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Carbohydrate Polymers 69 (2007) 419–425
Carbohydrate Polymers
www.elsevier.com/locate/carbpol

Preparation and rheological properties of deoxycholate-chitosan and carboxymethyl-chitosan in aqueous systems

Hong Tao Pang [a], Xi Guang Chen [a,b,*], Hyun Jin Park [b], Dong Su Cha [b], John F. Kennedy [c]

[a] College of Marine Life Science, Ocean University of China, Qingdao 266003, PR China
[b] The Graduate School of Biotechnology, Korea University, Seoul 136-701, South Korea
[c] Birmingham Carbohydrate and Protein Technology Group, Chembiotech Laboratories, University of Birmingham Research Park, Vincent Drive, Birmingham B15 2SQ, UK

Received 29 September 2006; received in revised form 22 December 2006; accepted 23 December 2006
Available online 19 January 2007

Abstract

Self-aggregation and micelles of amphiphatic polymers are important functions in application, and their properties are influenced by the parent hydrophilic polymer and the hydrophobic groups. In this paper, deoxycholate-chitosan (DE-chitosan) and carboxymethyl-chitosan (CM-chitosan) were prepared from chitosan (MW, 28 kDa; DD, 90%). The effects of acid, pH and ionic concentrations on aggregation behaviors of the polymers in aqueous systems were measured, respectively. The ionic strength was found to enhance the aggregation of DE-chitosan, but had no influence to CM-chitosan and chitosan itself. The modification of hydrophobic groups and hydrophilic groups influenced the rheology properties of both parent chitosan and the derivatives. The assemblage of DE-chitosan in solution can be controlled by an oil/water (methylene chloride/DE-chitosan solution) emulsification process using methylene chloride. The micelles of DE-chitosan formed nano-micelles particles of 200–600 nm in diameter which can be used to encapsulate lipid-soluble compounds for drug dispersing and delivery releasing.

Keywords: Chitosan; Rheology; Modification; Micelle; Nanoparticle

1. Introduction

Self-aggregations of water-soluble amphiphatic polymers in solution are important in various biomedical and pharmaceutical applications. This aggregation process, caused by incorporation of hydrophobic organic substances into micelles, can play very important roles such as adsorption, transfer, and slow release of drug or nutrient in applications in treatment of humans. Interesting physicochemical properties of amphiphatic polymers are directly related to the intra- and inter-molecular associations between the hydrophobic functionalities in the aqueous solution within a certain concentration range (Desbrieres, Martinez, & Rinaudo, 1996; Kohori, Yokoyama, Sakai, & Okano, 2002). Various hydrophobilized polymers have been synthesized by modifying an organic group on a hydrophilic polymer, and the aggregations and micelle behaviors had been extensively studied (Desbrieres et al., 1996). In the structure of amphiphatic polymers, the hydrophilic parent polymer had more tendency to become hydrated and dissolve in water, and the hydrophobic groups had more tendency to repel the water environment by assembling polymer (in the solution) (Akiyoshi, Deguchi, Tajima, Nishikawa, & Sunamoto, 1997). The self-aggregation behavior of the derivative, however, affected by the parent polymer and the modifying groups were not clear.

Chitosan (poly-1,4-linked β-D-glucosamine) is a cationic polysaccharide made by alkaline *N*-deacetylation of chitin. As one of the most abundant natural polymers, it has attracted much attention in various fields as a result of its

* Corresponding author.
E-mail addresses: xgchen@ouc.edu.cn (X.G. Chen), hjpark@korea.ac.kr (H.J. Park), jfk@chembiotech.ac.uk (J.F. Kennedy).

