Atomic Force Microscopy Observations of the Topography of Regenerated Silk Fibroin Aggregations

Tao Huang, Peng Ren, Bo Huo

National Microgravity Laboratory, Institute of Mechanics, Chinese Academy of Sciences, Beijing 100080, People's Republic of China

Received 3 January 2007; accepted 23 March 2007
DOI 10.1002/app.26591
Published online 5 September 2007 in Wiley InterScience (www.interscience.wiley.com).

ABSTRACT: How fibroin molecules fold themselves and further self-assemble into aggregations with specific structures when the solution concentration increases is the key to understanding the natural silk-forming process of the silkworm. A regenerated *Bombyx mori* silk fibroin solution was prepared, and serially diluted solutions were coated on aminated coverslips. Atomic force microscopy (AFM) observations of the topography of fibroin molecules revealed a transformation from rodlike aggregations 100–200 nm long to small globules 50 nm in diameter with decreasing concentrations. When the incubation duration increased, the aggregations of fibroin molecules showed a self-assembling process, which was measured with AFM. In particular, after the molecules were incubated for more than 20 min, rodlike micelles formed and were distributed evenly on the surface of the aminated slides. Flow chamber technology was used to study the effect of the shear loading on the topography of the fibroin molecular aggregations. After a shear loading was applied, larger rodlike particles formed at a higher incubation concentration in comparison with those at a lower concentration and were obviously oriented along the direction of fluid flow. © 2007 Wiley Periodicals, Inc. J Appl Polym Sci 106: 4054–4059, 2007

Key words: atomic force microscopy (AFM); morphology; proteins; self-assembly; shear

INTRODUCTION

To produce protein fibers with extraordinary mechanical properties, since the 1980s, people have paid a lot of attention to silkworms and spiders, which can spin silk fibers with strength comparable to and extensibility even better than those of Kevlar, an excellent and widely used artificial silk fiber.[1] Unlike the spinning process of most artificial silk fibers, for which a high pressure, a high temperature, and a strong acid or alkaline chemical reagent are required,[2] silkworms and spiders secret fibroin in their silk glands and spin silk at atmospheric pressure, a low speed, and room temperature. It is well recognized that during spinning, a conformational transition from a predominantly α-helix or disorder structure to a β-sheet structure occurs.[3] This transition is regulated by factors such as the specific amino acid sequences of fibroin, the concentration of the fibroin solution, and the shear stress field around the fibroin molecules.

For the silkworm larvae in the fifth instar stadium, fibroin is secreted in posterior silk glands and stored in posterior and middle silk glands. In *Bombyx mori*, this procedure generally lasts for 7–9 days before the silk fibers are spun, during which the volume of the posterior and middle silk glands gradually becomes larger, and the concentration of the fibroin gel solution also increases up to 20–30 wt %.[4,5] Why fibroin molecules are dissolvable in such highly doped solutions is a confusing problem.

In terms of optical technologies, some evidence has been found indicating that silkworms and spiders make use of liquid crystals to spin silk fibers.[6–8] Fibroin molecules aggregate into rodlike structures and orient regularly, preventing entanglement with one another. However, it is still unclear how fibroin molecules form the rodlike aggregations. On the basis of the characteristics of the repeated hydrophobic and hydrophilic amino acid segments of *B. mori*'s fibroin heavy chains,[9,10] some researchers have assumed that silk fibroin (SF) molecules organize into pseudomicelle or soaplike structures that form globular and gel states during processing in the middle and posterior glands.[11] This molecular structure makes fibroin molecules dissolvable in highly concentrated solutions and prevents the proteins from irreversibly crystallizing too early before the spinning process.

Correspondence to: B. Huo, Department of Biomedical Engineering, Columbia University, New York, NY 10027 (bh2247@columbia.edu).

Contract grant sponsor: National Science Foundation of China; contract grant numbers: 30300076, 10332060.

Contract grant sponsor: Open Project Program of the Key Laboratory of Molecular Engineering of Polymers at Fudan University (Ministry of Education).

Journal of Applied Polymer Science, Vol. 106, 4054–4059 (2007)
© 2007 Wiley Periodicals, Inc.

