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Guiding the behaviors of human umbilical vein endothelial cells with patterned silk fibroin films

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ABSTRACT

Silk fibroin is an ideal blood vessel substitute due to its advantageous qualities including variable size, good suture retention, low thrombogenicity, non-toxicity, non-immunogenicity, biocompatibility, and controllable biodegradation. In this study, silk fibroin films with a variety of surface patterns (*e.g.* square wells, round wells plus square pillars, square pillars, and gratings) were prepared for *in vitro* characterization of human umbilical vein endothelial cell's (HUVEC) response. The affects of biomimetic length-scale topographic cues on the cell orientation/elongation, proliferation, and cell-substrate interactions have been investigated. The density of cells is significantly decreased in response to the grating patterns (70 ± 3 nm depth, 600 ± 8 nm pitch) and the square pillars (333 ± 42 nm gap). Most notably, we observed the contact guidance response of filopodia of cells cultured on the surface of round wells plus square pillars. Overall, our data demonstrates that the patterned silk fibroin films have an impact on the behaviors of human umbilical vein endothelial cells.

1. Introduction

Extracellular matrix (ECM) consisting of cell-secreted proteins, polysaccharides, and complex three-dimensional micro/nano-scale topographies plays an important role in affecting the behaviors of cells [1–4]. It is well known that cells respond to micro/nano-scale topographies and exhibit different behaviors in many aspects including adhesion, proliferation, migration and apoptosis [5–9]. Studies of cellular responses to topographies of varying geometry and length scale are thus of great importance to understand cell biology and to promote applications of patterned substrates in tissue engineering [10,11].

Vascular endothelial cells are critical for forming the inner lining of major blood vessel which plays an important role in regulating blood pressure and preventing coagulation. A feature of vascular endothelial cells is that they fasten themselves to the underlying stroma through a particular specialized ECM, the basement membrane [12,13]. The abundant features of basement membranes, which include cell-secreted proteins, displayed functional groups, a battery of trophic agents and other cytoactive factors, can regulate the fundamental behaviors of endothelial cells [8,14–16]. In order to replicate native basement membrane and eventually improve vascular prosthetics, many synthetic or biologically derived substrates that simulate the native basement membrane have been developed to regulate endothelial cell behavior [8,17,18]. Surface topography is one of the key parameters that have been focused on to investigate the endothelial cell responses. Different substrates such as polydimethylsiloxane (PDMS) [8,19–21] and poly-(methyl methacrylate) (PMMA) [22–24], which were patterned by lithographic techniques, have been explored for *in vitro* applications. Biodegradable polymers such as poly(L-lactic acid) (PLLA) are being explored for potential *in vivo* applications [25]. Most of them, however, have significant drawbacks such as bulk degradation upon implantation and rigid mechanical properties. Rigid mechanical materials can cause localized inflammation in the dynamic *in vivo* environment [26–28].

Silk fibroin from *Bombyx mori* silkworm cocoons is biocompatible and possesses excellent features such as tunable mechanical properties and ambient aqueous processing. In addition, silk fibroin is implantable due to its non-immunogenic response and controllable degradation rates [29–31]. Thus, silk fibroin has been

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increasingly used as a biomaterial in a wide range of forms such as films, sponges, hydrogels and solid blocks for applications in tissue engineering and regenerative medicine [32,33]. Furthermore, silk fibroin film is able to be easily patterned with soft or nanoimprint lithography techniques [34,35].

The successful design of blood vessels should critically consider the blood pressure, the compatibility with adjacent host vessels, and the ability of sustaining cyclic loading and anti-thrombotic lining [36]. In this aspect, silk fibroin is an ideal blood vessel substitute and demonstrates an advantage over other anti-thrombotic materials with its excellent resistance to high shear stress and blood flow pressure [37,38]. In fact, it has been formed into microtubules with different inner diameters, porosities, mechanical strengths, and diffusivities for blood vessel engineering [31,36]. Additionally, the silk fibroin's elastic modulus is within a range that does not cause deleterious effects for cells. Other materials used for similar purposes with more rigid mechanical properties have been shown to cause localized inflammation *in vivo* [26–28]. Therefore, it would be advantageous to design silk fibroin films with micro/nano-scale topographies that are appropriate for future *in situ* tissue integration and regeneration. Such films have the potential to become the preferred vascular stents for *in vivo* implantation. It has been reported that patterned silk fibroin films can be developed for corneal tissue engineering applications, and the micro/nano-scale topographies play important roles in the wound-healing responses, such as corneal epithelial and fibroblast attachment, proliferation and alignment [29,30,39]. However, it is not clear regarding the vascular endothelial growth on silk substrates, especially taking into consideration the varying geometries and sizes of substrate topography.

