CHAPTER III
EXPERIMENTAL

3.1 Materials

The shell of *Penaeus merguiensis* shrimps was kindly provided by Surapon Food Public Co., Ltd. Silk fibre (*Bombyx Mori*) was degummed by treatment with 0.5% Na_2CO_3 at 100°C for 30 min, followed by washing with boiling distilled water. The degummed silk was dried at 60°C for 24 h in an oven. Aferwards, the silk fibroin was dissolved in a triad solvent $CaCl_2$: EtOH: H_2O with mole ratio of 1:2:8 at 100°C for 15 min. The silk solution was then dialyzed against distilled water for 7 days. The solution was next filtered through the sintered glass filter and subsequently diluted to achieve a concentration of 1% w/w.

Sodium hydroxide 50% w/w solution was kindly supplied by KPT Cooperation (Thailand). Glacial acetic acid 99.9% w/w purchased from J.T. Baker was analytical grade. Glutaraldehyde 50% w/w was purchased from Fluka.

Salicylic acid was purchased from Ajax Chemicals, Australia. Theophylline was purchased from Shanghai Wandai Pharmaceutical Co., Ltd., China. Diclofenac sodium was purchased from Tangyin Yongqi Chemical Industry Co., Ltd., China. Amoxicillin trihydrate was purchased from Anibiotics Co., Ltd., Spain. Other reagents are analytical grade and used without further purification.

3.2 Equipments

3.2.1 Capillary Viscometer

The viscosity-average molecular weight of chitosan was determined by using Cannon Ubbelohde-type number 50 of capillary viscometer.

3.2.2 FTIR Spectrophotometer

The FTIR spectrum of chitosan was recorded with Vector 3.2 Bruker FTIR Spectrophotometer with 16 scans at a resolution of 4 cm^{-1}. A frequency of

4000-400 cm^{-1} was observed by using deuterated triglycerinesulfate detector (DTGS) with specific detectivity of 1×10^{9} $cm\cdot Hz^{1/2}\cdot w^{-1}$.

3.2.3 UV/Visible Spectrophotometer

The amount of drug releasing from chitosan films and the blend films at pH 5.5 was determined by using Perkin Elmer UV/visible Spectrophotometer model Lambda10.

3.3 Methodology

3.3.1 Chitin Preparation

Chitin was prepared from shrimp shell by decalcification and deproteinization to remove calcium carbonate and protein, respectively. The shrimp shells were cleaned and dried under sunlight before grinding into small pieces. Shrimp shell chips were treated by immersion in 1 N HCl solution for 2 days with occasional stirring. The decalcified product was washed with distilled water until neutral. Deproteinization was followed by boiling in 4% w/w of NaOH solution at 80-90°C for 4 h. After NaOH solution was decanted, the chips were washed with deionized water until neutral. The product obtained was dried at 60°C in a convective oven for 24 h.

3.3.2 Chitosan Preparation

Chitin was deacetylated by heating in 50% w/w NaOH solution containing 0.5% w/w sodium borohydride ($NaBH_4$) to prevent depolymerization. The ratio of chitin to NaOH solution was 1 g of chitin in 10 ml of NaOH solution. The deacetylation was performed in an autoclave at 110°C for 1 h. The deacetylated product obtained was washed exhaustedly with deionized water until neutral. The resulting chitosan flakes was dried in an oven at 60°C for 24 h.

3.3.3 Degree of Deacetylation of Chitosan

The degree of deacetylation of chitosan was determined, based on an IR spectroscopic method reported by Sannan (1978). About 3 mg of chitosan powder, passed through a 200-mesh sieve, was mechanically mixed with 400 mg of potassium bromide to prepare a KBr disk. An infrared spectrum was recorded in a range from 4000 to 400 cm^{-1}. The absorbances at 2878 cm^{-1} (the C-H band) and 1550 (the amideII band) were used to determine the degree of deacetylation. The degree of deacetylation was calculated from the equation 3.1.

$$D = 98.03-34.68(A_{1550}/A_{2878}) \quad (3.1)$$

where D = degree of deacetylation (%)

A_{1550} = absorbance at 1550 cm^{-1}

A_{2878} = absorbance at 2878 cm^{-1}.

3.3.4 Viscosity-Average Molecular Weight of Chitosan

Chitosan solutions of different concentrations (0.00, 0.0125, 0.025, 0.050, 0.075 and 0.1g/100ml) in 0.2 M acetic acid: 0.1 M sodium acetate were prepared. An Ubbelohde viscometer was filled with 10 ml of sample, which maintained the temperature at 30°C. The sample was passed through the capillary once before the running times were measured. Each sample was measured 3 times. The running times of solvent and solutions were used to calculate the relative viscosity, specific viscosity, and reduced viscosity. The reduced viscosity was plotted against the concentration and the intrinsic viscosity determined from the intercept. The corresponding equations are:

$$\text{Relative viscosity } (\eta_{rel}) = t/t_s \quad (3.2)$$

$$\text{Specific viscosity } (\eta_{sp}) = (t/t_s)-1 \quad (3.3)$$

$$\text{Reduced viscosity } (\eta_{red}) = \eta_{sp}/C \quad (3.4)$$

$$\text{Intrinsic viscosity } [\eta] = (\eta_{red})_{c\rightarrow 0} \quad (3.5)$$

where t is the flow time in seconds of chitosan solution, t_s is the flow time in seconds of solvent and C is the concentration of chitosan solution in g/100 ml.

