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$CaCl_2$-포름산 용액에서 재생한 피브릴을 사용한 습식방사 실크 피브로인 필라멘트의 제조 및 특성

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Preparation and Property of Wet-spun Silk Fibroin Filaments with Fibrils Regenerated by Dissolving in $CaCl_2$-Formic Acid

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Abstract: In this work, silk fibroin (SF) filament with fibrils regenerated by dissolving in $CaCl_2$-formic acid (FA) was prepared by wet-spun method at room temperature. Different from traditional dissolved methods, SF solutions obtained by dissolving in $CaCl_2$-FA preserved fibrils, which have been recognized as the key to the high performance of native silk. The morphology of SF filament was analyzed, very dense filaments with smooth surface and circular, nanofibrils could be observed in longitudinal and cross-sections of filaments. Moreover, the breaking stress of samples was gradually increased with the increase of draw-down ratios. After 3 times drawing, the breaking stress and elongation at break of filament were 276.4±22.6 MPa and 40.8±3.1%, respectively. At the same time, the secondary structure of SF filament was typical β-sheet. In addition, SF filaments showed excellent degradation property, the mass lost of SF filament declined 42% after incubating in protease XIV solution. Above all, the human mesenchymal stem cells (hMSCs) adhered very well on the surface of the filaments, which demonstrated the good biocompatibility of SF filaments, was suitable for application in tissue engineering.

Keywords: silk fibroin fibers, $CaCl_2$-formic acid, fibril, morphology, biomaterials.

Introduction

Silk fibroin (SF) has been explored as a versatile protein biomaterial for the formation of fibers, films and porous scaffolds for various biomedical applications, due to its biocompatibility, slow degradability and robust mechanical properties.[1-4] The biocompatibility and biodegradability of the silk fibroin protein allow silk-based biomaterials to be used *in vivo*.[5] The dissolution of silk is a critical step in producing regenerated SF filaments.

The traditional dissolution solvents which degraded the SF restrict the production of regenerated SF filaments on many fields. Therefore, the way to construct the excellent mechanical properties of regenerated SF filament is still a problem for us. On the basis of our preceding work,[6-9] we report the novel preparation method of SF spinning solution which preserves the native fibrils by dissolving in $CaCl_2$-formic acid ($CaCl_2$-FA) solvent.[6]

In this paper, the SF filament was prepared by wet-spun method with water coagulation at room temperature. It was an environmentally and efficiently way to obtain filaments. Morphology, mechanical property, secondary structure, degradation property and biocompatibility of SF filaments were

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extensively investigated. Further more, the mechanism of SF dissolved in $CaCl_2$-FA solution was explained as well. Above all, in our previous studies, wet-spun silk fibroin scaffold consisted of wet-spun silk fibroin filaments with hierarchical structure for ligament tissue engineering was observed,[7] it was a simple and efficient method of constructing anterior cruciate ligament (ACL) silk scaffolds. Buiding from these observations, the present study aimed at engineering biocompatibile SF filaments, so the human mesenchymal stem cells (hMSCs) was selected to seed on SF filaments to investigated the possibility used *in vivo*.

Experimental

Materials and Preparation of SF Solution. *Bombyx mori* silk was purchased from Zhejiang, China. Sodium carbonate, calcium chloride, and formic acid (Sinopharm Chemical Reagent Co. Ltd., Shanghai, China) were analytical grade and used without further purification. PBS solution (poly(butylene succinate), pH=7.4) and protease XIV were bought from Sigma-Aldrich, USA.

Regenerated SF Filaments Preparation. Raw silk fibers were degummed with 0.05 wt% Na_2CO_3 solution at 100 °C for 30 min, rinsed thoroughly and dried. The extracted SF was dissolved in $CaCl_2$-FA solution with $CaCl_2$ concentration of 4 wt% for stirring 3 h at room temperature. The SF spinning solution was obtained and 12 wt% concentration.

