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Preparation of polymeric micelles based on chitosan bearing a small amount of highly hydrophobic groups

Gang-Biao Jiang, Daping Quan *, Kairong Liao, Haihua Wang

Institute of Polymer Science, School of Chemistry and Chemical Engineering, Sun Yat-sen University, Guangzhou 510275, China

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Abstract

A method has been developed to obtain micelles based on amphiphilic chitosan derivatives which were synthesized by grafting hydrophobic stearoyl, palmitoyl and octanoyl aliphatic chains onto molecules of chitosan with degrees of substitution from 0.9% to 29.6%. The *N*-fatty acylations were carried out by reacting carboxylic anhydride with chitosan in dimethyl sulfoxide. The chitosan derivative-based micelles were spherical as observed by transmission electron microscope (TEM). Their sizes were in the range of 140–278 nm as measured by dynamic light scattering (DLS). The micellar critical aggregation concentration (CAC) can reach 1.99×10^{-3} mg/mL, indicating that they are more stable upon dilution than micelles based on other chitosan derivatives such as deoxycholic acid-modified chitosan reported previously.

Keywords: Chitosan; *N*-Fatty acyl; Micelle

1. Introduction

Polymer micelles have been regarded as one class of the most promising carriers for delivering bioactive materials such as water-insoluble drugs, hormones and plasmid DNA. (Ngimhuang, Furukawa, Satoh, Furuike, & Sakairi, 2004). One important class of micelle-like polymer aggregates is hydrophobically modified natural polymers, i.e., polysaccharides with low grafting levels of hydrophobic groups such as bulky cholesteryl groups and long alkyl or acyl chains (Auzély-Velty & Rinaudo, 2002; Esquenet & Buhler, 2001; Nishikawa, Akiyoshi, & Sunamoto, 1994; Panmai, Prud'homme, Peiffer, Jockusch, & Turro, 2002; Park et al., 2004; Ramos, Rodríguez, Rodríguez, Heras, & Agulló, 2003; Yoshioka, Nonaka, Fukuda, & Kazama, 1995).

Chitosan, α-(1–4)-2-amino-2-deoxy-β-D-glucan, is the second most abundant biopolymer of renewable natural sources. Its unique characteristics such as biocompatibility, biodegradability, nontoxicity and positive charge make this biopolymer ideal as drug carrier (Janes, Calvo, & Alonso, 2001; Mura, Zerrouk, Mennini, Maestrelli, & Chemtob, 2003). Recently, polymer micelles prepared by hydrophobically modified chitosan were reported (Kwon et al., 2003; Liu et al., 2005; Lee, Jo, Kwon, Kim, & Jeong, 1998a, 1998b; Zhang et al., 2003). For example, Lee et al. (1998a, 1998b) obtained micelles through chitosan grafted deoxycholic acid in dilute acidic solution in the presence of 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC). However, the degree of substitution (DS) of these chitosan derivatives was relatively low (2.8–5.1%), leading to the high critical aggregation concentration (CAC 1.7×10^{-2}–4.1×10^{-2} mg/mL). Therefore, the formed micelles were unstable against dilution. Although Li and Kwon (2000) reported that the increase in the level of hydrophobic attachment led to high stability of the polymer micelles, Lee et al. (1998a) found that the derivatives with higher DS were difficult to be synthesized because the solubility of deoxycholic acid in the acidic reaction medium was very low. The same situation happened to obtain linolenic acid-

* Corresponding author. Tel.: +86 020 84114030; fax: +86 020 84112245.
E-mail address: cedc36@zsu.edu.cn (D. Quan).

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modified chitosan with higher DS (DS 1.8%, CAC 5×10^{-2} mg/mL) (Liu et al., 2005).

In this paper, a series of *N*-fatty acyl chitosans with acyl groups of different lengths were prepared, and their structures were clarified by means of FT-IR, ^{1}H NMR and X-ray diffraction. Their self-aggregation behaviors were examined by dynamic light scattering (DLS) and fluorescence spectrometry, and the morphologies of their aggregates were observed by transmission electron microscopy (TEM).

