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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, SF:CS ratio, and post-DEP freezing temperature. Scaffolds were characterized with polarized light microscopy (PLM) 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.

Keywords

Dielectrophoresis; Silk fibroin-chitosan scaffolds; Alignment; Nanofiber; Cell-substrate interaction; Vasculature

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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 (3D) 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 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 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 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 showed 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 3D 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 3D via repulsion from two-dimensional (2D) 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 (10V_{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 $\nabla \cdot (\epsilon_0 \epsilon_r E) = \rho_v$ 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 dodecylsulfate 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 then dissolved in a calcium nitrate tetrahydrate-methanol solution (molar ratio

1:4:2 Ca:H₂O: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 castellated electrodes that were fabricated on top of glass microscope slides (Fig. 1A). The electrodes were connected to an AC power supply. A 10V_{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 a –80 °C freezer with an isopropanol (IPA) bath container for 30 min to 45 min till 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 a 200× magnification using a polarizer, a crossed analyzer, and a red retardation plate ($\lambda = 530\text{--}560$ nm) attached to an Olympus IX70 microscope (Olympus, Center Valley, PA) [25]. Fibril self-assembly and alignment with polymer chains in 3D 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 a 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 percentage of parallel alignment in total aligned area was also included in analysis.

2.2.4. Scaffold topography evaluation using SEM—eSFCS samples were coated under vacuum using a Balzers MED 010 evaporator (TechnoTrade International, Manchester, NH) with platinum alloy, then immediately flash carbon coated under vacuum. The samples were transferred to a desiccator before being imaged with a JSM-5910

scanning electron microscope (JEOL USA, Inc., Peabody, MA) at an accelerating voltage of 5 kV. ImageJ was used to measure fibril size from SEM images.

2.2.5. Scaffold topography imaging and mechanical property evaluation using AFM

—eSFCS scaffold topography and mechanical properties were evaluated using a BioScope Catalyst AFM system (Bruker, Santa Barbara, CA). Contact-mode AFM imaging was conducted using silicon nitride probes (MLCT-C; Bruker) that had a spring constant of 0.03 N/m and a cone angle of 40°. The spring constant for the cantilever was calculated using the thermal vibration tuning method in the AFM software (NanoScope Analysis version 8.1; Bruker). Force-indentation curves were obtained in the ramp mode; the xy-scan velocity was 0.5 µm/s, and the tip-approaching velocity was 0.2 µm/s. The force applied to the surface of the scaffolds ranged from 0.1 nN to 5 nN.

After the force-indentation curves were measured, the data were analyzed with NanoScope Analysis software to obtain the elastic modulus. To determine the apparent elastic moduli of the scaffolds and cells, we used the Hertz model [28], which defines the elastic response of a homogeneous sample to a deformation with no contributions from viscous effects of the probe velocity as $F = kd = 2E \tan(\alpha) \delta^2 / \pi(1 - \nu^2)$ with $\delta = z - d$, where F is the applied force, k is the cantilever spring constant, z is the piezo height, and d is the cantilever deflection. The difference between z and d is the indentation δ . E is the sample apparent elastic modulus, and ν is the Poisson ratio that is assumed to be 0.5, given the relatively soft nature of the scaffolds [28]. The cantilever properties were defined by the opening cone angle α and the cantilever spring constant k . The slope of the force-indentation curve gives the apparent elastic modulus E for the cells and scaffolds. The accuracy of the “apparent” is limited by the sample properties (relative heterogeneity vs. homogeneity), measurement technique, parameters, and Hertzian assumptions.

2.3. Cell culture and integration with eSFCS scaffolds

2.3.1. Cell culture—HUVECs were purchased from Lonza (Walkersville, MD) and cultured in endothelial growth medium (Lonza) supplemented with bovine brain extract (Lonza). Human dermal fibroblasts (HFbs) were purchased from Cascade Biologics (Carlsbad, CA) and cultured in Medium 106 containing Low Serum Growth Supplement (Cascade Biologics). Cell cultures were maintained in 25-cm² flasks in a humidified incubator at 37°C with 5% CO₂. The culture medium was changed every other day. Cells were used within 4 passages.

