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# Colloidal Chitosan Nanospheres

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Chitosan was modified with hydrophobic N-phthaloyl and hydrophilic polyethylene glycol groups. The product provided a stable white colloidal solution in protic solvents including water. The colloidal particles were agglomerated spheres as observed by scanning electron microscope (SEM). Transmission electron microscopy (TEM) studies declared that the spheres were monodispersed in the sizes of hundred to few hundreds nanometer. The sphere size was dependent on the molecular weight of polyethylene glycol. Chitosan nanospheres showed the incorporation with stearylamine in high stability as determined by thermal analysis and infrared spectroscopy. The present work originally demonstrates that by simply adjusting the hydrophobic/hydrophilicity of chitosan chain, the self-assembly structure will be induced effectively to form spheres at the nanometer size without any specific processing techniques.
Key words: Chitosan, Nanospheres, Self-assembly, Colloidal Solution

## INTRODUCTION

Chitosan is an aminocopolysaccharide derived from the deacetylation of chitin, which is the second most abundant natural polysaccharide. Owing to the biodegradability, biocompatibility, bioactivity, and non-toxicity, chitin-chitosan is expected for the uses as a value-added material in pharmaceutical, biotechnological, and agricultural areas. Up to now, many reports and patents have shown chitosan applications in drug delivery system in the forms of films, beads, gels, and membranes obtained either from physical processing methods or chemical reaction pathways. Among those, there are several reports touch upon the processing of chitosan spheres in the sizes of 10~700 μm by some specific techniques such as suspension crosslinking [1], spray-drying coagulation [2], and emulsification/solvent evaporation [3]. Although the microsphere prodrugs can be achieved, the random networks limit the systematic controlled release level. In forming spheres, the use of crosslinking is also related to the toxicity of the crosslinkers. In addition, if the chemical reaction between chitosan and drug involved, the function of drug active site still remained has to be proven.

Core-corona structured polymeric spheres reported by Akashi et al. [4] is an alternative way to achieve micro or nanosphere prodrugs without the problems of crosslinkers and chemical reaction to destroy drug active sites. To our idea, the balancing hydrophobicity and hydrophilicity in the polymer chain is the key point to induce the sphere formation. This is an attractive strategy to extend to biopolymer as chitosan, hyaluronic acid, etc. In the past, we succeeded in introducing the hydrophobic and hydrophilic groups onto chitosan [5]. At this stage, we challenge the sphere-structured chitosan at micro or nanometer level obtained directly from chemical reactions without any specific processing techniques.

## EXPERIMENTAL

N-Phthaloylchitosan **2** was prepared as reported elsewhere [5-6] using chitosan **1** with degree of deacetylation (DD) = 0.9 and $M_v = 1.7 \times 10^5$ Dalton, provided by the Seafresh Chitosan (Lab) Company Limited, Thailand.

Anal. Calcd. for **2**, $(C_{14}H_{13}O_6N)_{0.8}(C_6H_{11}O_4N)_{0.1}(C_8H_{13}O_5N)_{0.1}$: (%) C, 56.17; H, 4.75; and N, 5.20. Found: (%) C, 56.18; H, 4.45; and N, 4.35: FT-IR (KBr, $cm^{-1}$) 3472 (OH), 1776 and 1714 (C=O anhydride), and 721 (aromatic ring): $^{13}C$ CP/MAS NMR (δ, ppm) 23.3 ($CH_3$), 57.0 (C-2), 64.7 (C-6), 73.2 (C-3, C-5), 80.5 (C-4), 100.4 (C-1), 131.1 (aromatic ring), and 169.1 (C=O): $^1H$-NMR (δ, ppm) 1.7 ($CH_3$ in acetamide), 3.4-5.0 (pyranose ring), and 7.6-7.7 (aromatic ring).