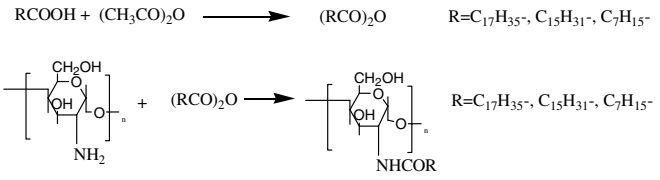
2. Experiments

2.1. Materials

Chitosan (M_w 190 kDa, 90–95% deacetylated) was obtained from Boao Biotechnology Co. (Shanghai, China). Stearic acid, palmitic acid, octanoic and acetic anhydride were purchased from Guangzhou Chemical Factory (Guangzhou, China). Pyrene was purchased from Aldrich Chemical Co. (USA). All reagents were of analytical grade and were used as received.

2.2. Synthesis of N-acyl chitosans (ALCSs)

Synthetic approach was carried out as described earlier (Jiang, Quan, Liao, & Wang, 2006), as shown in Scheme 1. The chitosan (2.0 g, 0.012 mol) was dissolved in 100 mL acetic acid (0.1 M), precipitated with 55 mL NaOH (0.2 M), and then collected by filtration and washed with water. The incompact or swollen chitosan powder was dispersed in 100 mL dimethyl sulfoxide (DMSO) with magnetic stirring, a fatty anhydride (0.024–0.048 mol), which was prepared through reaction of *n*-fatty acid with acetic anhydride (Xing, Xu, & Zhou, 1980), was added dropwise to the mixture and allowed to react at 60 °C for 8 h under stirring. The above solution was poured into acetone, and the precipitate was collected by filtration and washed five times with acetone and diethyl ether. The product was dried at 60 °C for 48 h under vacuum. The DS, defined as the number of the acyl groups per 100 sugar residues of chitosan, was determined by ^{1}H NMR using the ratio of acyl methyl protons ($\delta = 0.85$ ppm) to H-2 protons ($\delta = 2.89$ ppm) of chitosan. The numbers behind the sample codes SCS, PCS and OCS indicate the DS of the derivatives of chitosan coupling with stearoyl groups, palmitoyl groups and octanoyl groups, respectively.



Scheme 1. The synthesis of ALCSs.

2.3. Solubilities of ALCSs

Ten milligram of an ALCS sample was placed into a test tube with each of solvent 4 mL. After mixing with a vortex mixer and then with an ultrasonicator, the mixture was stored at room temperature for 5 days, and visually observed (Ngimhuang et al., 2004).

2.4. Characterization of ALCSs

FT-IR spectra were recorded on a Nicolet 670 FT-IR spectrometer (Thermo Nicolet, USA) in KBr discs. ^{1}H NMR spectra were performed on a Mercury Plus 300 MHz spectrometer (Varian, USA) using deuterated DMSO (DMSO-d_6).

X-ray powder diffractions were collected using a D/Max 2200 VPC diffraction meter (Rigaku, Japan) with Cu Kα radiation in the range 5°–40° (2θ) at 40 kV and 30 mA.

Fluorescence spectra were performed on a FLS920 Fluorescence spectrometer (Edinburgh Instruments Ltd., UK). A sample solution containing 6.0×10^{-7} M pyrene was excited using a xenon lamp. The concentration of sample solution was varied from 1.28×10^{-6} to 0.5 mg/mL. I_1 and I_3 were taken from the emission intensities of pyrene at 372 and 383 nm, respectively. The slit openings for excitation and emission were both set at 0.5 mm, the excitation wavelength (λ_{ex}) was 334 nm, and the spectra were accumulated with an integration time of 1 s/nm.