2.3.2. Co-culture of HFbs and HUVECs—HUVECs were seeded onto glass slides or eSFCS scaffolds at densities of 2.5×10^5 cells/cm². For cell co-culture, HFbs were cultured on either glass slides or eSFCS scaffolds to 100% confluency, and then HUVECs were seeded on top of the HFb layer and allowed to grow for 5 days. The culture medium was replaced every other day.

2.3.3. Cell immunostaining—Cell alignment/ patterning in the HUVEC single culture and co-culture with HFbs was examined by immunostaining 5 days after plating. On day 5 of co-culture, HUVECs were stained with mouse polyclonal anti-CD31 primary antibody

(1:300; Abcam, Cambridge, MA) followed by FITC goat-anti-mouse IgG (H+L) secondary antibody (1:500; Abcam). Images were captured with an Olympus IX81 confocal fluorescence microscope.

2.3.4. HUVEC mechanical property evaluation using AFM—The apparent elastic modulus of HUVECs cultured on glass slides and eSFCS scaffolds for 24 h was measured using a BioScope Catalyst AFM system (Bruker). Contact-mode AFM imaging used silicon nitride probes (DNP-10 D; Bruker) that had a spring constant of 0.06 N/m and a cone angle of 40°. Force-indentation curves were obtained in the ramp mode with a ramp velocity of 0.1 $\mu\text{m/s}$. The force applied to the cells ranged from 0.3 to 0.6 nN. Force-indentation curves were analyzed with NanoScope Analysis software to obtain the elastic modulus for each data point. The Hertz model was used to obtain the apparent elastic modulus of the cells as described in section 2.2.5.

2.4. Statistical Analysis

Data were summarized as means with standard deviations, and analyzed by the Student t-test and one-way ANOVA using SigmaStat software. p values of 0.05 were considered significant.

3. Results

3.1. Simulation of electric field distribution

Electrodes with triangular castellation arrays were used to establish a non-uniform electric field for DEP (Fig. 1). A 2D profile of four electrode units is shown in Fig. 1B. The units shown in purple were connected to a 10 V_{pp} input, and the units shown in grey were grounded. The electrode structure was divided into smaller elements by creating a fine mesh (Fig. 1C). The electrostatic model was applied to simulate the electrical potential distribution and electrical field distribution. The electrical potential decreased gradually from 10 V at the input electrode to 0 V at the grounded electrode in 3D space (Fig. 1D). A 3D non-uniform electrical field distribution was observed along the electrode edges (Fig. 1E). One-dimensional profiles of the electrical field distribution along the input electrode and grounded electrode are shown in Fig. 2A and B, respectively. This simulation revealed a 3D non-uniform electrical field along the electrodes. The electric field in the z direction was stronger than that in the x or y direction; the stronger field may have contributed to 3D DEP function about 20 μm above the electrode surface. However, the electric field in the center of the electrode gap was weak.

3.2. SF polymer chain alignment within eSFCS scaffolds

The schematic in Fig. 3A shows the positions of the polarizer (0°), analyzer (90°), and the red retardation plate (−45°) in the PLM. In eSFCS (50:50) scaffolds fabricated at 100 kHz (Fig. 3B), the SF polymer chain aligned parallel to the direction of the fibrils (blue). In contrast, eSFCS (75:25) scaffolds fabricated at 1 MHz (Fig. 3C) had a layered-network structure, with one layer of aligned fibrils (45° to the polarizer; blue) crossed with another layer of aligned fibrils (−45° to the polarizer; yellow-orange). In order to quantify SF

polymer chain alignment, 15 samples were prepared under each condition (broken samples not included for quantification).

