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The interaction between bovine serum albumin and the self-aggregated nanoparticles of cholesterol-modified *O*-carboxymethyl chitosan

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Abstract

The interaction between bovine serum albumin (BSA) and self-aggregated nanoparticles of cholesterol-modified *O*-carboxymethyl chitosan (CCMC) with different degrees of substitution (DS) of cholesterol moiety was studied by transmission electron microscopy (TEM), fluorescence quenching method and circular dichroism (CD) measurement. This interaction was started at the disaggregation of CCMC self-aggregated nanoparticles and reached equilibrium after 3–4 h. The apparent quenching constant (K_q) between BSA and CCMC self-aggregated nanoparticles calculated by the modified Stern–Volmer plot increased from 4.14×10^4 to 1.95×10^5 M^{-1} with DS of cholesterol moiety increasing from 3.2% to 9.8%, whereas the fraction of tryptophan residues in BSA molecule involved in the interaction decreased at the same time. Compared with free BSA, the relative α-helix content of BSA decreased and the unfolding of BSA by a denaturant such as urea was largely suppressed upon interaction with CCMC self-aggregated nanoparticles. DS of cholesterol moiety significantly affected the interaction between BSA and CCMC self-aggregated nanoparticles.

Keywords: Cholesterol modified *O*-carboxymethyl chitosan; Self-aggregated nanoparticle; Bovine serum albumin

1. Introduction

In living systems, association of various biomacromolecules such as proteins, polysaccharides or DNA is perfectly controlled by the intermolecular interactions such as ionic bonding, hydrogen bonding, and hydrophobic interaction [1,2]. The amphiphilic property of biomacromolecules is one important factor for their self-association in aqueous media [3]. In this sense, various polymeric amphiphiles were synthesized and the structure–function relationship of their self-assemblies has been extensively studied for understanding many biological phenomena [4–6] and for designing new materials in biotechnology and medicine [7–10]. The investigations of interaction between polymeric amphiphiles and proteins have been focused recently due to their significance for separation and stabilization of proteins [11–13], but the basic study of interaction between hydrophobically modified polysaccharides and proteins has seldom been done [14–16].

We have synthesized a novel kind of polymeric amphiphile – cholesterol-modified *O*-carboxymethyl chitosan (CCMC) and prepared its hydrogel nanoparticles by probe sonication in the aqueous media [17]. CCMC is amphiphilic in nature due to its hydrophilic polysaccharide backbone and hydrophobic cholesterol chains (Fig. 1a), and therefore they can form micelle or micelle-like particles (Fig. 1b) by self-aggregation in aqueous media like other polymeric amphiphiles [18–21]. As we illustrated previously [17], the hydrophobic microdomains are formed by the association of cholesterol

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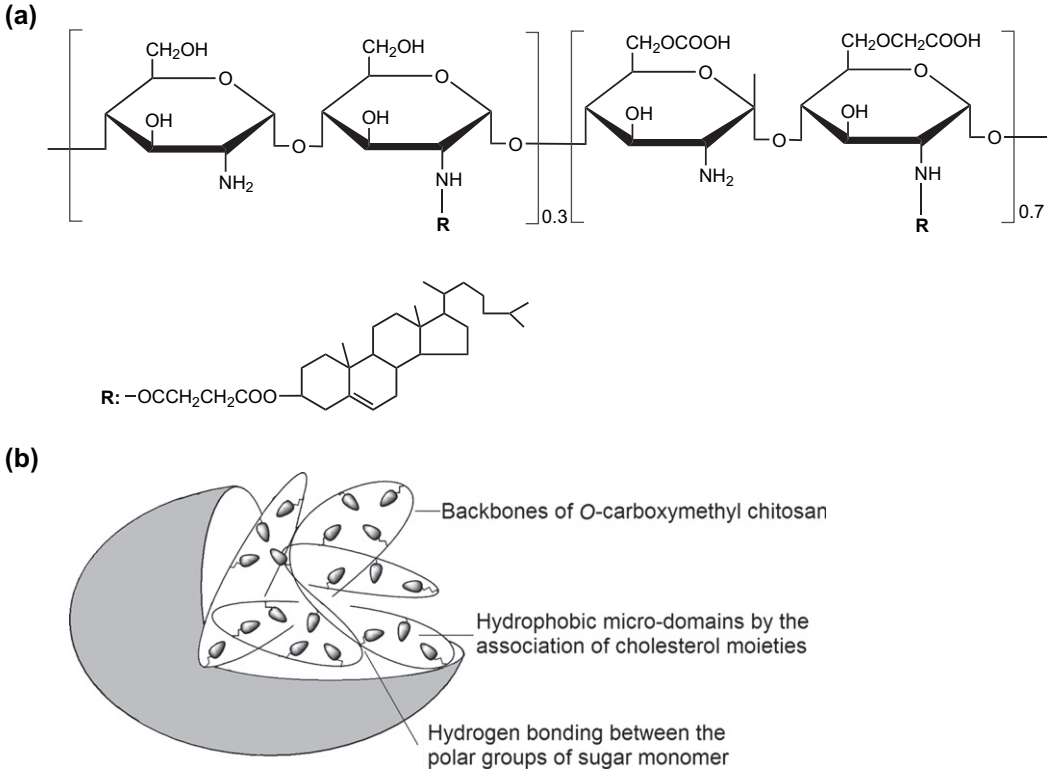