The hydrodynamic radii and polydispersity of ALCS micelles were determined by DLS at 25 °C using a BI-200SM Goniometer light scattering spectrophotometer (Brockhaven, USA). Distilled water solutions of an ALCS in glass cell were measured with a vertically polarized incident beam at 532 nm at a detector angle of 90°.

The morphologies and size distributions were observed by TEM using a JEM-2010 electron microscope (JEOL, Japan) operating at an accelerating voltage of 20–100 kV. Samples' negative staining was performed as follows: a drop of sample solutions (concentration = 1 mg/mL) was placed onto a 200 mesh copper grid coated with carbon, taped with a filter paper to remove surface water and air-dried for 5 min. These self-aggregates were deposited on the grid, followed by the application of methylamine tungstate negative staining. The samples were air-dried before measurement.

3. Results and discussion

3.1. Synthesis and characterization of ALCSs

The poor solubility of chitosan in water is mainly due to its high crystallinity and strong inter- or intra-molecular hydrogen bonding. Therefore, introduction of appropriate substituents into chitosan backbone may likely disrupt the inter- or intra-molecular hydrogen bonding of chitosan and

weaken its crystallinity as well (Zong, Kimura, Takahashi, & Yamane, 2000), and thus is in favor of solvating chitosan in water. However, an excessive hydrophobic substitution would generate water-insoluble derivatives due to strong hydrophobic interaction following a "hydrophobic self-assembling" model (Wang, McConaghy, Tetley, & Uchegbu, 2001).

In this study, a series of ALCSs with different DSs were prepared by heterogeneous reaction of anhydrides and swollen chitosan in DMSO while controlling the feed molar ratio of glucosamine residues to an anhydride. As shown in Table 1, the DSs of SCSs increased along with the increasing of feed molar ratio of stearic anhydride to chitosan. Moreover, the shorter of the acyl group length was, the higher DS of its acylated chitosan derivative was obtained.

The solubilities of ALCSs were tested before their spectroscopic characterization. As shown in Table 2, the ALCSs dissolved in water and DMSO and yielded transparent solutions. However, they were partly soluble in methanol and only swollen in ethanol.

Structure change of chitosan after acylation was confirmed by FT-IR spectra (Fig. 1). Chitosan has an absorption at 1574 cm^{-1} ascribed to the N–H bending vibration mode of nonacylated α-aminoglucose primary amines, and a very weak absorption at 1638 cm^{-1} due to the presence of the acetylated groups (Xu, McCarthy, Gross, & Kaplan, 1996). After *N*-acylation, the absorption at 1574 cm^{-1} almost disappeared even for SCS0.9, while prominent bands at 1638 and 1525 cm^{-1} were observed, which were assigned to the carbonyl stretching vibration mode of secondary amide (amide band I) and the bending vibration mode of the amide band II, respectively. No absorptions in the range of 1710–1760 cm^{-1} were observed in the spectra of the *N*-acylated chitosans, which are indicative of the existence of *O*-acyl ester groups (Wu, Zeng, Zhu, & Tong, 2005). Therefore, the above results clearly showed that *N*-acylated chitosans were obtained and the reaction was highly selective toward *N*-acylation. This phenomenon is consistent with the report by Félix, Hernández, Argüelles-Monal, and Goycoolea (2005). However, the acylated chitosans herein are different from those reported by Badawy et al. (2004) and Zong et al. (2000), who used acyl chloride to couple with soluble chitosan or swollen chitosan, and thus obtained *N*,*O*-acyl chitosan with higher DS.