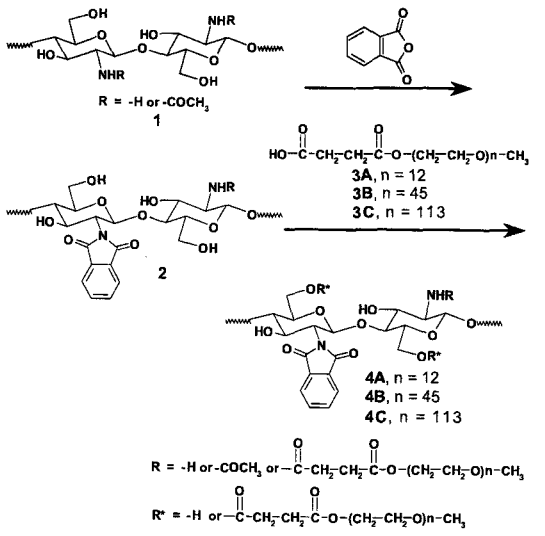
Poly(ethylene glycol) methyl ether (mPEG) terminated carboxylic group (mPEG-COOH, **3C**) was obtained from the reaction between mPEG ($M_n$ = 5,000 Dalton, 3.00 g, $6 \times 10^{-4}$ moles) and succinic anhydride (0.06 g, 1 mole equiv to mPEG) in DMF (2 mL) at 60 °C for overnight with a catalytic amount of pyridine. The mixture solution was extracted by diethyl ether and dried in vacuo to yield white powder of **3C**. Compounds **3A**, and **3B** were similarly prepared but using different molecular weights of mPEG, i.e., 550 and 2000, respectively.

FT-IR for **3** (KBr, $cm^{-1}$) 3472 (OH), 2875 (C-H stretching), 1736 (C=O), and 1105 (C-O-C): $^1H$-NMR (δ, ppm) 2.4 ($CH_2$ in succinic anhydride), 3.2 (O-$CH_3$), and 3.5 ($CH_2$ in PEG).

Compound **3C** (7.58 g, 0.40 moles equiv (40%) to

**2**) was mixed with **2** (1.00 g, $3.71 \times 10^{-3}$ moles) in 20 mL DMF solution containing 1-hydroxy -1H-benzotriazole, monohydrate (HOBt, 0.68 g, 3 moles equiv to **3C**). 1-Ethyl-3-(3-dimethyl aminopropyl)-carbodiimide, hydrochloride (WSC, 0.85 g, 3 moles equiv to **3C**) was added to react at 4°C for 30 min and then room temperature for overnight. The mixture was dialyzed in water for several days before thoroughly washed with methanol and dried in vacuo to obtain white particles of N-phthaloylchitosan-mPEG-COOH (compound **4C40**) (Scheme 1). A series of the compounds **4C02**, **4C05**, **4C10**, and **4C20** were also prepared by varying the amount of **3C** for 0.02, 0.05, 0.10, and 0.20 moles equiv to **2**, respectively.

Scheme 1




FT-IR for **4** (KBr, $cm^{-1}$) 3464 (OH), 2882 (C-H stretching), 1776 and 1714 (C=O anhydride), 1714 (C=O ester), and 721 (aromatic ring): $^1$H-NMR ($\delta$, ppm) 2.4 ($CH_2$ in succinic anhydride), 3.2 (O-$CH_3$), 3.5 ($CH_2$ in PEG), 2.8-4.7 (pyranose ring), and 7.6-7.8 (aromatic ring).

Stearylamine in isopropanol was prepared (0.02 mol/l) and used as a hydrophobic model molecule for incorporation in spheres. Compound **4C40** (0.15 g) was sonicated in isopropanol (5 mL) for few minutes before adding 5 mL of stearylamine-isopropanol solution. The mixture was slightly stirred for overnight. The precipitate was collected and re-dispersed in water. The white powder was kept and washed thoroughly with water and methanol before drying in vacuo. Hexylamine incorporation into **4C40** was done similarly.