Wet-spun Process. The schematic for the custom-made wet-spun apparatus built and used for spinning regenerated SF fibers in this study, as shown in Figure 1. All experiments were conducted at room temperature. The spinning solutions were loaded into 5 mL syringe, and the inner diameter of metal needle was 0.9 mm. The spinning parameters were as follows: the flow rate of solution was 5 mL h^{-1}; the length of wet-spun bath with water is 1 m; the take-up rate of the collector was 10 r min^{-1}. The resulting SF fibers (termed as-spun fiber) on the collector were immersed in water overnight for further solidification and removal of FA, and then were drafted with draw-down ratios of 2 and 3 times for use.

Measurement and Characterization. Scanning Electron Microscope (SEM): For SEM imaging, 2 μL SF solution (1 μg/mL) was dropped on a silica plate, and left to dry. Samples were sputter-coated with gold layer prior to imaging. The morphology of the fibrils and wet-spun fibers were observed using an SEM (Hitachi S-520, Japan) at 20 °C, 60 RH.

Mechanical Testing: Instron 5565 mechanical testing instrument (Instron, Norwood, MA) was used for single fiber testing (25±0.5 °C; 60±5% relative humidity; gauge length: 10 mm; cross-head speed: 10 mm/min). At least 10 measurements for each sample were performed in the testing. An average of ten measurements was reported as the mean±standard deviation for each sample. The cross-sectional areas of fibers were calculated by measuring at least 100 fibers at random using Image J. The stress of fibers was calculated by the following formula.

$$\text{Stress at break (MPa)} = \frac{\text{Breaking strength (cN)}}{\text{Cross-sectional area (mm}^2\text{)}}$$

FTIR: FTIR spectra of SF filaments was obtained using NicoLET5700 (Thermo Nicolet Company) in the spectral region of 600-2000 cm^{-1}, the fibers of 2 mg were pressed into potas-

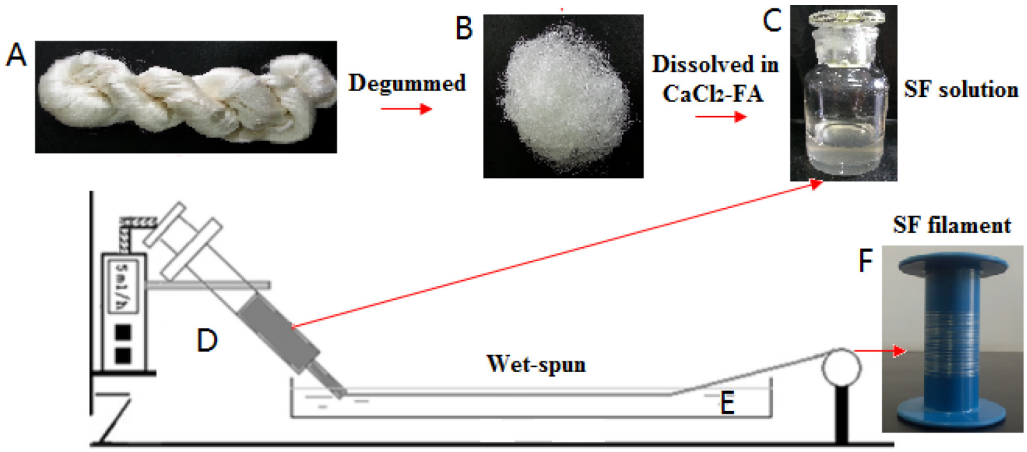

Figure 1. Schematic of custom-made wet-spun device for preparing SF filaments: (A) *Bombyx mori* silk; (B) degummed silk; (C) SF solution; (D) wet-spun device; (E) coagulation bath; (F) filaments.

sium bromide (KBr, 200 mg) pellets prior to data collection.

XRD: The secondary structure of SF filaments was analyzed with an X-ray diffraction instrument (X'Pert Pro MPD, PANalytical, Netherlands) in a transmittance mode.

Degradation Testing: SF filaments were incubated at 37 °C in 5 mL of PBS solution that contained 5 U mL^{-1} protease XIV at pH 7.4. Each solution contained an approximately equivalent mass (50±5 mg) of SF filaments. Solutions were replenished with enzyme and collected daily. At appointed time points, groups of samples were rinsed in distilled water and prepared for mass balance. Samples without enzyme but in PBS served as controls.