WILEY InterScience
DISCOVER SOMETHING GREAT

Although the suggested liquid-crystal model and micelle structure seem to provide good explanations for the liquidity and dissolvability of fibroin molecules, the research directly observing the topography of fibroin molecules has not been adequate to give a whole image of the conformational transition of fibroin molecules in silk glands. By using atomic force microscopy (AFM), Inoue et al.[12] observed a single rodlike molecule 60 nm long and 15 nm wide in an SF solution of 0.03 wt % obtained from the middle division of the silk gland of *B. mori*.[12] A rodlike nanostructure and high-order structures were also observed in fibroin gel directly taken from the silk gland of the *Samia Cynthia ricini* wild silkworm.[13] Yamada et al.[14] found networks and islandlike structures in films of regenerated SF by AFM.

In this study, we tried to use AFM to observe the topography of fibroin molecular aggregations to provide some direct images of the effect of the solution concentration on the molecular structures. We coated regenerated fibroin solutions with different concentrations on the surfaces of aminated coverslips. The incubation duration was also varied. Actually, the incubation process could be regarded as an aggregation process of molecules. We also provided preliminary results of AFM observations of fibroin molecular aggregations after the application of a shear loading by flow chamber technology. These results will be helpful in understanding what kinds of structures fibroin molecule aggregations may form within the silk glands of silkworms.

EXPERIMENTAL

Regenerated fibroin solution preparation

The *B. mori* cocoon silk was degummed in 5 g/L Na_2CO_3 at 98°C for 1 h. The degummed silk was rinsed in deionized water and then was dried in an oven at 65°C for 24 h. The dry silk was dissolved in a 9*M* LiBr solution at 40°C. After being filtered roughly by gauze to remove the undissolved silk, the obtained fibroin solution was dialyzed against deionized water with a cellulose dialysis bag for 3 days. The water was changed every 3 h. The concentration of the finally obtained regenerated fibroin solution was measured with a bicinchoninic acid (BCA) protein assay kit (no. 23225, Pierce Co.) and the OD280 method.[15] The concentration values measured by the two methods differed by less than 10%. During the preparation, the operation was performed gently to avoid shearing-induced aggregation and elongation of the fibroin molecules. The SF solution was stored at 4°C in a refrigerator before use.

Protein coating

High-quality coverslips were immersed in a piranha solution of 30% (v/v) hydrogen peroxide and 70% (v/v) sulfuric acid for 1 h at 98°C, rinsed in deionized water, and dried in air. The cleaned coverslips were immersed in a freshly prepared 2% solution of 3-aminopropyltriethoxysilane in acetone and then rinsed with acetone. To coat fibroin molecules, the aminated coverslips were immersed in SF solutions with different concentrations and then dried in air. The incubation duration was 30 s to 60 min.

Shear loading

The coverslips coated with fibroin molecules were placed in a custom-designed parallel-plate flow chamber. The height of the chamber was determined by the thickness of the gasket. A syringe pump was used to control the flux of pure water. The shear rate (γ) on the bottom of the flow chamber, that is, the surface of coverslip, was calculated with the following equation:

$$\gamma = \frac{6Q}{wh^2} \tag{1}$$

where w and h are the width and height of the chamber, respectively, and Q is the flux of pure water. After the applied shear loading, the coverslip was taken out and observed with AFM immediately.

AFM observations

The topography of the coated fibroin molecules on the coverslips was observed with AFM (Autoprobe CP, Thermomicroscopes). A V-shaped silicon nitride cantilever (Park Scientific Instruments) with a spring constant of 10 pN/nm was adopted. The typical radius of curvature of the AFM tip was 50 nm. In the AFM measurements, the contact mode was used, the scan rate was 1 Hz, and the set point was adjusted to be 1.5 nN. Twenty-five percent over the scan was obtained to get rid of edges and damaged boundaries of images. All the measurements were carried out at room temperature, and all the chemical compounds were at the laboratory level.