## RESULTS AND DISCUSSION

The degree of phthalimido group substitution of compound **2** was 0.89 as confirmed from EA. Compound **3** shows the peak at 2875 $cm^{-1}$ belonging to methylene group, 1736 $cm^{-1}$ for carbonyl, and 1105 $cm^{-1}$ for ether bond (C-O-C) (Fig. 1(b)). $^1$H NMR also confirmed that mPEG was terminated with COOH as seen from new peak at 2.4 ppm possessing to methylene protons in succinic anhydride. Here, compounds **2** and **3** were reacted in homogeneous system and the solution obtained was dialyzed for several days to obtain white precipitate of **4**. Compound **4** shows the increase in peak intensity at 2882 $cm^{-1}$ belonging to methylene groups implying the conjugation with mPEG is successful (Fig. 1(e)). $^1$H NMR also supported the mPEG conjugation as confirmed from the peaks at 2.4, 3.2, and 3.5 ppm referring to methylene protons of succinic anhydride, methoxy protons of mPEG terminal chain, and methylene protons of mPEG, respectively. By varying the amount of mPEG (Mn=113) up to 0.2 moles equiv to **2**, the degree of mPEG chain substitution is saturated at a certain level (about 0.07 chain per unit of chitosan as calculated from EA data) (Fig. 5B). This might be due to the strong mPEG chain-chain interaction to limit the reactivity.

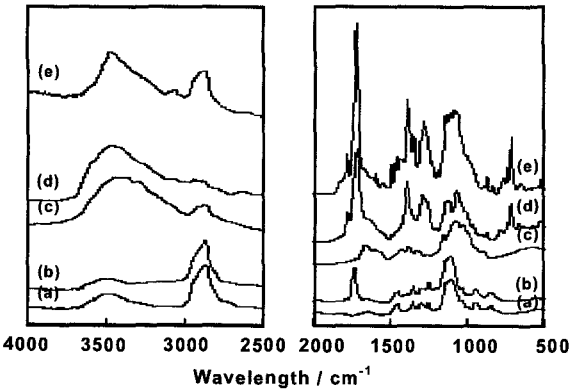



Fig. 1 FT-IR spectra of (a) mPEG ($M_n$ = 2000), (b) **3B**, (c) **1**, (d) **2**, and (e) **4C40**.

### Colloidal Formation and Effects of Solvents

Generally, chitosan and most of its derivatives are insoluble in water (Fig. 2(a)) and most organic solvents. After N-phthaloylchitosan was conjugated with mPEG to obtain **4**, a series of colloidal phenomena in various solvents were observed. Fig. 2(b)-(k) shows the appearance of **4** in protic and aprotic solvents, i.e., water, 1% aqueous acetic acid, methanol, ethanol, iso-propanol, DMF, DMSO, chloroform, toluene, and hexane. The solvents were selected based on the variation of dielectric constant and dipole moment to observe the colloidal induction by the solvent molecules. In the cases of protic solvents (i.e., water, 1% acetic acid, methanol, ethanol, and iso-propanol), the turbidity was clearly observed (Fig. 2(b)-(f)). We speculated that the colloidal phenomena were induced via the hydrogen bond between solvent molecules and mPEG chains of **4**, whereas N-phthalimido group acts as a hydrophobic segment. The appearance of the milky solution was remained for more than a week at ambient. The stability of **4** might come from the steric hindrance of mPEG chains, which are sticking out and repulsing each other. For aprotic solvents (i.e., DMF, DMSO, toluene, n-hexane,

chloroform), it should be note that **4** is completely dissolved in DMF and DMSO (Fig. 2(h)-(i)); however, it is insoluble in toluene and n-hexane (Fig. 2(j)-(k)). This might be related to the dielectric constant and dipole moment of each aprotic solvent. The complete dissolvation of **4** in DMF and DMSO might occur under ionic interaction between solvent molecules and chitosan at both mPEG chains and phthalimido groups ($\delta^-$ at oxygen atoms). For n-hexane and toluene, the precipitation of **4** might result from the lack of solute-solvent interactions. As shown in

Figure 2(g), **4** forms colloidal solution in chloroform. This might come from the fact that the dipole moment of chloroform compensates the dielectric constant value and induces the partially ionic interaction with **4**.

