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APPENDICES

APPENDIX A

Determination of Swelling Ratio

Water absorption of SF hydrogels

The degree of swelling ratio of the SF films and SF hydrogels were determined with amount of water content. The SF and blend films were immersed in DI-RO water at room temperature and in various type of gel former. The swelling ratio was calculated from the equation described below.

Swelling Test of SF hydrogel (%)

$$Swelling\ ratio\ (\%\ SR) = \frac{W_s - W_d}{W_d} \times 100$$

where W_s = weight of dried films (g)

W_d = weight of swollen films after immersing in the water (g)

For example:

From the value appears in **Table A.1**, at [SF] = 10 g/L

W_s = 0.0790 g

W_d = 0.0410 g

$$\% \text{ Swelling ratio} = \frac{(0.0790 - 0.0410)}{0.0410} \times 100$$

$$= 92$$

Table A.1 The weights of dried and swollen films of SF films and also the swelling ratio are summarized as follows:

SF (g/L)	I			II			III			Avg. (%SR)
	W_d (g)	W_s (g)	%SR	W_d (g)	W_s (g)	%SR	W_d (g)	W_s (g)	%SR	
10	0.0410	0.0790	92.7	0.0380	0.0770	102.6	0.0380	0.0630	65.8	87.03
20	0.0380	0.0700	84.2	0.0370	0.0630	70.3	0.0420	0.0900	114.2	89.59
30	0.0350	0.0630	80.0	0.0410	0.0930	126.8	0.0380	0.0650	71.0	92.63
40	0.0410	0.0590	43.9	0.0360	0.0550	52.8	0.0380	0.0580	52.6	49.77
50	0.0410	0.0710	73.2	0.0350	0.0520	48.6	0.0420	0.0550	31.0	50.90

Table A.2 The weights of dried and swollen films of SF/CP hydrogel films and also the swelling ratio are summarized as follows:

CP (g/L)	I			II			III			Avg. (%SR)
	W_d (g)	W_s (g)	%SR	W_d (g)	W_s (g)	%SR	W_d (g)	W_s (g)	%SR	
1	0.0520	0.122	134.6	0.0530	0.135	154.7	0.0550	0.156	183.6	157.7
3	0.0530	0.187	252.8	0.0530	0.205	286.8	0.0530	0.150	183.0	240.9
5	0.0500	0.185	270.0	0.0510	0.171	235.3	0.0510	0.196	284.3	263.2
6	0.051	0.214	319.6	0.0540	0.229	324.1	0.0500	0.214	328.0	323.9

Table A.3 The weights of dried and swollen films of SF/PL hydrogel films and also the swelling ratio are summarized as follows:

PL (g/L)	I			II			III			Avg. (%SR)
	W_d (g)	W_s (g)	%SR	W_d (g)	W_s (g)	%SR	W_d (g)	W_s (g)	%SR	
10	0.0520	0.0960	84.6	0.0520	0.100	92.3	0.0520	0.0970	86.5	87.8
30	0.0540	0.109	101.8	0.0530	0.106	100.0	0.0540	0.115	113.0	104.9
50	0.0520	0.118	126.9	0.0540	0.131	142.6	0.0540	0.121	124.1	131.2
80	0.0540	0.136	151.8	0.0510	0.125	145.1	0.0510	0.126	147.1	148.0
100	0.0540	0.130	140.7	0.0540	0.130	140.7	0.0550	0.170	209.1	163.5
200	0.0550	0.144	161.8	0.0550	0.150	172.7	0.0550	0.149	170.9	168.5

Table A.4 The weights of dried and swollen films of SF/HPMC hydrogel films and the also swelling ratio are summarized as follows:

HPMC (g/L)	I			II			III			Avg. (%SR)
	W_d (g)	W_s (g)	%SR	W_d (g)	W_s (g)	%SR	W_d (g)	W_s (g)	%SR	
10	0.0530	0.0970	83.0	0.0530	0.0970	83.0	0.0540	0.101	87.0	84.4
30	0.0540	0.107	98.2	0.0540	0.107	98.2	0.0540	0.100	85.2	93.8
50	0.0520	0.118	126.9	0.0530	0.125	135.8	0.0530	0.122	130.2	131.0
100	0.0520	0.185	255.8	0.0530	0.177	234.0	0.0520	0.174	234.6	241.4

APPENDIX B

Determination of SF Protein Release

Absorbability constant determination of silk fibroin solution

The SF solution with concentration in the range of 0.1-2.0 g/L were prepared and used for studying the relationship between SF concentration and absorbance of SF solution. The absorbances of SF solutions were measured at 275 nm at room temperature. The result from this measuring is shown in **Table B.1** and the calibration curve plotted from this result is shown in **Fig. B.1**.

