CHAPTER III
EXPERIMENTAL

3.1 Materials

Chitosan with a degree of deacetylation (%DD) 95 and molecular weight of 48,000 and 227,000 were locally supplied from the Seafreash Chitosan (Lab) Company Limited, Bangkok, Thailand. 1-Hydroxybenzotriazole monohydrate (HOBt·H_2O) and 1-ethyl-3-(3-dimethylaminopropyl-carbodiimide) hydrochloride (EDC·HCl) or water-soluble carbodiimide hydrochloride (WSC·HCl) were purchased from Wako Pure Chemical Industries Co. Ltd., Japan. Succinic anhydride and cholic acid (CA) were from Fluka Chemika, Switzerland. Poly(ethylene glycol) monomethyl ether (mPEG, M_n 1100, 2000 and 5000 Da) were purchased from Sigma-Aldrich, Inc., USA. Allergen extract (D. pteronyssinus) was purchased from ALK ABELLÓ, USA. Methanol, ethanol and acetone were purchased from Carlo Erba Reagenti, Italy. All chemicals were used without further purification.

3.2 Equipments

3.2.1 Stuctural Analysis

Qualitative analysis by Fourier transform infrared spectrophotometer (FTIR) was obtained from a Bruker Equinox 55/S with 32 scans at a resolution of 4 cm^{-1}. A frequency range of 4000-400 cm^{-1} was observed using a deuterated triglycinesulfate detector (DTGD) with a specific detectivity, D*, of 1×10^9 $cm.Hz^{1/2}$ w^{-1}. ^1H Nuclear magnetic resonance (NMR) was obtained from a 400 MHz JEOL JNM-GSX spectrometer.

3.2.2 Thermal Analysis

A Dupont thermal gravimetric analyzer was applied using a Perkin Elmer Pyris Diamond with N_2 flowing rate of 20 mL/min and a heating rate of 10 °C/min from 50 °C to 500 °C.

3.2.3 Morphology Observation

The morphology was investigated by using a JEOL/JSM 5200 scanning electron microscope (SEM) at 15 kV serviced by faculty of science Chulalongkorn university. Sample preparation was done by dispersing sample in water and drying. JEM-200CX transmission electron microscope (TEM) was service by faculty of science, Mahidol university.

3.2.4 Particle Size Measurement

Particle size was investigated by using Zetasizer nanoZS Malvern Instrument with He-Ne lazer source in wavelength 632.8 nm using backscattering detector.

3.3 Methodology

3.3.1 Chitosan-HOBt Aqueous Solution, **1**

Chitosan-HOBt aqueous solution was prepared as reported previously (J. Fungkangwanwong et al., 2006). In brief, chitosan (0.1 g, 0.61 mmol) was vigorously stirred with a mole of HOBt·H_2O (0.094 g, 0.61 mmol) to that of chitosan in deionized water (10 ml) at ambient temperature until the clear solution, **1**, was obtained.

3.3.2 Preparation of Chitosan-mPEG-Cholic Acid, **2**

The preparation of carboxyl terminated poly(ethylene glycol) methyl ether (mPEG-COOH) was reported elsewhere (Yoksan et al., 2003). In brief, mPEG (M_n 5000, 25 g, 5 mmol) was reacted with succinic anhydride (0.5 g, 5 mmol) in the presence of a catalytic amount of pyridine at 65 ^{o}C for 24 hours. The mixture was reprecipitated in diethyl ether, washed several times with diethyl ether and dried *in vacuo* to obtain mPEG-COOH.

The solution of **1** (10 ml) was heated at 60 °C and mixed with mPEG-COOH (1.5237 g, 0.3 mmol). CA (0.1226 g, 0.3 mmol) in ethanol (8 ml) and WSC·HCl (0.1178 g, 0.6 mmol) in ethanol (5 ml) were then added to the solution **1**. The reaction was allowed at 60 °C for 24 hours. The solvent was removed and the viscous solution obtained was reprecipitated in acetone and washed with methanol

several times and dried *in vacuo* to obtain **2a**. The compounds **2b**, **2c**, **2d** and **2e** were also prepared by varying mole ratio of chitosan:mPEG-COOH:CA, as shown in Table 3.1.

Moreover, Chitosan with Mw 227,000 and mPEG with Mw 1100 and 2000 were used to prepared **3**, **4** and **5**, respectively, in the same procedure as **2**

Table 3.1 Mole ratio of chitosan:mPEG-COOH:CA in preparing **2**, **3**, **4** and **5**

Compound	Mole Ratio of chitosan:mPEG-COOH:CA
2a, 3a, 4, 5	1:0.5:0.5
2b, 3b	1:0.5:0.25
2c, 3c	1:0.5:0.1
2d, 3d	1:0.25:0.5
2e, 3e	1:0.1:0.5

2 : For chitosan with Mw = 48,000, and mPEG with Mw = 5000

3 : For chitosan with Mw = 227,000, and mPEG with Mw = 5000

4 : For chitosan with Mw = 48,000, and mPEG with Mw = 1100

5 : For chitosan with Mw = 48,000, and mPEG with Mw = 2000

FT-IR (ZnSe, cm^{-1}): 3443 (br, OH), 2881 (s, CH), 1734 (m, C=O ester), 1659 (m, amide I), 1552 (m, amide II), 1467 (m, C-C cyclohexane bending), 1153-895 (s, pyranose ring and ether linkage of mPEG).

^{1}H-NMR (D_2O, ppm): δ_H 0.52 (s, CH_3 of cholic acid), 0.69 (s, CH_3 of cholic acid), 0.8 (s, CH_3 of cholic acid), 0.89-1.76 (m, CH_2 of cholic acid), 1.85 (s, H-Ac of chitosan), 2.68 (s, H-C-2 of chitosan), 3.18 (s, CH_3-O of mPEG), 3.23-3.79 (m, H-C3-C6 of chitosan and OCH_2CH_2 of mPEG), 4.09 (t, CH_2-OCO of mPEG).

3.3.3 Allergen Incorporation

Compound **2a** was dissolved in water and steriled by filtration through a 0.22 micron membrane filter. An equal weight of chitosan solution and allergen was mixed and vigorously stirred for 20 s. The chitosan-allergen solution

was investigated morphology by TEM and the in vitro immune response by lymphocyte transformation test (LTT).

For **3**, allergen incorporation was done by mixing **3** 0.1 g with allergen 10 μg and vigorous stirring for 20 s. The chitosan-allergen solution was investigated morphology by TEM.

Scheme 3.1