



CHAPTER III

EXPERIMENTAL

The experiments were divided into 2 parts. The emulsifying capability of PCTS, emulsion type, emulsion stability, and physical mechanism of unstable emulsions were investigated in Part 3.1. Main factors such as pH, ionic strength, and temperature on destabilized mechanism were investigated in Part 3.2.

3.1 Emulsification properties of PCTS

3.1.1 Chemicals and Materials

Chitosan with Molecular weight 250000 and degree of deacetylation of 85 (determined by $^1\text{H-NMR}$ (see appendix A), Varian, Unity Inova, USA) was purchased from Seafresh Chitosan (Lab) Co., Ltd. (Bangkok, Thailand). Methanesulfonic acid and phosphorus pentoxide were supplied from Acros Organics (NJ, USA). Sodium hydroxide was obtained from Ajax Chemicals (Sydney, Australia). Commercial grade of acetone and ethanol were purchased from RCI Labscan (Bangkok, Thailand). Hydrochloric acid was obtained from JT Baker Chemical Co (NJ, USA). Mineral oil (density of 0.8 g/cm^3) was purchased from Witco (Houston, USA). Sodium chloride was provided from Merck (Darmstadt, Germany). Fluorescein isothiocyanate (FITC) was purchased from Fluka (Deisenhofen, Germany). All chemicals and solvents were used as received. Distilled water was used in all experiments. Dialysis tube, CelluSep T2, with molecular weight cut off 6000-8000 was purchased from Membrane Filtration Products, Inc., USA

3.1.2 Synthesis of sodium phosphorylated chitosan and characterization

Synthesis of sodium phosphorylated chitosan

Sodium phosphorylated chitosan (PCTS) was synthesized as follows. [124]. Briefly, chitosan was reacted with phosphorous pentoxide at 0.1 equivalent moles to chitosan residue in methanesulfonic acid at $0-5^\circ\text{C}$ for 3 hrs. The mixture was

precipitated in acetone, washed several times in ethanol and dried in the oven at 40°C. In order to improve water solubility of phosphorylated chitosan, it was dissolved in distilled water and dialyzed against distilled water and 0.1 M sodium hydroxide, respectively. The dialyzed product was then neutralized with 0.1 M hydrochloric acid, following with dialysis in distilled water to remove by product of sodium chloride. The water soluble PCTS was obtained after lyophilization.

To evaluate the effect of degree of substitution on the emulsifying efficiency of PCTS, so PCTS was synthesized at various degree of substitution (DS) followed the above mentioned method but vary the amount of phosphorous pentoxide at 0.1, 1, and 2, equivalent mole to chitosan residue, respectively. The obtained PCTS was named as PCTS0.1, PCTS1 and PCTS2, respectively.

● Characterization of PCTS

The structure of PCTS was analyzed by Fourier transform infrared (FT-IR) spectrometer. FT-IR spectroscopy was carried out by grinding the samples with KBr at ratio of sample to KBr of 1:100, using Nicolet 6700, Thermo Scientific, USA (number of scan of 16 and accumulating every 4 cm⁻¹).

The number average molecular weight (M_n) and the weight average molecular weight (M_w) of PCTS were determined by gel permeation chromatography (GPC). Chromatograms were recorded and integrated using the Class-VP Version 6.14 (Shimadzu, Japan) chromatographic software on a system equipped with PL aquagel-OH 30 column using distilled water as the eluent at 40°C and flow rate 1ml/min. Pullulan was used as the standard for calibration.

The degree of substitution of phosphate group on chitosan residue was determined using a scanning electron microscope (JSM-5800, JEOL, Japan) equipped with an energy-dispersive X-ray spectrometer (SEM-EDX) and was calculated in the following equation:

$$\text{Degree of substitution} = \frac{\%P}{\%N} \quad (3.1)$$

Where %P was phosphorus content of phosphoric groups, %N was nitrogen content in chitosan residue.

3.1.3 Determination of Hydrophilic-Lipophilic Balance (HLB) of PCTS

A HLB was determined in order to investigate the balance of strength of the hydrophilic and lipophilic moiety in emulsifier molecule. The HLB value was determined with method of Becher [64] by measuring toluene drop diameter laid over the known HLB emulsifier solutions. Briefly, sodium dodecyl sulphate (HLB 40, [122]) and Tween 20 (HLB 16.7, [123]) were mixed at different ratios to obtain different HLB values in the range of 16.7 to 40. The HLB value of combination emulsifiers was calculated from the sum of each weight of the individual emulsifier multiplied with HLB value of it [124] (see appendix B). Then, toluene 0.5 ml was dropped onto the mixed emulsifier solution. The diameter of toluene drop laid on emulsifier solution was measured. The experiment had done in triplicate. The toluene drops diameter in emulsifier solutions with various HLB were plotted as calibration curve. In order to determine HLB number of PCTS0.1, the diameters of a toluene drop was measured onto the surface of 1% (w/v) PCTS0.1 aqueous solution. HLB of PCTS was calculated from the above calibration curve.