Topographic data were analyzed with Imaging Process 2.1 software. The roughness values were reported as the root-mean-square (RMS) deviation (S_q) of the surface heights from the mean surface plane:

$$S_q = \sqrt{\frac{1}{n}\sum_{i}^{n}(Z_i - \bar{Z})^2} \tag{2}$$

where Z_i is the height of the ith point ($i = 1 \ldots n$) in an image and $\overline{Z}$ is the mean height.

RESULTS AND DISCUSSION

After the SF solution was prepared, the fibroin molecules were coated on the aminated slides. The con-

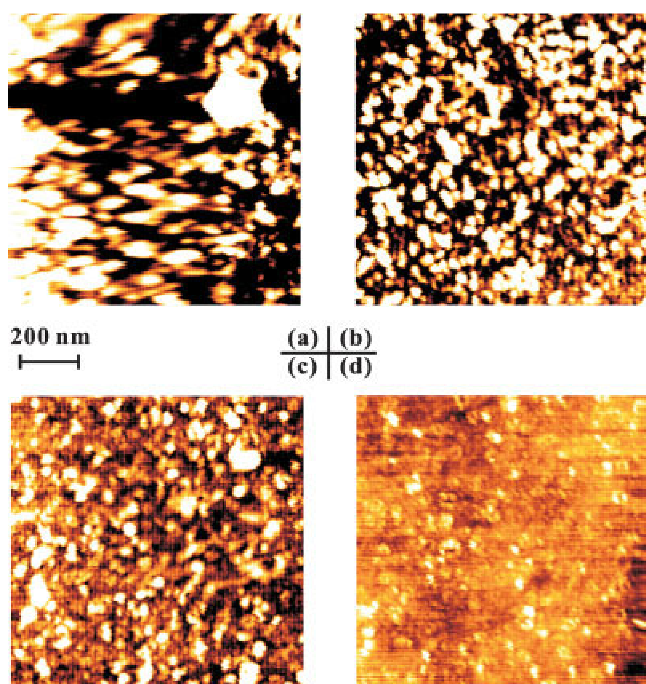

Figure 1 AFM observations of fibroin solutions with different concentrations: (a) 4.686, (b) 0.586, (c) 0.073, and (d) 0.009 mg/mL. The incubation duration for all solutions was 30 s. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

centrations of the incubation solutions varied from 4.686 to 0.005 mg/mL, and the incubation duration was 30 s. Figure 1 displays four typical AFM images with different concentrations. For the original SF solution of 4.686 mg/mL, the fibroin molecules aggregated into rodlike particles [Fig. 1(a)]. The size of the particles varied, with lengths of 100–200 nm and widths of 20–50 nm. When the original solution was diluted eightfold to 0.586 mg/mL [Fig. 1(b)], rodlike particles decreased, and some globules with a diameter of 50 nm could be observed on the surface of the slide. When the concentration decreased up to 0.073 mg/mL [Fig. 1(c)], the rodlike aggregations disappeared, and the globules were relatively dispersed. The globules were distributed more sparsely on the slides when the solution was diluted to 0.009 mg/mL [Fig. 1(d)]. These observations imply that if the solution were diluted to some concentration, the intermolecular entanglement would be broken, and the globules might be single molecules that folded themselves or oligomers such as dimers or trimers. The self-folding of a single fibroin molecule may produce more β-sheet structure than entangled molecules because of fibroin's specific amino acid sequences.[11] The circular dichroism results may support the assumption that there was a conformational transition from an α helix or random coil to a β sheet when the concentration changed from 0.586 to 0.073 mg/mL.[16]

Inoue et al.[12] obtained fibroin gel directly from the middle silk gland of *B. mori* and made use of AFM to observe the molecular structure for solution concentrations of 0.03, 0.18, and 0.64 wt %. They found that the fibroin molecule is rodlike at a lower concentration, and the rod is 60 nm long and 15 nm wide. If the concentration increases, these rodlike molecules can aggregate into threadlike particles. We obtained a fibroin sample by regenerating a silk fiber in a concentrated aqueous salt solution of LiBr. The globular micelles were observed in a more diluted regenerated SF solution. Some researchers also have observed globular micelles with 100–200-nm diameters in regenerated SF solutions blended with poly (ethylene oxide) in a ratio of 4 : 1. Why the fibroin molecules fold themselves as globular particles in regenerated SF solutions but not as rodlike ones in natural gel solutions should be further studied.