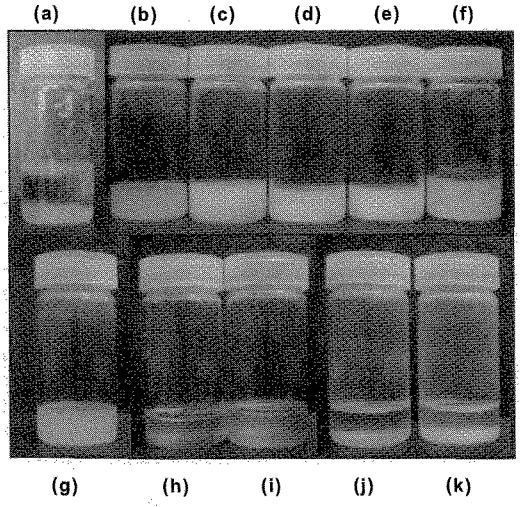



Fig. 2 Appearance of compound **1** in water (a) and compound **4C40** in water (b), 1% aqueous acetic acid (c), methanol (d), ethanol (e), iso-propanol (f), chloroform (g), DMSO (h), DMF (i), toluene (j), and n-hexane (k).

Nanospheres Clarification

Fig. 3(a)-(b) clarifies SEM photographs of chitosan and its derivatives. It was found that **1** shows irregular flake (Fig. 3(a)), while **4** performs the well-defined agglomerated spheres. The size was averagely 200 nm for **4C40** (Fig. 3(b)).

In order to reveal the information about individual sphere and/or its primary structure, TEM was applied. Fig. 4 illustrates TEM photographs of **4**, the images of **4** confirm the round shape particle with different sizes. It was found that the sizes of **4A40** (Fig. 4(a)) and **4C40** (Fig. 4(b)) are 400 and 85 nm, respectively.

Serizawa et al. [7] reported that the small size of polystyrene-mPEG core-corona nanospheres was induced by the long mPEG chain. For the present work, we speculate the similar structure which chitosan moiety acts as a core and mPEG chain as a corona. The size of the nanospheres, thus, should be related to the chain length of mPEG. Here, DLS was further applied to confirm the effect of mPEG chain length and its feed content in controlling the size of nanospheres.

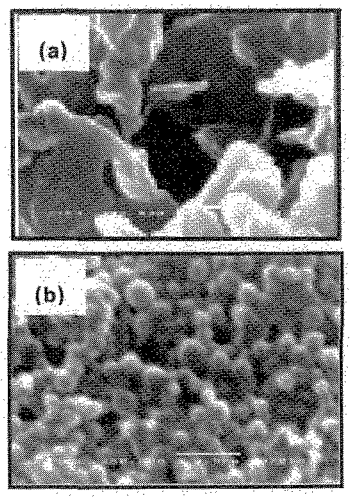



Fig. 3 SEM photographs at 25 kV of **1** (15,000×) (a) and **4C40** (50,000×) (b).

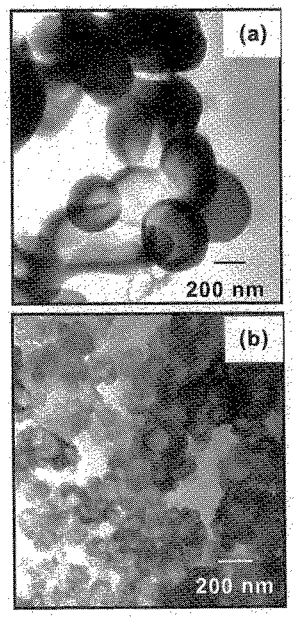



Fig. 4 TEM photographs at 30,000× of **4A40** (a) and **4C40**(b).

As shown in Figure 5, the $M_n$ of **3** affected on the sphere size, i.e., the longer the chain of mPEG, the smaller the sphere size. The sphere sizes were in average of 300-600 nm.

