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Preparation and characterization of self-aggregated nanoparticles of cholesterol-modified *O*-carboxymethyl chitosan conjugates

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Abstract

A series of cholesterol-modified *O*-carboxymethyl chitosan (CCMC) conjugates with different degrees of substitution (DS) of cholesterol moiety were synthesized by the succinyl linkages and characterized by Fourier transform infrared (FTIR), proton nuclear magnetic resonance (^{1}H NMR) and elemental analysis. CCMC conjugates were amphiphilic in nature and their self-aggregation behavior in aqueous media was evaluated by the fluorescence probe technique. CCMC self-aggregated nanoparticles were prepared by probe sonication in water and analyzed by dynamic laser light-scattering (DLLS), zeta potential and transmission electron microscopy (TEM) technologies. These novel nanoparticles were almost spherical in shape, and their size, ranging from 234.9 to 100.1 nm, could be controlled by DS of cholesterol moiety. The zeta potentials of CCMC self-aggregated nanoparticles were negative, and the absolute values decreased with increasing DS of the cholesterol moiety. This study also compared the morphology and the stability of self-aggregated nanoparticles between CCMC and cholesterol-modified chitosan (CHCS). The results showed that the negatively charged carboxymethyl groups are advantageous for the formation of well-shaped and stable self-aggregated nanoparticles.

Keywords: Cholesterol; *O*-Carboxymethyl chitosan; Polymeric amphiphile; Self-aggregated nanoparticles

1. Introduction

Polymeric amphiphiles consisting of hydrophilic and hydrophobic segments can form micelles or micelle-like self-assemblies with a hydrophobic core and a hydrophilic shell due to non-covalent association arising from intra- and/or intermolecular interactions among hydrophobic segments in aqueous media (Dalhaimer, Bermudez, & Discher, 2004; Mortensen, 2001; Rotureau, Chassenieux, Dellacherie, & Durand, 2005; Whitesides, Mathias, & Sato, 1991). Because these self-assemblies have potential uses in biotechnology and medicine due to their unique supramolecular structures (Akiyoshi, 2002; Jones & Leroux, 1999; Kimura, Kidchob, & Imanishi, 2001; Nagarajan, 2001; Ould-Ouali et al., 2004; Torchilin, 2005), various polymeric amphiphiles have been synthesized and their physicochemical characteristics have been studied extensively (Harada & Kataoka, 1999; Ouchi et al., 2002; Qiu, Zhang, Ruegsegger, & Marchant, 1998; Zhu & Nichifor, 2002). Recently, many efforts have been performed to prepare biodegradable and non-toxic polymeric amphiphiles on the basis of natural biomaterials such as polysaccharides (Akiyoshi & Sunamoto, 1996).

Water-soluble polysaccharides such as pullulan, dextran and heparin, modified with hydrophobic groups, e.g. long alkyl, alkyl acyl and deoxycholic acid, can form the monodisperse self-aggregated nanoparticles by sonication in aqueous media, and the morphology of these nanoparticles can be controlled by the chemical structure of hydrophobically modified polysaccharides, e.g. the molecular weight,

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the types and the degree of substitution (DS) of the hydrophobic groups (Akiyoshi, Deguchi, Moriguchi, Yamaguchi, & Sunamoto, 1993; Akiyoshi, Deguchi, Tajima, Nishikawa, & Sunamoto, 1995; Akiyoshi, Deguchi, Tajima, Nishikawa, & Sunamoto, 1997; Nichifor, Lopes, & Carpov, 1999; Park et al., 2004). Several investigations have demonstrated that this kind of self-aggregated nanoparticles is suitable for trapping hydrophobic drugs and some biomacromolecules such as proteins and genes to improve their stability, control their release and intensify their bioactivity (Akiyoshi et al., 1998; Kurian, Zschoche, & Kennedy, 1996; Nishikawa, Akiyoshi, & Sunamoto, 1994).

Chitosan, a homopolymer of (1,4)-linked 2-amino-2-deoxy-β-glucan, is produced by the deacetylation of chitin that is the second most abundant, renewable natural polysaccharide after cellulose. Chitosan and its derivatives have been used in many biomedical applications due to their good bioproperties such as biocompatibility, biodegradability, non-toxicity and bioadhesivity (Fini & Orienti, 2003; Ravi Kumar, Muzzarelli, Muzzarelli, Sashiwa, & Domb, 2004). Hydrophobically modified chitosan derivatives are being studied, with the main focus on various alkylated chitosan (Liu, Zhang, Sun, Sun, & Yao, 2003a; Liu, Zhang, Sun, & Yao, 2003b), acylated chitosan, e.g. linolenic acid-modified chitosan (Chen, Lee, & Park, 2003; Liu, Desai, Chen, & Park, 2005), palmitoyl chitosan (Sludden, Uchegbu, & Schaetzlein, 2000; Tien & Lacroix, 2003; Wang, McConaghy, Tetley, & Uchegbu, 2001), and deoxycholic acid-modified chitosan (DAMC) (Lee, Jo, Kwon, Kim, & Jeong, 1998; Lee & Jo, 1998; Lee, Kim, Kwon, & Jeong, 2000; Lee, Kwon, Jo, & Jeong, 2005). However, due to the rigidity of the molecular chains in water, it is difficult for hydrophobically modified chitosan derivatives to form perfect spherical-shaped self-aggregated nanoparticles (Kim, Gihm, & Park, 2001). Therefore, we believe that changing the rigidity of chitosan molecular chains by hydrophilic modification will improve the morphology of this kind of self-aggregated nanoparticles.