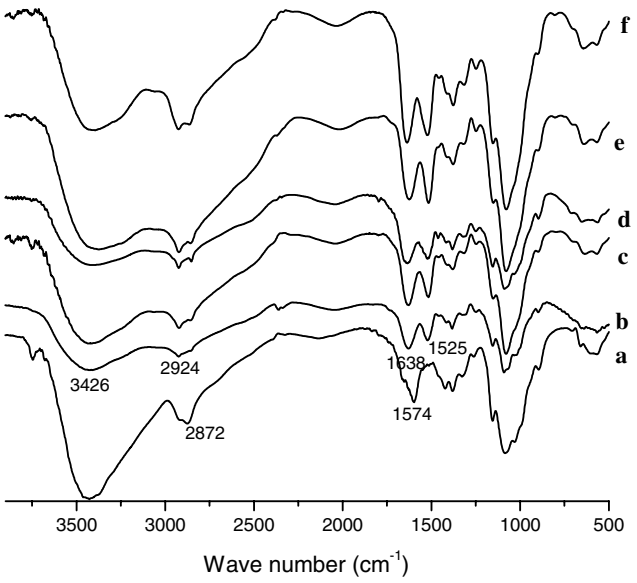


Fig. 1. FT-IR spectra of (a) CS, (b) SCS0.9, (c) SCS8.2, (d) SCS13.5, (e) PCS14.2 and (f) OCS29.6.

Table 1
Effects of feed molar ratios on DSs of ALCSs

Sample	Feed molar ratio[a]	Yield[b] (%)	DS[c] (%)
SCS0.9	1.0:1.0	84	0.9
SCS8.2	1.0:2.5	77	8.2
SCS13.5	1.0:3.0	73	13.5
PCS14.2	1.0:3.0	75	14.2
OCS29.6	1.0:3.0	77	29.6

[a] Molar ratio of chitosan vs acid anhydride in feed.
[b] Yield estimated by weight recovery in accordance with theoretical weight of products (theoretical weight = $W_{chitosan} + W_{Chitosan} \cdot DS \cdot M_{RCO-}/161$).
[c] DSs determined from the integration resonance ratio of the CH_3 groups within acyl blocks vs H-2 of chitosan residues detected by ^1H NMR.

The ^1H NMR spectra of ALCSs are shown in Fig. 2. Proton assignments are as follows: $\delta_{0.85} = CH_3$ (stearoyl), $\delta_{1.23} = CH_2$ (stearoyl), $\delta_{1.49} = CH_2$ (stearoyl deshielded by carbonyl, β to carbonyl), $\delta_{1.85} = CH_2$ (stearoyl deshielded by carbonyl, α to carbonyl), $\delta_{2.08} = CH_3$ (acetyl), $\delta_{2.88}$ to a H-2 proton, $\delta_{3.34–3.71}$ to the ring protons (H-3, 4,5,6,6′) overlapped with H_2O, $\delta_{4.95–5.44}$ to the H-1 protons and $\delta_{8.54}$ to N–H. The resonance intensities of CH_3 and $–CH_2–$ of acyl chain groups varied directly with the DS,

Table 2
Solubilities of chitosan and ALCSs in various solvents

Sample	Water	DMSO	Methanol	Ethanol	Acetone	THF	Dichloromethane
CS	▼	▼	▼	▼	▼	▼	▼
SCS0.9	□	○	◇	■	▼	▼	▼
SCS8.2	□	○	◇	■	▼	▼	▼
SCS13.5	□	○	◇	■	▼	▼	▼
PCS14.2	□	○	◇	■	▼	▼	▼
OCS29.6	□	○	◇	■	▼	▼	▼

□, highly soluble; ○, soluble; ◇, partly soluble; ■, swelling; ▼, insoluble.

