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ORIGINAL CONTRIBUTION

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Controlled hydrophobic/hydrophilic chitosan: colloidal phenomena and nanosphere formation

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Abstract *N*-Phthaloylchitosan-grafted poly(ethylene glycol) methyl ether (mPEG) gives a milky solution when dispersed in water and a series of solvents. The appearance of turbidity depends on the types of solvents, i.e., protic and aprotic solvents. *N*-Phthaloylchitosan-grafted mPEG shows an aggregation of sphere-like particles as observed by scanning electron microscopy. Transmission electron microscopy indicates that the spheres are at the nano level. When the chain length of mPEG is as high as 5×10^3 Da the sphere size becomes as small as 80–100 nm on average as observed by transmission electron microscopy. By simply adjusting the hydrophobicity/hydrophilicity of the chitosan chain, a stable nanosphere can be obtained directly.

Keywords Chitosan nanospheres · Homogeneous reaction · *N*-Phthaloylchitosan-grafted poly(ethylene glycol) methyl ether · Self-aggregation · Colloidal phenomena

Introduction

For the few past decades, the rapid progress in analytical instrument has brought us to an understanding about the performance of polymer chains at the molecular level [1]. Up to now, there have been several reports about a well-defined colloidal structure at the micro and/or nano scale where novel properties are discovered [2]. In some cases, the colloid is induced by the self-aggregated polymer [2] to form microspheres and/or nanospheres under specific conditions, such as the hydrophobicity/hydrophilicity [3], ionic strength [2], and hydrogen-bonding network [4]. Recently, nanospheres have received much attention as a material for advanced applications, especially catalysts [5] and drug delivery systems (DDS) [3]. For DDS, nanospheres are attractive since the drug incorporation can be achieved without the active sites being destroyed by harsh reactions in conjugation steps, and at the same time the self-aggregate is eventually formed without a cross-linker. Akashi et al. [6] proposed a polystyrene core–corona sphere which showed the ability to immobilize peptide drugs and antibodies. Here, it can be expected that microspheres and/or nanospheres obtained from a biopolymer backbone have the advantages of biodegradability [7], biocompatibility [8], bioactivity [9], and nontoxicity [10]. Chitosan is an aminopolysaccharide in a deacetylated form of chitin, which is the second most abundant natural polymer. Recently, the production of chitosan spheres for DDS was achieved by some specific processing techniques, such as suspension cross-linking [11], spray-drying coagulation [12], emulsification/solvent evaporation [13]. The spheres were obtained in the range 10–700 μm. Up to now, there has been no report about chitosan spheres at the micro and/or the nano level obtained by chemical modification. The functionalized chitosan studied by Ouchi et al. [14] might be the foremost approach showing the initiation of self-aggregation of chitosan. For the past few years, we have concentrated on the modification of chitosan based on the

balancing of polarity on the chain [15] in order to obtain novel derivatives. Through a specific molecular structure combining with the interaction induced from chitosan and solvent molecules, we expect to achieve spheres of a controllable size with high stability in solvents, especially water. The present work is, thus, focused on a controlled-structured chitosan by simply introducing hydrophobic/hydrophilic groups to induce spheres of nano size, which are hardly obtained by general processing techniques.

Experimental

Materials

Chitosan with 90% deacetylation ($M_v = 1.7\times10^5$ Da) was provided by Seafresh Chitosan (Lab), Co., Thailand. Phthalic anhydride and succinic anhydride were purchased from Fluka Chemika, Switzerland. Poly(ethylene glycol) methyl ethers (mPEG, M_n 550 and 5,000 Da) were obtained from Aldrich Chemical Company, USA. 1-Ethyl-3-(3′-dimethylaminopropyl)carbodiimide, hereinafter abbreviated as water soluble carbodiimide (WSCI), was purchased from TCI, Japan. 1-Hydroxy-1*H*-benzotriazole monohydrate (HOBt) was obtained from BDH Laboratory Supplies, UK. All chemicals were used without further purification.

