# CHAPTER IV
# CHITOSAN FUNCTIONALIZATION IN WATER: A NOVEL APPROACH TO DEVELOP A CHITOSAN-ALLERGEN DELIVERY SYSTEM

## 4.1 Abstract

A water soluble chitosan derivative prepared by functionalizing chitosan with poly(ethylene glycol) monomethyl ether (mPEG) and cholic acid (CA) in a water-ethanol system for the use as allergen delivery system is proposed. The derivative is water soluble depending on the molecular weight of chitosan and mPEG and the mole ratios of chitosan:mPEG:CA. The structural analyses by Fourier transform infrared spectroscopy (FTIR) and $^{1}H$ nuclear magnetic resonance spectroscopy ($^{1}H$ NMR) confirm the conjugation of mPEG and CA with substitution degree to be about 20-30%. The derivative performs nanospherical shape with size 40 nm whereas the incorporation of a model allergen, the house dust mite, by mixing the aqueous solutions of the derivative and the house dust mite initiates larger nanosphere size to be 200 nm as observed by transmission electron microscope (TEM). A preliminary in vitro immune response test based on lymphocyte transformation shows that the lymphocytes cultured in CS-mPEG-CA incorporated with allergen have less proliferation than the one in allergen as observed from microscope.

Keywords: Chitosan, Nanosphere, Adjuvant, Allergen delivery system

## 4.2 Introduction

Over the past few decades, prevalence of diseases involving immune system has been increasing significantly, such as allergic diseases which the sensitization to allergen is the major culprit causing inflammation in the end organs. The known allergens are dust-mite, pet dander, cockroach, molds and pollen. One effective treatment is allergen immunotherapy or 'allergy shot', which the immune system is gradually tolerated to the sensitized allergen by a series of increasing administration of allergen. The methods of administrations are injecting, sublingual or swallowing, etc. resulting in decrease allergic sensitization. However, allergen is administered with an adjuvant, of which the function is related to increase allergen activity in stimulating the immune response, for example alum, an effective adjuvant used in human system. For the fact that alum cannot be naturally metabolized by human organ system, and adverse reactions (e.g., granulomas) at injection sites were reported[1], so the development of the biocompatible adjuvant has received much attention for the past several years.

Natural polymer such as alginate, amino acid, starch and chitosan were proposed for adjuvants.[2,3] The basic properties of chitosan about the biodegradability, non-toxicity, non-irritation, immune response enhancement and transport of drug across the epithelium make it to be an attractive material.[4, 5, 6]

Although chitosan is a potential material, it has poor solubility in water and most organic solvents except acid. It is well-dissolved in acetic acid, however, the solution damages the cells when we consider the use of chitosan as an adjuvant and the incorporation with allergen.[7] Considering the requirements of a material for adjuvant, chitosan needs (i) derivatization for delivery system without contamination of organic solvents, (ii) effective allergen incorporation and (iii) hydrophilicity or water solubility for clinical treatment.

Choic acid is a bile acid which is in a family of steroid compounds. Previously, it is used as a hydrophobic molecule in modifying the nanoparticle for drug delivery system.[8, 9] It is important to point out that the functionalization of chitosan with cholic acid may bring the balance of hydrophilic and hydrophobic sites to form spherical structure either nano- or micro- sizes, and give the synergistic effects in interacting with allergen. There, the good balance of hydrophobicity and

hydrophilicity also merits the water solubility as well as the allergen incorporation which is one of the requirements for clinical use. Based on this viewpoint, methoxy end capped polyethylene glycol (mPEG) is, then, considered as a hydrophilic part to further modify chitosan. The use of mPEG also covers the biocompatibility and non-toxicity including the water based system which is the requirement for medical application.

### 4.3 Experimental

**Materials.** Chitosan with a degree of deacetylation (%DD) 95 and molecular weight (Mw) of 48,000 and 227,000 were locally supplied from the SEAFRESH (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, $M_n$ 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.

**Equipments.** Fourier transform infrared (FTIR) spectrum was carried out by using a Bruker Equinox 55/S with 32 scans at a resolution of 4 $cm^{-1}$ in a frequency range of 4000-400 $cm^{-1}$ using a deuterated triglycinesulfate detector (DTGD) with a specific detectivity, D*, of $1\times10^9$ $cm.Hz^{1/2}$ $w^{-1}$. $^1H$ Nuclear magnetic resonance (NMR) was obtained from a 400 MHz JEOL JNM-GSX spectrometer. 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. The morphology was investigated by using a JEOL/JSM 6480 scanning electron microscope (SEM) at 50 kV and JEM-200CX transmission electron microscope (TEM) at 80 kV. Particle size was measured by Zetasizer nanoZS Malvern Instrument with He-Ne lazer source using backscattering detector.

