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Preparation of non-woven nanofibers of *Bombyx mori* silk, *Samia cynthia ricini* silk and recombinant hybrid silk with electrospinning method

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Abstract

Electrospinning is a good method to obtain nanoscale fibers from polymer solutions. In this paper, we successfully prepared non-woven nanofibers of *Bombyx mori* and *Samia cynthia ricini* silk fibroins, and of the recombinant hybrid fiber involving the crystalline domain of *B. mori* silk and non-crystalline domain of *S. c. ricini* silk from hexafluoroacetone (HFA) solution using electrospinning method. ^{13}C cross polarization/magic angle spinning NMR spectroscopy was used to monitor the structural change of silk fibroins together with the detection of the residual HFA during the process of the fiber formation. Scanning electronic microscope was used to determine the diameters and their distributions of the fibers.

Keywords: Electrospinning; Non-woven nanofiber; Silk fibroin

1. Introduction

The research and development of electrospinning method has been rapidly increased during the past few years [1–10]. From a polymer solution a charged jet is created, when the electrical force overcomes surface tension. The jet typically develops a bending instability and then solidifies to form fibers, which measures in the range of nanometers to 1 μm. Most studies so far have been done with polymer solutions that had always been used as fibers, such as poly(ethylene oxide), poly(ethylene terephthalate) and others [1,4,6,8,9].

Recently, many researchers in fiber and biomedical fields have been of interest in natural silk fibers because of its outstanding mechanical properties [11,12]. Although many organisms can produce silk, silkworms and spiders are two main producers of silk. Among silkworms, the domesticated *Bombyx mori* is well known and has been of interest for at least 5000 years due to its excellent characters as textile fiber. The wild silkworms such as *Samia cynthia ricini* or *A. pernyi* can also produce silk fiber, but whose primary structure is different considerably from *B. mori* silk fiber [13]. And therefore the properties of the fibers are different among them. Most recently, the silk-like proteins with special functions has been produced using genetically engineering method by incorporating, for example, Arg-Gly-Asp (from fibronectin) unit or Gly-Val-Pro-Gly-Val (from elastin) repeated units into the silk fibroin chain [14–19]. The possibility to produce these recombinant silk-like proteins using biotechnology has provided an impetus for the efforts in both the biosynthesis and the spinning of these protein-based biopolymers.

In our previous paper [12], we tried to prepare regenerated silk fibers with HFA-hydrate as a spinning solvent with wet-spinning method. The films prepared from *B. mori* or *S. c. ricini* silk fibroins can be dissolved in HFA-hydrate very well without decomposition. We could prepare regenerated *B. mori* silk fibers with good physical properties, but could not prepare the regenerated *S. c. ricini* silk fibers [12].

In this paper, we will confirm optimized process of electrospinning for preparing nanofibers of *B. mori* and *S. c. ricini* silks, and of the recombinant hybrid silk with narrow distributions of the diameters of the fibers from the HFA solutions. In addition, the structural characterization of these fibers will be performed using ^{13}C CP/MAS NMR.

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2. Experimental section

2.1. Materials

The cocoons of *B. mori* were degummed three times with 0.5% (w/v) Marseilles soap solution at 100 °C for 30 min and washed with distilled water in order to remove another silk protein, sericin, from the surface of silk fibers. The silk fibroin fibers were then dissolved in 9 M LiBr (Wako Pure Chemical Industries, Ltd) at 40 °C. After dialysis against distilled water for 4 days, the solution was gently evaporated by airflow to a concentration of less than 2 wt%. The aqueous solution of silk fibroin was cast onto plastic plates to prepare silk films in the silk I form. The cocoons of *S. c. ricini* were degummed five times with 0.5 wt% sodium carbonate (Wako Pure Chemical Industries, Ltd) to get silk fibroin fibers. HFA-hydrate (HFA-trihydrate: $HFA\cdot 3H_2O$) used here was of analytical grade (Tokyo Kasei Kogyo Co., Ltd) and used for the solvents of the silk films or silk fibers. The *B. mori* silk fibroin/HFA solution and *S. c. ricini* silk fibroin/HFA solution were transferred to a pump fitted with a stainless steel 80 mesh screens to get a uniform solution. From the solutions of the concentrations of 10, 7, 5, 3, and 2 wt%, we tried to obtain a fiber by the electrospinning method.