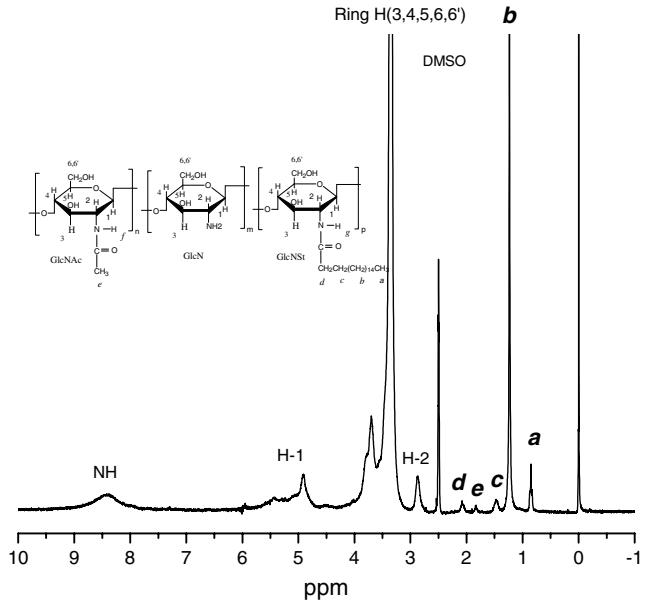


Fig. 2. ^{1}H NMR spectrum of SCS13.5 in DMSO-d_6.

while the resonance intensity of H-2 did not alter with the degree of hydrophobic substitution. Therefore, the ratios of $I_{CH3}/3I_{H\text{-}2}$ were used to determine the DS values of ALCSs.

3.2. The properties of ALCSs

Compared with the nonacylated chitosan, which had obvious crystalline diffractions at $2\theta = 20°$ and 10°, ALCSs showed a broad diffraction around $2\theta = 25°$ (Fig. 3). These results suggested that the crystalline structure of chitosan had been disrupted and its crystallinity had been weakened by the introduction of acyl substituents. Similar results were reported by Zong et al. (2000).

The aggregation behavior of ALCSs in aqueous medium was determined by fluorescence spectrometry using

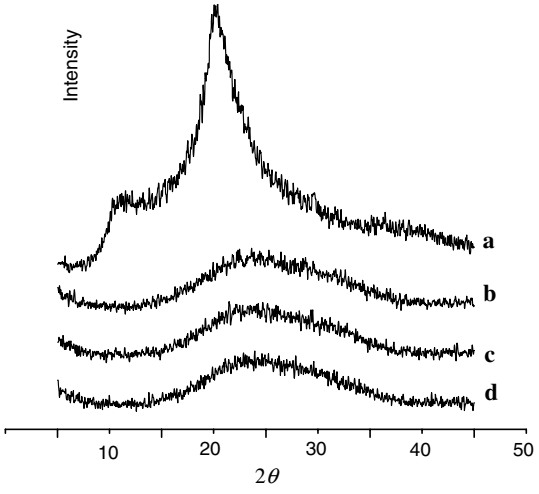


Fig. 3. WAXD patterns of (a) CS, (b) SCS0.9, (c) SCS8.2 and (d) SCS13.5.

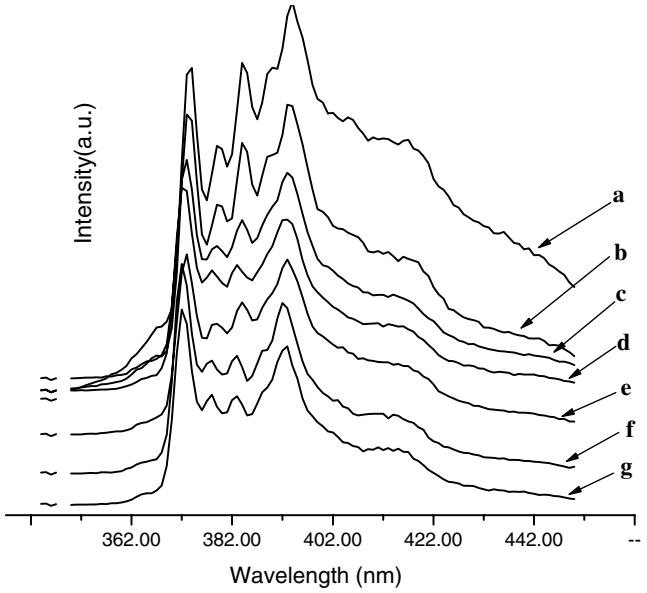


Fig. 4. Emission spectra of pyrene (6.0×10^{-7} mg/mL) as a function of SCS8.2 concentration (a) 2 mg/mL, (b) 0.4 mg/mL, (c) 0.08 mg/mL, (d) 0.016 mg/mL, (e) 0.0032 mg/mL, (f) 0.0064 mg/mL and (g) 0.000128 mg/mL in water.

