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Effect of processing temperature on the morphology of silk membranes

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Dedicated to Professor Imanishi on the occasion of his retirement

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Abstract

A concise literature survey concerning the processing and uses of silk membranes is presented in this note together with initial observations of new morphological data for the effect of processing temperature on morphology. Liquid silk from the middle section of the Middle Division of the silk gland of *Bombyx mori* was cast onto glass plates at 20, 40, 50, 60 and 80 °C. Silk from the anterior and posterior sections was cast at 20 °C. Samples cast at 20 °C exhibit particles, grains, nanofibrils and an irregular morphology. Each exhibits approximately the same dimensions for all the samples. Samples cast above 20 °C do not exhibit the irregular morphology. Samples cast above 50 °C exhibit larger grains and larger, more densely packed nanofibrils. All these changes might result from conversion of the amorphous structure to the β-pleated structure (Silk II). The nanofibrils appear to be self-assembled bio-nanofibrils. Membranes of regenerated fibroin treated with aqueous methanol solution exhibit grains and apparent nanofibrils. Opportunities for further work are pointed out.

Keywords: Membranes; Morphology; Liquid silk

1. Introduction

Silks can be processed into various forms including gels, powders, fibers, and membranes. Although silks have been used for more than 5000 years [1,2], interest in membranes has grown only within the last few decades [3–82].

Transparent membranes of silk fibroin have been processed with liquid silk from the gland, as well as solutions made with silk fibers dissolved in alkali salt solutions, other types of solutions, and organic solvents. Blend solutions of two different fibroins or one fibroin with cellulose, polyvinyl alcohol, polyurethane, cellulose acetate, chitosan, sodium polyglutamate, polyethyleneglycol, sodium alginate, and *S*-carboxymethyl keratin have also been used. A variety of processes have been employed. These include casting followed by air-drying or freeze drying, mechanical shearing, compression, the Langmuir–Blodgett technique, and bubble blowing. Processing conditions such as solution concentration, solution temperature, quenching temperature, drying rate, drying temperature, drying time, the presence of an electric field, pH, the presence of certain enzymes and the type of solvent can be used to control the molecular conformation in the resulting membrane. Casting onto different surfaces such as polyethylene, glass, polytetrafluoroethylene, polypropylene, polycarbonate, polystyrene, acrylics, polyvinylchloride, polyacrylonitrile, mercury, nylon 66, polyvinylidene chloride, and platinum wire also can affect the conformation [3–17,19–48,50–52,54–62,64–69,71,73–82].

Post processing treatment parameters have included: dry heat, steam heat, stretching, stretching rate, stretch ratio, and the application of pressure. Other parameters have included the application of hydrophilic polar organic solvents and their water solutions, application of salt solutions and hot water as well as application time. Solid membranes and porous membranes have been made. There has been work on the fibroin of *Bombyx mori*, *Antheraea yamamai*, *Antheraea pernyi*, *Dictyoploca japonica*, *Attacus ricini*, and a little work on sericin from *B. mori* [3–8,11–15,17–19,27,28,30,32–34,39–51,53,54,57–60,62,63,65,67,68,71,73–76,78–80,82].

Membranes have been made for a variety of reasons. One is to hold various molecules such as different enzymes for