**mPEG and CA conjuagated chitosan** (Scheme 4.1). Chitosan was dissolved with HOBt in aqueous solution **1** as reported by Fungkangwanwong *et al.*[10]

Carboxyl terminated poly(ethylene glycol) methyl ether (mPEG-COOH) was prepared as reported by Yoksan *et al.*[11] Chitosan-HOBt solution (10 ml, 0.61 mmol) was mixed with mPEG-COOH (1.5237 g, 0.3 mmol), CA (0.1226 g, 0.3 mmol) in ethanol 8 ml. Then, WSC·HCl (0.1178 g, 0.6 mmol) in ethanol (5 ml) was added into the solution. The homogeneous solution was allowed reacting at 60 °C for 24 hours. The solvent was removed and reprecipitated in acetone. The crude product was washed with methanol several times and dried *in vacuo* to obtain **2a**. The compounds **2b**, **2c**, **2d** and **2e** were also prepared by varying the mole ratios of chitosan:mPEG-COOH:CA as shown in Table 4.1.

Chitosan with Mw 227,000 and mPEG with Mw 1100, and 2000 were also used to prepare. Chitosan with Mw 227,000 and mPEG with Mw 1100 and 2000 were used instead.

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

$^1$H-NMR ($D_2O$, ppm): $\delta_H$ 0.52 (3H, s, $CH_3$ of cholic acid), 0.69 (3H, s, $CH_3$ of cholic acid), 0.8 (3H, s, $CH_3$ of cholic acid), 0.89-1.76 (m, $CH_2$ of cholic acid), 1.85 (3H, s, H-Ac of CS), 2.51 (4H, d, $COCH_2CH_2CO$), 2.68 (1H, s, H-C2 of chitosan), 3.18 (3H, s, $CH_3$-O), 3.23-3.79 (m, H-C3-C6 of chitosan and $OCH_2CH_2$ of mPEG), 4.09 (2H, d, $CH_2OCO$ of mPEG).

**Scheme 4.1**

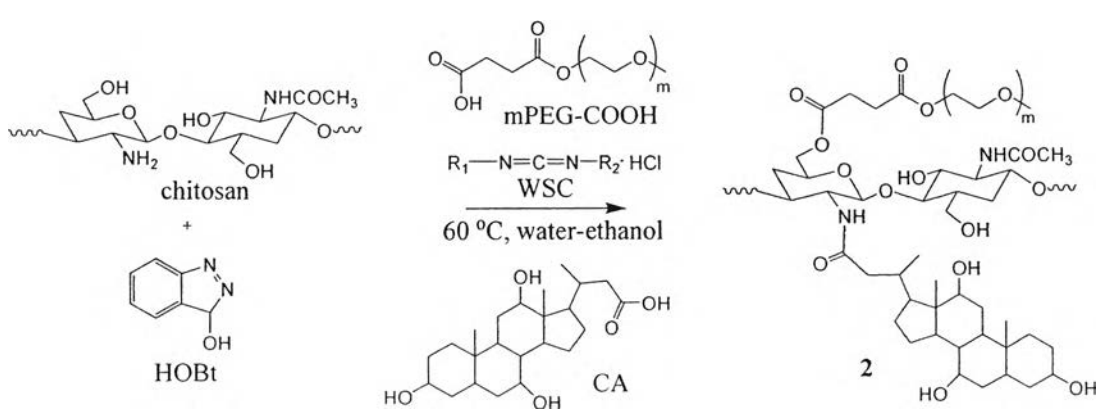

**Table 4.1** Mole ratios of chitosan:mPEG-COOH:CA in preparing **2**, **3**, **4** and **5**

| Compound | Mole Ratio of chitosan:mPEG-COOH:CA |
|---|---|
| **2a**, **3a**, **4a**, **5a** | 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.

**Allergen incorporation.** **2a** was dissolved in water and steriled by filtrating through a 0.22 micron membrane. An equal weight of chitosan solution and allergen was mixed and vigorously stirred for 20 s. The chitosan-allergen solution was investigated for the morphology by using TEM and for the in vitro immune response by testing the lymphocyte transformation (LTT). For **3**, as the sample was difficult to soluble in water, the solution of **3** obtained from the reaction (0.1g) was mixed with allergen (10 μg) and vigorously stirred for 20 s. The chitosan-allergen solution obtained was investigated morphology by TEM.

### 4.4 Results and Discussion

Our preliminary synthesis work clarified that the conjugation of cholic acid or deoxycholic acid onto chitosan gave the product which is difficult to dissolve in solvents, especially water or polar solvent. This obstructed the following step of the introduction of polyethylene glycol. Here, the conjugation with cholic acid and poly(ethylene glycol) was carried out in a single step to find that the reaction gave the gel and/or water soluble species depending on the conditions as detailed in follows.