3.1.4 Determination of Critical Micelle Concentration (CMC) of PCTS

In order to investigate the lowest concentration for PCTS0.1 to form micelle, critical micelle concentration was determined by measuring surface tension against PCTS solution for various concentrations. Surface tension has reported as one of the most popular means for determining CMC of emulsifier [124]

The Du Noüy ring method was used to measure the surface tension of PCTS solution at 25°C by using Dataphysics DCAT 21 dynamic tensiometer (Filderstadt, Germany). The concentrations of PCTS solutions were varied in the range of 0.00-

0.53%w/v. Then the surface tension of PCTS0.1 was plotted against concentrations of PCTS0.1, an inflection point was corresponded to the CMC. The experiment had done in triplicate.

3.1.5 Emulsion preparation

The water soluble PCTS0.1 aqueous solution was used as aqueous phase, while mineral oil was used as oil phase. Emulsification had been done in two steps, coarse and fine. The coarse emulsification was done with ultra turrax (Model T25, IKA Werk, Germany) at 3400 rpm for 5 min at room temperature. The obtained coarse emulsion was then fine homogenized with high pressure homogenizer (EmulsiFlex C3, Avestin Inc., Canada) at 1500 bar for 6 cycles.

3.1.6 Preliminary Emulsion Type Evaluation of PCTS

The water soluble PCTS0.1 aqueous solutions were used as aqueous phase, whereas mineral oil was used as oil phase. The concentration of PCTS0.1 was fixed at 1%w/v. The aqueous solution to oil ratios were varied to 2:8, 4:6, 5:5, 6:4 and 8:2 in order to determine a suitable ratios for emulsification. Emulsification had been done following to the above mention. The preferable emulsion type was evaluated from emulsion index, surface mean diameter (d_{32}), including droplet size distribution. Emulsion index can be calculated from ratio of the obtained emulsion volume to the total liquid volume as shown in the following equation.

$$\text{Emulsion index} = \frac{\text{Emulsion volume}}{\text{Total volume}} \quad (3.2)$$

Furthermore, the emulsion drops in distilled water or mineral oil, so called drop test, was observed to preliminary investigate the continuous phase [36].

3.1.7 Determination of emulsifying efficiency and emulsion stability

The emulsifying efficiency of PCTS0.1 was investigated in terms of emulsion droplet size. The stability of emulsion was determined in terms of storage appearance alteration and droplet size alteration. The emulsions were prepared using various concentrations of PCTS0.1 aqueous solutions in the range of 0.06-5.00% w/v. Emulsification had been done following as described above. All the experiments had done in triplicate.

3.1.7.1 Emulsifying efficiency

The emulsifying efficiency of PCTS0.1 was investigated in terms of emulsion droplet size. The droplet size was measured at 25°C by dynamic light scattering spectrometer (NanoZS, Malvern Instrument Ltd., UK). A 10 µL of fresh prepared emulsion (within 24 hours) was diluted in distilled water 10,000 times. The diluted emulsion was then shaken with vortex to prevent aggregation of individual droplet and avoid multiple scattering effects [125]. Particle size was reported as surface mean diameter, d_{32} . The value of d_{32} is defined as:

$$d_{32} = \frac{\sum n_i d_i^3}{\sum n_i d_i^2} \quad (3.2)$$

where n_i is the number of droplet with diameter d_i

Furthermore, the PCTS distribution at interface between oil and water phase was visualized using confocal scanning laser microscopy (CLSM). PCTS was labeled with fluorescein isothiocyanate and visualized the fluorescent emission.

- **Fluorescence labeling of PCTS**

In order to visualize the emulsion droplets, PCTS was labeled with fluorescent dye. A fluorescein isothiocyanate, FITC, 10 mg was dissolved in distilled

water at a concentration 2 mg/ml, and then the dye solution was added to 50 ml of the 4% (w/v) PCTS0.1 aqueous solution. The sample was stirred at room temperature overnight. The free FITC was removed by dialyzed against distilled water for 3 days. The FITC-labeled PCTS0.1 was obtained after lyophilization. The labeled reaction schemes for the PCTS0.1 with the fluorescein markers are shown in Figure 3.1.