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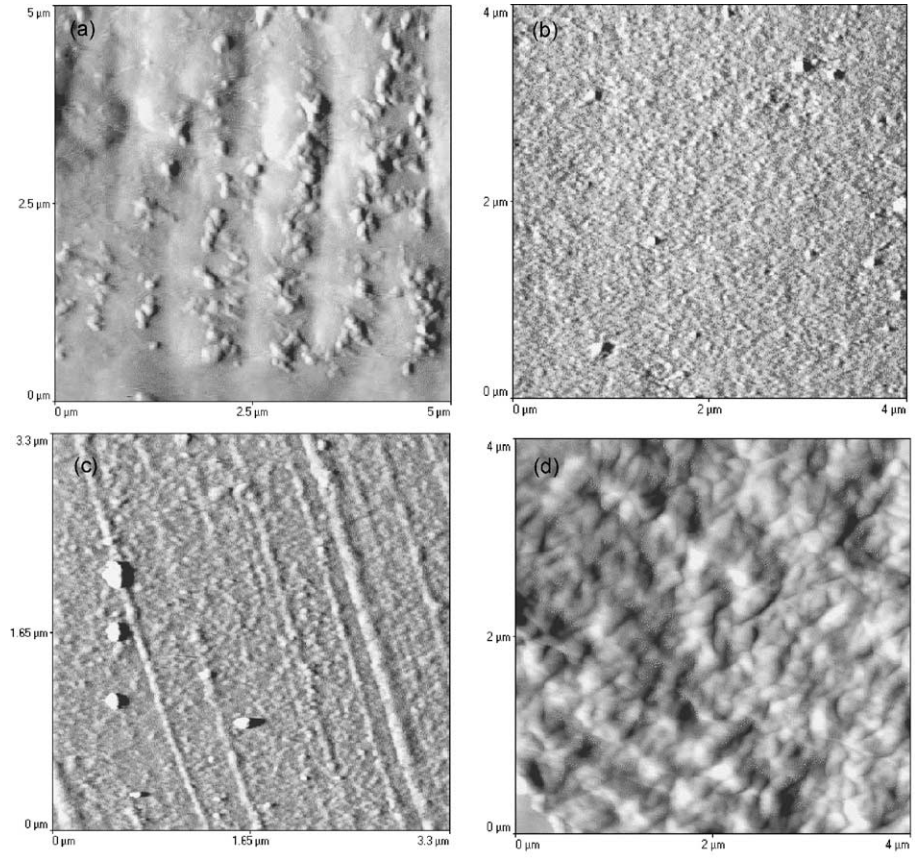

Fig. 1. AFM images of morphologies observed in membranes prepared with liquid silk from the gland of *B. mori*, Mp20: (a) particles; (b) grains; (c) thinly packed nanofibrils; and (d) the irregular morphology.

application in various bio-sensors and other detectors. The guest molecules are trapped when the conformation of the fibroin is converted from amorphous or Silk I to Silk II. The entrapment is physical rather than covalent and, thus, has a minimal effect on activity. The entrapment is also stable, thus providing long-term activity of the detectors. Certain blends have been shown to improve the anti-thrombogenic properties of silk. Silk membranes have been investigated for controlled release of drugs and as a platform for growing animal cells [3,6–9,15,20–34,43,49,56,57–61,68,69,71–73,79,82].

Membranes also have been used as materials for the study of the Silk I, Silk II and amorphous conformations, as well as the transformations among them under the influence of the processing and post processing parameters discussed above. Spectroscopic, thermal, light scattering, density, contact angle, crystallinity, transparency, X-ray, birefringence, and microscopic measurements have been made. Other properties, such as mechanical, swelling, thermal expansion, ion permeability, as well as the diffusion and solubility of small molecules, have been measured because of their relevance to potential applications. There have been relatively few morphological observations and these have not been detailed especially with respect to the effect of processing temperature [3–5,8–15,17–39,40–60,62–67, 69,71,74–82]. Therefore, this note reports initial observations of new morphological data for the effect of casting temperature on morphology.

2. Experimental

2.1. Sample preparation

2.1.1. Liquid fibroin

The liquid fibroin samples were taken from the Middle Division (storage) of the silk glands of mature silkworms of *B. mori* one day before spinning. The Middle Division was cut into three sections: the posterior section, Mp (toward the worm's synthesis area), the middle section, Mm, and the anterior section, Ma (toward the worm's spinnerets). The sericin was removed by washing thoroughly under de-ionized running water [37,39]. Aqueous solutions of fibroin were prepared from each section by dispersing the silk gel in nanopure water. The solutions were then decanted and filtered. Membranes of Mm were formed by casting the solutions (about 0.35 wt%) onto glass plates and annealing/drying at 20, 40, 50, 60, or 80 °C (Mm20, Mm40, Mm50, Mm60, and Mm80, respectively). Membranes of Ma and Mp were formed by casting the solutions onto glass plates

