CHAPTER VI
AN APPROACH OF CHITOSAN NANOSPERES TO PROTEIN CARRIER

6.1 Abstract

Chitosan nanospheres were succeeded in preparing by grafting phthalic anhydride and mPEG on chitosan chain and self-assembly formation as core-corona structure. The reformed nanospheres with mean diameter of 40–50 nm and surface charge of -30 mV is successful in incorporating both of negatively charge catalase, BSA, OVA, α-lactalbumin and positively charged lysozyme. It is found that repulsive force of electrostatic interaction plays an important role in incorporating of BSA, OVA, and α-lactalbumin (incorporating efficiency < 50%). Hydropathy index is the key factor to determine hydrophilicity of lysozyme and catalase. van der Waals attraction possible to be the reason for the incorporation of lysozyme and catalase at hydrophilic corona of nanospheres.

Keywords: chitosan; nanospheres; protein; hydopathy index; van der Waals force; electrostatic interaction

6.2 Introduction

In recent years, the field of protein and peptide engineering has been enormous developed. With the tremendous growth of biotechnology and recent sequencing of the human genome, it is possible to produce significant numbers of therapeutically active proteins [1]. The challenging task in the development of protein pharmaceuticals is to deal with physical and chemical instabilities of proteins. The traditional way for protein pharmaceuticals is administrated through injection rather than taken orally due to their instability [2]. Problems for taking protein oral administration are acid catalyzed degradation in stomach, proteolytic break down in the GI tract, poor permeability across the gastrointestinal mucosa and first-pass metabolism during transfer across the absorption barrier and in the liver must be overcome for the efficient delivery of drugs into the blood stream [3]. In the design of oral delivery of peptide or protein drugs, nanoparticles have been studied extensively. Due to nanoparticles can not only improve the stability of therapeutic

agents against enzymatic degradation and control the release of therapeutic agents, but they can also be delivered to distant target sites either by localized delivery or they can be conjugated to a biospecific ligand which could direct them to the target tissue or organ. Various types of polymeric nanoparticles were studied such as poly-∈-carpolactone (PCL) [4], polylactide (PLA) [5], poly(lactide-co-glycolide) (PLGA) [6], and poly(γ-glutamic acid) (γ-PGA) [7] due to their biocompatibility, biodegradability, and ability to prolong drug release behavior.

Chitosan is a (1, 4)-linked 2-amino-2-deoxy-β-D-glucan and can be obtained from chitin by alkaline or enzymatic deacetylation. Chitosan possess useful characteristic that offer advantages for the possibility of clinical use [8]. Moreover, chitosan has a special feature of adhering to the mucosal surface and transiently opening the tight junction between epithelial cells. However, the poor water solubility of chitosan poses a barrier for it to perform the mucoadhesive and adsorption enhancing properties in the small intestine, which is the main absorptive region of the GI tract [9]. To overcome these drawbacks, many studies have been done. *N*-Trimethyl chitosan (TMC) is a permanently quaternized chitosan derivative with improved aqueous solubility compare to native chitosan. TMC with a degree of quaternization 60% (TMC60) can enhance the permeability of the intestinal tight junction more than quaternization 40% [10]. Also, TMC was found to be a good absorption for peptide drug [11].

Considering effective nanoparticulate delivery system, the nanoparticles size and loading must be adjust carefully, and protein stability during preparation and release must be ensured. Guest molecule such as drug, protein, and peptide can either be entrapped in the polymer matrix, encapsulated in a liquid core, surround by shell-like polymer membrane, or bound to the particle surface by adsorption [12]. In the case of protein adsorption process, both Coulomb (electrostatic) and van der Waals interactions (hydrophobic) are thought to be governing factors for the adsorption of proteins [13].

Our group succeeded in preparing mPEG grafted phthaloylchitosan to obtain chitosan nanospheres via self-assembly process [14–15]. The chitosan nanospheres have mean diameter of 237 nm and zeta potential is -54 mV as observed by DLS.