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doi:10.1016/j.carbpol.2006.12.026

biological activity, biocompatibility and biodegradability in combination with its low toxicity (Dodane & Vilivalam, 1998; Xie, Xu, & Liu, 2001). Chitosan has widely been hydrophobically modified and used in the study of micelle and self-aggregation, such as deoxycholate-modified chitosan (Lee, Kwon, Kim, Jo, & Jeong, 1998), PEG-chitosan (Sugimoto, Morimoto, Sashiwa, Saimoto, & Shigemasa, 1998), fatty acyl-modified chitosan (Chen, Lee, & Park, 2003; Liu, Desai, Chen, & Park, 2005; Zhang & Hirano, 1995), glutaraldehyde-modified chitosan and others (Agarwal & Gupta, 1995). The well-regulated micelles or self-assembled particles formed by chitosan and the hydrophobic derivatives were important nano-carriers for some compounds such as drugs, enzyme, and DNA (Lee et al., 1998; Liu et al., 2005). Chitosan without hydrophobic modification also has been proved to be a fine self-aggregation substance in solution (Amiji, 1995). The aggregation characteristics of the parent chitosan molecule, inevitably, have influence on the properties of its derivatives and in particular the molecular state and hydrophobically modified chitosan in aqueous solution.

Modification of chitosan by introduction of hydrophilic groups is known to have a function in improving the chitosan's solubility in water, such as carboxymethyl-chitosan (CM-chitosan), hydroxyethyl-chitosan (HE-chitosan), and PEG-chitosan. Such chitosan derivatives also have the phenomena of self-assembly in aqueous solution (Sugimoto et al., 1998). The back bone of the parent chitosan will obviously influence the hydrophilic groups when determining the rheological properties in solution. However, the relationship between the parent chitosan and the modified group, including hydrophobic groups and hydrophilic groups, in the display of properties of rheology and self-aggregation in aqueous solutions is not clear. To achieve some investigation of this, deoxycholate-chitosan (DE-chitosan) and carboxymethyl-chitosan (CM-chitosan) were prepared, and their rheological properties in aqueous systems, particularly with respect to the effects of acid, pH and ionic concentrations on aggregation behaviors of the polymers in solution have been examined. Additionally, since DE-chitosan has a hydrophobic group in the molecule, it has functional ability of surface activity to form micelles in higher concentration in 'solution'. So the formation and characteristics of micelles particles of the DE-chitosan in aqueous system have been measured to show the potential in the application of nano-encapsulation to drug delivery.

2. Materials and methods

2.1. Materials

Chitosan, 100 mesh, degree of deacetylation 90%, molecular weight 28 kDa (viscosity–average molecular weight Mη), was obtained from Biotech. Co., (Mokpo, Korea). Monochloroacetic acid, deoxycholic acid, pyrene, and 1-ethyl-3-(3-dimethylaminopropyyl)carbodiimide (EDC) were purchased from Sigma (St. Louis, USA).

2.2. Deoxycholate-chitosan preparation

Deoxycholate-chitosan (DE-chitosan) was prepared by the method of Lee et al. (1998). In brief: deoxycholic acid (1 g) in methanol (10 ml) was added to a 1% w/w chitosan solution in 1% aqueous acetic acid (100 ml) followed by dropwise addition of EDC (0.5 g in 5 ml methanol) with stirring at room temperature. After 48 h, the reaction mixture was poured into the methanol/ammonia solution (7:3, v/v). The precipitate was filtered off, sequentially washed thoroughly with distilled water, methanol, and ether, and then followed by vacuum drying at room temperature.

2.3. Carboxymethyl-chitosan preparation

Carboxymethyl-chitosan (CM-chitosan) was prepared by the previously described method (Chen & Park, 2003). In brief: chitosan (1.0 g), sodium hydroxide (1.35 g), and solvents (distilled water, 2.0 ml; isopropanol, 8.0 ml) were added into a flask (50 ml). The temperature was controlled by a water bath (Thermo-controller, Comabiotech. Co., Korea). Monochloroacetic acid (1.5 g) was dissolved in isopropanol (2.0 ml), and added into the flask by dropping equably and reacted for 4 h, then stopped by adding 70% v/v aqueous ethanol (20 ml). The solid was filtered off and rinsed in 70–90% ethyl alcohol to desalt and dewater, and vacuum dried.