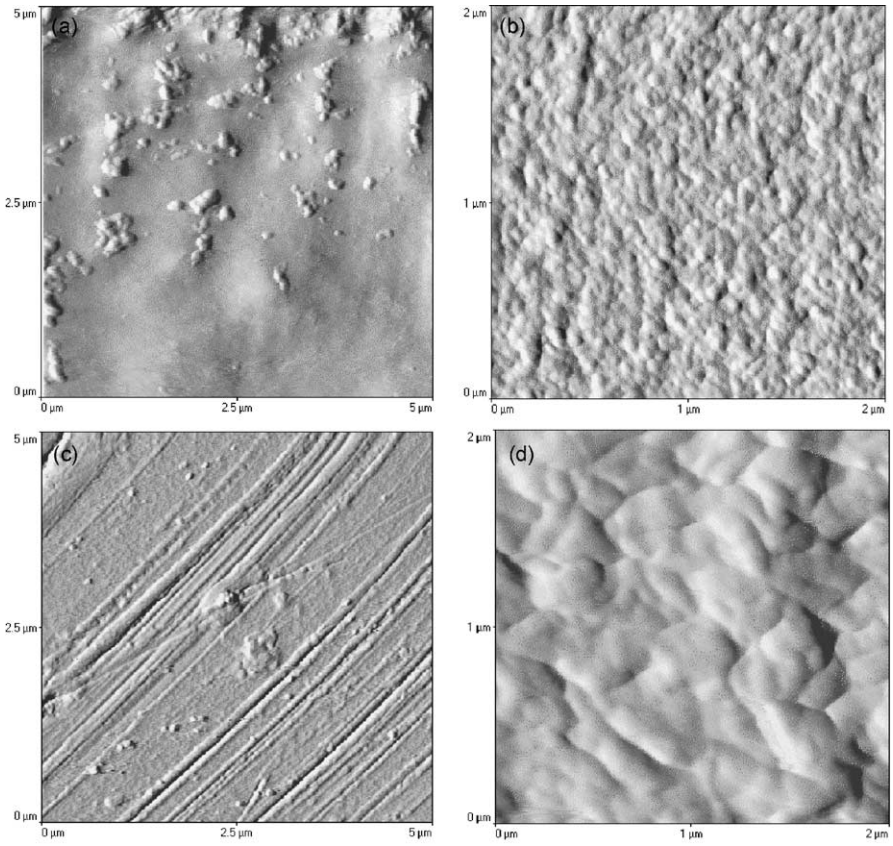

Fig. 2. AFM images of the morphologies observed in membranes prepared with liquid silk from the gland of *B. mori*, Mm20: (a) particles; (b) grains; (c) nanofibrils; and (d) the irregular morphology.

and annealing/drying at 20 °C (Ma20 and Mp20). All the membranes were dried uncovered in contact with air. The average thickness was 15–35 μm.

2.1.2. Regenerated fibroin

Regenerated silk fibroin membranes were prepared by the following procedure. The cocoon silk of *B. mori* was degummed with aqueous boiling 0.5% (w/v) Na_2CO_3 solution and subsequently dissolved in calcium chloride/ethanol/water solution (1/2/8 mol ratio) at 70 °C for 2 h. After the solution was filtered, dialysis was continued for 3 days against running ion-exchanged water to remove $CaCl_2$ and ions present in the fibroin [83] using a cellulose semipermeable membrane. Thin membranes of the fibroin were cast onto polystyrene petri dishes and dried at 30 °C in air for 48 h (R30). After that, the membranes were treated with a 75 vol% aqueous methanol solution for 15 min and dried. They were 20 μm thick.

2.2. Microscopy

Scanning electron microscopy (SEM) was performed with either a Hitachi S-4700 low voltage high resolution SEM (LVHRSEM) operating at 0.5 keV without a conductive-coating on the sample or a more conventional SEM operating at 15 keV. The LVHRSEM was equipped for X-ray Energy Dispersive Spectroscopy (EDS). Samples were held in place on the sample holders with silver paint. An optical lever type Atomic Force Microscope (AFM), Topo-Metrix 2010, was used in the repulsive contact mode at ambient conditions. Small pieces of the membranes were mounted on the sample holder with two-sided tape. Images were obtained with a constant force of approximately 10^{-10}–10^{-9} N. Cantilevers with a silicon nitride tip of approximately 50 nm radius were used. The scanning frequency was 2 or 3 Hz with 400 data points being taken on each of the scan lines. Measurements of the observed features were made using image analysis software built into the AFM operating system. Since the dimensions covered a broad range, the *approximate* minima and maxima are presented.

