CHAPTER III
EXPERIMENTAL

3.1 Materials

3.1.1 Materials and Chemicals

Chitin whisker was prepared from the shells of *Metapenaeus dobsoni* shrimp, which were kindly provided by Surapon Foods Public Co., Ltd. (Thailand). Analytical grade hydrochloric acid (HCl) 37 %w/w and analytical grade anhydrous sodium hydroxide pellets (NaOH) were purchased from RCI Labscan Limited (Thailand). Pluronic F-127 and phosphate-buffered saline (PBS) were purchased from Sigma Aldrich and dialysis tube for drug release was purchased from Spectrum Laboratories (spectra), which has a molecular weight cutoff of 3,500 Da. Methylene blue C.I. 52015 (UNILAB), Methyl orange C.I. 13025 (LABCHEM) and insulin were used as the model compounds. The chemical structures of the dyes and insulin are illustrated in Figure 3.1.

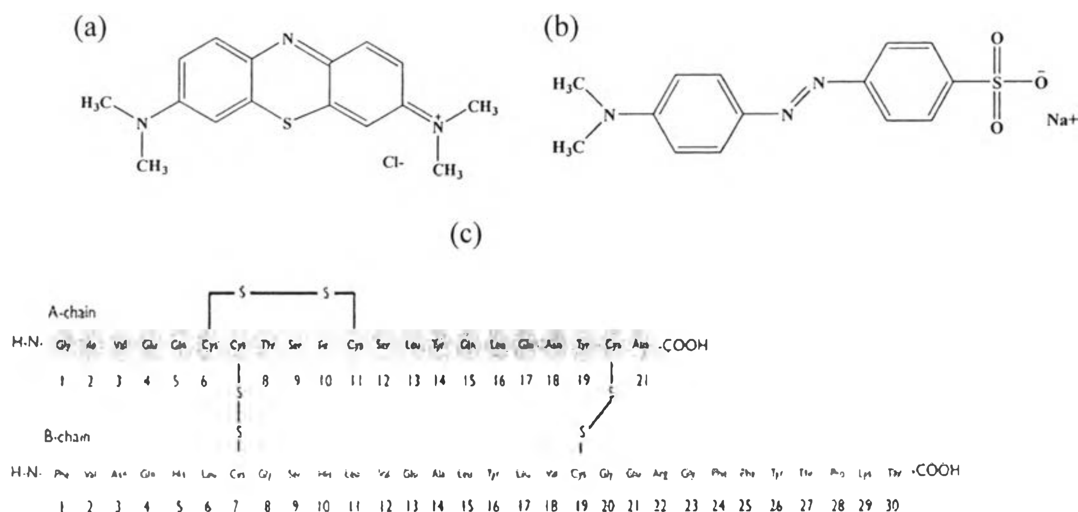

Figure 3.1 The chemical structures of (a) methylene blue (cationic dye), (b) methyl orange (anionic dye) and (c) insulin.

3.2 Methodology

3.2.1 Preparation of Chitin

Chitin was prepared from shrimp shell by decalcification and deproteinization to remove calcium carbonate and protein, respectively. These processes were carried out according to the procedure described by Shimahara and Takigushi, (1988). To prepare chitin, shrimp shells were first cleaned and dried under sunlight for a few days. Then, chitin was ground into small pieces. 1 kg of dried shrimp shells was immersed in 10 liters of a 1 N HCl solution with occasional stirring at room temperature for 2 days. The acidic solution was changed daily. The decalcified shrimp shells were subsequently neutralized by distilled water and dried at 60 °C for 48 hours. The decalcified shrimp shells were further deproteinized in a 4 % w/v NaOH solution at a ratio of NaOH solution to shrimp shells of 10:1 with continuous stirring at 80 °C for 4 hours. The obtained chitin was filtered, neutralized by distilled water, and dried at 60 °C in a convective oven for 24 hours.

3.2.2 Preparation of Chitin Whisker (CTW) Suspension

Chitin whisker suspension was prepared by acid hydrolysis based on the method of Dufresne *et* al., (2001, 2002, 2003). Chitin flakes were hydrolyzed with 3N HCl, which the ratio of chitin to HCl was 1 g of chitin to 30 ml of HCl, under vigorous stirring at 104°C for 6 hours. The suspension was later diluted with distilled water, followed by centrifugation at 10,000 rpm for 10 minutes. This process was repeated for three times to remove HCl. Afterward, the suspension was dialyzed in distilled water until neutral. The dispersion of chitin whiskers in the suspension was accomplished by ultrasonication for 10 minutes. The suspension was stored in a refrigerator before use.

3.2.3 Preparation of Pluronic Solution and Pluronic/Chitin Whisker Composite Gel

Pluronic solution was prepared by Schmolka's cold method (Schmolka, 1972). Briefly, an appropriate amount of the pluronic copolymer was slowly added into cold distilled water with constant agitation using magnetic stirrer. After that the solution was kept in a refrigerator for at least 24 hours to ensure complete dissolution. Then the pluronic solution was mixed with chitin whisker

suspension by varying the weight ratio of chitin whisker to pluronic. The pluronic/chitin whisker suspension was incubated at 37 °C to induce gel formation.

