In Vitro Biocompatibility of Electrospun Silk Fibroin Mats with Schwann Cells

Shanqin Xu,[1] Xiaoli Yan,[2] Yahong Zhao,[2] Wei Wang,[2] Yumin Yang[2]

[1]*School of Textile and Clothing, Nantong University, 19 Qixiu Road, Nantong, JS 226001, China*
[2]*Jiangsu Key Laboratory of Neuroregeneration, Nantong University, 19 Qixiu Road, Nantong, JS 226001, China*

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ABSTRACT: In this study, electrospinning was used to fabricate silk-fibroin (SF)-based mats, which served as substrates for the culturing of rat Schwann cells. Microscopic observation and physical parameter measurements revealed that the electrospun SF mats had a nanofibrous structure with favorable physical properties. Fourier transform infrared analysis provided chemical characterization of the molecular confirmation of the SF proteins in the mats. The morphology and immunocytochemistry showed that the mats supported the survival and growth of the cultured Schwann cells, and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide analysis indicated that the electrospun SF mat extract had no cytotoxic effects on Schwann cell proliferation. Collectively, all of the results suggest that the electrospun SF mats might become a candidate scaffold for tissue-engineered nerve grafts to promote peripheral nerve regeneration. © 2010 Wiley Periodicals, Inc. J Appl Polym Sci 119: 3490–3494, 2011

Key words: biocompatibility biomaterials; nanofiber

INTRODUCTION

For tissue engineering applications, electrospun fibrous scaffolds (matrices) have attracted research interest in recent years because they have good surface properties suitable for cell attachment and growth and, thus, raise the possibility of mimicking the extracellular matrix architecture, which would be favorable for tissue regeneration.[1]

Peripheral nerve injuries are a commonly encountered clinical problem. Surgical repair with nerve grafts is usually required for the management of transection injuries.[2] To develop tissue engineering nerve grafts as a promising alternative to classical autologous nerve grafts, a wide range of scaffolds with different structures have been prepared with various biomaterials. Among naturally derived biomaterials, silk fibroin (SF) has proven to possess favorable physicochemical and biological properties and has, thus, found increasing applications in the fabrication of tissue engineering scaffolds.[3] Although electrospun SF scaffolds have been explored for various areas of tissue engineering, to our knowledge, they have not been extensively studied for nerve tissue engineering applications.[4]

In this study, electrospinning was used to fabricate SF mats. We characterized the SF mats with scanning electron microscopy (SEM) and Fourier transform infrared (FTIR), and we measured the physical parameters, including thickness, fibers average diameter, pore size, and porosity, of the mats. Then, we cultured rat Schwann cells on the SF mats. The survival and growth of Schwann cells after culturing were investigated by morphology, immunocytochemistry, and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) analysis, with an attempt to evaluate the *in vitro* biocompatibility of the electrospun SF mats with Schwann cells.

EXPERIMENTAL

Preparation of the electrospun SF mats

Raw silk fibers (from *Bombyx mori* cocoons) were bought from Xinyuan Sericulture Co. (Hai'an, Jiangsu, China). The SF aqueous solution was prepared as previously described.[5] Degummed SF fibers were first dissolved in a tertiary solvent system of $CaCl_2/H_2O/EtOH$ solution (molar ratio = 1 : 8 : 2) at 80°C for 1 h and then dialyzed against distilled water in a cellulose tube (molecular cutoff = 12,000–14,000) at room temperature for 3 days. The final SF

Shanqin Xu and Xiaoli Yan contributed equally to this work.

Correspondence to: Y. Yang (yangym@ntu.edu.cn).

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aqueous solution was spread on stainless steel dishes to generate the air-dried regenerated SF membranes, which were further dissolved in 98% (w/w) formic acid to obtain a 13% (wt/wt) SF spinning solution.

The homemade electrospinning setup used in this study was made up of a high-voltage supplier, a capillary needle serving as an anode, and a grounded collector serving as a cathode. A high electric potential (20 kV) was applied to the anode with a needle tip (i.d. = 0.9 mm), into which the droplet of SF spinning solution was loaded. The resulting electrospun mat was formed on the stainless steel meshes. The distance between the needle tip and the steel collector ranged from 7 to 13 cm. A constant volume flow rate (0.3 mL/h) was maintained by a syringe pump, which kept the SF spinning solution in the needle.