pyrene as a fluorescence probe. Fig. 4 shows the fluorescence emission spectra of pyrene incorporated into self-aggregates of SCS8.2 in water at 25 °C. Emission spectra of pyrene monomer strongly depend on the polarity of the microenvironment. If micelles or other hydrophobic microdomains are formed in an aqueous solution, the pyrene preferably lies close to these microdomains and shows strong emission, while its emission is weak while quenched in polar media. In polar solvent, there is an enhancement in the intensity of I_1 (at 372 nm), whereas no effect is seen on that of I_3 (at 383 nm). The intensity ratio of I_1/I_3, therefore, can be used to determine the critical micelle concentrations (CACs) of various surfactants (Amiji, 1995). The change of the intensity ratio is shown in Fig. 5. At low concentrations of ALCSs, the ratios were close to those observed in water and other polar solvents, and a linear decrease was observed with the addition of polymeric amphiphiles as its concentration was higher than the CACs. The CACs were determined by the interception of two straight lines of each ALCS. The CAC values of SCS13.5, SCS8.2 and PCS14.2 are roughly 2 orders of magnitude lower than those of low molecular weight surfactants (e.g., 0.52 mg/mL for cetyltrimethyl ammoniumbromide) and other chitosan derivatives, e.g., deoxycholic acid-modified chitosan (in the range of 1.7×10^{-2} to 4.1×10^{-2} mg/mL) (Lee et al., 1998a), linolenic acid-modified chitosan (5×10^{-2} mg/mL) (Liu et al., 2005), and reductive derivative of 3-*O*-dodecyl-D-glucose coupled with chitosan (0.1 mg/mL in neutral aqueous media) (Ngimhuang et al., 2004). The lower CAC values of ALCSs indicate that a small amount of ALCSs can form self-aggregates and maintain the stability in dilute solution.

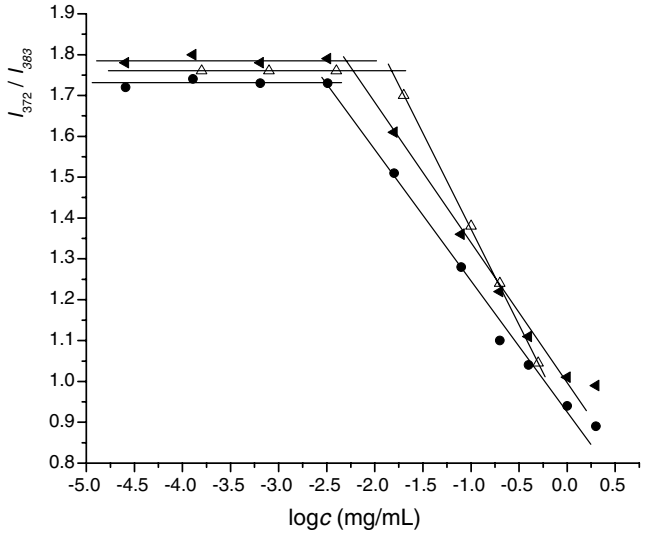


Fig. 5. Intensity ratio (I_{372}/I_{383}) from pyrene emission spectra of SCSs as a function of concentration in water: (Δ) SCS0.9, (◀) SCCS8.2 and (●) SCS13.5.

