



Article

# Effect of Silver Sulfadiazine and Metallic Ions on Properties of Thai Silk Fibroin/Gelatin Films for Anti-Bacterial Applications

Patchara Umprasert<sup>1</sup>, Siriporn Damrongsakkul<sup>2,3,4</sup>, Supansa Yodmuang<sup>1,2,4,5</sup>,  
and Juthamas Ratanavaraporn<sup>1,2,4\*</sup>

<sup>1</sup> Biomedical Engineering Program, Faculty of Engineering, Chulalongkorn University, Bangkok, Thailand

<sup>2</sup> Biomedical Engineering Research Center, Faculty of Engineering, Chulalongkorn University, Bangkok, Thailand

<sup>3</sup> Department of Chemical Engineering, Faculty of Engineering, Chulalongkorn University, Bangkok, Thailand

<sup>4</sup> Biomedical Engineering for Medical and Health Research Unit, Faculty of Engineering, Chulalongkorn University, Bangkok, Thailand

<sup>5</sup> Research Affairs, Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand

\*E-mail: [juthamas.r@chula.ac.th](mailto:juthamas.r@chula.ac.th) (Corresponding author)

**Abstract.** This study aimed to develop Thai silk fibroin/gelatin (SF/GA) films incorporating various concentrations of silver sulfadiazine (SSD) and to investigate the effects of SSD and metallic ions (Ag(I) and Cu(II)) on chemical conformation of the SF/GA films. We found that the incorporation of SSD in the films changed the conformation of silk fibroin protein by increasing  $\beta$ -sheet content. The same phenomena was also observed with the other 2 metallic ions, Ag(I) and Cu(II), incorporated in the SF/GA films. This effect of metallic ions on the SF conformation transition in the SF/GA films may be similar to the phenomenon occurred during natural spinning process of the *Bombyx mori* silkworm. The SF/GA films incorporating SSD had therefore more stability, less water-insoluble fraction and extended degradation rate than the SF/GA films without SSD due to the higher content of stable  $\beta$ -sheet conformation. When cultured with L929 mouse fibroblast cells according to ISO 10993 part 5 standard, the SF/GA films incorporating SSD at all concentrations showed no cytotoxicity. The SSD released from the films also showed obvious anti-bacterial activity against *Staphylococcus aureus* (Gram positive) and *Escherichia coli* (Gram negative) bacteria. We suggested that the SF/GA films incorporating SSD may be useful for some medical applications in which anti-bacterial effect was required.

**Keywords:** Silk fibroin, gelatin, silver sulfadiazine, silver ion, copper ion.

**ENGINEERING JOURNAL** Volume 25 Issue 5

Received 23 September 2020

Accepted 1 May 2021

Published 31 May 2021

Online at <https://engj.org/>

DOI:10.4186/ej.2021.25.1.13

## 1. Introduction

Nowadays, an interest in natural-derived biomaterials for medical applications is growing worldwide. Natural-derived biomaterials are demonstrated to show biocompatibility, biodegradability and biological tissue imitation rather than synthetic polymers. Silk is one of the most attractive natural-derived biomaterials that received much interest in biomedical researches recently. It is a fibrous protein produced from silkworms, spiders, or other insects. Silk protein from *Bombyx mori* silkworms consists of two major protein components, which are sericin and fibroin. Silk fibroin has been shown to have excellent mechanical properties and slow degradation rate comparing to other natural-derived biomaterials while generates minimal inflammatory and immune responses [1-2]. However, silk fibroin itself shows poor biological activities, the blending of silk fibroin with other biological active polymers may be required in some applications. Gelatin is a denatured collagen which possesses biocompatibility and Arg-Gly-Asp (RGD) sequence to promote cell attachment and proliferation [3]. In our previous works, gelatin was introduced to blend with Thai silk fibroin and fabricated into various forms such as porous scaffold, microsphere, and nanofiber [4-6]. We showed that the degradation rate, mechanical, physical and biological properties of the blends could be modulated by the blending ratio of silk fibroin and gelatin. Silk fibroin enhanced mechanical properties and extended degradation rate while gelatin improved biological activities of cells on the blend system. In this study, we introduced silver sulfadiazine (SSD), a cationic antibiotic for wound infection treatment, into the blended silk fibroin/gelatin (SF/GA) films. The effects of incorporated SSD on the chemical structure and protein confirmation of the SF/GA films were investigated. The other 2 metallic ions (Ag(I) and Cu(II)) were also selected to incorporate in the SF/GA films, and the conformational change of the films was further explored. In addition, water swelling ability, solubility, degradability, and SSD release profiles of SF/GA films incorporating SSD were evaluated. *In vitro* cytotoxicity and anti-bacterial activity of SF/GA films incorporating SSD were studied to confirm their potential for medical applications.