2.4. NMR spectroscopy

^{1}H NMR spectra of samples, at a concentration of 20 mg/ml were recorded on a Brukker ARX 300 spectrometer in D_2O at 25 °C. For water-insoluble samples, CD_3OOD (99.9%) was added (1%, v/v). The measurement conditions were as follows: a spectral window of 500 Hz, 32 k data points, a pulse angle of 30°, an acquisition time of 2.03 s, and 32 scans with a delay of 1 s between scans (Shigemasa, Matsuura, Sashiwa, & Saimoto, 1996).

2.5. Fluorescence determination

Pyrene, purified by repeated recrystallization from methanol, in ethanol (0.04 mg/ml, 20 μL) was pipetted into a test tube and the ethanol was dried off under a stream of pure nitrogen gas. Polymer solution (2 ml) with the desired final concentration was added into the test tube, with a final concentration of pyrene of 2 μM. The mixture solution containing pyrene was incubated for 3 h in water bath at 65 °C, and then shaken over night at room temperature. Pyrene emission spectra were obtained using a Shimadzu RF-5301PC fluorescence spectrophotometer (Shimadzu Co., Kyoto, Japan). The probe was excited at 343 nm and the emission spectrum was collected in the range of 360–500 nm at an integration time of 1.0 s. The excitation and emission slit opening were 15 and 1.5 nm, respectively (Amiji, 1995).

2.6. Preparation and properties of the nano-micelles particles (Uchegbu et al., 2001)

DE-chitosan (10 mg) was dissolved in 0.1 M acetic acid/1 M sodium acetate solution (2 ml), methylene chloride (80 μL) was added, and the mixture homogenized (5 min, 13,000 rpm) with a ULTRA-TURRAX T-25 dispersing machine (Kushu Kika Kogyo Co., Japan). This solution was held under vacuum for 30 min at room temperature to remove methylene chloride and then 0.25% w/w aqueous sodium tripolyphosphate (STPP, 1 ml) was added as cross-linking reagent. The solution was stirred for 1 h at 30–40 rpm, then used for nanoparticles characteristics determinations.

To determine the encapsulation properties of the DE-chitosan, tocopherol acetate (liquid form) and stearic acid methyl ester (solid form), as lipid-soluble model compounds, were firstly incorporated in methylene chloride (10 g/L), and then these methylene chloride solutions were added to the DE-chitosan solution (DE-chitosan 5 mg/ml in 0.1 M acetic acid/1 M sodium acetate) in the ratio of 1% (v/v). Subsequent emulsification was as previously described (homogenized 5 min at 13,000 rpm). After cross-linking as above, the solution was centrifuged (9000*g* to remove the particles, and the supernatant was used to determine the incorporation of drug into the nanoparticles. This is based on the fact that during the encapsulation process some of the drug was enveloped in the core of the nanoparticles, there was some drug un-enveloped. This free un-enveloped drug was in the supernatant and could be determined by HPLC to calculate by difference the amount of the drug in the particles. The transmittances of the resulted solutions were recorded on a DU-650 spectrophotometer (BECKMAN Co., USA) using a quartz cell with an optical path length of 1 cm at 600 nm. The particle size was measured using Zetasier S (Malvern Instrument, Malvern, UK) at detector angle 90°, 670 nm and 25.2 °C in a 1 cm^2 quartz cell.