We measured the RMS roughness values of blank and aminated slide surfaces using AFM; they were around 0.5 nm. When SF solutions with different concentrations were incubated on coverslips for a constant duration of 30 s, the RMS roughness of the AFM images varied significantly (Fig. 2). For the highest incubation concentration, the RMS value was about 1.5 nm, and this meant that the molecules formed a very flat structure with good self-assembly. When the SF solution was diluted, the RMS values gradually increased, indicating the appearance of more separate aggregations. Further decreasing the concentrations of the SF solutions made the corresponding RMS values reach a peak and then decrease, and this was similar to the topography observations (Fig. 1); that is, large aggregations

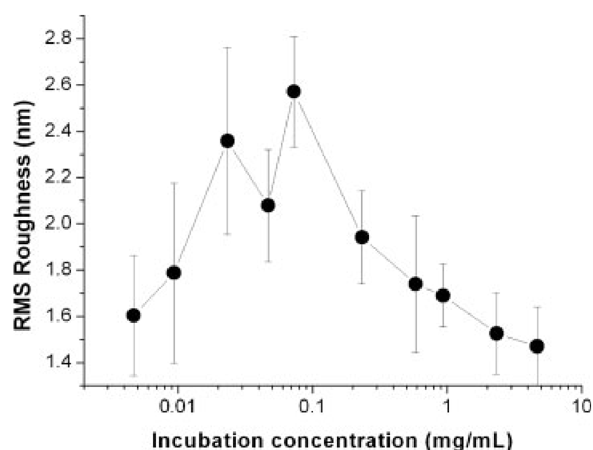

Figure 2 RMS roughness of AFM images for fibroin solutions with different concentrations. The data are presented as the mean/standard deviation of not less than five images randomly measured in the same coverslip. The incubation duration for all solutions was 30 s. The data points are connected by a line to reveal the variation tendency.

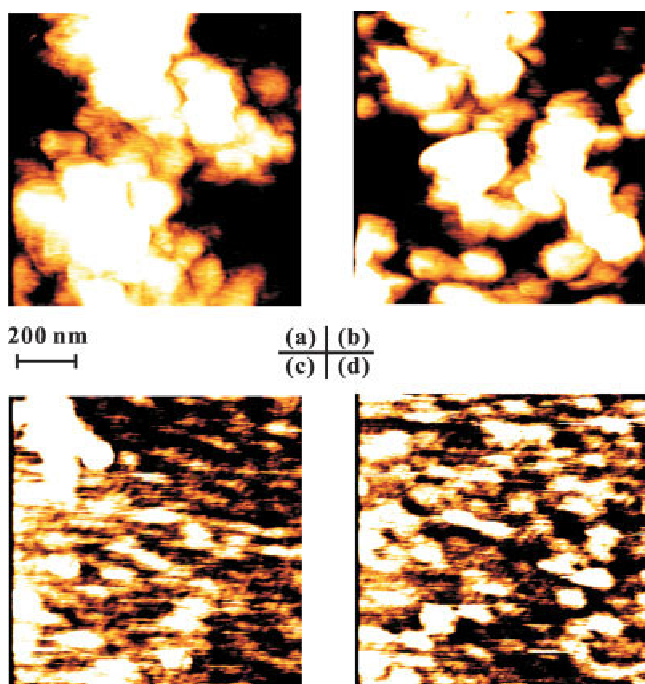

Figure 3 AFM observations of 0.026 mg/mL fibroin solutions with different incubation durations: (a) 5, (b) 10, (c) 20, and (d) 60 min. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

became smaller dispersed globules during this dilution process. The standard deviations of RMS became large when the concentrations decreased, especially around the maximum RMS values. This implies that when the interaction between molecules is reduced, the self-assembly of molecules is inhibited, and molecular aggregations become random and inhomogeneous even in one test.