Recently, our group reported not only an incorporation of model guest molecule i.e. hexylamine, cyclohexylamine, nonylamine, pentadecylamine, stearylamine and stearic acid [16] but also an encapsulation of anticancer drug i.e. camptothecin [17–18], all-*trans*-rectinoic acid [19].

In this study, we aimed to develop amphiphilic chitosan nanospheres for protein delivery systems. In order to understand the driving force for incorporation protein with nanospheres, the factors which effect to the incorporation efficiency are evaluated by studying the relations of incorporation efficiency to surface charge and hydropathy index of protein.

6.3 Experimental Section

Materials

Chitosan (deacetylation degree (%DD) = 95 and Mv = 3.37 x 10^5 Dalton) was provided by the Seafresh Chitosan (Lab) Company Limited, Thailand. Phthalic anhydride, polyethylene glycol monomethyl ether (mPEG, Mn = 5000), and succinic anhydride were purchased from Fluka Chemika, Switzerland. 1-Ethyl-3-(3'-dimethylaminopropyl)carbodiimide, hydrochloride (EDC) and 1-hydroxy-1H-benzotriazole, monohydrate (HOBt) were obtained from TCI, Japan, and *N,N*-dimethylformamide (DMF) and dimethylsulfoxide (DMSO) were supplied by LabScan, Ireland. All proteins (albumin from chicken egg white (OVA), albumin from bovine serum (BSA), α-lactalbumin from bovine milk, catalase from bovine liver, and lysozyme from chicken egg white) were purchased from Sigma-aldrich, Germany. All chemicals were used without further purification.

Preparation of chitosan nanospheres. mPEG grafted phthaloylchitosan was prepared as reported previously [14–15]. Briefly, phthaloylchitosan (0.3 g) was reacted with mPEG terminated with carboxylic acid (mPEG-COOH; 2.45 g, 0.4 mmol equivalent to phthaloylchitosan) in 30 ml of DMF. A specific amount of HOBt (0.22 g, 1.2 mmol equivalent to mPEG-COOH) with EDC (0.28 g, 1.2 mmol equivalent to mPEG-COOH) were added and the mixture was stirred at room temperature overnight. The solution was dialyzed using dialysis tubing cellulose membrane (12400 molecular weight cut off) against water. Colloidal solution of

mPEG grafted phthaloylchitosan was observed. White powder was collected by washing with water, centrifugation, and drying in vacuum (scheme 6.1).

mPEG grafted phthaloylchitosan; FT-IR (KBr, cm^{-1}): 3469 (OH), 2990 (C-H stretching), 1777 and 1712 (C=O anhydride), and 721 (aromatic ring): ^1H NMR (δ, ppm): 2.1 (OCH_3), 2.4 (CH_2 in succinic anhydride), 3.2–5.0 (H2–H6 of GluN unit in chitosan), 5.5 (H1 of GluN unit of chitosan), and 7.4–8.3 (C_6H_5).

Preparation of protein incorporated chitosan nanospheres. Chitosan nanospheres were dissolved in DMSO to have concentration of 4 mg/ml. A 200 µl of polymer solution was pipetted into 200 µl protein solution (0.5, 1, 2, and 4 mg/ml in 0.15 M NaCl, pH 6.5). The colloidal solution was centrifuged with 14500 rpm, 10 min at room temperature and washed with 400 µl mQ 2 times. The obtained protein-incorporated nanospheres were suspended in mQ of 0.2 ml. Protein incorporation was determined by Lowry's method. The suspension of nanosphere was added to DMSO with ratio of 9:1 (DMSO:H_2O) and protein content was determined by UV spectrophotometer (Model 680 Microplate Reader, Bio-Rad Laboratories (UK) Ltd) at λ=750 nm. Chitosan nanospheres were not detected by the Lowry's method. The incorporation efficiency of protein into the nanospheres was calculated as (amount of incorporated protein / initial protein loading) x 100.