Sample preparation

An amount of mPEG ($M_n = 5{,}000$ Da, 6×10^{-3} mol) was reacted with succinic anhydride (1 mol eqivalent to mPEG) in *N,N*-dimethylformamide (DMF) at 60 °C overnight. The mixture was reprecipitated in diethyl ether to obtain mPEG–COOH, **2a**. Compound **2a** (0.40 mol equivalent to *N*-phthaloylchitosan, **1** [16]) was stirred with **1** (3.71×10^{-3} mol) at room temperature overnight in DMF containing HOBt (3 mol equivalent to **2a**) and WSCI (3 mol equivalent to **2a**). The mixture was dialyzed in water and washed thoroughly with methanol to obtain white particles, **3a**. Compounds **2b** and **3b** were prepared similarly to **2a** and **3a**, respectively but using mPEG with $M_n = 550$ Da. The *N*-phthalimido group was removed as reported [16] to yield **4a** (Scheme 1).

Characterization

Compound **1**: Anal. Calcd. for $(C_{14}H_{13}O_6N)_{0.8}(C_6H_{11}O_4N)_{0.1}(C_8H_{13}O_5N)_{0.1}$ (%): C, 56.17; H, 4.75; N, 5.20. Found (%): C, 56.18; H, 4.45; N, 4.35. FTIR (KBr, cm^{-1}): 3,472 (OH), 1,776 and 1,714 (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). ^{1}H NMR (δ, ppm): 1.7 (CH_3 in acetamide), 3.4–5.0 (pyranose ring), and 7.6- 7.7 (aromatic ring).

Compound **2a**: FTIR (KBr, cm^{-1}): 3,472 (OH), 2,875 (C–H stretching), 1,736 (C=O), and 1,105 (C–O–C). ^{1}H NMR (δ, ppm): 2.4 (CH_2 in succinic anhydride), 3.2 (O–CH3), and 3.5 (CH_2 in PEG).

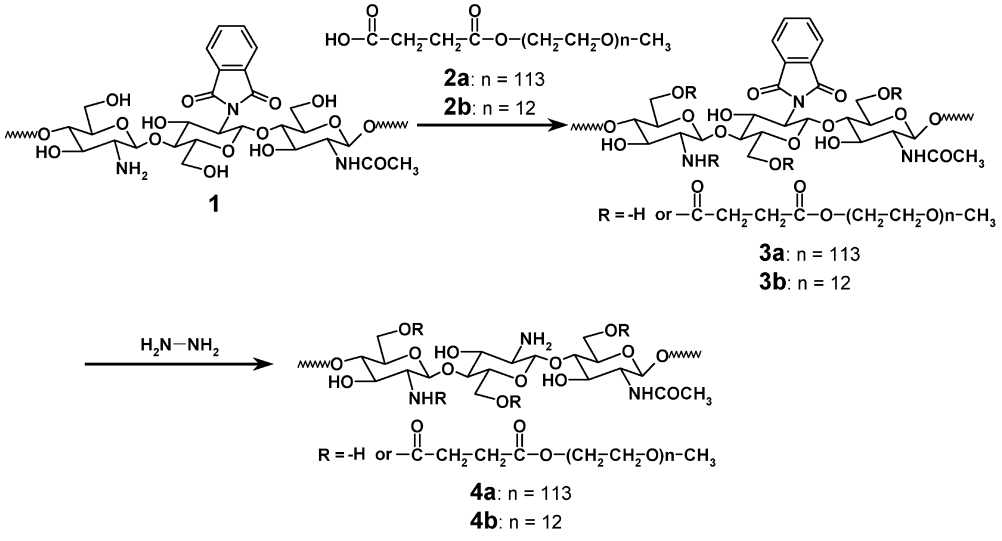
Compound **3a**: Anal. Calcd. for $(C_{14}H_{13}O_6N)_{0.509}(C_{245}H_{471}O_{122}N)_{0.291}(C_6H_{11}O_4N)_{0.027}(C_{468}H_{927}O_{236}N)_{0.073}(C_8H_{13}O_5N)_{0.064}(C_{239}H_{471}O_{12}N)_{0.036}$ (%): C, 54.67; H, 8.58; N, 0.52. Found (%): C, 56.22; H, 4.82; N, 6.82. FTIR (KBr, cm^{-1}) 3,464 (OH), 2,882 (C–H stretching), 1,776 and 1,714 (C=O anhydride), 1,714 (C=O ester), and 721 (aromatic ring). ^{1}H NMR (δ, ppm): 2.4 (CH_2 in succinic anhydride), 3.2 (O–CH3), 3.5 (CH_2 in PEG), 2.8–4.7 (pyranose ring), and 7.6–7.8 (aromatic ring).

