CHAPTER II

EXPERIMENTAL

2.1 **Materials and Chemicals**

Chitosan (MW of 110,000) was purchased from seafresh Chitosan (Lab), Co., Thailand.

N,N'-Dimethyl formamide, dimethyl sulfoxide used in syntheses and spectroscopic works were reagent or analytical grades purchased from Labscan (Bangkok, Thailand). Methanol, ethanol, hexane were purified from commercial grade solvents prior to use by distillation.

1-Ethyl-3(3-dimethylaminopropyl)carbodiimide(EDCI), 4-methoxycinnamic acid and 1-Hydroxy benzotriazole (HOBt) were purchased from Acros Organics (New Jersey, USA). Methoxy poly(ethylene glycol) methyl ether (mPEG) MW of 5,000 was purchased from Fluka Chemical company (Buchs, Switzerland). Phthalic anhydride was purchased from Carlo Erba Reagent (Val de Reuil, France). Pyridine was purchased from sigma (Sigma chemical Co. Ltd, USA.). 2-ethylhexyl-p-methoxycinnamate (EHMC, Eucolex 2292) was obtained from Merck Co. Ltd. (Darmstadt, Germany). Astaxanthin was obtained from Gowell Co., Ltd. (Thailand). Ascorbyl palmitate was obtained from Adinop Co., Ltd. (Thailand). Membranes used for dialysis experiments were seamless cellulose tubing, molecular weight cut off 12,400 Dalton, size 36/32 100 ft (Viskase Companies, Inc., Japan). Centrifugal filter devices molecular weight cut off (MWCO) 10,000 Dalton (Amicon Ultra-15, Millipore, Ireland) used for experiment was purchased from Millipore (Ireland).

2.2 **Instruments and Equipments**

The FT-IR spectra were recorded on a Nicolet Fourier Transform Infrared spectrophotometer: Impact 41.0 (Nicolet Instruments Technologies, Inc. WI, U.S.A.). The ^{1}H-NMR spectra was obtained in deuterated dimethylsulfoxide (DMSO-d_6) and N,N' dimethylformamide (DMF-d_7) with tetramethylsilane (TMS) as an internal reference using Varian Mercury spectrometer which operated at 400.00 MHz (Variance Company, USA.). Ultraviolet absorption spectra were obtained with the aids of an HP 8453 UV/VIS spectrophotometer (Agilent Technologies, CA, U.S.A.) using 1 cm-pathlength quartz cell.

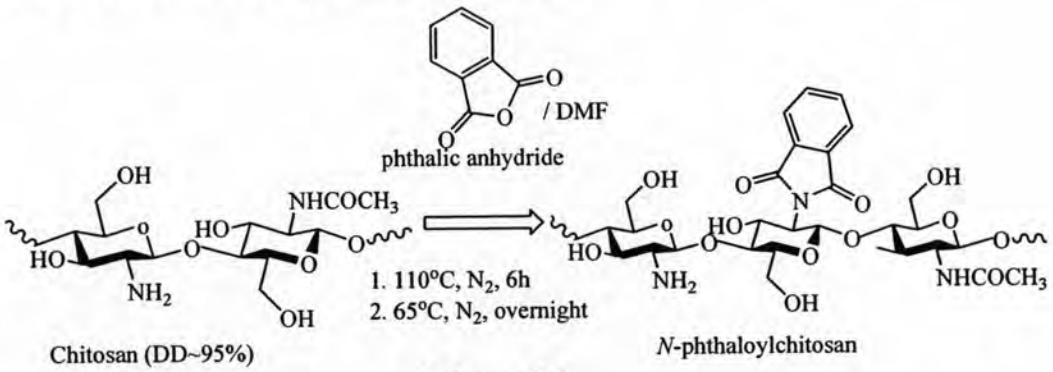
Thin layer chromatography (TLC) was performed on aluminium sheets precoated with silica gel (Merck Kieselgel 60 F_{254}) (Merck KgaA, Darmstadt, Germany). For UV radiation, broad band UVA (320-400 nm) was generated by the FSX24T12/BL/HO (PUVA) lamp (National Biological Corporation, Twinsburg, Ohio, U.S.A.) and broad band UVB (280-320 nm) was generated by the FSX24T12/BL/HO lamp (National Biological Corporation, Twinsburg, Ohio, USA). UV Irradiance was measured using an UVA-400C and an UVB-5ooC power meter (National Biological Corporation, Twinsburg, Ohio, USA).

