CHAPTER IV
AMPHIPHILIC CHITOSAN NANOSPHERES: FACTORS TO CONTROL NANOSPHERE FORMATION AND STUDIES ON LIDOCAINE MODEL DRUG INCORPORATION

4.1 Abstract

Chitosan grafted with hydrophobic and hydrophilic groups initiates the formation of amphiphilic chitosan nanospheres. The molecular weight of mPEG plays an important role to control the particle size. As compared to mPEG 2000, which gives a bimodal nanosphere (~200, and ~300 nm), mPEG 5000 initiates a monodispersed nanosphere with the smaller size (150 nm). In aqueous solution, the nanosphere surface is negatively charged resulting in a well dispersion in neutral to high pH but a significant precipitation in low pH. A model drug incorporation using lidocaine is successful when amphiphilic chitosan nanospheres were dissolved in good solvent followed by allowing mixing with drug solution before dialysis. The particle size of the drug incorporated chitosan is significantly increased, that is, from 100–150 nm to approximately 400–500 nm when the amount of incorporated lidocaine was about 0.68 mg per mg of lidocaine-loaded nanosphere.

Keywords: pH responsive; nanospheres; chitosan

4.2 Introduction

Amphiphilic copolymers consisting of hydrophilic and hydrophobic segments are known to form micelles with the hydrophobic inner core and hydrophilic outer shell. The basic driving force for micelle formation is the decrease in surface tension of which the inter-corona chain repulsion as well as the chain stretching of the hydrophobic cores play an important role in minimizing the interfacial energy [1–3]. Zhang *et al.* [4] reported the preparation of amphiphilic block copolymers based on methoxy poly(ethylene glycol) (mPEG) and poly(capolactone-*b*-D,L-lactide) (P(CL-DLLA)). The size of the hydrodynamic diameter was found to be based on hydrophobic content in copolymers. Hydrophilic outer shell also plays an important role in controlling the size. For example, chitosan grafted with amphiphilic

polycaprolacton-*b*-mPEG (PCL-*b*-MPEG) showed the self-aggregation to form particles and the particle size decreased as grafting chain of PCL-*b*-MPEG increased [5]. In addition, the size of the particles is found to increase with the length of the grafted chain branching.

Previously, our group reported the preparation of the amphiphilic chitosan nanospheres by simply grafting phthalic anhydride as a hydrophobic group and polyethylene glycol as a hydrophilic chain to obtain core–corona structured chitosan. The nanospheres were formed depending on the types of the solvent whereas their self-assemble phenomena are confirmed under the polar solvents [6,7]. Although we clarified that our amphiphilic chitosan nanospheres were in the sizes of 80 nm to 500 nm, the factors involved in controlling the size and shape are yet to be done. Herein, we systematically prepared chitosan nanospheres by varying the degree of deacetylation of chitosan and the molecular weight of PEG to investigate how these factors governed the nanospheres in size and shape. The work also extends to a study of hydrophilic drug incorporation using lidocaine as a model drug to understand how hydrophobic core and hydrophilic corona play their roles in drug incorporation.

4.3 Experimental Section

Materials

Chitosan with deacetylation percent (%DD) of 80, 85, 90, and 95 with the corresponding viscosity average molecular weights (Mv) of 8.70×10^5, 5.75×10^5, 5.78×10^5, and 3.37×10^5 Dalton, respectively, were the gifts from Seafresh Chitosan (Lab) Company Limited, Thailand. Phthalic anhydride, polyethylene glycol monomethyl ether (mPEG, Mn = 2000 and 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 bought from TCI, Japan. *N,N*-dimethylformamide (DMF), dimethylsulfoxide (DMSO) were purchased from LabScan, Ireland. Lidocaine was purchased from Aldrich Chemical Company, Inc., USA. All chemicals were used without further purification.

Instruments and equipments

Qualitative FT-IR spectra were recorded by a Thermo Nicolet Nexus 670 (Madison, USA) with 32 scans at a resolution of 4 cm^{-1}. A frequency range of 4000–400 cm^{-1} was observed using a deuterated triglycinesulfate detector with a specific detectivity, D^*, of 1×10^9 cm $Hz^{1/2}$ w^{-1}. Phthaloylation degree and mPEGylation degree were determined by using a Varian Mercury-400BB proton nuclear magnetic resonance (^1H NMR) spectrometer at room temperature in DMF, d_7. Drug incorporation content was determined by using a JEOL JNM-GSX-400 ^1H NMR spectrometer at room temperature in DMSO, d_6. The morphologies were observed by using a JSM-6700F (JEOL, Japan) and an H-7650 TEM (Hitachi High-Technology Corporation, Japan) at an operating voltage of 100 kV. The size and zeta potential of the dispersed particles in water and buffer solution were determined at 25°C by using a Malvern Zetasizer Nano ZS.

Phthaloylchitosan, PhCS. PhCS was prepared as reported previously [6,7]. In brief, chitosan (%DD = 85, 3.0 g) was reacted with phthalic anhydride (13.28 g, 5 mol equivalent to pyranose rings) in DMF (30 ml) at 100 °C under vacuum for 6 h. The temperature was reduced to 60 °C under nitrogen atmosphere and left overnight. The insoluble fraction was filtered and the solution was reprecipitated in cold water. The precipitates were collected, washed with ethanol three times, and dried in vacuum to give pale yellowish powder of **PhCS 85** (Scheme 4.1). Chitosan with %DD = 80, 90, and 95 were prepared in the same procedures to obtain **PhCS 80**, **PhCS 90**, and **PhCS 95**, respectively.

FT-IR (KBr, cm^{-1}): 3469 (OH), 2640 (free carboxyl), 1777 and 1712 (C=O anhydride), 1261–1287 (C-O-C in ester), and 721 (aromatic ring)

^1H NMR (δ, ppm): 3.2–5.0 (H2–H6 of GluN unit in chitosan), 2.1 (OCH_3), 5.5 (H1 of GluN unit of chitosan), and 7.4–8.3 (C_6H_5).

Poly(ethylene glycol) methyl ether terminated with carboxyl group (mPEG-COOH; Mn of 2000 and 5000, mPEG 2k and mPEG 5k). mPEG (Mn = 2000, 3 g) was reacted with succinic anhydride (0.15 g, 1 mole equivalent to mPEG) in DMF (10 ml) with a catalytic amount of pyridine at 60 °C overnight. The solution

obtained was concentrated and precipitated in diethyl ether before drying in vacuo to obtain **mPEG 2k**. Similary, **mPEG 5k** was prepared.

