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Characterization of Sericin Biomaterial from Silk Cocoon Waste

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Abstract

Cocoons of the *Bombyx mori* silkworm are mainly composed of twin tread fibroins with sericin surrounding them. The fibroin fiber is used for the silk fabric industry, while the sericin is removed for preparing soft and shiny silk fibers. Thus, degumming waste solution with sericin from silk cocoon was obtained from silk manufacturer. The sericin powder can be extracted from cocoon waste by boiling it in water and then hydrolyzed by citric acid. We have succeeded in preparation the self-assembly films based on electrostatic interaction between sericin and cationic amphiphile (DDAB). X-ray photoelectron spectroscopy and atomic force microscope results indicate that this film has a lamellar multilayered structure with layers of sericin being separated by lipid bilayers of DDAB. The tensile properties of this sericin/DDAB film correspond with the structure of film.

1. Introduction

The silk fibers of *Bombyx mori* silkworm are composed mainly of a fibrous core twin tread of protein fibroin (70-80%) with sericin (20-30%) surrounding it with other impurities such as wax, color pigments, and inorganic components [1-2]. Fibroin is the protein that forms the filaments of silkworm silk and gives it unique physical and chemical properties. Sericins, on the other hand, are the group of proteins, which bring together the fibroin filaments. The cocoons of the silkworm are mainly composed by these two proteins. Generally, the fibroin is a highly water insoluble protein and is solubilized only in concentrated salt solutions, while the sericin protein is solubilized in hot water [3]. The sericin is a family of water-soluble proteins found in silkworms and performs the function of gluing the fibroin fibers together and imparting strength to them in the cocoons. The silk sericin and other impurities cause hardness and coarseness, and mask the luster of silk fibroin. Hence, removing of sericin and other impurities is a necessary process to prepare soft, shiny, and whitened silk fibers ready to be dyed [4].

Waste from the silk degumming process contains high levels of sericin, this sericin can be extracted as describing elsewhere [5-6]. Blending of sericin with other polymers such as polyurethanes has been reported for production of biodegradable polymers. Sericin can be coated on fibers to prevent abrasive skin injuries, development of rashes and promote hydrophilicity on fiber surface. These sericin coated fibers were absorbent and did not cause skin rash [7]. The use of sericin as an antioxidant and antimicrobial for polluted air treatment was also reported [8-9]. Sericin coated cotton or polyester fabric increased Kubelka Monk function (K/S) values of the dye with an increase in the concentration of sericin [10-11]. Sericin and milk whey were modified with diisocyanate, toluene-2,4-diisocyanate or hexamethylene diisocyanate as a crosslinking agent, in order to apply for a coating agent of polyester fabrics.

Generally, the method to prepare the sericin powder affects on its molecular weight. Vaithamsat *et al.* proposed a method to extract the sericin powder from degumming waste solution using filtration, freeze-drying and grounding into fine powder [12]. They obtained the sericin powder in 2-10 kDa and in 1-3 kDa with enzymatic hydrolysis. Wu *et al.* worked on a method to extract the sericin from degumming waste water: compressed and then spray-dried to obtain the sericin powder. Next, the sericin powder was dissoled in chilled water and ethanol. Then, the solution was lyophilyzed and freeze-dried to obtain the sericin powder with its diversity of molecular weight distribution during 6-1,600 kDa [13].

The sericin powder's preparation based on previous method was modified to control its molecular weight. The objective of this present work is to extract the sericin powder from cocoon waste water and then characterize it in order to obtain the optimun molecular weight of sericin powder for using in biomaterial applications. Moreover, there are limited of research work on the sericin biomaterial application. Supramolecular self-assemblies are new trends for the applications of biomaterials [14-18] and structure of nano-materials [19-22]. We demonstrate herein the formation and structure of supramolecular films using electrostatic interaction between sericin and cationic amphiphile as didodecyldimethylammonium bromide (DDAB). We develope the preparation method of this self-standing film based on our previous work on nucleic acid/DDAB film [23-24].

2. Experimental

2.1. Materials

Silk cocoon waste was obtained from Shinano Kenji (Thailand) Co., Ltd. Didodecyldimethylammonium bromide (DDAB) was purchased from TCI America. All chemicals were used as received.