Fig. 1. Chemical structure of (a) CCMC and (b) schematic representation [27] of CCMC self-aggregated nanoparticle formed by hydrophobic association.

moieties and the backbones of *O*-carboxymethyl chitosan coil to form the hydrophilic shells outside these hydrophobic micro-domains, 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 CCMC molecules.

In subsequent study, we found CCMC self-aggregated nanoparticles can bind some water-soluble proteins such as bovine serum albumin (BSA), which is very similar to the complexation between BSA and cholesterol-bearing pullulan (CHP) self-assembled hydrogel nanoparticles that was described by Nishikawa and his co-workers [14]. CCMC has a chemical structure similar to CHP such as hydrophilic polysaccharide backbones and hydrophobic side chains of cholesterol moiety, and therefore we believed that CCMC self-aggregated nanoparticles would be used as a host of a macromolecular guest such as proteins like CHP self-aggregated nanoparticles. Whereas, as a hydrophobically modified derivative of *O*-carboxymethyl chitosan, CCMC has its unique characteristics produced from the good biological and physicochemical properties of *O*-carboxymethyl chitosan such as biocompatibility, biodegradability, low-toxicity and its polyelectrolytical nature [22–24].

In this paper, the interaction between BSA and CCMC self-aggregated nanoparticles was systemically studied using transmission electron microscopy (TEM), fluorescence spectroscopy and circular dichroism (CD), and the affection of DS of cholesterol moiety on the interaction was also discussed in order to obtain further information about the potential application of CCMC self-aggregated nanoparticles being used as the protein carrier.

2. Experimental methods

2.1. Materials

Cholesterol-modified *O*-carboxymethyl chitosan (CCMC) was synthesized according to the method previously reported [17]. *O*-Carboxymethyl chitosan (CMC, molecular weight was 6.78×10^4 Da and DS of carboxymethyl groups was 70%) was substituted by 3.2, 6.9 and 9.8 cholesterol moieties per 100 glucosamine units of chitosan. These CCMCs were coded as CCMC-3.2, CCMC-6.9 and CCMC-9.8, respectively. Fatty acid-free bovine serum albumin (BSA) was purchased from Sigma Chemicals (St. Louis, MO). All other chemical reagents in the study were of analytical grade and were obtained from commercial sources.

2.2. Preparation of CCMC self-aggregated nanoparticles

CCMC self-aggregated nanoparticles were prepared by the sonication method which we previously described [17]. CCMC was 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 using a pulse function (pulse on 2.0 s, pulse off 2.0 s). The sonication step was repeated three times until the

desired size value had been reached. Then, the sample solution was filtered through a filter (0.45 μm, Millipore) to remove dust and impurity. The morphology of CCMC self-aggregated nanoparticles was observed by a transmission electron microscope (TEM, Tecnai G^2 20 S-Twin, USA) at an accelerating voltage of 80 kV, and the mean diameter of nanoparticles was measured by 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.

2.3. Preparation of CCMC–BSA interactant

The BSA solution was added to the above CCMC self-aggregated nanoparticle solution, followed by incubation at 25 °C for 24 h. Then, the mixture was centrifuged at 30,000*g* at 4 °C for 30 min to separate un-interacted nanoparticles. The changes in CCMC self-aggregated nanoparticles upon interaction with BSA were observed by naked eyes and TEM (Tecnai G^2 20 S-Twin, USA).

2.4. Fluorescence measurement

Fluorescence spectra and fluorescence intensities of free BSA and CCMC–BSA interactants were recorded on a fluorescence spectrophotometer (Shimadzu RF-4500, Japan). The tryptophan chromophore in BSA molecule was excited at 295 nm and the emission spectra were recorded in the range of 310–450 nm at an integration time of 1.0 s [25]. The excitation and emission slit openings were both 2.5 nm. All experiments were carried out at 25 °C and the pHs of sample solutions were in the range of 5.5–6.0.