Table B.1 Absorbances of SF solutions at various concentrations

SF concentration (g/L)	Absorbance at 275 nm
0.1	0.04860
0.5	0.2055
1.0	0.4059
2.0	0.8125

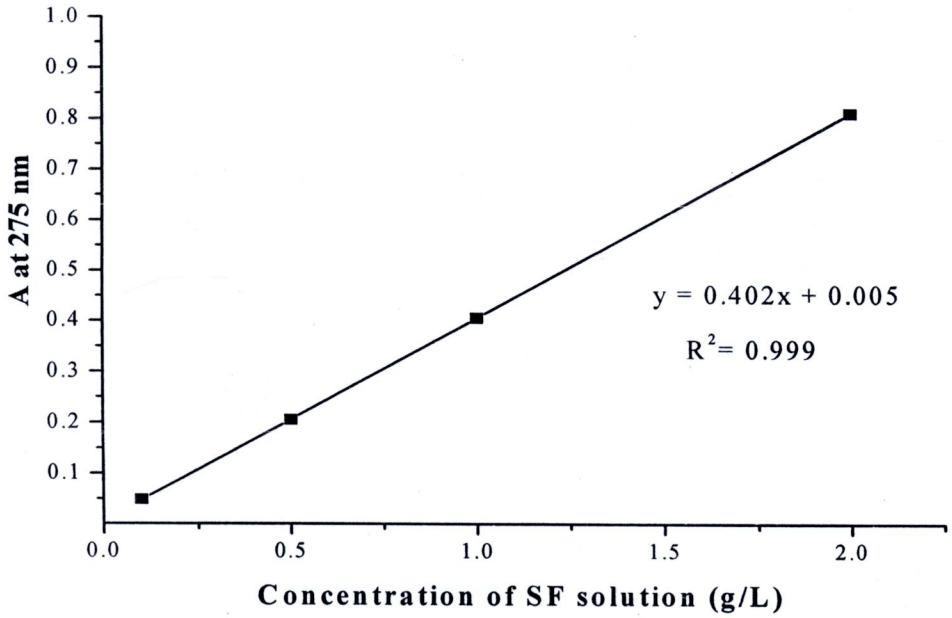

Fig. B.1 Calibration curve of SF solution

According to Beer's Law:

$$A = \varepsilon bC$$

Where A = absorbance

ε = molar absorbtivity

b = cell length (1 cm)

C = fibroin concentration (mg/mL)

From the equation, the slope of a plot between the absorbance and SF concentration is the absorption constant (b=1 cm).

Equation of calibration curve: $y = 0.402x + 0.005$

When y = absorbance and x = SF concentration (g/L)

$$\% \text{ Protein release} = \frac{\text{Detected SF concentration in the solution (g/L)}}{\text{Initial SF concentration (g/L)}} \times 100$$

For example:

Result from **Table B.2**

$$0.0315 = 0.402x + 0.005$$

$$x = 0.0659$$

From the result in **Table B.2,** at $[SF_I] = 30$ g/L

$$x = 0.0659$$

$$y = 0.0315$$

$$\% \ Protein\ release = \frac{0.0659(g/L)}{30\ (g/L)} \times 100$$

$$= 0.022$$

The results from this measuring are shown in **Table B.2-B.4**

Table B.2 The protein release of SF hydrogel with Carbopol prepared at various concentrations and fixed 30 g/L SF concentration