2.2. Characterization

Scanning electron microscope or SEM (Philips 525M) at an excitation energy of 30 kV was used to study the morphology of the sericin powder.

Thermogravimetric analysis (METTLER TGA/sDTA851e) goes up to 600°C. Nitrogen was used as a carrier gas (10 mL.min^{-1}).

Fourier-transformed infrared spectroscopy or FTIR (Nicolet Magna 850) in transmission mode was used to determine the chemical structure of the films. Infrared laser source passed the films through the detection.

The mechanical properties of the films were recorded on a standard tensile testing machine (Instron). The tensile test was carried out on dumbbell specimens with a crosshead speed of 0.5 in.min^{-1}. The dumbbell specimens were produced from the obtained self-assembling films with a thickness of 50 μm. Each of the films was tested at least three times.

The AFM experiments were performed using a MultiMode microscope equipped with a Nanoscope IIIA controller. The AFM images were recorded in tapping mode under ambient conditions using a moderate force ratio to avoid contact loss. Experiments were performed using a commercial silicon tip (NSC12) with resonance frequency f = 150–200 kHz and spring constant k = 4–8 N.m^{-1}. Each film sample was typically cast three times and analyzed in multiple regions. Images were processed and analyzed using NanoScope software. Raw data were levelled by a first and second order plane fit correction to remove the sample tilt. All spectroscopic experiments were carried out in air without a specific close chamber, which would control the environment. However the humidity was constantly controlled in the laboratory and oscillates in the range 30-40%.

X-ray Photoelectron Spectroscopy (XPS). XPS measurements were performed on a Kratos Axis Ultra using a monochromatic Al Kα source. Film surfaces were investigated at 90° (normal detection) and 30° (60° off-normal) between sample film and detector. Tilting the sample increases the distance that electrons heading in the direction of the detector axis must travel to exit the sample surface, making the XPS experiment more surface sensitive. When a sample is flat (90° with respect to the X-ray beam), electrons can escape from a depth of about 100 Å. When the sample is tilted at 60° with respect to the beam, electrons can only escape from a depth of about 20 Å. Decomposition of high-resolution spectra, which have been normalized by fixing the carbon peak at 285.0 eV, is performed using a least-squares fitting program. The nitrogen spectra of the different species are decomposed at different binding energies: quaternary nitrogen (positively charged nitrogen) at 402.3 eV, aliphatic nitrogen at 400.2 eV, and aromatic nitrogen at 398.9 eV.

3. Results and Discussion

Waste water from the degumming process was treated under the condition of Sothornvit *et al.*: dilution of waste water by distilled water in 10 times and then stock it at 115°C for 1 h [6]. Then, the solution was digested by 1.25% citric acid at 85°C for 60 min [25]. Finally, sericin solution was transformed to be powder using spray dry technic. The molecular weight of this type of sericin powder was 132 kDa using SDS-PAGE technic. The composition of amino acids in sericin powder was determined by amino acid analyzer which is presented in Table 1.

The sericin powder obtained by extraction from hot water and then digested with citric acid gives the short amino acid molecules which reduces the viscosity of the sericin solution. The color of this type of sericin presents brightness (L* =

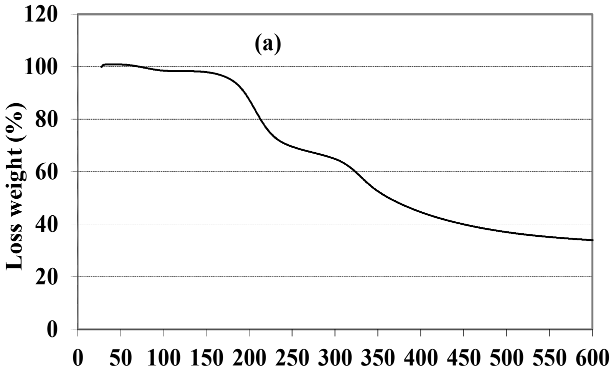
87.7), redness (b* = 2.0) and yellowness (c* = 13.1).