## 2. Materials and Methods

### 2.1. Materials

The cocoons of Thai silkworms “Nangnoi Srisaket 1” were kindly supplied by Queen Sirikit Sericulture Center, Nakhonratchasima province, Thailand. The regenerated silk fibroin (SF) solution was prepared following the method previously described by Lerdchai et al. [7]. Type A gelatin (GA) was obtained from Nitta Gelatin Co., Osaka, Japan. Silver sulfadiazine (MW = 357.14 g/mol) was purchased from Sigma-Aldrich laborchemikalien, Germany. Other chemicals used in this study were of analytical grade.

### 2.2. Preparation of SF/GA Films Incorporating SSD

The regenerated SF solution was mixed with GA solution to prepare the blended SF/GA (50/50) solution at 4% w/w. SSD at various concentrations (0, 0.12, 1.16 and 2.16% w/w) was added to the blended silk fibroin/gelatin solution. The blended solution was stirred for 10 min and cast in a teflon mold and air-dried under darkness. After 2-3 days, the SF/GA films incorporating SSD were obtained. The same procedures were performed to prepare the SF/GA films incorporating AgCl or CuCl<sub>2</sub>. The 0.87% w/w AgCl and 1.03% w/w CuCl<sub>2</sub> were used in order to obtain equivalent molar to that of 2.16% SSD.

### 2.3. Physico-chemical Characterization of SF/GA Films Incorporating SSD

#### 2.3.1. FTIR analysis

The functional groups and secondary structure of protein presented in the SF/GA films incorporating SSD, AgCl, and CuCl<sub>2</sub> were determined using Fourier transform infrared (FTIR) spectroscopy (Perkin-Elmer, USA). The analysis was based on the identification of absorption bands (ATR mode) concerned with the vibrations of functional groups presented in the films. The secondary structures relevant to the amide I region were quantified by Fourier self-deconvolution and peak fitting using OMNIC™ software (Thermo Scientific, USA). Peaks were fit using 11 Gaussian line shape profiles assigned to positions reported in previous work [8]. The  $\beta$ -sheet structure of SF was assigned at 1608-1637 and 1697-1700 cm<sup>-1</sup>, and the relative structures (tyrosine, random coil, alpha helix, and  $\beta$ -turn) were calculated from the ratio of area under the curve for  $\beta$ -sheet peaks to that of the whole deconvoluted spectra.

#### 2.3.2. Water swelling test

The dried films were cut into 5 mm x 10 mm and weighed. The thickness of dried films was measured using a micrometer. Then, the films were immersed in phosphate buffer saline (PBS, pH 7.4) at 37°C for 24 h. The swollen films were weighed and the swollen thickness of the films was measured. The percentage of increased thickness and swelling ratio of the films were calculated based on their initial dried thickness and dried weight, respectively.

#### 2.3.3. Evaluation of water-insoluble fraction

The films were cut into 5 mm x 10 mm and weighed. Then, the films were immersed in deionized water at 37°C for 24 h. After that, the films were dried in an oven at 60°C, for 24 h. The remained dried films were weighed. The water insoluble fraction of the films was calculated based on their initial dried weight.

## 2.4. *In vitro* Degradation Test of SF/GA Films Incorporating SSD

The dried films were immersed in protease enzyme solution (pH 7.4, conc. 1 unit/ml) at 37°C. The enzyme solution was refreshed every other day. At the predetermined time points, the remained films were collected and dried in an oven at 60°C. The weights of remained dried films were measured. The percentage of weight remaining of each film was calculated based on their initial dried weight.

## 2.5. *In vitro* Release Test of SSD from SF/GA Films

*In vitro* release test was performed by immersing the films in two media; PBS solution (pH 7.4) and protease enzyme solution (pH 7.4, conc. 1 unit/ml), at 37°C. At the predetermined time points, the supernatant was collected to measure the optical density of SSD at a wavelength of 240 nm [9]. The concentration of SSD released from films was determined from the standard curve of SSD prepared at known concentrations. The cumulative release profile of SSD along the study was plotted (n = 3).

## 2.6. *In vitro* Cytotoxicity Test of SF/GA Films Incorporating SSD

The cytotoxicity of SF/GA films incorporating SSD against L929 mouse fibroblast cells was evaluated using the indirect method according to ISO 10993-Part 5 (1992). The films were incubated in Dulbecco's Modified Eagle powder medium (DMEM) without fetal bovine serum at 37°C, 5% CO<sub>2</sub> for 24 h to prepare the film's extracts. The concentration of film's extract solution (100%) was diluted to three different concentrations (50%, 25%, and 12.5%) by culture medium. L929 mouse fibroblast cells were seeded at a density of 26,000 cells/cm<sup>2</sup> into a 24-well plate and incubated at 37°C, 5% CO<sub>2</sub> for 24 h to allow cell attachment. After that, the medium was removed and replaced with the following groups; (1) DMEM without serum (negative control), (2) DMEM with 10% serum (control), (3) DMEM containing 20 ppm zinc acetate (positive control), (4) the film's extract solution (100%), (5) the film's extract solution (50%), (6) the film's extract solution (25%), and (7) the film's extract solution (12.5%). Viability of cells was determined using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay [10].