**mPEG and CA conjuagated chitosan**. Chitosan (Mw 48,000) was conjugated with mPEG and cholic acid in the chitosan-HOBt aqueous system. The reaction was carried out in a homogeneous system at 60 °C for a day using WSC coupling reagent. The products obtained were transparent solution. The solvent was evaporated out and the viscous solution was obtained. The crude product was purified by reprecipitated in acetone and washed with methanol several times. At that time, it forms gel which due to there is some trace of good solvent, water, in the system. It was, then, dried *in vacuo*. The products **2a**, **2b** and **2c** obtained were soluble in water whereas **2a** was the best water soluble. This reflected the loss of inter- and intra- molecular hydrogen bonding network between chitosan chains and the enhancement of hydrophilicity of molecule. The product **2d** and **2e** cannot be soluble in water because they have not enough hydrophilic groups. The solubility of the products is summarized in Table 4.2.

**Table 4.2** Solubility of **2a**, **2b**, **2c**, **2d** and **2e**

| | Water | $CH_3COOH$ | Methanol | Ethanol | Acetone | Chloroform | DMF | DMSO | Hexane |
|---|---|---|---|---|---|---|---|---|---|
| **2a** | ✓ | ✓ | × | × | × | × | × | × | × |
| **2b** | ✓ | ✓ | × | × | × | × | × | × | × |
| **2c** | ✓ | ✓ | × | × | × | × | × | × | × |
| **2d** | ○ | × | × | × | × | × | × | × | × |
| **2e** | ○ | × | × | × | × | × | × | × | × |

✓ = soluble, ○ = swell and × = non-soluble.

Moreover, it was found that the solubility of the product is also influenced by molecular weight of chitosan and mPEG. When chitosan with molecular weight 227,000 was used and the mole ratio of chitosan:mPEG:CA were 1:05:0.5, 1:0.5:0.25, 1:0.5:0.1, 1:0.25:0.5 and 1:0.1:0.5, the gel product, **3a-e**, were obtained (Figure 4.1).

The pictures of **3b-e** are not shown here. The products swell in water. This may due to the intermolecular hydrogen bond network between mPEG chains and cholic acid groups. For mPEG with molecular weight 1100 and 2000 were used, the gel-like products, **4a** and **5a**, were also obtained under the mole ratio 1:0.5:0.5. This implied how the molecular weight of mPEG is an important factor in water solubility.

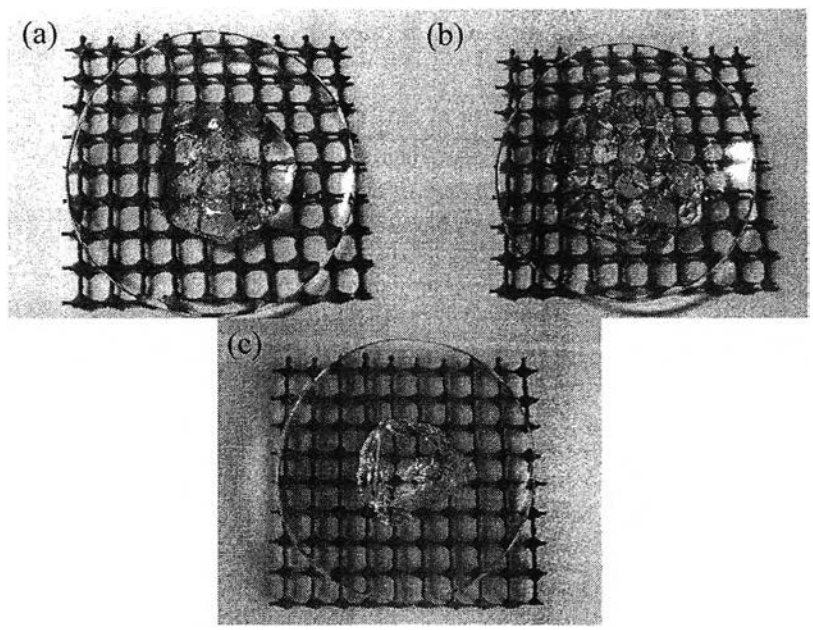


**Figure 4.1**. Gelation of (a) **3a**, (b) **4a** and (c) **5a**.

**Structural characterization**

Chitosan shows characteristic peaks at 3368 $cm^{-1}$(OH), 2881 $cm^{-1}$(CH stretching), 1644 $cm^{-1}$(amide I), 1597 $cm^{-1}$(amide II), 1153-895 $cm^{-1}$(pyranose ring). After reacting with cholic acid and mPEG-COOH for a day, a significant increase of the peak at 2881 $cm^{-1}$ for CH stretching and at 1659 $cm^{-1}$ for amide I and at 1552 $cm^{-1}$ for amide II are identified. The two new peaks at 1734 $cm^{-1}$ for ester group and at 1467 $cm^{-1}$ for cyclohexane are found implying the successful reaction. FTIR spectra of the **2b**, **2c**, **2d** and **2e** were similar to that of **2a**.

