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The outermost surface properties of silk fibroin films reflect ethanol-treatment conditions used in biomaterial preparation

Dohiko Terada [a], Yoshiyuki Yokoyama [a], Shinya Hattori [b], Hisatoshi Kobayashi [b], Yasushi Tamada [c,1,*]

[a] Toyama Industrial Technology Center, Futagami-cho 150, Takaoka, Toyama, Japan
[b] National Institute for Materials Science, Sengen 1-2-1, Tsukuba, Ibaraki, Japan
[c] National Institute of Agrobiological Sciences, Ohwashi 1-2, Tsukuba, Ibaraki, Japan

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ABSTRACT

Silk fibroin has attracted interest as a biomaterial, given its many excellent properties. Cell attachment to silk substrates is usually weaker than to standard culture dishes, and cells cultured on silk films or hydrogels typically form spheroids and micro-aggregates. However, too little is known about the higher order structures and behavior of fibroin under different conditions to explain the features of silk fibroin as a culture substrate. For instance, different biomaterial surfaces, with distinct effects on cell culture, can be achieved by varying the conditions of crystallization by alcohol immersion. Here, we show that treatment of fibroin film with <80% ethanol results in a jelly-like, hydrated hydrogel as the outermost surface layer; fibroblasts preferably aggregate, rather than attach individually to such a hydrogel surface, and therefore aggregate into spheroids. In contrast, a fibroin film treated with >90% ethanol has a harder surface than the <80% ethanol-treated fibroin, to which individual cells prefer to attach (and then expand on the surface), rather than to aggregate. We discuss the influence of alcohol concentration on the surface properties, based on surface analysis of the films. The surface analysis involved assessment of static and dynamic contact angles, zeta potential, changes in crystallinity and microscopic morphology of electrospun fibers, and texture changes of the outermost surface at a nanometer-scale captured by a scanning probe microscope.

1. Introduction

Silk has historically had varied uses; for instance, silk threads have been utilized not only for producing textiles, but also as sutures. Recently, the utilization of silk has not been limited to its use as a thread. Silk can be processed into different shapes, such as films [1] sponges [2], nanofibers [3], and nanoparticles [4], and is being investigated as a new material in various fields. In particular, its various excellent material properties have encouraged many researchers to study it as a biomaterial [5–7].

Despite a long history of basic and practical silk research, the nature of silk has not yet been elucidated. For instance, silk fibroin films are known to show specific interaction with cells cultured thereon [8–11]. Cell attachment to silk substrates is usually weaker than to standard culture dishes [12], and cells cultured on silk films or hydrogels typically form spheroids and micro-aggregates. Recently, a characteristic, mobile behavior of fibroblasts on silk fibroin films was reported: marked cell mobility was observed on silk fibroin films, as compared to that on glass, a fibronectin-coated surface, and a typical cell culture plastic dish [13]. Furthermore, increased expression of genes encoding extracellular matrix proteins, which corresponds to mobility, was found in the former fibroblasts. Thus, these unique characteristics would be endowed by the surface properties of the fibroin films.

Regenerative silk fibroin materials, such as as-cast films and as-electrospun fibers, are amorphous and water-soluble. Usually, they are treated with organic reagents, such as alcohol, to prevent their solubilization in the cell culture medium. Servoli et al. showed that silk fibroin films could be modified by means of methanol treatment, thereby providing different surfaces for cell culture [14]. Their results showed that our current understanding of the primary structure of fibroin is insufficient to elucidate the features of silk fibroin as culture substrates, and it remains unclear why the initial attachment behavior and the mobility of cells cultured on silk fibroin surfaces are different from those cultured on conventional surfaces.

On the other hand, cell adhesion and proliferation are strictly affected by the physico-chemical nature of the substrate surface, such as wettability [15–18] and surface charges [19,20], in addition to surface roughness [21,22] and stiffness [23–25]. Cells prefer to adhere to surfaces with moderate wettability, on which the water contact angle is

Abbreviations: CI, crystallinity index; IR, infrared; PEO, poly(ethylene oxide); RO, reverse osmosis; SEM, scanning electron microscopy; SPM, scanning probe microscopy.

* Corresponding author at: Shinshu University, Tokida 3-15-1, Ueda, Nagano, Japan.
E-mail address: ytamada@shinshu-u.ac.jp (Y. Tamada).
[1] Present address: Shinshu University, Faculty of Textile Science and Technology, Tokida 3-15-1, Ueda City, Nagano 386-8567, Japan.

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around 60–70°. On more hydrophilic and more hydrophobic surfaces, cell adhesion and proliferation tend to decrease, while surfaces with a higher charge allow more cell adhesion. However, to date, the changes caused by various alcohol treatments to the physico-chemical properties of a fibroin surface, and the effect this has on cell adhesion in particular, have not been investigated in depth. In other words, the influence of alcohol treatments on silk properties has been studied using bulky samples, cast films with a thickness of several to tens of micrometers; consequently, the structural modification of the outermost surface, at a depth of tens of nanometer, remains unknown [26,27]. Accordingly, it is not known whether silk substrates that have been prepared by various methods may have different effects on the cells cultured thereon.

In this paper, we studied the physico-chemical properties of fibroin surfaces that had been prepared under various conditions, and evaluated the influence of the treatment conditions on the structural changes, surface textures, and morphology of the cells cultured thereon. We also discuss the changes to the state of the outermost surface of the fibroin materials caused by various alcohol treatments and the influence thereof on cell morphology. Our results provide greater understanding of silk fibroin, and yield new insights into its application as a biomaterial.