## 2.7. *In vitro* Antibacterial Test of SSD Released from SF/GA Films

*In vitro* antibacterial activity of SSD released from SF/GA films was evaluated against two strains of bacteria, *Staphylococcus aureus* (ATCC 25923, gram-positive) and *Escherichia coli* (ATCC 25922, gram-negative) using the disc diffusion method (CLSI) [11]. The bacterial strains were cultured on a Mueller Hinton agar plate at 37°C for 24 h to obtain 2 × 10<sup>6</sup> colony forming units (CFU). Then, three

swabs were applied on the surface of the Mueller Hinton agar plate. The ethylene oxide-sterilized films were placed on the agar plate and incubated at 37°C. After 24 h, the diameter of inhibition zone was measured. The SF/GA film without SSD were used as a control (n = 3).

## 2.8. Statistical Analysis

All quantitative data were shown as mean ± standard deviation. The statistical significance was determined by one-way analysis of variance (ANOVA). A value of p < 0.05 was considered to be significantly different.

## 3. Results and Discussion

### 3.1. Chemical Structure of SF/GA Films Incorporating SSD, AgCl or CuCl<sub>2</sub>

The FTIR spectra of the SF/GA film without SSD and those incorporating SSD are shown in Fig. 1(a). The characteristic peaks of proteins including hydroxyl group (3270 cm<sup>-1</sup>), amide I (1650 cm<sup>-1</sup>), amide II (1550 cm<sup>-1</sup>), and amide III (1274 cm<sup>-1</sup>), are found in all SF/GA films [13]. The films incorporating SSD showed peaks of pyrimidine groups (1415 cm<sup>-1</sup>), sulfur dioxide group (1100 cm<sup>-1</sup>), and S-N group (1077, 977, 833 cm<sup>-1</sup>) of SSD. These peaks were clearly seen in the films incorporating high concentration of SSD (2.16% w/w). This result confirmed the presence of SSD in SF/GA films. The percentages of secondary structures of each film obtained from deconvolution of the amide I band of the FTIR spectra are demonstrated in Table 1. For the SF/GA film without SSD, the percentages of tyrosine, β-sheet, random coil, alpha helix, and β-turn were 1.94, 36.81, 31.82, 9.88, and 19.55%, respectively. Incorporation of SSD slightly increased percentage of β-sheet structure of the films to 39-45%, compared to that of the SF/GA film without SSD (36.81%). Because SSD composed of silver (Ag) ion, we then chose other 2 metallic ions (Ag(I) and Cu(II) in the forms of AgCl and CuCl<sub>2</sub>) to incorporate in SF/GA films and studied their effects on the chemical conformation change of the films. The FTIR spectra of SF/GA films incorporating 0.87% w/w AgCl or 1.03% w/w CuCl<sub>2</sub> were similar to that of SF/GA film (Fig. 1(b)). However, after deconvolution of the amide I band, the percentage of β-sheet structure of SF/GA films incorporating either 0.87% w/w AgCl (40.66%) or 1.03% w/w CuCl<sub>2</sub> (43.15%) was increased from that of SF/GA film (36.81%) (Table 1). This clearly elucidated that metallic ions enhanced the secondary structure of silk fibroin protein. The effects of metallic ions on the change in the secondary structure of silk fibroin were studied by several works [12-14]. Zong X.H. et al. reported that the addition of Cu(II) obviously influenced the SF conformation transition in Cu(II)-SF complex membranes. Raman spectra showed that the amide I band of the SF shifted from 1657 to 1667 cm<sup>-1</sup> when the concentration of Cu(II) is increased, implying that the β-sheet conformation was getting an increase [12].

They explained that Cu(II) may form a stable coordination with SF via the AHGGYSGY peptides in the heavy chain of *B. mori* silk fibroin. Interestingly, there are some reports concerning changes in metallic ion content in different parts of the silk secretory pathway [13, 14]. Zhou L. et al. demonstrated that the copper content increased from the posterior part to the anterior part of the silk gland, and further increased in the silk fiber [15]. They also investigated the effect of copper on the conformation transition of the regenerated silk fibroin when dialyzed concentrated silk fibroin aqueous solution against  $\text{CuSO}_4$ . It was found that the dilute solutions of  $\text{CuSO}_4$  could induce the conformation transition in aqueous solutions of silk fibroin from random coil/helical conformation to  $\beta$ -sheet. They further investigated the concentrations of six metal elements (Na, K, Mg, Ca, Cu, and Zn) at different stages in the silk secretory pathway in the *Bombyx mori* silkworm [16]. The results showed that the contents of metal element (exception of Ca) increased from the posterior part to the anterior part of silk gland. Mg(II), Cu(ii), and Zn(II) ions appeared to be favorable to  $\beta$ -sheet formation. This may be suggested that metallic ions have important functions in the natural spinning process [13, 14]. Furthermore, some studies demonstrated that Cu(II) can induce the formation of  $\beta$ -sheet structure in other proteins [17, 18].