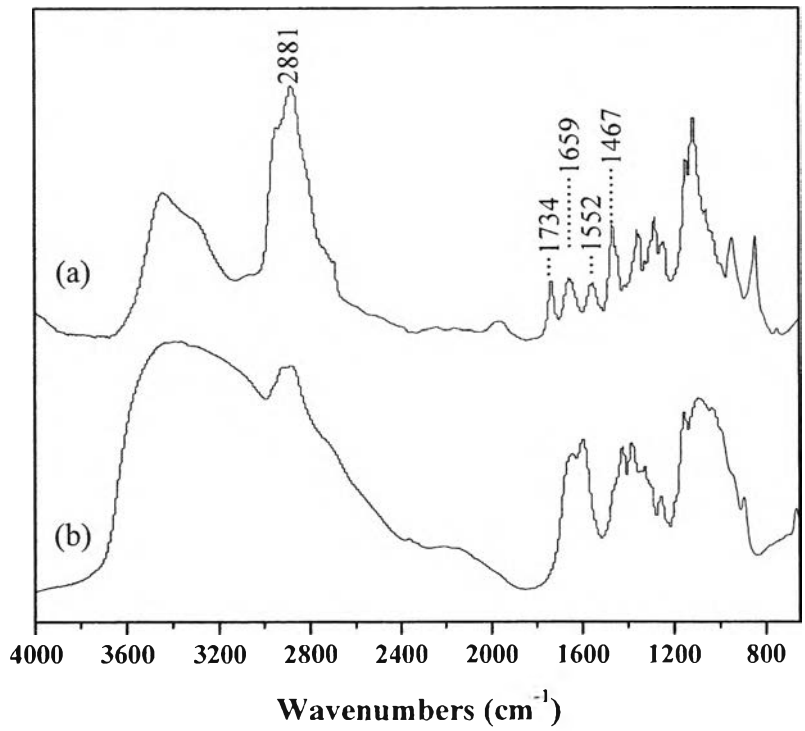


**Figure 4.2**. FTIR spectra of (a) chitosan and (b) **2a**.

The $^1$H NMR operating at room temperature was applied to analyze the structure of **2a**. Figure 4.3 shows the chemical shift of **2a** in $D_2O$ with 0.51 ppm (-$CH_3$ of cholic acid), 0.71-1.58 ppm (-$CH_2$- and -CH- of cholic acid), 1.86 ppm (NHAc), 2.51 ppm ($COCH_2CH_2CO$ of mPEG) 2.68 ppm (H2 of GluN unit in chitosan), 3.18 ppm ($CH_3$-O- in mPEG) and 3.32-3.72 ppm ($CH_2$-O- in mPEG and H3-H6 of pyranose ring) and 4.09 ppm ($CH_2OCO$ of mPEG). The degree of substitution (DS) was calculated by using the integration of H at C-2 position of chitosan as an internal standard peak. The DS of mPEG was found to be 31.81% based on the $COCH_2CH_2CO$ integration for 0.28 at 2.51 ppm and the DS of CA was 21.21% based on the $CH_3$- integration for 0.14 at 0.52 ppm. In the cases of **2b** and **2c**, they were hardly soluble in $D_2O$ and **2d** and **2e** were partially swelled so they could not be characterized by this technique.