2. Materials and methods

2.1. Materials

Degummed silk threads were kindly donated by Dr. Chiyuki Takabayashi at the National Institute of Agrobiological Sciences, Okaya, Japan. Guaranteed-grade lithium bromide, ethanol, methanol, and Giemsa stain solution were purchased from Wako Pure Chemical Industries, Ltd., Osaka, Japan. Dialysis tubing (MWCO; 5000–8000), poly(ethylene oxide), PEO (Mw = 900,000 g/mol), and a BCA protein assay kit were purchased from Funakoshi, Tokyo, Japan, Sigma-Aldrich, MO, USA, and Takara Bio, Inc., Shiga, Japan, respectively. Fibroblasts (NIH3T3) were purchased from RIKEN, Japan. Eagle MEM was obtained from NISSUI Pharmaceutical Co., Ltd., Japan. Fetal bovine serum and antibiotics were purchased from Gibco, Life Technologies Corp., USA.

2.2. Sample preparation

We prepared an aqueous solution of fibroin as previously reported [28]. Degummed silk fibers were dissolved in 9 M lithium bromide solution, with stirring for 6 h, at room temperature. The concentration of silk was adjusted to about 0.06 g/ml. The prepared solution was packed in dialysis tubes and dialyzed against reverse osmosis (RO) water. The RO water was replaced twice a day for three days. Then, the dialysis tubes were placed in a desiccator to concentrate the fibroin solution. The concentration was confirmed using the BCA kit and was adjusted to the prescribed concentrations for each experiment by diluting if necessary.

We prepared samples using two different methods: spin coating and electrospinning. Films prepared by spin coating were supplied for static and dynamic contact angle measurements, zeta potential measurement, scanning electron microscope (SEM) observation, scanning probe microscope (SPM) observation, and cell culture tests. Electrospun fibers were evaluated by infrared (IR) spectroscopy measurement and SEM observation. In this paper, the non-woven mat of electrospun fibers is not described as a film; rather, “film” refers to the spin coated film throughout this paper.

A 1% fibroin solution was placed on a glass substrate and the glass was spun initially at 1500 rpm for 5 s, and then at 3000 rpm for 30 s. For the cell culture tests, a circular cover glass ($\varphi = 15$ mm) was used. For contact angle measurement, zeta potential measurement, and scanning probe microscope observation, a standard slide glass was used. Coated glasses were dried overnight in an incubator at 25 °C.

For electrospinning, the concentration of aqueous fibroin solution was adjusted to 80 mg/ml. A PEO solution of 5 wt.% was added to the fibroin solution at a volume ratio of 4:1. The mixture was stirred very gently in a refrigerator, as described elsewhere [29]. Electrospinning was performed using a commercial electrospinning system (NANON, MECC Ltd., Fukuoka, Japan). A voltage of 12 kV was applied to a 25-gauge stainless needle, as spinneret, from which a spinning solution was extruded at 0.3 ml/h. The distance between the needle tip and a grounded flat corrector was fixed at 120 mm. Electrospinning was carried out at room temperature and a relative humidity of less than 30%.

2.3. Ethanol treatment

As-prepared films and as-spun fibers were immersed in ethanol solutions with various concentrations, viz., 70, 80, and 90% (volume percentage), at 25 °C for 24 h. After the treatment time, the films were rinsed in RO water and stored in RO water until required for each experiment. These treated samples were supplied for contact angle measurements, zeta potential measurement, SEM and SPM observations, and cell culture tests.

To evaluate the influence of the ethanol concentration and treatment time on the crystallinity of electrospun fibers, fibers were dried rapidly by exposure to an air blast immediately after removal from the ethanol baths filled with 70, 80, and 90% ethanol solutions, at the predetermined time-points, namely, 10 min, 1 h, 3 h, 6 h, and 24 h. This drying process was employed to prevent the crystallinity of the treated fibers from rising further.

2.4. Static contact angle measurement

For the static contact angle measurement, spin-coated films treated with 70, 80, and 90% ethanol solutions for 24 h were used. As shown in Section 3.3, the crystallinity nearly rose to equilibrium after 24 h of ethanol treatment; a treatment time of 24 h was therefore adopted for preparing samples for static and dynamic contact angle measurements. The spin-coated glass was lightly exposed to a dry nitrogen breeze to remove water from the surface. The static contact angle was measured using the sessile drop method. A 4-μl drop of water was placed slowly onto the coated surface and the shape of the water drop was captured on a digital video camera by using a contact-angle measurement system (FTÅ188, First Ten Ångstroms Inc., USA). The time profile of change in contact angles was plotted and the values extrapolated to 0 s from the first order approximation line calculated from a linearly decreasing range. At least 30 measurements were made for each treatment condition, and the results statistically analyzed with Student's *t*-test. p-Values of <0.05 were considered significant.

2.5. Dynamic contact angle measurement

For the dynamic contact angle measurement, spin-coated films treated with 70, 80, and 90% ethanol solutions for 24 h were used. The spin-coated glass was exposed to a light dry nitrogen breeze to blow off dripping water from the surface. A 10-μl drop of water was placed onto a coated surface and allowed to settle for 30 s. Then, the coated glass was tilted at a rate of 1.146°/s to a vertical position, and maintained in this position for 30 s. The shape of the water drop imaged using a digital video camera. Time profiles of advancing and reducing contact angles were plotted by processing the digital video. Hysteresis, namely the difference between advancing and reducing contact angles, was obtained from a steadily declining region in the time profile. Five measurements were obtained for each treatment condition.