Preparation and Application of Porous Silk Fibroin Materials

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SYNOPSIS

This article deals with the preparation and morphological characterization of porous materials obtained by freezing and lyophilizing silk fibroin solutions. When an aqueous silk solution is frozen at different temperatures (−18, −45, and −80°C), the average pore size decreases with lowering of the freezing temperature. Silk fibroin aggregates obtained in these conditions exhibit a sheetlike structure. By lowering the pH from neutrality to 4.01 and 2.65, the morphology of the solid phase changes from a sheet to a fiber structure. The average pore size is smaller at pH 4.01 than is the former value corresponding to the isoelectric point of silk fibroin. The addition of different amounts of methanol to the silk solution results in a sharp fall of the average pore size and hardens the material, as a consequence of the high packing density of the fibroin molecules. Silk fibroin aggregates prepared in these conditions exhibit a typical fibrous structure. A drug-delivery system was prepared by incorporating acetylsalicyclic acid into a porous silk fibroin carrier, and the kinetics of the drug release was studied. © 1994 John Wiley & Sons, Inc.

INTRODUCTION

Silk fibroin is an attractive natural fibrous polymer produced by different species of silkworms. Among the wide variety of silks, that produced by the species *Bombyx mori* (domestic silk) has been extensively investigated.[1,2] Besides the use as a textile fiber, silk fibroin has been recently studied as a starting material for nontextile applications. These include enzyme immobilization for the preparation of biosensors[3] and the production of oxygen-permeable membranes.[4] However, the scientific interest in this field is growing rapidly; hence, the number of new applications is believed to be increasing more and more. Some inferior physical and mechanical properties of silk fibroin membranes can be improved by blending with other natural or synthetic polymers, such as sodium polyglutamate,[5] sodium alginate,[6] chitosan,[7] cellulose,[8] and poly(vinyl alcohol).[9]

Silk fibroin exhibits some interesting advantages compared to other biopolymers. High-purity silk fibroin can be easily obtained from cocoons in fiber form. The fibers can be dissolved by means of neutral concentrated salt solutions (LiBr, LiSCN, $CaCl_2$), without inducing hydrolytic degradation. Aqueous silk solutions represent a good starting material for the preparation of different kinds of fibroin-based materials, such as gel, powder, porous membranes, and homogeneous membranes. The solubility of silk membranes can be controlled by immersion in water/methanol solutions and by means of other physicochemical treatments.[10] Homogeneous membranes exhibit a very dense structure, due to the fact that during evaporation the polymer concentration increases, reaching a high packing density. Hirabayashi et al.[11] reported some properties of silk fibroin gels and characterized the coarsely porous structure of the solid material obtained from them.

The use of porous substrates is highly attractive for several biotechnological and biomedical applications, because the high surface area available and the presence of a network of interconnected pores can favor processes related to diffusion, permeation, enzyme–substrate reaction, etc. Moreover, porous polymeric materials can be successfully exploited as carriers for the controlled delivery of drugs.[12] Various kinds of natural and synthetic polymers can be em-

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ployed in the preparation of these devices, the choice being based on the physicochemical requirements of the drug–polymer system considered, especially as concerns the incorporation of the drug, the administration site, and the kinetics of delivery.

This article deals with the preparation of porous materials by freezing and lyophilizing silk fibroin solutions. The influence of different parameters on membrane morphology and pore-size distribution were studied by modifying the freezing temperature and the starting pH of silk solution and by adding organic solvents to the fibroin aqueous solution. The properties of a drug-delivery system, prepared by incorporating acetylsalicyclic acid into a porous silk fibroin carrier, were preliminarily investigated by measuring the kinetics of the drug release.

EXPERIMENTAL

Materials

Degummed silk fibers were obtained from *Bombyx mori* cocoons by treatment with a 0.2% aqueous solution of Marseille soap containing 0.05% Na_2CO_3, at 98°C for 30 min, followed by washing with distilled water and drying at room temperature. The regenerated silk fibroin solution was prepared by dissolving the fibers with a 9*M* LiBr solution at 50°C for 15 min. The salt was completely removed by dialysis against distilled water for 3 days at 5°C. The final concentration of silk fibroin was adjusted by slowly drying the solution in air.

