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Regenerated Silk Fibroin Nanofibrous Matrices Treated with 75% Ethanol Vapor for Tissue-Engineering Applications

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Abstract

As an excellent biocompatible and biodegradable protein polymer, silk fibroin (SF) has found wide applications, particularly serving as therapeutic agent for tissue-engineering applications, on which both post-spin treatment and sterilization processing are crucial to drug-loaded matrices. To find a safe, effective and appropriate post-spin treatment and sterilization approach for drug-loaded biomaterial matrices is one of the major problems in the field of tissue engineering at present. In this work, a simple, safe and effective approach skillfully integrating post-spin treatment with sterilization processing was developed to drug-loaded SF nanofibrous matrices. Electrospun SF nanofibrous matrices from its aqueous solution were post-treated with 75% ethanol vapor. ^{13}C-NMR and WAXD analysis demonstrated that such post-spin treatment rendered the structure of SF nanofibrous matrices transform from the silk I form to the silk II form. Furthermore, biological assays suggested that as-treated SF nanofibrous matrices significantly promoted the development of murine connective tissue fibroblasts. Skillfully integrated with novel sterilization processing, 75% ethanol vapor treatment could be a potential approach to designing and fabricating diverse drug-loaded SF nanofibrous matrices serving as therapeutic agents for tissue-engineering applications in that it can effectively protect the drug from losing compared with traditional post-spin treatment and sterilization processing.

Keywords

Electrospinning, silk fibroin, nanofiber, post-spin treatment, sterilization processing, green processing

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1. Introduction

Silk fibroin (SF) is a naturally occurring protein polymer with abundant resource which has been clinically explored as sutures for centuries [1]. SF exhibits diverse impressive properties, such as excellent biocompatibility and biodegradability, remarkable oxygen and water vapor permeability, low inflammatory response, and so on [2–9]. Thus far, SF has been extensively explored for varied applications with different processing approaches, including lyophilizing [10, 11], casting [12] and electrospinning [13]. Among these processing approaches, the electrospinning of SF has already been reported in the literature and it has been demonstrated that SF nanofibrous matrices have versatile advantages and potentials for biomedical applications [14–17]. Specifically, electrospun SF nanofibrous matrices have resemblance with extracellular matrix (ECM) in fibrillar structure and composition, which could significantly support and promote the development of cell in the field of tissue engineering and regenerative medicine [18–22].

Although the previous studies have demonstrated the advantages of electrospun SF nanofibrous matrices [14–17], varied organic solvents are involved, which are potentially toxic to human body and unfriendly to environment. Few studies concerning green processing of SF (i.e., no harmful substance is involved throughout the whole processing) have been reported. Interestingly, electrospun nanofibers from aqueous SF solution have been successfully fabricated. However, the structure of SF nanofibrous matrices was converted, typically adopting the traditional treatment based on methanol [6, 13, 18], which is also potentially toxic and unfriendly to organism and environment. To date, varied post-spin treatment approaches to SF nanofibrous matrices have been developed, including soaking in pure ethanol [23] and treatment with water vapor [24]. However, the soaking approach has great limitations for the application of SF as diverse drug-loaded therapeutic agents in that some drugs could be lost at different amounts with this treatment. For water vapor treatment, although it is not harmful to organism and environment, it is still difficult to find an appropriate sterilization process for drug-loaded SF nanofibrous matrices for tissue-engineering applications.

In the present work, we aimed to find a simple, safe and effective post-spin treatment and sterilization processing for electrospun SF nanofibers, especially drug-loaded SF nanofibrous matrices, which could be beneficial to designing and fabricating diverse drug-loaded SF nanofibrous matrices serving as therapeutic agents for tissue-engineering applications.

