CHAPTER III
EXPERIMENTAL

3.1 Materials

Chitin was prepared from shrimp shell (*Penaeus merguiensis*) that was kindly supplied from Surapol Food Public Co., Ltd. by the method of Shimahara and Takigushi (1988). The degree of deacetylation of chitin was determined by infrared spectroscopic measurement according to the method of Sannan *et al.* (1978) was 24.14%. Chitin was ground to powder with the size of 71-75 μm before use. Glutaraldehyde (50% w/w) was purchased from Fluka. Monochloroacetic acid, glacial acetic acid (99.9% w/w) and sodium hydroxide were analytical grade. Theophylline was purchased from Shanghai Wandai Pharmaceuticals, China. Diclofenac sodium was purchased from Tangyin Yongqi Chemical Industry Co., Ltd., China. Salicylic acid was purchased from Ajax Chemicals, Australia. Amoxicillin trihydrate was purchased from Antibiotics Co., Ltd., Spain.

3.2 Equipment

3.2.1 Restch Sieving Machine

The chitin powder was sieved by using Restch Sieving Machine type Vibro and chitin with the size of 71-75 μm was collected for using in the experiment.

3.2.2 Capillary Viscometer

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

3.2.3 Elemental Analysis

The degree of substitution of CM-chitin was estimated by elemental analysis using PERKIN ELMER Series II CHNS/O Analyzer 2400.

3.2.4 FTIR Spectrophotometer

The FTIR spectrum of silk fibroin/CM-chitin blend films were recorded with a Bruker FTIR Spectrophotometer, model Vector 3.0, with 16 scans at a resolution of 4 cm^{-1}. The samples with the thickness of 10 μm were attached to the sample frames. A frequency of 4000-400 $cm^{-1}$was observed by using deuterated triglycinesulfate detector (DTGS) with specific detectivity of 1 x 10^9 $cm.Hz^{1/2}.W^{-1}$.

3.2.5 UV/Visible Spectrophotometer

The amount of drug release from CM-chitin films and blend films at pH 2.0, 5.5, and 7.2 was determined by using UV/Visible Spectrophotometer model Lambda10, Perkin Elmer.

3.3 Methodology

3.3.1 Preparation of Chitin

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 the decalcified product in 4% w/w of NaOH solution at 80-90°C for 4 h. After NaOH solution was decanted, the product was washed with deionized water until neutral and dried at 60°C in a convective oven for 24 h.

3.3.2 Viscosity-Average Molecular Weight of Chitin

The different concentrations (0.00625, 0.0125, 0.025, 0.05 and 0.01 g/100 ml) of Chitin solutions dissolved in 5% LiCl/N,N-dimethylacetamide were prepared. All of samples were passed through filter papers before use. The Ubbelohde viscometer was filled with 10 ml of sample solution and then equilibrated in water bath, which was maintained the temperature at 30°C. The sample was

passed through the capillary once before the running time was measured. Each sample was measured 5 times. The running times of solvent and solutions were used to calculate the relative viscosity, specific viscosity, and reduced viscosity, by the following equations:

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

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

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

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

where t is the running time of CM-chitin solution, t_s is the running time of solvent and C is the concentration in g/100 ml.

The viscosity-average molecular weight of CM-chitin was determined based on Mark-Houwink equation. The K and a values were $8.93x10^{-4}$ and 0.71, respectively, according to Kaneko *et al.*, (1982).

$$[\eta] = 8.93x10^{-4}M^{0.71} \quad (3.6)$$

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

3.3.3 Degree of Deacetylation of Chitin

The method used to determine the degree of deacetylation of chitin is based on quantitative infrared spectroscopic technique (Sannan *et al.*, 1978). About 3 mg of chitin powder, passed through a 200-mesh sieve, was mechanically mixed with 400 mg of potassium bromide powder 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 cm^{-1} (the amide II band) were evaluated by the

baseline method. The degree of deacetylation (DD) was calculated from the following equation:

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

where DD = Degree of deacetylation (%)

A_{1550} = Absorbance at 1550 cm^{-1} (the amide II band)

A_{2878} = Absorbance at 2878 cm^{-1} (the C-H band)

3.3.4 Preparation of CM-chitin

CM-chitin was prepared by the method of Hirano (1988). Alkaline chitin was prepared by suspending chitin power (4 g) in 42% NaOH solution (80 ml). After the suspension was allowed in desiccator for 30 min under reduced pressure, crush ice (160 g) was added and the mixture was mechanically stirred for 30 min in an ice bath to dissolve chitin. A viscous alkaline chitin solution was obtained. For successful synthesis of CM-chitin, the concentration of NaOH solution should not be less than 14 %. Monochloroacetic acid solution was prepared by dissolving in 14% NaOH solution in an ice bath and was added dropwise into the alkaline chitin solution with stirring over 30 min. After standing overnight at room temperature, the mixture was neutralized with acetic acid under cooling in an ice bath and dialyzed against running water for 2 days, followed by dialysis against distilled water for 1 day. The dialysate was centrifuged at 5000 rpm for 20 min, in order to remove insoluble material, and the supernatant was added to 3 volumes of acetone. After standing overnight, the precipitate was collected by centrifugation and washed with acetone. The product was resuspended in ethanol and collected by filtration. After drying at room temperature, CM-chitin sodium salt was obtained.