3. Results and discussion

3.1. Synthesis and characterization of DE-chitosan and CM-chitosan

The yield of DE-chitosan was 1.15 g (98% mole basis based on glucosamine residue), and CM-chitosan was 1.08 g (78% mole basis based on glucosamine residue). The information of the molecular structure of chitosan, DE-chitosan, and CM-chitosan were confirmed by ^{1}H NMRs (Fig. 1). Proton assignment of chitosan: $\delta_{1.8}=CH_3$ (acetyl group); $\delta_{2.7}=CH$ (carbon 2 of sugar); $\delta_{3.1-3.4}=CH+CH_2$ (carbon 3–6 of sugar); $\delta_{3.9}=CH$ (carbon 1 of sugar). Proton assignment of DE-chitosan: $\delta_{1.0}=CH_3$ (methyl group of deoxycholate); $\delta_{1.8}=CH_3$ (acetyl group of chitosan); $\delta_{2.8}=CH$ (carbon 2 of chitosan); $\delta_{3.0}=CH_2$ (deoxycholate protons); $\delta_{3.9}=CH$ (carbon 1 of chitosan). Proton assignment of CM-chitosan: $\delta_{1.8}=CH_3$ (acetyl group); $\delta_{2.65}=CH$ (carbon 2 of sugar); $\delta_{2.8}=CH_2$ (carboxymethyl group on carbon 2 of sugar); $\delta_{3.05-3.4}=CH+CH_2$ (carbon 3–6 of sugar); $\delta_{3.5-3.65}=CH_2$ (carboxymethyl groups on carbon 3, 6 of sugar); $\delta_{3.8}=CH_2$ (carboxymethyl group on carbon 3 of sugar); $\delta_{4.0-4.2}=CH$ (carbon 1 of sugar) (Hjerde, Varum, Grasdalen, Tokura, & Smidsrod, 1997; Uchegbu et al., 2001).

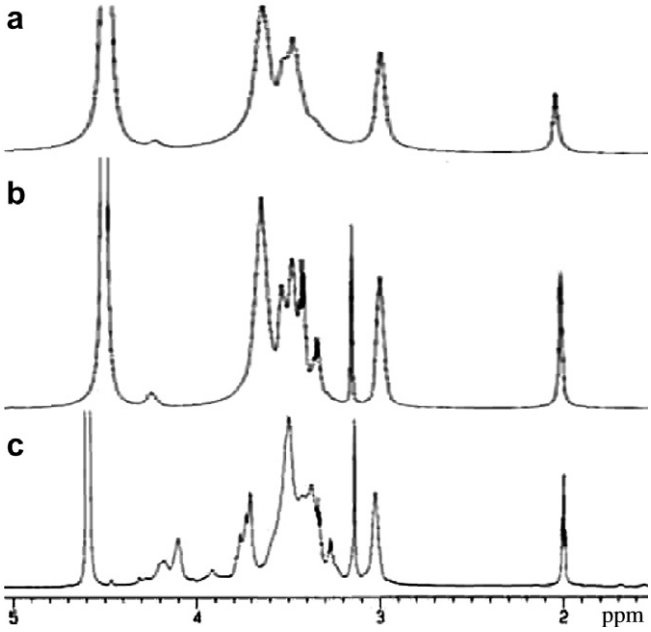

Fig. 1. ^{1}H NMR of chitosan (a), DE-chitosan (b), and CM-chitosan (c) in D_2O and CD_3COOD.

The degrees of substitution by carboxymethyl and deoxycholate group, calculated from the ^{1}H NMR spectrum, were 1.04 and 0.051, respectively.

3.2. Self-aggregation and fluorescence spectroscopy

The self-aggregation propensities of DE-chitosan, CM-chitosan and the parent chitosan were checked with pyrene which is a single molecule fluorescence probe. According to their molecular structure, the polyelectrolytes bearing a hydrophobic group show tendency for intra-molecular assembling, such as the cholesterol derivatives were reported to have properties of forming liquid crystals (Nagano & Seki, 2002) and gelation of organic fluids (Wang, Geiger, Chen, Swanson, & Whitten, 2000). In polar solvent, the associated hydrophobic domains are expected to form a non-polar microenvironment to pyrene (Li, Jiang, Zhang, & Fang, 1997). In the fluorescence spectrum of pyrene, peak I (at 372 nm) was sensitive to the change of microenvironment around its molecule in the solution, and peak III (at 384 nm) was stable and constant. With the assembling of the hydrophobic group around the molecule of pyrene, there is an enhancement in the intensity of peak I, where no effect is seen on the intensity of peak III. The III/I ratio, therefore, was used to study the change in the environmental polarity of amphiphatic molecules upon association in aqueous solution,

and to determine the critical micelle concentrations (Chen, Jiang, Zhang, & Zhou, 1999).