The purpose of this study is to investigate the relationship between the various micro/nano-scale topographical structures of silk fibroin films and the behavior of human umbilical vein endothelial cells (HUVECs). Silk fibroin films containing different surface topographic features have been prepared in this study. Such patterned silk films have never before been used as a culture substrate for human umbilical vein endothelial cells. The design of silk fibroin films with micro/nano-scale topography allows for the study of fundamental endothelial cell behaviors including orientation and alignment, proliferation, and attachment. This will aid in the development of novel strategies in tissue engineering and will ultimately advance the development of cardiovascular prosthetics.

2. Materials and methods

2.1. Preparation of aqueous silk solutions

Aqueous silk solutions were obtained using previously published protocols with slight modifications [40]. Cocoons from *B. mori* silkworm were boiled twice for 30 min in an aqueous solution of 0.02 M Na_2CO_3 (Sigma-Aldrich). The silk fibers were rinsed thoroughly with distilled water three times with room temperature water to remove the glue-like sericin proteins and then dried at room temperature overnight. The purified silk fibers were dissolved in a mixed solution of $CaCl_2$ (Sigma-Aldrich): water: ethanol (in a 1:8:2 molar ratio) at 72 °C. The solution was put into a dialysis cassette (3.5k MWCO, 5–15 ml capacity) and dialyzed against distilled water for three days. The distilled water was replaced for at least five times during the dialysis. The solution was centrifuged at 10,000 rpm for 20 min at 4 °C. The final solution had a relative concentration of 4–5% (w/v), and was stored at 4 °C.

2.2. Fabrication of silk fibroin films with micro/nano-scale surface topographies

Homemade substrates with micro/nano-scale patterns were prepared by electron beam lithography using polymethylmethacrylate (PMMA) as photoresist. The thickness of PMMA resist on silicon wafer was 400 nm. Each patterned field consists of an array of features over a total area of 16 mm^2. To generate clean silk fibroin films, the aqueous silk solution was filtered through a 0.45 μm pore size syringe filter before using. Patterned silk films with controllable film thickness were prepared by pouring aqueous silk solution onto the patterned silicon substrates bearing PMMA micro/nano-structures and drying overnight at room temperature. The replicated pattern types include square wells, square pillars, round wells plus square pillars, and gratings. The geometries of the patterns are summarized in Tables 1 and 2. In order to make the films water insoluble, the silk fibroin films were treated with 90% methanol for about 5 h to induce β-sheet transition. The silk fibroin films were subsequently degassed for 1 h under vacuum and dried for at least 24 h. The patterned silk fibroin films were placed into 48-well plates. The plates were sterilized with 75% ethanol for 30 min. Each film was washed with three separate aliquots of aseptic dH_2O. The samples were left in the final aseptic dH_2O wash until ready for cell seeding.

Table 1
Average dimensions of the structured surfaces for the replicated pattern types, $n = 5$.

Types	Width (nm ± SD)	Gap (nm ± SD)	Depth (nm ± SD)
Square wells	1033 ± 57	967 ± 57	357 ± 40
Round wells[a] plus	291 ± 31[a]	513 ± 47.9[a]	97 ± 15[a]
square pillars[b]	486 ± 24[b]	333 ± 42[b]	380 ± 20[b]
Square pillars	1167 ± 57	293 ± 11	373 ± 25

[a] The size of round wells.
[b] The size of square pillars in Round wells plus square pillars.

2.3. Cell culture

HUVECs were cultured in RPMI medium 1640 (Hyclone, UT, USA) containing 10% FBS, 100 U/ml penicillin, and 0.1 mg/ml streptomycin at 37 °C with 98% humidity and 5% CO_2 in air. Cells were harvested by trypsinization at approximately 80–90% confluence.

2.4. Cell alignment and orientation analysis

HUVECs were seeded on the silk films at a density of 8000 cells/cm^2 as previously described [29]. Briefly, to characterize the alignment and orientation, microscopic images were acquired on the second day with an inverted fluorescence microscope (IX-71, Olympus). The orientations of the HUVECs were analyzed with ImagePro 6 software. The orientation angle was determined by measuring the angle differences between the longest axis within the cell borders and the orientation of gratings. Over one day in culture, the angle difference between the longest axis of the cell boundary and the groove direction was measured from all existing cells shown in 10× microscopic images. Cells were considered aligned with gratings when this angle was between 0 and 10°. Cell elongation is defined as the ratio between the length and breadth of each cell. Cells were considered elongated if this factor was higher than 1.3.

2.5. Cell proliferation

HUVECs were plated at a density of 10,000 cells/cm^2. One day after plating, cells on each pattern were imaged and counted at 10× magnification using an inverted microscope (IX-71, Olympus). Cells were cultured for 3 days at which time the cells were imaged using an inverted microscope (IX-71, Olympus). Cell counts were obtained from images using ImagePro 6 software. Each experiment was repeated for at least three times.