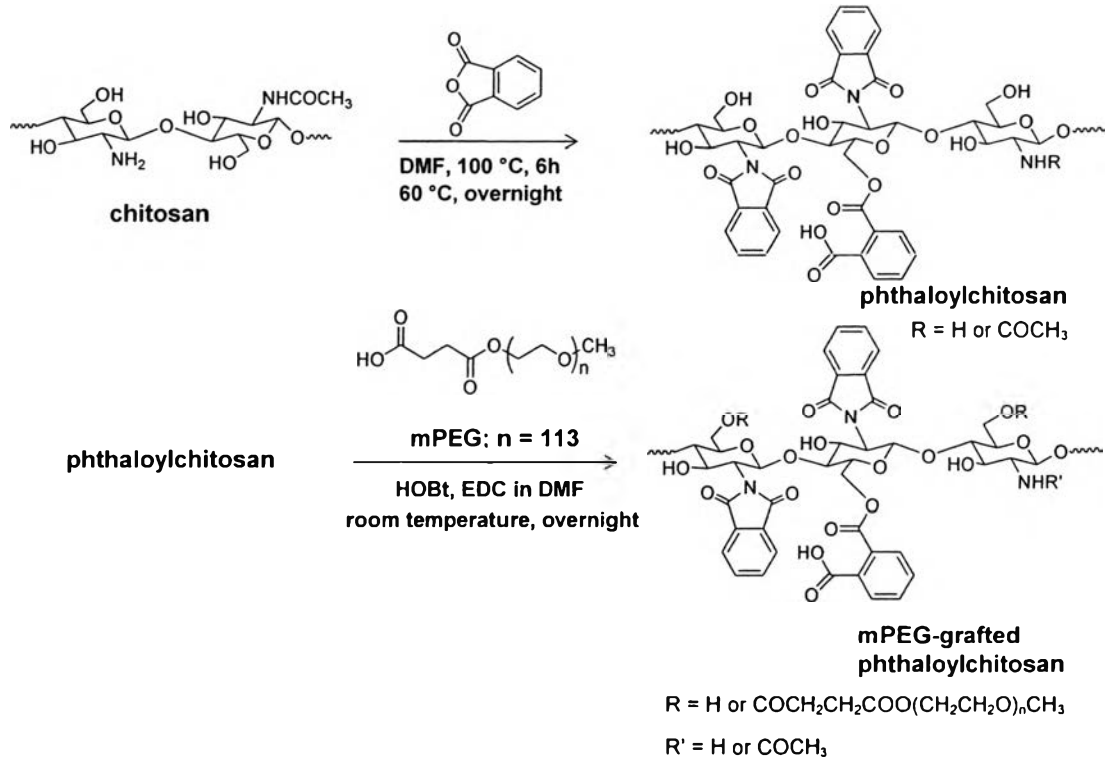
Morphology of protein incorporated nanospheres. A suspension of protein-incorporated nanospheres was dropped on carbon-coated copper grid (300 meshes). Excess water was removed in desiccator at room temperature. The dried grid containing the nanospheres was visualized using an H-7650 transmission electron microscope (Hitachi High-Technology Corporation, Japan).

Zeta potential measurements. Suspension of protein-incorporated nanospheres containing 4 mg/ml protein loading was diluted with 10 % (v/v) and measured by using Zetasizer Nano ZS (Malvern Instruments, UK) at 25 °C.