FT-IR (ZnSe, cm^{-1}): 3494 (OH), 2871 (C-H stretching), 1736 (C=O), and 1113 (C-O-C).

mPEG grafted PhCS. PhCS 85 (1.0 g) was stirred with **mPEG 2k** (3.04 g, 0.4 moles equivalent to **PhCS 85**) in DMF solution (20 ml). HOBt (0.59 g, 3 moles equivalent to **mPEG 2k**) was added and stirred at room temperature until the solution became clear. EDC (0.89 g, 3 moles equivalent to **mPEG 2k**) was added and stirred overnight. The mixture was dialyzed using a dialysis tubing cellulose membrane (12400 molecular weight cut off) against distilled water to obtain the product **CSN 85-mPEG 2k** in white colloidal solution form. The product was collected in solid form by centrifuging, washing with ethanol, and drying in vacuo. Similarly, **CSN 80-mPEG 2k**, **CSN90-mPEG 2k**, and **CSN95-mPEG 2k**, including **CSN 80-mPEG 5k**, **CSN 85-mPEG 5k**, **CSN90-mPEG 5k**, and **CSN95-mPEG 5k**, were prepared.

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): 3.2–5.0 (H2–H6 of GluN unit in chitosan), 2.1 (OCH_3), 5.5 (H1 of GluN unit of chitosan), and 7.4–8.3 (C_6H_5).

Amphiphilic chitosan nanospheres observation. CSN-mPEG were dispersed in deionized water (0.2 mg/ml) followed by centrifuging and collecting the precipitated particles. One ml of citrate buffer solution (pH=3 and 5) was added before observing nanosphere size and charges. The buffers, tris buffer: pH=7, borate buffer: pH=10 and KCl/ NaOH: pH=12 were also used. The concentration of all buffers is 0.05 M.

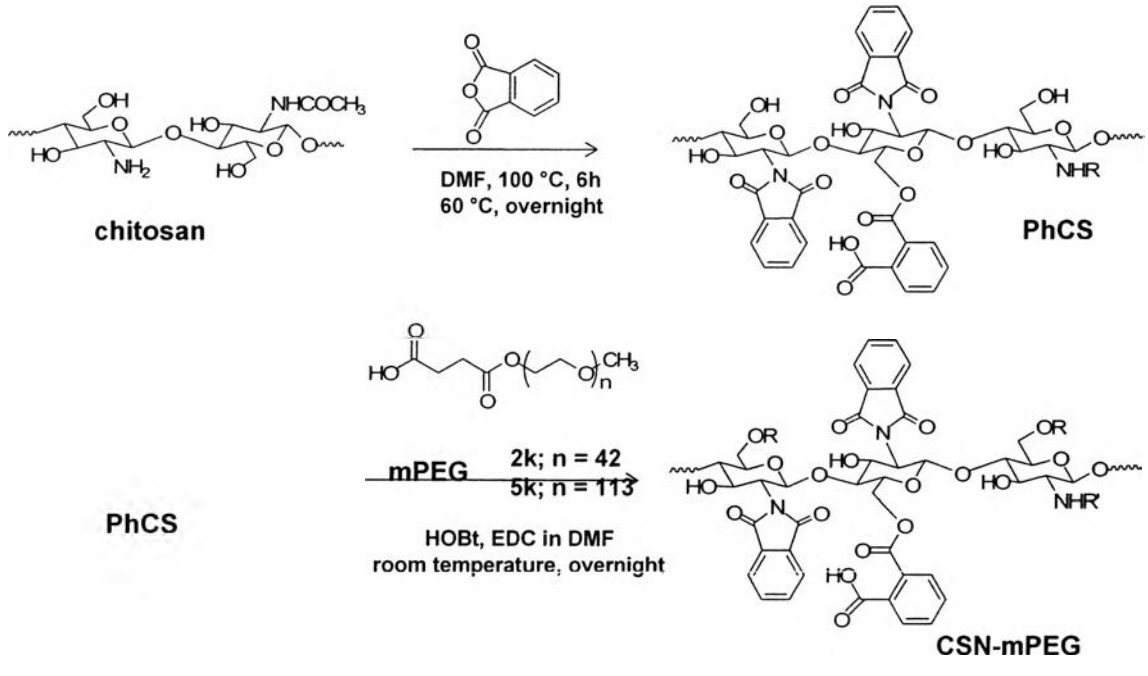
Treatment of CSN-mPEG in aqueous by urea. A colloidal solution of **CSN 95-mPEG 5k** (0.4 mg/ml) was added to urea (10 ml of 2M) solution and stirred for 1 hr. The colloidal solution was dialyzed using a spectra/pore membrane (50000 molecular weight cut off) against distilled water. The dialysis was repeated for 3 times with fresh distilled water.

Model Drug Incorporation. The incorporation of a model drug, lidocaine, was carried out in homogeneous system [8]. In brief, the **CSN-mPEG** (20 mg) were

dissolved in DMSO (3 ml) followed by adding lidocaine into the solution (with an amount of 10, 20, 50, 100, and 200 wt% of **CSN-mPEG**). The mixture was stirred at room temperature for 6 hr before using Spectra/Por® cellulose membrane (10000 molecular weight cut off) to dialyze against distilled water (2 L). The suspension was lyophilized to obtain white powder.

4.4 Results and Discussion

In the past, we succeeded in preparing chitosan nanospheres under core–corona structure by conjugating chitosan with phthaloyl group as hydrophobic core and PEG chain as hydrophilic corona (Scheme 4.1).



Scheme 4.1 Preparation of **CSN-mPEG**

The detailed analysis has proven that the core–corona nanospheres were induced by polar solvents [7]. Initially amino group on chitosan unit was substituted with phthalimido group before grafting with carboxyl terminated mPEG using EDC as conjugating agent. It is important to note that all reactions were carried out in homogeneous DMF or DMSO solution. There, the nanosphere formation was accomplished after the homogeneous solution was dialyzed against water. This could

be confirmed by naked eyes when the solution in dialysis tube gradually became turbid. The further analysis by TEM showed that the colloidal solution consists of nanospherical particles with the size of ~85 nm [6,7]. This might be due to the self-assembly structure which hydrophobic parts form core structure whereas the hydrophilic corona takes part in interacting with water.

Effect of %DD on degree of phthaloylation and degree of mPEGylation. In order to clarify the effect of %DD in forming nanosphere, four different chitosans i.e. 80, 85, 90, and 95 (%DD) were used. As %DD involves in the very first step of phthalimido substitution which initiates the hydrophobicity to chitosan, here, a quantitative analysis by using ^{1}H NMR was carried out. The substitution degree was evaluated by using the integral ratio of aromatic proton to pyranose protons (C2–C6). Table 4.1 shows the phthaloylation degree of chitosan with different %DD. All **PhCS** show a similar level of substitution degree which is about 48%. The phthaloylation degree suggests us that the chemoselective protection of amino group of chitosan by reacting with phthalic anhydride tends to saturated at a certain level. It is important to note that the FT-IR spectrum of **PhCS** not only showed the anhydride peaks at 1777 and 1712 cm^{-1} but also the weak free carboxyl band at 2640 cm^{-1} as well as the ester peak at 1261–1287 cm^{-1}. As the anhydride peak overlaps the phthalimido peak, Kurita *et al.* [9] reported that the ester peak and free carboxyl band are useful to confirm partial O-phthaloylation (Scheme 4.1).