### 3.2. Water Swelling Ability of SF/GA Films Incorporating SSD

The SF/GA film without SSD after immersed in PBS solution for 24 h showed the percentage of increased thickness at around 54.27% while those incorporating SSD had less percentage of increased thickness (36.62-41.30%), as presented in Table 2. It was likely that higher concentration of SSD resulted in less percentage of increased thickness. As measured by weight, after immersed in PBS solution SF/GA films without SSD

showed the highest swelling ratio at 3.24. Incorporation of SSD significantly reduced the swelling ratio of SF/GA films to 2.03-2.38 in a concentration dependent manner. The less water swelling ratio of the films incorporating SSD than the film without SSD was possibly due to the higher content of dense  $\beta$ -sheet structure as reported in Table 1. Furthermore, the electrostatic interaction between the positive ion of Ag(I) and the negative-charged segment of SF molecules may be occurred. This interaction would reduce water affinity of the films [19, 20].

### 3.3. Water-insoluble Fraction of SF/GA Films Incorporating SSD

Percentage of water-insoluble fraction of SF/GA films without SSD was only 38.54% (water solubilized fraction  $\sim 62\%$ ) while that of the films incorporating SSD were increased to 70.84-78.26% (water solubilized fraction  $\sim 22$ -29%) (Table 3). The water-insoluble fraction of the films incorporating SSD tended to increase with the increasing SSD concentration. The results supported that the SF/GA films incorporating SSD were stabilized by  $\beta$ -sheet structure (Table 1).

### 3.4. *In vitro* Degradation Profiles of SF/GA Films Incorporating SSD

All SF/GA films were degraded gradually after incubated in protease enzyme solution along 14 days (Fig. 2). The degradation rates of the film without SSD and the film incorporating 0.12% w/w SSD were faster than that of the films incorporating 1.16 and 2.16% w/w SSD. After 3 days of incubation, only 20-25% of the film without SSD and the film incorporating 0.12% w/w SSD remained while the films incorporating 1.16 and 2.16% w/w SSD remained 48-53%. The film without SSD and the film incorporating 0.12% w/w SSD were completely

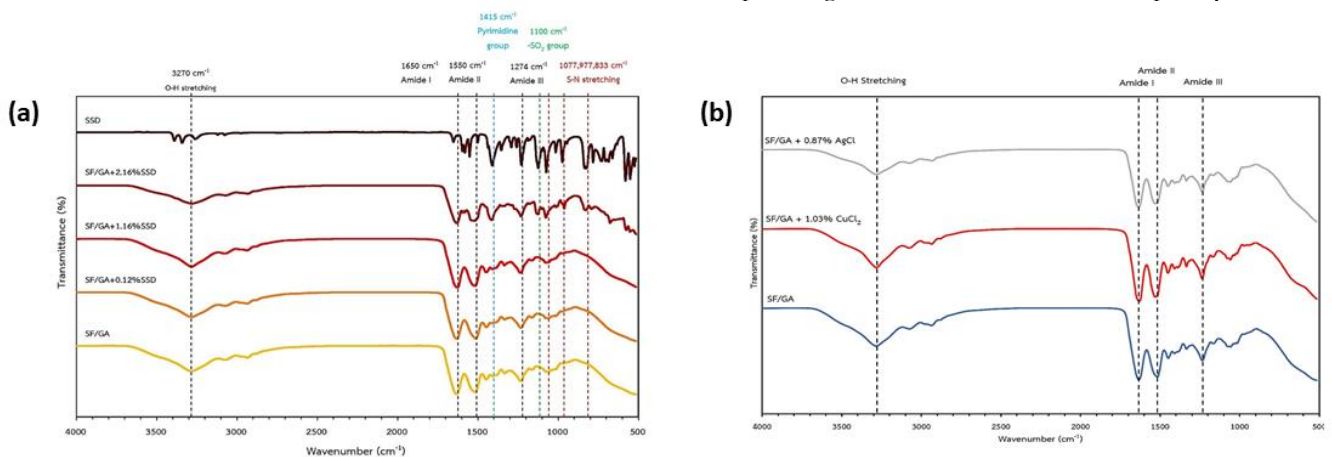


Fig. 1. FTIR spectra of (a) SSD (—) and SF/GA films incorporating 0 (—), 0.12 (—), 1.16 (—) and 2.16% w/w SSD (—), (b) SF/GA films (—) and SF/GA films incorporating 0.87% w/w AgCl (—) or 1.03% w/w  $\text{CuCl}_2$  (—).