CP (g/L)	Absorbance (λ_{max} 275 nm)			Avg. Abs.	Conc. (g/L	SF Release (%)
	Trial I	Trial II	Trial III			
0	0.0524	0.0302	0.0120	0.0315	0.0659	0.0022
1	0.0497	0.0243	0.0228	0.0323	0.0678	0.0023
3	0.0590	0.0343	0.0266	0.0400	0.0870	0.0029
5	0.0545	0.0239	0.0251	0.0345	0.0734	0.0024
6	0.0238	0.0225	0.0366	0.0276	0.0563	0.0019

Table B.3 The protein release of SF hydrogel with Poloxamer prepared at various concentrations and fixed 30 g/L SF concentration

Poloxamer (g/L)	Absorbance (λ_{max} 275 nm)			Avg. Abs.	Conc. (g/L)	SF Release (%)
	Trial I	Trial II	Trial III			
0	0.0524	0.0302	0.0120	0.0315	0.0659	0.0022
30	0.0444	0.0309	0.0237	0.0330	0.0697	0.0023
50	0.0640	0.0621	0.0617	0.0626	0.1433	0.0048
80	0.0591	0.0290	0.0271	0.0384	0.0831	0.0028
100	0.0426	0.0306	0.0214	0.0315	0.0660	0.0022
200	0.0523	0.0251	0.0251	0.0342	0.0726	0.0024

Table B.4 The protein release of SF hydrogel with HPMC prepared at various concentrations and fixed 30 g/L SF concentration

HPMC (g/L)	Absorbance (λ_{max} 275 nm)			Avg. Abs.	Conc. (g/L)	SF Release (%)
	Trial I	Trial II	Trial III			
0	0.0524	0.0302	0.0120	0.0315	0.0659	0.0022
10	0.0336	0.0207	0.0181	0.0241	0.0476	0.0016
30	0.0578	0.0254	0.0184	0.0339	0.0718	0.0024
50	0.0500	0.0237	0.0143	0.0293	0.0605	0.0020
100	0.0422	0.0398	0.0154	0.0325	0.0683	0.0023

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CHARACTERIZATION OF SILK FIBROIN HYDROGELS PREPARED WITH CARBOPOL AND POLOXAMER

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Abstract

Preparations of silk fibroin hydrogels with gel formers were carried out by adding the crosslinking agents, Carbopol and Poloxamer, into fibroin solution. Then the mixture solution was left on polystyrene dish at 60°C until the film dried. The cross section of the hydrogel films, investigated through a Scanning Electron Microscope, exhibited the influence of crosslinker on film porosity. The conformation structures of the silk fibroin hydrogels prepared with different gel formers were investigated by Fourier Transform Infrared Spectroscopy. Results from UV-visible absorption measurements on the dissolution of the hydrogels showed that the prepared silk fibroin hydrogels had high water resistance. In addition, the crosslinker could remarkably improve the compliance and tenacity of the film hydrogels. So the silk fibroin based blend materials extended the range of biomaterial properties that could promote their uses in biomedical applications such as drug release and transdermal systems.

Keywords: Silk fibroin, Hydrogel, Carbopol, Poloxamer

Introduction

Hydrogels are composed of hydrophilic homopolymer or copolymer networks and can swell in the presence of water or physiological fluids. Chemical crosslinks (covalent bonds) or physical junctions (e.g. secondary forces, crystallite formation, chain entanglements) provide the hydrogels' unique swelling behavior and three-dimensional structure. Hydrogels have been a topic of extensive research in the past decades and their properties as for example their high water content and the possible control over the swelling kinetics make them very attractive for biomedical applications [1].

Many natural polymers have been shown to exhibit gelation upon temperature change. Researchers have used them alone in combination with synthetic polymers to fabricate thermally responsive hydrogel with desired properties. In relation on this characteristic the silk fibroin

(SF) can be a material for the production of natural hydrogels with a biocompatibility, biodegradability, high thermal stability, minimal inflammatory reaction and good water vapor permeability, it has been applied as biomaterials for many purposes such as enzyme immobilization, antithromboplastic materials, dialysis membranes and soft contact lenses [2-3].