Zeta potential was obtained with zetasizer nano series instrument (Zs, Malvern instruments, United Kingdom). Centrifuge used for experiments was obtained from Beckmann Coulter instrument (Allegra 64R centrifuge Beckman Coulter, Beckman Coulter, Inc, Japan). TEM and SEM photographs were obtained from JEM-2100 (JEOL, Japan) and JSM-6400 (JEOL, Japan), respectively.

A. Synthesis of nanoparticles

2.3 **mPEG–phthaloylchitosan Nanoparticles (d)** [83]

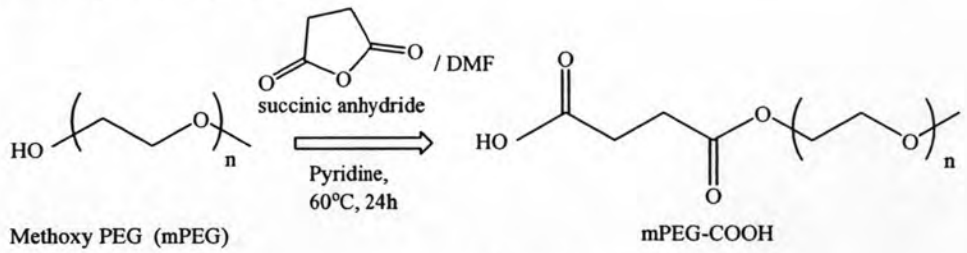
2.3.1 Preparation of *N*-Phthaloylchitosan (**b**)



Scheme 2.1

Chitosan (3.00 g) was reacted with phthalic anhydride (13.623 g, 5 moles equivalent to pyranose rings) in *N,N'*-dimethylformamide (DMF) (20 mL) at 110°C under nitrogen for 6 h. After that, temperature was reduced to 65°C and the mixture was left for 12 h. The solution was poured into ice water and the precipitate was collected, washed with methanol (2×100 mL) and dried to give pale brown product of **b** (Scheme 2.1). The product was analyzed by ^{1}H-NMR, IR and UV-VIS spectroscopy, respectively.

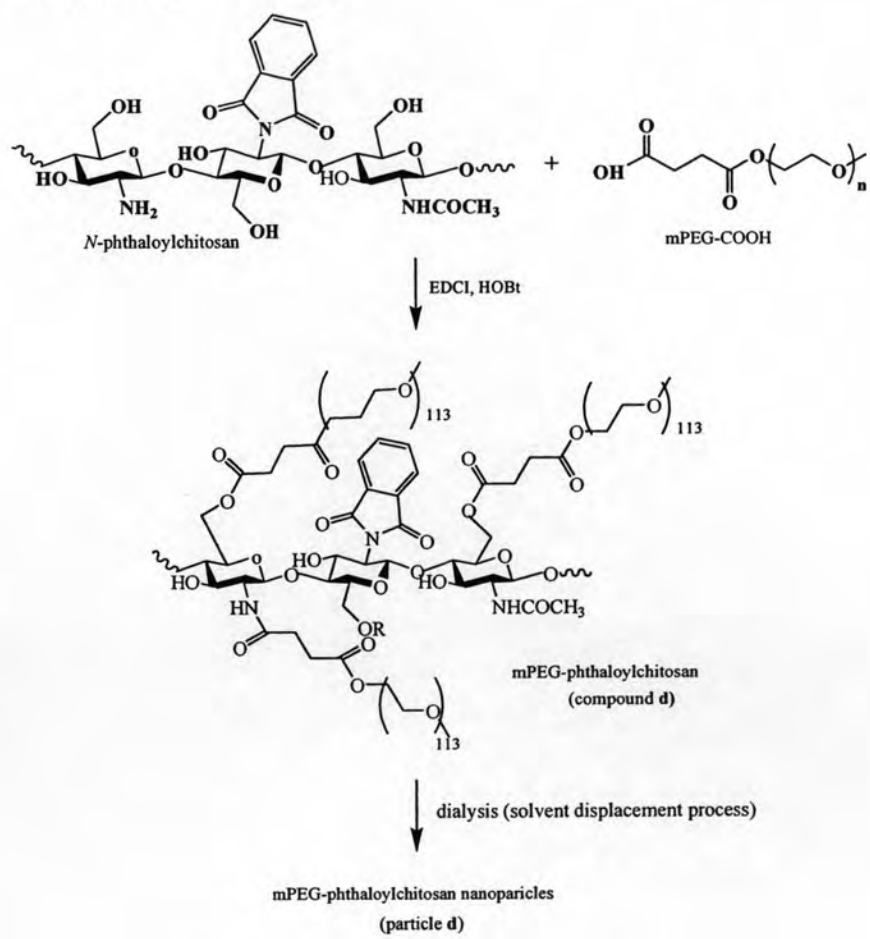
2.3.2 Preparation of Poly(ethylene glycol) methyl ether terminated with Carboxylic Groups (**c**)