In this study, chitosan was hydrophilically modified by *O*-carboxymethylation to increase the flexibility of chitosan molecular chains in water (Yin, Hou, Jiang, & Gu, 2004; Zhao, Yuan, & Chang, 2004), then followed by the hydrophobic modification with cholesterol to yield novel polymeric amphiphiles, cholesterol-modified *O*-carboxymethyl chitosan (CCMC) conjugates with different DS of cholesterol moiety, which were used to prepare self-aggregated nanoparticles in water by probe sonication. Cholesterol is an indispensable structural building block in cells and plays a major role in many body functions. Cholesterol is often used to hydrophobically modify biomaterials due to its rigid and highly hydrophobic sterol skeleton (Akiyoshi, Yamaguchi, & Sunamoto, 1991; Akiyoshi et al., 1993; Akiyoshi et al., 1998; Chern, Chiu, & Chuang, 2004; Nishikawa et al., 1994). It has been reported that cholesterol-bearing polysaccharides can form more stable nanoparticles compared with palmitoyl-bearing polysaccharides (Akiyoshi et al., 1991). Therefore, we chose cholesterol as the hydrophobically modifying groups. Furthermore, we also investigated the relationships among the chemical structure, the amphiphilic property and the morphology of CCMC self-aggregated nanoparticles in this study.

2. Experimental methods

2.1. Materials

Cholesterol, succinic anhydride and 1,3-dicyclohexyl carbodiimide (DCC) were obtained from SINOPHARM Chemical Reagent Co., Ltd. *N*-Hydroxyl succinimide (NHS) was obtained from Sigma without further purification. Pyrene was obtained from Aldrich and purified by recrystallization from absolute ethanol. Chitosan (deacetylation degree was 92%, viscosity average molecular weight was 8.0×10^4 Da) was obtained from Yuhuan Ocean Biochemical Co., Ltd. All other chemicals were analytical grade and were obtained from Aldrich.

2.2. Synthesis of cholesterol succinate (CHS)

Synthesis was carried out according to the method previously reported (Shaikh, Maldar, Lonikar, Rajan, & Ponrathnam, 1998). Briefly, cholesterol (5.0 g, 13.0 mmol) was mixed with succinic anhydride (3.6 g, 36.0 mmol) in 20 mL of pyridine. After reaction for 24 h at room temperature, the mixture was precipitated in the ice dilute hydrochloric acid solution. The white powder of CHS (5.1 g, 10.5 mmol) was obtained by recrystallization in tetrahydrofuran (THF) and ethanol.

IR (KBr, cm^{-1}): 3442 (O—H stretch), 1709, 1732 (C═O stretch of succinyl group), 1180 (C—O—C stretch). ^{1}HNMR ($CDCl_3$ with TMS, ppm): 0.67 (3H, ***s***, cholesterol 18-H_3), 0.8–2.4 (28H, cholesterol 1-H_2, 2-H_2, 4-H_2, 7-H_2, 8-H_1, 9-H_1, 11-H_2, 12-H_2, 14-H_1, 15-H_2, 16-H_2, 17-H_1, 20-H_1, 22-H_2, 23-H_2, 24-H_2 and 25-H_1), 0.86 (6H, ***d***, $J = 9$ Hz, cholesterol 26-H_3 and 27-H_3), 0.91 (3H, ***d***, J = 7 Hz, cholesterol 21-H_3), 1.02 (3H, ***s***, cholesterol 19-H_3), 2.72 (2H, ***m***, $COCH_2$), 2.93 (2H, ***m***, CH_2CO), 4.63 (1H, ***m***, cholesterol 3-H_1), 5.37 (1H, ***m***, cholesterol 6-H_1).

2.3. Synthesis of NHS-activated cholesterol succinate (CSN)

NHS (1.2 g, 10 m mol) was mixed with CHS (4.9 g, 10 mmol) and DCC (2.1 g, 10 mmol) in 50 mL THF. The mixture was reacted for 24 h at room temperature. The precipitated dicyclohexyl urea was removed by filtration. The filtrate was evaporated, and the white powder of CSN (5.8 g, 7.0 mmol) was recrystallized in THF and ethanol.

IR (KBr, cm^{-1}): 1816, 1782 (C═O stretch of the NHS carbonyls), 1745 (C═O stretch of succinyl groups in the CHS molecule), 1213 (C—N—C stretch) and 1180 (C—O—C stretch). ^{1}HNMR ($CDCl_3$ with TMS, ppm): 0.67 (3H, ***s***, cholesterol 18-H_3), 0.7–2.4 (28H, cholesterol 1-H_2, 2-H_2, 4-H_2, 7-H_2, 8-H_1, 9-H_1, 11-H_2, 12-H_2, 14-H_1, 15-H_2,

16-H_2, 17-H_1, 20-H_1, 22-H_2, 23-H_2, 24-H_2, and 25-H_1), 0.86 (6H, ***d***, $J = 9$ Hz, cholesterol 26-H_3 and 27-H_3), 0.98 (3H, ***d***, $J = 7$ Hz, cholesterol 21-H_3), 1.03 (3H, ***s***, cholesterol 19-H_3), 2.72 (2H, ***m***, $COCH_2$), 2.84 (4H, ***s***, CH_2CH_2), 2.95 (2H, ***m***, CH_2CO), 4.65 (lH, ***m***, cholesterol 3-H_1), 5.37 (lH, ***m***, cholesterol 6-H_1).

2.4. Synthesis of CMCS

CMCS was prepared and analyzed as previously described (Abreu & Campana-Filho, 2005; Chen et al., 2003; Chen & Park, 2003). Briefly, chitosan (10 g, 0.06 mol) was alkalized in 50% aqueous NaOH (50 mL) at −18 °C for 8 h, followed reacting with chloroacetic acid (25 g, 0.26 mol) in ethanol solution (300 mL) at 45 °C for 4 h. The precipitate in the reaction mixture was filtered and washed with a solution of ethanol/water (3/1). The white powder of CMCS was obtained after drying. DS of carboxymethyl groups was determined by the potentiometric titration method (Ge & Luo, 2005), and the average molecular weight was calculated from the viscosity method (Nishimura, Nishi, Tokura, Nishimura, & Azuma, 1986).