The fluorescence spectra of pyrene in DE-chitosan solution with the concentrations of (A) 10.0, (B) 1.0, and (C) 0.1 mg/ml in 0.1 M acetic acid are shown in Fig. 2. At a concentration of 0.1 or lower, the III/I ratio is constant at 0.55. This is close to the values of 0.6 reported by Amiji (1995), 0.71 reported by Uchegbu et al. (2001) and 0.63 reported by Huang, Dunn, & Zink (2000). When the concentration was increased to 1.0 mg/ml or above, the III/I ratio increased (Fig. 3). That means that at lower concentrations of the polymers there were no change in the polarity environment of pyrene in the solution, whereas at concentrations over 1.0 mg/ml, DE-chitosan exhibited intra-molecular and inter-molecular aggregation to form micelles in the solution around the pyrene molecule (Lee et al., 1998).

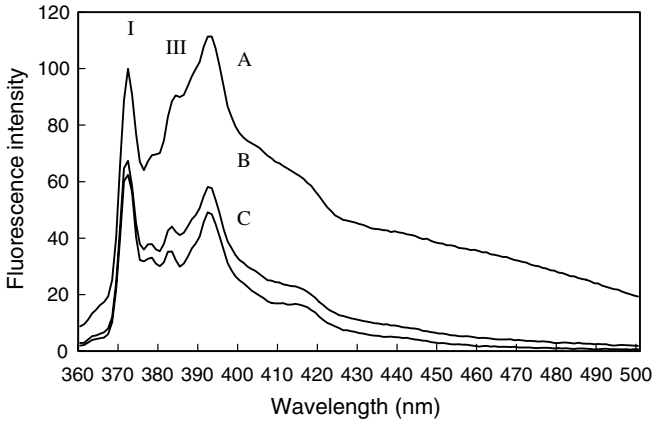

Fig. 2. Fluorescence spectra of pyrene in DE-chitosan with the concentration of (A) 10 mg/ml; (B) 1.0 mg/ml; and (C) 0.1 mg/ml in 0.1 M acetic acid with 2.0 μM pyrene.

By the same method, the pyrene fluorescence characteristics in (0.1 M) acetic acid solution of different concentrations of chitosan and CM-chitosan with different concentrations were measured (Fig. 3). Chitosan, at concentrations over 0.1 mg/ml, is known to exhibit self-aggregation in solution. Amiji (1995) had reported this phenomenon, and was ascribed to the containing inter-molecular hydrophobic interaction between 2-acetamino-2-deoxy-D-glucopyranose (GlcNAc) residues in the polymer chain. Fig. 3 shows that the ratio of III/I from CM-chitosan was changed at the concentration 0.1 mg/ml, the result means CM-chitosan had aggregation properties in the solution. The rheological properties of CM-chitosan in this solution had no significant difference from those of DE-chitosan, and chitosan (concentration to III/I ratio) as shown in Fig. 3. This is interesting since chitosan was a control, to compare the different between the hydrophobic modification and hydrophilic modification, and to see the role of the back bone of the chitosan molecule in the rheology properties). The results show that the structure of parent chitosan plays an important role in the aggregation properties of the modified chitosan. The reason maybe the substitution degree of the deoxycholic group in the chain of DE-chitosan was too low [0.051] and so its molecular self-assembling cannot operate to form micelles.

The graph of III/I ratio for different concentrations of chitosan, DE-chitosan and CM-chitosan in 0.1 M hydrochloric acid was exactly the same as that observed in 0.1 M acetic acid (Fig. 4). The acid had no effect on the self-aggregation behavior. The change of III/I ratio in CM-chitosan solutions of pH 1.0, pH 7.0, and pH 10 are shown in Fig. 5. The graphs of the spectra of III/I ratio in each pH of solution were close.