Scheme 2.2

Poly(ethylene glycol) methyl ether (mPEG, M_n = 5,000 Dalton, 3.00 g, 0.6×10^{-3} moles) was reacted with succinic anhydride (0.06 g, 0.6×10^{-3} moles) in DMF (1 mL) at 60°C for 17 h in the presence of a catalytic amount of pyridine (~1-2 drops). The mixture solution was reprecipitated in diethyl ether and dried to yield white powder of mPEG-COOH, **c** (Scheme 2.2). The product was subjected to ^{1}H-NMR and IR analyses.

2.3.3 mPEG-phthaloylchitosan (**d**)



Scheme 2.3

Compound **c** (7.58 g, 0.40 moles equivalent to **b**) was stirred with **b** (1.00 g, 3.71×10^{-3} moles) in 20 mL of DMF solution containing 1-hydroxybenzotriazole (HOBt, 0.68 g, 5.03×10^{-3} moles) at room temperature until homogeneous. 1-Etyl-3-(3-dimethylaminopropyl)-carbodiimide, hydrochloride (EDCI, 0.85 g, 4.43×10^{-3} moles) was added to the reaction mixture and the mixture was maintained at 4°C for 1 h and then at room temperature for 12 h. The mixture was dialyzed against water for 5 days (15×1,000 mL change of water) and then centrifuged for 45 min at 43,000×g before thoroughly washed with methanol and dried to obtain white particle, **d** (Scheme 3). The product was analyzed by ^{1}H-NMR, IR and UV-VIS spectroscopy. In addition, the particle size distribution, zeta potential, stability and anti-microbial of the particles were also determined.

Rapid solvent displacement

20 mL solution of compound **d** in DMF was dropped in water 500 mL and the precipitate formed was centrifuged for 45 min at 43,000×g before thoroughly washed with methanol and dried to obtain pale brown particle, **f**. The product was analyzed by SEM.

Dialyses at various concentrations

Particle d (0.06 g, 0.006g and 0.0006 g, respectively) was dissolved in 10 mL of DMF solution and the obtained solution was transferred into dialysis bags and placed in a beaker containing 1,000 mL water. The solution was dialyzed against water for 5 days (15×1,000 mL change of water). The solution was analyzed by SEM and TEM.

2.3.4 Encapsulation of EHMC into Particle **d**.

2.3.4.1 Solvent displacement method

Dialysis against H_2O/ EtOH mixtures

EHMC-loaded-nanoparticle **d** induced with various H_2O:EtOH ratio were prepared by displacing DMF with H_2O/ EtOH mixtures. Particle **d** (0.26 g) was dissolved in 45 mL of DMF solution. EHMC (0.542 g, 0.0415 M) was added into the solution. The solution was divided into three dialysis bags. The three bags were separately placed into beakers containing 1,000 mL of H_2O: EtOH at the ratio of 100:0, 80:20, 50:50, respectively. This solution was dialyzed in each beaker for 5 days (15×1,000 mL change of H_2O: EtOH) and then centrifuged for 45 min at 43,000×g. The precipitated was analyzed for EHMC content using UV-VIS spectroscopy as described in Appendix B.

EHMC-encapsulated-particle ***d***

EHMC-loaded-nanoparticle **d** was prepared by the solvent displacement procedure. Particle **d** (0.06 g) and EHMC (0.121 g) were dissolved in 10 mL of N,N'-dimethylformamide. This solution of the polymer and EHMC was poured into a dialysis bag and placed in a beaker containing 1,000 mL of H_2O. The solution was dialyzed against water for 5 days (15×1,000 mL change of water) and then centrifuged for 45 min at 43,000×g before quickly washed with methanol. The precipitate was analyzed by SEM, TEM and UV-VIS spectroscopy, respectively.