2.5. Circular dichroism (CD) measurement

CD spectra of free BSA and CCMC–BSA interactants were recorded in a range of 200–250 nm on a circular dichroism spectrometer (JASCO J-810, Japan) at 25 °C. A relative α-helix content of BSA molecule was calculated as follows [26]:

$$\%\ \alpha\text{-Helix} = \frac{-[\theta]_{208} - 4000}{33{,}000 - 4000} \quad (1)$$

where $[\theta]_{208}$ is the mean residue ellipticity in deg cm^2 dmol^{-1} at 208 nm.

In order to assess the stability of CCMC–BSA interactant against the denaturant, an appropriate amount of urea was added to the sample solution. Then, unfolding of BSA induced by urea was monitored by the change of mean residual ellipticity at 222 nm as a function of the urea concentration.

Table 1
Characterization of CCMC self-aggregated nanoparticles and the Stern–Volmer quenching parameters of CCMC–BSA interactants

Samples	CH DS[a] (mol%)	Diameter[b] (nm)	Polydispersity index[b]	f_a[c]	K_q[c] (M^{-1})
CCMC-3.2	3.2	305.8	0.178 ± 0.056	0.96	4.14×10^4
CCMC-6.9	6.9	198.4	0.107 ± 0.045	0.95	5.40×10^4
CCMC-9.8	9.8	109.0	0.162 ± 0.073	0.57	1.95×10^5

[a] Degree of substitution of cholesterol moiety.
[b] The size and size distribution (mean value ± standard deviation) measured by the dynamic laser light scattering with three times.
[c] The fraction of fluorophore (protein) accessible to the quencher and the apparent quenching constant determined from modified Stern–Volmer plot.

3. Results and discussion

3.1. CCMC self-aggregated nanoparticles

Three CCMC samples formed monodisperse self-aggregated nanoparticles by probe sonication in aqueous media. In the preparation period, the size of CCMC self-aggregated 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. CCMC self-aggregated nanoparticles imaged by TEM (Fig. 2) had a nearly spherical shape with good structural integrity and their size (Table 1) measured by DLLS decreased from 305.8 to 109.0 nm with DS of cholesterol moiety increasing from 3.2% to 9.8%, which was consistent with

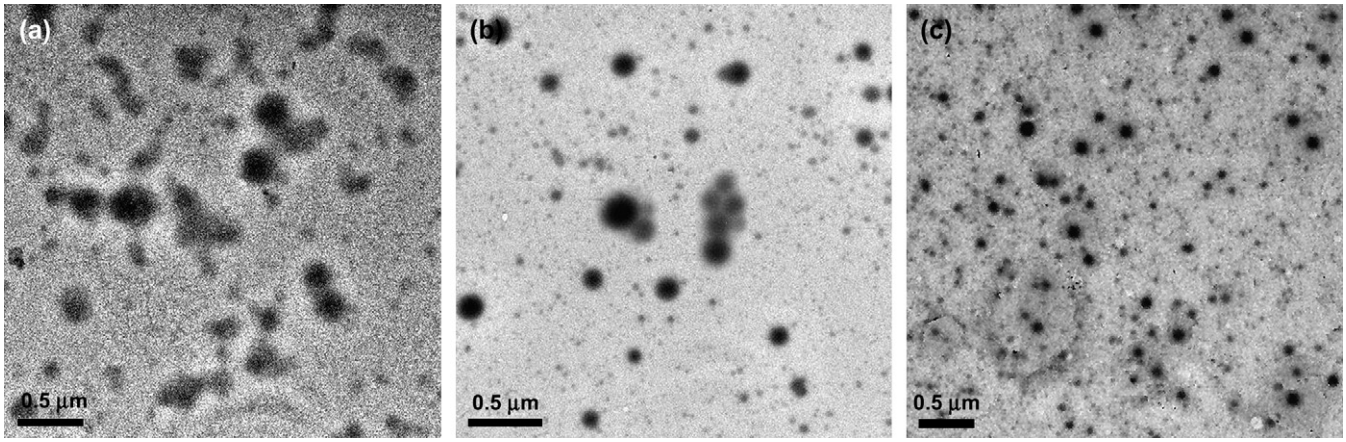

Fig. 2. TEM images of self-aggregated nanoparticles of (a) CCMC-3.2, (b) CCMC-6.9 and (c) CCMC-9.8 by probe sonication in water, bar = 500 nm.

other self-aggregate systems of hydrophobically modified polysaccharides that were previously reported [27–29].