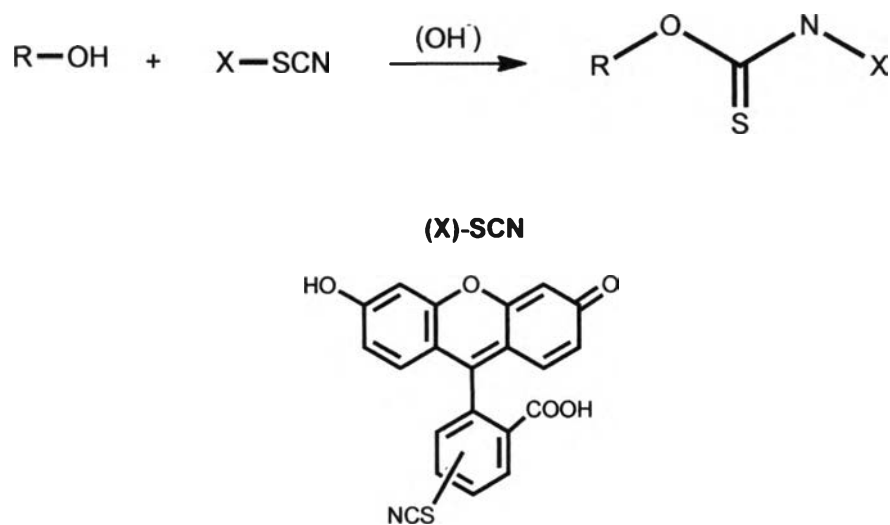


Figure 3.1 Reaction schemes for the labeling procedures of the PCTS0.1 with the fluorescence markers. X-SCN, fluorescein isothiocyanate [applied from 126]

- **Confocal laser scanning microscopy**

The visualization of droplets was evaluated with confocal scanning laser microscope. This method can investigate the location, distribution of PCTS within the droplets. Furthermore, an image of the sample can be shown in three dimensional reconstructions. Hence, computational image analysis enables the visualization and characterization of PCTS not only on the surface of the droplet, but also inside the droplet in order to clarify type of emulsion as well as physical mechanisms such as flocculation, coalescence, creaming, Ostwald ripening. The emulsions were diluted with

distilled water at 0.01%w/v. The diluted emulsions were examined by using a confocal laser scanning microscope (FluoView™ FV1000; Olympus, Japan) consisting of an Olympus IX81 microscope and an argon ion laser to visualize the fluorescein-labeled PCTS surrounding the emulsion droplet at magnification 40x. The imaging software incorporated with the confocal laser scanning microscope was the Olympus FV10-ASW 1.7 Viewer software (Olympus, Japan).

3.1.7.2 Emulsion stability

Emulsion stability was investigated in terms of storage appearance alteration and droplet size alteration.

3.1.7.2.1 *Storage appearance alteration*

The obtained emulsions were kept in standard test tube (diameter 13 mm, height 120 mm) at room temperature. The appearances of the fresh emulsions and that of after storage for 7 and 90 days were observed by naked eyes. Phase separations of the emulsions were recorded in terms of normalized height.

3.1.7.2.2 *Droplet size alteration*

The alteration on particle size after storage was investigated in order to assess the short term and long term stability of the emulsion. The droplet sizes of emulsion were measured again after storage for 7 days and 90 days at room temperature.

The particle size alteration was reported as the droplet size difference at time t from the droplet size of fresh prepared emulsion as calculated from the following equation.

$$\Delta D = D_t - D_f \quad (3.3)$$

Where ΔD , D_t , D_f were droplet size difference, droplet size at time t , and droplet size of fresh emulsion, respectively.

3.1.8 Investigation of physical mechanism of unstable emulsion

Physical mechanism of an unstable emulsion was investigated in order to find out the main reasons making emulsion destabilization. Physical mechanism of the unstable emulsion can be classified into two main categories; (i) reversible process consisting of creaming, sedimentation, flocculation and (ii) irreversible process consisting of coalescence, Ostwald ripening. Those processes obviously show different phenomena. Therefore, the physical mechanism of the unstable emulsion was determined by visualization the PCTS distribution on emulsion droplet and determination of reversibility phenomena under pH variation.

3.1.8.1 Visualization of the PCTS distribution on the emulsion droplet

In order to visualize the PCTS distribution on emulsion droplet after storage emulsion at room temperature for 7 days, PCTS was labeled with fluorescent dye and observed under confocal scanning laser microscope following method as described above.

3.1.8.2 Determination of physical reversibility of the unstable emulsion under pH variation

Owing to PCTS was an amphoteric molecule having both positive and negative charged functional groups, thus, charged alteration corresponding to change in environmental pH was considered as main factor influenced to physical mechanism of the unstable emulsions. Furthermore, ionic levels were also considered as a factor

influenced to physical mechanism of the unstable emulsions. Therefore, the unstable emulsions forming at given PCTS concentration and PCTS ionic levels were selected to investigate the physical reversibility under pH variation in terms of surface charges alteration and droplet size alteration.