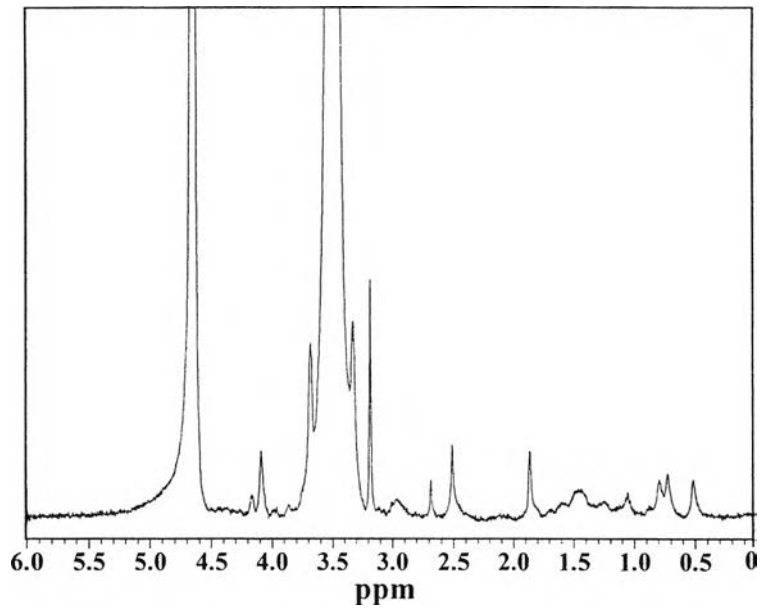


**Figure 4.3**. NMR spectrum of **2a**.

**Thermal properties**

Figure 4.4 shows the thermal stability of **2a-2e** as compared to that of chitosan. The weight loss for 10% starting from 60 °C to 100 °C refered to the moisture and water content. Compound **2** shows the two degradation steps. The first degradation step is from 215 °C to 240 which might come from the degradation at amide bonds or ester bonds to result in some small molecules vaporized. The degradation step is from 380 °C and proceeded continuously to as high as 430 °C whereas chitosan shows the degradation from 290-310 °C. The discussion is supported based on the result from TG-FTIR (Figure 4.5). In general, the introduction of functional group onto chitosan might disturb the packing structure and as a result the degradation temperature might decrease. In this case, we suspect that the hydrogen bonds or hydrophobic-hydrophobic interaction between cholic acid parts and the hydrophilic-hydrophilic interaction of mPEG induce the thermal stability. The ash content of chitosan about 40% whereas that of **2a-2e** were 3-15% ash content. This might be related to the degradation. The nearly complete degradation of chitosan-mPEG-cholic acid might results from the changing of its structure from high crystallinity to more amorphous structure.