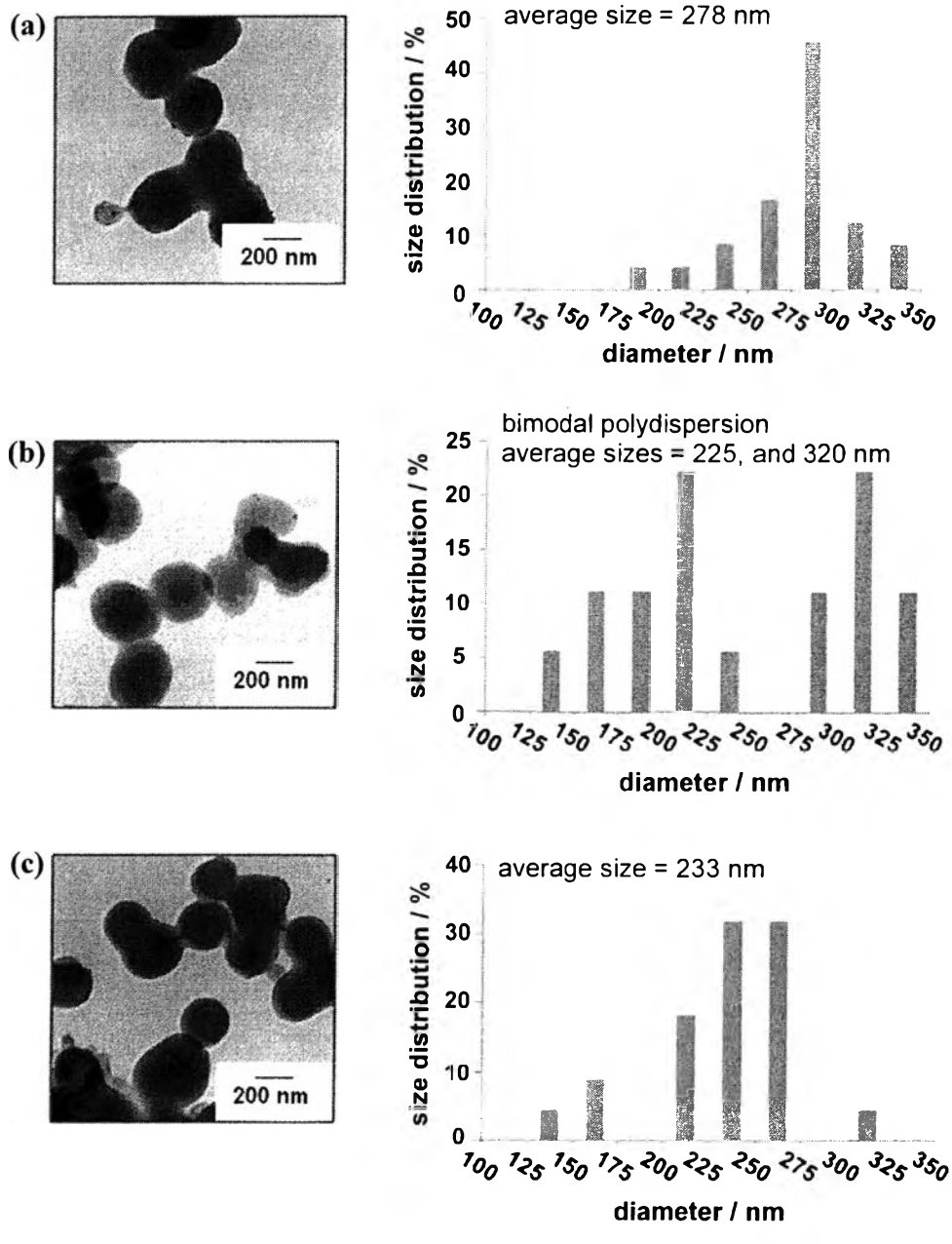
Table 4.1 Phthaloylation, grafting of mPEG on chitosan, and surface charges of **CSN-mPEG**

Sample	Substitution of phthaloylation (%)[a]	Grafting of mPEG (%)[a]		Zeta potential (mV)[b]	
		2k	**5k**	**2k**	**5k**
CSN 80-mPEG	46.6	48.9	7.5	-35.0	-52.3
CSN 85-mPEG	47.7	59.8	7.3	-50.1	-45.8
CSN 90-mPEG	50.5	35.4	17.5	-51.0	-41.3
CSN 95-mPEG	48.1	43.4	18.0	-49.8	-54.0

determined by [a] ^{1}H NMR, [b] Zetasizer Nano ZS

Although the introduction of **mPEG** onto **PhCS** is possible at both the remaining amino (C-2) and hydroxyl groups (C-6), in the past, we found that the substitution of mPEG-COOH at C-6 was dominant as the amount of **mPEG** in the system increased [10]. Here, the dependency of **mPEG** substitution degree to **mPEG** chain length is further investigated by using two different molecular weights of **mPEG**, i.e. 2000 and 5000 Dalton and clarifying the substitution degree by using ^{1}H NMR. As the chemical shifts of methylene proton belonging to **mPEG** and proton at C-2 to C-6 of pyranose ring are overlapping to each other, the integration value of the total methylene protons was subtracted with 6 protons of pyranose ring to calculate the grafting degree of **mPEG** [11]. As shown in Table 4.1, for **mPEG 2k**, the grafting is about 35–60% whereas for **mPEG 5k**, the grafting is only 7–18%. The low grafting degree of **mPEG 5k** reflects the bulkiness of **mPEG** chain length to hindrance the reaction. This is similar to the case reported by Lu *et al.* [5] when polyethylene glycol-co-polycarprolactone was grafted onto chitosan. In addition, the substitution degree of **mPEG** is rather constant even the chitosans with different %DD were used.

Effect of %DD and grafting degree of mPEG to spherical size. It is important to investigate how those substitution degrees of phthalimido group and **mPEG** chain effect to the nanosphere formation and its consequent size and size distribution. Figure 1 shows the chitosan nanospheres obtained from grafting **mPEG 2k** on each **PhCS**.