Table 1. *Type and amount of amino acid composition in the sericin powder.*

Type of amino acid	Amino acid (%) in 100 g of sericin
Aspartic acid	17.7
Serine	32.7
Glutamic acid	7.3
Glycine	9.9
Histidine	1.8
Arginine	6.2
Threonine	5.5
Alanine	3.7
Proline	0.6
Tyrosine	4.6
Valine	3.1
Methionine	0.1
Lysine	3.1
Isoleucine	1.0
Leucine	1.4
Phenylalanine	1.1

We found that the main components of sericin are serine, aspartic acid and glycine. Serine presents strong polar molecule from the effect of hydroxyl group which gives the interesting properties for sericin [26]. For aspartic acid and glycine, they are important for structural group in sericin. In addition, the hydrophilic amino acids make sericin more soluble than fibroin and also absorb moisture well [13].

Concerning the thermal property of sericin powder, the thermal gravimetric analysis (TGA) was used. Figure 1 (a) shows that the sericin powder loses around 5 wt% of water at 100°C. After that, the sericin still loses more weight at temperature close to 200°C which comes from the degradation of peptide bonds and then amino acids respectively [27-28]. Finally, the sericin is completely degraded around 300°C. Morphology of the sericin powder was presented in Figure 1 (b) using scanning electron microscope (SEM). We found that the surface of powder is rough certainly due to the acid digestion. Most of powder particles look like hollow spheres that are shrunken based on their preparation of spray dry technic. The sericin powder is heterogeneous with the large distribution of size.



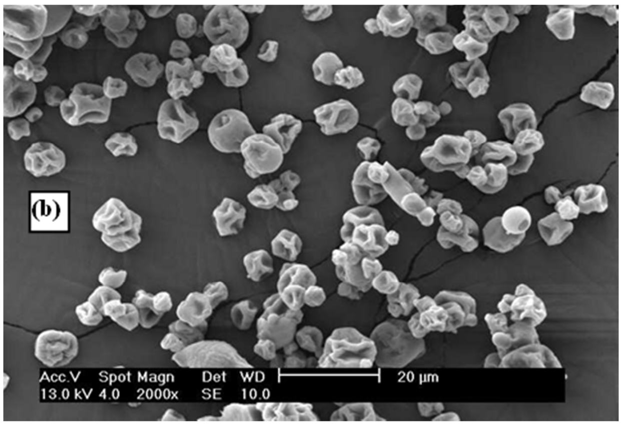


Fig. 1. *(a) TGA spectrum of sericin and (b) SEM photography of sericin at x2000.*

The sericin powder was dissolved by hot water, and then was mixed with an equivalent aqueous solution of 1.1 mol cationic amphiphile or DDAB. The reaction mixture of complex formation was first stirred at room temperature for 3 h, and the water insoluble complexes were then collected by centrifugation and lyophilization. The obtained complexes were compressed at 100°C under 100 bars for 5 min, the formation of self-assembled transparent films were obtained with a thickness of several micrometers. The obtained films were formed in one-to-one stoichiometry of an anionic amino acid in sericin and the cationic amphiphile, which was confirmed by elemental analysis: 57% C, 11% H and 8% N corresponds to the theoretical calculation (56% C, 11% H and 9% N).

The structure of the lipid sericin film was explored by other surface characterization techniques such as X-ray Photoelectron Spectroscopy (XPS) which quantifies the chemical composition of the film. In an angle-dependent mode, XPS measurements allow the determination of the chemical composition at different depths in the material. By lowering the angle between sample and detector from 90° (normal) to 30° (60° off-normal), the analysis depth into the sericin/DDAB film could be varied from approximately 100 Å down to 20 Å respectively. The chemical composition of the uppermost layer (or surface layer), as well as the layers beneath it, can therefore be determined. All XPS data obtained were correlated with the NIST XPS database. Nitrogen was chosen as the control to measure with XPS because it was the only atom with a different chemical environment in both the sericin and the lipid. While the head group of the lipid DDAB contains a quaternary amine, the sericin only contains aromatic and aliphatic nitrogen atoms. The difference in chemical binding of nitrogen atoms was reflected in the binding energy.