Compound **4a**: FTIR (KBr, cm^{-1}): 3,412 (OH), 2,882 (C–H stretching), 1,714 (C=O ester), 1,654 (amide I), 1,549 (amide II), and 895 (pyranose ring). ^{1}H NMR (δ, ppm): 1.8 (CH_3 in acetamide), 2.4 (CH_2 in succinic anhydride), 2.9 (O–CH3), 3.3 (CH_2 in PEG), 3.2–5.0 (pyranose ring), and 7.6–7.7 (aromatic ring).

Results and discussion

In the first step, *N*-phthaloylchitosan, **1** (phthalimido group substitution of 80%) was prepared in order to have a homogeneous reaction system. At the same time, to achieve controlled hydrophilic/hydrophobic chitosan, we consider the phthalimido group as a hydrophobic segment and mPEG as a hydrophilic one. However, the mPEG introduction onto the chitosan chain requires a conjugating or coupling agent. Here, WSCI was selected as a conjugating agent. The mPEG was modified to be mPEG–COOH, **2** with succinic anhydride to enhance

Scheme 1



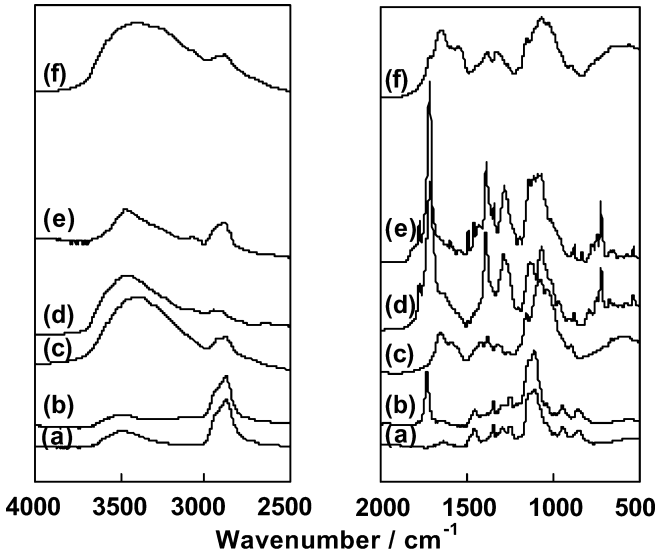


Fig. 1 Fourier transform IR spectra of poly(ethylene glycol) methyl ether (*a*), **2a** (*b*), chitosan (*c*), **1** (*d*), **3a** (*e*), and **4a** (*f*)

the reactivity. Comparing Fig. 1 spectra (*a*) and (*b*), the peak at 1,736 cm^{-1} confirms the conjugating structure of **2a** via ester bonds. In the next step, **2a** was introduced onto **1** to obtain white particles, **3a**. The amount of mPEG incorporated was determined by elemental analysis. Although the molar ratio of mPEG–COOH was varied, the amount of mPEG attached onto chitosan was saturated at a certain level. For example, the conjugation of **2a** onto **3a** was limited at 0.03 mol equivalent to pyranose rings. At present, we are studying the factor to control the mPEG conjugation. In the final step, the amino groups of chitosan were recovered to obtain **4a**. By comparing the performance of **3a** and **4a**, we can clarify the effect of the hydrophobic/hydrophilic chain involved in colloidal phenomena and sphere formation (see Effect of amino group).