After the electrospun SF mats were removed from the stainless steel meshes, they were inserted into 100% alcohol for 10 min to induce conformational transition; they were then washed with distilled water at 37°C for 72 h to remove residual formic acid. The treated mats had to be sterilized with a hyperbaric method and were then equilibrated in Dulbecco's modified Eagle's medium (DMEM)/F12 culture medium (Gibco, Carlsbad, CA) for 30 min before use.

Preparation of the electrospun SF mat extract

After sterilization, the electrospun SF mats were placed in plain medium consisting of DMEM/F12 (Gibco) supplemented with 10% fetal bovine serum (FBS), according to the ratio of a 6-cm^2 area of the electrospun SF mats to a 1-mL volume of plain medium. The extraction procedure was conducted at 37°C for 72 h. The obtained mat extract had to be used within a 24-h period.

Characterization and measurements of the electrospun SF mats

For morphological observation, the electrospun SF mats were coated with gold with a JFC-1100 unit (JEOL, Inc., Tokyo, Japan) before they were examined under a Philips XL-30 scanning electron microscope (Eindhoven, The Netherlands).

A liquid displacement method was used to measure the porosity of the electrospun SF mats.[6] Hexane was used as a displacement liquid because it permeated through the electrospun SF mats without swelling or shrinking the matrix. The electrospun SF mats with constant mass were immersed in a known volume of hexane (V_1) in a graduated cylinder for 5 min. The total volume of hexane plus the hexane-impregnated electrospun SF mats was recorded as V_2. The hexane-impregnated electrospun SF mats were then removed from the cylinder, and the residual hexane volume was recorded as V_3. The total volume of the electrospun SF mats was calculated as follows:

$$\text{Total volume} = (V_2 - V_1) + (V_1 - V_3) = V_2 - V_3$$

where $V_2 - V_1$ is the volume of the electrospun SF mats and $V_1 - V_3$ is the volume of hexane within the electrospun SF mats. The porosity of the electrospun SF mats was determined with the following formula:

$$\text{Porosity}(\%) = (V_1 - V_3)/(V_2 - V_3)$$

The average fiber diameter and average pore size were obtained by analysis of the SEM images with a custom code image analysis program (Adobe Photoshop 7.0) as described previously.[7]

For chemical characterization, the mats were ground and pelleted with dried KBr; this was followed by analysis with a model Nexus 870 FTIR spectrophotometer (Nicolet Instruments Co., Madison, WI) in the range 400–4000 cm^{-1}.

Cell culture

Neonatal Sprague–Dawley rats (1–2 day old) were provided by the experimental animal center of Nantong University. The rat Schwann cells were harvested as previously described.[8] After 7 days of incubation, the subcultured Schwann cells were planted onto the substrate made up of electrospun SF mats, which had been placed onto a 24-well culture plate and soaked in DMEM/F12 culture medium supplemented with 15% FBS to allow for further cell culturing and various assays.

Light and electron microscopy

After the rat Schwann cells were cultured on the electrospun SF mats for designated times, they were observed under an inverted microscope (Olympus, Tokyo, Japan) or under SEM, for which the pretreatment of the samples was conducted as previously described.

Immunocytochemistry

After they were cultured on the electrospun SF mats, the rat Schwann cells were fixed and subjected to immunocytochemistry. Mouse anti-S-100 antibody (1:1200 dilutions, Sigma, St. Louis, MO), as a primary antibody, and fluorescein isothiocyanate labeled goat anti-mouse immunoglobulin G (1:400 dilutions, Sigma), as a secondary antibody, were

