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Characterization of dielectrophoresis-aligned nanofibrous silk fibroin–chitosan scaffold and its interactions with endothelial cells for tissue engineering applications

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ABSTRACT

Aligned three-dimensional nanofibrous silk fibroin–chitosan (eSFCS) scaffolds were fabricated using dielectrophoresis (DEP) by investigating the effects of alternating current frequency, the presence of ions, the SF:CS ratio and the post-DEP freezing temperature. Scaffolds were characterized with polarized light microscopy to analyze SF polymer chain alignment, atomic force microscopy (AFM) to measure the apparent elastic modulus, and scanning electron microscopy and AFM to analyze scaffold topography. The interaction of human umbilical vein endothelial cells (HUVECs) with eSFCS scaffolds was assessed using immunostaining to assess cell patterning and AFM to measure the apparent elastic modulus of the cells. The eSFCS (50:50) samples prepared at 10 MHz with NaCl had the highest percentage of aligned area as compared to other conditions. As DEP frequency increased from 100 kHz to 10 MHz, fibril sizes decreased significantly. eSFCS (50:50) scaffolds fabricated at 10 MHz in the presence of 5 mM NaCl had a fibril size of 77.96 ± 4.69 nm and an apparent elastic modulus of 39.9 ± 22.4 kPa. HUVECs on eSFCS scaffolds formed aligned and branched capillary-like vascular structures. The elastic modulus of HUVEC cultured on eSFCS was 6.36 ± 2.37 kPa. DEP is a potential tool for fabrication of SFCS scaffolds with aligned nanofibrous structures that can guide vasculature in tissue engineering and repair.

1. Introduction

Natural polymers have been successfully used as scaffold materials for tissue engineering [1]. In particular, *Bombyx mori* silk fibroin (SF) has been investigated for surgical implantation owing to its biocompatibility, relatively low thrombogenicity, low inflammatory response, degradation kinetics, high tensile strength with flexibility, and permeability to oxygen and water [2–4]. Another polymer used as a scaffold is the naturally occurring polysaccharide chitosan (CS), a partially deacetylated product of chitin. CS, which has been applied clinically as hemostatic wound dressing [5], is generally inert in vivo, has favorable degradation kinetics and mimics the glycosaminoglycan component of the extracellular matrix (ECM). Researchers have explored blending SF and CS to develop three-dimensional (3-D) SFCS scaffolds that mimic the in vivo extracellular matrix [6–8]. In addition to their excellent biocompatibility, SFCS scaffolds have biological, structural and mechanical properties that can be adjusted to meet specific clinical needs. The first generation of SFCS scaffolds have produced promising outcomes both in vitro and in vivo in repairing abdominal wall defects, healing skin wounds and regenerating bone and tracheal cartilage [9–13].

In vitro studies have shown that nanofibrous structures affect cellular morphology and various cellular activities, including cell attachment, proliferation and differentiation [14]. In particular, recent studies have suggested that aligned nanostructures enhance endothelial cell capillary networks in vitro, which fulfills an important need for neovascularization in tissue engineering [15,16]. The first generation of SFCS scaffolds were smooth sheet-like structures with microfibrillar extensions that lacked the nanofibrous architecture found in the native ECM. Various methods have been used to fabricate nanofibrous scaffolds, including electrospinning, phase separation and self-assembly [1,17,18].

Our laboratory previously investigated the use of dielectrophoresis (DEP) to create nanofibrous structures in SFCS scaffolds [25] by manipulating current frequency and applied voltage to generate a non-uniform electric field on a microfabricated gold electrode. The electric field resulted in movement of particles in solution on the electrode surface due to polarization effects [26]. In the

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presence of a field gradient, an alternating current (AC) electric field induces positive DEP force (toward the high field intensity region) or negative DEP force (toward the low field intensity region). Recent work by our group and others has shown that dielectrophoresis (DEP) is a promising technique for fabricating nanofibrous scaffolds.

DEP is a non-destructive electrokinetic mechanism with great potential for manipulation of micro- or nanoparticles such as DNA, proteins, nanotubes and nanoparticles in aqueous solutions [19–24]. Allowing scaling for massively parallel electronic manipulation of bioparticles, DEP has become an important technique in the field of microfluidics for separating DNA, viruses and bacterial spores. Recent studies have shown that DEP can be used to align actin filaments into nanofibers in vitro [19,24]. The previous study's model of SF fibrils self-assembly in a 3-D SFCS scaffold using DEP was based on exposing rod-shaped particles in solution to an inhomogeneous alternating electric field, generating a time-averaged, translational DEP force due to induced dipolar effects. Small-radius (<100 nm) molecules experience DEP attraction to electrode tips even at high frequencies. Molecular assembly into solid fibers of sufficiently large radius results in a sharp decrease in crossover frequency and negative DEP. The threshold radius for which the crossover frequency drops off rapidly is determined by the suspension medium conditions. The model showed that it should be possible to concentrate and orient small-radius molecules in solution by using strong attractive DEP forces at the electrode tips and repel larger-radius fibers toward low-field regions between the electrodes in the bay region. The proposed mechanism of fiber assembly is orientation of molecules in 3-D via repulsion from two-dimensional (2-D) electrode planes due to positive DEP in high-field regions at localized electrode tips and movement away from electrode tip surface structures due to negative DEP. In addition to experimentally applying DEP to a SFCS solution to fabricate nanofibrous SFCS scaffolds and aligned structures, we studied interactions of endothelial with stem cells on these scaffolds [25].