2.2. Electrospinning and post-spin treatments

In the electrospinning process, a high electric potential is applied to a droplet of a silk solution at the tip of a plastic capillary in which a Pt wire is used as an electrode. When the electric force from the applied field becomes larger than the surface tension of the droplet, a charged jet of the solution is formed and ejected in the direction of the applied field. The jet dries as the solvent is evaporated in the air. The dried fibers are collected on a receiving conducting mesh. The collecting mesh was placed at distance of 10–15 cm from the capillary tip. And additionally the capillary axis had to be tilted about 5–10° from horizontal direction. A voltage of 15–30 kV was applied to the wire in the capillary through a high voltage power supply, while the receiving mesh was grounded. The process was carried out at room temperature, since for the fluids, viscoelastic forces can stabilize the jet.

Methanol was used as a solvent to remove HFA from the electronspun fiber and induce the structural change in the post-spin stage. The non-woven fibers were soaked in a methanol bath overnight to allow HFA to diffuse from the fiber, and then the non-woven fibers were dried in the vacuum overnight at room temperature.

2.3. SEM observation

We used SEM to measure the fiber diameters together with the distribution. The samples were coated by 3 nm Pt and observed using a FE-SEM (Hitachi S-900). The sample is stuck on an alumni sheet or stainless mesh that is used as a collection sheet. The size of the samples is a 3 mm × 3 mm sheet. The fiber diameter distributions of the non-woven fibers are sampled from 100 positions of fiber-crossing in the SEM pictures.

2.4. Solid-state ^{13}C CP/MAS NMR measurements

The ^{13}C solid-state CP/MAS NMR spectra of the non-woven fibers prepared by electrospinning method were recorded on a Chemagnetics CMX 400 MHz spectrometer using a cross-polarization pulse sequence and with magic-angle spinning at 10 kHz. The experimental conditions were as follows, ^{1}H 90° pulse of 4 μs, ^{13}C 90° pulse of 4 μs, contact time of 1 ms and pulse delay of 5 s. The ^{13}C chemical shifts were calibrated indirectly through the adamantane peak observed upfield (28.8 ppm relative to tetramethylsilane).

2.5. Mechanical properties measurements

The stress–strain curve was recorded on a tensile testing machine (Auto COM/AC-50 T. S. E.) at room temperature under room humidity. The non-woven fiber samples with 10 mm width and 30 mm original length were prepared. The thickness of the samples was from 0.11 to 0.35 mm. Then, the distance between two crossheads was set to be 10 mm and the rate of the crosshead was 2 mm/min.

3. Results and discussion

3.1. Best condition for preparation of non-woven silk fibers

The best condition for producing a stable polymer jet and obtaining non-woven silk fibers will be examined. *B. mori* silk solutions of 7, 5 and 3 wt% formed a stable jet, and 1.0–1.6 kV/cm electronic field was condition for the fiber formation. For the 2 wt% solution, since the viscosity is too low to get stable drops and jet, we could not get the fibers. A similar observation was reported for PEO/H_2O solutions with low viscosity [9]. Figs. 1 and 2 show the SEM photos of the non-woven *B. mori* silk fibers and the diameter distributions of the silk fibers obtained from the elctro-spinning method, respectively. In these four cases, thin and rod-like fibers with diameters ranged from about 100–1000 nm were obtained. The mean of the fiber diameters is the smallest and the fiber diameter distribution is the narrowest for the 3 wt% case. The thick and branched fibers along with broader distribution of the diameters were prepared for the fibers from the solutions from 5 to 7 wt% due to their high solution viscosity compared with the 3 wt% solution. Thus, the best condition for nanoscale non-woven *B. mori* fiber formation is 3 wt% concentration and 1 kV/cm electronic field. Methanol was used as coagulant solvent in the post treatment process. The silk fiber collected on the stainless mesh was soaked in a methanol bath