Three series of porous silk fibroin materials were prepared as follows: In the first series (A), fractions of a 2.2% (w/v) silk fibroin solution were frozen at different temperatures (−18, −45, and −80°C) and then lyophilized under reduced pressure (10^{-2} mmHg), using a Tozai Tsusho lyophilizer. The initial temperature of the frozen gel sample was −10°C, which increased to 25°C in 12 h by the controlled heating system. The second series (B) was prepared from a 0.8% (w/v) silk fibroin solution coagulated at different pH. One sample was brought to pH 2.65 by addition of acetic acid to the liquid silk and stored at 5°C for 12 h until coagulation occurred. The coagulated silk fibroin was then collected by decantation, frozen at −80°C, and lyophilized as described above. Another sample was adjusted to pH 4.01 and by dialysis against a phtalic acid solution (pH 5) for 12 h at 5°C. The coagulum was then frozen at −80°C and lyophilized. The third series (C) was prepared from a 0.6% (w/v) silk fibroin solution. Fixed volumes of water/methanol solutions were added in order to attain a final methanol concentration ranging from 10 to 40% (v/v). The mixed solutions were stored at 5°C for 1 day until coagulation occurred. The coagulum was then collected by decantation, frozen at −18°C, and lyophilized. One sample of 0.6% (w/v) silk fibroin solution was rapidly coagulated by dialysis against 100% methanol at 5°C for 1 day. After centrifugation at 3500 rpm for 10 min, the coagulum was frozen at −45°C and lyophilized.

For the drug-delivery test, the drug-containing silk membrane was prepared by carefully adding 50 mL of liquid silk (1.5% w/v) to 100 mL of acetylsalicyclic acid (9.2 mg) solution. The mixed solution was dialyzed against water at pH 4.0 by acetic acid, at 5°C for 12 h, to promote coagulation. After centrifugation at 3000 rpm for 20 min, the coagulum was frozen at −80°C and then lyophilized.

Measurements

The porosity of the different samples of silk fibroin materials was evaluated by measuring the pore-size distribution using a scanning electron microscope (JEOL 333S). Cross sections were cut and examined at 15 kV acceleration voltage after gold coating. Samples were first observed at low magnification, then four fields at 500× were selected and photographed for pore-size measurement. About 20 pores per each sample were measured manually, referring to the scale marker on the micrographs for calculating the original pore size.

The drug-delivery test was carried out spectrophotometrically, using a Shimadzu UV-200S spectrophotometer. The porous silk fibroin carrier, 36 mg, containing the drug was immersed into 3 mL of distilled water directly in the measuring quartz cell. The concentration of acetylsalicylic acid released into the solution was determined by measuring the UV absorbance at a wavelength of 206.9 nm as a function of time.

RESULTS AND DISCUSSION

Morphology of Porous Silk Fibroin Materials

Effect of the Freezing Temperature

The influence of different freezing temperatures on the porosity of silk fibroin materials was studied by freezing aqueous silk solutions at −18, −45, and −80°C. The cross-sectional morphology of the membranes obtained after lyophilization was examined by scanning electron microscopy (Fig. 1).