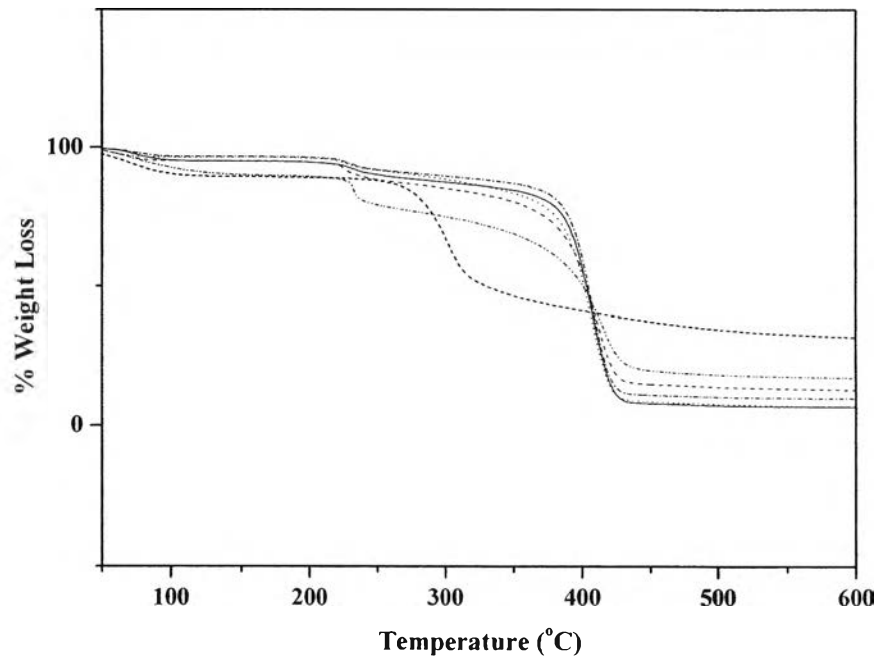


**Figure 4.4**. TGA thermograms of chitosan (—), **2a** (···), **2b** (—), **2c** (···), **2d** (···) and **2e** (···).

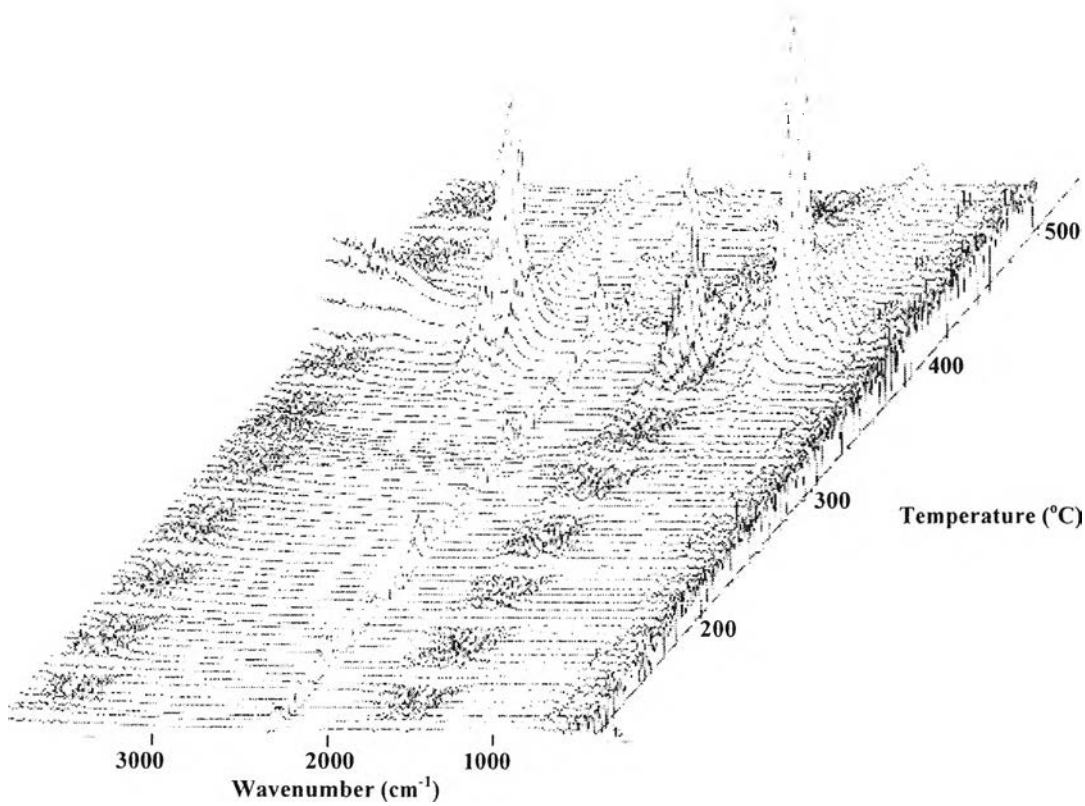


**Figure 4.5**. TG-FTIR spectra of **2a** from room temperature to 500 °C.

**Morphology**

Figure 4.6 shows the morphology of **2a**. The nanosphere formed in water with 40 nm by size is confirmed. Previously, chitosan nanoparticles can be prepared by several methods e.g. isotropic gelation[12], self assemble polyelectrolyte[13], etc. Due to the limitation of chitosan, especially the difficulty in dissolving in most organic solvents and water, the homogeneous reaction of chitosan was, thus, carried out in acetic acid. In this work, the preparation of chitosan nanoparticles is prepared in one-pot synthesis, which mPEG and CA are subjected to the reaction, in 56% ethanol/aqueous system. After allergen incorporation, **2a** (Figure 4.5 (c)) shows the increase in size as compared to that of before allergen incorporation. This implied the successful incorporation of allergen.

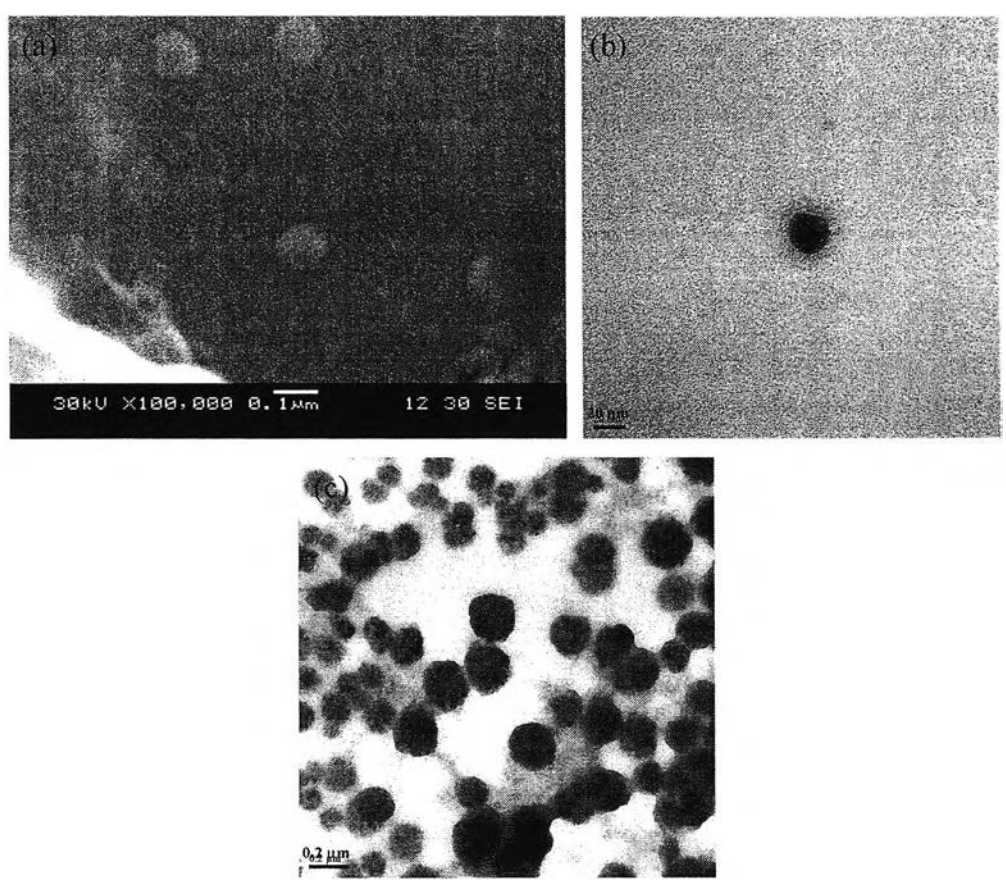


**Figure 4.6**. (a) SEM micrograph of **2a** with 100,000x magnification and (b) TEM micrographs of **2a** with 80,000x magnification before allergen incorporation and (c) with 9,600x magnification after allergen incorporation.