When a fibroin solution is incubated on a slide for a longer time, more molecules may deposit on other ones, and the deposing process may redisplay the process of molecular aggregation in silk glands. An SF solution of 0.026 mg/mL was incubated on aminated slides 3 days after the preparation. The incubation duration differed (5, 10, 20, and 60 min). An interesting self-assembly process for fibroin molecules was found in the AFM images (Fig. 3). When the solution was incubated for 5 or 10 min [Fig. 3(a,b)], the molecules aggregated into larger particles than those after 30 s of incubation, and their size was about 100–200 nm. The particle size was similar to that in a solution of 4.686 mg/mL with 30 s of incubation, and this means that a longer incubation duration is actually equivalent to a higher concentration. When the incubation duration increased [Fig. 3(c,d)], the molecular layer became flat and not like the rough surface of the shorter incubation duration. A larger fraction of rodlike aggregations was found. This observation implied that the molecules approached one another with an increasing incubation duration and that molecular interactions made the molecules self-assemble into rodlike aggregations even though no shear stress was exerted.

When the incubation duration was increased, the RMS roughness of the AFM images increased first and then decreased significantly (see Fig. 4). The concentration of the SF solution in Figure 4 was smaller, that is, 0.026 mg/mL, and the globules with a diameter of 50 nm might have been dispersed in the solution before incubation [see Fig. 1(c)]. At the beginning of the incubation, some globules close to the aminated surface were grasped by the amine group. Other globules were deposited on the former grabbed ones or on the blank surface. Nevertheless, it seems that the interaction between the globules was stronger than that between the globules and surface because at this time the roughness increased dramatically, revealing that more nucleation between the globules occurred. Furthermore, when more fibroin molecules were deposited, the whole aminated surface was covered by aggregations, and the interactions between these larger aggregations became strong. Therefore, the molecular aggregations adjusted their structure to fit the enhancement of this interaction, which was reacted by the reduction of the RMS roughness of the protein film profile.

The fibroin globules or micelles expose most hydrophilic amino acids to the surrounding water. Because there are many more hydrophobic amino acids than hydrophilic ones in the *B. mori* fibroin heavy chains, a lot of hydrophobic amino acids are also exposed to water. It is known that hydrogen bonds are easy to form between the hydrophobic amino acids with small residues, so fibroin molecules may aggregate together, although this aggrega-

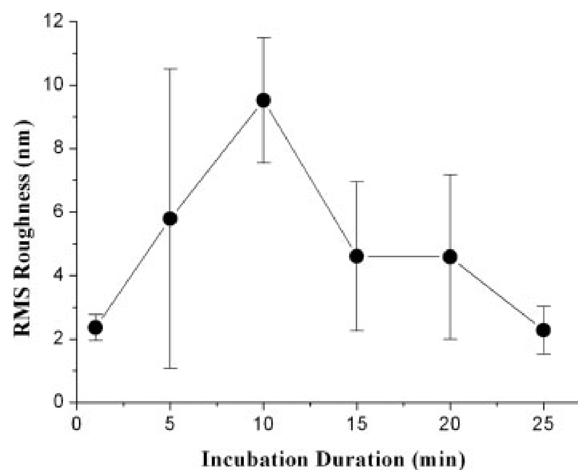

Figure 4 RMS roughness of AFM images for 0.026 mg/mL fibroin solutions with different incubation durations. The data are presented as the mean/standard deviation of eight images randomly measured in the same coverslip. The data points are connected by a line to reveal the variation tendency.

tion may be easily broken by Brownian motion. It is reasonable to imagine that this aggregation process may be regulated by self-assembly.

The concentration of the SF gel increases from the middle division to the anterior division of the silk gland of *B. mori*, so the previous analyses show that the fibroin molecules may first fold themselves at a low concentration because of the hydrophobic and hydrophilic interactions. Even when the molecules approach one another, they form only aggregations with weak intermolecular connections. These structural characteristics make the SF gel with a high concentration run through the silk gland without entanglement, which usually will degrade the conditions of artificial spinning. More interestingly, rodlike aggregations of fibroin molecules were self-assembled under the longer incubation duration even when no shear force was exerted. The mechanism of this phenomenon is worthy of further study.