2.5. Synthesis of CCMC conjugates

CHS was coupled to CMCS by a NHS-mediated reaction through the formation of amide linkages similar to the reaction of glycol chitason and palmitic acid *N*-hydroxysuccinimide (Noble, Gray, Sadiq, & Uchegbu, 1999). The ratios of reactants varied as shown in Table 1 to give CCMC-1, CCMC-2 and CCMC-3 of increasing hydrophobicity. Briefly, for preparation of CCMC-1, CMCS (400 mg) was dissolved in a pH 8.3 sodium bicarbonate solution (50 mL), and a solution of CSN (500 mg) in THF (300 mL) was added drop-wise to alkaline solution of CMCS over 30 min. The mixture was reacted for 72 h at room temperature, and then the produced precipitate was filtered and washed with THF, acetone and diethyl ether, respectively, to give the white powder. This powder was dispersed in 30 mL water and exhaustively dialyzed (Millipore dialysis tube, molecular weight cutoff 12–14 kDa) against water (3 L) for 2 days with 6 exchanges, and then dried by freeze-drying to obtain the white, cotton wool-like product of CCMC-1 (0.39 g). DS of cholesterol moiety defined as the amount of the cholesterol moieties per 100 glucosamine units of chitosan was calculated according to the nitrogen content which determined by elemental analysis.

Table 1
Synthesis of CCMC conjugates with different DS of the cholesterol moiety

Samples	CMCS[a] (g)	CSN[b] (g)	Elemental analysis (%)			CH DS[c] (mol%)
			C	H	N	
CCMC-1	0.400	0.500	46.06	6.02	5.54	6.9
CCMC-2	0.400	0.700	47.76	6.22	5.26	9.8
CCMC-3	0.400	1.00	49.20	6.40	5.02	12.5

[a] *O*-Carboxymethyl chitosan.
[b] NHS-activated ester of cholesterol succinate.
[c] Degree of substitution of cholesterol moiety.

2.6. Fourier transform infrared (FT-IR) spectroscopy

The IR spectra of CMCS and CCMC conjugates were obtained as KBr pellets on FT-IR spectrometer (Nicolet NEXUS 470-ESP, USA) at room temperature.

2.7. Nuclear magnetic resonance (NMR) spectroscopy

The ^1H NMR spectra of CMCS and CCMC conjugates in the solution of D_2O/CD_3COOD (100/2, v/v) were acquired at 60 °C using a 600 MHz spectrometer (JEOL JNM-ECP 600, Japan).

2.8. Preparation of CCMC self-aggregated nanoparticles

CCMC conjugates were, respectively, dispersed in water under gentle shaking at 37 °C for 48 h, followed by sonication using a probe type sonifier (Automatic Ultrasonic Processor UH-500A, China) at 100 W for 2 min. The sonication step was repeated three times until the desired size had been reached. To prevent heat build-up during the sonication, the pulse function was used (pulse on 2.0 s, pulse off 2.0 s). The sample solutions were filtered through a filter (1.0 μm, Millipore) to remove dust and impurity.

2.9. Self-aggregation behavior of CCMC conjugates

The pyrene solutions (1.0×10^{-4} M) in methanol were added into the test tubes and evaporated under a stream of nitrogen gas to remove the solvents. Then, the solutions of CCMC self-assembly nanoparticles were added into the test tubes. The final concentration of pyrene in a sample solution was 1.0×10^{-6} M, which was nearly equal to the solubility of pyrene in water at 22 °C (Wilhelm et al., 1991). The mixtures were sonicated for 30 min in an ultrasonic bath and shaken in a shaking air bath (HZQ-C, China) for 1 h at room temperature. Pyrene emission spectra were recorded on a fluorescence spectrophotometer (Shimadzu RF-4500, Japan). The probe was excited at 343 nm, and the emission spectra were recorded in the range of 350–500 nm at an integration time of 1.0 s. The excitation and emission slit opening were 10 and 2.5 nm, respectively.

2.10. Particle size distribution and zeta potential

The sizes and size distributions of CCMC self-aggregated nanoparticles in water were determined using dynamic laser light-scattering (DLLS) with a digital autocorrelator (Brookhaven BI-90 Plus, USA) at a scattering angle of 90°, a wavelength of 633 nm and a temperature of 25 ± 0.1 °C. The zeta potentials of CCMC self-aggregated nanoparticles in water were measured using an electrophoretic light-scattering spectrometer (Brookhaven BI-Zetaplus, USA).

2.11. Transmission electron microscopy (TEM)

To observe the morphology of CCMC self-aggregated nanoparticles, sample solutions (0.5 mg/mL) were dropped onto the carbon-coated 300 mesh copper grids. Then, the grids were air-dried and imaged using a transmission electron microscope (Tecnai G^2 20 S-Twin, USA) at an accelerating voltage of 80 kV.

3. Results and discussion

3.1. Preparation of CCMC conjugates

As shown in Fig. 1, for the synthesis of CCMC conjugates, a carboxyl group was initially introduced to cholesterol molecule using succinic anhydride (Shaikh et al., 1998), and then chemically coupled with the primary amino group of *O*-carboxymethyl chitosan (CMCS) by formation of an amide bond using "zero length" crosslinker of *N*-hydroxyl succinimide (NHS) (Noble et al., 1999). CMCS (deacetylation degree was 92%; DS of carboxymethyl groups was 70%; average molecular weight was near 6.78×10^4 Da) was prepared and analyzed as previously described (Abreu & Campana-Filho, 2005; Chen et al., 2003; Ge & Luo, 2005; Nishimura et al., 1986). CCMC conjugates with different DS of cholesterol moiety were prepared by controlling the ratio of CSN to CMCS, which is listed in Table 1.