3.2.1. Effect of SF to CS ratio on total alignment—The percentages of total alignment in total observable area for eSFCS (50:50) and eSFCS (75:25) scaffolds prepared at 10 kHz, 100 kHz, and 1 MHz were compared (Fig. 4A). The percentage of total alignment per total observable area was significantly higher in eSFCS (50:50) scaffolds fabricated at 100 kHz than in eSFCS (50:50) samples fabricated at 10 kHz and 1 MHz ($p < 0.05$). The percentage of total alignment per total observable area was significantly higher in eSFCS (75:25) scaffolds fabricated at 100 kHz as compared to eSFCS (75:25) scaffolds fabricated at 10 kHz and 1 MHz ($p < 0.05$). At the DEP AC frequency of 10 kHz and 1 MHz, eSFCS (50:50) had significantly higher percent of total alignment per total observable area than eSFCS (75:25) ($p < 0.05$), however, at 100 kHz eSFCS (50:50) and eSFCS (75:25) had similar percent of total alignment in the total observable area (Fig. 4A).

3.2.2. Effect of DEP frequency on parallel alignment—Parallel alignment was dominant in eSFCS (50:50) scaffolds fabricated at all frequencies, whereas in eSFCS (75:25) scaffolds the parallel alignment was dominant only in samples fabricated at 100 kHz ($p < 0.05$) (Fig. 4B). At the DEP AC frequency of 10 kHz and 1 MHz, the percentage of parallel alignment per total aligned area was significantly higher for eSFCS (50:50) than for eSFCS (75:25) samples ($p < 0.05$). However, at 100 kHz, there was no significant difference between eSFCS (50:50) and eSFCS (75:25) (Fig. 4B).

eSFCS (50:50) scaffolds fabricated at DEP AC frequencies of 10 kHz ($n = 10$), 100 kHz ($n = 11$), 1 MHz ($n = 10$), and 10 MHz ($n = 9$) had high percentages of parallel alignment per total aligned area (Fig. 4B). eSFCS (50:50) scaffolds fabricated at 10 and 100 kHz, $97 \pm 6\%$ and $94 \pm 10\%$ of the alignment was parallel, respectively and at 10 MHz percent of parallel alignment in total aligned area was not significantly different.

3.2.3. Effect of NaCl on parallel alignment of 50:50 eSFCS Scaffolds—Since percent parallel alignment per total aligned area was the highest for eSFCS (50:50), the effect of salt at 100 kHz, 1 MHz, and 10 MHz was assessed (Fig. 4C). The presence of 5mM NaCl significantly increased the percent of parallel alignment per total observable area, $38 \pm 12\%$ in the 100 kHz scaffolds ($n = 9$) as compared to $25 \pm 8\%$ in the 100 kHz samples ($n = 13$) prepared without NaCl ($p < 0.05$). Results showed that the percent area of parallel alignment was significantly higher ($p < 0.05$) in eSFCS (50:50) scaffolds fabricated at 10 MHz ($34 \pm 9\%$, $n = 13$) than in eSFCS (50:50) scaffolds fabricated at 100 kHz ($25 \pm 8\%$, $n = 13$) and 1 MHz ($20 \pm 11\%$, $n = 7$). DEP frequencies ranging from 100 kHz to 10 MHz did not significantly affect parallel alignment in eSFCS samples prepared with NaCl.

3.2.4. Effect of post-DEP freezing temperature on parallel alignment—In our previously published studies, SF-based scaffolds were all fabricated at -80°C . However, when we first fabricated eSFCS scaffolds at -80°C , cracks formed in scaffolds, which compromised the practical application in tissue engineering. Thus, The IPA bath was used to eliminate cracking of the scaffolds due to freezing at -80°C . In addition, at -20°C there was no cracking without the IPA; and with the IPA bath the freezing time increased significantly