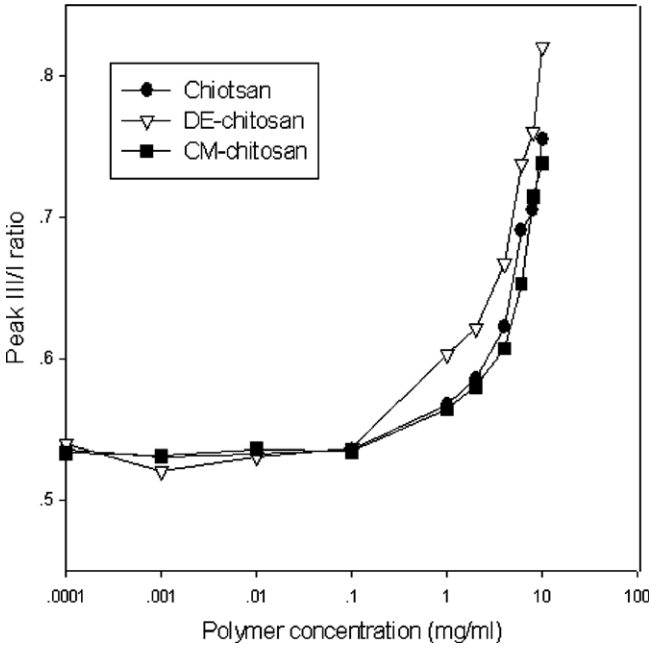

Fig. 3. Peak III/I ratio of pyrene fluorescence as a function of chitosan (●); DE-chitosan (▽); and CM-chitosan (■) concentration in 0.1 M acetic acid.

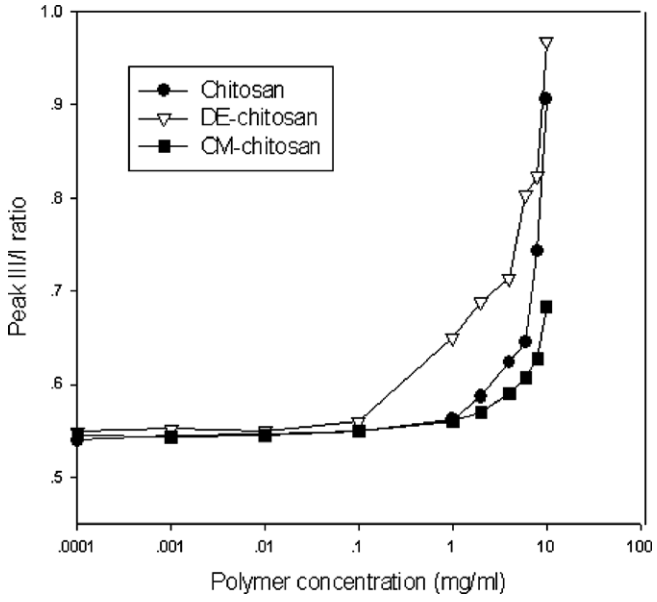

Fig. 4. Peak III/I ratio of pyrene fluorescence as a function of chitosan (●); DE-chitosan (▽); and CM-chitosan (■) concentration in 0.1 M hydrochloric acid with 2.0 μM pyrene.

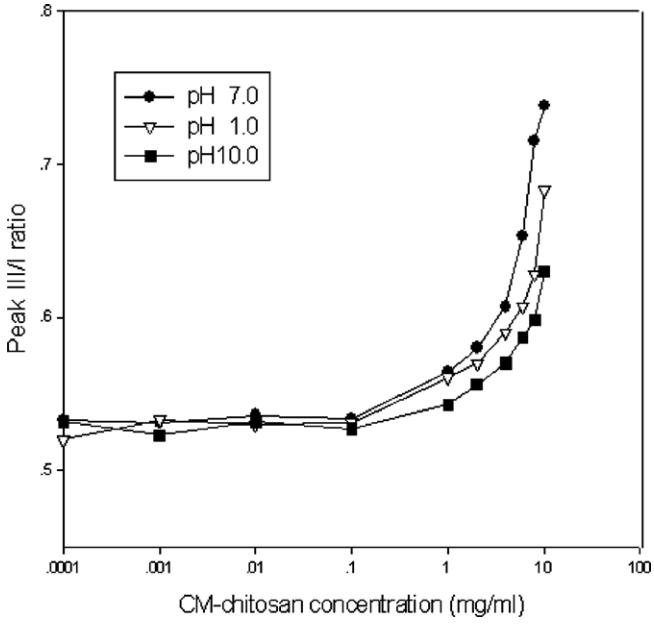

Fig. 5. Peak III/I ratio of pyrene fluorescence as a functional of CM-chitosan concentration in 2.0 μM aqueous pyrene solution of pH 1.0 (▽); pH 7.0 (●); and pH 10 (■).