3. Results

3.1. Liquid fibroin

All the membranes prepared from liquid silk were transparent. Initial AFM observations revealed a range of different finer scale morphologies. Therefore, about 100 images were made of each of the seven membranes in order to ensure a good sampling of the types of morphology present.

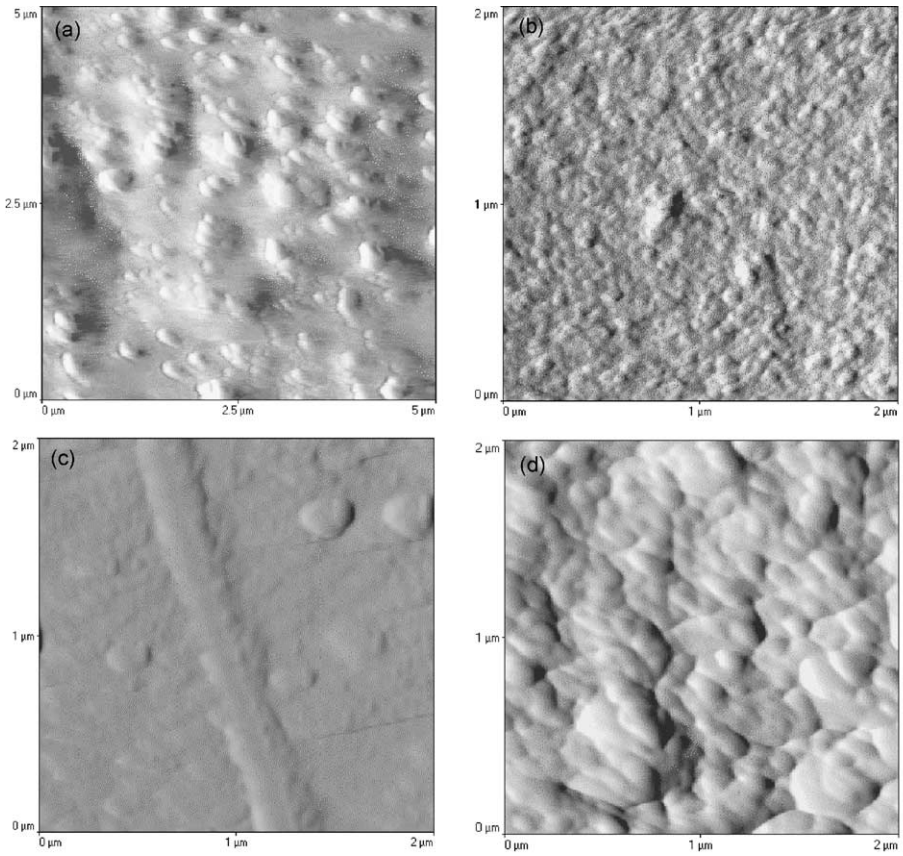

Fig. 3. AFM images of the morphologies observed in membranes prepared with liquid silk from the gland of *B. mori*, Ma20: (a) particles; (b) grains; (c) a less densely packed and completely different nanofibril structure; and (d) the irregular morphology.

While there was a broad distribution of morphologies, the results fell into four major categories.

The morphologies observed are shown in Fig. 1 for Mp20. One type is relatively small particles which are sometimes found to be relatively isolated and sometimes somewhat aggregated in small arrays (Fig. 1a). EDS analysis showed that they are organic. Illustrative of one of the other types of morphology are the densely packed grains (Fig. 1b), which somewhat resemble the internal surfaces of regenerated fibroin [10,12]. Another morphology is illustrated by the not so well developed, relatively small and thinly packed nanofibrils in Fig. 1c. A fourth example is given by the irregular morphology shown in Fig. 1d. Features akin to all those above are shown for Mm20 in Fig. 2. Similar features also are exhibited by Ma20 in Fig. 3. The nanofibrils in this sample are, however, less densely packed and rather different (Fig. 3c). The data in Table 1 show that with the exception of these nanofibrils, all the membranes cast at 20 °C exhibit features which are similar and of similar size. (Note that dimensions are not given for the irregular morphology which are difficult to characterize.) The different morphologies were found more or less uniformly over the surface of these three membranes as were the morphologies of the other membranes discussed below.