2. Materials and Methods

2.1. Materials

Cocoons of *Bombyx mori* silkworm were purchased from Jiaxing Silk. Murine connective tissue fibroblasts (L929) were obtained from Institute of Biochemistry and Cell Biology (Chinese Academy of Sciences). All other reagents were of analytical

grade or higher. Ultrapure water (Rephile Shanghai Bioscience & Technology) was used throughout this study.

2.2. Preparation of Regenerated SF (RSF)

Cocoons were boiled in an aqueous solution of 0.5% (w/w) Na_2CO_3 for three times 30 min and then sufficiently rinsed with warm ultrapure water to remove sericin proteins. The degummed silk, dried overnight at 40°C, was dissolved in a ternary solvent system of $CaCl_2/H_2O/CH_3CH_2OH$ solution (1:8:2 in molar ratio) at 65°C until a clear solution was obtained. The silk solution was dialyzed against an excess of ultrapure water with cellulose tube (molecular weight cutoff 14 000, Sigma-Aldrich) at room temperature for 3 days, refreshing the water phase with 4 times each day. After filtering the silk solution, RSF sponges were obtained by lyophilization.

2.3. Electrospinning

2.3.1. Fabrication of SF Nanofibrous Matrices

According to the previous study [18], RSF sponge was dissolved into ultrapure water and constantly incubated under slight agitation with a magnetic stirrer for 8 h at ambient temperature to obtain a clear RSF solution (30 wt%). After storage in a refrigerator at 4°C for 1 h, the uniform solution was filled into a 2.5 ml plastic syringe capped with a 7-gauge blunt needle (ID = 0.21 mm). The syringe was located in syringe pump (Model 789100C, Cole-Parmer Instrument) with a fixed rate of 0.3 ml/h. A voltage of 20 kV generated with a high voltage power supply (BGG6-358, Bmeico) was applied to the needle, a ground aluminum foil as collector, and the distance between needle tip and collector was 20 cm. The electrospinning experiments were performed at room temperature. In order to improve the water-resistant property, the samples were treated with 75% ethanol vapor at room temperature for 24 h and then dried in a fume hood at ambient temperature for 24 h.

2.3.2. The Preparation of SF/Rhodamine B Nanofibrous Matrices

Briefly, RSF sponges and rhodamine B powder (0.5 wt% based on the weight of RSF sponges) were dissolved in ultrapure water to obtain a 30 wt% RSF solution. The rhodamine B-loaded RSF nanofibrous matrices were fabricated under the same conditions by the electrospinning of the RSF aqueous solution mentioned above.

2.4. Post-Spin Processing

2.4.1. Traditional Post-Spin Treatment and Sterilization

Rhodamine B/SF composite nanofibrous matrices (5 mg) were immersed in 5 ml 90% aqueous methanol solution for 1 h to induce a structure change in the nanofibrous matrices. Following drying, nanofibrous matrices were sterilized in 5 ml 70% aqueous ethanol solution for 1 h. The amount of rhodamine B lost in aqueous methanol and ethanol solution was determined using an UV-Vis spectrophotometer (WFZ UV-2102, Unique Technology) at an optical wavelength of 550 nm against the predetermined calibration curve.

2.4.2. Post-Spin Treatment and Sterilization with 75% Ethanol Vapor

Rhodamine B/SF nanofibrous matrices (5 mg) were treated with 75% ethanol vapor at room temperature for 24 h, and were dried in a fume hood at ambient temperature for 2 h. To quantify the lost rhodamine B, as-treated nanofibrous matrices were sufficiently dissolved in 5 ml of aqueous LiBr (9 M) at 50°C, and the amount of rhodamine B was determined with the same procedure as above.

Meanwhile, to quantify the rhodamine B loaded into 5 mg nanofibrous matrices, composite nanofibrous matrices without further treatment were sufficiently dissolved in 5 ml aqueous LiBr (9 M) at 50°C, and the amount of rhodamine B was determined with the same procedure as above.