3.2. Interaction between CCMC self-aggregated nanoparticles and BSA

When CCMC self-aggregated nanoparticles interacted with BSA, the change in their macro- and microscopic characteristics occurred. As shown in Fig. 3, CCMC-6.9 formed spherical nanoparticles by self-aggregation in the aqueous media (a); but after interacted with BSA, a great deal of white turbidity rapidly produced in the mixture and the shape of CCMC-6.9 self-aggregated nanoparticles changed from spherical to amorphous (b); then the white turbidity gradually disappeared with the disappearance of CCMC-6.9 self-aggregated nanoparticles (c). Considering that CCMC self-aggregated nanoparticles were formed by the hydrophobic association of cholesterol moieties [17], the disaggregation of CCMC self-aggregated nanoparticles indicated that the hydrophobic association of cholesterol moieties changed with the addition of BSA. We believed that this was due to the interaction between CCMC and BSA molecules, and the subsequent Stern–Volmer analysis confirmed it. CCMC-3.2 and CCMC-9.8 self-aggregated nanoparticles also showed the similar changes when they interacted with BSA.

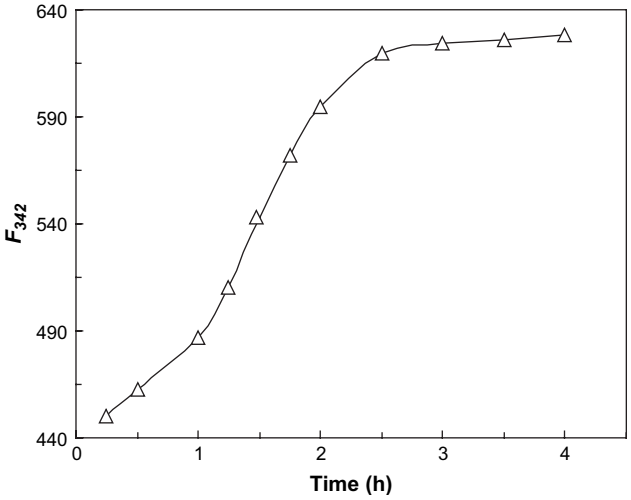

Fig. 4. Emission intensity of the mixed solution of BSA (1.0×10^{-5} mol L^{-1}) and CCMC-6.9 self-aggregated nanoparticles (1.0×10^{-5} mol L^{-1}) at 324 nm as a function of time.

Fig. 4 shows the emission intensity of the mixed solution of CCMC-6.9 self-aggregated nanoparticles and BSA at 342 nm