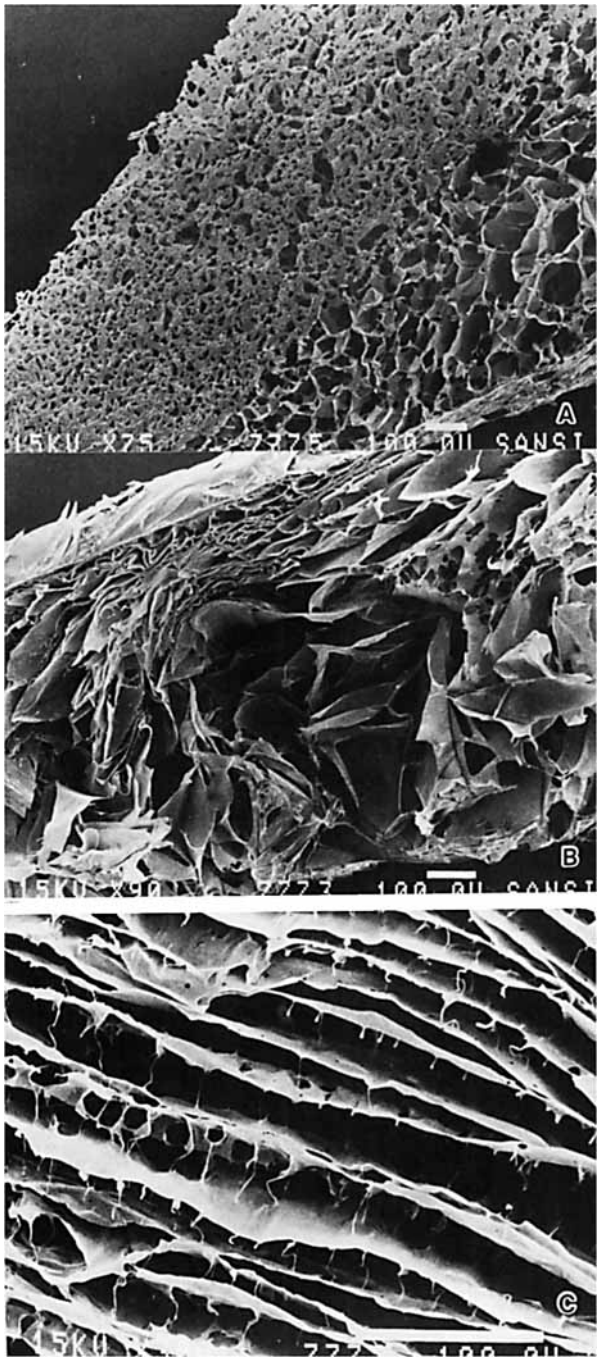

Figure 1 SEM photographs of porous silk fibroin membranes prepared by freezing aqueous silk solutions at different temperatures: (A) −18°C; (B) −45°C; (C) −80°C.

The sample frozen at −18°C (a) exhibits a coarsely porous structure, the pores becoming larger near the air surface. The change in shape from a closed to an open sponge observed on approaching the top layer of the membrane could be attributed either to the coagulation of silk fibroin and migration of water during freezing or to the expansion of the material that occurred during the lyophilization process. The SEM photograph of the silk membrane obtained after freezing at −45°C shows that silk fibroin aggregates preferentially take a leaf shape. This kind of structure, partially visible also in the coarse pore region of the sample frozen at −18°C, delimitates variously shaped voids, probably interconnected among them. The sample frozen at −80°C presents a clearly defined sheet structure, with large fibroin sheets running parallel to each other and connected by very thin bridges made of the same material.

The pore-size distribution of the different materials was measured on the basis of the SEM images and plotted as a function of the freezing temperature (Fig. 2). These data show that the pore size is characterized by a large variability within the same sample, as shown by the width of the error bars. The average values tend to decrease with lowering the freezing temperature, the drop being more significant in the range from −45 to −80°C.

The above SEM observations point out that both the morphology and the average pore size of porous silk fibroin membranes seem to be markedly affected by the freezing temperature to which liquid silk has been subjected. These results can be explained in terms of temperature gradient between the fibroin solution and the cooling system, the other parameters (sample size and starting temperature) being constant. On cooling, the fibroin solution becomes less stable and tends to demix as a result of the coagulation of protein chains. The higher the cooling rate, the more aggregate formation is favored, due

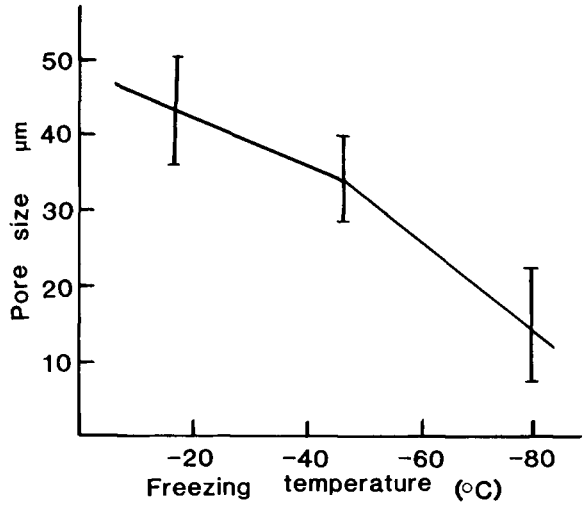

Figure 2 Changes in average pore size as a function of the freezing temperature.

to the rapid denaturation of fibroin molecules, accompanied by squeezing water out of the coagulated protein mass. On lyophilizing, the small ice crystals formed by water leave small-to-medium size pores. This effect might explain the decrease in average pore size as the freezing temperature decreases down to −80°C. On the contrary, at a lower cooling rate, the formation of protein aggregates takes place slowly enough to produce a less compact fibroin structure with large voids occupied by water, in such a way that larger ice crystals are formed, and, consequently, larger pores are left after lyophilization.

The typical sheet and/or leaf structure of silk fibroin aggregates obtained by quenching at −45 and −80°C is worthy of some comment. It has been reported that the molecular conformation of silk fibroin in aqueous solution is random coil and that fibroin particles of lamellar shape are present.[13] If liquid silk is frozen by quenching in the temperature range from −2 to −20°C, β-form crystals are obtained, whereas random coil remains the prevailing molecular conformation when it is frozen at a temperature below −20°C.[10] On the basis of these findings and taking into account the morphological characterization discussed above, we elucidated that the formation of sheetlike aggregates might be favored when silk fibroin in a random coil conformation is frozen at a temperature lower than −20°C.