Fig. 2 shows the IR spectra of CMCS and CCMC-1. The peak assignment of CMCS (Fig. 2(a)) is as follows (cm^{-1}): 3455 (O—H stretch overlapped with N—H stretch), 2922 and 2879 (C—H stretch), 1650–1550 (C═O stretch of carboxyl methyl group overlapped with amide N—H bend), 1409 (C—H bend), 1327 (C—N stretch), 1155 (bridge O stretch), and 1077 (C—O stretch). Compared with CMCS, the peaks of CCMC-1 (Fig. 2(b)) at 1650–1550 cm^{-1} (C—O stretch of succinyl group and carboxyl methyl group overlapped with N—H bend) and 1409 cm^{-1} (C—H bend) observably increased, which confirmed the formation of an amide linkage between the primary amino groups of CMCS and the carboxyl groups of cholesterol succinate.

Fig. 3 shows the ^1H NMR spectra of CMCS and CCMC-1. The proton assignment of CMCS (Fig. 3(a)) is as follows (ppm): 2.06 (CH_3, acetamido group of chitosan), 3.22 (C*H*, carbon 2 of glucosamine ring), 3.40 (C*H*, carbon 2 of glucosamine ring with the substituted amino group), 3.7–4.0 (C*H*, carbon 3, 4 and 6 of glucosamine ring), 4.26 (CH_2, carboxymethyl group), 4.34 (C*H*, carbon 5 of glucosamine ring), 4.91 (C*H*, carbon 1 of glucosamine ring). According to what has been previously reported (Muzzarelli, Ilari, & Petraluro, 1994), the appearance of the small proton signal at 3.4 ppm was due to mono-substitution of

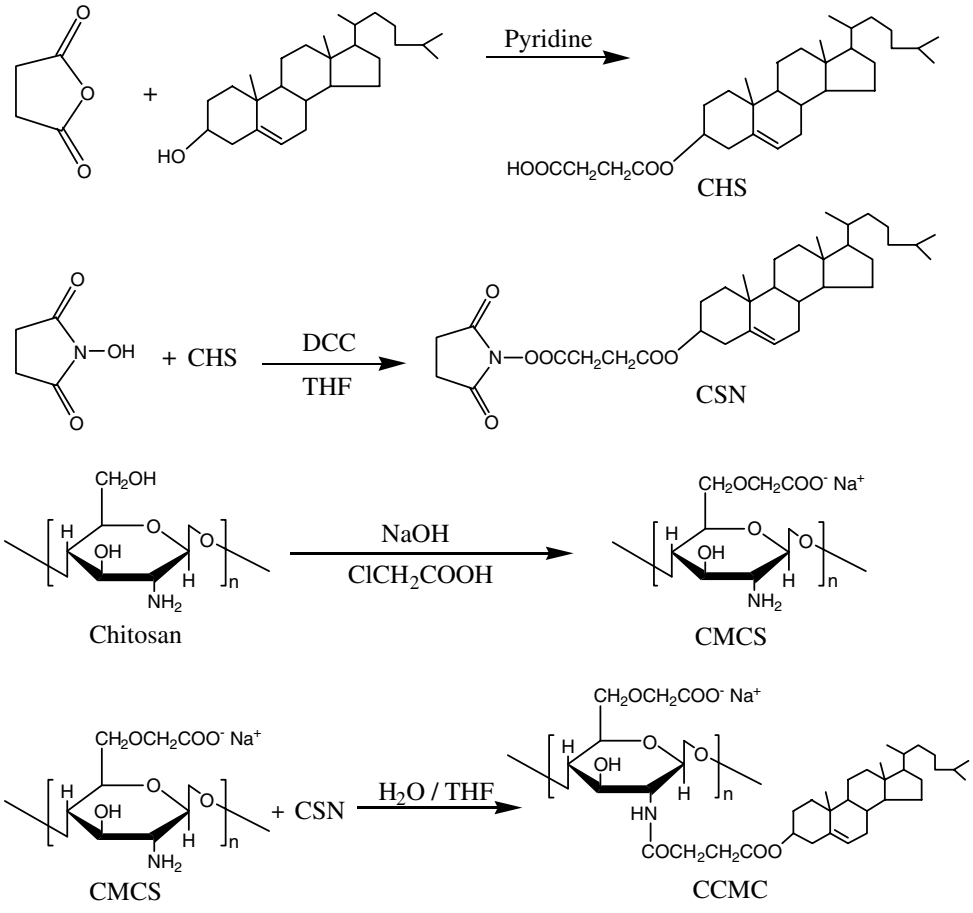

Fig. 1. Schematic illustration for the synthesis of CCMC conjugates.

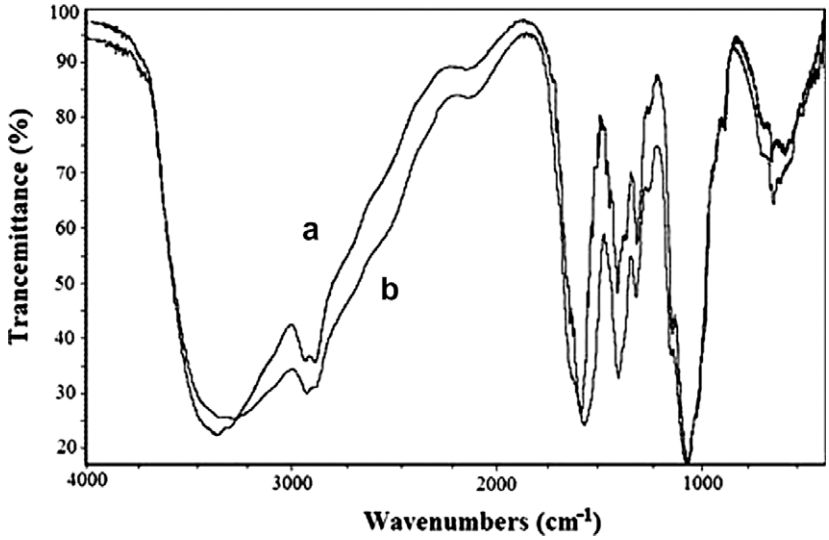

Fig. 2. IR spectra of (a) CMCS and (b) CCMC-1.