Table 1
Range of dimensions (nm) of morphological features found in membranes cast at 20 °C

Sample	Features		
	Particles	Grains	Nanofibrils
Mp20	90–270	60–160	50–200
Mm20	70–250	50–170	50–260
Ma20	90–310	50–150	See text

The results for Mm40 and Mm50 are shown in Figs. 4 and 5. They are not too different from those for Mm20 with the exception that the irregular morphology of Mm20 in Fig. 2d is not observed in the samples cast at higher temperatures. For Mm60, the particles in Fig. 6a are qualitatively similar to those discussed above. The grains in Fig. 6b are similar to, but somewhat larger than those presented above for Mm20, Mm40 and Mm50 (Table 2). The nanofibrils in Fig. 6c are different, being larger, and more densely packed than those in Figs. 2c, 4c and 5c. The morphological features of Mm80, similar to those of Mm60 with more developed nanofibrils in Fig. 7c. Since the nanofibrils in Fig. 7c appear to have a substructure, the overall dimension was taken as the upper range and the substructures dimension as the lower range in Table 2.

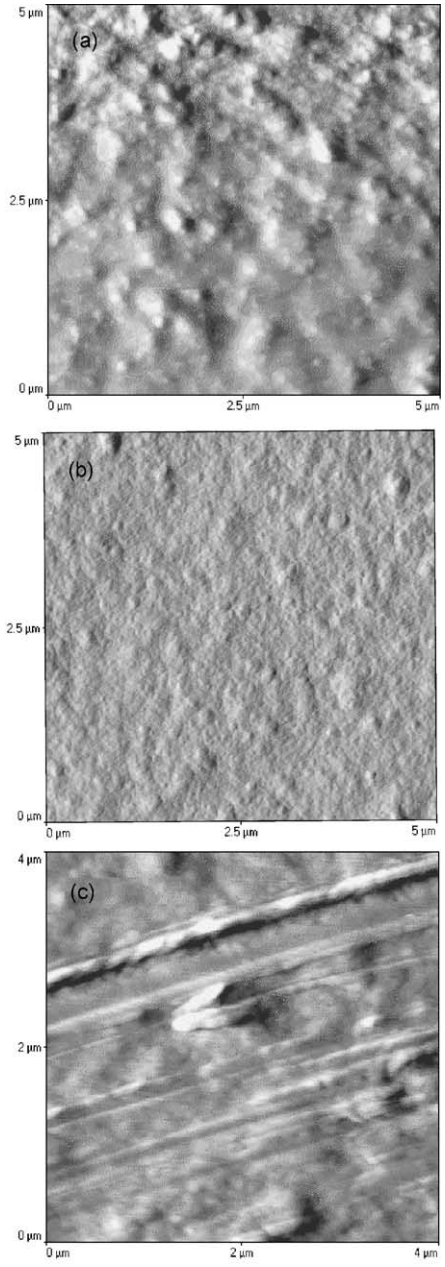

Fig. 4. AFM images of the morphologies observed in membranes prepared with liquid silk from the gland of *B. mori*, Mm40: (a) particles; (b) grains; and (c) nanofibrils.