Cell Seeding and Culture: SF filaments were cut into disks with length 2 mm, transferred to 24-well plates and then sterilized by γ radiation. The filaments were incubated with the culture medium overnight, and then seeded with hMSCs at a density of 1.0×10^5 hMSCs. The cells were allowed to adhere to the filaments for 3 h and then the cell-filaments complexes were covered with 150 μL of culture medium. The culture medium was changed every 3 days up to the indicated time points.

Cell Morphology: The cell morphology on the filaments was observed by SEM. hMSCs were cultured for 10 days on the filaments, then fixed with 2.5% glutaraldehyde for 3 h at room temperature, rinsed three times with PBS and dehydrated in a gradient of alcohol (50, 60, 70, 80, 90, 100%). Samples were then lyophilized, coated with gold and observed by SEM (Hitachi S-520, Japan).

Results and Discussion

Mechanism of SF Dissolved in $CaCl_2$-FA Solution. Usually, the nanofibril structure is completely decomposed to molecules during silk dissolution in traditional solvent (such as $CaCl_2$-C_2H_5OH-H_2O), in this study, the traditional dissolution solvents were replaced by $CaCl_2$-FA, different from the traditional dissolution solvents, silk showed a completely different dissolution behavior with preservation of fibrils instead of generating molecules with reduced size and fully dissolved,[10,11] the mechanism of SF dissolved in $CaCl_2$-FA solution was shown in Figure 2.

As we know, FA can not dissolve native silk at all. However, the adding of a small quantity of inorganic salts into FA makes it an effective solvent for dissolving degummed silk.[12] After dissolved in the $CaCl_2$-FA solution, fiber expansions and hydrogen bonds between molecules were weakened, the β-sheet structures were destroyed into amorphous structure, while with fibril preservation. Therefore, the $CaCl_2$-FA blend solvent was a new solvent system for silk dissolution. The solvent system could dissolve silk at room temperature, more

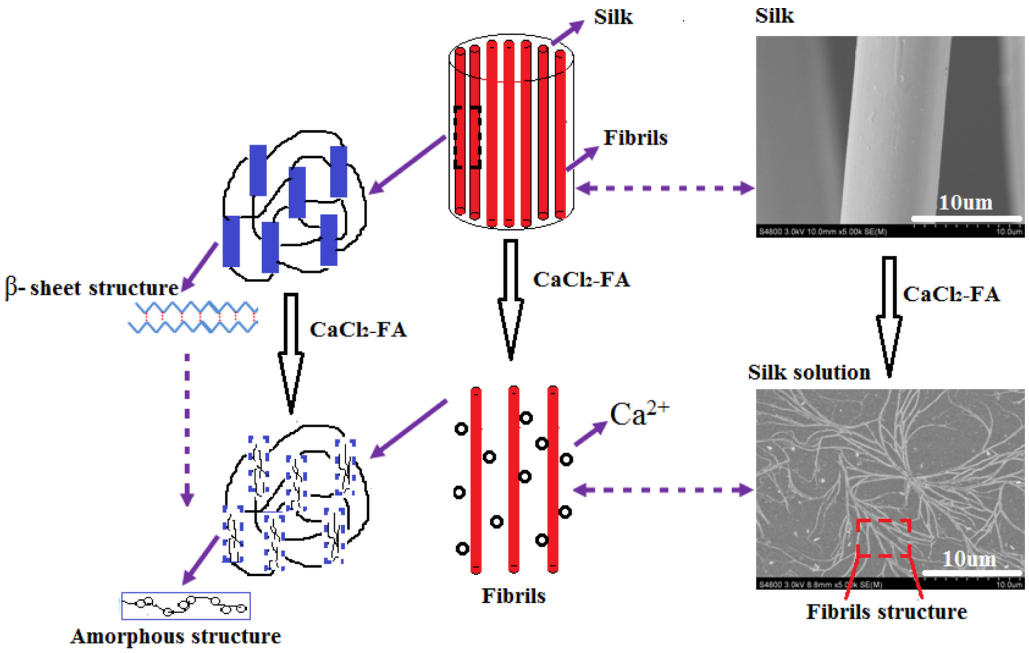

Figure 2. Mechanism of SF dissolved in $CaCl_2$-FA solution.

