# CHAPTER III
# EXPERIMENTAL

## 3.1 Materials

Low molecular weight chitosan (LCS) was a gift from Chitin Research Center, Chulalongkorn University. 1-Hydroxybenzotriazole monohydrate ($HOBt{\cdot}H_2O$) and 1-ethyl-3-(3-dimethylaminopropyl-carbodiimide)hydrochloride ($EDC{\cdot}HCl$) were purchased from Wako Pure Chemical Industries Co., Ltd., Japan. DL-Phenylalanine (Phe) and succinic anhydride were obtained from Fluka Chemika, Switzerland. Poly(ethylene glycol)methyl ether (mPEG, $M_n$ 5000 Da) was supplied by Sigma-Aldrich, Inc., USA. Allergen extract (*D.pteronyssius*) was purchased from ALK ABELLÓ, USA. Methanol was obtained from Carlo Erba reagent, Italy. All chemicals were used as received without further purification.

## 3.2 Instruments and Equipment

Qualitative FT-IR spectra were recorded by a Bruker Equinox 55/S with 32 scans at resolution of 4 $cm^{-1}$ in a frequency range of 4000-650 $cm^{-1}$ using a deuterated triglycinesulfate detector (DTGS) with a specific detectivity, D*, of $1 \times 10^9$ cm Hz ½ $w^{-1}$. Degree of substitution was determined by using a Bruker Ultrashield 500 Plus NMR system. Concentration of allergen was determined by a Shimadzu UV-Vis spectrophotometer (UV-1800) and a Thermo Separation Products High performance liquid chromatography (HPLC). The Z-average diameter of samples was determined by a Malvern Zetasizer Nano Series (Malvern Instruments Ltd.) with a detection angle of 173°, dynamic light scattering (DLS). Transmission electron micrographs were observed by an H-7650 Hitachi transmission electron microscope at 100 kV. A Perkin Elmer Pyris Diamond thermogravimetric/differential thermal analyzer was used with a $N_2$ flow and a heating rate of 10 °C/min from 30-600 °C. Packing structure was determined by a

Rigaku D/MAX 2200 X-Ray wide angle diffractometer using a scan range 5°-90° 2θ with a scan step of 0.05° 2θ and a scan speed of 5° 2θ /min.

### 3.3 Methodology

#### 3.3.1 Poly(ethylene glycol)Methyl Ether Terminated with a Carboxyl Group (mPEG-COOH)

mPEG ($M_n$ = 5000 Da, 3 g) was reacted with succinic anhydride (0.06 g, 1 mole equivalent to mPEG) at 60°C overnight in the presence of a catalytic amount of pyridine. The solution obtained was concentrated and precipitated in diethyl ether before drying in vacuo to obtain mPEG-COOH.

FT-IR (ZnSe, $cm^{-1}$): 3503 (OH), 2864 (C-H stretching), 1733 (C=O), and 1112 (C-O-C).

#### 3.3.2 Phe and mPEG conjugated Chitosan, 2

LCS (0.1 g, 1 mole) was vigorously stirred with HOBt (0.08 g, 1 mole equivalent to LCS) in deionized water 20 ml at ambient temperature until the solution became clear. LCS-HOBt solution was mixed with Phe (0.10 g, 1 mole equivalent to LCS) and mPEG-COOH (2.95 g, 0.1 mole equivalent to LCS) in deionized water 10 ml followed by adding EDC (0.11g, 1 mole equivalent to LCS) into the solution. The homogeneous solution was carried out at ambient temperature overnight. The crude product was dialyzed, lyophilized, washed with methanol, and dried under vacuum to obtain LCS-Phe-mPEG, **2**. Other reactions with different mole ratios of Phe (0.5, 1.0, 1.5, 2.0, 2.5, and 3.0 equivalent to LCS) and mPEG-COOH (0, 0.1, 0.2, 0.3, 0.4, and 0.5 equivalent to LCS) were also prepared in the same procedures as **2**.

FT-IR (KBr, $cm^{-1}$): 3446 (OH), 2884 (CH stretching), 1653 (amide I), 1559 (amide II), 1594 ($-NH_2$), 1153-894 (pyranose ring and ether linkage of mPEG), 1457 ($-CH_2$, $-CH_3$ in mPEG), 750 (benzene ring of Phe).

$^1H$ NMR (2%$CD_3COOD/D_2O$, ppm): δ 1.99 (H-Ac of chitosan), 3.104 (H-2 of pyranose ring), 3.39-3.82 (H-3 to H-6 of pyranose ring and H-e of

mPEG), 2.59 (H-d of mPEG), 3.28 (H-f of mPEG), 2.80 (H-b of Phe), 7.53-7.80 (H-c of Phe).

### 3.3.3 Allergen Incorporation

The incorporation of allergen was carried out in water. A concentration of chitosan solution (1 mg/ml) and allergen (21.7μg/ml) was mixed and vigorously stirred for a minute at ambient temperature. The concentration of entrapped allergen was calculated from a difference between concentration of loading allergen and concentration of allergen in supernatants by UV-Vis spectroscopy (Bradford protein assay at a UV-Vis wavelength at 595 nm) and HPLC.