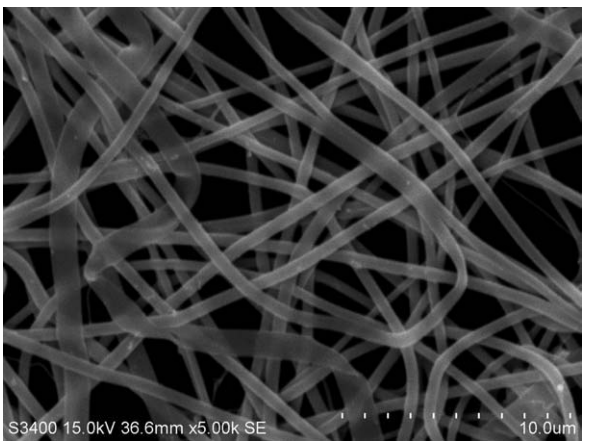

Figure 1 SEM micrograph of the electrospun SF mats.

used. The cells were also stained with 5 μg/mL Hoeechst 33342 (Sigma). Finally, the cell samples were observed under a confocal laser scanning microscope.

MTT assay

After incubation in plain DMEM/F12 medium supplemented with 15% FBS or in the mat extract, the cell viability of the Schwann cells was assessed by MTT assay as previously described.[9] The data were expressed as Means ± Standard deviations and were analyzed by a one-way analysis of variance. Statistical significance was accepted at the 0.05 confidence level.

RESULTS AND DISCUSSION

Morphological observation

SEM showed that the electrospun SF mats were composed of a large number of randomly oriented fibers, which were interconnected to create a three-dimensional porous network structure (Fig. 1). Although a few fibers were over 1 μm in diameter, the diameter of most fibers ranged from 200 to 800 nm. The mat fibers possessed circular cross sections and smooth surfaces. Therefore, the morphological observation confirmed the nanofibrous structure of the mats.

According to the statistical analysis of the fiber diameter distribution, the average fiber diameter was 420 ± 110 nm. The other physical parameters, such as the thickness, pore diameter, and porosity of the electrospun SF mats, are listed in Table I. The pore size (pore diameter) and porosity are measures for the permeability of tissue engineering scaffolds, and they should be appropriated for material exchange between inside and outside the scaffold and for blocking the ingrowth of tissue cells from the outside. The physical parameters of the electrospun SF mats, together with the nanofibrous structure of the mats, ensured that the mats would mimic the natural extracellular matrix architecture and, thereby, meet the requirements for peripheral nerve regeneration.

TABLE I
Some Physical Parameters of the Electrospun SF Mats

Average diameter (nm)	Thickness (μm)	Pore diameter (μm)	Porosity (%)
420 ± 110	1.6 ± 0.2	18 ± 2	65–68

Chemical characterization

FTIR spectroscopy was used to investigate the molecular conformations of the SF proteins.[10] As is known, in the FTIR spectrum of SF-based matrices, two absorption bands at 1660.0–1650.0 cm^{-1} (C = 0 stretching in amide I) and 1545.0–1535.0 cm^{-1} (N—H deformation in amide II) are associated with the random coil conformation, whereas two absorption bands at 1640.0–1620.0 cm^{-1} (amide I) and 1525.0–1515.0 cm^{-1} (amide II) are associated with the β-sheet conformation.[11]

Figure 2 provides a comparison of the FTIR spectra among three different SF-based matrices. The three FTIR curves were similar to each other in shape; this confirmed an identical chemical composition for them. However, slight differences in the peak wave number were noted for three FTIR curves because of conformation transitions that took place during the fabrication process of the electrospun SF

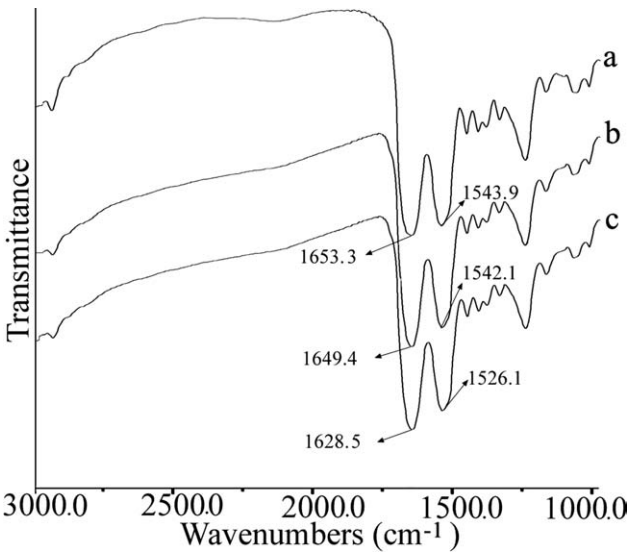

Figure 2 FTIR spectra of the (a) air-dried SF membranes, (b) untreated electrospun SF mats, and (c) alcohol-treated electrospun SF mats.