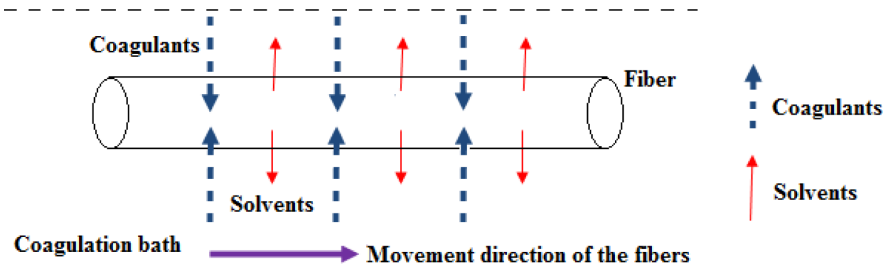

Figure 3. Double diffusion process in wet-spun.

importantly, the natural nanofibrillar structure was preserved and the β-sheet crystal structure was destroyed.

Description of the Wet-spun Process and Morphology of SF Filaments. Schematic of custom-made wet-spun device for preparing SF filaments was depicted in Figure 1. In the wet-spun process, the solution was spun into water and transformed into a partial gel state, as shown in Figure 3, and then with the action of double diffusion, as shown in Figure 3. The filament solidified with the diffusion and dialysis of spinning solvents, under suitable solidification conditions, which diffusion coefficient and the solidification rate achieve a relatively balanced, solvents and salts diffused outward from the spinning solution, coagulant diffused inward to the fiber to form a filament.

SF filaments were successfully prepared by using water as the coagulation bath, which was a simple, efficient, and environmentally compatible process. In addition, transparent SF solution was obtained through dissolving degummed silk in $CaCl_2$-FA solvent, and many fibrils were observed in spinning solution. This dissolution method dissolved silk by breaking hydrogen bonds in the crystalline region of SF while preserving the fibrils structures.[6] After wet-spinning, SEM images of the longitudinal views and cross-section of the SF filaments were shown in Figure 4. We could observe the fibrils existed in the filaments with smooth surface and circular, and it confirmed that the fibrils aggregate into filament. The hierarchical structure (molecular orientation, β-sheet crystals, nanofibrils) was considered the origin of the high performance of native silk.[13] In this paper, it was surprised to discover the fibrils in the solutions and filaments. It inferred that with wet-spun process, fibrils in the solutions easily re-assembled into filaments due to physical shear and coagulation in water.[6] Therefore, it was a feasible method to achieve superior properties for regenerated SF filaments.

Mechanical Performance. Compared with previous reports,[14,15] the as-spun filaments prepared by dissolving in

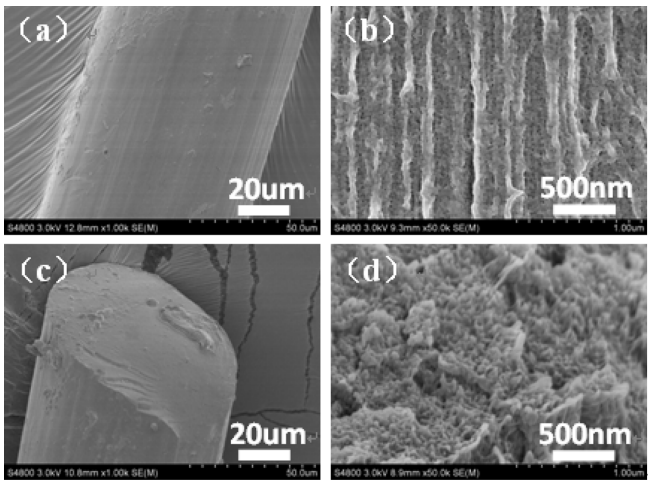

Figure 4. SEM images of SF filaments. Longitudinal views (a,×1000; b,×50000) and cross-section views (c,×1000; d,×50000)

