CHAPTER III
EXPERIMENTAL

3.1 Material

3.1.1 Silk and Coconut oil

Raw silk fiber, Bombyx Mori, was obtained from Thai Sanetsu (Thailand). Virgin coconut oil was purchased from Maprawthai., Thailand.

3.1.2 Other Chemical

Escherichia coli and Staphylococcus aureus were purchased from Microbiological resource centre, Thailand Institute of Scientific and Technological Research (TISTR). Nutrient Broth (Approximate formula per liter: Beef extract 3.0g and Peptone 5.0g) was purchased from Difco™. Agar powders were bacteriological grade and purchased from Himedia. Analytical grade Ethanol, Methanol, Chloroform and Glacial acetic acid 99.7% w/w were purchased from Labscan Asia Co., Ltd., Thailand. Laboratory grade Calcium chloride ($CaCl_2$), Sodium chloride (NaCl) and Sodium acetate hydrated ($CH_3.COONa.3H_2O$) were purchased from Ajex Finechem. Pluronic f68 (Polyoxyethylene-Polyoxypropylene block copolymer) was purchased from S.M. Chemical. Laboratory grade Sodium carbonate was purchased from Fisher Scientific.

3.2 Equipment

3.2.1 UV-Vible Spectrophotometer

The released oil was determined by using UV-Vible Spectrophotometer (Barascientific, model UV-1800 spectrophotometer).

3.2.2 Scanning electron microscopy (SEM)

The sample sheets were removed oil by imersing in 100% chloroform for 5 h. Then the sample sheets were immersed in 100% ethanol for 15 min and changed to 50% ethanol for 15 min and changed to water for 15 min. The sample

sheets were dried by freeze-drying method to preserve the morphology of the sample sheets. Cross section and surface morphology of the sample sheets were observed by using JEOL, JSM-5410LV scanning microscope (SEM) at 15 kV.

3.2.3 Fourier transform infrared spectroscopy (FTIR)

The FTIR spectrum of pure silk fibroin, methanol-treated oil-incorporated silk fibroin, Coconut oil, and Pluronic f68 were recorded with a Thermo Nicolet, Nexus 670 FT-IR Spectrophotometer, with 32 scan at a resolution of 4 cm^{-1}. A frequency range of 4000-400 cm^{-1} was observed by using deuterated triglycinesulfate detector (DTGS) with specific detectivity of 1 x 10^9 $cm.Hz^{1/2}.W^{-1}$.

3.2.4 Thermogravimetric Analysis (TGA)

The thermal property of oil-incorporated silk fibroin was examined by using a thermogravimetric analyzer (TG-DTA, Perkin Elmer, Thailand) in the temperature range of 30 to 950 °C at a heating rate of 10 °C/min, under a nitrogen atmosphere (50 cm^3/min). The mass of the sample pan was continuously recorded as a function of temperature.

3.2.5 Optical microscopy (OPM

Stability of the oil in water emulsions at different concentrations of silk and oil were confirmed by using Olympus optical microscope at a magnification of 40 to observe the emulsion that are stained with oil soluble dye (SudanIII) and water soluble dye (Phenol red).

3.3 Methodology

3.3.1 Preparation of aqueous silk fibroin solution

The raw silk fibers of Bombyx mori were boiled for 15 min in an aqueous solution of 0.05% Na_2CO_3. The boiling process was repeated two times to remove sericin. The fibers were then rinsed thoroughly with hot water and dried at 40 °C overnight. After drying, the degummed silk fibers were dissolved in a solvent mixture of $CaCl_2$: ethanol: H_2O (molar ratio = 1:2:8) at 78 °C. The undissolved

particle was filtered out using filter cloth. The filtrated solution was subsequently dialyzed in distilled water for 4 days by changing the media daily, followed by centrifugation at 10,000 rpm for 10 min. (Wongpanit et al., 2007)

3.3.2 Preparation of coconut oil-incorporated silk fibroin sheets

3.3.2.1 Preparation of emulsions

Pluronic f68 was prepared at desirable concentration by dissolve in water at different concentration to investigate the concentrations that give complete emulsion of oil in silk solution. The emulsion was prepared by preparing homogeneous mixture of Pluronic f68 at different concentrations and 4% w/v silk solution and then 15.33 % w/v coconut oil was added at mixture solution. The mixture was homogenized by using vortex at steady force for 30 seconds and observed emulsion stability after left for 24 hrs. (Tomoko et al., 2004; Canselier et al., 2002)

3.3.2.2 Processing of the emulsion

3.3.2.2.1 Freeze drying method

The emulsion of silk fibroin and coconut oil were put into the Teflon mold and then frozen in refrigerator at -40 °C for 12 h. The ice/silk composites were dried with a freeze-dryer, leaving a porous matrix. After the porous matrices were obtained, they were treated in saturated vapor methanol for about 3 h to induce crystallization and water-stability. The water insoluble three-dimensional silk fibroin incorporated coconut oil scaffolds were then prepared following methanol evaporating at room temperature. (Qiang et al., 2005)

3.3.2.2.2 Vacuum drying method

The emulsion of fibroin and coconut oil were put into the Teflon mold and left at room temperature over night and then put into vacuum for remove water at room temperature, leaving the emulsion films. After the emulsion films were obtained, they were treated in saturated vapor methanol for about 3 h to induce crystallization and water-stability. The water insoluble three-dimensional silk fibroin incorporated coconut oil sheets were then prepared following methanol evaporating at room temperature. (Qiang et al., 2005)

3.3.2.3 Silk fibroin coating

The methanol-treated oil-incorporated silk fibroin sheet was immersed in 10 ml of 15% (w/v) silk solution and the suspension was gently shaken for 1 min at room temperature and was dried in air. The coated oil-incorporated silk fibroin sheets were treated with methanol again. Double-coated and triple-coated oil-incorporated silk fibroin sheets were prepared by repeating the above coating procedure.