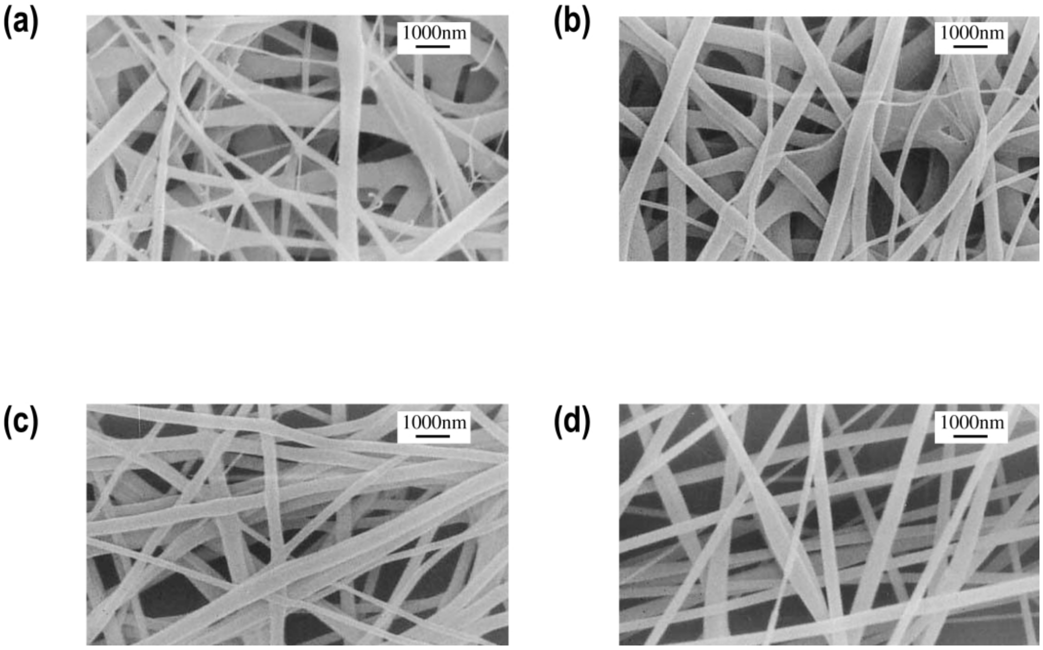

Fig. 1. Scanning electron micrographs of non-woven silk fibers from (a) 7 wt% *B. mori*/HFA solution, 1.3 kV/cm; (b) 5 wt% *B. mori*/HFA solution, 1.6 kV/cm; (c) 5 wt% *B. mori*/HFA solution, 1.0 kV/cm and (d) 3 wt% *B. mori*/HFA solution, 1.0 kV/cm.

overnight at room temperature to remove the residual HFA from the fiber and induce the structural change. Then the silk fibers were dried in the vacuum for two days to remove HFA completely. We also applied this process of non-woven fiber formation to preparation of non-woven *S. c. ricini* fiber. The optimum condition for the fiber formation was 10 wt% and 1 kV/cm, and the prepared non-woven fiber is shown in Fig. 3. Since it is difficult to prepare the fiber of *S. c. ricini* silk fibroin by using the typical wet-spinning process even in the case of HFA as a solvent, the electro-spinning method is especially useful in preparation of the fiber of *S. c. ricini* silk fibroin.

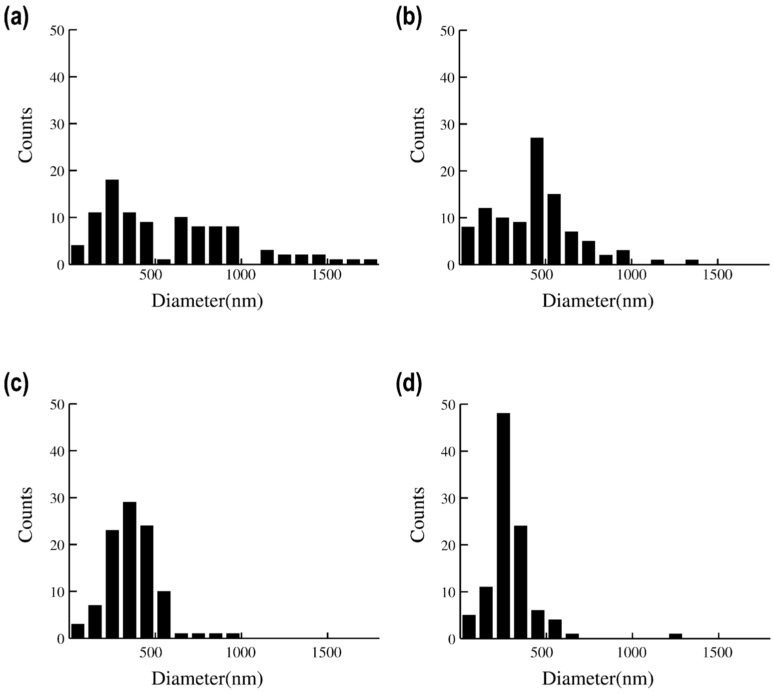

Fig. 2. Distributions of the diameters of non-woven silk fibers from (a) 7 wt% *B. mori*/HFA solution, 1.3 kV/cm; (b) 5 wt% *B. mori*/HFA solution, 1.6 kV/cm; (c) 5 wt% *B. mori*/HFA solution, 1.0 kV/cm and (d) 3 wt% *B. mori*/HFA solution, 1.0 kV/cm.