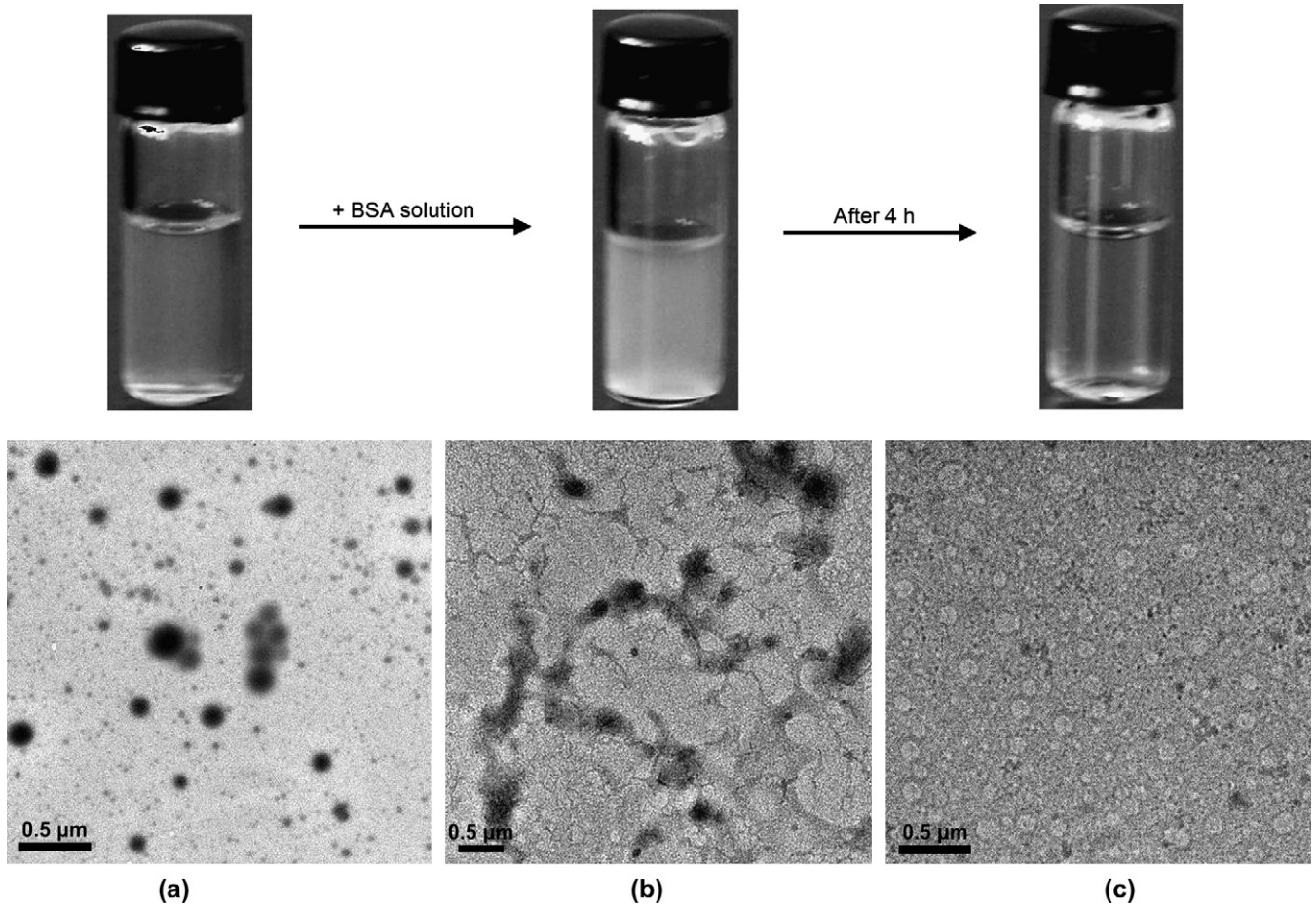

Fig. 3. The photographs and TEM images of (a) CCMC-6.9 self-aggregated nanoparticle solution, (b) the mixed solution of CCMC-6.9 self-aggregated nanoparticles and BSA, and (c) the interactant of CCMC-6.9 self-aggregated nanoparticles with BSA.

as a function of time. The emission intensity was very low at the beginning due to the white turbidity produced in the mixture. As the white turbidity gradually disappeared, the emission intensity increased and reached equilibrium after 3–4 h, which deduced that the interaction between CCMC-6.9 self-aggregated nanoparticles and BSA was a rapid process. CCMC-3.2 and CCMC-9.8 self-aggregated nanoparticles also showed a similar trend when interacted with BSA.

The fluorescence quenching method [25,30–32] was used to study the interactions between CCMC self-aggregated nanoparticles and BSA in this paper. As shown in Fig. 5, free BSA and CCMC-6.9–BSA interactant gave the fluorescence emission spectra with a maximum at 342 nm due to the tryptophan residues in BSA molecule, and the emission intensity decreased with the increase of CCMC-6.9 concentration, which indicated that CCMC-6.9 molecules quenched the fluorescence of the tryptophan residues. Eftink has reported that the maximum emission wavelength of the tryptophan residue is very sensitive to the polarity of its environment (e.g. in polar environment $\lambda_{max} \approx 350$ nm, while in non-polar environment $\lambda_{max} \approx 320$ nm) [25]. Fig. 5 shows the maximum emission wavelength of the tryptophan residues almost retained at about 340 nm when BSA interacted with CCMC-6.9 self-aggregated nanoparticles, implying that the polarity of micro-environment around the tryptophan residues did not change in the interaction process. It further proved that the disaggregation of hydrophobic microdomains of CCMC self-aggregated nanoparticles formed by the association of cholesterol moieties. CCMC-3.2 and CCMC-9.8 self-aggregated nanoparticles gave the similar fluorescence emission spectra when they interacted with BSA.

3.3. Stern–Volmer analysis

Fluorescence quenching has been widely used as a powerful tool to reveal the accessibility of fluorophores in the protein matrix to quenchers. Two quenching processes are known: static and dynamic quenching. Both of them require molecular contact between the fluorophore and the quencher. Static quenching refers to formation of a non-fluorescent fluorophore–quencher interactant. Dynamic quenching refers to the quencher diffusion to the fluorophore during the lifetime of the excited state and upon contact, the fluorophore returns to the ground state, without emission of a photon [33]. Both static and dynamic processes are described by the Stern–Volmer expression [34]:

$$F_0/F = 1 + K_{SV}[Q] \quad (2)$$

where F_0 and F are the fluorescence intensities at 342 nm in the absence and presence of quencher, respectively, $[Q]$ is the quencher concentration and K_{SV} is the Stern–Volmer quenching constant. As shown in Fig. 6, the Stern–Volmer plots of F_0/F versus the concentrations of CCMC displayed