6.4 Results and Discussion

Chitosan nanospheres. We succeeded in preparing amphiphilic chitosan by grafting phthalic anhydride and mPEG on chitosan chain in homogeneous DMF solution. During dialysis in water, the solution gradually increased turbidity. This implied self-assembled formation of mPEG-grafted phthaloylchitosan. TEM

micrograph confirmed spherical structure of mPEG-grafted phthaloylchitosan with diameter about 200 nm [14–15]. This compound was called as chitosan nanospheres. Qualitative and quantitative analysis were carried out by using FT-IR, and ^{1}H NMR. FT-IR spectrum of phthaloylchitosan shown not only N-phthaloylation but also O-phthaloylation which could be observed at 2640 cm^{-1} (free carboxyl band) as well as at 1261–1287 cm^{-1} (ester peak). Kurita *et al.* reported that the ester peak and free carboxyl band are useful to confirm partial O-phthalolylation [20] (Scheme 6.1). ^{1}H NMR was used to determine phthaloylation degree and grafting degree of mPEG on chitosan which were 48% and 18%, respectively. Our chitosan nanospheres represented significantly negative charge on surface (-54 mV). This might be due to the carboxyl group obtained from O-phthaloylation performed as anionic shell. Gref *et al.* reported PLA nanospheres which had surface charge around -55 mV because the ionization of PLA carboxylic end groups at the particle surface in the presence of water [21].



Scheme 6.1 Preparation steps of chitosan nanospheres

Protein-incorporated nanospheres. As chitosan nanospheres are soluble in DMF or DMSO and self-assemble to form nanosphere in water, chitosan nanospheres were dissolved in DMSO and pipette into protein solution. During reformation of nanospheres, protein may be entrapped with core or adsorbed on the surface of nanospheres. The possibilities of interaction between protein and nanospheres will be mentioned later.

The first model protein was lysozyme. Although lysozyme is well-known as hydrolysis enzyme for chitosan, it rarely effects to chitosan with deacetylation degree higher than 94 [22]. Vårum *et al.* reported that susceptibility degradation was decreased as deacetyation degree increased due to decreasing of N-acetylglucosamine sequence was attributable to crucial recognition by lysozyme [23]. A simple and effective method to determine protein incorporation is Lowry's method. This process was achieved by staining incorporateded protein with Folin Phenol reagent [24]. The result showed that lysozyme was successful to incorporate with chitosan nanospheres. The amount of incorporated lysozyme was increased as initial loading increased (Figure 6.1). Other proteins with difference in molecular weight and isoelectric point (pI) were investigated (Table 6.1).

Table 6.1 Model protein for incorporating with chitosan nanospheres

Protein	Molecular weight x 10^3 (g/mol)	pI	Hydropathy index[a]
Catalase	232	5.5	-1.19
BSA	69	4.9	5.13
OVA	45	4.6	-2.62
Lysozyme	14.5	11.0	-9.28
α-lactalbumin	14.0	5.0	3.63

[a] by calculating using MPEx 3.0 [29]

Catalase, OVA, α-lactalbumin, and BSA were incorporated with nanospheres same procedure as lysozyme. Figure 6.1 shows the success of proteins incorporation with chitosan nanospheres. The amount of incorporated protein was increased as initial protein loading increased.