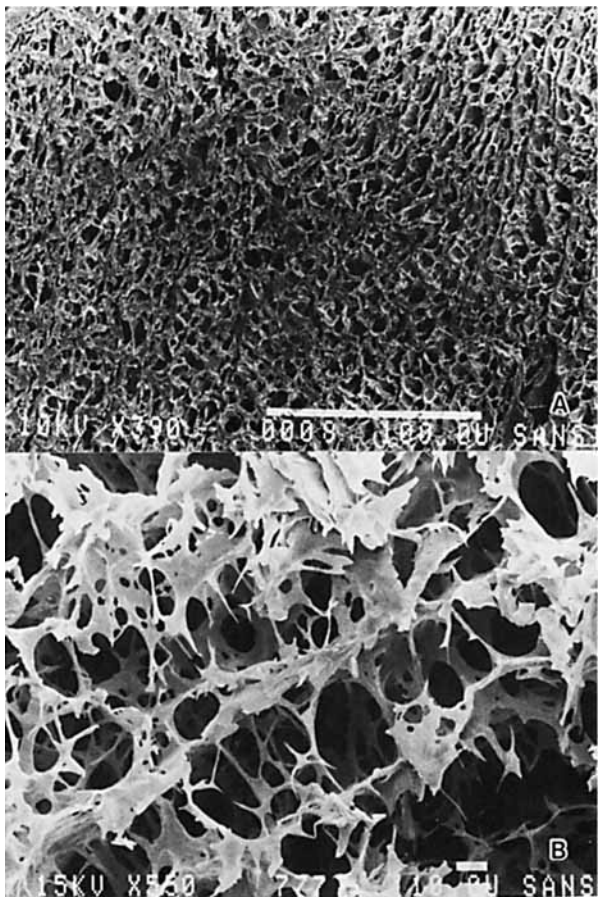

Figure 3 SEM photographs at (A) low and (B) high magnification of porous silk fibroin materials obtained from the silk solution coagulated at pH 4.01.

Effect of the PH of Fibroin Solution

The cross section of the porous silk material obtained by lowering the pH to 4.01 is shown in Figure 3. The morphology of the sample examined at low magnification (A) can be described as a closed-cell structure, like a honeycomb, with pores characterized by a quite high degree of regularity in size and shape. The same sample observed at higher magnification (B) shows interesting details. It is worth mentioning the complicated three-dimensional network of small-to-medium-size pores formed by the fibroin material, which consists of flat aggregates interconnected by very thin filaments. This sample shows an interesting transition of aggregate morphology from leaf to fiber shape, probably due to the pH value at which gelation of silk fibroin occurred.

The sample prepared at pH 2.65 is characterized by pores more heterogeneous in size (Fig. 4). At low magnification (A), it is possible to see the presence of thin sheets running parallel to each other, as observed in the sample frozen at −80°C from a neutral water solution. However, significant differences can be noted, such as the close connection among the sheets provided by a dense network of fibrous material, which holds the sheets together. This feature is emphasized at higher magnification (B). Moreover, it is possible to observe also that the sheets exhibit a fibrillar structure, suggesting that the aggregation of fibroin molecules to form fibrous structures is the prevailing phenomenon taking place on cooling liquid silk at acidic pH.

The graph of Figure 5, obtained by plotting the average pore size as a function of the pH of liquid silk, shows that the values tend to decrease as the pH approaches the isoelectric point of silk fibroin (pH 5). This confirms that gelation and the consequent aggregate formation are promoted at pH = pI. On increasing the pH toward neutrality, no significant changes in average pore size are detectable, suggesting that this parameter is no more influenced by the pH of liquid silk. As discussed in the previous paragraph, the freezing temperature is much more effective in determining the ultimate porosity of materials prepared from neutral silk fibroin solutions.