(samples were not frozen after 45 min). Thus, the -20°C scaffolds were fabricated without the IPA. Taking all these in consideration, eSFCS (75:25) scaffolds were prepared at a post-DEP freezing temperature of -80°C with IPA bath and -20°C without IPA bath. Samples fabricated at 100 kHz, 1 MHz, and 20 MHz and frozen at -20°C had percentages of parallel alignment in the total observable area of $25 \pm 16\%$ ($n = 6$), $24 \pm 13\%$ ($n = 6$), and $28 \pm 13\%$ ($n = 6$), respectively. Scaffolds fabricated at 10 kHz, 100 kHz, and 1 MHz and frozen at -80°C had percentages of parallel alignment within the total observable area of $12 \pm 9\%$ ($n = 10$), $51 \pm 29\%$ ($n = 6$), and $1 \pm 0\%$ ($n = 6$), respectively. The amount of parallel alignment fabricated at AC frequencies ranging from 10 kHz to 20 MHz differed significantly ($p < 0.05$). The percentage of parallel alignment in eSFCS (75:25) scaffolds fabricated at identical frequencies but frozen at different temperatures differed significantly ($p < 0.05$). The percentage of parallel alignment in total observable area was significantly lower in samples prepared at 100 kHz and frozen at -20°C ($25 \pm 16\%$, $n = 6$) than in samples prepared at 100 kHz and frozen at -80°C ($51 \pm 29\%$, $n = 6$) ($p < 0.05$).

3.3. Characterization of eSFCS scaffold nanofeatures

SEM and AFM were used to assess the surface properties (such as texture and fibril size) of the eSFCS scaffolds. Representative SEM images of eSFCS scaffolds with fibrous structures are shown in Fig. 5A–C. Fibril sizes obtained from the SEM images are summarized in Fig. 5D. Fibril size decreased with increase in frequency. The fibrils in eSFCS scaffolds prepared with or without NaCl at a DEP frequency of 100 kHz ($0.43 \pm 0.11\ \mu\text{m}$ and $0.53 \pm 0.13\ \mu\text{m}$, respectively) were significantly larger ($p < 0.05$) than those in scaffolds prepared at a DEP frequency of 10 MHz ($0.27 \pm 0.09\ \mu\text{m}$ and $0.30 \pm 0.09\ \mu\text{m}$, respectively). The presence of NaCl did not affect fibril size significantly. Representative AFM images of eSFCS scaffolds fabricated at 10 MHz in the presence of 5 mM NaCl revealed fibril bundles consisting of fine nanofibers ($77.96 \pm 4.69\ \text{nm}$) (Fig. 6).

3.4. Characterization of eSFCS scaffold apparent elastic modulus

We used AFM to obtain force-indentation curves (Fig. 7A) and the Hertz model to calculate the apparent elastic modulus of eSFCS (50:50) samples fabricated at 10 MHz in the presence of 5 mM NaCl. Fig. 7B shows the effect of indentation depth on the derived apparent elastic modulus of eSFCS scaffolds. For example, the indentation depth of 12 nm resulted in an apparent modulus of 34.8 kPa, while the indentation depth of 50 nm resulted in an apparent modulus of 44.6 MPa. The apparent elastic moduli of eSFCS samples measured under different loading forces are shown in Fig. 7C. When the loading force was 0.1 nN, the apparent elastic modulus was $39.9 \pm 22.4\ \text{kPa}$ ($n = 55$). As the force increased to 1 nN and above, the apparent modulus increased.

3.5. Interaction of HUVECs with eSFCS scaffolds

Based on PLM evaluation SEM examination, eSFCS scaffolds (50:50, 10 MHz, 5 mM NaCl) showed the highest level of parallel alignment in total observed area and the finest fibril size within scaffolds. Thus, eSFCS scaffolds (50:50, 10 MHz, 5 mM NaCl) were selected for to assess the endothelial cell interactions with the eSFCS scaffold.

The mechanical properties of HUVECs on eSFCS scaffolds were assessed using the AFM. The force-indentation curves of HUVECs cultured on glass slides and eSFCS scaffolds are shown in Fig. 8A and B. With the Hertz model, the apparent elastic modulus of HUVECs cultured on glass slides was 7.42 ± 0.46 kPa ($n = 12$), and the apparent elastic modulus of HUVECs cultured on eSFCS scaffolds was 6.36 ± 2.37 kPa ($n = 12$) ($p = 0.07$) (Fig. 8C). There was no significant difference in the apparent elastic modulus of HUVECs cultured on glass slide versus eSFCS scaffold although cells on eSFCS scaffolds appear to be slightly less stiff than the cells cultured on glass slide.