Table 1. Percentage of secondary structures of SF/GA films incorporating 0, 0.12, 1.16 and 2.16% w/w SSD or 0.87% w/w AgCl or 1.03% w/w CuCl<sub>2</sub>, as obtained from the deconvolution of the amide I band of the FTIR spectra.

Wavenumber (cm <sup>-1</sup> )	Secondary structure	Percentage of secondary structure					
		SF/GA film	SF/GA film + 0.12% SSD	SF/GA film + 1.16% SSD	SF/GA film + 2.16% SSD	SF/GA film + 0.87% AgCl	SF/GA film + 1.03% CuCl <sub>2</sub>
1590-1605	Tyrosine	1.94	1.66	2.14	12.36	4.07	12.23
1608-1637, 1697-1700	Beta sheet	<b>36.81</b>	<b>39.67</b>	<b>45.49</b>	<b>43.78</b>	<b>40.66</b>	<b>43.15</b>
1638-1653	Random coil	31.82	26.50	26.42	17.63	30.16	23.36
1654-1659	Alpha helix	9.88	9.79	8.47	7.01	10.93	7.47
1660-1696	Beta turn	19.55	22.38	17.48	19.22	14.18	13.79

Table 2. Percentage of increased thickness and swelling ratio of SF/GA films incorporating 0, 0.12, 1.16 and 2.16% w/w SSD after incubated in PBS solution (pH 7.4) at 37°C for 24 h (\* represent significant difference at  $p < 0.05$  when comparing to the value of SF/GA film without SSD).

	Percentage of increased thickness (%)	Swelling ratio (by wight)
SF+GA film + 0% SSD	54.27±0.96	3.24±0.08
0.12%SSD	41.30±1.29*	2.38±0.02*
1.16%SSD	40.09±1.37*	2.10±0.04*
2.16%SSD	36.62±0.40*	2.03±0.01*

Table 3. Water-insoluble fraction of SF/GA films incorporating 0, 0.12, 1.16 and 2.16% w/w SSD or 0.87% w/w AgCl or 1.03% w/w CuCl<sub>2</sub> after incubated in deionized water (pH 6.5) at 37°C for 24 h (\* represent significant difference at  $p < 0.05$  when comparing to the value of SF/GA film without SSD).

Sample	Water-insoluble fraction (%)
SF/GA film+ 0% SSD	38.54±3.79
0.12% SSD	70.84±2.12*
1.16% SSD	72.87±4.10*
2.16% SSD	78.26±1.18*

degraded with 14 days of incubation, however, some amount (10-20%) of the films incorporating 1.16 and 2.16% w/w SSD were remained. In correspondence with previous data, the SF/GA films incorporating SSD had stable  $\beta$ -sheet structure, then the degradation rate was extended.

### 3.5. *In vitro* Release of SSD from SF/GA Films

SSD was released rapidly from all films after incubated in either PBS or protease enzyme solutions (Fig. 3). The significant difference in SSD release profiles of all films was not observed. Although the incorporation of SSD stabilized SF/GA films, SSD may bind with the SF and GA molecules in the films via only the weak electrostatic interaction. Therefore, SSD would burst from the films rapidly.

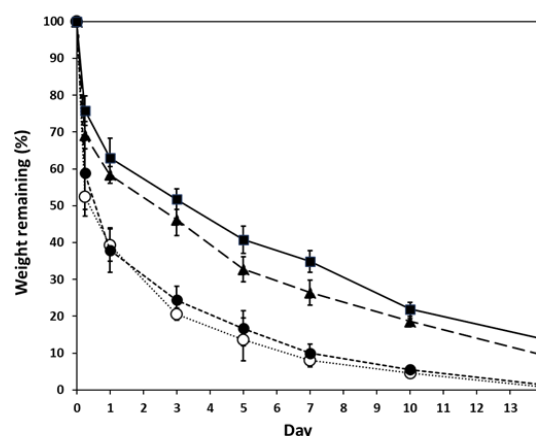


Fig. 2. Degradation profiles of SF/GA films incorporating 0 (---○---), 0.12 (---●---), 1.16 (—▲—) and 2.16% w/w SSD (—■—) when incubated in protease enzyme solution (pH 7.4, conc. 1 unit/ml) at 37°C.