SF hydrogels have been prepared from aqueous SF solution and are formed from β-sheet structures. The pH of the SF solution have some impacts on the rate of solution gelation. Gelation of a 3% solution was obtained in two days at pH 3-4, compared with eight days as required from a solution with pH 5-12. Moreover, the results of *in vivo* studies with fibroin membrane used as a wound dressing showed that SF has no toxicity and irritation. However, the porous sponge form of SF is brittle, whereas its application to biomedical materials also requires sufficient mechanical strengths. Improvements in mechanical properties of SF-based materials have been sought by blending with other synthetic or natural polymers, including poly (sodium glutamate), sodium alginate, chitosan and cellulose [4].

Carbopol, a high molecular weight cross-linked polymer of acrylic acid capable, is of forming a hydrogel in aqueous solutions depending on the degree of hydration of the carboxyl group in Carbopol (Figure 1). Although Carbopol has many advantages as a candidate for an extended-release tablet matrix, e.g. a good gel-forming ability and muco-adhesive property, there are few reports on the application of Carbopol to the extended-release dosage forms. This might be due to the ionic nature and high sensitivity of Carbopol to the pH of the medium. When Carbopol is exposed to water, the polymer begins to uncoil, generating an increase in viscosity and gel formation. In an alkaline environment, the carboxyl groups ionize, generating negative charge along the polymer backbone. Electrostatic repulsion of the negative charge causes uncoiling and expansion of the molecule which results in polymer swelling and gel formation [5-6].

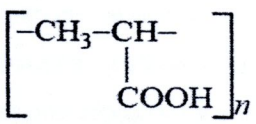

Figure 1 Structural formula of Carbopol.

Poloxamers are nonionic triblock copolymers composed of a central hydrophobic chain of polyoxypropylene (poly(propylene oxide)) flanked by two hydrophilic chains of polyoxyethylene (poly(ethylene oxide)). Poloxamer is also known by its trade name as Pluronic.

The Poloxamer (Figure 2), consisting of more than 30 different non-ionic surface-active agents, are ABA-type triblock copolymers composed of polyethylene oxide$_a$-polypropylene oxide$_b$-polyethylene oxide$_a$ [PEO$_a$-PPO$_b$-PEO$_a$] and are used as substitutes for skin in standardized third-degree thermal burns. Poloxamer hydrogels have also been used in a variety of biomedical fields such as medical, pharmaceutical, and cosmetic systems. The experimental modulation

of wound healing suggested that the non-ionic poloxamer could significantly enhance the rate of wound healing through yet unknown mechanism, possibly by stimulating the epithelial growth factor [7-8].

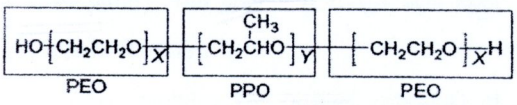

Figure 2 Structure of poloxamer 188 (X=79, Y=28).

In this study, the above mentioned gel formers were used in mixing with SF to act as cross-linking agents in order to form SF hydrogels. These gel formers are expected to enhance mechanical strength of SF in different degrees providing that the resulting hydrogels have appropriate gel properties for further applications.

Material and Methods

Materials

Carbopol 940 and Poloxamer 188 were kindly supplied by Bangkok Lab and Cosmetic Ltd. Both polymers were used without further purification. Deionized water was used in all sample preparation.

Preparation of the SF powder

Silk fibers were degummed with 0.5% w/v Na_2CO_3 solution at 98-100°C. After that, the degummed SF was dissolved in a ternary solvent system of calcium chloride, water and ethanol at mole ratio of 1: 8: 2 at 110-115°C for 2 h. This SF solution was dialyzed in distilled water using cellulose tubular membrane for three days to remove the natural salts, then followed by filtration and freeze- drying.

Preparation of SF solution

SF solutions with concentrations over 3% w was prepared by dissolving SF powder in deionized water.

Preparation of gel former solutions

Carbopol 940 was dispersed into the deionized water at various concentrations. The resulting mixture was stirred for 1-2 h. at room temperature and neutralized with triethanolamine (TEA) to give a Carbopol gel matrix with pH value of 7.

Poloxamer 188 were added into cold water at various concentrations and stirred with a magnetic stirrer. All solutions were stored at 7÷C before use.