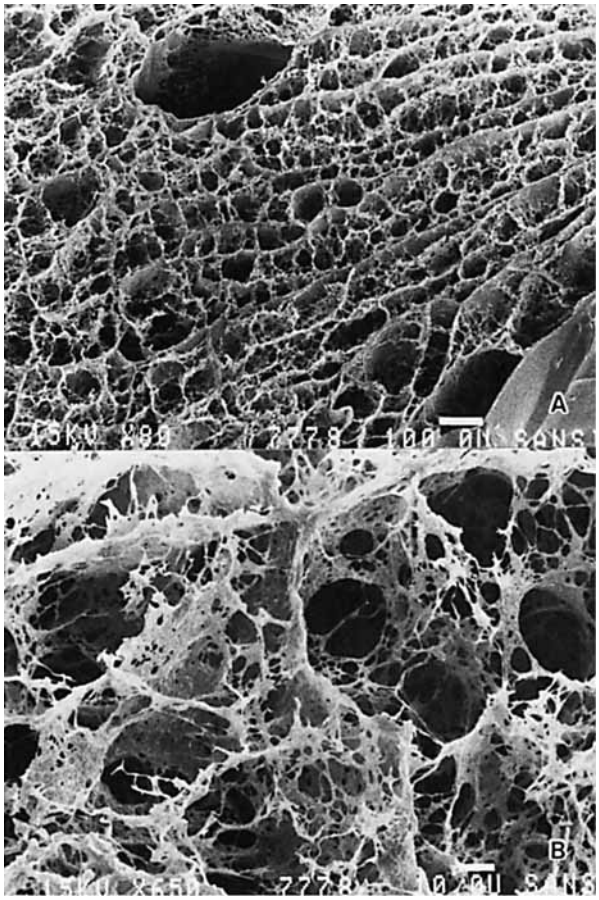

Figure 4 SEM photographs at (A) low and (B) high magnification of porous silk fibroin material obtained from the aqueous silk solution coagulated at pH 2.65.

The transition from sheetlike to fiberlike morphology observed on decreasing the pH of liquid silk can be related to the solvent used. Magoshi et al.[10] included acetic acid among the solvents able to induce conformational transition of liquid silk from random coil to the β structure. Therefore, it can be elucidated that gelation of liquid silk in these conditions might occur through crystallization and that the formation of fibrous aggregates is characteristic of this process, whereas aggregates growing from silk fibroin in a random coil form preferentially exhibit a sheetlike morphology.

Effect of the Treatment with Methanol Solution

The SEM photographs (Fig. 6) of silk fibroin materials prepared from liquid silk, to which different amounts of methanol have been added, show interesting morphological features attributable to specific effects of the organic solvent. The sheetlike aggregates typical of the control samples obtained from pure water (A) are transformed into fibrous aggregates as soon as methanol concentration attains 20% (C). The higher the amount of the organic solvent, the denser the three-dimensional fibrous network [(D)-(F)].

The behavior of porosity is consistent with the above morphological observations (Fig. 7). The average pore size of silk fibroin membranes decreases sharply with increasing methanol concentration, the pores becoming 10–20 times smaller than in the control sample. Accordingly, the compactness of the material increases. Although the control sample and that treated with 10% methanol are as soft as a sponge, the others become stiffer and harder.

The addition of methanol induces drastic changes in the material texture. Methanol is a poor solvent for silk fibroin, which is able to promote aggregate formation leading to irreversible gelation of liquid silk. The methanol–water–fibroin mixture can be regarded as a ternary system (nonsolvent–solvent–polymer), in which the structure formation process taking place on freezing depends on the concentration of each component. In these kinds of ternary systems, phase separation leading to membrane formation can occur by liquid–liquid demixing and by aggregate formation,[14-17] the former mechanism resulting in larger pores, as a consequence of the nucleation and growth of the diluted phase before gelation of the polymer chains occurs and a rigid polymeric structure is formed. In our system, the balance between these two processes is controlled by the water/methanol ratio, the fibroin concentration being constant. The SEM photographs, as well as the behavior of porosity, show that aggregate formation

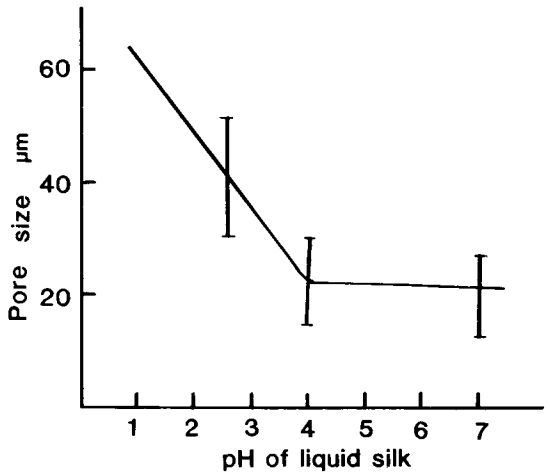

Figure 5 Changes in average pore size as a function of the starting pH value of the silk solution.