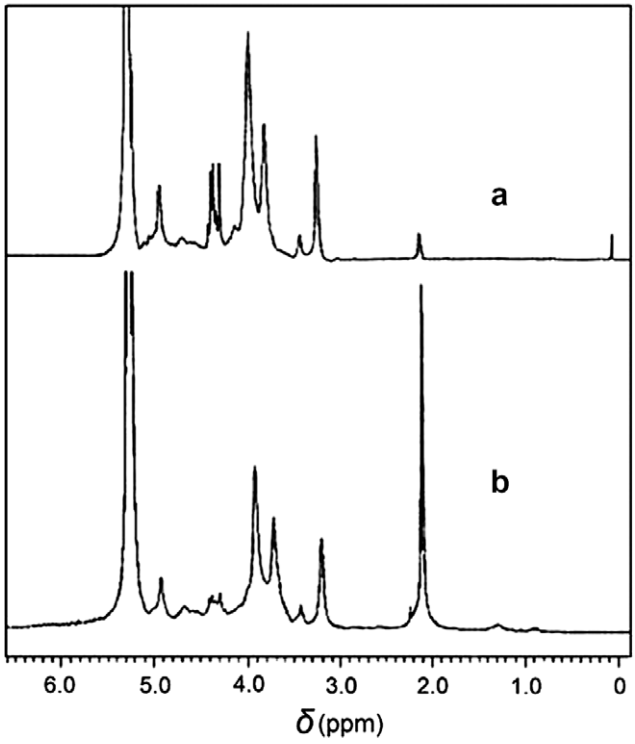

Fig. 3. ^{1}H NMR spectra of (a) CMCS and(b) CCMC-1.

the amino groups of chitosan, which indicated that less than 10% of chitosan amino groups were also carboxymethylated during the occurrence of *O*-carboxymethylation. The characteristic proton signals of CCMC-1 (Fig. 3(b)) appeared in the range of 0.5–3.0 ppm. Compared with CMCS, the evident increase of the proton signal at around 2.1 ppm and the appearance of the new signals at 0.86 and 1.30 ppm indicated that cholesterol succinate covalently linked to CMCS.

Although the proton signals in the ^{1}H NMR spectra of CCMC conjugates confirmed the presence of cholesterol moieties, DS of cholesterol moiety could not be exactly calculated by comparing the ratio of cholesterol protons to sugar protons due to the self-aggregation of CCMC conjugates in the aqueous phase. In our study, the signal intensity of the characteristic cholesterol protons was not in proportion to the DS of the cholesterol moiety and sometimes even a lower signal intensity was observed corresponding to a higher DS of cholesterol moiety, which suggested that cholesterol moieties aggregated to form hydrophobic micro-domains to minimize their interaction in D_2O. This trend in the ^{1}H NMR spectra was consistent with other polymeric amphiphiles that formed micelles or aggregates in the aqueous phase (Kim, Lee, & Kang, 2000; Park et al., 2004). Therefore, DS of cholesterol moiety of CCMC conjugates were determined by elemental analysis in this study and listed in Table 1.

3.2. Self-aggregation behavior of CCMC conjugates

The fluorescence probe technique was used to study the self-aggregation behavior of CCMC conjugates on a molecular level, where pyrene was chosen as a fluorescence probe. Pyrene shows only a small fluorescence intensity in a polar environment (water) due to its poor solubility and self-quenching, but strongly emits radiation when the hydrophobic micro-domains are formed in an aqueous solution because the pyrene molecules prefer to be close to (or inside) these hydrophobic micro-domains. Therefore, pyrene is often used as a fluorescence probe to monitor the self-aggregation behavior of surfactants and/or polymers (Glushko, Thaler, & Karp, 1981). Fig. 4 shows the fluorescence spectra of pyrene at various concentrations of CCMC-1 in water after sonication. The total fluorescence intensity of pyrene evidently increased with the concentration of CCMC-1 increasing, which implied that CCMC-1 molecules aggregated to form hydrophobic micro-domains and the pyrene molecules transferred from aqueous media

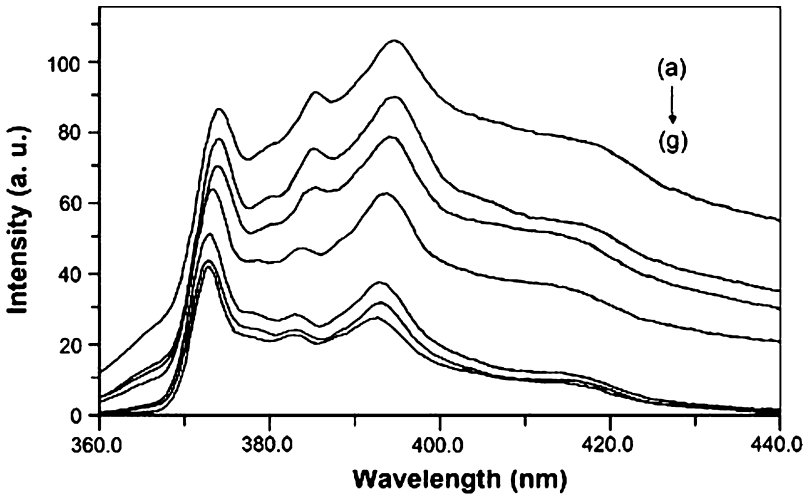

Fig. 4. Emission spectra of pyrene (1.0×10^{-6} mol/L) in CCMC-1 solution with concentrations (mg/mL) of (a) 1.0, (b) 0.5, (c) 0.1, (d) 0.05, (e) 0.025, (f) 0.01 and (g) 0.005 in distilled water.

into these micro-domains. This result was similar to what previously reported for other polymeric amphiphiles (Lee et al., 1998; Liu et al., 2005).