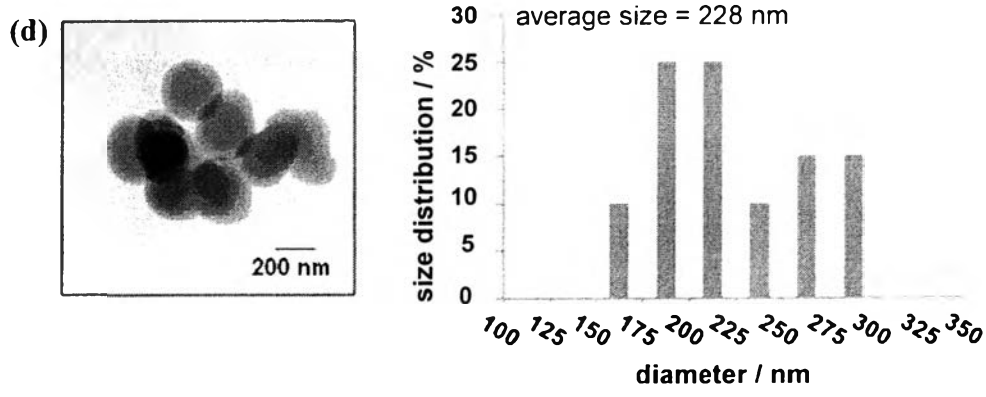
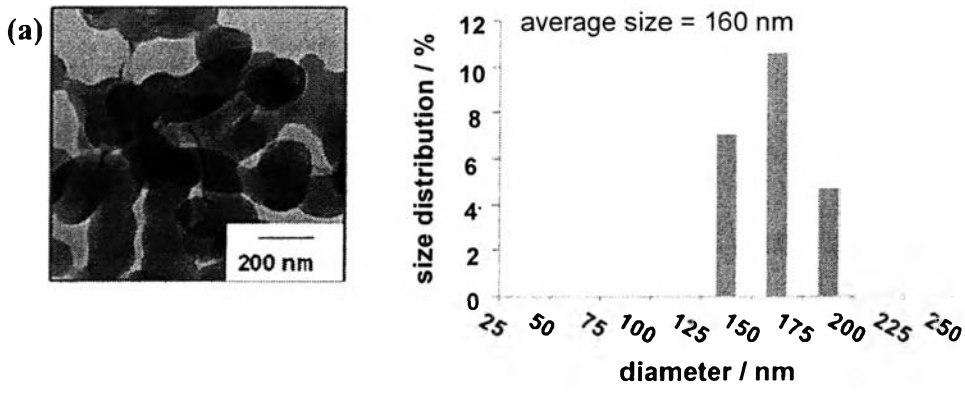


Figure 4.1 Transmission electron micrographs and size distribution of **CSN-mPEG** obtained from **mPEG 2k** grafted on **PhCS** ; (a) **CSN 80-mPEG 2k**, (b) **CSN 85-mPEG 2k**, (c) **CSN 90-mPEG 2k**, and (d) **CSN 95-mPEG 2k**.

The micrographs confirm the spherical morphology with some aggregation. The diameter of each individual nanosphere was carefully measured to evaluate the size distribution. The diameters of nanospheres are in the range of 200–300 nm and they show a decrease tendency in spherical diameter as %DD increased. In the case of **CSN 80-mPEG 2k**, a monodisperse of an average size of 278 nm is observed. For **CSN 95-mPEG 2k**, the average size is about 228 nm. When **mPEG 5k** was grafted to **PhCS** the spherical size of **CSN-mPEG 5k** is dramatically decreased (Figure 2) as compared to the **CSN-mPEG 2k**. It is important to note that, according to the size distribution evaluated in Figures 1 and 2, **mPEG 5k** not only induces nanospheres but also gives a good monodispersion at the level of 150–160 nm.



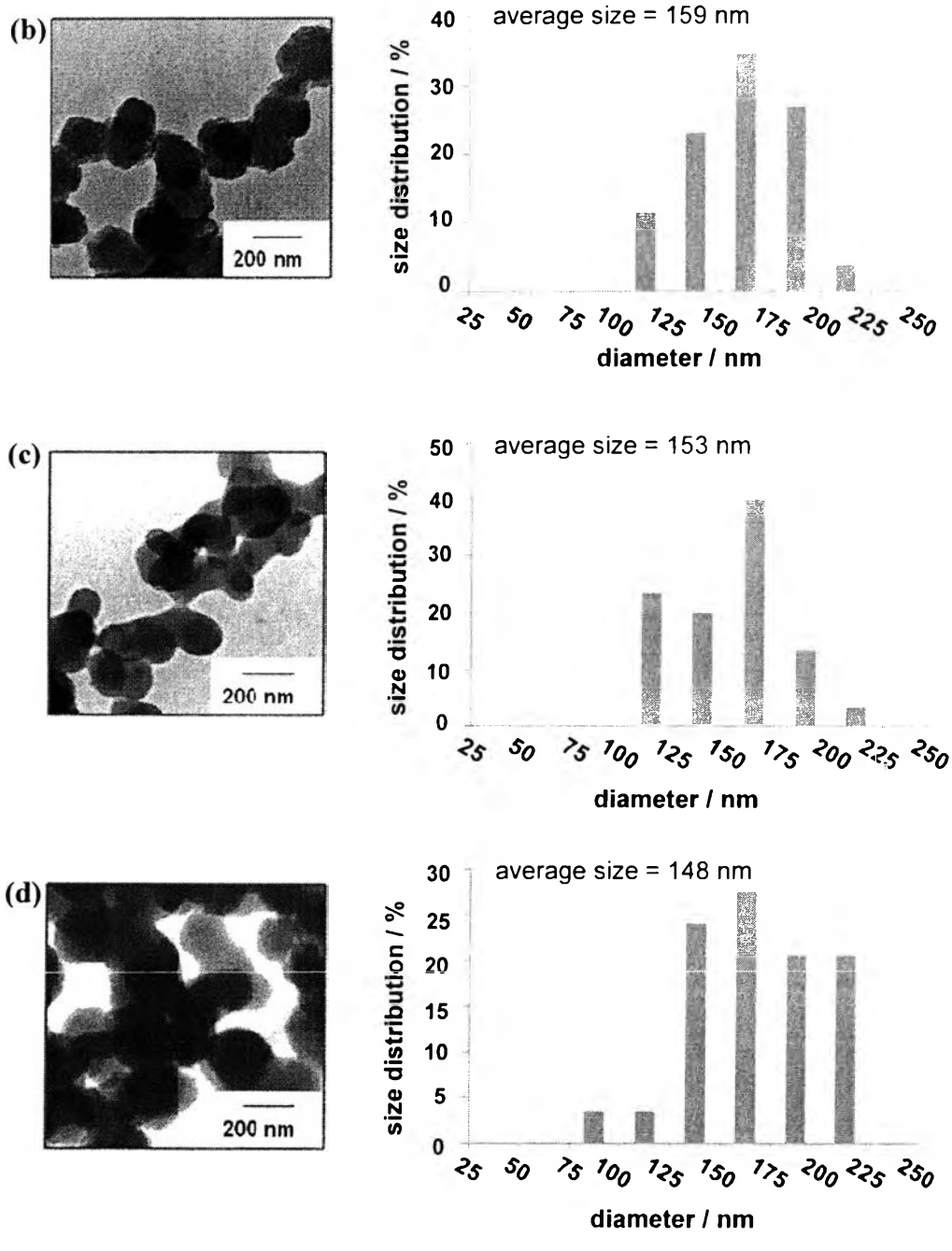


Figure 4.2 Transmission electron micrographs and size distribution of **CSN-mPEG** obtained from **mPEG 5k** grafted on **PhCS**; (a) **CSN 80-mPEG 5k**, (b) **CSN 85-mPEG 5k**, (c) **CSN 90-mPEG 5k**, and (d) **CSN 95-mPEG 5k**.