By using flow chamber technology, we applied a shear loading to fibroin molecules coated on an aminated coverslip surface, and the incubation concentrations were 5.86 and 0.73 mg/mL. The rodlike molecular aggregations oriented obviously along the direction of fluid flow for both incubation concentrations [Fig. 5(a,b)]. For the high incubation concentration of 5.86 mg/mL, however, rodlike particles 700 nm long and 80 nm in diameter were formed, and they were much larger than those particles 200 nm long and 40 nm in diameter for the low incubation concentration of 0.73 mg/mL. A comparison with an AFM image of a longitudinal section of *B. mori* silk fiber [Fig. 5(c)][17] shows that the dimensions and orientation of the rodlike particles for a high concentration in our results are very similar to those of the fibrils in natural silk fiber, although the concentration of the SF solution coated on the coverslip is much lower than that in the silk gland of *B. mori*, which is about 30 wt %, that is, about 300 mg/mL.[4]

When the fibroin gel runs through the middle division to the anterior one of the silk gland, the wall shear stress gradually increases because of the decreasing gland tube diameter. Here we want to simply estimate the wall shear stress in the silk gland. On the basis of fluid mechanics theory, in a laminar flow tube, the wall shear rate at some position x can be expressed as $\gamma_0(x) = 4v_sR_s^2/R^3(x)$, where $R(x)$ is the radius of the tube at x and v_s and R_s are the flow rate and tube radius at some specific position, respectively. Here we assume that the radius of the spinneret of *B. mori* is about 8 μm in terms of the radius of the silk fiber,[18] and the spinning speed is 10 mm/s.[19] The radii of the anterior and middle divisions of the silk gland of *B. mori* are about 0.1 and 3 mm, respectively. Thus, the wall shear rates at the spinneret, anterior, and middle divisions of the silk gland can be calculated to be 5000, 2.4×10^{-5}, and 9×10^{-5} s^{-1}, respectively. Thus, in this study, the shear rate of 200 s^{-1} applied to fibroin molecules in the flow chamber is close to that in the middle and anterior divisions of the silk gland.

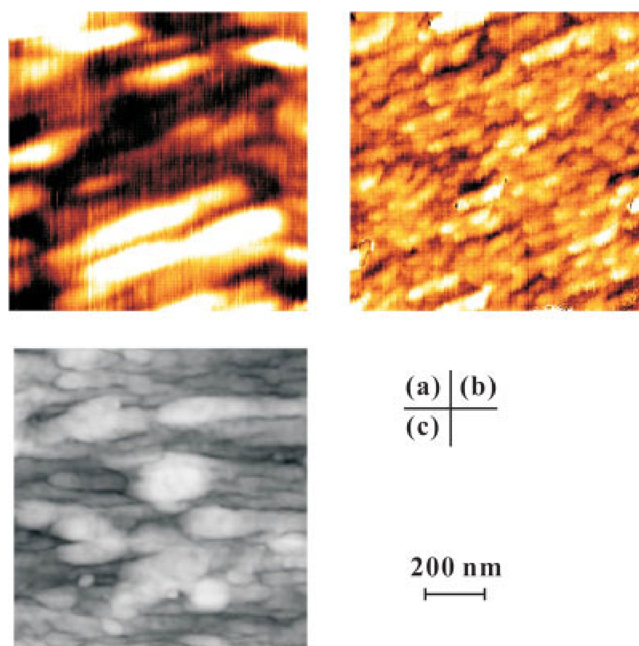

Figure 5 (a,b) AFM observations for shear-loaded fibroin solutions with concentrations of 5.86 and 0.73 mg/mL, respectively. The shear rate was 200 s^{-1}, and the shear loading duration was 30 s. The fibroin was incubated on the coverslips for 30 s. (c) AFM image of a longitudinal section of *B. mori* silk. (Reprinted with permission from Poza et al.[17] Copyright 2002 Elsevier Science Ltd.) [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

In this study, we aminated the coverslip surface to adsorb the molecules or molecular aggregations that gradually were deposited on the surface. For *B. mori*, those amino acids with free carboxyl groups that can bind with amino groups are small and are only located in the hydrophilic and amorphous domains,[9] which are exposed to the environmental solution in the exterior surface of fibroin molecule granules.[11] Thus, those amino groups on the coverslips can grasp only the residues sparsely distributed in the exterior surface and do not destroy the structure of the granules or aggregations.