Model Drug Incorporation

It is important to clarify how we can apply chitosan sphere as a novel material for polymeric prodrug. Here, drug conjugation is done via three steps: (i) dispersing spheres in iso-propanol to allow some hydrophobic aligning on the surface, (ii) mixing with stearylamine model drug, and (iii) collecting the spheres and re-dispersing in water. By those steps, we assured that stearylamine was incorporated via hydrophobic-hydrophobic interaction at molecular level.

Drug conjugation is succeeded as confirmed by FT-IR at 2878 $cm^{-1}$ (Fig. 6(A)). Thermogravimetric analysis indicates the degradation temperature of stearylamine at 310°C (Fig. 6(B)) and percent incorporation to be 15.4.

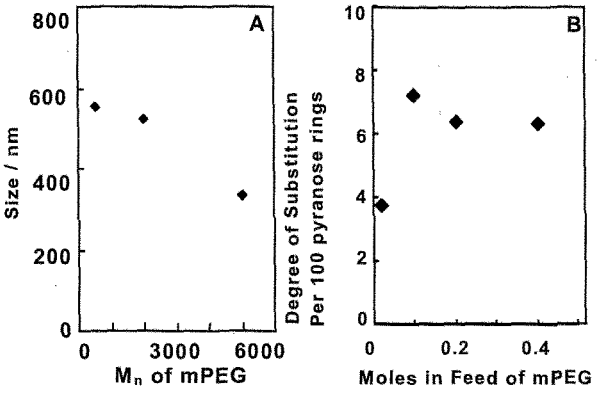



Fig. 5 (A) Size of **4** as a function of $M_n$ of mPEG (feed content of **3** = 0.40 moles), (B) Degree of substitution per 100 pyranose rings as a function of mPEG amount.

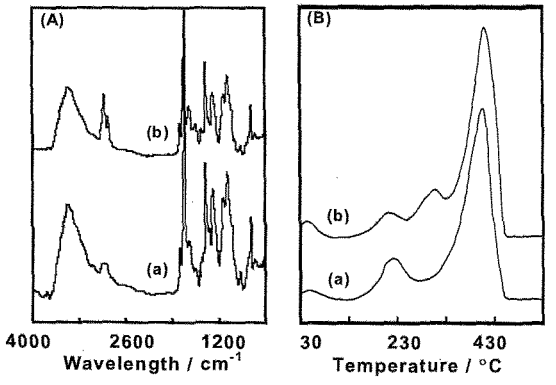



Fig. 6 FT-IR spectra (A) and TGA thermograms (B) of **4C40** (a) and **4C40** after stearylamine incorporation (b).

The sphere size after drug incorporation was increased significantly. For example, the sphere agglomerates shown in Figure 3 (b) change from 200 nm to 1000 nm after incorporating with stearylamine (Figure 7 (b)) as observed by SEM. Agglomerate spheres incorporated with hexylamine shows the size of 800 nm (Figure 7 (a)) implying that the longer the chain of amine, the larger the sphere size.

## CONCLUSIONS

The present work demonstrates that by grafting hydrophilic chains, mPEG, to hydrophobic polymer, N-phthaloylchitosan, we could obtain sphere particles directly from the reaction without any specific processing techniques. Colloidal phenomena and nanosphere formation were induced by the polarity of the media. The chain length of hydrophilic mPEG was the factors to control the sphere sizes. The chitosan nanospheres showed the hydrophobic drug incorporation as identified from the model case of long chain alkylamine.

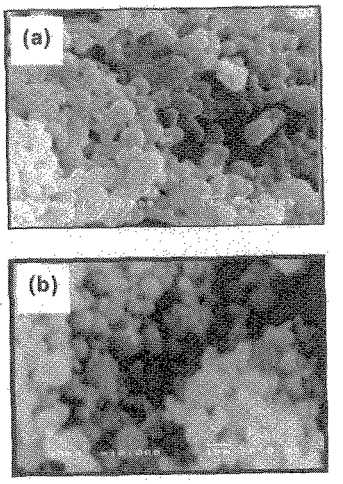



Fig. 7 SEM photographs at 20 kV of **4C40** after incorporating with hexylamine (a) and stearyl amine (b).

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