There are five vibrational peaks in the pyrene emission spectra. The intensity ratio of the first peak (372 nm) and the third peak (385 nm) I_{372}/I_{385} is quite sensitive to the polarity of the microenvironment. This is due to the fact that increasing the polarity of the medium induces an increase of the intensity of the first peak I_{372}, corresponding to the forbidden transition, while the intensity of the third peak I_{385}, corresponding to the allowed transition, is unchanged. As a result, the value of the ratio is higher in more polar media (Magny, Iliopolous, Zana, & Audebert, 1994). Therefore, the critical aggregation concentration (CAC), which is the threshold concentration of self-aggregate formation by intra- and/or intermolecular association, can be determined from the change of the I_{372}/I_{385} value of pyrene in the presence of polymeric amphiphiles. Fig. 5 illustrates the changes of the I_{372}/I_{385} values as a function of the concentrations of CCMC conjugates and CMCS. For CCMC conjugate, the I_{372}/I_{385} values were close to the value (1.87) of pyrene in water at low concentrations (Dong & Winnik, 1984), and then followed by a linear decrease with further increasing concentration, and therefore the CAC could be determined by the intercept of two straight lines (Lee et al., 1998). As shown in Table 2, the CAC of CCMC conjugates are lower than the critical micelle concentrations of many low-molecular-weight surfactants, e.g. sodium dodecyl sulfate (Rahman & Brown, 2003). However, for CMCS, the I_{372}/I_{385} value decreased slightly only at high concentration, which indicated it was difficult for CMCS molecules to aggregate in this range of concentrations. It could be deduced from the comparison between CMCS and CCMC conjugates that the aggregation of CCMC conjugates in water was due to the hydrophobic interactions of cholesterol moieties. Table 2 also shows the CAC of CCMC conjugates decreased from 0.03 to 0.006 mg/mL with DS of cholesterol moiety increasing from 6.9% to 12.5%. This was because the increase of hydrophobicity made CCMC molecules easier to aggregate in water.

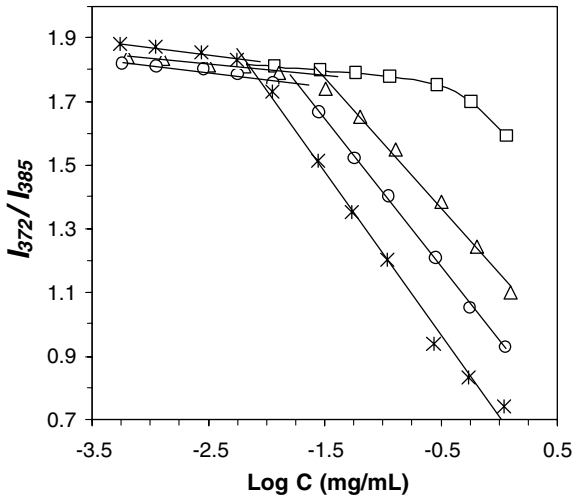

Fig. 5. Change of the intensity ratio (I_{372}/I_{385}) from excitation spectra of pyrene (1.0×10^{-6} mol/L) with various concentrations of CCMC-1 (–△–), CCMC-2 (–○–), CCMC-3 (–∗–) and CMCS (–□–) in distilled water.

3.3. Preparation and characterization of CCMC self-aggregated nanoparticles

CCMC conjugates formed monodisperse self-aggregated nanoparticles by probe sonicating their dispersions in water at the concentration of 0.5 mg/mL. On probe sonication, the size of nanoparticles was found to decrease with increasing sonication time, reaching a limiting value after a maximum of 10 min. All samples were sonicated until the limiting size values had been reached. Fig. 6 shows the transmission electron microscopy (TEM) images of CCMC self-aggregated nanoparticles. These nanoparticles were almost spherical in shape and their size decreased from 180 nm to less than 50 nm with DS of cholesterol moiety increasing from 6.9% to 12.5%. However, as shown in Table 2, the sizes

Table 2
Characterization of CCMC self-aggregated nanoparticles

Samples	CAC[a] × 10^2 (mg/mL)	Diameter[b] (nm)	Polydispersity index[b]	Zeta potential[c] (mV)
CCMC-1	3.00	234.9 ± 2.5	0.092 ± 0.041	−23.19 ± 4.59
CCMC-2	1.33	189.3 ± 0.4	0.167 ± 0.024	−19.92 ± 0.46
CCMC-3	0.65	100.1 ± 1.9	0.170 ± 0.073	−11.15 ± 1.57

[a] Critical aggregation concentration determined from I372/I385 data.
[b] The size and size distribution (mean value ± standard deviation) determined by the dynamic laser light-scattering with three times.
[c] The zeta potential (mean value ± standard deviation) measured by an electrophoretic light-scattering spectrometer with three times.

of CCMC self-aggregated nanoparticles measured by dynamic laser light-scattering (DLLS) rangedfrom 234.9 to 100.1 nm, which were obviously larger than the sizes determined by TEM. We believed this was mainly due to two factors. First, TEM depicted the size in the dried state of the sample, whereas DLLS measured the size in the hydrated state of the sample, so that the size measured by DLLS was a hydrodynamic diameter and had a larger value because of solvent effect (Liu et al., 2005; Prabha, Zhou, Panyam, & Labhasetwar, 2002). Second, CCMC self-aggregated nanoparticles tended to agglomerate in water while the size decreased owing to the increase of the surface energy, which resulted in the larger size than that depicted by TEM such as CCMC-3.