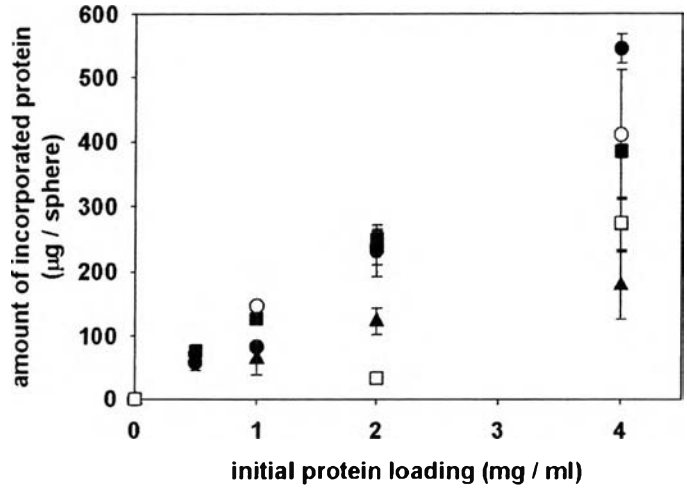
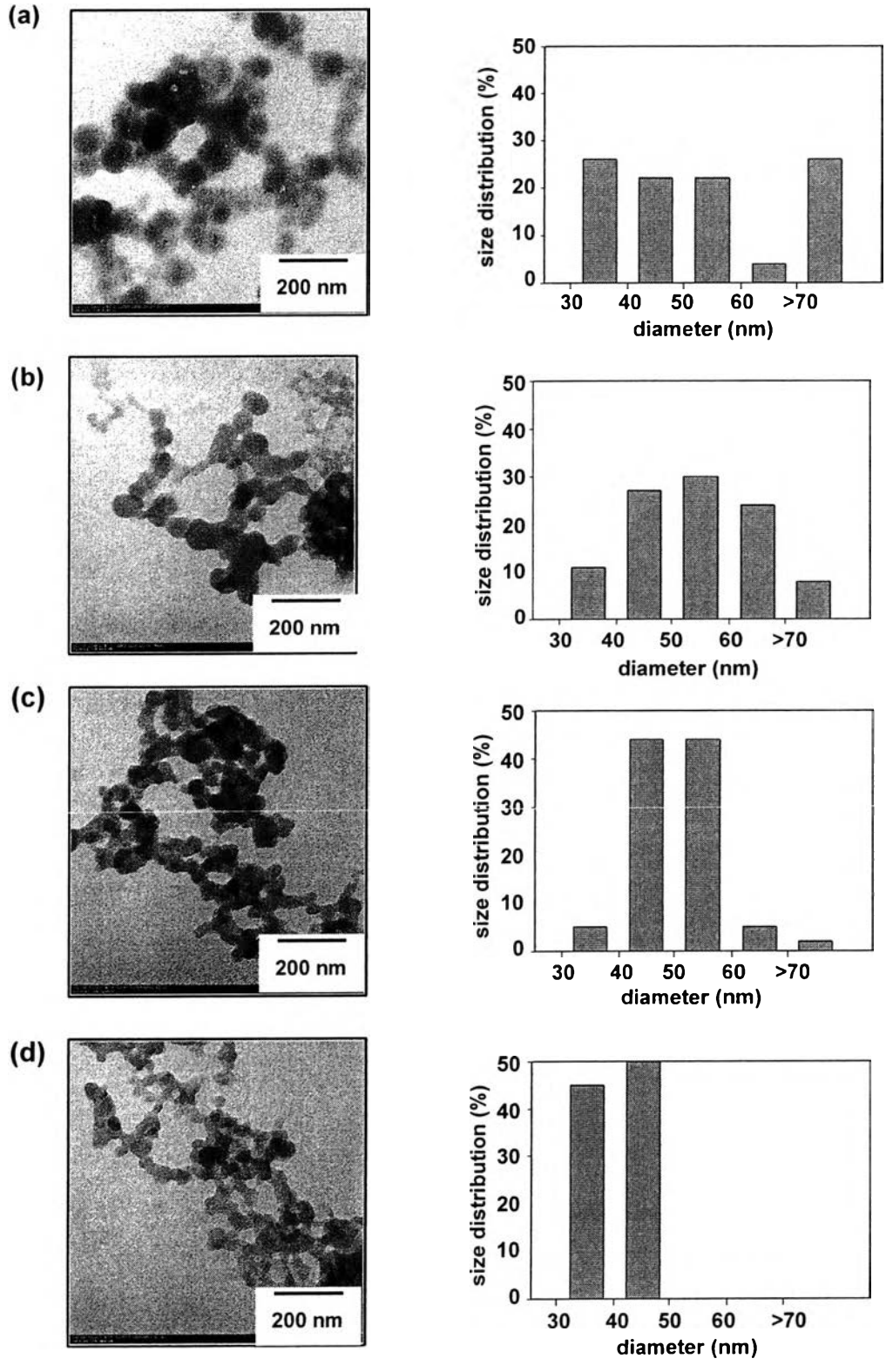


Figure 6.1 Effect of initial protein loading: (●) lysozyme, (○) catalase, (■) OVA, (□) α-lactalbumin, and (Δ) BSA to amount of incorporated protein with nanospheres.