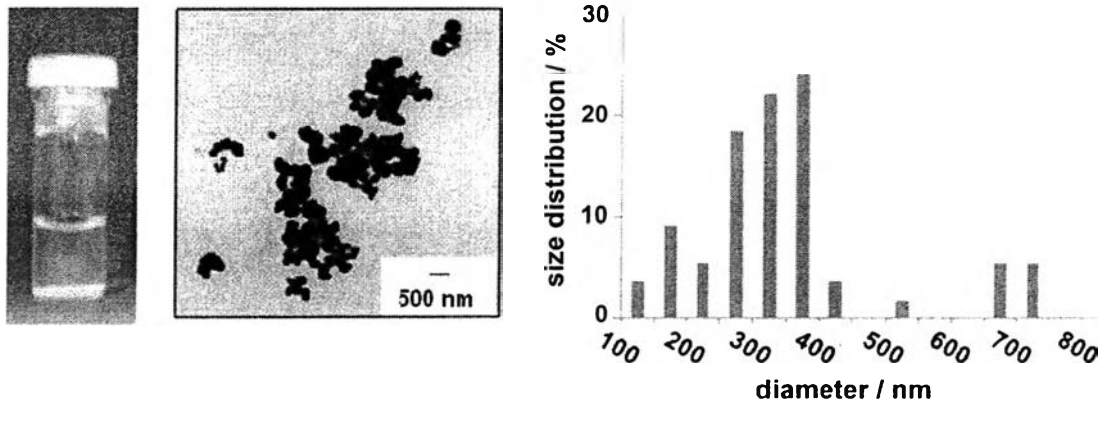
This implies that the molecular weight of **mPEG** plays an important role in controlling the spherical size although the substitution degree was limited only at 7–

20% (Table 4.1). It is important to raise the point that TEM micrographs give the details of nanospheres in solid state. Although the sample preparation for TEM was done in highly diluted condition, as shown in Figures 1 and 2, the nanospheres tended to be aggregated.

The DLS was further applied to obtain the information of the nanospheres in aqueous solution, especially the size. It was found that the nanospheres in the solution state and in the solid state showed the difference in size. TEM images show aggregation of nanospheres even in the dilute condition, accordingly, DLS results present particles size of both individual and aggregate diameters. The **CSN-mPEG 2k** in aqueous solution shows the sizes in the range of 165–423 nm whereas the **CSN-mPEG 5k** shows the smaller sizes in the range of 115–292 nm. Here, it is clear that the molecular weight of **mPEG** was a dominant factor to control nanosphere size.

Surface charge and self-assembly formation nanosphere. Table 4.1 shows another important point. That is the zeta potentials of the nanosphere surfaces obtained from the grafting of **mPEG 2k** and **mPEG 5k** are highly negative. However, the negative charge values seem not to be related to the %DD and the molecular weight of **mPEG**. There, the factors initiated the surface charge was further investigated by dispersing the nanospheres in various pHs. As shown in Figure 3, at low pH (pH = 3), the **CSN 95-mPEG 5k** totally precipitates out from the solution. When the pH is increased (pH = 5–7), the solution becomes colloidal. The turbidity of the colloidal solution gradually decreases when the pH of the solution is further increased (pH > 9).

(a)



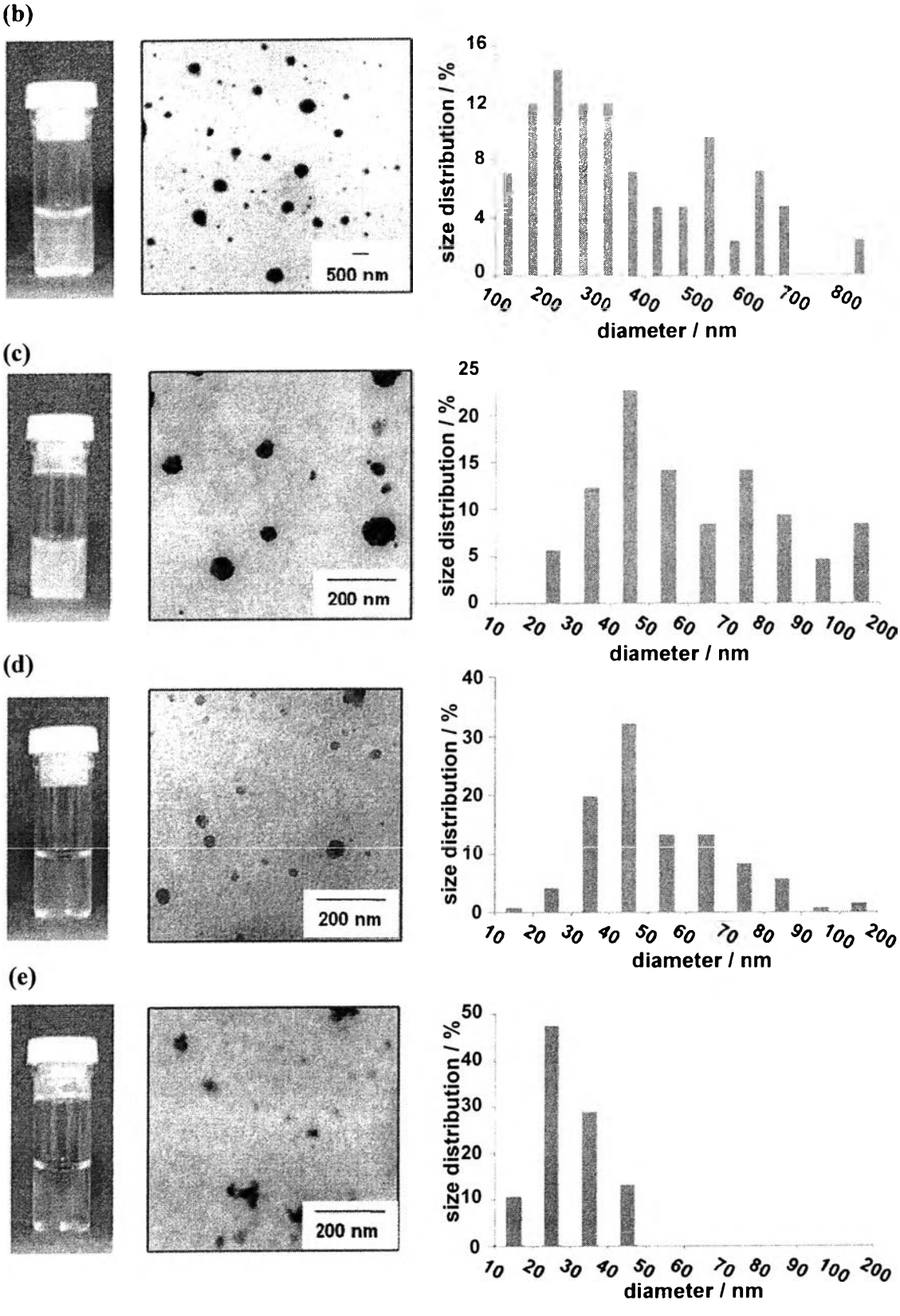


Figure 4.3. Appearances, TEM micrographs, and size distribution of **CSN 95-mPEG 5k** in pH (a) 3, (b) 5, (c) 7, (d) 10, and (e) 12.