$CaCl_2$-FA exhibited higher strength and extensibility, it was contributed to the fibrils structure in the SF solution. In the process of wet-spun, the orientation of the as-spun fiber was limited, a large number of pores and irregular molecular chain entanglement, so the surface of fibers was fragile and poor mechanical property. Post-drawing is an effective methods to improve the mechanical properties of regenerated silk materials.[16,17] After post-drawing, the mechanical properties were drastically improved with increased draw ratio due to the positive change in molecular orientation, suggesting a close relationship between mechanical properties and secondary structure. When as-spun filaments were drafted by different draw-down ratios, the breaking stress of SF filaments gradually increased with the increase of draw-down ratios, as shown in Figure 5.

Under the low draw-down ratio, the force was mainly acting on the ordered structure in amorphous regions, after gradually ordered structure parallel to the fiber axis, the mechanical property of the fibers reach up to a certain value. With increased of draw ratio, the positive change in molecular ori-

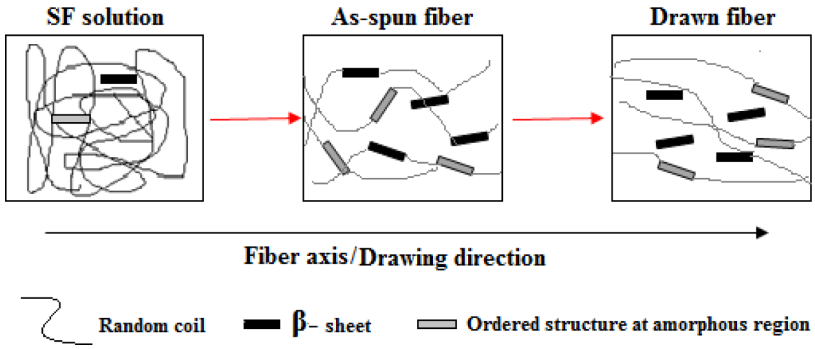

Figure 5. Schematic illustration of SF filaments structure changed in process of drawing.

Table 1. Mechanical Properties of SF Filaments with Varied Drawing Ratios

Sample	Diameter (μm)	Stress at break (MPa)	Strain at break (%)
as-spun (×1)	58.2±4.6	97.2±15.7	16.8±6.3
SF×2 (×2)	32.2±5.2	148.3±26.2	29.4±6.3
SF×3 (×3)	20.6±3.8	276.4±22.6	40.8±3.1
Native silk	12.5±1.2	364.6±42.5	18.2±2.3

entation was improved further, as shown in Table 1. After 3 times drawing, the breaking stress and elongation at break of filament were 276.4±22.6 MPa and 40.8±3.1%, respectively. This phenomenon is attributed the development molecular orientation in post-drafting process.

The remarkable mechanical properties of native silk are attributed to its hierarchical structure,[13] such as β-sheet nanocrystals[18,19] and nanofibrils.[20] In this study, the traditional dissolution solvents were replaced by $CaCl_2$-FA, in which silk showed a completely different dissolution behavior with preservation of fibrils instead of generating molecules with reduced size. It illustrated that fibrils were an important factors to improve the mechanical properties of regenerated SF materials.

Secondary Structure. Secondary structure of SF filaments during wet-spun were determined by FTIR (Figure 6(A)) and XRD (Figure 6(B)). The filaments showed a strong peak at 1626 cm^{-1} in the amide I region, 1528 cm^{-1} in the amide II region and 1236 cm^{-1} in the amide III region was indicative of silk II conformation, corresponding to β-sheet.[21] The filaments exhibited 2θ peak at 9.7°, 19.7°, 19.9° and 20.4°, which were assigned to silk II structure.[22] The results of FTIR and XRD indicated indirectly and directly the mainly amorphous structure of silk in $CaCl_2$-FA, which was transformed into silk II structure after wet-spun. It illustrated that water infiltration and physical shear promoted fibrils aggregation and the structural transition to the insoluble crystalline β-sheet, which could produce SF filaments with high mechanical properties and a hierarchical structure.

