CHAPTER III
EXPERIMENTAL

3.1 Materials

The shell of Penaeus merguiensis shrimps was kindly provided by Surapon Food Public Co. Ltd. Aniline monomer was purchased from Merck Co. and distilled under a reduced pressure prior to use.

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.

Ammonium peroxodisulfate of AR grade from Merck Co. was used as a oxidant. The hydrochloric acid of AR grade from Labscan was use as dopant. AR grade chemicals of N-methyl-2-pyrrolidone (NMP), acetic acid, ammonia solution, and methanol were used without further pretreatment.

3.2 Equipment

3.2.1 Capillary Viscometer

The viscosity-average molecular weight of chitosan was determined by using Cannon Ubbelohde-type number 75 of capillary viscometer.

3.2.2 FTIR Spectrophotometer

The FTIR spectra of chitosan, polyaniline, and polyaniline/chitosan blend films were recorded with Thermo Nicolet Nexus 670 FTIR Spectroscopy with 16 scans at a resolution of 4 cm^{-1}. A frequency range of 4000-400 cm^{-1} was observed by using a 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

UV-Visible spectrum of a synthesized polyaniline was obtained from a Shimadzu UV-VIS spectrometer model 2550 in the wavelength range 200-800 nm. The light source was a deuterium lamp. N-Methyl-2-pyrrolidone was used as the solvent to prepare the sample polyaniline solution at the concentration of 0.3 g/l.

The amount of drug release from polyaniline/chitosan blend films at pH 5.5 and 37°C was determined at the maximum wave length of drug model (296 nm) by using the calibration curve with various concentrations of a drug model in range of 0.004-0.012 mg/ml.

3.2.4 Lloyd Tensile Tester

The mechanical tests of chitosan and the blend films with different polyaniline loading were determined for a dried rectangular film (10 x 1 cm) by stress-strain technique using Universal Testing Machine (Lloyd, Model LRX) at room temperature. The thickness of the blend films was in the range of 20-25 μm. A strain rate of 25 $mm \cdot min^{-1}$ and gauge length of 50 mm was employed.

3.2.5 Thermogravimetric Analyzer (TGA)

Thermogravimetric analysis (TGA) used to evaluate the thermal stability and determined the decomposition temperature of chitosan, polyaniline, and the blend films was Dupont Instrument TGA 5.1 model 2950. The temperature range studied was 30-600°C at a heating rate of 10°C/min under a nitrogen gas atmosphere.

3.2.6 Different Scanning Calorimeter (DSC)

Differential scanning calorimetry (DSC) of chitosan, polyaniline, and the blend samples was carried out in the temperature range of 30-600°C using Mettler-Toledo DSC 822 at a heating rate of 10 °C/min under a nitrogen gas atmosphere.

3.2.7 Scanning Electron Microscope (SEM)

A scanning electron microscope (JOEL model JSM-5800LV) is used to investigate the surface and cross-sectional morphology of polyaniline/chitosan blend films. For the surface morphology, the film samples were cut into small pieces and adhered on a brass-stub by using an adhesive tape. The samples on stub are coated with thin layer of gold by using a JFC-1100E ion sputtering device. The surface morphology of the blend films is obtained by using an acceleration voltage of 10 kV with a magnification of 2000 times. Meanwhile, the cross-sectional fracture surfaces of the blend films were achieved by cooling in liquid nitrogen before adhering on a brass-stub and coating with gold. The magnification of cross-sectional morphology of blend films was 3000 times.

3.2.8 X-Ray Diffractometer (XRD)

The X-ray diffraction (Rigaku, model D/MAX-2000) was used to characterize the crystalline structure of chitosan, polyaniline, and their blend films. The measurements were carried out in the continuous mode with a scan speed of 5^{o}/min covering the angles 2θ between 5 and 50^{o}. Cu $K\alpha_1$ was used as the X-ray source.

3.2.9 Electrometer

The electrical conductivity of the blend films was measured at room temperature and 50% relative humidity using a conventional two-probe technique with 6517A Electrometer/ High Resistance Meter (Keithley, model 7517A).