2.5. Characterization of Nanofibrous Matrices

The morphology of electrospun SF nanofibers was observed using scanning electronic microscope (SEM; JSM-5600, Japan) at an accelerated voltage of 10 kV. The samples were sputtered with a thick gold film of 10 nm prior to the measurement. The average fiber diameter was determined from over 60 random measurements on a typical SEM image using Image J software (National Institutes of Health).

^{13}C-NMR experiments were performed at room temperature using a NMR spectrometer (Bruker AV400) with a ^{13}C resonance frequency of 100 MHz, contact time of 1.0 ms and pulse delay time of 4.0 s.

Wide-angle X-ray diffraction (WAXD) experiments at ambient temperature were carried out using an X-ray diffractometer (Riga Ku) within the scanning region of 2θ (5–60°), with Cu K_α radiation ($\lambda = 1.5418$ Å) at 40 kV and 40 mA.

2.6. Cell Viability Assay

In this work, L929 cells were used to investigate the effects of SF nanofibrous matrices treated with 75% ethanol vapor on the cell viability *in vitro*. L929 cells were maintained in RPMI 1640 (Gibco) supplemented with 10% fetal bovine serum (FBS) (Gibco). The culture was incubated in humidified atmosphere incubator containing 5% CO_2 at 37°C.

SF nanofibrous matrices were deposited on circular glass cover slips (14 mm in diameter) for cell study. As-prepared SF nanofibrous matrices were converted from the silk I to the silk II form under the atmosphere of 75% ethanol vapor for 24 h. The 75% ethanol vapor treated nanofibrous matrices were placed in a 24-well cell-culture plate with no further sterilization for cell viability assay. With autoclaved steel rings on the top of nanofibrous matrices, they were completely immersed in medium and the total volume of medium was 400 μl per well during the assay.

The relative cell viability of SF nanofibrous matrices with 75% ethanol vapor treatment was assessed using the 3-[4,5-dimehyl-2-thiazolyl]-2,5-diphenyl tetrazolium bromide (MTT, Sigma) viability assay, hematoxylin and eosin (HE) double staining method against L929 cells and SEM. L929 cells were directly plated on SF nanofibrous matrices ($n = 4$) with glass cover slips and tissue culture plates TCPs) as controls in the 24-well culture plate at a density of 1.5×10^4 cells/well

and medium was replaced every 3 days. MTT assay: at day 1, day 3, day 5 and day 7, medium was aspirated and each well was supplemented with 360 µl serum-free Dulbecco's Modified Eagle's Medium (DMEM, Gibco) and 40 µl MTT assay stock solution (5 mg/ml in phosphate-buffered saline, PBS). After incubating the cells for 4 h, the medium was thoroughly removed and 400 µl dimethylsulfoxide (DMSO) was added to each well. Along with formazan crystals being sufficiently dissolved, 100 µl of solution was taken into a 96-well plate and the absorbance was determined using an Enzyme-labeled Instrument (MK3, Thermo) at 570 nm.

Hematoxylin–eosin (HE) double staining: L929 cells were fixed in 4% paraformaldehyde for 20 min and rinsed with ultrapure water twice, 3 days following incubation. Then, L929 cells were stained in hematoxylin for 10 min, followed by washing with running tap water. After being dehydrated with 95% alcohol, L929 cells were stained with eosin for 2 min and dehydrated with 70% alcohol, then allowed to dry. The stained cells were imaged at $\times 400$ magnification using an Olympus phase-contrast microscope (Model 1×70).

To further observe the morphology of L929 cells, the cell–scaffold composite was scanned using SEM. The sample was extensively rinsed twice with PBS and fixed with 2.5% glutaraldehyde in PBS for 2 h at 4°C. After being sufficiently washed in PBS three times (20 min each wash), the sample was consecutively dehydrated in 30, 50, 70, 80, 90, 95 and 100% ethanol (7 min for each concentration) and allowed to air dry in a fume hood. Then, the samples were sputtered with gold prior to observation using an SEM at voltage of 15 kV.