The aggregation behaviour of chitosan, DE-chitosan and CM-chitosan was influenced by the solution ionic strength (Tables 1 and 2). When the ionic strength increased from 0.00 to 1.0 M (Table 1, NaCl; Table 2, NaAc) all of the polymers had the same tendency in the changes of the peak III/I ratio in their solution. Two Tables 1 and 2 did not show any difference between NaCl and NaAc in the rheological properties of the solution of each polymer. At each ionic concentration in Tables 1and 2, the organic acid (HAc, 0.1 M) and inorganic acid (HCl, 0.1 M) show similar results in the peak III/I ratio for each polymer; that means that the acid solution (0.10 M) had no different influence on the rheological properties between HAc and HCl. In addition, as the ionic concentration increased from 0.00 to 0.10 M (NaCl in Table 1, NaAc in Table 2), all of the polymers almost had no change in the peak III/I ratio, meaning that ionic strength at lower concentration (below 0.10 M) had no influence on the rheology properties.

However, when the ionic concentration increased to 1.0 M (Tables 1 and 2), only DE-chitosans were obviously increased in the peak III/I ratio compared to their ratios in the solution of lower ionic concentration; the data for chitosan and CM-chitosan in the same conditions were not found to change. From Table 1, with the increase of NaCl from 0.10 to 1.00 M, the peak III/I ratio in the DE-chitosan solution were increased from 0.698 to 0.780 (an increase of 11.7%) and from 0.766 to 0.846 (10.4% increase), respectively. From Table 2, on increase of NaAc from 0.10 to 1.00 M, the peak III/I ratios in the DE-chitosan solution were increased from 0.694 to 0.824 (increase 18.7%) and from 0.749 to 0.837 (increase of 11.7%), respectively. It was shown that in higher ionic concentrations (over 1.00 M for NaCl and NaAc) only DE-chitosan was clearly influenced in the rheological properties and resulted in acceleration of molecular self-aggregation in the solutions.

These results show that the ionic strength of the medium enhances the hydrophobic modified group association, but does not enhance the parent chitosan and the hydrophilic group association.

Table 1
Effect of ionic strength (NaCl) on the peak III/I ratio of pyrene fluorescence in chitosan solution[a]

Polymer and solution[b]	Sodium chloride concentration (M)			
	0.00	0.01	0.10	1.0
Chitosan in acetic acid	0.646	0.642	0.622	0.631
Chitosan in hydrochloric acid	0.648	0.671	0.669	0.666
DE-chitosan in acetic acid	0.699	0.734	0.698	0.780
DE-chitosan in hydrochloric acid	0.765	0.767	0.766	0.846
CM-chitosan in water	0.642	0.635	0.661	0.656
CM-chitosan in hydrochloric acid	0.599	0.602	0.604	0.616
CM-chitosan in sodium hydroxide	0.601	0.605	0.597	0.606

[a] The final concentration of pyrene dissolved in the polymer solution was 2.0 μM.
[b] The polymer solution with a concentration of 5.0 mg/ml was prepared in 0.1 M acetic acid, or in 0.1 M hydrochloric acid, or in 0.1 M sodium hydroxide, and or in distilled water.