3.2.4 Preparation of dyes stocks

The dyes used in this study were Methylene blue, a cationic dye and Methyl orange, an anionic dye. The dye stock solutions were prepared by dissolving dyes in the solvent to the concentration of 1000 mg/l. The experimental solvent is the distilled water. The known concentrations of the dyes were dilute from the dyes stocks to make the calibration curve. The absorbance of each solution was measured at the maximum wavelength of each solvent. Consequently, the dye stock was diluted to 1 ppm as an initial concentration.

3.2.5 Evaluation of model drug release characteristics

The pluronic/chitin whisker composite gel was added 0.5 ml of dye solutions at low temperature to prepare the sample. The *in vitro* release studies were carried out in 100 ml of phosphate buffer saline (PBS) solution pH 7.4 at 37 °C. A 1 ml of release media was taken out at a specific time interval and replaced with the same volume of fresh media. The concentrations of dye solutions were determined by the UV-VIS Spectrophotometer. A system having two separated compartments was used to study the release of drug from the gels as shown in Figure 3.2, (Paavola *et* al., 1997). In this system, cellulose membrane, with the molecular weight cut off equal to 3500 Da, was used to separate the gel in the donor compartment from the PBS solution. The area of the membrane was 4.90 cm^2 and the PBS solution in the acceptor compartment was stirred with a magnetic bar at 250 rpm. The release behaviors of dyes from dye solution, neat pluronic gel, and pluronic/chitin whisker composite gels containing in the donor compartment were evaluated. The solutions in the acceptor compartment were taken to determine the released dye concentrations at the specific time interval until 72 hours.

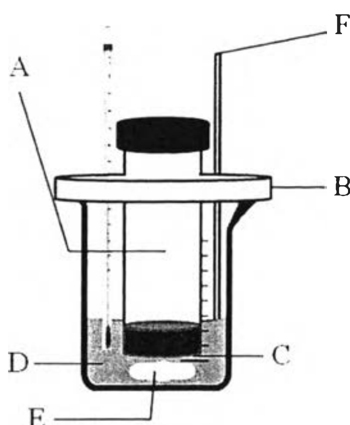

Figure 3.2 Experimental set-up for the dye release measurement. A : Glass cylinder (donor compartment) ; B : Plexiglass cover ; C : Membrane ; D : PBS solution (acceptor compartment) ; E : Stirring magnetic ; F : Sampling port.

3.3 Characterization

3.3.1 Fourier Transform Infrared Spectroscopy (FTIR)

Chemical structures of chitin, chitin whisker were confirmed by FTIR. The characterization was performed with Thermo Nicolet Nexus 670 spectrophotometer using total 64 scans at a resolution of 4 cm^{-1} for each sample. A frequency range of 4000-400 cm^{-1} was observed. Degree of deacetylation of chitin was determined following the method of Sannan *et* al. (1977) which estimated from the absorbance ratio of amide II band at 1550 cm^{-1} and C-H band at 2878 cm^{-1} as following equation:

$$DD = 101-35.71(Abs_{1550}/Abs_{2878}) \quad (1)$$

3.3.2 Sol-gel Transition (Sol-gel Temperature)

The appearance of pluronic solution with the various concentrations after incubated at different temperatures. The sol-gel phase transition temperature was recorded using a vial tilting method. A 10 ml test tube with a diameter of 13 mm were used to prepare various concentrations of pluronic solution. The gel state was determined by tilting the test tube at 45 degree from the ground. If the solution did not flow in 1 min, it was regarded as "gel phase" and if it flowed, it called "sol phase".

3.3.3 Scanning Electron Microscopy (SEM)

For the morphological study, sample solutions were dropped onto foil, followed by drying in convection oven. After that, dry samples were placed onto stubs, coated with platinum using a JEOL JFC-1100 sputtering device, and observed for their microscopic morphology using JEOL JSM-5200 scanning electron microscopy (SEM).

3.3.4 Transmission Electron Microscopy (TEM)

The transmission electron microscope observations were observed by JEOL model JEM 2100 operating voltage of 200 kV. The CTW suspension were dilute with distilled water into the concentration at 0.006 %w/v. The samples were prepared by air-drying the particles onto a carbon-coated copper grid and air-dried.

3.3.5 Stability of Composite Gel

The samples were then dried in convection oven and measured the weight remaining after immersion in PBS solution pH 7.4 at 37 °C. The gel stability of samples in wet state was also evaluated in term of weight remaining by the following equations:

$$\text{Weight remaining (\%)} = 100 - \{[\frac{(W_0 - W_t)}{W_0}]\times 100\} \quad (2)$$

where W_0 is original dry weight and W_t is the weight of the dry gel after incubation for a given time period.

3.3.6 UV-VIS Spectroscopy

The UV-VIS Spectrophotometer was used to examine the model drug concentration at the various times by using a wavelength 664 nm, 465 nm and 260 nm for testing MB, MO and insulin, respectively. The absorbances were converted to the amount of drug released using a calibration curve based on standard solution in PBS pH 7.4. The release data of drug were plotted against the releasing time.