Particle size. The reformation of nanospheres was done under precipitation process. Spherical structure of nanospheres was remained, although aggregation of nanospheres could be observed as shown in Figure 6.2(a). An attempt to measure diameter of nanospheres was done by measuring individual diameter from TEM micrographs. The diameter of nanospheres was reported as size distribution. Mean of nanosphere size was ~46 nm (Figure 6.2(a)). The particle size above 70 nm refers to an aggregation of particles. It should be noted that the difference in nanosphere size from dialysis method might be due to the effect of polymer concentration. Liu *et al.* reported an increasing in size of PLA spheres when PLA concentration increased [25].

Changing in particle size observed by TEM also reflected the effect of protein incorporation. Figure 6.2(b–f) shows that the aggregated nanospheres still retained their spherical shape. Size distribution of all proteins tended to increase in size (mean diameter of incorporated lysozyme was ~53 nm, α-lactalbumin was ~51 nm, BSA

was ~61 nm, and ~52 nm) except OVA-incorporated nanospheres, which provided mean diameter about 41 nm.



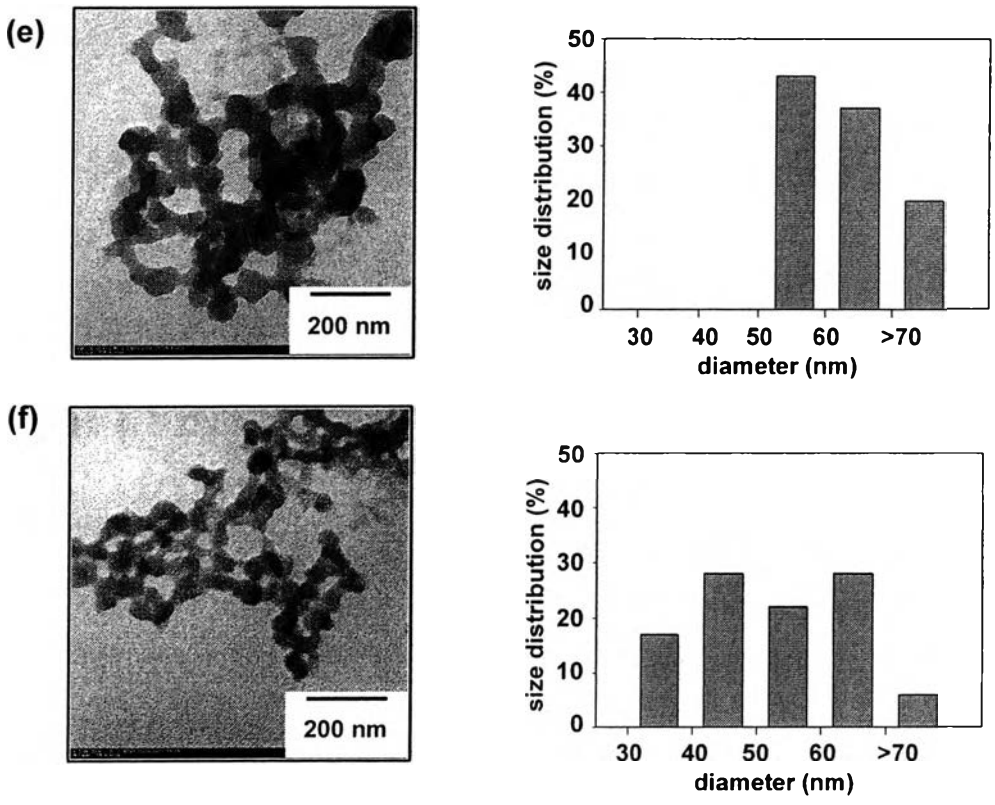


Figure 6.2 TEM micrographs and size distribution of (a) reformed nanospheres and (b)–(f) protein-incorporated nanospheres containing 4 mg/ml protein loading; (b) lysozyme, (c) α-lactalbumin, (d) OVA, (e) BSA, and (f) catalase.