2.7. Statistical Analysis

All experiments were performed at least three times, and all values were reported as the mean and standard deviation. Statistical comparisons were determined using one-way ANOVA carried out in SPSS software. In all assessments, the difference between two sets of data was considered statistically significant when $P < 0.05$.

3. Results and Discussion

3.1. Morphology of Electrospun Nanofibrous Matrices

At the concentration of 30 wt%, smooth nanofibers with an average fiber diameter of 713.9 ± 91.2 nm ($n \geqslant 60$) were obtained and the diameter distribution histogram of SF nanofibers showed that the fibers had a relatively homogeneous morphology (Fig. 1).

After soaking the matrices in PBS for 4 days, nanofibrous matrices with 75% ethanol vapor treatment had no significant change of morphology (Fig. 2), indicating that treated nanofibers had a remarkable water-resistant property.

3.2. ^{13}C-NMR Spectroscopy Analysis

^{13}C-NMR spectroscopy is a powerful analytical tool for verifying the major structure of *Bombyx mori* SF [25]. Previous studies had already documented that chem-

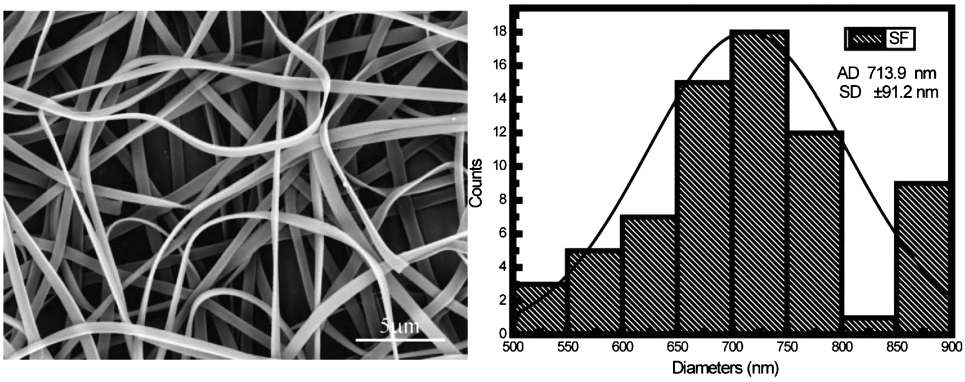

Figure 1. SEM micrograph and diameter distribution histogram of electrospun SF nanofibers at the concentration of 30 wt%.

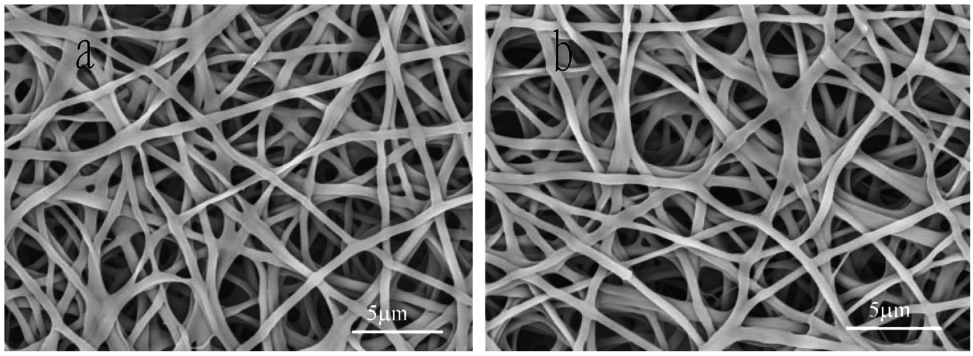

Figure 2. SEM micrograph of as-spun SF nanofibrous matrices treated with 75% ethanol vapor. (a) After treatment with 75% ethanol vapor for 24 h and (b) after treatment with 75% ethanol vapor and soaking in PBS for 4 days.