Preparation of hydrogels

A 3% w/v SF solution was mixed together with gel formers by mixing appropriate amounts of the components to obtain blends with different compositions. The mixtures were stored for 24 h to promote full swelling. The homogeneous solutions were poured into a PET plate and then dried in a vacuum oven at 60°C for 12 h. Moreover, the blend films were treated with 80% ethanol for 15 min at room temperature, the solvent (EtOH) was then slowly evaporated. After solvent evaporation, the blend films were stored in a desiccator until analysis.

Characterization method

The cross-sectional morphologies of the samples were observed with a Scanning Electron Microscopy (SEM) instrument (Jeol,

JSM-5410LV, Japan). The specimens were sputter-coated with gold.

Fourier Transform Infrared Spectroscopy (FTIR) spectra of the samples were measured with a FTIR spectrometer (Perkin Elmer, Spectrum Rx I, England). The scanning range was kept from 4000-400 cm^{-1}.

The solubility (% protein release) of the SF hydrogels were determined by soaking the samples in deionized water for 15 min. The release of SF from blending was measured with a UV-VIS spectrometer (Shimadzu, UV-1700, Japan) at the optimal wavelength for SF detection.

Results

Morphology of SF hydrogels

The cross-section of SF hydrogel and blend films were determined using SEM (Figure 3) to verify the compatibility of SF with the gel formers within the blend.

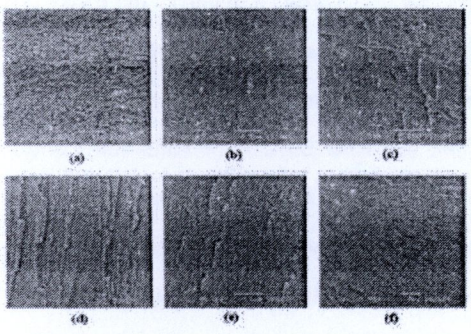

Figure 3 SEM cross-sectional images: (a) SF hydrogel, (b) SF/Carbopol blend, (c) SF/Poloxamer blend, (d) SF hydrogel treated with EtOH (e) SF/Carbopol blend treated with EtOH and (f) SF/Poloxamer blend treated with EtOH.

Figure 3a shows non-porous and dense morphology of the untreated SF hydrogel, whereas the EtOH treated SF hydrogel exhibits a sheet-like morphology (Figure 3d). Figures 3b and 3c show the cross-sectional SEM images of SF hydrogel prepared by blending SF with Carbopol and Poloxamer, respectively. The morphology of blending SF/Poloxamer hydrogel was more sheet-like than SF/Carbopol hydrogel. From Figures 3d, 3e and 3f, the SF hydrogel was treated with EtOH, it was found that the cross-sectional structure of the SF hydrogel with added gel formers (Figures 3e and 3f) became smoother than pure SF hydrogel (Figure 3d). This finding indicates that the gel formers help improve the morphological transition. When the SF film is immersed in aqueous ethanol, water firstly causes of the swelling of the amorphous region of the protein through interruption of hydrogen bonds, then ethanol penetrates the swollen region, generating an hydrophobic environment and making the hydrophobic molecule chain segments in the random coils of SF to get closer to each other and form a crystal nucleus. Finally, stable β-sheet conformation is formed by growth of crystal nucleus and rearrangement of hydrogen bonds [9].

FTIR Measurement

FTIR spectra of pure components and different compositions of SF and gel formers blend are shown in Figures 4 and 5.

Figure 4a, the absorption bands observed for SF powder at 1687 cm^{-1}(amide I), 1526 cm^{-1}

(amide II) and 1239 cm^{-1} (amide III) were assigned to random coil structure. The sorption bands for SF film treated with ethanol had frequencies of 1663 cm^{-1}(amide I), 1553 cm^{-1} (amide II) and 1232 cm^{-1}(amide III) which are the characteristics of β-sheet conformation, as shown in Figure 4(d).

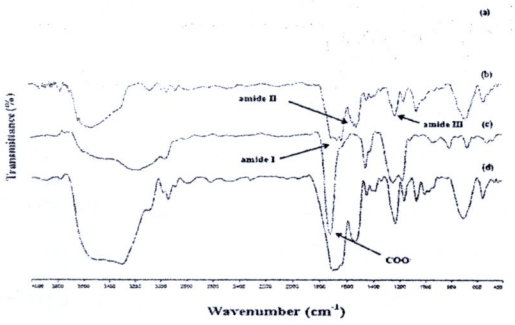

Figure 4 FTIR spectra of (a) SF powder, (b) SF/Carbopol blend, (c) Carbopol powder, (d) SF film treated with EtOH.