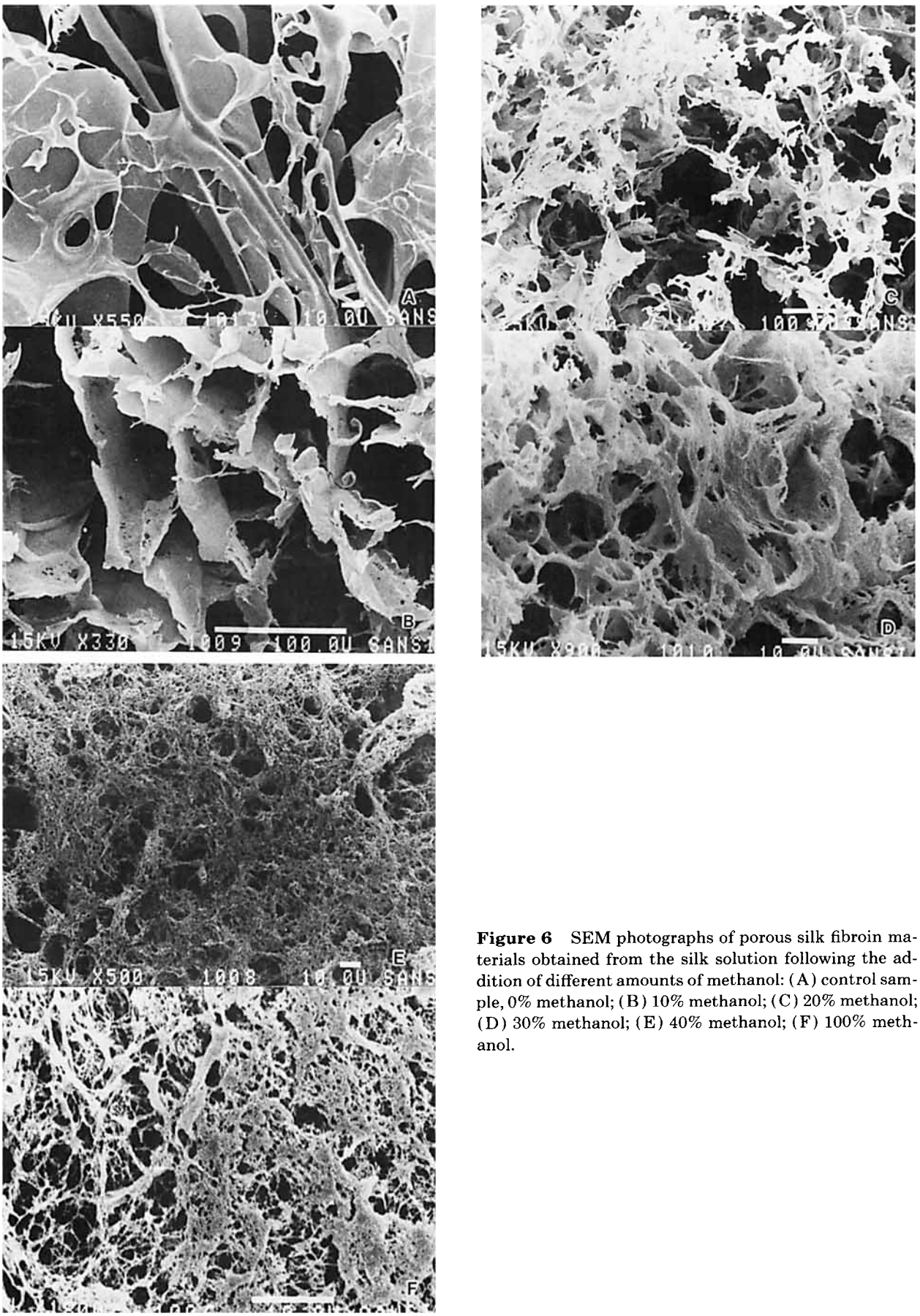

Figure 6 SEM photographs of porous silk fibroin materials obtained from the silk solution following the addition of different amounts of methanol: (A) control sample, 0% methanol; (B) 10% methanol; (C) 20% methanol; (D) 30% methanol; (E) 40% methanol; (F) 100% methanol.

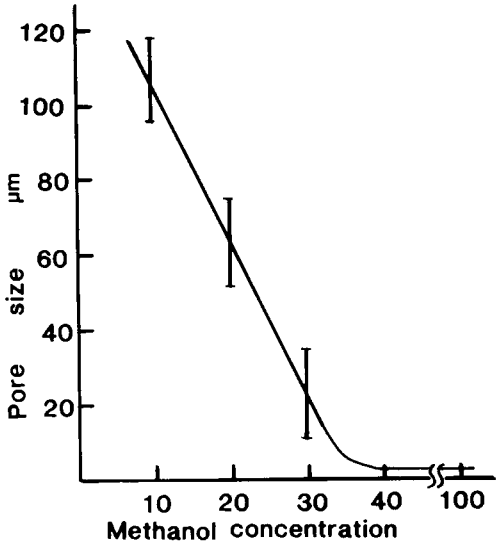

Figure 7 Changes in average pore size as a function of the amount of methanol added to the silk solution.

by gelation seems to be the most favored process at high methanol concentration [Fig. 6(E) and (F)]. It is worth mentioning that the solid phase is hindered from attaining a high degree of density, due to the low polymer concentration of the starting silk fibroin.