HUVEC interactions with eSFCS without other biochemical stimuli were examined to assess the effect of fibril alignment on microvessel assembly as a function of the aligned scaffold surface. HUVECs cultured on glass slides for 5 days reached confluency and had no vessel/capillary-like structures or branches (Fig. 9A) whereas HUVECs co-cultured with HFbs on glass slides formed vessel/capillary like structures (Fig. 9B). Single cell culture of HUVECs (Fig. 9C and E) and co-culture with HFbs (Fig. 9D and F) on eSFCS scaffolds for 5 days both resulted in vessel/capillary-like structures and branching of the vascular structures.

4. Discussion

In this study, PLM was utilized to examine silk fibroin polymer chain alignment in a macro-scale. The relationship between polymer chain alignment and fiber alignment could be straightforwardly observed as shown in Fig. 3. Based on PLM and SEM examination, our study revealed that when DEP is used to fabricate nanofibrous eSFCS scaffolds, the AC frequency, presence of salt, SF:CS ratio, and post-DEP freezing temperature affect SF polymer chain alignment and fibril size in the eSFCS scaffolds. In order to gain more insights on the functional groups in a micro-scale, Fourier transform infrared spectroscopy (FTIR) /Raman spectroscopy will be employed in future studies to better understand DEP effects on SF-based scaffold fabrication.

Our finding that post-DEP freezing temperature affects SF polymer chain alignment within eSFCS scaffolds is in agreement with previous studies showing that the initial freezing temperature and freezing rate affect the architecture and mechanical properties of the 3D scaffolds [6, 14, 29]. SF polymer chain parallel alignment in eSFCS (75:25) samples was DEP frequency dependent at -80°C but not at -20°C , although the difference could also be a function of the use of an IPA bath for freezing at -80°C and not for freezing -20°C freezing. SF polymer chain alignment analysis based on PLM images indicated significant differences in SF polymer chain parallel alignment between eSFCS (75:25) samples prepared at -80°C (with an IPA bath) and eSFCS (75:25) samples prepared at -20°C (without an IPA bath) at the same AC frequencies. The effects of fast freezing may overcome the effect of AC frequency on SF patterning, possibly as a result of directional ice crystal formation during the freezing process. However, we only explored two temperature conditions in this study (-80°C with IPA bath and -20°C). In our future studies, systematic exploration of a wide range of temperatures will be included to provide more insights into the possible mechanisms.

Similar to Gobin et al [6], we found that the SF:CS ratio can affect the properties of eSFCS scaffolds. In that study, a higher proportion of SF in the SFCS solution significantly increased the ultimate tensile strength and elastic modulus of the SFCS scaffolds [6]. Increasing the CS content increased the water capacity of the SFCS solutions. In our study, eSFCS (50:50) scaffolds fabricated at frequencies ranging from 10 kHz to 1 MHz had larger aligned areas than did eSFCS (75:25) scaffolds fabricated at the same frequencies. Parallel alignment was dominant in all eSFCS (50:50) samples, regardless of the frequencies at which the samples were fabricated, whereas dominant parallel alignment occurred in eSFCS (75:25) samples fabricated at a frequency of 100 kHz only.

AC frequency and the presence of salt in SFCS solution affected SF polymer chain alignment and fibril size in eSFCS scaffolds. The percentage of total alignment areas in eSFCS (50:50) samples was AC frequency dependent, and the presence of NaCl in the SFCS (50:50) solution increased parallel alignment. At a certain frequency, DEP may work synergistically with intermolecular forces (e.g., hydrogen bonds) to induce parallel alignment. The presence of NaCl may facilitate the breaking of intermolecular hydrogen bonds in random coil structures and enhance intermolecular hydrogen bonding, thereby causing polymer chain alignment[30]. We also found that higher DEP frequencies resulted in smaller fibrils within eSFCS (50:50) scaffolds with fibril bundles consisting of fine nanofibrils.

Solvents used to dissolve silk fibroin-chitosan play an important role in scaffold properties [31]. $\text{Ca}(\text{NO}_3)_2$ -MeOH- H_2O system was used to dissolve silk and fabricated the eSFCS scaffolds. Regenerated SF aqueous solution using a $\text{Ca}(\text{NO}_3)_2$ -MeOH- H_2O system and LiBr-EtOH- H_2O system at room temperature showed different protein conformation in resultant scaffolds[31]. In addition, silk films fabricated using the LiBr method also results in residual LiBr crystals that are found embedded in the film. Apparently, the dialysis process is unable to remove all of the LiBr salt from the SF solution [32].