CONCLUSIONS

A regenerated *B. mori* SF solution was prepared, and serially diluted solutions were coated on aminated coverslips. This study was focused on the direct observation of the topography of fibroin molecular aggregations for different solution concentrations and different incubation durations with AFM. Preliminary results of AFM observations of fibroin molecular aggregations under shear loading were also presented.

As the solution concentration decreased, the fibroin molecules revealed a transformation from rodlike aggregations 100–200 nm long to small globules 50 nm in diameter. When the concentration decreased to 0.073 mg/mL, the rodlike aggregations disappeared, and the sparsely distributed globules might have been single molecules or oligomers such as dimers or trimers.

The incubation duration could redisplay the concentrating process of the fibroin molecules. When the duration increased, the molecular layer on the coverslips gradually became flat from the originally rough surface, and this implied that as the molecules approached one another, the molecular interaction made the molecules self-assemble into rodlike aggregations even without shear stress. The measurements of the RMS roughness supported this conclusion.

By using flow chamber technology, we applied a shear loading to the fibroin molecules coated on the aminated coverslips. Under the application of the shear loading, the fibroin molecules formed larger rodlike particles, and these particles oriented obviously along the direction of fluid flow. The incubation concentration of the SF solution could influence the size of the particles.

References

1. Stokstad, E. Science 2000, 287, 1378.
2. Salem, D. R. Structure Formation in Polymeric Fibers; Hanser-Gardner: Munich, 2000.
3. Ayub, Z. H.; Arai, M.; Hirabayashi, K. Polymer 1994, 35, 2197.
4. Tsukada, M.; Obo, M.; Kato, H. J Appl Polym Sci 1996, 60, 1619.
5. Matsumoto, A.; Chen, J.; Colletee, A. L.; Kim, U. J.; Altman, G. H.; Cebe, P.; Kaplan, D. L. J Phys Chem B 2006, 110, 21630.
6. Braun, F. N.; Viney, C. Int J Biol Macromol 2003, 32, 59.
7. Vollrath, F.; Knight, D. P. Nature 2001, 410, 541.
8. Willcox, P. J.; Gido, S. P.; Muller, W. Macromolecules 1996, 29, 5106.
9. Zhou, C. Z.; Confalonieri, F.; Medina, N. Nucleic Acids Res 2000, 28, 2413.
10. Bini, E.; Knight, D. P.; Kaplan, D. L. J Mol Biol 2004, 335, 27.
11. Jin, H. J.; Kaplan, D. L. Nature 2003, 424, 1057.
12. Inoue, S.; Magoshi, J.; Tanaka, T.; Tanaka, T.; Magoshi, Y.; Becker, M. J Polym Sci Part B: Polym Phys 2000, 38, 1436.
13. Inoue, S.; Tsuda, H.; Tanaka, T.; Kobayashi, M.; Magoshi, Y.; Magoshi, J. Nano Lett 2003, 3, 1329.
14. Yamada, K.; Tsuboi, Y.; Itaya, A. Thin Solid Film 2003, 440, 208.
15. Pace, C. N.; Vajdos, F.; Fee, L. Protein Sci 1995, 4, 2411.
16. Li, G. Y.; Zhou, P.; Shao, Z. Z.; Xie, X. Eur J Biochem 2001, 268, 6600.
17. Poza, P.; Perez-Rigueiro, J.; Elices, M.; Llorca, J. Eng Fract Mech 2002, 69, 1035.
18. Huo, B.; Zhai, Y.; Cui, F. Z. Acta Polym Sinica 2002, 3, 261.
19. Shao, Z. Z.; Vollrath, F. Nature 2002, 418, 741.