Although our previous work provided proof of concept for using DEP to create aligned nanofibrous SFCS scaffolds, little is known about the effects of system parameters such as voltage, AC frequency and solution ionic concentration on the DEP-processed SFCS scaffolds (eSFCS). In the present study, we investigated the effects of AC frequency, sodium chloride (NaCl) presence, SF:CS ratio, and post-DEP freezing temperature on scaffold properties. We used polarized light microscopy (PLM) to analyze SF polymer chain alignment within the SFCS scaffolds and scanning electron microscopy (SEM) and atomic force microscopy (AFM) to analyze the topography of the scaffolds. The interaction of human umbilical vein endothelial cells (HUVECs) with the eSFCS scaffolds was studied using AFM and immunostaining to determine the cell mechanical properties and patterning on the eSFCS scaffolds, respectively.

2. Materials and methods

2.1. Simulation of electric field distribution

Electrodes (200 nm thick) fabricated with gold on glass slides with triangular castellation array geometry (Fig. 1A) were connected to an AC power supply (10 V_{pp} sine wave). Four pieces of castellation arrays were treated as a unit for simulation. Electrical potential (V) and electrical field (E) distributions were studied by simulation using COMSOL Multiphysics 4.1 (COMSOL, Burlington, MA). The electrostatic model was applied for simulation at $V_0 = 10$ volts based on the equations $\nabla\cdot(\varepsilon_0\varepsilon_r E) = \rho_v$ and $E = -\nabla V$, where ρ_v is the charge density, ε_r is the relative permittivity for the electrode material and ε_0 is the permittivity for the free space.

2.2. Scaffold fabrication and characterization

2.2.1. Preparation of SFCS solutions

SFCS solutions were prepared as described previously [6]. Briefly, raw silk (*Bombyx mori* silkworm), donated by Dr. S. Hudson, North Carolina State University, Raleigh, NC, originally obtained from Sao Paulo, Brazil via Korean National Sericulture and Entomology Research Institute, was degummed in 0.25% (w/v) sodium carbonate and 0.25% (w/v) sodium dodecyl sulfate at 100 °C for 1 h, then in distilled water at 100 °C for 1 h. The degummed silk was washed in running distilled water, air-dried and dissolved in a calcium nitrate tetrahydrate–methanol solution (molar ratio 1:4:2 $Ca:H_2O:MeOH$) at 65 °C for 3 h with continuous stirring to create 3.66% (w/v) SF solution. CS (82.7% deacetylation: Sigma–Aldrich) was dissolved in 2% acetic acid solution at the same concentration as was the SF solution. To make SFCS solutions, the SF solution and CS solution were blended at a ratio of 3:1 (75:25 samples) or 1:1 (50:50 samples) and mixed for 15 min. The mixtures were subjected to dialysis (MWCO 6–8 kDa) in deionized water for 3 days. The final solution was clear and homogeneous, and stored at 4 °C till use.

2.2.2. Fabrication of eSFCS scaffolds by DEP

eSFCS scaffolds were fabricated from SFCS solutions using a procedure similar to the one previously described [25]. Briefly, a 1.0 ml SFCS solution was pipetted onto triangular castellation electrodes that were fabricated on top of glass microscope slides (Fig. 1A). The electrodes were connected to an AC power supply. A 10 V_{pp} sine wave (10 kHz, 100 kHz, 1 MHz, 10 MHz and 20 MHz) was applied to the samples for 45 min at room temperature; the samples were then directly transferred to a −20 °C or −80 °C freezer with an isopropanol (IPA) bath container for 30–45 min until the samples were frozen. Frozen samples were lyophilized overnight and crystallized in a 50:50 (v/v) methanol:sodium hydroxide (1 N) solution for 15 min. To study the effects of ions on SF alignment, a 200 mM NaCl solution was added to a 50:50 SFCS solution until a final NaCl concentration of 5 mM was achieved [27].

2.2.3. Scaffold evaluation using PLM

eSFCS samples were imaged at 200× magnification using a polarizer, a crossed analyzer and a red retardation plate (λ = 530–560 nm) attached to an Olympus IX70 microscope (Olympus, Center Valley, PA) [25]. Fibril self-assembly and alignment with polymer chains in 3-D eSFCS scaffolds on the DEP electrode were assessed using PLM. Fibrils appeared blue or yellow-orange when they aligned in the direction of SF polymer chain formation. Fibrils that aligned parallel to the SF polymer chains, at a 45° angle to the polarizer-analyzer and perpendicular to the red retardation plate appeared blue; when rotated parallel to the red retardation plate, the same fibrils appeared yellow-orange. Parallel alignment was defined as fibril and polymer chain alignment at 45° to the polarizer-analyzer (with the fibrils appearing blue), whereas non-parallel alignment was defined as fibril and polymer chain alignment at −45° to the polarizer-analyzer (with the fibrils appearing yellow-orange).

The imaging processing program Image J (National Institutes of Health) was used to analyze the alignment area in the scaffold samples (three or four areas per sample). A total of four measurements were made: parallel aligned area (blue fibrils), non-parallel aligned area (yellow-orange fibrils), total aligned area (blue and yellow-orange fibrils) and total observable area at 200× magnification. Total aligned area was defined as the sum of parallel aligned area and nonparallel aligned area. The percentage of parallel alignment was defined as the ratio of parallel aligned area over the total observable area. The percentage of total alignment was defined as the ratio of total aligned area over the total observable area. The

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