3.3.5 Degree of Substitution of CM-chitin

The degree of substitution was estimated by elemental analysis with combustion at 950°C. The sample (1-2 mg) was filled in tin foil and analyzed under air with oxygen as a combustion gas (flow rate of 20 ml/min) and with He as a carrier gas (flow rate of 200 ml/min).

3.3.6 Viscosity-Average Molecular Weight of CM-chitin

The different concentrations (0.00625, 0.0125, 0.025, 0.05 and 0.01 g/100 ml) of CM-chitin solutions dissolved in 0.1 M NaCl were prepared. All of samples were passed through filter papers before using. The Ubbelohde viscometer was filled with 10 ml of sample solution and then equilibrated in water bath, which was maintained the temperature at 25°C. The sample was passed through the capillary once before the running time was measured. Each sample was measured 5 times. The running times of solvent and solutions were used to calculate the relative viscosity, specific viscosity, and reduced viscosity, by the following equations:

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

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

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

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

where t is the running time of CM-chitin solution, t_s is the running time of solvent and C is the concentration in g/100 ml.

The viscosity-average molecular weight of CM-chitin was determined based on Mark-Houwink equation. The K and a values were$7.92x10^{-5}$ and –1, respectively, according to Kaneko *et al.*, (1982).

$$[\eta] = 7.92x10^{-5}M^{-1} \quad (3.6)$$

where $[\eta]$ = Intrinsic viscosity

M = Viscosity-average molecular weight

3.3.7 Gel Permeation Chromatography (GPC)

A 60ul of CM-chitin aqueous solution was injected into Hitachi Liquid Chromatograph which is composed of pump system (Hitachi L-7100), 60 °C of

Column oven (Hitachi L-7300), with Shodex Ohpak KB-800, RI Detector (Hitachi L-7490) and Chromato analyzer (Hitachi D-2500). Data were further analyzed by Hitachi SC-8020 program to estimate molecular weight and molecular weight distribution (Mw/Mn). Standard polulan was applied to makeup working curve to estimate molecular weight accurately.

3.3.8 Swelling Behavior

The blend films were cut into disk form with diameter of 16 mm and 25-30 μm in thickness. The weights of the completely dried samples were measured, and the samples were immersed in different pH buffer solutions i.e., pH 2.0, pH 5.5 and pH 7.2 at 37°C. The degrees of swelling of these samples were calculated from the following equation (Wang *et al.*, 1997):

$$\text{Degree of swelling (\%)} = \frac{Ws - Wd}{Wd} \times 100 \qquad (3.7)$$

where Ws and Wd denote the weight of swollen and dried film, respectively.

3.3.9 Preparation of Silk Fibroin Solution

Raw silk fiber (*Bombyx mori*) was degummed by heating in 0.5% Na_2CO_3 solution at 100°C for 1 h followed by washing with boiling water and drying at 60°C for 24 h in an oven. Degummed silk fibroin 6 g was then dissolved in 94 g of 1:2:8 by mole of $CaCl_2$:EtOH:H_2O solvent system at 100°C for 15 minutes (Chen *et al.*, 1994). The resulting silk fibroin solution was filtered through the sinter glass filter and subsequently dialyzed against distilled water for 7 days. The dialyzed silk fibroin solution was filtered and diluted to achieve a concentration of 1% w/w.

3.3.10 Preparation of CM-chitin Solution

CM-chitin solution was prepared by dissolution of CM-chitin in distilled water to obtain the concentration of 1% by weight. The solution was stirred continuously overnight at room temperature.

3.3.11 Preparation of Durg-Loaded Blend Films

Solutions containing CM-chitin and silk fibroin were prepared by mixing various ratios of 1% w/w of silk fibroin solution and 1% w/w of CM-chitin solution. The model drugs (theophylline, diclofenac sodium, salicylic acid and amoxicillin trihydrate) were added to the blend solutions to reach a concentration of 0.1% w/w. Glutaraldehyde, used as crosslinking agent, was added to the blend solutions at the amount of 0.01%. The blend solution containing a model drug was stirred slowly for 12 h and then 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.

3.3.12 Drug Release Studies

To study the release characteristics of model drugs from the films, drug-loaded blend films were immersed in buffer solutions at pH 2.0, pH 5.5 and pH 7.2. At time intervals, 1-mL aliquots were withdrawn and assayed for the amount of drug released. Theophylline, diclofenac sodium, amoxicillin trihydrate and salicylic acid released in the solutions were determined by a UV-visible spectrophotometer (Perkin Elmer, Lambda 10) at 272, 275, 272, 299 nm, respectively, using calibration curves for each drug. The experiments were performed in triplicate. The percentages of released drugs were average values of repeated three experiments.