To understand the phenomena, the changes in the particle morphology including the spherical size and the size distribution were observed. The size of the nanospheres precipitated in pH 3 is about 300–400 nm (Figure 4.3(a)). For pH 5, a certain amount of the particles is dispersed in the solution and some is precipitated at the bottom (Figure 4.3(b)). The size distribution is bimodal of which the smaller ones are 200–300 nm and the bigger ones are 500 nm. The colloidal solution in pH 7 gives the mean spherical size of 40–50 nm. In the case of pH 10, the size distribution is nearly the same as the nanospheres in pH 7 but with a significantly narrow distribution. This might lead to a decrease in turbidity which could be observed by the naked eyes when the pH was higher. For pH 12, the solution becomes clear, and at that time, the micrograph shows the regular particles of **CSN 95-mPEG 5k** with the sizes of 20–30 nm (Figure 4.3(e)). The results imply an ionic interaction of chitosan which its amphiphilic structure is under the self-assembly system controlled by the pH.

Points related to negative charge and spherical size reduction in basic condition. Chitosan is known as a cationic polysaccharide where the pKa is about 6.5. It is important to note that the **CSN-mPEG** performs anionic property and easily changes its nanospherical size with the pH. For example, as shown in Figure 4.4, the zeta potential of **CSN-mPEG** shows the negative charge starting from pH 3.

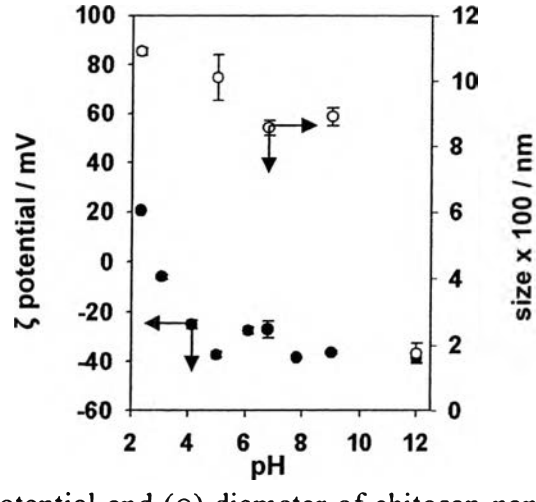
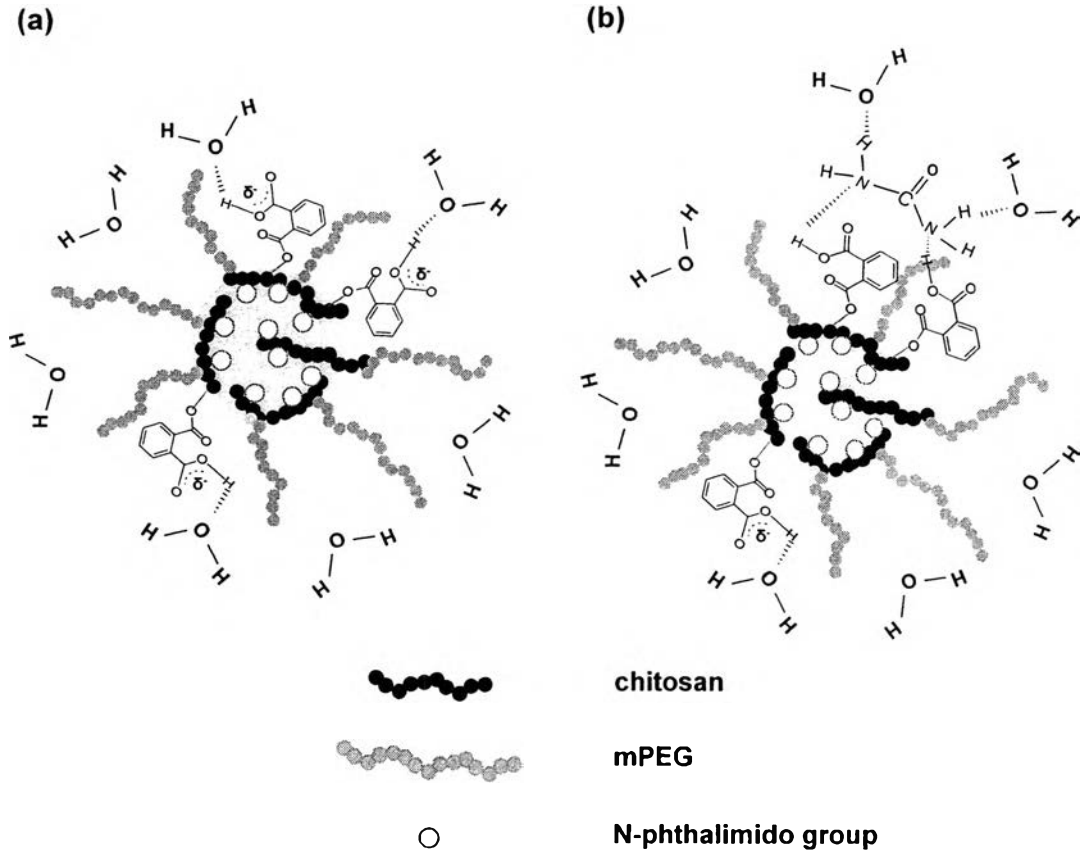


Figure 4.4 (●) Zeta potential and (○) diameter of chitosan nanospheres determined by DLS as a function of pH levels.

In order to trace the particle size changes along with pH and zeta potential in the solution, DLS was applied. The nanosphere size obtained was found to

dramatically decrease while the surface charges become more and more negative along with an increment of pH. It is important to note that nanospheres tend to aggregate easily therefore nanosphere size obtained by DLS is different from diluted sample in TEM. In our case, the fact that the phthaloylation in DMF results in the carboxylic acid group formation (Scheme 4.1), the zeta potential of **PhCS 95** was decreased significantly from 23 mV (chitosan) [12] to 3 mV. We suspected that after **PhCS 95** was conjugated with **mPEG 5k**, the hydrophilicity was enhanced and at that time the nanosphere formation in aqueous base, where the hydrogen bond with water molecules is favored, might allow the carboxylic acid to align on the surface of the nanospheres. As a result, the charge of **CSN 95-mPEG 5k** drastically decreased to about -40 to -50 mV.