The aggregated silk fibroin retained exhibits a fibrous structure at a methanol/water ratio of > 20%. The structure-enhancing effect of methanol on liquid silk has been reported by several authors.[10,13,18] The addition of methanol to liquid silk induces the transition from random coil to β-sheet conformation, this effect increasing with solvent concentration. Hence, we suggest that gelation of silk fibroin in water/methanol solutions is likely to occur through a crystallization mechanism.[17] From the morphological point of view, silk fibroin with the β-sheet structure leads to the formation of fibrous aggregates, as already discussed in the previous paragraph.

Application of a Porous Silk as a Drug-delivery System

A drug-delivery system was prepared by incorporating acetylsalicylic acid into a porous silk fibroin carrier. The drug was not covalently bonded to the polymeric substrate, but only physically dispersed into it. The cross-section morphology of this material is shown in Figure 8. The average pore size is 4.8 μm (SD: 2.8 μm), largely lower than that of the sample coagulated at pH 4 without acetylsalicylic acid (see Fig. 5). This fact may be attributed to the

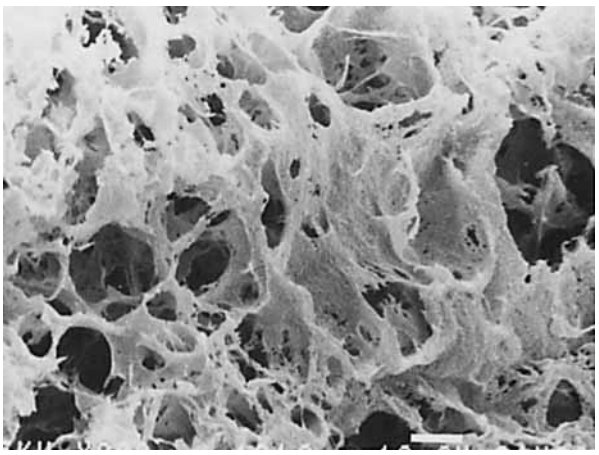

Figure 8 The cross-section morphology of a drug-delivery system prepared by incorporating acetylsalicylic acid into a porous silk fibroin carrier.

higher concentration of the starting fibroin solution (1.5% w/v) and to the centrifugation at 3000 rpm, which made the coagulum more close-packed. The effectiveness of the silk membrane as a drug carrier was studied by measuring the kinetics of drug release. Figure 9 shows that acetylsalicylic acid rapidly diffuses into the external solution within the first 2 h. The concentration of the drug in solution then increases more slowly than during the first stage, attaining a rate of diffusion that remains almost constant for several hours. Equilibrium has not been attained within the time range explored in our experiment.

The preliminary results reported in this article demonstrate the potential suitability of porous silk as a carrier for the preparation of a drug-delivery system. More extensive investigations are needed in order to design a device able to deliver the required amounts of a drug in the therapeutic range for a programmed short-, medium-, or long-term therapy. The diffusion of the drug from the carrier into the

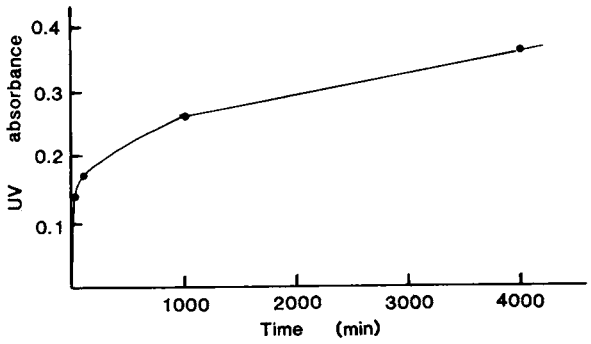

Figure 9 Kinetics of acetylsalicylic acid release of porous silk fibroin material.

external solution is governed by various parameters, such as the physicochemical properties of the polymeric substrate (amphoteric character, hydrophilicity–hydrophobicity ratio, swelling power, degree of solubility, etc.) and its morphological characteristics (available surface area, particle size, pore shape, and size), which permit one to determine the performances of the system, aiming to attain a controllable blood level of the drug.[19]

CONCLUSION

Silk fibroin materials with a variable extent of porosity can be prepared in different ways, either by changing some simple parameters, such as the temperature at which liquid silk is frozen and the pH of the starting fibroin solution, or by adding fixed amounts of an organic solvent (methanol) to liquid silk. Moreover, it has been shown that pore size and shape, as well as the structure and morphology of fibroin aggregates, are determined not only by the kinetics of gelation, but also by the molecular conformation of silk fibroin, the porous rather than leaf-shape structure being favored when transition from the random coil to the β-form is promoted.