Colloidal phenomena and the effect of solvents

Chitosan and most of its derivatives are insoluble in organic solvents and water. It is important to note that although the *N*-phthalimido group had not yet been removed, soon after the mPEG was conjugated onto **1** to obtain **3a**, the compound gave a milky solution in water and the turbidity was maintained even after 4–5 days as shown in Fig. 2a. This appearance might come from the fact that there are some hydrations by hydrogen bonds between water and **3a** (at oxygen atoms of mPEG) (Scheme 2 case a). The dispersion stability of **3a** also reflects the function of PEG chains in causing steric hindrance to obstruct chitosan chain packing. The colloidal phenomena were observed from the turbidity in a series of solvents as shown in Fig. 2a. It was found that the turbidity level largely depended on the types of solvents, i.e., protic and aprotic ones. In order to understand how the turbidity is related to the interaction between **3a** and solvent molecules, we tabulated the values of the dielectric constant and dipole moment as well as the appearance of the turbidity. Here, the effects are focused on the hydrogen bonding and polar–polar interaction. In the cases of protic solvents (1% acetic

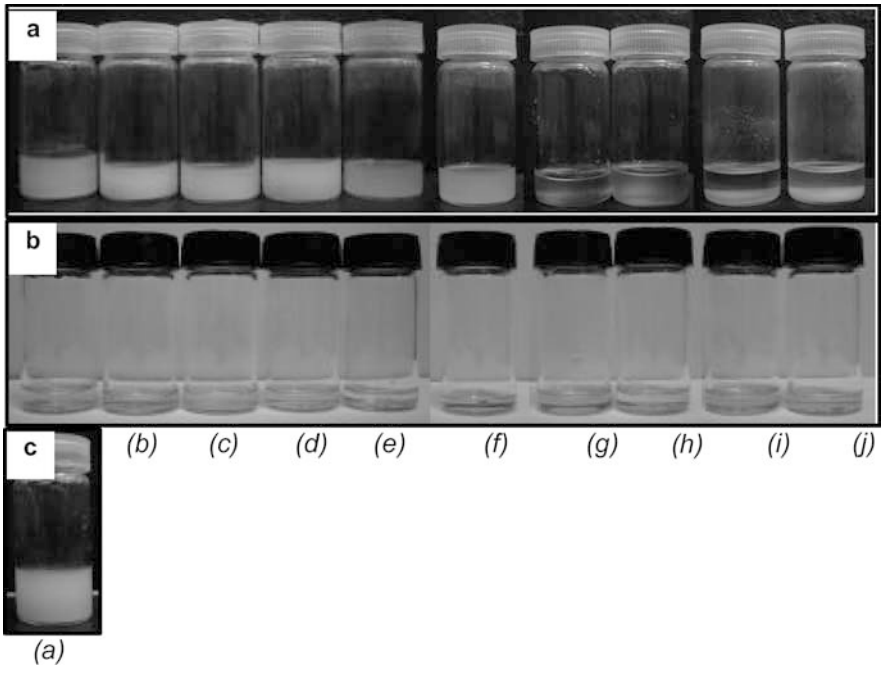
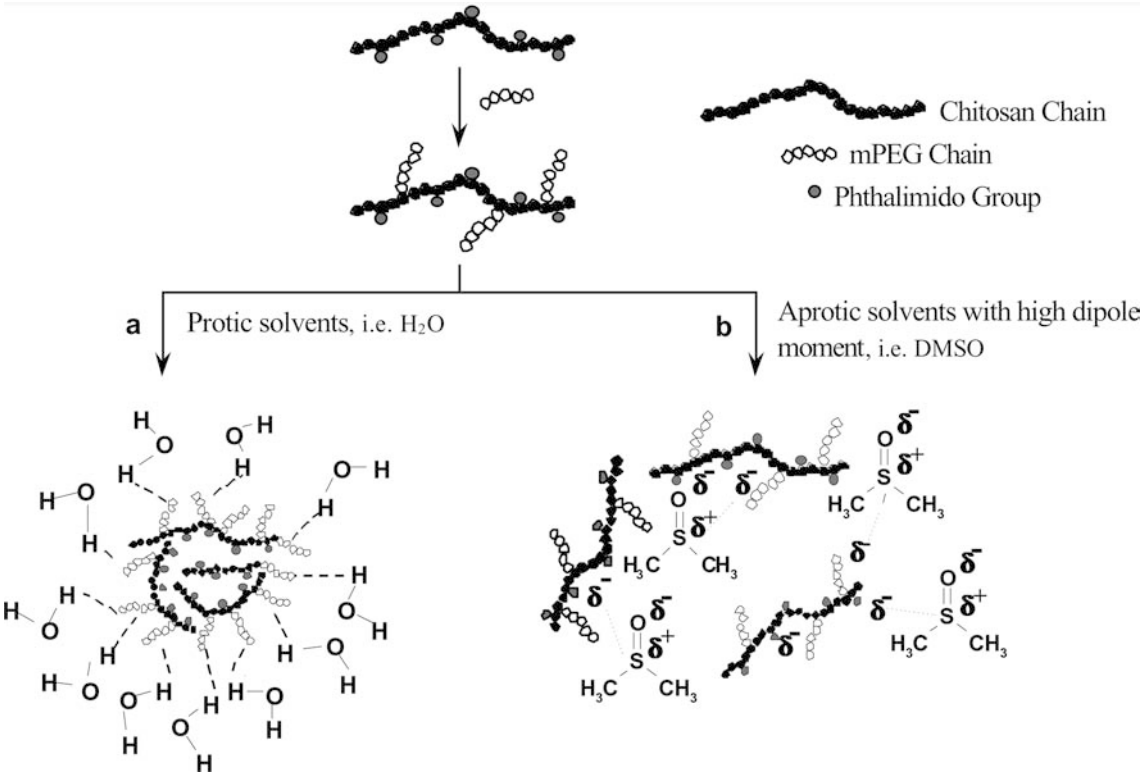


Fig. 2 Appearance of **a 3a**, **b 4a**, and **c 1** dispersed in various solvents: water (*a*), 1% acetic acid (*b*), methanol (*c*), ethanol (*d*), 2-propanol (*e*), chloroform (*f*), dimethyl sulfoxide (*g*), *N*,*N*-dimethylformamide (*h*), toluene (*i*), and *n*-hexane (*j*)