ical shifts of C_α (within the range of 48.0–50.0 ppm) and C_β (within the region of 18.5–20.5 ppm) in the Ala residue of SF molecule were assigned to silk II form (in which β-sheet conformation is favored), and within the range of 50.0–53.0 ppm and 14.5–17.5 ppm, respectively, represented silk I (which typically presents random coil conformation) [25]. In this study, the chemical shifts of Ala C_α and C_β were 50.5 ppm and 16.9 ppm (SF nanofibrous matrices), and 49.2 ppm and 19.4 ppm (electrospun SF nanofibrous matrices with treatment of 75% ethanol vapor), respectively (Fig. 3). The results indicated that 75% ethanol vapor treatment converted the structure of SF nanofibrous matrices from silk I into silk II form.

3.3. WAXD Spectra Analysis

The WAXD spectra of electrospun SF nanofibrous matrices and treated fibrous matrices are shown in Fig. 4. The peaks of electrospun SF matrices treated with 75% ethanol vapor were seen at 20.4 and 24.6°, in agreement with the characteristics of the silk II crystalline structure [18, 26, 27], whereas the WAXD pattern of electrospun SF matrices showed only a broad scattering central peak, at 21.9°, which

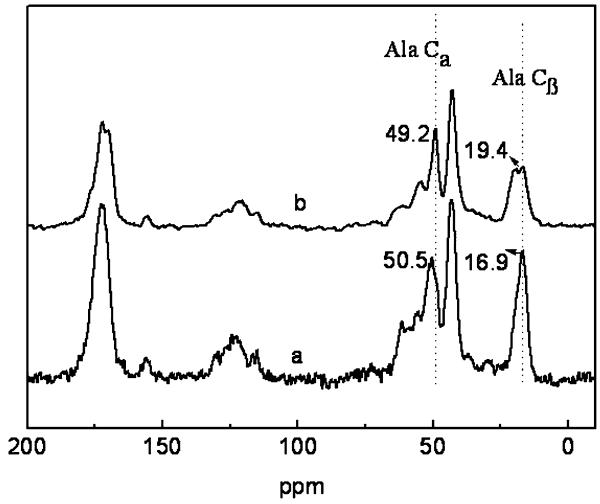

Figure 3. ^{13}C-NMR spectra of (a) SF nanofibrous matrices and (b) SF nanofibrous matrices with 75% ethanol vapor treatment.

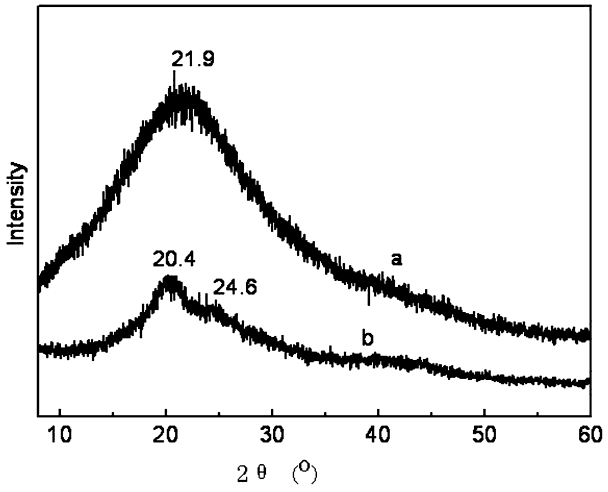

Figure 4. WAXD curves of (a) electrospun SF nanofibrous matrices and (b) electrospun SF nanofibrous matrices with treatment of 75% ethanol vapor.

corresponded to the characteristic of an amorphous state [18, 23, 26]. The results suggested that SF nanofibrous matrices treated with 75% ethanol vapor underwent a structure transformation from silk I into silk II, which was consistent with the results of ^{13}C-NMR spectroscopy analysis.