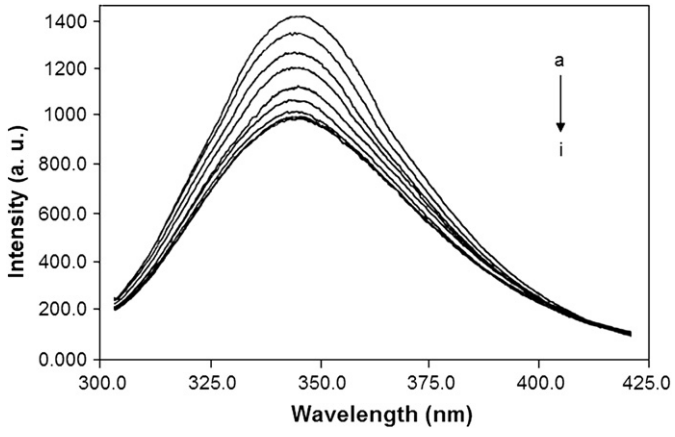

Fig. 5. The fluorescence spectra of BSA (1.0×10^{-5} mol L^{-1}) in the presence of various concentrations of CCMC-6.9: (a) 0, (b) 1.78×10^{-6}, (c) 3.56×10^{-6}, (d) 4.45×10^{-6}, (e) 5.34×10^{-6}, (f) 6.23×10^{-6}, (g) 7.12×10^{-6}, (h) 8.01×10^{-6}, (i) 8.9×10^{-6} mol L^{-1}.

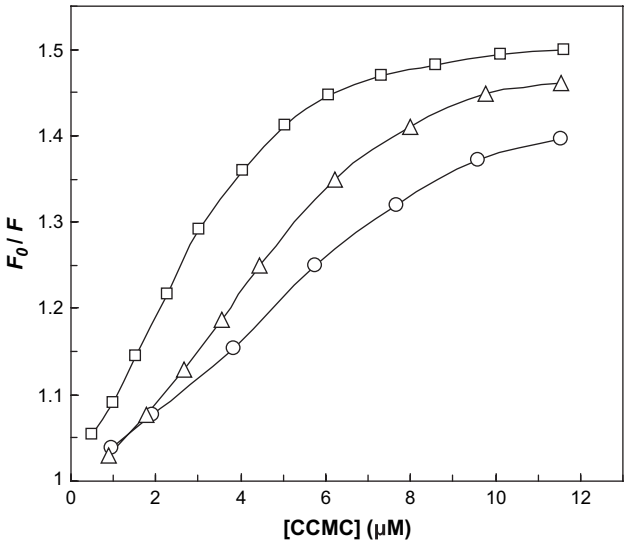

Fig. 6. Stern–Volmer plots at low concentrations of CCMC-3.2 (–○–), CCMC-6.9 (–△–) and CCMC-9.8 (–□–), respectively. The concentration of BSA was 1.0×10^{-5} mol L^{-1}.

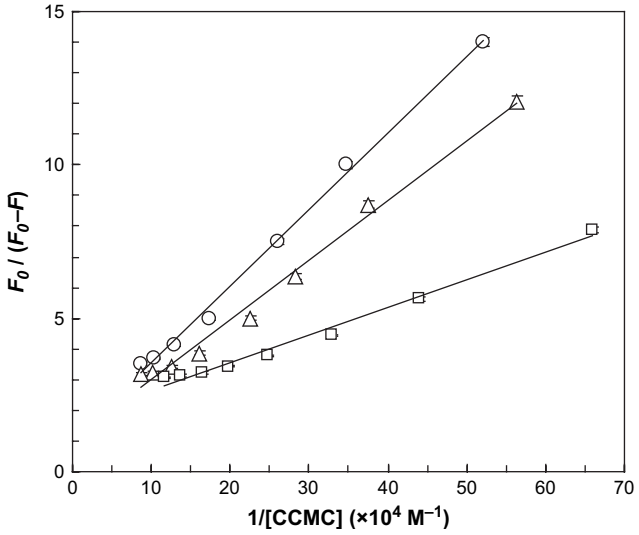

Fig. 7. Plots ($n = 5$) of $F_0/(F_0 - F)$ versus 1/[CCMC] for CCMC-3.2 (–○–), CCMC-6.9 (–△–) and CCMC-9.8 (–□–), respectively. The concentration of BSA was 1.0×10^{-5} mol L^{-1}.