To confirm this, **CSN 95-mPEG 5k** was treated in urea solution. As urea may substitute the hydrogen bond between solute and solvent [13,14], we expected to see the shift of negative charge to more positive values due to the decrease of carboxylic acid group existed on the **CSN 95-mPEG 5k** surface. It was founded that zeta potential was drastically increased from -54 mV (without urea treatment) to about -30 mV (with urea dispersion). Scheme 4.2 shows a speculated core-corona structure of **CSN-mPEG** which the carboxylic acid obtained from phthaloylation is on the surface as a part of corona when the hydrogen bond is favorable. By adding urea solution in the system, a certain amount of carboxylic acid is obstructed and as consequence, the zeta potential is increased to more positive values.



Scheme 4.2 Schematic representation of (a) hydrogen bond between nanospheres in aqueous buffer, and (b) hydrogen bond between nanospheres and urea.

Model drug incorporation. Here, lidocaine was used as a model drug to study the incorporation with chitosan nanospheres. As lidocaine is hydrophilic, the incorporation was carried out in DMSO solution. The amphiphilic chitosan nanospheres were completely dissolved in DMSO followed by mixing with lidocaine. The solution obtained was dialyzed in water to reform the nanospheres. Although lidocaine can be qualitatively and quantitatively analyzed by UV technique based on the peak at 262 nm [15,16], the fact that the nanospheres give the broad peaks at 289, 332, and 346 nm to obstruct the precise analysis. The analysis, then, was done by ^{1}H NMR technique. Lidocaine shows the chemical shifts (ppm) at 9.3 (NH, H-c), 7.0 (C_6H_3, H-a), 3.1 (CH_2, H-d), 2.6 (CH_2, H-e), 2.1 (CH_3, H-b), and 1.2 (CH_3, H-f) (Figure 4.5(a)). Figure 4.5(b) shows the spectrum of chitosan nanospheres after lidocaine incorporation. Based on the aromatic proton peaks at 7.4–8.3 ppm of

CSN-mPEG and methyl proton peak at H-b and H-f = 2.1 and 1.2 ppm, respectively of lidocaine, the amount of incorporated lidocaine was calculated.

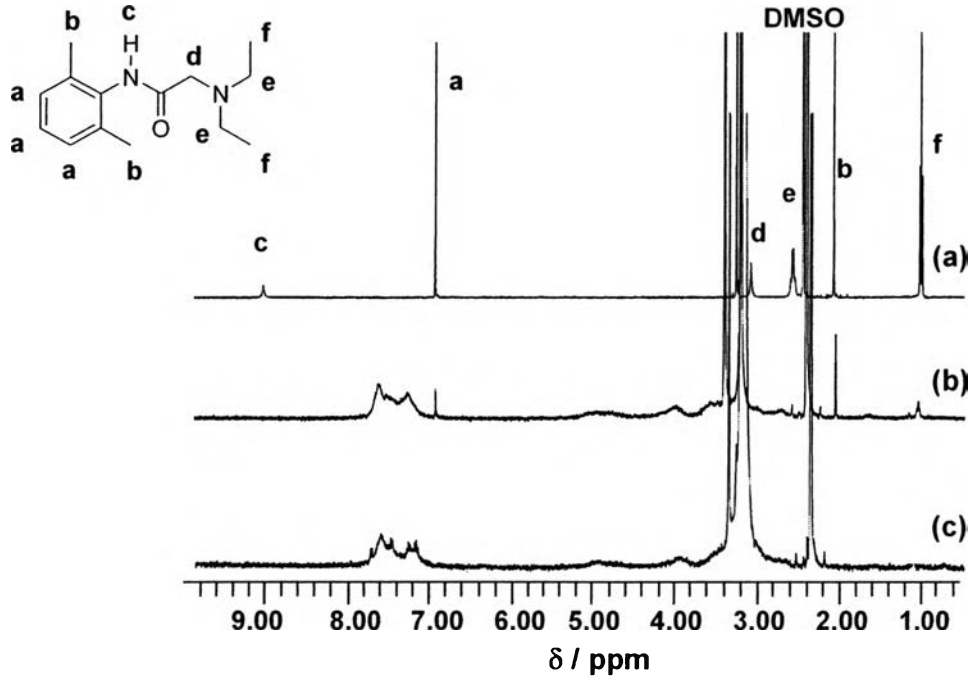


Figure 4.5 ^{1}H NMR spectra of (a) lidocaine, (b) 50 wt% loaded lidocaine in **CSN 85-mPEG 5k**, and (c) **CSN 85-mPEG 5k** (unloaded nanospheres).

Figure 4.6 summarizes the amount of the incorporated lidocaine with different % lidocaine loading amount for all types of **CSN-mPEG**. The incorporation of lidocaine in the **CSN-mPEG** might be related to the hydrophilicity of mPEG chain and lidocaine. The **CSN 85-mPEG 5k** shows the highest amount of lidocaine incorporation. The loading condition with 200 wt% of lidocaine gives the most significant loading amount in all types of **CSN-mPEG**.

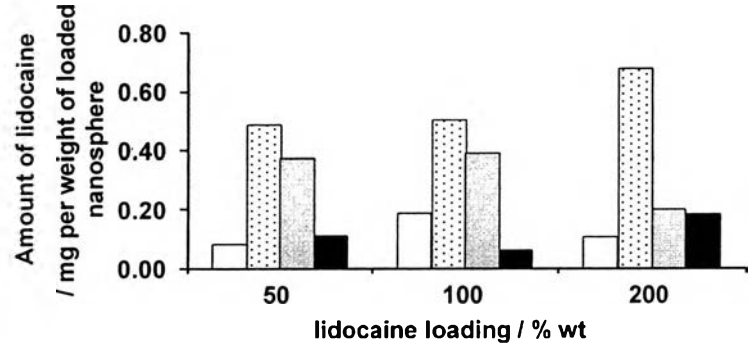
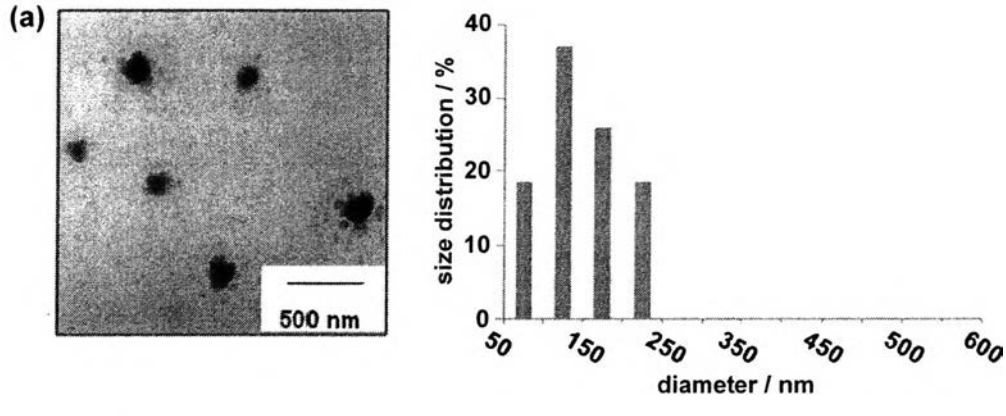


Figure 4.6 Amount of lidocaine incorporated with; (□) **CSN 80-mPEG 5k**, (□) **CSN 85-mPEG 5k**, (□)**CSN 90-mPEG 5k**, and (■)**CSN 95-mPEG 5k**.