Scheme 2

acid, methanol, ethanol, and 2-propanol), the turbidity was clearly observed (Fig. 2a). This might come from the hydrogen-bond formation as detailed in the case of water. When it comes to 2-propanol, the hydrophobicity of the solvent might reduce the hydration; as a result, the turbidity becomes insignificant. For aprotic solvents, **3a** is completely dissolved in DMF and dimethyl sulfoxide (DMSO), however, it is insoluble in toluene and *n*-hexane. The reason why the turbidity could not be seen in the cases of DMF and DMSO may be due to both a high dielectric constant and a high dipole moment (Table 1). Scheme 2 case b illustrates the complete dissolution of **3a** in DMSO when a polar–polar interaction occurs between solvent molecules (δ^+ at the sulfur atom) and chitosan at not only mPEG chains but also phthalimido groups (δ^- at oxygen atoms). In the cases of *n*-hexane and toluene, the precipitation of **3a** might come from the lack of both hydrogen bonds and polar–polar interactions (Fig. 2, Table 1). It is important to clarify the dispersion stability of **3a** in chloroform. The low dielectric constant of chloroform implies difficulties in hydrogen-bond formation with **3a**. On the other hand, a certain value of the dipole moment ($\mu = 1.04$) suggests a charge-separated structure of chloroform to favor the interaction with **3a**. As a result, the turbidity in chloroform was less than in aprotic solvents.