As shown in Table 2, the zeta potentials of CCMC self-aggregated nanoparticles in water were negative, suggesting that the negatively charged carboxymethyl groups in CCMC molecules were mainly distributed on the surface of self-aggregated nanoparticles due to their hydrophilic property. Furthermore, the absolute value of the zeta potential of CCMC self-aggregated nanoparticles decreased from 23.19 to 11.15 mV with DS of cholesterol moiety increasing from 6.9% to 12.5%. This was another reason that resulted in the agglomeration of CCMC self-aggregated nanoparticles in water.

3.4. Formation mechanism of CCMC self-aggregated nanoparticles

It could be deduced from above results that self-aggregation behavior of CCMC conjugates was due to the strong hydrophobic interaction of cholesterol moieties, and DS of cholesterol moiety played an important role in the size and the surface property of CCMC self-aggregated nanoparticles. To further investigate the effect of carboxymethyl groups in the formation of CCMC self-aggregated nanoparticles, we compared the structure and the stability of self-aggregated nanoparticles between CCMC-1 and cholesterol-modified chitosan (CHCS). CHCS was prepared by the same synthesis method of CCMC-1 using chitosan as the raw material. DS of cholesterol moiety of CHCS determined by elemental analysis was 7.2%, which was similar to CCMC-1, so that the carboxymethyl groups were the only difference in chemical structure between CCMC-1 and CHCS.

CHCS also formed monodisperse self-aggregated nanoparticles by probe sonication in water. The size of these nanoparticles measured by DLLS was 417.2 ± 18.0 nm with a polydispersity index of 0.317 ± 0.036. TEM images (Fig. 7(a_1) and (a_2)) show that CHCS self-aggregated nanoparticles were irregularly spherical in shape with a bumpy surface. However, CCMC-1 self-aggregated nanoparticles had a smaller size (mean diameter 234.9 ± 2.5 nm), a more narrow size distribution (polydispersity index 0.092 ± 0.041) measured by DLLS, a smoother surface and a more compact structure shown in TEM images (Fig. 7(b_1) and (b_2)).

CCMC-1 self-aggregated nanoparticles exhibited good stability on storage for at least 1 month at refrigerator temperature (4–8 °C), and no sediment was observed after this time. However, in CHCS self-aggregated nanoparticle solution, some flocs were clearly observed after two weeks.

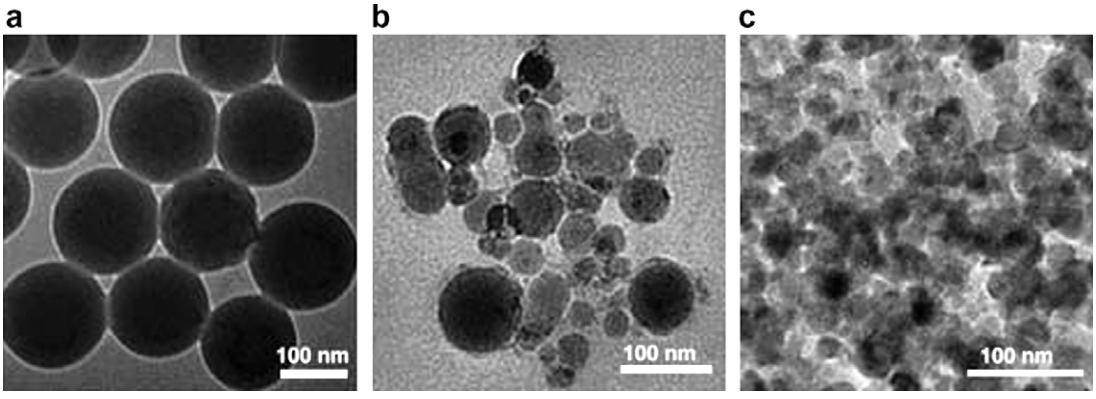

Fig. 6. TEM images of self-aggregated nanoparticles prepared, respectively, from (a) CCMC-1, (b) CCMC-2 and (c) CCMC-3 by probe sonication in water.