The viscosity average molecular weight of chitosan was determined based on the Mark-Houwink equation (Lee *et al.*, 1974)

$$[\eta] = 7.52 \times 10^{-4} M^{1.0016} \quad (3.6)$$

where $[\eta]$ is the intrinsic viscosity and M is viscosity average molecular weight.

3.3.5 Chitosan Solution Preparation

Chitosan flake was dried at 110°C for 1 h before use. Chitosan solution was prepared by dissolution of chitosan in 1% w/w acetic acid. The chitosan solution was allowed to stand overnight at room temperature to get rid of air bubbles before preparation of films.

3.3.6 Crosslinked Drug-Contained Blend Films Preparation

Solutions containing chitosan and silk fibroin were prepared by mixing various ratios of 1% w/w of silk fibroin solution and 1% w/w of chitosan solution. Glutaraldehyde, used as crosslinking agent, was added to the blend solutions at the amount of 0.01 mole/glucosamine unit of chitosan. The model drugs (theophylline, diclofenac sodium, salicylic acid and amoxicillin trihydrate) were added to the blend solutions to reach a concentration of 1.0% w/w. The blend solution containing a model drug was stirred slowly for 12 h, residues some amounts of drug that over the solubility of drug, and left overnight to get rid of air bubbles before casting onto clean dry petri dishes in a dust-free atmosphere at room temperature. The films were allowed to dry at ambient temperature for 72 h and then stored over silica in a desiccator before use.

3.3.7 Skin Preparation

Permeation experiments were performed with full-thickness pig skin which were excised from a side of pigs. The whole pig skins were surgically removed and cleaned with sterile normal saline. The subcutaneous fat, tissue, blood vessel and epidermal hair were carefully removed by blunt section. The skin was

free of obvious holes or defects. The full thickness skin was cleaned with normal saline and finally with distilled water, blotted dry, wrapped with aluminium foil and stored frozen before use. To perform in-vitro skin permeation experiment, full thickness skin was thawed at room temperature and cut into pieces (peripheral of circumference cell cap area) and a unit of drug-loaded blend films was applied onto the stratum corneum surface of the skin and then mounted individually between the half-cells.

3.3.8 Spectrophotometric Analysis of Model Drug

UV/visible Spectrophotometer model Lambda10 (Perkin Elmer) was employed to determine the maximum spectra of model drugs (theophylline, salicylic acid, diclofenac sodium and amoxicillin trihydrate). It was performed by scanning the UV absorption in a wavelength range of 350-200 nm. Model drugs in aqueous solution was prepared for scanning the maximum absorption wavelength. The procedure was done at an ambient condition with a scan speed of 240 nm/min. The characteristic peaks were observed for theophylline, salicylic acid, diclofenac sodium and amoxicillin trihydrate at maximum wavelength of 272, 299, 275 and 272 nm, respectively. The absorbance values at the maximum wavelength of model drugs were read and the correspondent medel drug concentrations were calculated from the calibration curve. The calibration curves were plotted between the concentrations of drugs and the absorbance. The various concentrations of drug were in range 0.1-1 mg/100 ml.

3.3.9 *In vitro* Skin Permeation of Drug

The *in vitro* skin permeation of drug from prepared membrane was studied using a modified Franz diffusion cell. The full-thinkness pig skin was mounted onto the receptor compartment with the stratum corneum side facing upward into the donor compartment and the dermal side facing downward into the receptor compartment. A unit of drug-contained blend film was placed over the skin and the whole assembly was clamped together with the donor cap on the top. The receptor compartment was then filled with the acetate buffer solution pH 5.5 constantly stirred using a magnetic stirrer and manintained at 37°C by a circulating

waterbath. A portion (0.5 ml each) of buffer solutions were withdrawn from the receptor compartment at predetermined time intervals of ¼, ½, ¾, 1, 1½, 2, 2½, 3, 4, 5, 6, 8, 10, 12, 14, 16, 18, 20, 22 and 24 hours; the samples were replaced with an equal volume of freshly prepared pH 5.5 acetate buffer solutins (drug-free). The drug concentrations in these samples were determined by the UV/visible spectrophotometer method.

3.3.10 Kinetics

The graphs between drug release and time from time zero to t were plotted for zero order kinetic. The graphs between drug release and $(time)^{1/2}$ were plotted for Higuchi's model. The linear correlation coefficient (R^2) of zero order kinetic was calculated to compare to the linear correlation coefficient of Higuchi's model. The one is close to the value one of correlation coefficient. Those data was fitted to that model.