Table 1 Evaluation of colloidal formation of **3a** in various solvents relating to dielectric constant and dipole moment

Solvents	Dielectric constant at 25 °C/D [18]	Dipole moment/D [18]	Appearance[a]
Water	78.54	1.85	±
Methanol	32.63	1.70	±
Ethanol	24.30	1.69	±
2-Propanol	15.80	1.58	±
Chloroform	4.81	1.04	±
Dimethyl sulfoxide	47.00	3.96	+
N,*N*-Dimethylformamide	36.70	3.82	+
Toluene	2.38	0.38	–
n-Hexane	1.89	0.08	–

[a] ± colloidal formation; + solvation; – precipitation

Formation of spheres of nano sizes

Figure 3a shows that the chitosan starting material consisted of irregular flakes. However, after phthaloylation, the compound obtained (1) appears to have a partially round shape (Fig. 3b), implying the sphere initiation by the hydrophobic phthaloimido groups (see Effect of amino group). The spherical particles are significant after mPEG incorporation as seen in the cases of **3a** and **3b** (Fig. 3c–e). The average sizes were observed to be 200 nm for **3a** (Fig. 3c, d) and 400 nm for **3b** (Fig. 3e) as determined by scanning electron

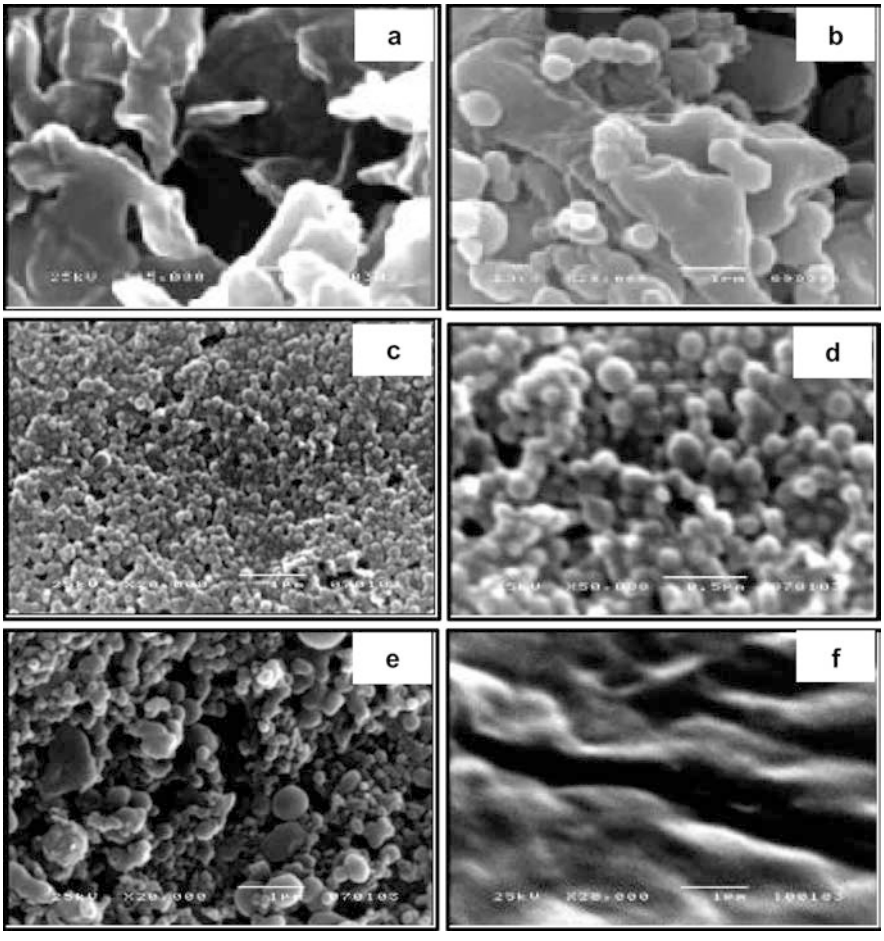


Fig. 3 Scanning electron microscopy photographs at 25 kV of **a** chitosan (×15,000), **b 1** (×20,000), **c 3a** (20,000×), **d 3a** (×50,000), **e 3b** (×20,000), and **f 4a** (×20,000)

microscopy (SEM). The observation from transmission electron microscopy (TEM) gives us information about the individual particle to determine the precise structure and size (Fig. 4). Compounds **3a** and **3b** are identified to be spheres with average sizes of 80 and 400 nm, respectively. The different sphere size might relate to the chain length of mPEG. Serizawa et al. [17] reported polystyrene–mPEG core–corona nanospheres where the longer mPEG chain gave the smaller spheres. Here, we also found that the higher the molecular weight of mPEG is, the smaller the sphere size will be as characterized by TEM. Although the drying process in sample preparation for either SEM or TEM may cause physical structure deformation, the results obtained indicated the regular