The amount of quaternary nitrogen increased when we tilted the sample from 90° to 30° for sericin/DDAB film (Table 2), which indicates that the upper layer is formed by the DDAB lipid layer. However, we did not find the aromatic nitrogen in both angles or both depths. In the present film, some amino acids with aromatic nitrogen as histidine and proline are present in sericin but at small amounts (1.82%

and 0.60% respectively). On the other hand, the amount of aliphatic nitrogen decreased when we tilted the sample. This means that the layer beneath DDAB layer is sericin. This result is compatible with the result from nucleic acid/DDAB films [19].

Table 2. *Percentage of different nitrogen species on the film surfaces as revealed by XPS.*

Nitrogen species (N)	Sericin/DDAB film	
	90° (depth 100 Å)	30° (depth 20 Å)
Aromatic N (%)	0.00	0.00
Aliphatic N (%)	29.27	21.57
Quaternary N or N^+ (%)	70.73	78.43

The AFM experiments were performed to investigate the morphology of the film using a Digital Instrument Nanoscope IIIA under ambient conditions and using a commercial silicon tip. All the sample films were analyzed in the tapping mode. It seems that there are step-like structures with different height in the sericin/DDAB film (Fig. 2). This result is in good agreement with the previous works [24, 29]. The good organization of step-like structure in the long DNA/DDAB film can be found. However, the short RNA/DDAB film can also create the step-like structure but less organization.

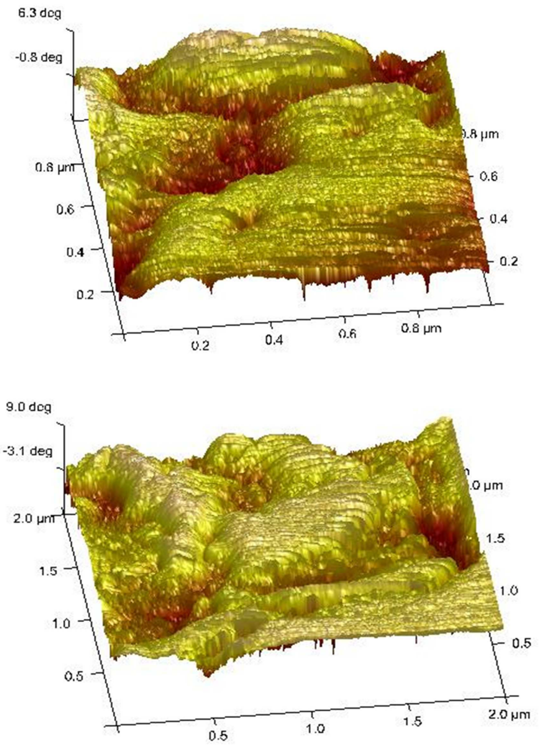


Fig. 2. *AFM phase images of sericin/DDAB films.*

The functional group of sericin/DDAB film was characterized by FTIR spectrophotometer (Fig. 3). We found that the spectrum of sericin/DDAB film displays main peaks at 3279 cm^{-1} for hydroxyl groups, 1660-1670 cm^{-1} for Amide I, 1530-1540 cm^{-1} for Amide II, and 1234 cm^{-1} for Amide III [30-32]. Moreover, we found a peak at 1380 cm^{-1} for the interaction between hydroxyl group and serine [32]. We still found the important spectrums of sericin/DDAB film at 3040-3030 cm^{-1} for quaternary amine (DDAB), 2930 and 2855 cm^{-1} for the stretching of CH_3 and CH_2, 1470 cm^{-1} for bending of H-C-H, 824 and 720 cm^{-1} CH_2 rocking [33-34].