It is noticed that the CAC values of ALCSs decreased with increasing DS (Table 3). This phenomenon is consistent with the results reported by Li and Kwon (2000). On the contrary, the CAC of OCS29.6 is much greater than those of SCS13.5, SCS8.2 and PCS14.2, although its DS is much higher than the latter, indicating that the acyl chain length has great effect on the CAC value. It was also pointed out by Laschewsky (1995) that aggregation could be promoted by an increase in hydrophobic group with a minimum chain length of eight carbons. In our viewpoint, the formation of micelles by self-aggregation of ALCSs in the aqueous solution is basically determined by their amphiphilicity, and this is related to the chain length of acyls and the DS of ALCSs. According to this suggestion, the amphiphilicity of chitosans modified by octanoyl groups is weaker because the length of octanoyl group is shorter and thus its hydrophobicity is poorer than those of stearoyl and palmitoyl groups. Therefore, the OCS29.6 is not easy to self-aggregate and forms micelles in aqueous solution. Moreover, the SCSs and PCS14.2 possessing suitable hydrophobic chain length and DS have lower CAC values. These results indicate that the stability of the micellar structure can be controlled by adjusting the balance between hydrophobic acyl groups and hydrophilic chitosan in an ALCS molecule.

It can be seen from Table 3 that the lower the CAC value, the smaller the size of the self-aggregates. This indicates that an ALCS with suitable length of acyl groups and DS will self-aggregate easily in aqueous medium and forms more compact hydrophobic cores as a result of stronger van der Waals forces among the hydrophobic chains. In the case of OCS29.6, the van der Waals interaction among the octanoyl groups is weaker because the octanoyl chain is shorter and consequently less compact self-aggregates were produced.

The molecular weight of chitosan is another factor affecting the size of micelles. Kwon et al. (2003) and Wang et al. (2001) reported that the increase of chitosan in molecular weight would result in an increase in the size of self-aggregates. Although the molecular weight of the starting material chitosan ($M_w = 1.9 \times 10^5$) used here is much higher than those of deoxycholic acid-modified chitosan ($M_w = 7 \times 10^4$) (Lee et al., 1998a) and linolenic acid-modified chitosan (low molecular weight) (Liu et al., 2005), the mean diameters (140–144 nm) of SCS8.2, SCS13.5 and PCS14.2 micelles are smaller than those of deoxycholic acid-modified chitosan (159–180 nm) (Lee et al., 1998a) and linolenic acid-modified chitosan (211 nm) (Liu et al., 2005). This may be attributed to the bulky deoxycholic group which is unfavorable for the deoxycholic acid-modified chitosan to forming tight hydrophobic cores. In contrast, the lower DS of linolenic acid-modified chitosan (1.8%) and higher rigidity of linolenic group compared to the saturated fatty acyl groups studied here were assumed to be the major reasons of forming those larger aggregates.

As shown in Table 4, with increasing the concentration of NaCl, the CAC values of ALCSs decreased because that sodium chloride facilitated self-aggregation of acylated chitosans in aqueous medium (Lee et al., 1998a). It also shows that the CAC values of ALCSs decreased with the increasing of the pH value of the medium, indicating that the self-aggregation of acylated chitosans is affected significantly by the protonation extent of amino groups of the acylated chitosans.

Overall, to obtain small size micelles with tightly packed cores, adequate long hydrophobic groups and properly high DS are prerequisite, and only under such conditions,

Table 3
Effects of DSs and substituents on the properties of self-aggregates of ALCSs

Sample	CAC[a] × 10^3 (mg/mL)	d[b] (nm)	Polydispersity[b]
SCS0.9	16.20	211	0.163
SCS8.2	4.98	144	0.251
SCS13.5	3.24	143	0.190
PCS14.2	1.99	140	0.219
OCS29.6	25.50	287	0.398

[a] Determined by fluorescence spectrometry.
[b] The size and the polydispersity of self-aggregates determined by DLS.