3.3 Methodology

3.3.1 Synthesis of Polyaniline in Emeraldine Base Form

The polyaniline in emeraldine base form (EB form) was prepared according to the method described by Cao *et al.* (Cao, 1989). In a typical procedure, 20.4 g of freshly distilled aniline monomer was dissolved in 230 ml of 1.5 M HCl and the solution was cooled to below 5^{o}C. A pre-cooled solution, at a temperature below 5^{o}C, containing 25 g $NH_4(S_2O_8)$ in 250 ml of 1.5 M HCl was slowly poured into the monomer solution for 1 h with vigorous stirring. The reaction temperature was maintained at 0-5^{o}C for 4 hours. A dark green precipitate was recovered from reaction mixture filtered under reduce pressure and then washed thoroughly with distilled water and methanol in ratio 80:20 until the washing liquid was completely colorless and neutral. The precipitate product was treated with 300ml. of 3% ammonia solution at room temperature for 2 hours. Upon filtering, washing with distilled water, and dring under dynamic vacuum at ambient temperature for 48 hours, the blue powder of polyaniline in emeraldine base form was obtained.

3.3.2 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 1N 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.

3.3.3 Preparation of Chitosan

Chitosan 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.

3.3.4 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 cm^{-1} (the amideII band) were used to quantitate the degree of deacetylation. The degree of deacetylation was calculated from the equation 3.1.

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

where D = degree of deacetylation (%)

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

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

3.3.5 Viscosity-Average Molecular Weight of Chitosan

Chitosan solution of different concentration (0.00, 0.0125, 0.025, 0.050, 0.075, and 0.1 g/100 ml) in 0.2 M acetic acid: 0.1 M sodium acetate was 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:

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

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

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

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

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 (Wang *et al.*, 1991)

$$[\eta] = 6.59 \times 10^{-5} M_v^{0.88} \quad (3.6)$$

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

3.3.6 Preparation of Polyniline/Chitosan Blend Film

The emeraldine base form of polyaniline was dissolved in N-methyl-2-pyrrolidone (NMP) to obtain 1 wt% solution. A 2 wt% solution of chitosan was obtained by dissolving chitosan in 2% acetic acid with vigorous stirring. The 0.01 mole % of glutaraldehyde, as a crosslinking agent, was added to the chitosan solution. Depending on the desired fraction of polyaniline in the final product, an appropriated amount of polyaniline /NMP solution was mixed with a 2 wt% solution of chitosan. This mixture solution was stirred for 12 hours at room temperature. The resulting solution was cast onto a stainless steel mould and dried at 55°C for 12 hours. Finally, the films were lift off the mould and stored in desiccators prior to use.

3.3.7 Doping of Polyaniline/Chitosan Blend Film

The polyaniline/chitosan blend films were doped with aqueous HCl solution of different concentration and using various doping time. Subsequently, the

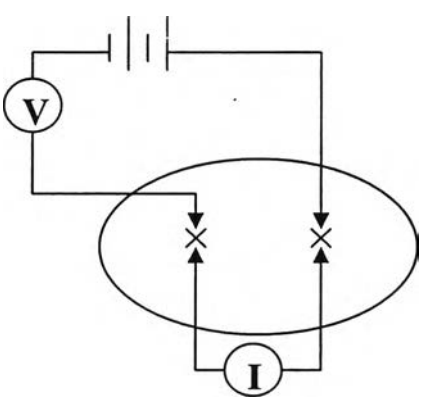
films were placed between two sheets of filter paper and dried under dynamic vacuum at ambient temperature for 48 hours.

3.3.8 Mechanical Properties

The elastic modulus, tensile strength, and elongation at break of chitosan and polyaniline/chitosan blend films were measured by Lloyd Tensile Tester by following the standard ASTM D882 using the gauge length of 50 mm and an extension rate of 25 mm/min at room temperature. Test films were cut in the dimension of 10 mm × 100 mm and the thickness of the films were in the range of 20-25 μm. Then the films were dried under dynamic vacuum at room temperature for 48 h before testing.