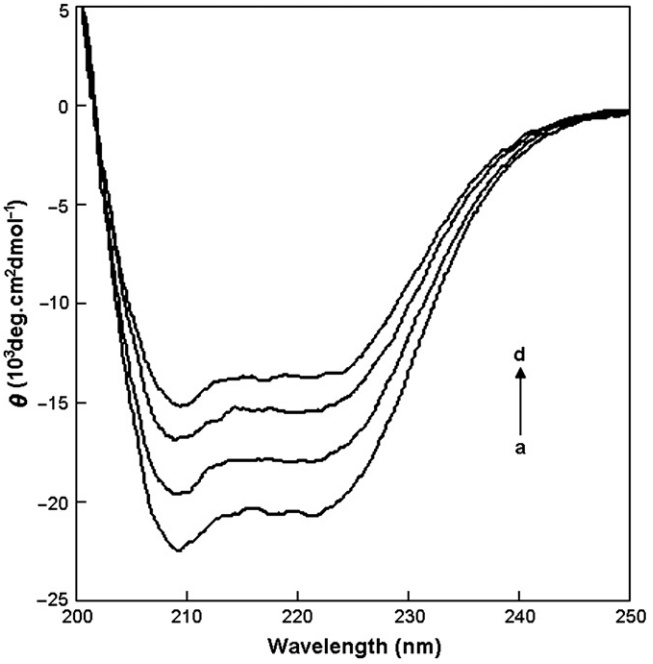

Fig. 8. CD spectra of free BSA (a), CCMC-3.2–BSA interactant (b), CCMC-6.9–BSA interactant (c) and CCMC-9.8–BSA interactant (d) in water at 25 °C.

a downward curvature (negative deviation from a straight line), suggesting the presence of a static component in the quenching mechanism [35].

According to Eftink and Ghiron [35], an upward curvature in the Stern–Volmer plot indicates that both tryptophan residues of BSA are exposed to the quencher and the quenching constants of both tryptophan residues are nearly identical, while a downward curvature indicates that the fluorophore population is heterogeneous and only a fraction of fluorophores contributes to the fluorescence signal. Therefore, the fluorescence quenching data were also analyzed by the modified Stern–Volmer plot [36]:

$$F_0/(F_0 - F) = 1/f_a + 1/(f_a K_q[Q]) \quad (3)$$

where f_a represents the fraction of fluorophore (protein) accessible to the quencher and K_q is the apparent quenching constant; F_0, F and $[Q]$ are the same as in Eq. (2).

From the plots of $F_0/(F_0 - F)$ versus 1/[CCMC] (Fig. 7), the values of f_a and K_q were calculated and listed in Table 1. The value of f_a was close to unity at low DS of cholesterol moiety such as f_a (0.96 and 0.95) corresponding to CCMC-3.2 and CCMC-6.9, indicating that both tryptophan residues of BSA were involved in the interaction. However, with DS of cholesterol moiety further increasing, the value of f_a decreased, e.g. f_a (0.60) corresponding to CCMC-9.8, which indicated that part tryptophan residues of BSA were involved in the interaction. Moreover, Table 1 also shows the value of K_q increased from 4.14×10^4 to 1.95×10^5 M^{-1} with DS of cholesterol moiety of CCMC increasing from 3.2% to 9.8%. All above results implied that DS of cholesterol moiety affected the quenching reaction between BSA and CCMC self-aggregated nanoparticles. So it could be deduced that the hydrophobic interactions among cholesterol moieties of CCMC and hydrophobic domains of BSA perhaps play an important role in the formation of CCMC–BSA interactant.

3.4. Structure of CCMC–BSA interactant

Circular dichroism (CD) spectroscopy is often used to estimate the conformation of proteins [26,37]. In this paper, we used CD to determine the conformational changes of BSA due to its interaction with CCMC self-aggregated nanoparticles. In CCMC self-aggregated nanoparticle itself, no CD was observed under the condition employed. The CD spectra of the free and the interacted BSA in the near ultraviolet region are