The results obtained in this work are summarized in Table I and show that the addition of different amounts of methanol to silk fibroin solutions is most effective in controlling pore size. Moreover, if a close-packed porous material is required, higher fibroin concentrations (> 2% w/v), separation of the coagulum by centrifugation, and lower freezing temperatures (−80°C; liquid nitrogen) should be used in combination with the organic solvent. The texture of the porous material changes from soft to hard and brittle on increasing methanol concentration above 50% w/v. These parameters represent a powerful tool for controlling the structure and morphology of porous silk materials and allow us to foresee that different fibroin-based devices can be modeled in order to meet specific requirements, such as those of drug-delivery systems.

Table I Porosity of Silk Fibroin Materials Prepared Under Different Conditions

Samples	Mean Value (μm)	SD (μm)
Series A[a]		
−18°C	43.5	3.4
−45°C	34.0	3.1
−80°C	14.4	3.8
Series B[b]		
pH 2.7	40.4	7.1
pH 4.0	22.7	5.5
pH 7.0	21.2	3.8
Series C[c]		
10% v/v MetOH	106.0	7.3
20% v/v MetOH	63.0	5.5
30% v/v MetOH	23.0	6.2
100% v/v MetOH	2.5	0.7

[a] 2.2% (w/v) silk fibroin solution.
[b] 0.8% (w/v) silk fibroin solution treated at different pH, decanted, and frozen at −80°C.
[c] 0.6% (w/v) silk fibroin solution treated with methanol, decanted, and frozen at −18°C.

REFERENCES

1. F. Lucas, J. T. B. Shaw, and S. G. Smith, *Adv. Protein Chem.*, **13,** 107 (1958).
2. R. D. B. Fraser and T. P. MacRae, *Conformation in Fibrous Protein,* Academic Press, New York, 1973, p. 293.
3. M. Demura, T. Komura, T. Hirade, and T. Asakura, *Sen-i Gakkaishi,* **46,** 391 (1990).
4. N. Minoura, M. Tsukada, and M. Nagura, *Biomaterials,* **11,** 430 (1990).
5. C. X. Liang and K. Hirabayashi, *Sen-i Gakkaishi,* **46,** 535 (1990).
6. C. X. Liang and K. Hirabayashi, *J. Appl. Polym. Sci.,* **45,** 1937 (1992).
7. C. X. Liang and K. Hirabayashi, *Sen-i Gakkaishi,* **47,** 334 (1991).
8. M. Romanó, G. Freddi, M. R. Massafra, and F. Boniardi, in *Proceedings of the 4th European Symposium on Polymer Blends,* May 24–29, 1993, Capri, Italy, p. 381.
9. M. Tsukada, G. Freddi, and J. S. Crighton, *J. Polym. Sci. Polym. Phys. Ed.,* **32,** 243 (1994).
10. J. Magoshi, Y. Magoshi, and S. Nakamura, *J. Appl. Polym. Sci. Appl. Polym. Symp.,* **41,** 187 (1985).
11. Z. H. Ayabu, K. Hirabayashi, and M. Arai, *Sen-i Gakkaishi,* **48,** 141 (1992).
12. Z. B. Salama, T. Collins, and H. Jaeger, *BFE,* **9,** 235 (1992).
13. M. Canetti, A. Seves, F. Secundo, and G. Vecchio, *Biopolymers,* **28,** 1613 (1989).
14. M. H. V. Mulder, J. Oude Hendrikman, J. G. Wijmans, and C. A. Smolders, *J. Appl. Polym. Sci.,* **30,** 2805 (1985).
15. A. J. Reuvers, F. W. Altena, and C. A. Smolders, *J. Polym. Sci. Polym. Phys. Ed.,* **24,** 793 (1986).
16. A. J. Reuvers and C. A. Smolders, *J. Membr. Sci.,* **34,** 45 (1987).
17. W. R. Burghardt, L. Yilmaz, and A. J. McHugh, *Polymer,* **28,** 2085 (1987).
18. E. Iizuka and J. T. Yang, *Biochemistry,* **7,** 2218 (1968).
19. E. Ghezzo, L. Benedetti, and L. Callegaro, *Chimicaoggi,* **Jan.–Feb.,** p. 13 (1991).

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