For **3**, the sample preparation was done by dropping solutions of **3a-e** on the stub and drying *in vacuo*. Figure 4.7 shows the differences of **3a** before and after allergen incorporation. Before allergen incorporation, **3a** shows the morphology of film. After allergen incorporation, the morphology was changed into sphere, especially in the cases of **3c** and **3e**. This may be due to the interaction between **3c** or **3e** and allergen to form self-assemble. In addition, when the amount of allergen incorporated is increased, the morphology was also changed and, as a consequence, allergen particles were observed on the surface (Figure 4.8).

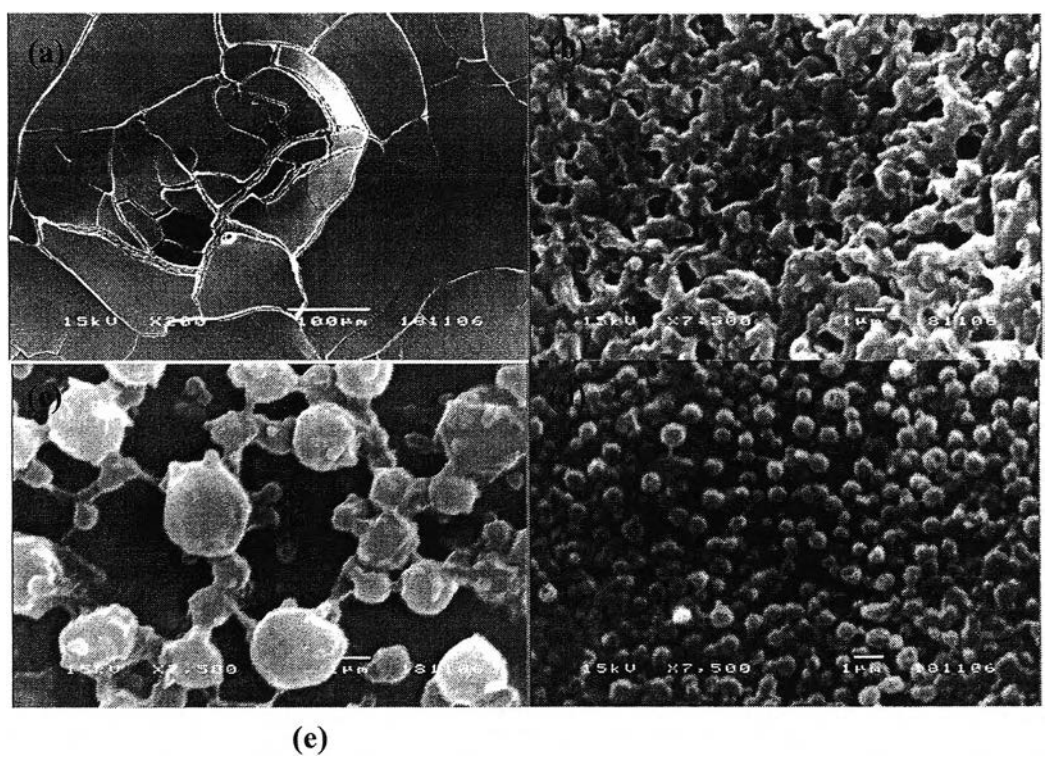


**Figure 4.7**. SEM micrographs of (a) **3a** with 200x magnification before allergen incorporation, after incorporation of (b) **3a** with 7,500x magnification, (c) **3c** with 7,500x magnification, and (d) **3e** with 7,500x magnification.

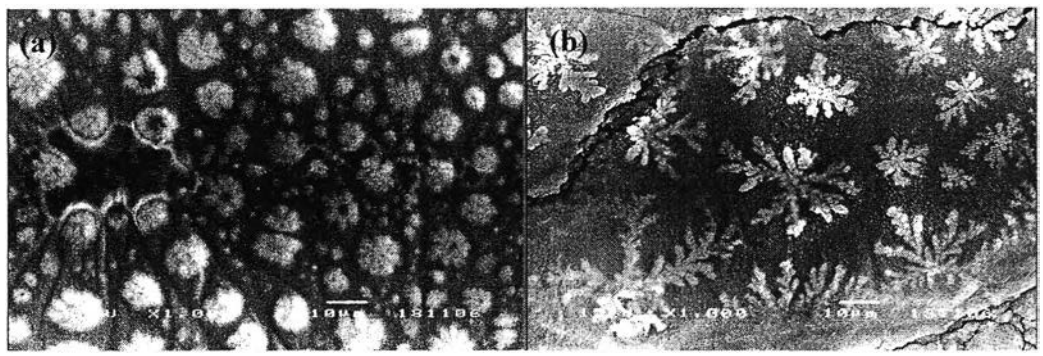


**Figure 4.8**. SEM micrographs of (a) allergen with 1,000x magnification and (b) **3c** with 1,000x magnification after allergen incorporation.