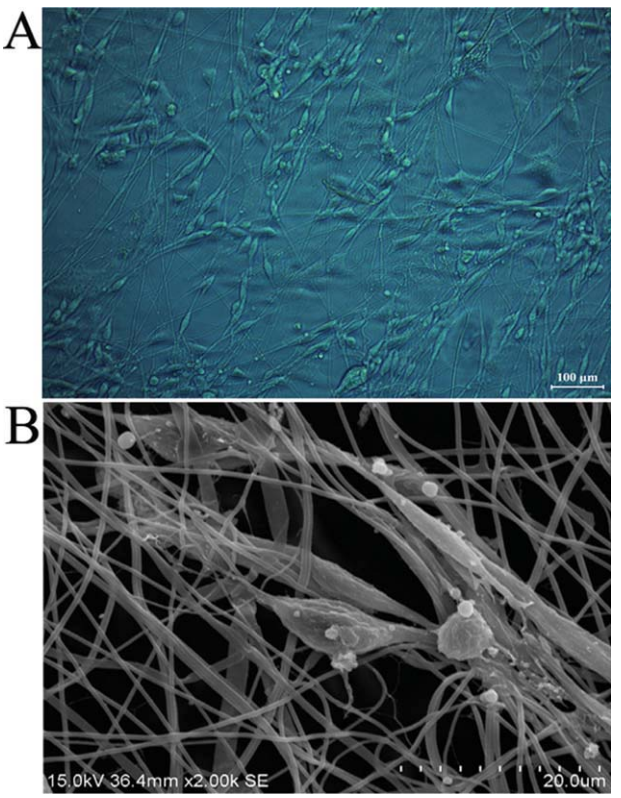

Figure 3 (A) Light and (B) SEM micrographs of the Schwann cells cultured on the electrospun SF mats for 7 days. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

mats.[12] Air-dried regenerated SF membranes exhibited a random-coil-predominant conformation in terms of the two peaks at 1653.3 (amide I) and 1543.9 cm^{-1} (amide II) in the FTIR curve [Fig. 2(a)]; the conformational transition from the random coils to β sheets was related to the untreated electrospun SF mats, as evidenced by the two peaks at 1649.4 (amide I) and 1542.2 cm^{-1} (amide II) in the FTIR curve [Fig. 2(b)]. In contrast, the alcohol-treated electrospun SF mats displayed a water-insoluble β-sheet-enriched confirmation, as shown by the two peaks at 1628.5 (amide I) and 1526.1 cm^{-1} (amide II) in the FTIR curve [Fig. 2(c)].

The chemical characterization by FTIR analysis indicated that posttreatment by alcohol was an important procedure in making electrospun SF mats suitable for tissue engineering applications because alcohol posttreatment induced crystallization and conformational transition of the SF proteins and enhanced the water insolubility of the electrospun SF mats.

Neural cell affinity of the electrospun SF mats

Schwann cells are primary structural and functional neural cells, which play a crucial role in peripheral nerve regeneration through the production of growth factors and the excretion of extracellular matrix.[13] After peripheral nerves are damaged, Schwann cells are involved in Wallerian degeneration and the formation of Büngner bands. They are capable of forming myelin sheaths around axons and of guiding axon growth to establish a precise enervation of targets.[14] The ideal scaffold for the preparation of tissue engineering nerve grafts must be beneficial for the attachment, expansion, and migration of Schwann cells and must create an appropriate microenvironment for axon growth and regeneration.