The apparent elastic moduli of HUVECs cultured on glass slides (7.42 ± 0.46 kPa) and HUVECs cultured on eSFCS scaffolds (6.36 ± 2.37 kPa) differed only slightly ($p = 0.07$) although the glass substrate is much stiffer than the eSFCS scaffolds. The stiffness of the endothelial cells on glass substrate is similar to the endothelial cell stiffness reported by Mathur et al. (6.8 ± 0.4 kPa). The thin eSFCS scaffold may contribute to the measured elastic modulus of endothelial cells [33]. Altman et al. showed that the apparent elastic modulus of adipose tissue-derived stem cells was higher on stacked-sheet SFCS scaffolds than those of cells adherent to the stiffer glass slides due to higher stress fiber formation [9]. Engler et al. reported that cells adherent on stiff substrates results in stiffer cells and the elasticity directs stem cell differentiation [34]. While the apparent elastic modulus of scaffolds affects cellular response to scaffolds via the cell adhesion complexes and the cytoskeleton where molecular pathways affect cellular activities, other studies have shown that when endothelial cells make cell-cell contact, such as the case in microvessel formation, the effect of substrate stiffness on cell shape and the cytoskeleton is eliminated [35, 36].

Neovascularization is an important aspect of tissue regeneration for the integration of implanted scaffolds with surrounding tissues at the reconstruction sites. eSFCS scaffolds

induced HUVECs to form vessel/capillary-like structures similar to HUVECs cultured on human fibroblast as compared to the cells on glass slide that did not. A previous study found that aligned nanostructures on cell culture substrates affect HUVEC alignment and induce capillary-like structure formation *in vitro* [15]. DEP force-driven polymer chain alignment may contribute to cell alignment and patterning similar to HUVECs cultured on human fibroblast. In our future studies, the effects of eSFCS scaffolds on vasculature guidance will be quantified to provide more information on scaffold design and fabrication for tissue engineering application.

5. Conclusion

This study investigated the effects of AC frequency, presence of salt, SF:CS ratio, and post-DEP freezing temperature on eSFCS scaffold properties. Endothelial cell interactions with eSFCS scaffolds were studied to understand vascular guidance via biomaterial surfaces. The eSFCS (50:50) samples prepared at 10 MHz with NaCl had the highest percentage of aligned area than scaffolds prepared with other SFCS ratios and DEP frequencies. As DEP frequency increased from 100 kHz to 10 MHz, fibril sizes decreased significantly. HUVECs on eSFCS scaffolds formed aligned and branched capillary-like vascular structures. DEP is a potential tool for fabrication of eSFCS scaffolds with aligned nano-fibrous structures. eSFCS scaffolds can be used to guide the vasculature and have applications for tissue engineering and repair of critical-size defects.

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Fig. 1.

(A) Application of DEP to fabricate SFCS scaffolds. The eSFCS scaffolds were frozen at -80°C in an IPA bath container. (B) 2D profile of electrode arrays. The electrodes shown in blue were connected to an AC input (10 V_{pp}). (C) 3D mesh profile of the electrode structure. Extra fine mesh was generated for accurate simulation. (D) 3D electrical potential distribution. The electrical potential ranged from -10 V to 10 V . (E) 3D electrical field distribution. The magnified portion of the image shows the electrical field distribution along the electrode in 3D space.

Fig. 2.

One-dimensional profiles of electrical field distribution along the electrode connected to the input (A, upper panel) and along the grounded electrode (B, upper panel). In the plots below, the blue, green, and red lines represent the electric field components in the x, y, and z directions, respectively.

Fig. 3.