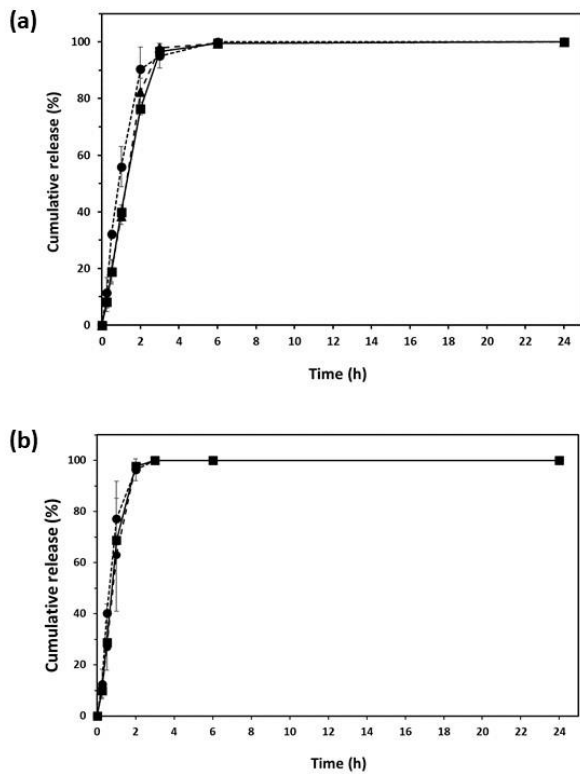


Fig. 3. Cumulative release profiles of SSD from SF/GA films incorporating 0.12 (---●---), 1.16 (---▲---) and 2.16% w/w SSD (—■—) when incubated in (a) PBS solution (pH 7.4) and (b) protease enzyme solution (pH 7.4, conc. 1 unit/ml) at 37°C.

### 3.6. Cell Cytotoxicity of SF/GA Films Incorporating SSD

Viability percentages of L929 cells cultured in extracted medium of all SF/GA films incorporating SSD at various dilutions for 24 h were higher than 70% (Fig. 4), indicating non-cytotoxicity of the films against L929 cells according to ISO 10993-Part 5 standard. The cell cultured in serum-free DMEM and DMEM with 10% serum served as negative control groups showed complete viability, respectively. On the other hand, DMEM with zinc acetate as a positive control group was toxic to L929 cells by lowering viability of cells down to 10%. The result confirmed the non-cytotoxicity of SF/GA films incorporated with SSD.

### 3.7. 3.7 Anti-bacterial Activity of SF/GA Films Incorporating SSD

To verify anti-bacterial activity of SSD incorporated in SF/GA films, two types of bacteria which are *Staphylococcus aureus* (Gram positive bacteria) and *Escherichia coli* (Gram negative bacteria) were selected to culture with SF/GA films and SF/GA films incorporating SSD (Fig. 5). Inhibition zone was not observed on SF/GA film without SSD for both bacteria. On the other hand, inhibition zones of both bacteria cultured with SF/GA film incorporating 1.16% and 2.16% SSD were around 2.6-3.2 cm. According to the Performance standards for antimicrobial susceptibility of Clinical and Laboratory Standards Institute (CLSI), the inhibition zone over 1.5 cm is evaluated as susceptible level of bacteria [11]. Therefore, this clearly demonstrated the anti-bacterial effect of SF/GA films incorporating SSD which could be useful in medical applications.

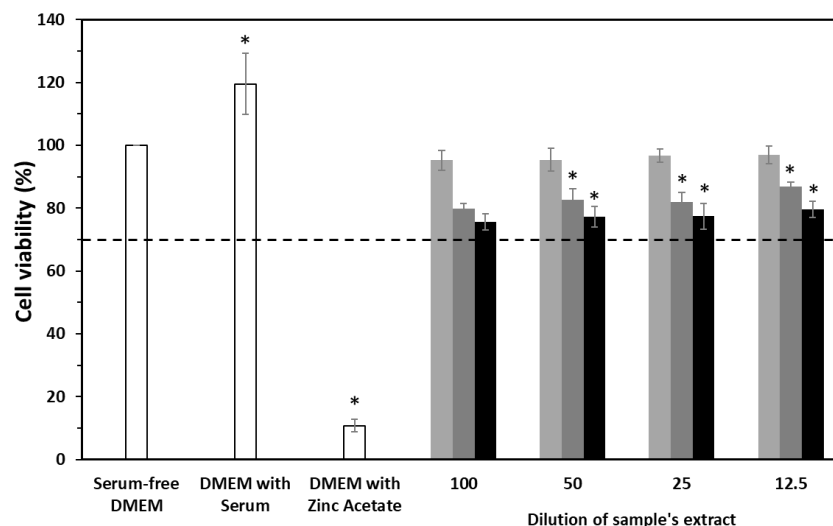


Fig. 4. Percentage of L929 cell viability after cultured with the extracts of SF/GA films (■) and SF/GA films incorporating 1.16% (■), 2.16% w/w SSD (■) at different dilutions (100, 50, 25 and 12.5%) at 37°C for 24 h (\* represent significant difference at  $p < 0.05$  when comparing to the value of serum-free DMEM group).