Table 4
Effects of NaCl concentration and pH value of the media on the properties of SCS8.2 self-aggregates at 25 °C[a]

Concentration of NaCl (mol/L)	pH	CAC[b] × 10^3 (mg/mL)
0	7.0	4.98
0.01	7.0	4.87
0.1	7.0	4.63
1.0	7.0	2.84
0	3.5	7.24
0	9.0	2.07

[a] The concentrations of SCS8.2 were from 2.0 to 1.28×10^{-4} mg/mL.
[b] Determined by fluorescence spectrometry.

the preparation of self-aggregates with low CAC would become possible. TEM observations are shown in Fig. 6. The shapes of the ALCS micelles were observed as approximately spherical. The diameters of aggregates for SCS8.2, SCS13.5 and PCS14.2 were in the range of 20–50 nm, whereas those for samples of SCS0.9 and OCS29.6 lay in 50–80 nm. Compared with the results in Table 3, it can be found that the sizes of the aggregates evaluated by TEM are much smaller than those measured by DLS, and this may be ascribed to the difference in the state of the aggregates. Because before TEM observations, samples were dried and the aggregates shrank in this process, therefore, the sizes of the aggregates are smaller than those in aqueous medium.

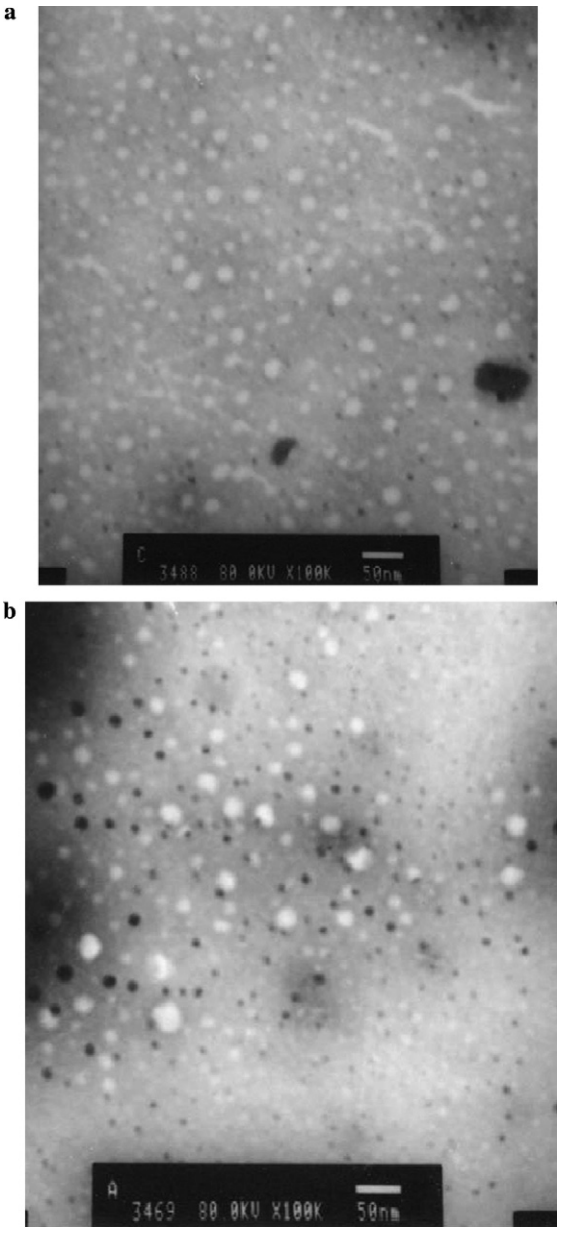


Fig. 6. TEM micrographs of self-aggregates based on (a) PCS14.2 and (b) SCS8.2.

4. Conclusion

Polymer micelles based on amphiphilic chitosan derivatives by grafting hydrophobic long acyl chains with different DSs have been prepared and they can form nanoparticles via self-aggregation in water. The CAC values and the size of the micelles are mainly determined by the amphiphilicity of ALCSs. It is proposed that micelles with smaller size could be obtained with high molecular weight of chitosan by *N*-acylating it with suitable hydrophobic groups and DS.

Acknowledgements

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