3.1.8.2.1 ζ -Potential measurements

The ζ -Potential or surface charge of droplet was evaluated by zetasizer (NanoZS, Malvern Instrument Ltd., Worcestershire, UK) at 25 °C. A 10 μL of fresh prepared emulsion (within 24 hours) or 7 days storage emulsion was diluted in various pH (1-12) aqueous solutions, which were adjusted by 0.1 M HCl or 0.1 M NaOH, to a final droplet concentration of 0.0001% v/v. Diluted emulsion was injected into cell. ζ -Potential was determined by measuring the direction and velocity of droplets in the applied electric field. ζ -Potential value shows the difference in potential of charge between the slip plane of the double layer near the droplet surface and the bulk solution.

3.1.8.2.2 Determination of droplet size and droplet size distribution of emulsion at various pH

Droplet size and droplet size distribution of emulsion at various pH was determined following the above mentioned method and using PCTS with various ionic levels as emulsifiers. A 10 μL of fresh prepared emulsion (within 24 hours) or 7 days storage emulsion was diluted in various pH aqueous solutions 10,000 times (final droplet concentration of 0.0001% v/v) . The pH of aqueous solution was adjusted by 0.1 M HCl or 0.1 M NaOH.

Meanwhile, Droplet size distribution was represented by plotting as curve of volume intensity (i.e. number of particles) against diameter of droplets.

3.2 Effect of systematic conditions (pH, ionic strength, and temperature) on emulsion stabilization

3.2.1 Chemicals and Materials

PCTS0.1, PCTS1, and PCTS2 were synthesized in our laboratory following the above mentioned method. Sodium hydroxide was obtained from Ajax Chemicals (Sydney, Australia). Hydrochloric acid was obtained from JT Baker Chemical Co (NJ, USA). Mineral oil (density 0.8 g/cm^3) was purchased from Witco (Houston, USA). Sodium chloride was provided from Merck (Darmstadt, Germany). All chemicals and solvents were used as received.

3.2.2 Preparation of emulsion

The emulsions were prepared using PCTS as emulsifiers which was dissolved in an aqueous phase at fixed concentration of 1%w/v PCTS. PCTS with various degrees of phosphate substitution (PCTS 0.1, PCTS 1, or PCTS 2) were used in this experiment. A mineral oil (oil phase) was gradually added into the aqueous solution. High pressure homogenizer (EmulsiFlex C3, Avestin Inc., Canada) was used to mix those two phases at 500 psi 3 cycles and to homogenize into fine emulsion at 1500 psi 6 cycles. All emulsions were prepared at ratio of oil to water of 2 to 8.

3.2.3 Physical mechanism of the emulsion under various conditions

Physical mechanism is a phenomenon describing on how emulsion changes its stability or emulsion destabilization. The physical mechanism of the emulsion prepared from different PCTS having various degree of phosphate substitution was investigated under changing the environmental conditions such as pH, ionic strength and temperature. Various pH solutions were prepared by the combination of 0.1 M HCl and 0.1 M NaOH. Various ionic strength solutions were prepared with various concentrations of NaCl in the range of 0-1000 mM. The temperature of distilled water

was varied at 30°C, 40°C, 50°C, 60°C, and 70°C. Emulsion destability was determined in terms of surface charge, droplet size and droplet size distribution.

3.2.3.1 ζ -Potential measurements

The ζ -Potential or surface charge of droplet was evaluated by zetasizer (NanoZS, Malvern Instrument Ltd., Worcestershire, UK) at 25 °C. A 10 μL of fresh prepared emulsion (within 24 hours) was diluted in aqueous solution of varying pH (1-12) or NaCl concentration (0-1000 mM) or various temperature (30°C -70°C) to a final emulsion concentration of 0.0001% v/v. Diluted emulsions were injected into cell. ζ -Potential was determined by measuring the direction and velocity of droplets in an applied electric field. ζ -Potential value shows the difference in potential of charge between the slip plane of the double layer near the droplet surface and the bulk solution.

3.2.3.2 Determination of droplet size and droplet size distribution of emulsion

The droplet size was measured at 25°C by dynamic light scattering spectrometer (NanoZS, Malvern Instrument Ltd., Worcestershire, UK). A 10 μL of fresh prepared emulsion (within 24 hours) was diluted in aqueous solution of varying pH (1-12) or NaCl concentration (0-1000 mM) or various temperature (30°C -70°C) to a final emulsion concentration of 0.0001% v/v. The diluted emulsion was then shaken with vortex to prevent aggregation of individual droplet and avoid multiple scattering effects. Particle size was reported as surface mean diameter, d_{32} . The value of d_{32} is defined as:

$$d_{32} = \frac{\sum_i n_i d_i^3}{\sum_i n_i d_i^2} \quad (3.2)$$

where n_i is the number of droplet with diameter d_i ,

Meanwhile, Droplet size distribution was represented by plotting as curve of volume intensity (or in other words was the amount of particles) against diameter of droplet.