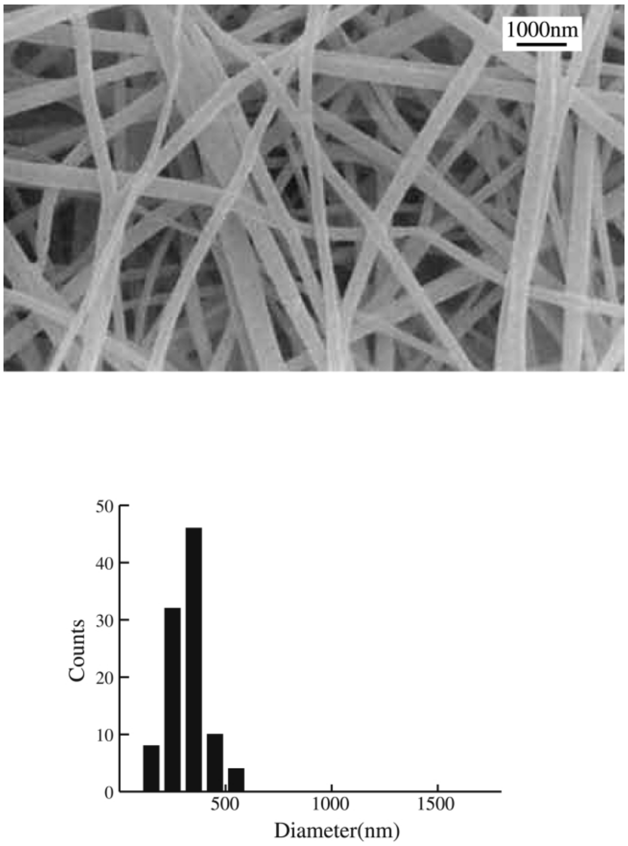

Fig. 3. Scanning electron micrograph of non-woven silk fiber from 10 wt% *S. c. ricini*/HFA solution at 1 kV/cm and distribution of the diameter of the fiber.

3.2. Characterization of the non-woven silk fibers

Thus far, ^{13}C CP/MAS NMR has been used to determine silk I (type II β-turn structure) as the structure before spinning [20,21] and silk II (heterogeneous structure, but mainly anti parallel β-sheet [22]) as the structure after spinning of *B. mori* silk fibroin. Similarly, this solid state NMR method was also used to identify the α-helix structure before spinning and silk II (mainly, anti parallel β-sheet) as the structure after spinning of *S. c. ricini* silk fibroin [23–25]. Fig. 4 shows ^{13}C CP/MAS NMR spectra of the regenerated *B. mori* silk fibers prepared by the electrospinning method from the HFA solution, (a) as spun and (b) after methanol treatment. In Fig. 4(a), the ^{13}C chemical shifts were 16.2 ppm for Ala Cβ, 52.9 ppm for Ala Cα and 173.1 ppm for Ala C=O carbons, and these peaks were relatively broad. This indicates that the Ala residues take random coil structure [26–29]. There are also three peaks from HFA used as a solvent at 90.7, 116 and 122 ppm, and thus the fiber contains a small amounts of HFA molecules [12]. In Fig. 4(b) for the fibers after the methanol immersion, the ^{13}C chemical shifts were changed to 20.2 ppm for Ala Cβ, 48.9 ppm for Ala Cα and172.3 ppm for Ala C=O carbons. Thus, the Ala residues take typical silk II structure [22–24,26–28]. The peak of HFA was disappeared, means that HFA molecules were removed completely after methanol treatment in the post-spin process.