The morphology and size distribution of **CSN-mPEG** before and after lidocaine loading was further observed by TEM. Figure 4.7 shows an example of **CSN 85-mPEG 5k** which the size is gradually increased from 100–150 nm before lidocaine incorporation to 150–200 nm and to 250–300 nm after incorporation with 50 and 100% loading, respectively. The size of **CSN 85-mPEG 5k** is significantly increased when the loading amount was 200 %wt and the mean spherical size is 400–450 nm.



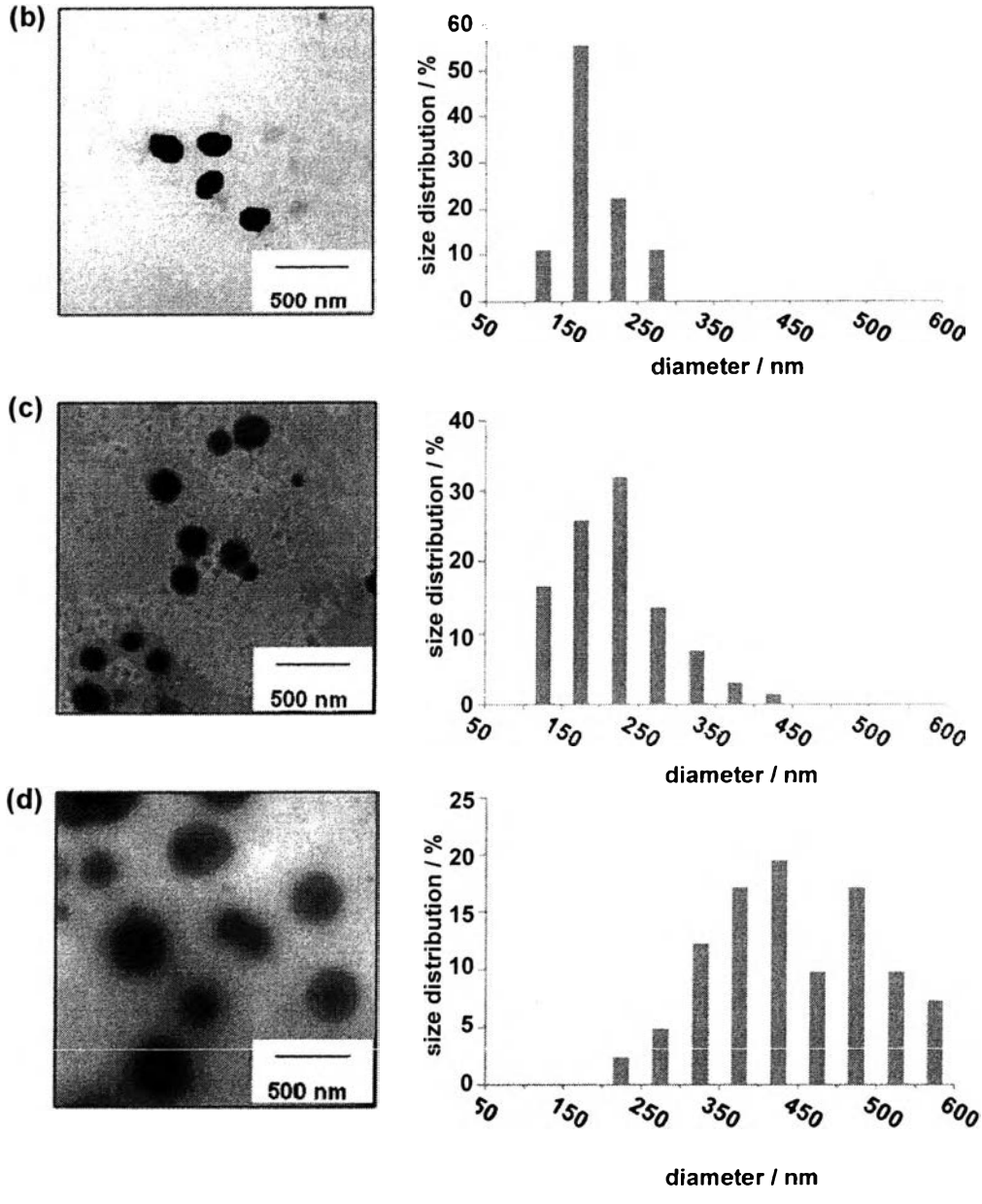


Figure 4.7 TEM micrographs and size distribution of (a) **CSN 85 mPEG 5k**, and **CSN 85 mPEG 5k** after loading with lidocaine; (b) 50%, (c) 100, and (d) 200 %wt.

The increase in size supports the successful lidocaine incorporation. At present, more studies related to the incorporation of lidocaine under pH effects, including the incorporation of other model drugs to represent the possible conditions such as ionic drug under ionic interaction, hydrophilic drug under hydrogen bond interaction, and hydrophobic drug under van der Waals interaction are in progress.

4.4 Conclusions

Previously, we reported a simple approach to prepare chitosan nanospheres. The present work emphasized on the factors related to the nanosphere formation and the morphologies. The substitution of phthalimido group at both of amino and partial hydroxyl group of chitosan was achieved at about 48% whereas the grafting of polyethylene glycol at hydroxyl group in the following step was accomplished at 18-60% depending on the molecular weight of mPEG. Both of phthaloylation and mPEG grafting tend to be constant even using the chitosans with different %DD. The dominant factor to control nanosphere size was the molecular weight of mPEG of which a series of **CSN-mPEG 5k** gave a monodispersed nanosphere with the size of 150–160 nm. The chitosan nanospheres showed highly negative charged surface. The morphologies of the amphiphilic chitosan in various pHs reflected the self-assembly phenomena which the colloidal appearances became transparent in basic condition and at that time, the size of the nanospheres was as small as 20–30 nm. A model drug incorporation using lidocaine was successful by using the homogeneous system of DMSO solution. The nanospheres were found to increase their sizes as the % loading amount of lidocaine increased.

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4.6 References

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