In addition, the SF/Carbopol blend also exhibited absorption bands at 1662 cm^{-1}(amide I), 1525 cm^{-1} (amide II) and 1228 cm^{-1}(amide III), which were the characteristic absorption of β-sheet structure of SF, and the corresponding band intensities increased with the increase of interaction between SF and Carbopol. This band tends to change to a higher vibrational frequency with the increase of Carbopol composition, which indicates the change of acid-acid intramolecular hydrogen bonding of Carbopol by the acid-amine bonding of SF/Carbopol blend.

In the Carbopol spectra, a broad and intense band of pure Carbopol was observed. This band corresponds to the overlap of two bands at 3599 cm^{-1} and 3195 cm^{-1}which is related to the free hydroxyl group and the hydroxyl-forming intermolecular hydrogen bonding, respectively. The strong band at 1715 cm^{-1} is due to the carbonyl stretching of the carboxylic group of the Carbopol polymer (Figure 4c).

The resulting observation might be interpreted as the interaction between SF and Carbopol could be between the hydroxyls of amino acids of SF and the COO^- group of Carbopol when pH of the solution was between 6-7.

Interaction also occurred between SF and Poloxamer during the preparation SF/Poloxamer hydrogel. The FTIR spectra of the components are shown in Figure 5.

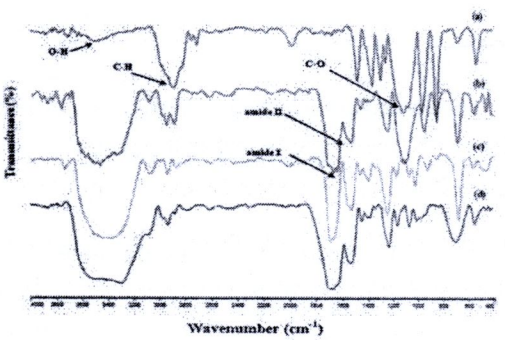

Figure 5 FTIR spectra of (a) Poloxamer powder, (b) SF/Poloxamer blend, (c) SF powder, and (d) SFfilm treated with EtOH.

FTIR spectrum of Poloxamer 188 (Figure 5a) exhibits characteristic IR bands at 3495 cm^{-1} (broad), 2885 cm^{-1} (intense) and 1106 cm^{-1} (intense) due to the stretching of O-H, C-H and C-O groups, respectively.

The peaks around 1110 cm^{-1} assigned to the characteristic C-O-C stretching band in Poloxamer appeared at 1106 cm^{-1} and 1107 cm^{-1} in the FTIR spectra of Figures 5a and 5b, respectively. The wave number is higher than the 1064 cm^{-1} observed in Figures 5c and 5d, attributed to the superposition of C-O stretching vibration of SF and Poloxamer in contrast to SF before the addition of Poloxamer.

After film blending, the intermolecular hydrogen bonds were formed by interaction between the hydroxyls of amino acids (Ser, Asp and Glu) on the SF chain and -OH of Poloxamer. The FTIR spectra of the blend film (Figure 5b) show that the amide I and amide II bands of SF at 1633 cm^{-1} and 1553 cm^{-1}, shift to approximately 1631 cm^{-1} and 1525 cm^{-1}, respectively. This result indicates that the conformation of SF was changed from random coil to β-sheet structure by addition of poloxamer, due to the formation of intermolecular hydrogen bonds between SF and Poloxamer.

FTIR spectra in Figures 4a and 5c will help confirm the effect of EtOH on inducing the conformational change of SF. Generally, untreated SF film possesses a structure that is water soluble which becomes less soluble after soaking in EtOH. Actually, water causes the swelling of SF hydrogel that lead to the conformational change from a dense α- helix form and uncoils the helices. Once the EtOH seeps into the pores of SF structure, this will cause the rearrangement of inter- and intramolecular hydrogen bonds converting its structure from random coil to β-sheet. Similar behavior has been reported for treatment with aqueous methanol [9].