2.3.4.2 Diffusion method

Particle **d** (0.06 g) was dispersed in 8 mL of water. EHMC (0.121 g, 0.0415 M) was dissolved in methanol solution (2 mL). And then, the methanolic EHMC solution (2 mL) was added dropwise into 8 mL of particle **d** aqueous solution and stirred for 30 days. The 1.5 mL of solution containing EHMC and particle d was withdrawn at 0 second, 4 hours, 10 days, 20 days and 30 days, respectively. The solution was centrifuged at 43,000×g for 45 min before quickly washed with methanol and dried. The product was analyzed for EHMC content using UV-VIS spectroscopy. The particle was also subjected to SEM analysis.

2.3.4.3 Photostability test

Photostability test of free EHMC and loaded EHMC was carried out by irradiating: the EHMC loaded nanoparticles suspension, and the unloaded nanoparticles suspension added with EHMC (free EHMC), with broad band UVB light (1.48 mW/ cm^2) for 0, 15 and 30 min, which correspond to the irradiances of 0, 22.5 and 45 mJ, respectively. The 10 mL of EHMC loaded particle aqueous suspension contained 26.7 mg of EHMC loaded in 120 mg of nanoparticles. The 10 mL of EHMC added unloaded particle suspension (free EHMC) contained 26.7 mg of free EHMC and 120 mg of nanoparticles. After specific UV exposure closed, both suspensions were withdrawn, quickly dried, dissolved in deuterated $CDCl_3$ and analyzed by NMR spectroscopy.

2.3.5 Encapsulation of Ascorbyl Palmitate into Nanoparticle **d**

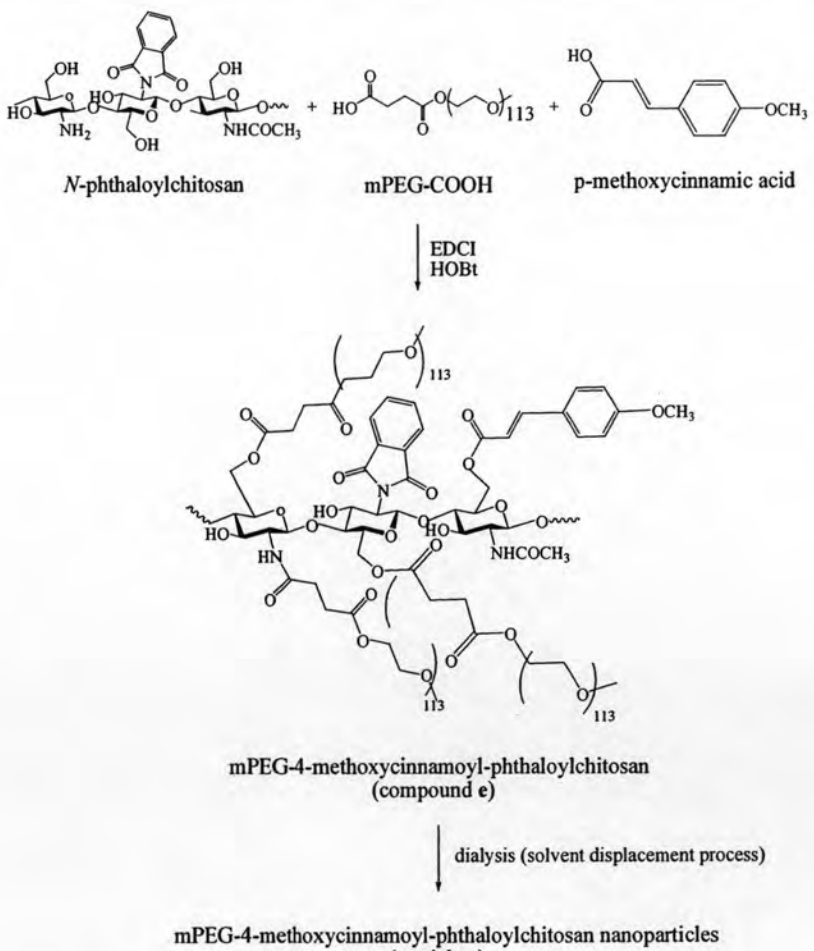
Ascorbyl palmitate-loaded-nanoparticle **d** was prepared by the solvent displacement procedure. Particle **d** (0.06 g) and ascorbyl palmitate (0.172 g) were dissolved in 10 mL of N,N'-dimethylformamide until the homogeneous was observed. This solution of ascorbyl palmitate was poured into a dialysis bag and placed in a beaker containing 1,000 mL of H_2O. The solution was dialyzed against water for 5

days (15×1,000 mL changes of water) and then centrifuged for 45 min at 43,000×g before quickly washed with methanol. The precipitated was analyzed by SEM, TEM and UV-VIS spectroscopy.