(A) Schematic of polarizer and analyzer positions for examining eSFCS scaffolds. (B and C) PLM images and corresponding phase contrast images of eSFCS samples. Both samples were frozen at -80°C after DEP processing. The blue arrow indicates SF polymer chain alignment along the fiber that was at 45° , this alignment was parallel to the direction of the sample, and it was defined as parallel alignment for analysis. The red arrow indicates SF polymer chain alignment along the fiber that was at -45° , this alignment was non-parallel to the direction of the sample, and it was defined as non-parallel alignment for analysis.

Fig. 4.

(A) Plot of percent of total alignment area in total observable area in eSFCS (75:25) scaffolds prepared at 10 kHz ($n = 10$), 100 kHz ($n = 6$), and 1 MHz ($n = 6$) and eSFCS (50:50) scaffolds prepared at 10 kHz ($n = 10$), 100 kHz ($n = 11$), and 1 MHz ($n = 10$). * indicates $p < 0.05$, compared with eSFCS 75:25 under the same frequency. # indicates $p < 0.05$, compared with eSFCS 50:50 under DEP frequency of 100 kHz. @ indicates $p < 0.05$, compared with eSFCS 75:25 under DEP frequency of 100 kHz. Scaffolds were prepared at a post-DEP freezing temperature of -80°C . (B) Percent of parallel alignment in total aligned

area for eSFCS (75:25) samples prepared at 10 kHz (n=10), 100 kHz (n=6), or 1 MHz (n=6) and eSFCS (50:50) samples at 10 kHz (n=10), 100 kHz (n=11), and 1 MHz (n=10). * indicates $p < 0.05$, compared with eSFCS 75:25 under the same frequency. @ indicates $p < 0.05$, compared with eSFCS 75:25 under DEP frequency of 100 kHz. \$ indicates $p < 0.05$, compared with eSFCS 75:25 under DEP frequency of 10 kHz. (C) Percent of parallel alignment area in total observable area in eSFCS (50:50) samples prepared with 5 mM NaCl at 100 kHz (n=9), 1 MHz (n=8), and 10 MHz (n=11) and without 5 mM NaCl at 100 kHz (n=13), 1 MHz (n=7), and 10 MHz (n=13). * indicates $p < 0.05$ compared with No NaCl group under the same frequency. # indicates $p < 0.05$ compared with 10 MHz group with NaCl.

Fig. 5. (A–C) SEM images of eSFCS scaffolds with fibrous structures. Fiber structures were indicated by yellow arrows. Samples were frozen at -80°C after DEP processing. (D) Summarization of fiber sizes measured from SEM images. * $p < 0.05$, compared with eSFCS (50:50) at 100 kHz without 5 mM NaCl; # $p < 0.05$, compared with eSFCS (50:50) at 100 kHz with 5 mM NaCl.

Fig. 6. AFM images of eSFCS scaffolds with nanofibrous structures. A 5 μm × 5 μm area was scanned. eSFCS (50:50) scaffolds were fabricated at a DEP frequency of 10 MHz in the presence of 5 mM NaCl. The samples were frozen at -80 °C after DEP processing.

Fig. 7.

(A): Typical force-indentation curves for the approach and retraction of an AFM probe on eSFCS scaffolds under different loading forces. (B): Elastic modulus measured at different indentations. (C): Elastic modulus measured under different loading forces. eSFCS (50:50) scaffolds were fabricated in the presence of 5 mM NaCl and frozen at -80°C after DEP processing. *: $p < 0.05$ as compared with elastic modulus measured under 0.1 nN. &: $p < 0.05$ as compared with elastic modulus measured under 0.5 nN.

Fig. 8. (A–B): Typical force-indentation curve of HUVECs on glass (A) or eSFCS scaffolds (B). (C): HUVEC elastic modulus on eSFCS scaffolds or glass. eSFCS (50:50) scaffolds were fabricated in the presence of 5 mM NaCl and frozen at -80°C after DEP processing.

Fig. 9.

Confocal images of HUVECs on glass (A) eSFCS scaffolds (C and E), and of HUVECs co-cultured with HFbs on glass (B) or eSFCS scaffolds (D and F). eSFCS (50:50) scaffolds were fabricated in the presence of 5 mM NaCl and frozen at -80°C after DEP processing.