size of the spherical particles. We speculate that the sphere formation might occur mainly from the aggregation of chitosan chains under the hydrophobic and hydrophilic interaction as shown in Scheme 2 case a rather than the swelling effects, and thus the spherical structures are maintained. As a result, in the case of protic solvents, the strong interaction induced by the solvent enhances the sphere formation as evidenced in Figs. 3c–e and 4.

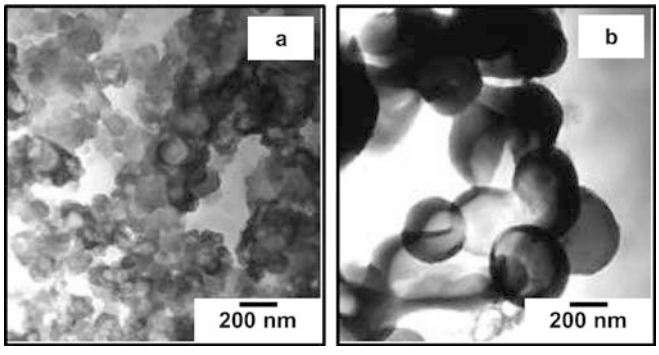


Fig. 4 Transmission electron microscopy photographs at ×30,000 of **a 3a** and **b 3b**

Effect of amino group

It should be noted that **1** also gives a milky solution in water (Fig. 2c). This might be due to the tendency to form self-aggregates based on the hydrophobic phthalimido groups on the chitosan chain. The stable dispersion under nanosphere formation is satisfied when the chitosan chains have both phthalimido groups and PEG

chains (see Formation of spheres of nano sizes). When phthalimido groups were removed from **3a** to obtain **4a**, the turbidity could not be observed anymore and the compound precipitated (Fig. 2b). In addition, compound **4a** dissolves very well in acetic acid. This might be related to the protonation of amino groups and the hydrogen bonds between water molecules and mPEG chains. Here, the SEM photograph also confirmed that **4a** does not show the spherelike structure (Fig. 3f).

Conclusion

N-Phthaloylchitosan-grafted mPEG was a good model to fulfill the conditions for colloidal phenomena where the nanospheres were induced. The stability of the appearance of the milky solution was enhanced in protic solvents where hydrogen bonds were accomplished. mPEG with $M_n = 5{,}000$ Da gives spheres with sizes about 80–100 nm, whereas mPEG with $M_n = 550$ Da provides spheres with an average size of 400–500 nm as determined by TEM. At present, we are studying the related derivatives, the effects of mPEG chain length and content as well as the potential applications.

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