**Particle size**

Dynamic light scattering was another technique to investigate particle size besides TEM technique. Average particle size of **2a** was 93 nm and 40 nm observed by dynamic light scattering and TEM, respectively. This can be assumed that the samples can swell in solution under dynamic light scattering, compared with solid state TEM. After allergen incorporation, particle size increased to be 196 nm which is the same tendency as observed by TEM.

**Immune response**

LTT is *in vitro* immune response test. It reveals a sensitization of T-cells by enhancing the proliferative response of peripheral blood mononuclear cells to a certain compound. Figure 4.9 shows the preliminary LTT test. After culturing the cells 72 hrs, it was found that **2a** incorporating with allergen stimulate human peripheral blood mononuclear cells (PBMC) to proliferate less than the dust mite. It is also important to note that the **2a** incorporated allergen showed more significant stimulation than pure **2a**. This might be related to the fact that that **2a** is compatible to cells and possible to be an adjuvant. However, the patterns of cytokine production and level of toxicity need to be clarified in further steps.

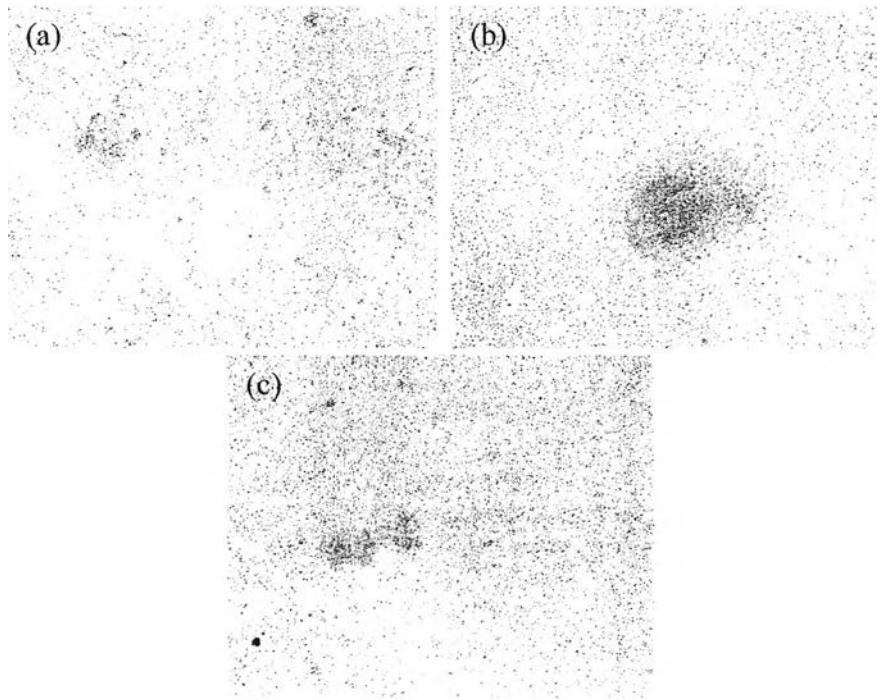


**Figure 4.9**. Micrographs of cells in *in vitro* culture with (a) **2a**, (b) dust mite, and (c) **2a** incorporating with allergen.

### 4.5 Conclusions

Water-soluble chitosan nanosphere can be easily prepared by functionalizing chitosan with mPEG and CA in water-ethanol system. The molecular weight of chitosan and of mPEG and the mole ratio of chitosan:mPEG:CA are the main factors to initiate the water solubility. The chitosan with Mw 48000 Dalton with 1:0.5:0.5 mole ratio of chitosan:mPEG:CA is the optimal condition in preparing water soluble derivative. The NMR confirmed that the chitosan was successfully conjugated with mPEG and CA with the degree of substitution 31.81 and 21.21%, respectively. The *in vitro* immune response was preliminarily investigated by LTT to find that CS-mPEG-CA incorporated with allergen tends to down regulate the cell response to allergen when compared to CS-mPEG-CA and allergen alone.

**4.6 Acknowledgements**

The authors thank Seafreash Chitosan (LAB) Company Limited, Thailand for chitosan material, Assoc. Prof. Buncha Pulpoka for NMR measurement and technical services, National Metal and Materials Technology Center (MTEC) for particle size measurement.

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