Degradation Properties. To functionally assess the degradation property of SF filament *in vitro*, they were incubated in protease XIV solution for 48 h. At the same time, SF fil-

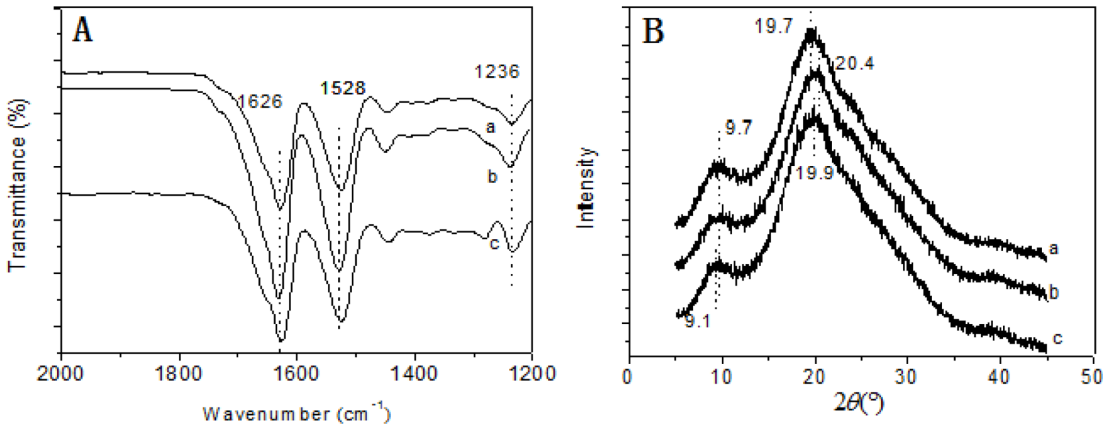

Figure 6. FTIR (A); XRD (B) results of SF filaments (a. as-spun; b. SF×2; c. SF×3).

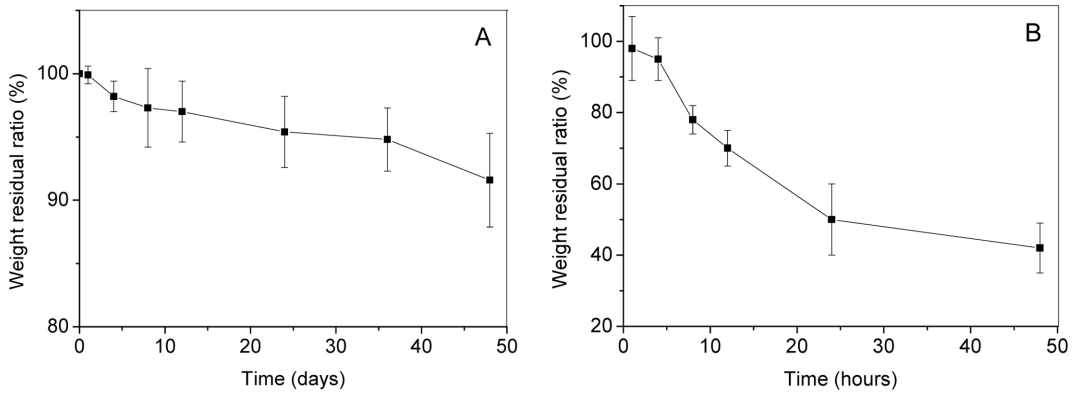

Figure 7. Weight of SF in degradation process in PBS solution (A); protease XIV solution (B).