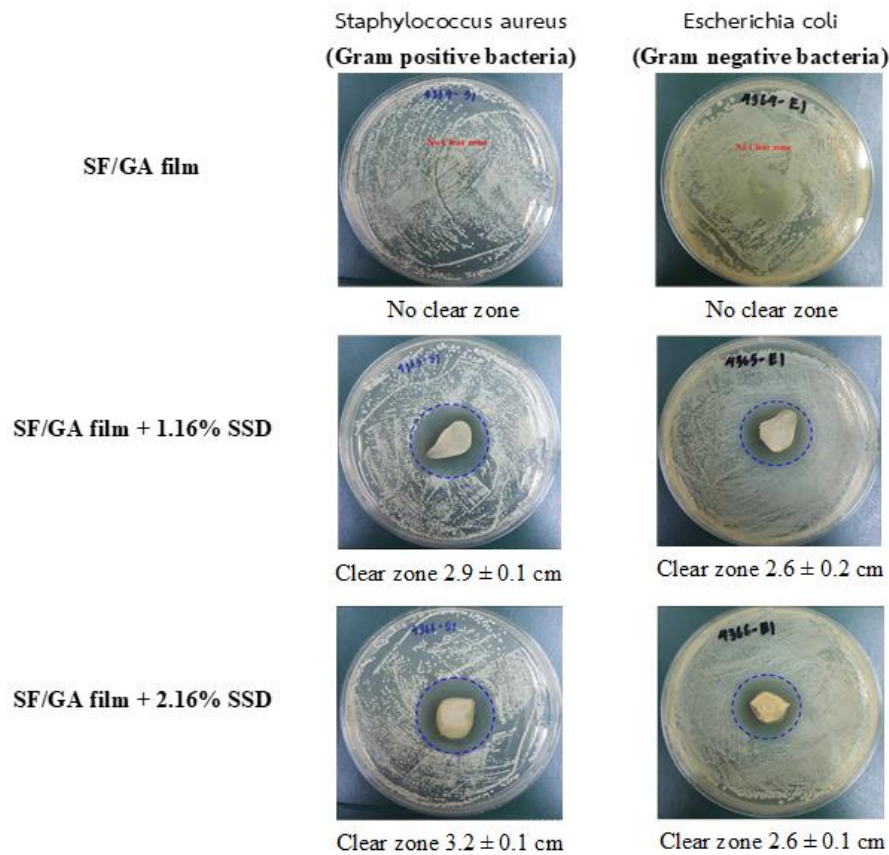


Fig. 5. Inhibition zone of *Staphylococcus aureus* (gram-positive) and *Escherichia coli* (gram-negative) bacteria when cultured with SF/GA film and SF/GA films incorporating 1.16% and 2.16% w/w SSD at 37°C for 24 h.

#### 4. Conclusion

The SF/GA films incorporating SSD at various concentrations were fabricated. Incorporation of SSD in the films led to the conformational change of silk fibroin by increasing content of the  $\beta$ -sheet structure. The same phenomena was further confirmed by the incorporation of other 2 metallic ions, Ag(I) from AgCl and Cu(II) from  $\text{CuCl}_2$ , in the SF/GA films. The SF/GA films were stabilized by SSD, leading to less water-insolubility and extended degradation rate, compared to the SF/GA film without SSD. However, SSD was burst released from the films. The SF/GA films incorporating SSD at all concentrations were non-cytotoxic to L929 mouse fibroblast cells when tested according to ISO 10993 part 5. The SSD released from the films showed an essential anti-bacterial activity against both *Staphylococcus aureus* (Gram positive) and *Escherichia coli* (Gram negative) bacteria. The SF/GA films incorporating SSD could be further applied in medical uses, such as anti-bacterial dressing.

#### Acknowledgement

Financial support from Chula Engineering's promoting research grant, Faculty of Engineering, Chulalongkorn University is gratefully acknowledged.

#### References

- [1] A. E. Thurber, F. G. Omenetto, and D. L. Kaplan, "In vivo bioresponses to silk proteins," *Biomaterials*, vol. 71, pp. 145-157, 2015.
- [2] H. Tungtasana, S. Shuangshoti, S. Kanokpanont, D. L. Kaplan, T. Bunaprasert, and S. Damrongsakkul, "Tissue response and biodegradation of composite scaffolds prepared from Thai silk fibroin, gelatin and hydroxyapatite," *J. Mater. Sci. Mater. Med.*, vol. 21, pp. 3151-3162, 2010.
- [3] C. Vepari and D. L. Kaplan, "Silk as a biomaterial," *Prog. Polym. Sci.*, vol. 32, pp. 991-1007, 2007.
- [4] P. Jetbumpenkul, P. Amornsudthiwat, S. Kanokpanont, and S. Damrongsakkul, "Balanced electrostatic blending approach – An alternative to chemical crosslinking of Thai silk fibroin/gelatin scaffold," *Int. J. Biol. Macromol.*, vol. 50, pp. 7-13, 2012.
- [5] J. Ratanavaraporn, S. Kanokpanont, and S. Damrongsakkul, "The development of injectable gelatin/silk fibroin microspheres for the dual delivery of curcumin and piperine," *J. Mater. Sci.: Mater. Med.*, vol. 25, pp. 401-410, 2013.
- [6] M. Okhawaia, R. Rangkupanb, S. Kanokpanonta, and S. Damrongsakkul, "Preparation of Thai silk fibroin/gelatin electrospun fiber mats for controlled release applications," *Int. J. Biol. Macromol.*, vol. 46, pp. 544-550, 2010.