3.3.9 Conductivity Measurement

Two-point probe technique was used to measure the sheet resistivity that measures of the ability of a layer to resist the conduction of electrical carriers within the thin film with can be converted into the specific conductivity. The schematic of two probe used in this work is shown in the Scheme 3.1. Probe tips made from silver were press against the surface of the film. The specific conductivity was obtained by introducing a voltage (V) to the tips and determined the current (I) across two tips. The specific conductivity was calculated from equation 3.7.



Scheme 3.1 Schematic of two-point probe.

$$\sigma = \frac{1}{\rho} = \frac{I}{K \times V \times t} \qquad (3.7)$$

where σ is the specific conductivity (S/cm), ρ is the specific resistivity (Ω·cm.), V is the applied voltage (V) to the tips, I is the measured current (A) across two tips, t is thickness of the films (cm), and K is the geometric correction factor which was obtained from the standard material, PANI/chitosan composite film with 40 wt% PANI content, whose specific conductivity are known from the Resistivity Test Figure (Keithley 8009). The calibration procedure details are given in Appendix G and H.

3.3.10 Preparation of Skin

Permeation experiments were performed with full-thickness pig skin which was 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 aluminum foil and stored frozen before use. To perform in-vitro 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.

3.3.11 Spectrophotometric Analysis of Model Drug

UV/Visible Spectrophotometer (Shimadzu model 2550) was employed to determined the maximum spectra of salicylic acid used as the model drug. It was performed by scanning the UV absorption in a wavelength range 400-200 nm. Model drug in aqueous solution were 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 each drug models. The absorbance value at the maximum wavelength of model drug was read and the correspondent model drug concentrations were calculated from the calibration curve with various drug concentrations in range of 0.004-0.012 mg/ml.

3.3.12 *In vitro* Skin Permeation of Drug

The *In vitro* skin permeation for electrically controlled release of drug from prepared membrane was studied via a modified Franz diffusion cell. The full-thickness pig skin was mounted onto the receptor compartment with the stratum corneal side facing upward into the donor compartment and the dermal side facing downward into the receptor compartment. The receptor compartment was filled with the acetate buffer solution pH 5.5 constantly stirred using a magnetic stirrer and maintained at 37°C by a circulating water bath. A unit of drug-contained blend film was placed over the skin followed by the copper plate used to distribute the electrical potential to over all position of the blend film. The electrodes for applying the electrical potential were connected to a modified Franz diffusion cell; one of them was connected with the copper plate, while another one was immersed in the acetate buffer at the sampling plot as shown in Figure 3.1. The whole assembly was clamped together with the donor cap on the top. A portion (0.3 ml each) of buffer solutions were withdrawn from the receptor compartment at predetermined time intervals of ¼, ½, ¾, 1, 1½, 2, 2 ½,3, 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 solution (drug-free). The drug concentrations in these samples were determined by the UV/Visible spectrophotometer method.

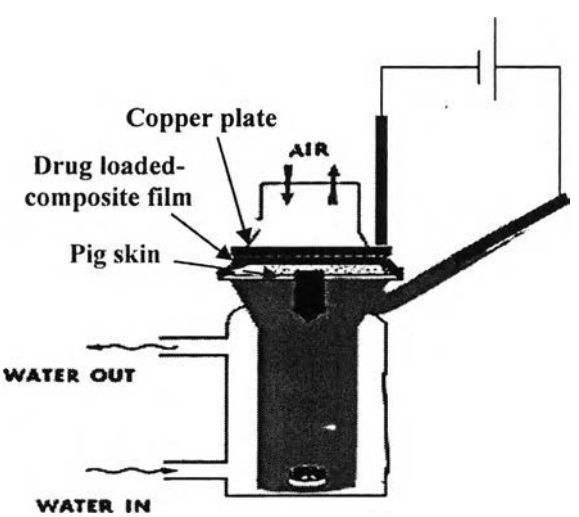


Figure 3.1 Modified Franz diffusion cell set-up for electrically controlled release system.