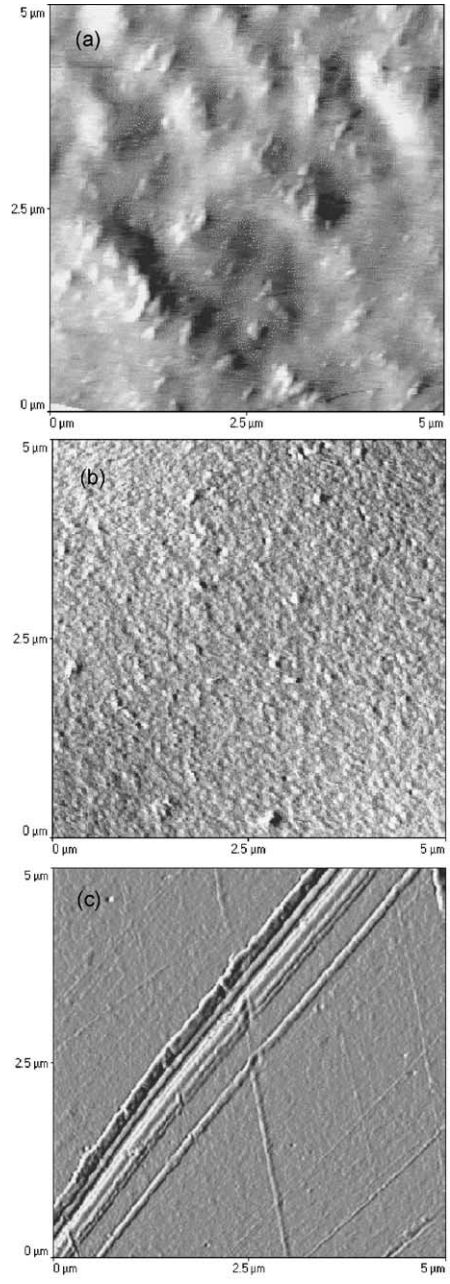

Fig. 5. AFM images of the morphologies observed in membranes prepared with liquid silk from the gland of *B. mori*, Mm50: (a) particles; (b) grains; and (c) nanofibrils.

In summary, the particles are nearly the same size through the whole range of casting temperatures and samples from different parts of the gland (Tables 1 and 2). The grains are nearly the same for samples from different parts of the gland cast at 20 °C. They become quite a bit larger for Mm at casting temperatures of 60 and 80 °C. With the exception of those cast with fibroin from the anterior part of the gland, the nanofibrils are similar in size for casting temperature of 20–50 °C. At the higher temperatures they increase markedly in development and size. The irregular morphology is also observed for all the samples cast at 20 °C.

3.2. Regenerated fibroin

These membranes did not exhibit either the small particles or the irregular morphology. They did, however, exhibit aspects of the other morphologies observed for the membranes made with liquid silk from the gland. For example, Fig. 8 shows an AFM image of grains which are somewhat similar to that shown in Fig. 7b and the other previous figures. Fig. 9 shows a LVHRSEM image of the grains also somewhat similar to those shown in Fig. 7b and the

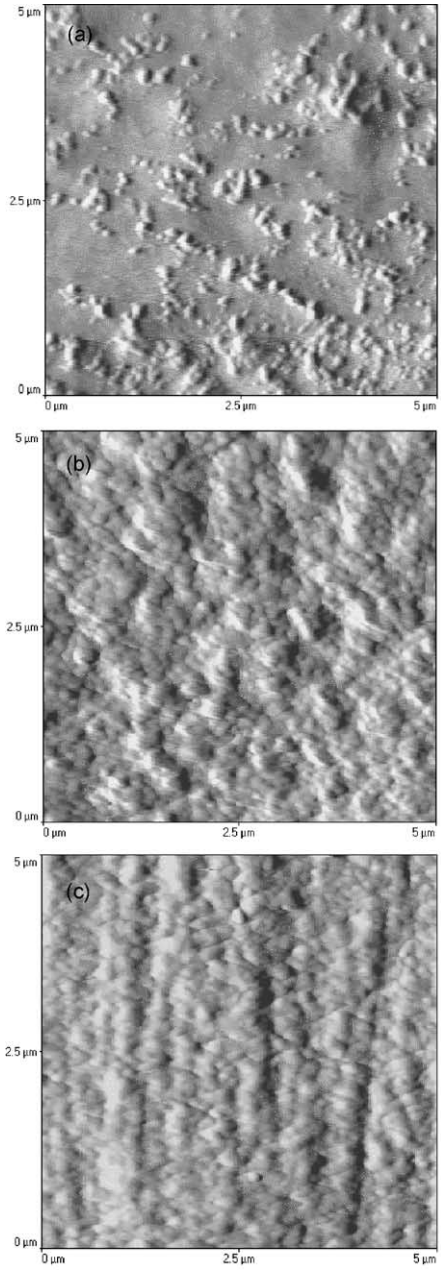

Fig. 6. AFM images of the morphologies observed in membranes prepared with liquid silk from the gland of *B. mori*, Mm60: (a) particles; (b) grains; and (c) more densely packed, larger, nanofibrils.