Possibilities interaction between incorporated protein and nanospheres. In most nanoparticles/nanospheres delivery systems, the drug carrying capacity is defined as incorporation or encapsulation efficiency. Up to this point, all of proteins have been proved the incorporation with chitosan nanospheres. Here, we come to the point what driving force is related to protein incorporation. Ability of nanospheres to incorporate with protein was presented as incorporation efficiency.

The possible interactions between protein and nanospheres are electrostatic interaction, van der Waals forces, hydrogen bond, and hydrophobic effect. In present work, all protein solutions were in NaCl at pH 6.5. Concerning pI of proteins (Table 6.1), catalase, BSA, OVA, and α-lactalbumin possessed negatively charged maclomolecules. The electrostatic interaction seemed to lie in the pI of protein and particles. Rezwan *et al.* presented correlation between amount of adsorbed protein on

oxide particles and zeta potential of particle surfaces by subdividing the plot into four quadrants according to attractive/repulsive force from zeta potential of particles. In order to investigate electrostatic interaction, zeta potential was the key factor to consider [26]. Zeta potential of reformed nanospheres was -30 mV. Change of zeta potential after protein incorporation was detected and plotted following Rezwan [26].

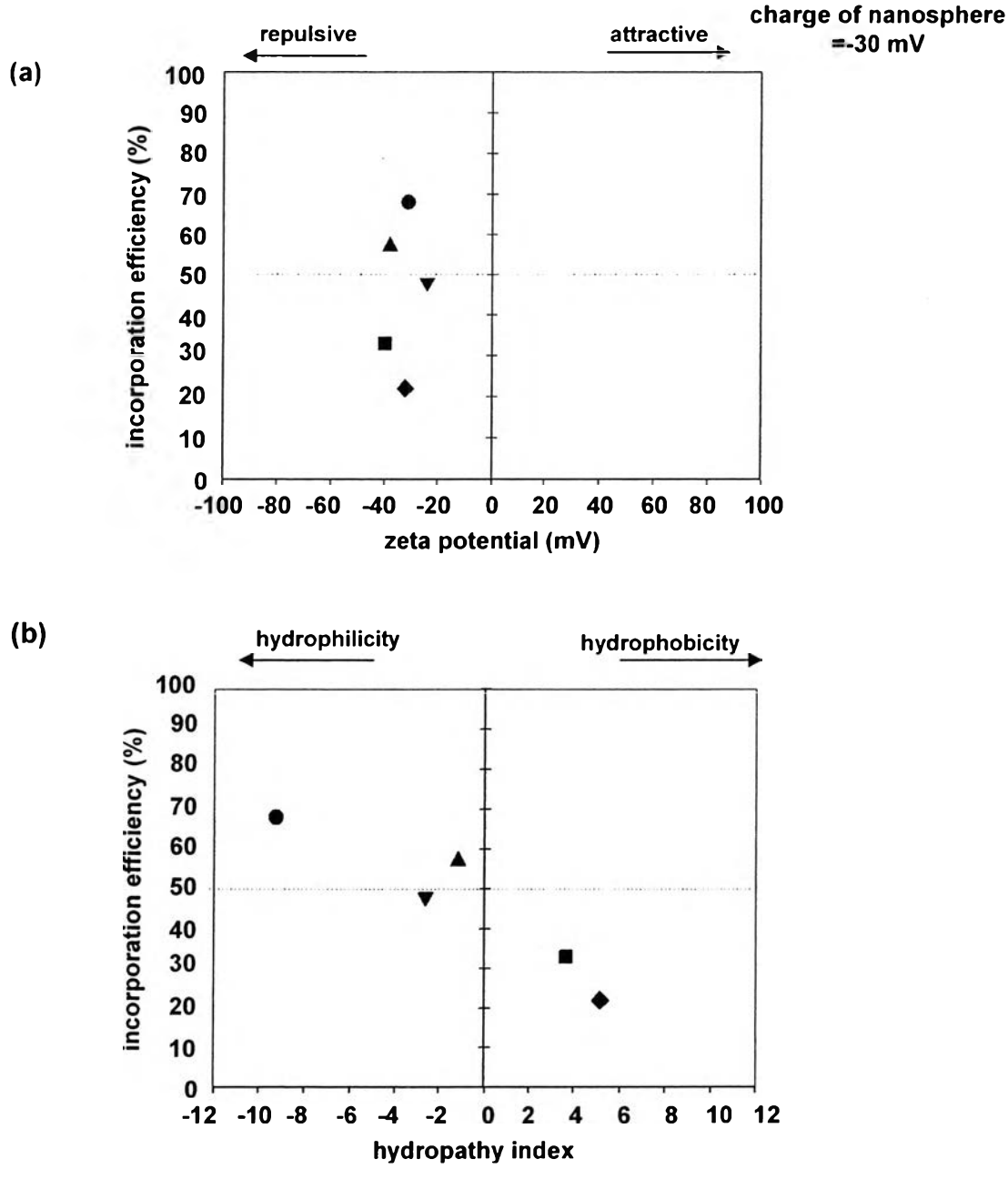


Figure 6.3 Correlation between incorporation efficiency and (a) zeta potential and (b) hydropathy index of protein; (●) lysozyme, (▲) catalase, (▼) OVA, (■) α-lactalbumin, and (♦) BSA.

Figure 6.3(a) shows correlation between incorporation efficiency and zeta potential of protein-loaded nanospheres. The plotted was subdivided into four quadrants I–IV. Quadrant I and III was effected by repulsive of liked charge and quadrant II and IV was effected by attractive of opposite charge. The data points of OVA, α-lactalbumin, and BSA are in the quadrant III (repulsive zeta potential, low incorporation efficiency, < 50 %). The result showed that the electrostatic interaction plays an important role in the incorporation of OVA, α-lactalbumin, and BSA with nanospheres. However, electrostatic interaction could not explain for catalase and lysozyme.