Water Resistance

The protein loss of SF from SF/gel former film was determined by measuring absorbance of the solubilized SF protein at 275 nm and then calculating the percent water solubility of the hydrogel. The results are shown in Figures 6-7.

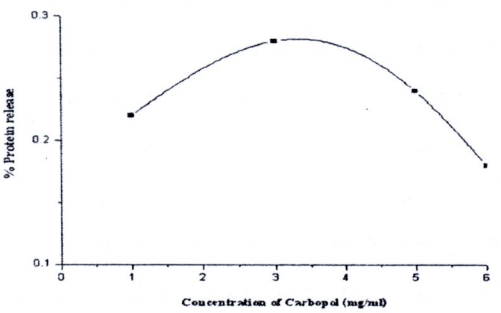

Figure 6 The effect of Carbopol concentrations on SF/Carbopol blend.

From Figure 6, % protein release of SF blended with Carbopol improves when the incubated concentration of Carbopol is increased. Having arrived at a maximum of 3 mg/mL, % protein release decreases when the concentrations of Carbopol are beyond 3 mg/mL. The finding might be explained as that the polymer chains could be able to spread enough when suitable concentration of Carbopol is given. However, too high concentration of Carbopol is useless because it causes the dissolution and disintegration in water.

The obtained results indicated that the SF/Carbopol hydrogel could not provide the suitable gelation of SF due to Carbopol polymers absorbed water and swelled to form a gelatinous network. Therefore, the polymer chain between SF and Carbopol loosens their hydrogen bonds which is greater in the extent than the blend with Poloxamer.

Figure 7 shows, likewise, the influence of Poloxamer concentrations on the % Protein release of SF/Poloxamer blend films after soaking in aqueous solution.

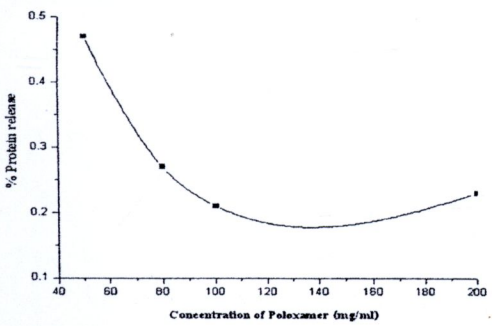

Figure 7 The effect of Poloxamer concentrations on SF/Poloxamer blend.

The result shows that the percent loss of SF protein from SF/Poloxamer blends decrease when the concentrations of Poloxamer are between 50-100 mg/mL and become nearly constant. At the point where protein release reaches minimum, it indicates that the best conditioned hydrogel is formed. The increase of polymer content, may cause an increase of cross-linking density in the hydrogel. But high concentration can interrupt the relaxation of polymer network, resulting in an obstruction of their swelling behavior.

Water solubility of SF hydrogel is a limiting factor that must be minimized for uses as biomedical material. The percentage loss of SF/Poloxamer hydrogel was reduced after treating with ethanol, thus it is highly effective in improving the water resistance.

Conclusions and Discussion

Silk fibroin hydrogel, which normally has poor mechanical properties and is not practically suitable for biomedical application, was modified in order to improve such properties by blending with Carbopol and Poloxamer. The characterization of the blend films suggested that the conformational change of SF hydrogel from the blending of SF and gel formers induced intermolecular hydrogen bonding between SF and the gel formers. The mechanical properties of the blend films were strongly affected by the gel formers content.

Acknowledgements

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THE RELEVANCE OF THE RESEARCH WORK TO THAILAND

Due to silk which is a natural protein having excellent properties including biodegradability, biocompatibility and low inflammatory reaction. Silk fibroin, a major component in silk, can be used in various forms depending on applications and it can be obtained easily from silk waste. Utilization of unused silk remaining from textile industry and damaged silk cocoons in various applications can create the values of such wastes. Therefore, the preparation of silk fibroin hydrogel with different gel formers done in this research work will, for sure, be another alternative of utilization. This study would be a guideline to improve other properties that can be further developed in production industry of medical materials and also result in the promotion of silk farm in the country.