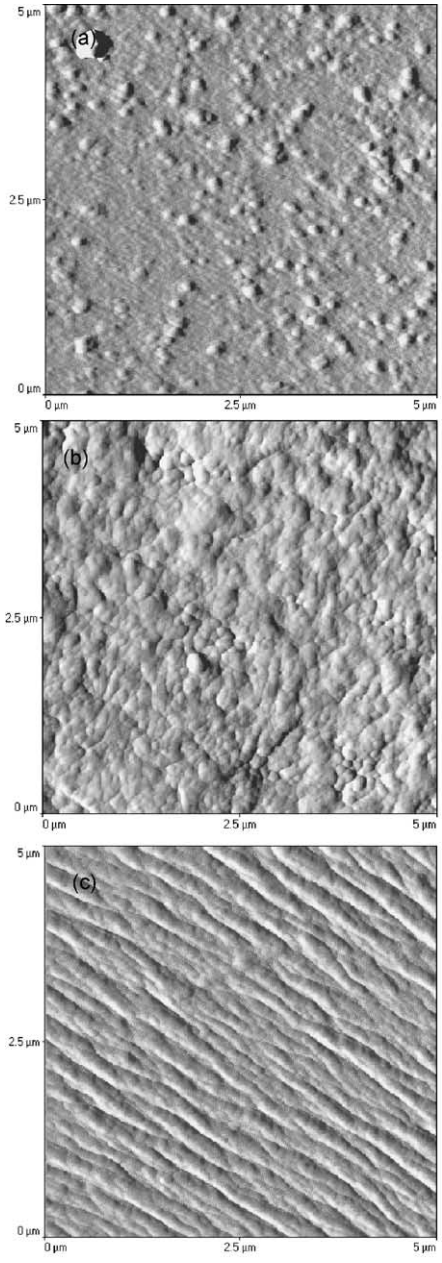

Fig. 7. AFM images of morphologies observed in membranes prepared with liquid silk from the gland of *B. mori*, Mm80: (a) particles; (b) grains; and (c) densely packed, more developed nanofibrils.

Table 2
Range of dimensions (nm) of morphological features found in samples of Mm cast at different temperatures

Sample	Features		
	Particles	Grains	Nanofibrils
Mm20	70–250	50–170	50–260
Mm40	90–270	60–210	60–210
Mm50	90–290	60–150	50–230
Mm60	70–240	80–410	120–420
Mm80	80–200	70–270	70–330

preceding figures. Finally, Fig. 10 shows an SEM image containing what appear to be nanofibrils.

4. Discussion

The statistics for the images must be treated with considerable caution. However, it is worth noting that the membranes produced at 20 °C exhibited the most images with particles whereas those produced at 80 °C exhibited the most images with nanofibrils and grains. The nanofibrils are similar to those in fibers spun by *B. mori* and other

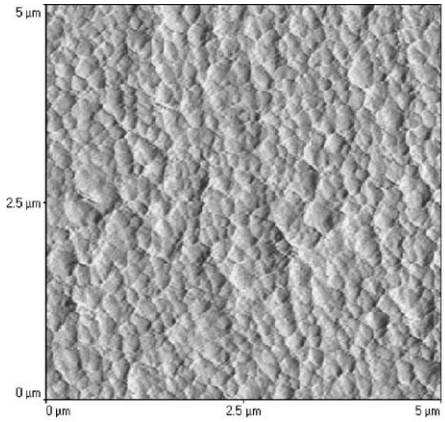

Fig. 8. AFM image of grains in a membrane prepared with regenerated fibroin of *B. mori*, R30.

silkworms [83,84], in that they are, for the most part, well oriented, densely packed, and exhibit diameters of ~70–330 nm. Their 'rough' surface features and their apparent entering and leaving the surface (Fig. 7) are similar to the nanofibrils on the surface of the degummed silk and less similar to those on the surface exposed by peeling [83,84]. Their composite structure is somewhat similar to that exposed by peeling fibers of *B. mori* to reveal the internal morphology [83]. They are larger than those reported for electrospinning [85,86].