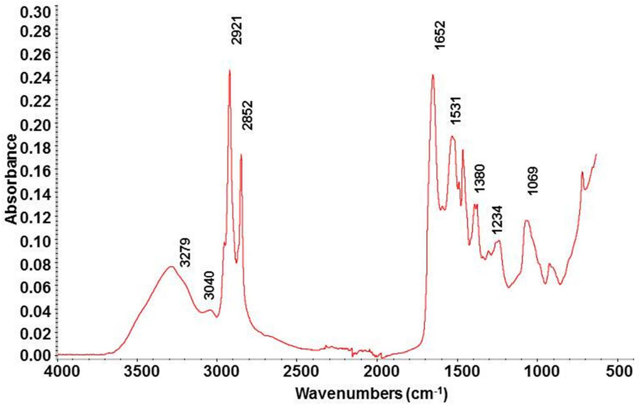


Fig. 3. *FTIR spectrum of sericin/DDAB film.*

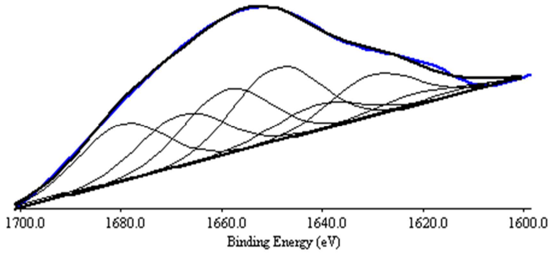


Fig. 4. *Decomposition of the FTIR peaks of Amide I of sericin/DDAB film in the 1600-1700 cm^{-1} range by means of the fitting curve processing of XPS analysis.*

Regarding to the Amide I peaks from FTIR, the relationship between stretching position of C=O in structure and orientation of secondary structure of amide have been investigated at different wavenumbers in Table 3 [35-36]. Based on the fit curve at 1600-1700 cm^{-1} of sericin sample using the XPS Peak processing (Fig. 4), the calculation of secondary structure of Amide I in sericin/DDAB film shows that random coil (28%) is the most structure in this type of film, while β-sheet (12%) is also found. The previous work stated that the sericin film with 30% random coil and 15% β-sheet gives the flexible film [37]. The other typs of secondary structure are also well-known for sericin.

Table 3. *Secondary structure of Amide I at different wavenumbers of sericin/DDAB film.*

Wavenumber (cm^{-1})	Assignment	Sericin/DDAB film (%)
1613, 1621	Aggregated	9
1630	β-sheet	12
1641, 1649	Random coil	28
1659	α-helix	18
1668, 1680	Turn	31
1695	Antiparallel β-sheet	2

We also investigated the macro-scale properties via tensile tests of the film. Mechanical properties were characterized using a tensile testing machine (Instron) according to ISO 527-1:1993 (E). The tensile test was carried out on dumbbell specimens using a crosshead speed of 0.5 in.min^{-1}. The dumbbell specimens were produced from the supramolecular films having 50 µm thickness approximately. Film sample was tested at least five times. Table 4 presented the tensile strength and elongation at break of our sericin film compare to typical polymers as polystyrene [38] and silicone rubber [39]. We found that sericin/DDAB film has better tensile strength than silicone rubber but lower than conventional plastic. However, this sericin/DDAB material has the longest deformation. The mechanical properties of the sericin/DDAB are in good agreement with the AFM results of nano-scale property. From the XPS and AFM points of view, the step features of sericin/DDAB film can make the film more stretchable due to the sliding of each layer independently, which is why it presents the high strain at break. This result displays the advantageous correlation of sericin nano-scale structure to the formation and properties of macro-scale film.

Table 4. *Mechanical properties of different materials.*

Material type	Tensile strength (MPa)	Elongation at break (%)	Young's modulus (GPa)
Sericin/DDAB	2	70	0.6
Polystyrene	43	10	3
Silicone rubber or PDMS	0.3	20	0.015

4. Conclusions

Sericin powder was extracted from the silk cocoon waste in order to use it as a biofilm. Sericin is interesting biomaterials which is able to self-assemble into supramolecular architectures through the formation of electrostatic interaction between anionic amino acid in sericin and cationic amphiphile. We have succeeded in preparation the self-standing film incorporating cationic lipids, creating complexes based on electrostatic interaction. XPS experiments and AFM results indicate that these films have a multilayered step-like structure. Interestingly, the tensile properties are compatible with the structure of film. Moreover, we will focus on the addressability and programmability of the sericin into this supramolecular film. We anticipate that this new biomaterial could have interesting applications as the functional material, biodegradable material, drug delivery material, etc.

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