We previously found that SF fibers have a good biocompatibility with Schwann cells and peripheral nerve tissues.[8] Compared with SF fibers, however, the physicochemical properties of the electrospun SF mats experienced great changes in the fabrication process. To explore whether processing with electrospinning and chemical posttreatment induced additional negative effects on neural cells, we evaluated the biocompatibility of the electrospun SF mats with Schwann cells *in vitro.*

After the Schwann cells were cultured on the electrospun SF mat for 7 days, light microscopy revealed that the Schwann cells connected to each other and showed adhesion, survival, and migration on the electrospun SF mat [Fig. 3(A)]. SEM microscopy revealed that the Schwann cells migrated along the surface of the mat and even penetrated into the mat pores [Fig. 3(B)] because of the high porosity of the mat.

S-100 is a common cell marker for Schwann cells. Anti-S-100 immunocytochemistry indicated that a number of S-100 positive cells, identified as Schwann cells, had normal cell morphology and closely attached to the electrospun SF mat, as evidenced by strong red fluorescence emission. In addition, the

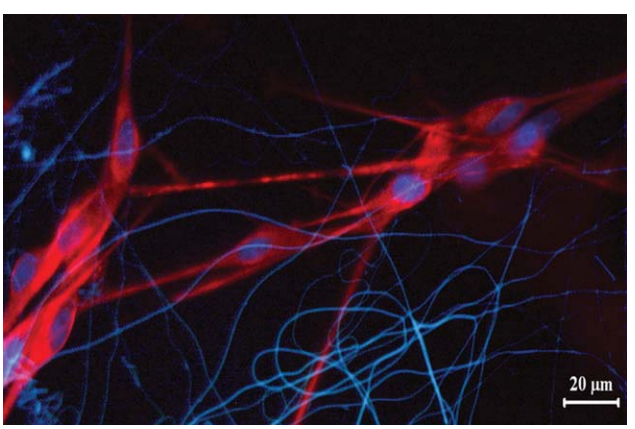

Figure 4 Immunocytochemistry with anti S-100 and Hoechst staining for the Schwann cells after 7-day culturing on the electrospun SF mats. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

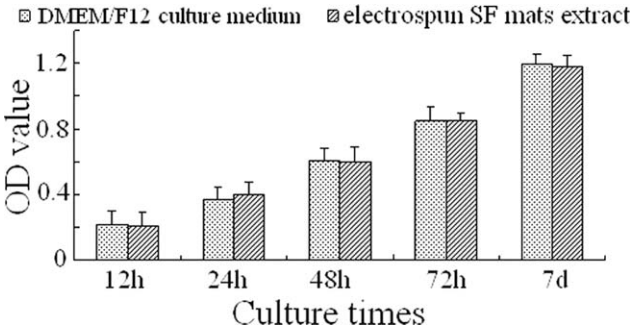

Figure 5 Changes in the cell viability of the Schwann cells, expressed by the optical density (OD), after the Schwann cells were cultured in plain DMEM/F12 (nutrient mixture product supplied by Gibco) medium or on the electrospun SF mats extracted for 12 h, 24 h, 48 h, 72 h, or 7 days. Analysis of variance was used for statistical analysis. All p values were larger than 0.05.

nuclei of the cells were shown by Hoechst staining (violet; Fig. 4).

The cytotoxicity test was very important for evaluating the biocompatibility of the electrospun SF mats with peripheral neural cells. MTT, as a quantitative measure of cell viability, was used to test the possible cytotoxic effects of the electrospun SF mats according to ISO 10993-5. The data indicated that the cell viability of the Schwann cells cultured in the electrospun SF mat extract was not significantly different from those cultured in the plain DMEM/F12 medium at different observation time points (Fig. 5). This analysis suggested that the electrospun SF mats, in an extract form, had no cytotoxic effects on the proliferation of the Schwann cells.

CONCLUSIONS

In this study, we fabricated SF mats by an electrospinning technique. The electrospun mats were found to possess a nanofibrous structure with favorable physical parameters. The electrospun SF mats supported the survival and growth of the Schwann cells cultured onto the mats, and the mat extract showed no significant cytotoxic effects on the proliferation of the Schwann cells. These results suggest that electrospun SF mats might become a candidate scaffold for tissue engineered nerve grafts to promote peripheral nerve regeneration.

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