Table 2
Effect of ionic strength (NaAc) on the peak III/I ratio of pyrene fluorescence in chitosan solution[a]

Polymer and solution[b]	Sodium acetate concentration (M)			
	0.00	0.01	0.10	1.0
Chitosan in acetic acid	0.651	0.643	0.635	0.671
Chitosan in hydrochloric acid	0.670	0.660	0.660	0.661
DE-chitosan in acetic acid	0.685	0.694	0.694	0.824
DE-chitosan in hydrochloric acid	0.747	0.734	0.749	0.837
CM-chitosan in water	0.639	0.647	0.620	0.628
CM-chitosan in hydrochloric acid	0.523	0.603	0.623	0.614
CM-chitosan in sodium hydroxide	0.590	0.585	0.594	0.662

[a] The final concentration of pyrene dissolved in the polymer solution was 2.0 μM.
[b] The polymer solution with a concentration of 5.0 mg/ml was prepared in 0.1 M acetic acid, or in 0.1 M hydrochloric acid, or in 0.1 M sodium hydroxide, and or in distilled water.

3.3. Nano-micelles particles formation of DE-chitosan

Sodium chloride enhanced the molecular self-aggregation of DE-chitosan in solution, but could not bring the DE-chitosan molecule into micelles formation. Methylene chloride (1%, v/v) was added into the DE-chitosan solution (1.0 mg/ml, contained 1.0 M NaCl) to make an influence to the formation of molecular aggregation of DE-chitosan by the method of emulsification in methylene chloride/DE-chitosan solution. After the emulsification with methylene chloride and cross-linking with STPP the aggregated DE-chitosan micelle were solidify to form the particles. The particles could be seen with Transmission Electron Micrograph (TEM), the sizes

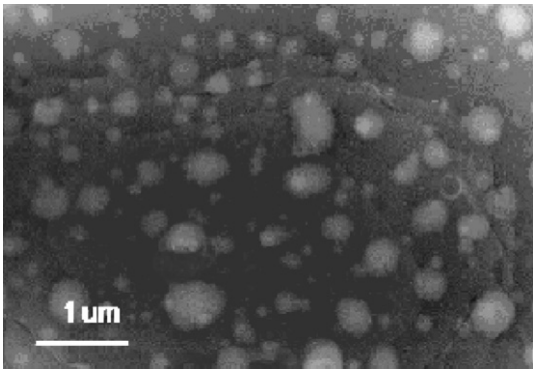

Fig. 6. Transmission electron micrograph of the nanoparticles of DE-chitosan. The specimen was prepared after emulsification of DE-chitosan solution with methylene chloride.

were 200–600 nm in diameter (seen in Fig. 6). The result shown that the methylene chloride emulsification made an important role in the controlling of micelles formation during the process of molecular aggregation of DE-chitosan. The methylene chloride representing the oil phase in the emulsion attracted the hydrophobic groups of DE-chitosan around the vacuole, and the chitosan moiety was at the outer part of the vacuole. After the methylene chloride was removed by vaporization under vacuum, the ordered micelles were believed to have become formed with the DE groups on the inner-core and chitosan in the outer-shell since the all of the process take placed in aqueous system.

Tocopherol acetate and stearic acid methyl ester were examined for its encapsulation efficiency in the DE-chitosan micelle-particles. The efficiency was found to be 50%, and 71%, respectively. The result means that the solid particles were micelle-capsules, and had ability to carry the lipidic compounds, had ability to equably disperse lipidic drugs into aqueous system with the nano-size. This property would bring DE-chitosan a great potential utility in the technology of drug nano-packaging.

4. Conclusions

Chitosan, CM-chitosan, and DE-chitosan had similar properties of self-aggregation in aqueous solutions. Ionic strength was found to have no influence on CM-chitosan as well as the parent chitosan in respect of the self-aggregation, but enhanced the aggregation properties of DE-chitosan in solution. The assemblage of DE-chitosan in sodium acetate solution could be controlled by means of emulsification of the DE-chitosan solution with methylene chloride. The emulsification of methylene chloride plays an important role in the formation of well-regulated micelles of DE-chitosan. From such micelles of DE-chitosan, solid capsules in nano-size of 200–600 nm diameter could be formed and these had the ability to encapsulate tocopherol acetate and stearic methyl ester. These results bring a potentially new technology to nano-capsule preparation and new lipid drug carriers.

Acknowledgments

We acknowledge the ISTCP(2006DFA33150), NSFC (30670566) and KOSEF (1999-2-220-009-4) for the financial support in this study.

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