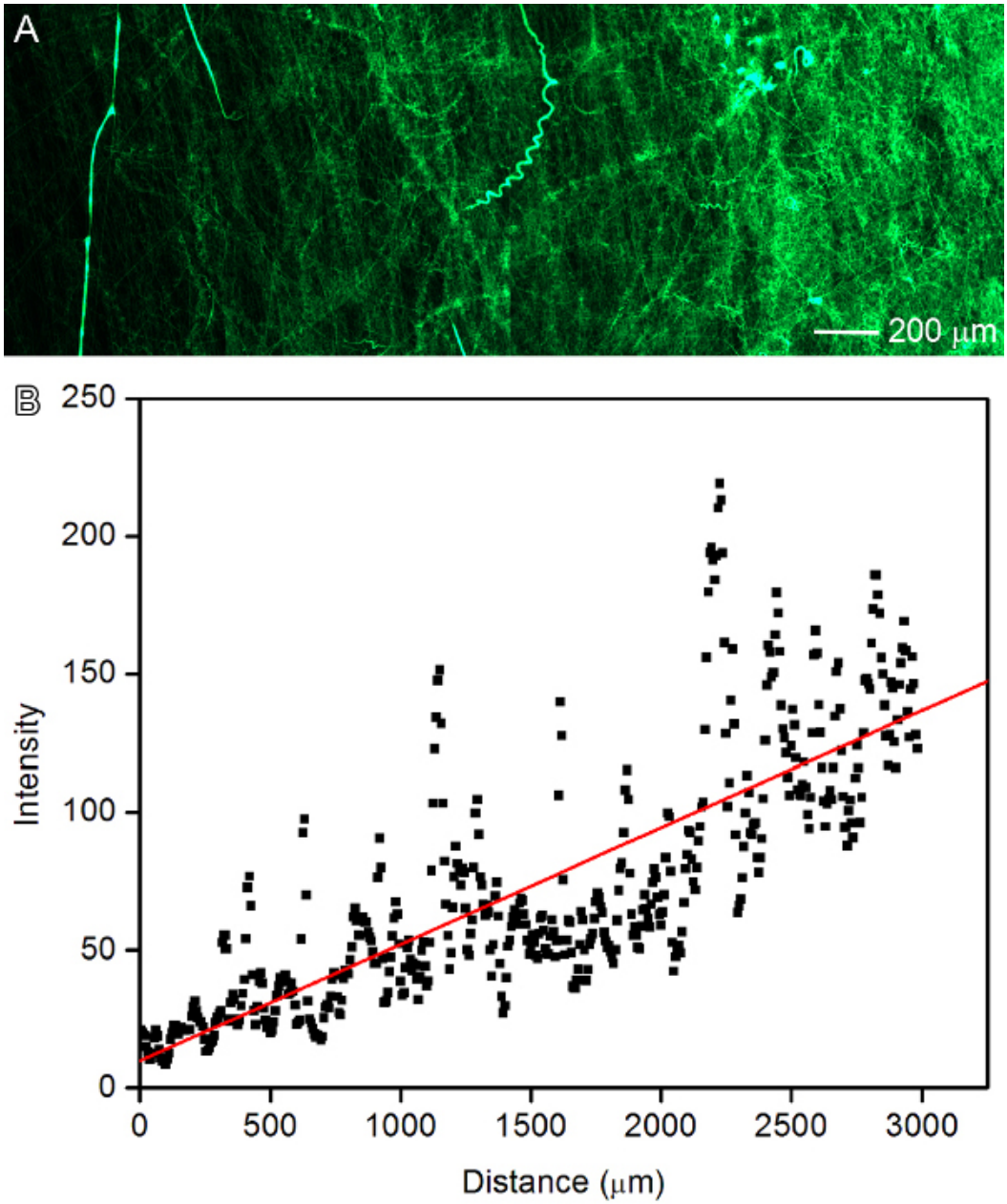

Figure 5: (A) Fluorescence microscopy image of Coumarin 6-encapsulated fibers. (B) The graph exhibits the fluorescent intensity across the scaffold. The linear increase signifies a gradual change in the chemical concentration through the scaffold. This figure has been reprinted from [10] Macromol. Biosci., 12, Xie, J., Ma, B., Michael, P. L. & Shuler, F. D. Fabrication of Nanofiber Scaffolds With Gradations in Fiber Organization and Their Potential Applications. 1336–1341, Copyright 2012, with permission from Wiley-VCH.

Discussion

The most critical part of the protocol is generation of the gradient scaffold. It is imperative that the mask covering the collector moves at a constant velocity so there is a gradual change within the fiber scaffold. The correct preparation of PCL solution is also important to ensure electrospinning success. Checking the fiber morphology prior to electrospinning is recommendable, especially after the encapsulation of Coumarin-6, which may require a higher voltage to electrospin correctly.

Furthermore, the protocol allows for fabrication of discrete gradations in fiber organization. However, this protocol yields variable thicknesses across the scaffold, which could potentially limit its mechanical applications. The area with increased random fiber deposition will be thicker than the aligned region. This protocol also does not extend to the recreation of the chemical composition at tendon-to-bone insertion site. Construction of mineral gradients is needed to successfully recreate the native mineral contents at the insertion sites. Fabrication of fiber scaffolds with gradients in mineral composition and organizational structure will be further explored to better replicate the complete native environment in a tendon-to-bone interfacial tissue.

As of now, this is the first protocol for fabrication of an electrospun nanofiber scaffold with an organizational gradient. Previous studies have fabricated scaffolds with both random and aligned fibers, but only as two separate sections, with an immediate change between the two organizations[9]. Gradient structures should allow for tailoring cell orientation and their extracellular matrix deposition and thus contribute to graded mechanical properties throughout the tissue constructs. Native tendon-to-bone insertion sites have reduced stiffness by decreasing the fiber alignment on one side[2], which can be replicated with our scaffold. Additionally the organizational gradient offers increased biomimicry of the extracellular matrix with the new ability to replicate the native fiber orientations. This structure offers more biomedical applications by the formation of chemical gradients through nanoencapsulation. New chemical gradients facilitate areas of study in cellular behavior, high throughput screening, and implant or implant coating design[10]. Additionally bone morphogenetic protein2 (BMP2), which is resembled by a dye molecule Coumarin-6 because of its ease of detection using fluorescence microscopy, could be encapsulated to further promote cellular differentiation at the end made of random nanofibers[10]. Encapsulation within the fiber gradient provides a method for control of its chemical concentration, as BMPs are only needed at the site of bone tissues.

Disclosures

The authors have nothing to disclose.

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