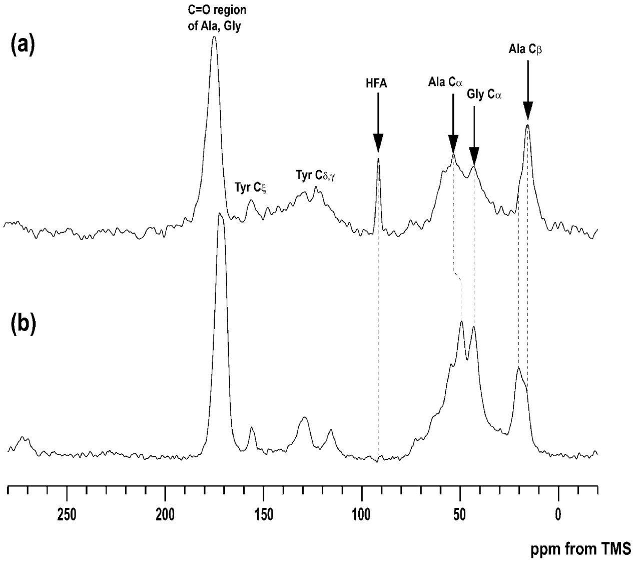

Fig. 4. ^{13}C CP/MAS NMR spectra of non-woven *B. mori* silk fibers prepared (a) as-spun and (b) after methanol treatment.

Fig. 5 shows ^{13}C CP/MAS NMR spectra of the regenerated *S. c. ricini* silk fibers prepared from the HFA solution by the electrospinning method, (a) as spun and (b) after methanol treatment. The ^{13}C chemical shifts were 15.0 ppm for Ala Cβ, 52.1 ppm for Ala Cα and 175.4 ppm for Ala C=O carbons in Fig. 5(a), which is slightly different from typical α-helical chemical shifts [27,28]. Actually, in Fig. 5(b), the ^{13}C chemical shifts were slightly changed to 15.9 ppm for Ala Cβ, 52.3 ppm for Ala Cα and 175.7 ppm

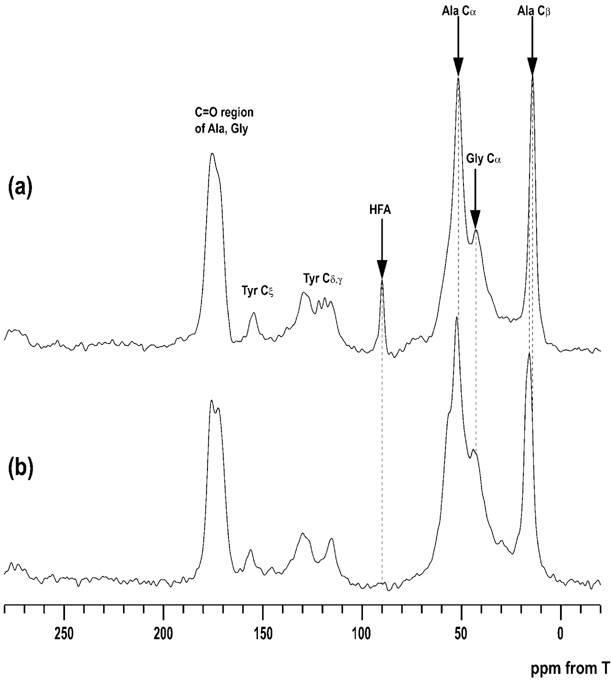

Fig. 5. ^{13}C CP/MAS NMR spectra of non-woven *S. c. ricini* silk fibers prepared (a) as-spun and (b) after the methanol treatment.

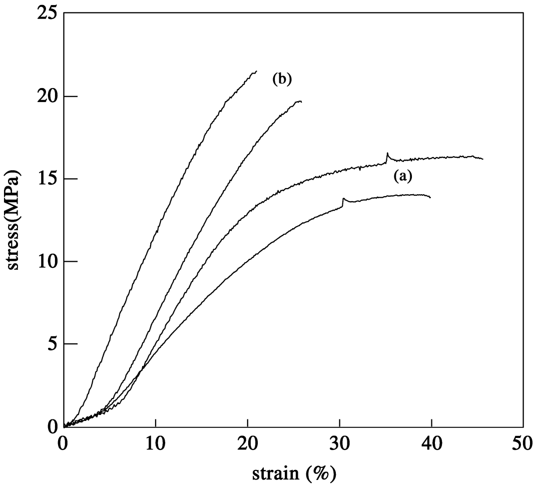

Fig. 6. Stress–strain curves of the non-woven silk fibers after the methanol treatment, (a) *B. mori* and (b) *S. c. ricini*. The stress–strain experiments were performed two times for each fiber.

for Ala C=O carbons, which is typical α-helical chemical shifts [23–25]. The peaks of HFA molecules were also observed at 90.4, 116 and 122 ppm in Fig. 5(a) [12]. Thus, the solvent effect from HFA still remains and seems to be the origin of such a small deviation from the typical α-helical chemical shifts. Contrary to the case of *B. mori* silk fiber, the structural change to anti parallel β-sheet does not occur after the methanol immersion. This means that densely packed structure is formed during the drying process of the *S. c. ricini* silk fibers before methanol immersion.