3.4. Cell Viability on SF Nanofibrous Matrices Treated with 75% Ethanol Vapor

The viability of L929 cells on the SF nanofibrous matrices treated with 75% ethanol vapor was first determined using the MTT assay, in which the cell number was indirectly quantified by absorbance. Cells at the same amount were seeded onto all

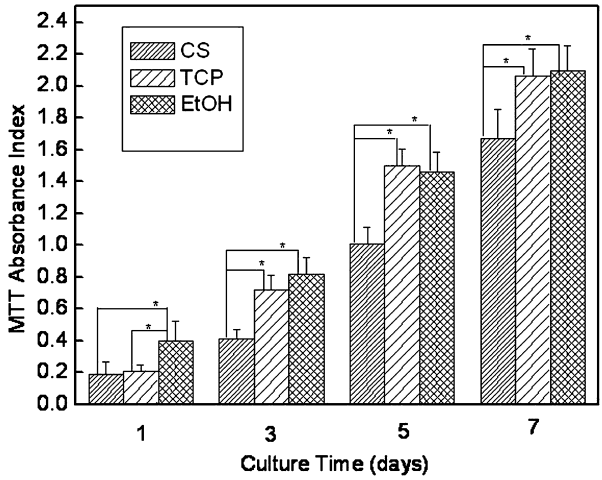

Figure 5. MTT analysis of L929 cells on electrospun SF nanofibrous matrices (CS, glass cover slips; TCP, tissue-culture plates; EtOH, nanofibrous matrices with 75% ethanol vapor treatment). Data are expressed as mean ± SD ($n = 4$). Statistical difference between groups is indicated ($^*P < 0.05$).

culture wells with or without matrices and glass cover slips. The results showed that the cell number on the SF nanofibrous matrices was higher than that on the glass cover slips ($P < 0.05$) on different days (Fig. 5). Moreover, the number of viable cells on glass cover slips, TCPs and SF nanofibrous matrices increased with increasing of culture time. It was obvious that after incubation for 1 day, the number of cells on SF nanofibrous matrices was higher than that on the TCPs, indicating that SF nanofibrous matrices were more beneficial to the initial cell adhesion as compared to TCPs. Additionally, the cell viability on the SF nanofibrous matrices was comparable to that on TCPs for any other day. These results implied that as-prepared SF nanofibrous matrices were beneficial to the adhesion and proliferation of L929 cells.

Cell behavior was also revealed with other approaches. The result of HE double staining (400×) (Fig. 6) showed that, after cultivation for 3 days, the cells on glass cover slips, TCPs and SF nanofibrous matrices typically took a spreading fusiform shape. Interestingly, cells and nanofibers appeared to assemble a cell–scaffold composite. To further investigate the interaction of cells and matrices, the composite was inspected using SEM at a high magnification (2000×) (Fig. 7). Obviously, cells on SF nanofibrous matrices had expanded filopodia to nanofibers around them, and exhibited a strong interaction with fibers. Furthermore, cells bridged mutually on the nanofibrous matrices, which could be beneficial to biochemical signal conduction of cells. These results indicated that as-treated SF nanofibrous matrices had a good cyto-compatibility.

3.5. Effects of Post-Spin Treatment and Sterilization Processing on Drugs

The results above suggested that 75% ethanol vapor treatment was not only an excellent post-spin treatment, but also a safe and effective sterilization processing. To