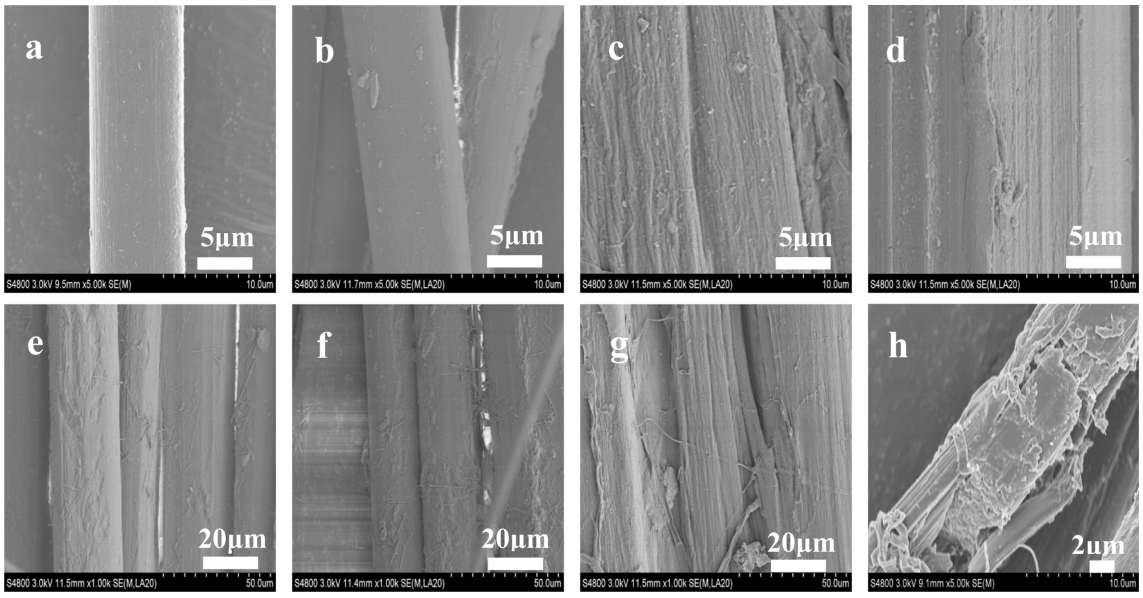

Figure 8. SEM of SF filament at different degradation times in PBS solution: (a) 1 d; (b) 15 d; (c) 30 d; (d) 48 d; and protease XIV solution: (e) 1 h; (f) 12 h; (g) 24 h; (h) 48 h.

aments were incubated in PBS solution for 48 days, serving as controls. The results showed SF filament dissolved slowly in PBS, with about 8.4% mass lost after 48 days (Figure 7(A)). However, after incubating in protease XIV solution, the mass lost decreased 42% after 48 h (Figure 7(B)). At appointed time points, the degradable SF filaments were rinsed and freeze-dried. SEM images depicted the degradation process of filaments in PBS and protease XIV solution, respectively (Figure 8(a)-(f)). It can be seen that SF filaments were seriously collapsed in PBS solution containing protease XIV rather than PBS solution. The degradation test results illustrated that SF filaments were degradable, additionally, the speeds of degradation in protease XIV solution was faster than in PBS solution.[7]

Biocompatibility of SF Filaments. Tissue engineered constructs have been considered in order to sustain cells at the implant site, the biocompatibility and biodegradability of the regenerated silk fibroin protein allow silk-based biomaterials to be used *in vivo*. In this paper, SF filaments were cultured with hMSCs to observe the cell growth. Figure 9 showed the growth state of hMSCs cultivated on SF filaments. The hMSCs adhered very well on the surface of the filaments at day 2, and the cell spread out on the surface of the filaments at day 10, and it suggesting that hMSCs grew very well on the SF filaments. The preliminary results of cell compatibility encourage further exploration of SF filaments as biomimetic scaffolds for application in tissue engineering.