A comparison of the stress–strain curves of the non-woven fibers between *B. mori* silk fibroin (a) and *S. c. ricini* silk fibroin (b) is shown in Fig. 6. The experiments were performed two times for each sample. In *B. mori* silk samples, the high maximum broken strength is 15 MPa and the larger elongation of 40%, while in *S. c. ricini* samples, the maximum broken strength is 20 MPa and smaller elongation of 20%. This difference in the stress–strain curves is different from those of these two kinds of silk fibers prepared as a single fiber [30]. However, it is difficult to correlate these stress–strain curves with the structures in atomic level because the curves of these non-woven fibers depend on the assembles of these fibers rather than the structural character of a fiber molecule.

3.3. Fiber formation of genetically engineered protein

Finally, we prepared non-woven fibers of recombinant hybrid silk protein prepared by genetically engineered method. The primary structure is [GGAGSGYGGGYGHGYGSDGG(GAGAGS)$_3$]$_6$, which is a hybrid of the crystalline region of *B. mori* silk fibroin and the Gly-rich region of *S. c. ricini* silk fibroin. Detailed method for the preparation will be published elsewhere [31]. The non-woven fiber prepared with electrospinning method was successfully obtained from only 25 mg sample. However, because of the limited amounts of the samples, only SEM of the non-woven fiber was observed as shown in Fig. 7. The diameter distribution is also shown. The very homogeneous fibers with small diameters like 100 nm was obtained.

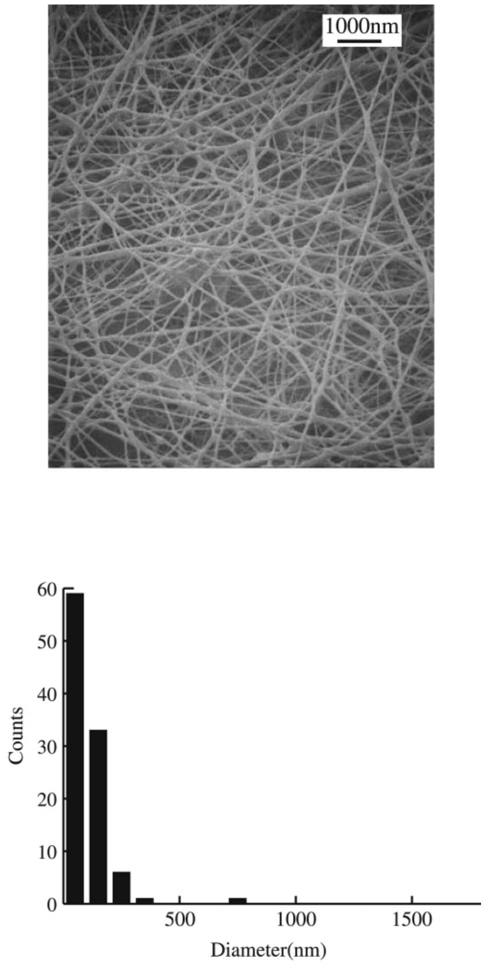

Fig. 7. Scanning electron micrograph of non-woven fiber prepared from the 12 wt% solution of the hybrid silk protein in HFA solution at 1 kV/cm. and distribution of the diameter of the fiber.

4. Conclusions

The electrospinnings of *B. mori* silk fibroin and *S. c. ricini* silk fibroin, and genetically engineered recombinant silk fibroin were performed with HFA-hydrate as a spinning solvent. The best spinning conditions and procedure are established; 3 wt% solution for *B. mori* silk and 10 wt% for *S. c. ricini* silk which are spun onto a stainless mesh at 1 kV/cm. As the post-spinning treatment, the regenerated silks were immersed in methanol, and then dried in vacuum. The solid-state ^{13}C CP/MAS NMR spectra were used to characterize the structure and check the trace of HFA molecules in the fibers.

Acknowledgements

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