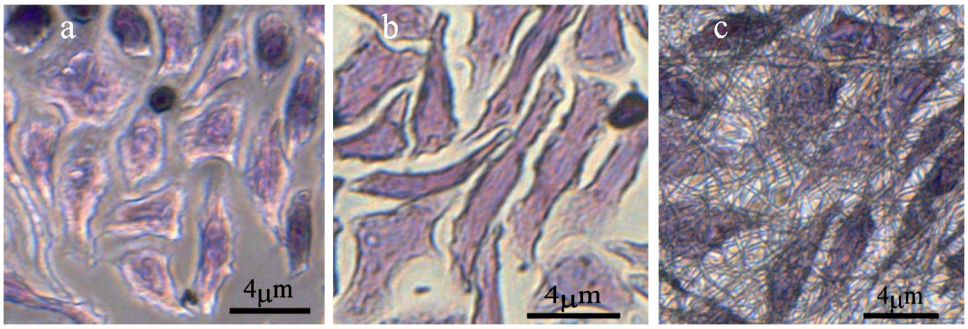

Figure 6. Hematoxylin and Eosin (HE, ×400 light microscope) staining images of L929 cells on electrospun SF nanofibrous matrices after culture for 3 days. (a) Glass cover slips, (b) TCPS and (c) SF nanofibrous matrices with 75% ethanol vapor treatment. This figure is published in colour in the online edition of this journal, which can be accessed *via* http://www.brill.nl/jbs

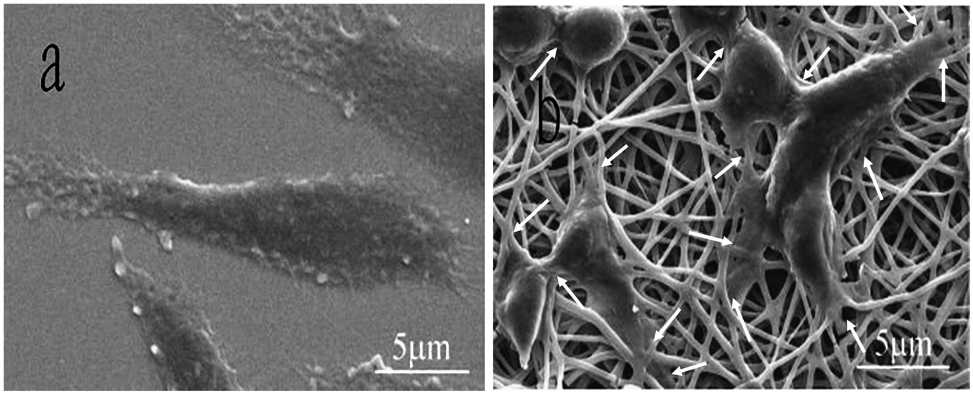

Figure 7. SEM images of L929 cells on electrospun SF nanofibrous matrices after culture for 3 days at a high magnification (2000×). (a) Glass cover slips and (b) SF nanofibrous matrices with 75% ethanol vapor treatment. Arrows indicate cell–cell interactions and cell–matrix interactions.

determine effects of this approach on drugs loaded into SF nanofibrous matrices, the traditional post-spin treatment (immersion in aqueous methanol solution) [2, 6, 24] and the traditional sterilization processing (immersion in aqueous ethanol solution) [13, 28] were selected as controls. After post-spin treatment and sterilization, the amount of rhodamine B lost was 79% (based on the actual amount of rhodamine B incorporated in rhodamine B-loaded SF nanofibrous matrices). With the traditional approach, however, only 18% was lost with 75% ethanol vapor processing (Table 1). The results illustrated that 75% ethanol vapor treatment effectively protected drugs from loss compared with the traditional post-spin treatment and sterilization processing.

4. Discussion

It is widely accepted that tissue engineering is an emerging methodology that reconstructs a tissue or organ *in vitro* and *in vivo* [29]. Therefore, as matrices for tissue

Table 1.
Effects of post-spin treatment and sterilization on the model drug

Type of nanofibrous matrix	Rhodamine B loss based on the actual amount of rhodamine B incorporated in rhodamine B-loaded SF nanofibrous matrices (%)
SF nanofibrous matrices with the traditional post-spin treatment and sterilization processing	79
SF nanofibrous matrices with 75% ethanol vapor treatment and sterilization processing	18

regeneration, varied properties of biomaterial scaffolds determine the process of tissue regeneration including good biodegradability against the new tissue growth, remarkable biocompatibility, low toxicity and so on, among which good biocompatibility and low toxicity are extremely important. However, in the fabrication of many matrices, diverse organic solvents are often involved, which are unfriendly to the human body and environment. Therefore, products from the green processing are extremely favored by humans, and capture the growing interesting of researchers all the time.