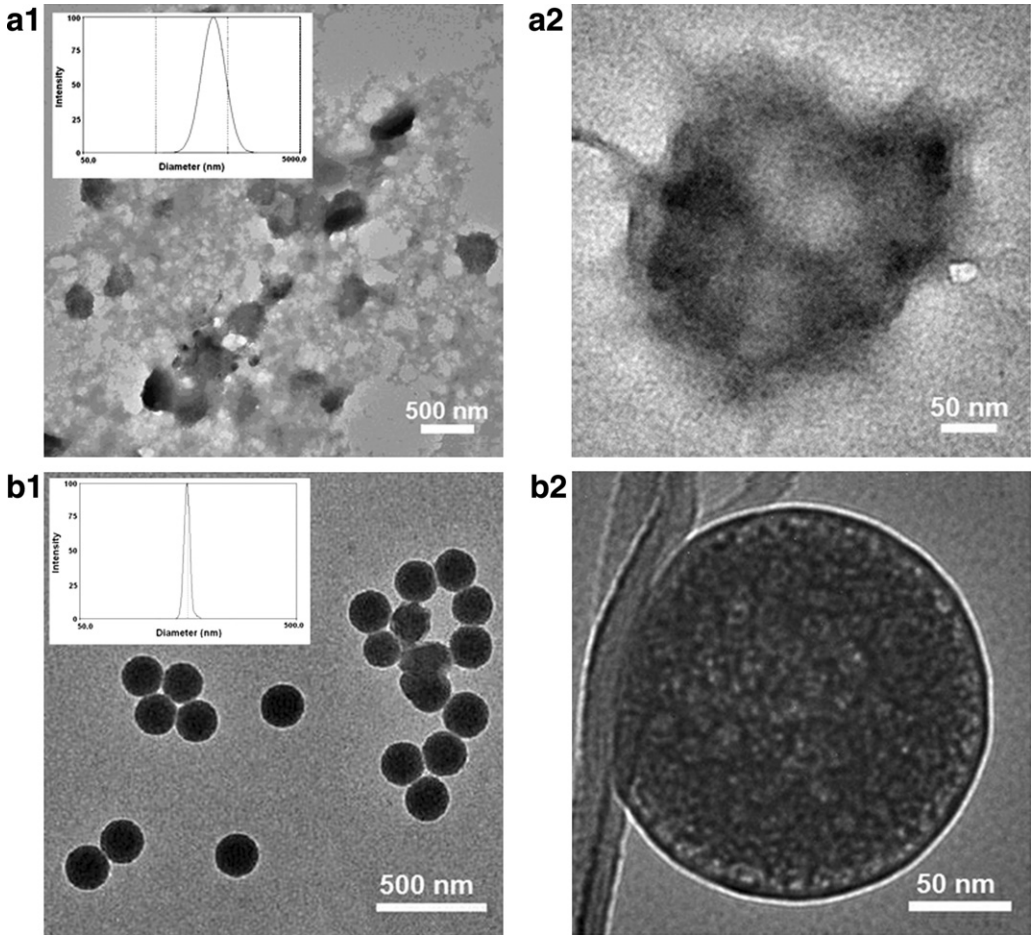

Fig. 7. TEM images and size distributions of self-aggregated nanoparticles prepared from CHCS (a_1 and a_2) and CCMC-1 (b_1 and b_2) by probe sonication in water.

Therefore, it was deduced that the negatively charged carboxymethyl groups, the only difference in chemical structure between CCMC-1 and CHCS, are advantageous to form the well-shaped and stable self-aggregated nanoparticles.

To explore the reason why the presence of carboxymethyl groups caused such a large difference between CCMC-1 and CHCS self-aggregated nanoparticles, we need to understand the poly-core model that was firstly proposed to illustrate the structure of self-aggregated nanoparticle of cholesterol-bearing pullulan (CHP) (Akiyoshi et al., 1997), but later was often used to explain other self-aggregated nanoparticles of hydrophobically modified polysaccharides due to its plausibility (Lee et al., 1998; Park et al., 2004). In Fig. 8, the structure of self-aggregated nanoparticle of hydrophobically modified polysaccharides based on the poly-core model is schematically illustrated. (Wang, Liu, Jiang, & Zhang, 2007). The hydrophobic microdomains are formed by the association of hydrophobic groups, and the polysaccharide backbones coil to form the hydrophilic shells outside these hydrophobic microdomains,

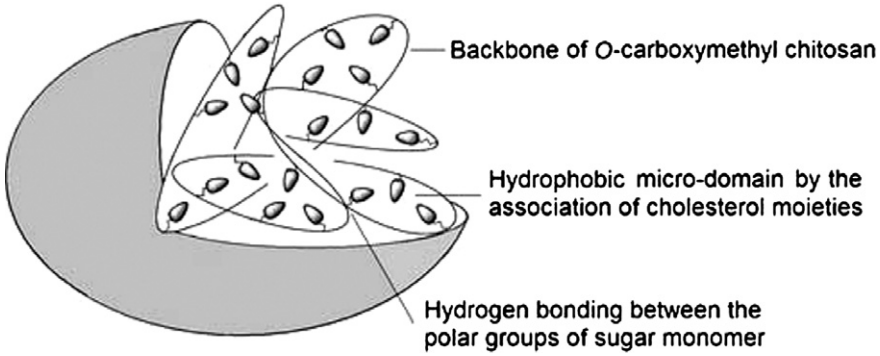

Fig. 8. Schematic representation of self-aggregated nanoparticle of hydrophobically modified polysaccharide (Wang et al., 2007).

thus a minimal energy state is attained in aqueous media. Furthermore, inter- and/or intramolecular hydrogen bondings among tightly packed polysaccharide backbones will promote the self-aggregation of hydrophobically modified polysaccharides. Therefore, it was deduced that not only the hydrophobic groups but also the hydrophilic polysaccharide backbones play important roles in the formation of self-aggregated nanoparticles.

It is well known that chitosan molecules have the chain rigidity arising to a great extent from various hydrogen bonds such as 3—OH···5—O (intramolecular) and 2—NH···6—O (intermolecular) (Ravi Kumar et al., 2004), so that it is difficult for CHCS molecules to self-aggregate to form the well-shaped nanoparticles that has been commonly reported for the other hydrophobically modified water-soluble polysaccharides such as cholesterol-bearing pullulan (Akiyoshi et al., 1997) and deoxycholic acid-modified heparin (Park et al., 2004). However, the molecular chains of *O*-carboxymethyl chitosan are highly flexible because the carboxymethylation prevents the formation of hydrogen bonds (2—NH···6—O). Hence, compared with CHCS, CCMC-1 molecules are easier to self-aggregate in water by the hydrophobic interactions of cholesterol moieties, and this is the main reason why CCMC self-aggregated nanoparticles have a more spherical shape, a smaller size and a more compact structure.

4. Conclusions

Novel self-aggregated nanoparticles were prepared from polymeric amphiphiles, cholesterol-modified *O*-carboxymethyl chitosan (CCMC) conjugates, and the relationships between the chemical structure, the amphiphilic property and the morphological characteristics of CCMC self-aggregated nanoparticles were investigated in this study. The formation of CCMC self-aggregated nanoparticles is due to the hydrophobic interactions of cholesterol moieties in aqueous media, and the negatively charged carboxymethyl groups also play an important role in the morphology and the stability of nanoparticles. This novel nanoparticle system is hoped to be used as a carrier of hydrophobic drugs and protein drugs, and the further investigations are in progress.

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