- [7] K. Lerdchai, J. Kitsongsermthon, J. Ratanavaraporn, S. Kanokpanont, and S. Damrongsakkul, "Thai silk fibroin/gelatin sponges for the dual controlled release of curcumin and docosahexaenoic acid for anticancer treatment," *J. Pharm. Sci.*, vol. 105, pp. 221–230, 2015.
- [8] C. Laomeephol, M. Guedes, H. Ferreira, R. L. Reis, S. Kanokpanont, S. Damrongsakkul, and N. M. Neves, "Phospholipid-induced silk fibroin hydrogels and their potential as cell carriers for tissue regeneration," *J. Tissue Eng. Regen. Med.*, vol. 14, pp. 160–172, 2020.
- [9] C. Rigo, M. Roman, I. Munivrana, V. Vindigni, B. Azzena, C. Barbante, and W. R. Cairns, "Characterization and evaluation of silver release from four different dressings used in burns care," *Burns*, vol. 38, no. 8, pp. 1131-42, 2012.
- [10] T. Mosmann, "Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays," *J. Immunol. Methods*, vol. 65, no. 1-2, pp. 55-56, 1983.
- [11] *Performance Standards for Antimicrobial Susceptibility Testing*, Clinical and Laboratory Standards Institute (CLSI), USA, 2013.
- [12] X. H. Zong, P. Zhou, Z. Z. Shao, S. M. Chen, X. Chen, B. W. Hu, F. Deng, and W. H. Yao, "Effect of pH and copper(II) on the conformation transitions of silk fibroin based on EPR, NMR, and Raman Spectroscopy," *Biochemistry*, vol. 43, no. 38, pp. 11932-11941, 2004.
- [13] T. Tanaka, J. Magoshi, Y. Magoshi, S.I. Inoue, M. Kobayashi, H. Tsuda, and S. Nakamura, "Acupuncture status in Canada: Summary of educational, social and legislative issues in Ontario global communications on acupuncture (17)," *Polym. Preprints*, vol. 42, pp. 623-623, 2001.
- [14] A. Ochi, K.S. Hossain, J. Magoshi, and N. Nemoto, "Rheology and dynamic light scattering of silk fibroin solution extracted from the middle division of *Bombyx mori* silkworm," *Biomacromolecules*, vol. 3, no. 6, pp. 1187-1196, 2002.
- [15] L. Zhou, X. Chen, Z. Shao, P. Zhou, D. P. Knight, and F. Vollrath, "Copper in the silk formation process of *Bombyx mori* silkworm," *FEBS Letters*, vol. 554, pp. 337-341, 2003.
- [16] L. Zhou, X. Chen, Z. Shao, Y. Huang, and D. P. Knight, "Effect of metallic ions on silk formation in the Mulberry silkworm, *Bombyx mori*," *J. Phys. Chem. B*, vol. 109, no. 35, pp. 16937-16945, 2005.
- [17] J. P. Schneider and J. W. Kelly, "Synthesis and efficacy of square planar copper complexes designed to nucleate .beta.-sheet structure," *J. Am. Chem. Soc.*, vol. 117, no. 9, pp. 2533-2546, 1995.
- [18] A. Lim, P.A. Guy, A. M. Makhov, M. J. Saderholm, M. Kroll, Y. B. Yan, R. J. Anderegg, J. D. Grith, and B. W. Erickson, "Engineering of betabellin 15D: Copper(II)-induced folding of a fibrillar beta-sandwich protein," *Lett. Pept. Sci.*, vol. 6, no. 1, pp. 3-14, 1999.
- [19] R. Wong, M. Ashton, and K. Dodou, "Effect of crosslinking agent concentration on the properties of unmedicated hydrogels," *Pharmaceutics*, vol. 7, no. 3, pp. 305-319, 2015.
- [20] H. V. Chavda and C. N. Patel, "Effect of crosslinker concentration on characteristics of superporous hydrogel," *Int. J. Pharm. Investig.*, vol. 1, no. 1, pp. 17–21, 2011.

**Patchara Umprasert**, photograph and biography not available at the time of publication.

**Siriporn Damrongsakkul**, photograph and biography not available at the time of publication.

**Supansa Yodmuang**, photograph and biography not available at the time of publication.

**Juthamas Ratanavaraporn**, photograph and biography not available at the time of publication.