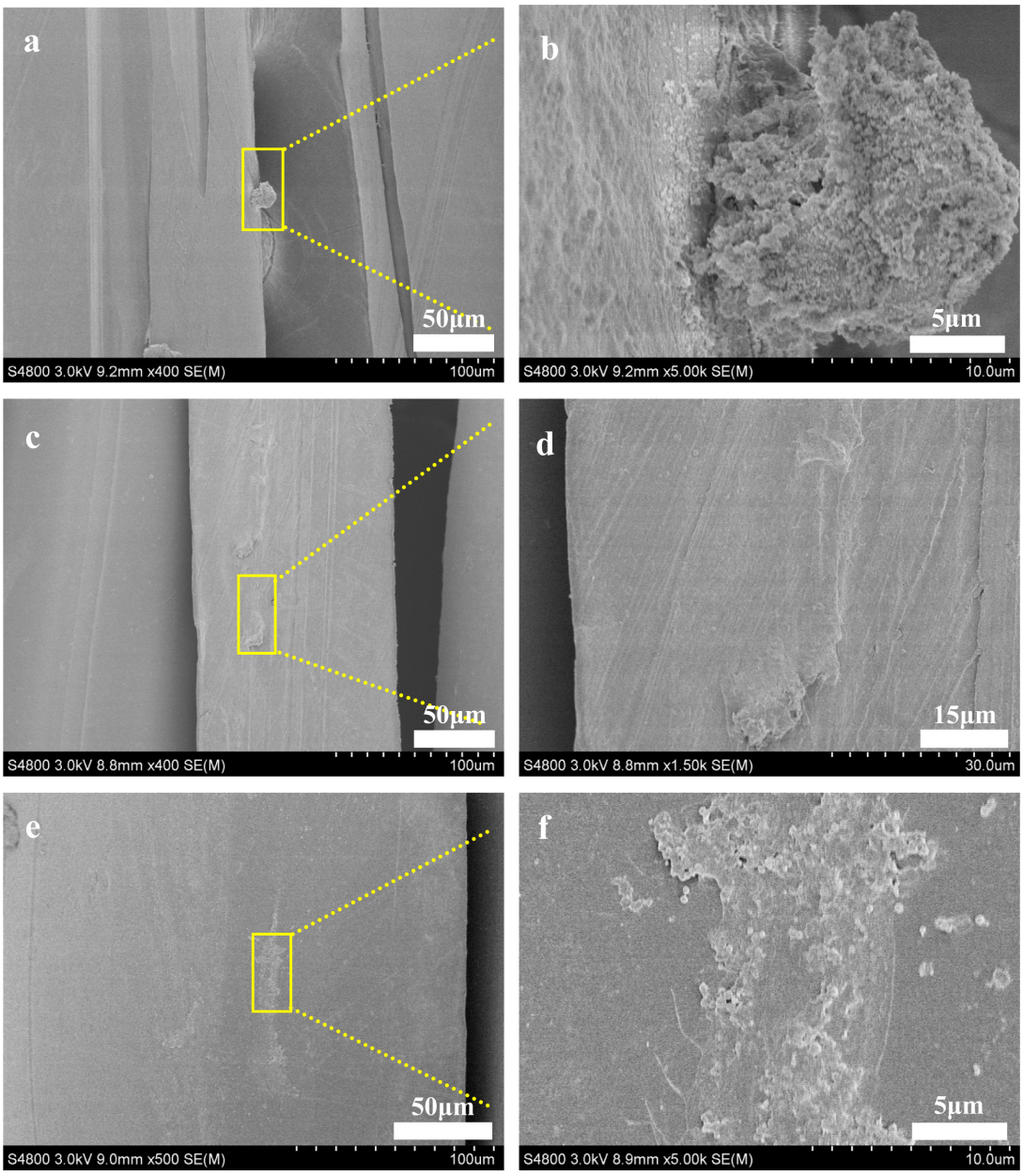

Figure 9. SEM images of hMSCs cultivated on SF filaments: (a) 2d×500; (b) 2d×5000; (c) 8d×500; (d) 8d×5000; (e) 10d×500; (f) 10d×5000.

Conclusions

In summary, SF filament with fibrils regenerated by dissolving in $CaCl_2$-FA was prepared by wet-spun method at room temperature. SF solutions obtained by dissolving in $CaCl_2$-FA preserved fibrils, which supply the key factor for preparing the high performance SF filament. After 3 times drawing, the breaking stress and elongation at break of filament were 276.4±22.6 MPa and 40.8±3.1%, respectively. Fibrils in SF solutions dissolved by $CaCl_2$-FA played an important role for its excellent mechanical property and drawability. In addition, the secondary structure of SF filament was typical β-sheet. SF filaments showed excellent degradation property, the mass lost of SF filament declined 42% after incubating in protease XIV solution. Above all, the hMSCs adhered very well on the surface of the filaments, which demonstrated the good biocompatibility of SF filaments, and it was suitable for application in tissue engineering. This method provided a new way to prepare flexible SF filament for biomedical application in future.

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