Noticeably, electrospun SF or SF hybrid nanofibers from aqueous solution have been successfully fabricated. However, the structure of SF nanofibrous matrices was induced from silk I form into silk II form typically adopting the traditional soaking treatment with pure methanol or aqueous methanol solution [6, 13, 18], which is potentially toxic and unfriendly to the human body and environment. It was recently reported that the structure of nanofibrous matrices from SF aqueous solution was successfully converted from silk I into silk II form by soaking SF matrices in pure ethanol [23], whereas the immersion treatment had great limitations for the application of SF as vehicle for diverse drug-loaded therapeutic agents in that with soaking treatment, plenty of solvent molecules at a high concentration sufficiently interacted with the drug, which made drug easily released into the solvent. Therefore, some drugs could be lost at different amounts.

Interestingly, the water vapor-induced structural conversion of SF nanofibrous matrices had been successfully developed [24]; however, as-treated SF nanofibrous matrices still needed additional appropriate sterilization treatment when serving as drug-loaded therapeutic agents for tissue-engineering applications. To find an appropriate sterilization approach for drug-loaded biomaterials is one of the major problems in the field of tissue engineering at present. For a long time, drug-loaded vehicles were often sterilized with traditional ethanol solvent immersion treatment, in which some drugs could release into the solution at different amounts [13, 28]. For drug-loaded matrices, the appropriate sterilization treatment should be developed to meet the need for use.

In the present work, we developed a simple, safe and effective post-spin treatment and sterilization approach (75% ethanol vapor treatment processing) to SF nanofibers from its aqueous solution, especially to drug-loaded SF nanofibrous matrices. By volatilization, water and ethanol molecules gently permeated through SF nanofibrous matrices, and interacted with SF nanofibrous matrices at a low concentration at ambient temperature, which effectively protected the drug from losing compared with traditional soaking treatment. Under the atmosphere of 75% ethanol vapor, the water-resistant ability of SF nanofibrous matrices was improved markedly. Meanwhile, the SF nanofibrous matrices were also effectively sterilized. Thus, this novel post-spin processing could be also a potential sterilization approach to other drug-loaded biomaterial matrices. This mild preparation process not only involved no harmful substance, but also preserved the bioactivity of SF, which could be beneficial to cell adhesion and proliferation, as shown by MTT. Therefore, our work indicated that 75% ethanol vapor treatment was not only an excellent post-spin treatment, but also an effective sterilization processing for SF nanofibrous matrices, especially for drug-loaded SF nanofibrous matrices serving as therapeutic agents for tissue-engineering applications.

5. Conclusions

In this work, a simple, safe and effective approach (75% ethanol vapor treatment processing), which skillfully integrated post-spin treatment with sterilization processing, was developed to drug-loaded SF nanofibrous matrices, which could be a potential sterilization process for other drug-loaded biomaterial matrices. The structural conversion (from silk I into silk II form) of SF nanofibrous matrices fabricated by green processing was successfully induced with 75% ethanol vapor, and the water-resistant ability of nanofibers was dramatically improved. Furthermore, such post-spin treatment which skillfully integrated with effective sterilization processing, effectively protected the drug from loss compared with the traditional post-spin treatment and sterilization processing, and as-treated SF nanofibrous matrices significantly promoted the development of L929 cells. These results suggest that 75% ethanol vapor treatment is not only an excellent post-spin processing, but also an effective sterilization approach to SF nanofibrous matrices, especially drug-loaded SF nanofibrous matrices, which could be beneficial to designing and fabricating diverse drug-loaded SF nanofibrous matrices serving as therapeutic agents for tissue-engineering applications.

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