2.3.6 Encapsulation of Astaxanthin into Nanoparticle **d**

Astaxanthin-loaded-nanoparticle **d** was prepared by the solvent displacement procedure. Particle **d** (0.06 g) and astaxanthin (0.248 g) were dissolved in 10 mL of N,N'-dimethylformamide until the homogeneous was observed. This solution of astaxanthin was poured into a dialysis bag and placed in a beaker containing 1,000 mL of H_2O. The solution was dialyzed against water for 5 days (15×1,000 mL change of water) and then centrifuged for 45 min at 43,000×g before thoroughly washed with methanol. The precipitated was analyzed by SEM, TEM and UV-VIS spectroscopy.

2.4 **mPEG-4-methoxycinnamoyl-phthaloylchitosan Nanoparticles (e)**



Scheme 2.4

Compound **b** (3.3 g) was stirred with 4-methoxycinnamic acid (0.6545 g, 3.67×10^{-3} moles) in 50 mL of DMF solution containing HOBt, 1.985 g, 3 moles equivalent to 4-methoxycinnamic acid) at room temperature. EDCI (2.112 g, 3 moles equivalent to 4-methoxycinnamic acid) was added at 4°C and the mixture was kept at 4°C for 1 h and then at room temperature for 12 h. After that, the mixture was charged with **c** (26.49 g, 5.19×10^{-3} moles) at room temperature and it was stirred, homogeneous solution was observed. Then, EDCI (2.82 g, 3 moles equivalent to poly(ethylene glycol) methyl ether terminated with carboxylic groups) was added at 4°C and the mixture was maintained at 4°C for 1 h and then at room temperature for 12 h. Then the solution was dialyzed against water (pH 7) for 5 days (15×1,000 mL change of water) and then centrifuged for 45 min at 43,000×g before quickly washed with methanol and dried to obtain pale brown particle, **e** (Scheme 4). The product was analyzed by ^{1}H-NMR, IR, UV-VIS spectroscopy, SEM and TEM. In addition, the particle size distribution and zeta potential were determined by zetaziser instrument.

2.4.1 Encapsulation of Ascorbyl Palmitate into Particle **e**

2.4.1.1 Solvent displacement method

Ascorbyl palmitate-loaded-nanoparticle **e** was prepared by the solvent displacement procedure. Particle **e** (0.06 g) and ascorbyl palmitate (0.172 g) were dissolved in 10 mL of N,N'-dimethylformamide. This solution was poured into a dialysis bag and placed in a beaker containing 1,000 mL of H_2O. The solution was dialyzed against water for 5 days (15×1,000 mL changes of water) and then centrifuged for 45 min at 43,000×g before quickly washed with methanol. The precipitated was analyzed by SEM, TEM and UV-VIS spectroscopy.

2.4.1.2 Diffusion method

Particle **e** (0.06 g) was dispersed in 8 mL of water. Ascorbyl-palmitate (0.172 g, 0.0415 M) was dissolved in methanol solution (2 mL). And then, the methanolic ascorbyl palmitate solution (2 mL) was added dropwise into 8 mL of particle **e** aqueous solution and stirred for 30 days. The 1.5 mL of solution containing ascorbyl palmitate and particle **e** was withdrawn at 0 second, 5 days, 10 days, 20 days and 30 days, respectively. The solution was centrifuged at 43,000×g for 45 min before quickly washed with methanol and dried. The product was analyzed for ascorbyl palmitate content using by UV-VIS spectroscopy. The particle was also subjected to SEM analysis.

2.4.2 Encapsulation of Astaxanthin into Particle **e**

Astaxanthin-loaded-nanoparticle **e** was prepared by the solvent displacement procedure. Particle **e** (0.06 g) and astaxanthin (0.248 g) were dissolved in 10 mL of N,N'-dimethylformamide. This solution of astaxanthin was poured into a dialysis bag and placed in a beaker containing 1,000 mL of H_2O. The solution was dialyzed against water for 5 days (15×1,000 mL changes of water) and then centrifuged for 45 min at 43,000×g before quickly washed with methanol. The precipitated was analyzed by SEM, TEM and UV-VIS spectroscopy.