Nanofibrils can be produced in a wide range of synthetic and natural polymers by a variety of methods [84]. In addition to the many methods mentioned in Ref. [84], such fibrils also can be formed by extruding thermoplastics through a row of fine orifices into two converging high velocity streams of heated gas [87]. Another method is to co-extrude a mixture of two polymers with an appropriate take up speed and degree of immiscibility [88–90]. The fibrils described here were not wittingly produced by any of those many methods which tend to extend the molecules. Especially, the fibrils described here differ from the fibrils produced by spiders and silkworms, in that the present ones were not produced by having the silk flow

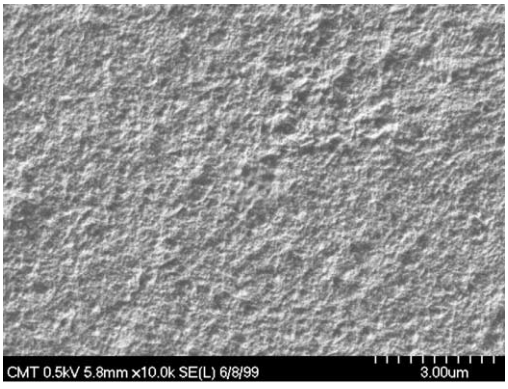

Fig. 9. LVHRSEM image of grains in a membrane prepared with regenerated fibroin of *B. mori*, R30.

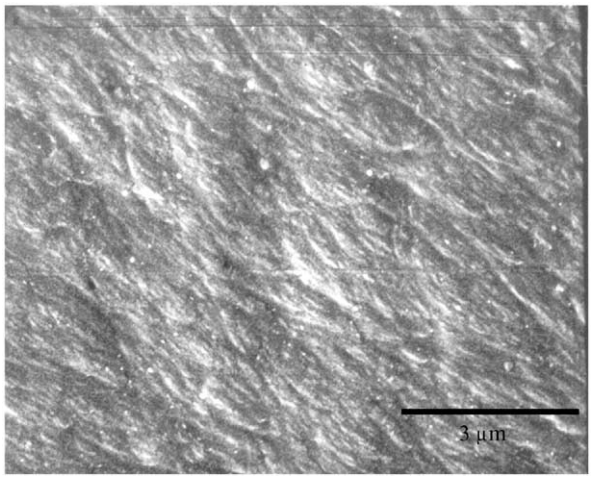

Fig. 10. SEM image of apparent nanofibrils in a membrane prepared with regenerated fibroin of *B. mori*, R30.

through the Middle Division and the long narrow spinnerets in the Anterior Division of the gland of the silkworm or the spider. They appear to be self-assembled bio-nanofibrils.

Beyond the frequency of observations mentioned above, there also is a general trend for the grains and the nanofibrils of Mm cast at 60 and 80 °C to be somewhat larger and more well-developed than those produced at 20 °C. Further, those produced at 40 and 50 °C were closer to those produced at 20 °C. It seems likely that these results might be associated with the conversion of the amorphous structure to Silk II at casting temperatures above 40 °C as indicated, for the solution concentration used, by the phase diagram established with X-ray and spectroscopic measurements [45]. (It seems possible that the conversion might also be associated with the absence of the irregular morphology at higher temperature.) Sheets of Silk II tend to curl, coil helically, and/or twist and can take on many different shapes [84,91]. The effect has also been observed by electron microscopy for single crystals and aggregates of single crystals [92] as well as by computational modeling of proteins including silk [93]. Thus, these effects also might play a role in the changes of the nature of the nanofibrils that occur at higher casting temperatures. Further, the conversion to these shapes might also provide mechanisms for the physical trapping of 'guest' molecules within silk membranes.

5. Conclusions

Membranes made with liquid silk from the Middle Division of the silk gland were cast at 20, 40, 50, 60, and 80 °C. These new observations revealed a broad range of morphological features. Four major classes were identified. These are particles, grains, nanofibrils, and an irregular morphology. The morphologies of all membranes prepared at 20 °C were somewhat similar with the exception of the rather

different nanofibrils of Ma20. The irregular morphology was not observed for casting temperatures above 20 °C. Membranes cast at temperatures above 50 °C exhibit larger grains and nanofibrils with the structure of the Mm80 nanofibrils being a composite one. In addition, the packing densities of the 60 and 80 °C nanofibrils were greater than those for 20, 40 and 50 °C. It is proposed that all these differences might be the result of conversion of the amorphous conformation to Silk II. It is further proposed that the curled, coiled, twisted, and other shapes which Silk II can take might lead to the physical entrapment of additive molecules. Thus, membranes containing smaller molecules are converted to Silk II for stability. Membranes of regenerated fibroin cast at 30 °C and treated with aqueous methanol solution exhibited grains and nanofibrils. There are a number of opportunities for further work including investigations of the effect of different solution concentrations, solvents, salts, drying conditions, etc.

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