The other possibility to consider is hydrophobic effect. The distribution of amino acid residues between the surface of a protein in contact with aqueous environment and its interior where the residues are withdrawn from water to different extents is commonly analyzed in terms of the hydrophobicity concept [27]. In this work, hydropathy index was parameter to identify hydrophobic of proteins. Hydropathy index is a scale combining hydrophobicity and hydrophilicity of R groups; it can be used to measure the tendency of an amino acid to seek an aqueous environment (- values) or a hydrophobic environment (+ values) [28]. White and Wimley designed the calculation method of hydropathy index based on principles of membrane protein stability [29]. The correlation between incorporation efficiency with hydropathy index was plotted (Figure 6.3(b)). The data points of lysozyme and catalase are in the quadrant I (hydrophilicity, high incorporation efficiency, > 50 %). The self-assembly of mPEG-grafted phthaloylchitosan is formed in water due to the hydrophobic phthalimido groups gathered in the core and the hydrophilic mPEG chains interacting with water molecules outside the core [15]. This supports the incorporation of lysozyme and catalase with hydrophilicity effect of protein and corona of nanosphere. This force is called as van der Waals attraction.

6.5 Conclusions

Succeeding in grafting mPEG on phthaloylchitosan provided chitosan nanospheres. Reformation of chitosan nanospheres via precipitation method gave nanosphere size of 40–50 nm and surface of -30 mV. This article shows success of

incorporation of protein with nanospheres. Electrostatic interaction plays an important role in the incorporating of OVA, α-lactalbumin, and BSA. Beside catalase and lysozyme are incorporate with nanospheres under van der Waals attraction due to hydrophilic property of R residues and nanosphere surface.

6.6 Acknowledgments

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