3.3.3 Study the possibility for using as wound dressing

3.3.3.1 Absorption ability

The samples are weighed and immersed in acetate buffer at pH 5.5 and shake in shaking incubator at 37°C at the speed of 50 rpm. At specific time intervals, the weight of the samples was noted after removing the acetate buffer and gently blotting the samples with a filter paper. Weights of samples were noted until equilibrium swelling was reached.

$$\text{Equilibrium fluid content} = \frac{[W_s - W_d]}{W_s} \times 100 \qquad (3.1)$$

Where W_s and W_d represent the weight of swollen and dry sample, respectively. All experiments was done at least in triplicate.

$$\text{Equilibrium degree of swelling} = \frac{[W_s - W_i]}{W_i} \times 100 \qquad (3.2)$$

Where W_i is the weight of initial sample (before being immersed in water).

3.3.3.2 Releasing property

Release of coconut oil from the oil-incorporated silk fibroin sheets was studied by put into 20 ml acetate buffer solution, pH 5.5 and shaken in water bath shaking at 37°C. All Samples was collected at studying time. The released coconut oils were expressed as the percentage of initial coconut oil in the oil-incorporated silk fibroin sheets that were quantified by the partition-gravimetric method using Chloroform as a solvent (APHA et al., 1992). Briefly, the oil-

incorporated silk fibroin sheet was disintegrated in 10 ml chloroform by centrifuging at 12,000 rpm for 10 min. Both initial coconut oil and released coconut oil were extracted with chloroform by shaking in separator funnel for 2 min. The separator funnel was left standing to obtain two separate phases. The lower layer (solvent phase) was drained through a funnel containing a filter paper (Whatman No. 40) and both the separator funnel and the filter paper were rinsed with the solvent. The upper layer (aqueous phase) was re-extracted a few more times using the same procedure. The solutions were determined amount of coconut oil at 240 nm by UV-VIS spectrophotometer. The filtered extracted phase was then taken to determine amount of coconut oil by UV-VIS spectrophotometer. The release data was fitted by using Eq. (3.3) (Ritger et al., 1987) in order to propose the possible release mechanism.

$$\frac{M_t}{M_\alpha} = Kt^n \qquad (3.3)$$

Where M_t corresponds to the amount of released oil at time t; M_α is the total amount of released oil that must be released at infinite time; K is a release constant that is related to the structural and geometric properties of the dosage form; n is the release exponent indicating the type of oil release mechanism. This equation has been used frequently in the literature, due to its utility in describing the relative importance of Fickian (n=0.5) and zero-order (n=1) mechanism in drug diffusion. In zero-order kinetics, the release rate remains constant until the entire active ingredient has been delivered. The term zero-order means time-independent rate and so as being independent of quantity of oil remaining.

3.3.3.3 Evaporative water loss

The samples were kept at 37 °C. After regular intervals of time, the weight of the oil-incorporated silk fibroin sheet was measured. Water loss was estimated by the following equation:

$$\text{Water loss (\%)} = \frac{W_t}{W_0} \times 100 \qquad (3.4)$$

Where W_0 and W_t are the initial weight after 24 h immersion time and weight after time (t), respectively

3.3.3.4 Weight loss

Weight loss of the oil-incorporated silk fibroin sheets were were plotted after incubation in acetate buffer solution (pH 5.5) for different periods. Percentage loss in weight was determined by the formula:

$$\text{Water loss (\%)} = \frac{W_i - W_t}{W_i} \times 100 \qquad (3.5)$$

Where W_i is the initial dry weight of the construct and W_t is the final weight after time (t). The experiment was performed in triplicate.

3.3.4 Antimicrobial Activity Testing

Antimicrobial activities of oil-incorporated silk fibroin sheets have been investigated against Escherichia coli and Staphylococcus aureus. The samples used for the antimicrobial activity assay were sterilized under UV light over a period of 30 min. In this work, two kinds of bacteria, Escherichia coli (E. coli) and Staphylococcus aureus (S. aureus) were used for test. The 1 ml bacteria were added to a bottle contains 9 ml of 0.85% sterile saline solution.(to control the osmotic pressure of bacteria). The solution was diluted to 9 ml of 0.85% sterile saline solution in other bottles until concentration was 10^{-6} colony units per ml (cfu/ml) for Escherichia coli (E. coli) and 10^{-6} colony units per ml (cfu/ml) for Staphylococcus aureus (S. aureus). Each bottle was added with samples. This experiment tested antimicrobial activity of the sample by comparing to pure silk, pure coconut oil. All bottles were shaken in shaking incubation at 37°C with the speed 150 rpm for 3 hours. The 100 μL of bacterial suspension was drawn from each tube, spread on a nutrient agar plate and incubated at 37 °C for 24 h for colony forming counts.

$$\text{Bacterial Reduction Rate (BRR)} = \frac{(N_1 - N_2)}{N_1} \times 100 \qquad (3.5)$$

Where N_1 is the number of colonies of blank control and N_2 is number of colonies of positive control