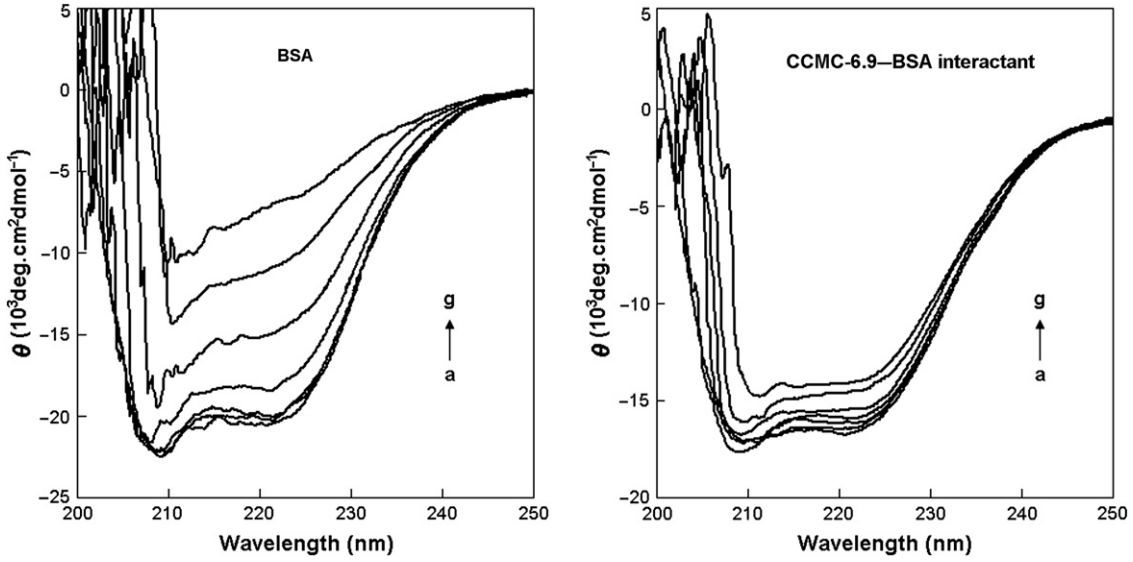

Fig. 9. CD spectra of free BSA, CCMC-6.9–BSA interactant in urea solution with concentrations of: (a) 0, (b) 1.0, (c) 2.0, (d) 3.0, (e) 4.0, (f) 5.0 and (g) 6.0 mol L^{-1}.

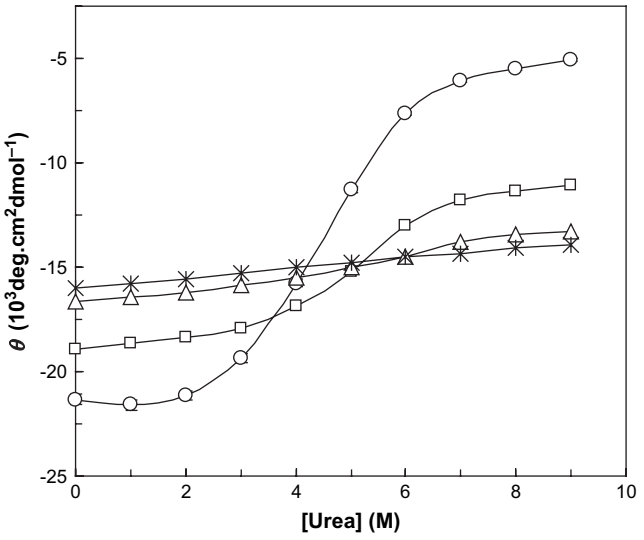

Fig. 10. Mean residual ellipticity (θ) at 222 nm of (−○−) free BSA, (−□−) CCMC-3.2−BSA interactant, (−△−) CCMC-6.9−BSA interactant and (−*−) CCMC-9.8−BSA interactant as a function of urea concentration.

shown in Fig. 8. The main features of BSA spectrum occurred at 208 and 222 nm were in accordance with that reported before [38−39]. As shown in Fig. 8, the CD spectra of BSA changed upon the interaction with CCMC self-aggregated nanoparticles. The helical content of free BSA is 64.2%. After the interaction, however, it decreased to 52.6% for CCMC-3.2, 44.3% for CCMC-6.9, and 41.9% for CCMC-9.8. So that it could be concluded that the higher order structure of BSA changed upon interaction with CCMC self-aggregated nanoparticles and this structural change was related to DS of cholesterol moiety.

3.5. Stability of interacted BSA against denaturant

According to Nishikawa's method [14], urea was chosen as a protein denaturant to evaluate the stability of CCMC−BSA interactants in this paper. CD spectra of free BSA and CCMC-6.9−BSA interactant in the presence of urea are shown in Fig. 9. Free BSA was gradually unfolded to form a random coil structure when the concentration of urea increased to 6 M. However, the CD spectra of CCMC-6.9−BSA interactant slightly changed under the same conditions, and the other CCMC−BSA interactants showed the similar changes. The difference in the mean residual ellipticity ($\Delta\theta$) at 222 nm between the absence and presence of 6 M urea was 13,700 deg cm^2 dmol^{-1} for free BSA, 6000 deg cm^2 dmol^{-1} for CCMC-3.2−BSA interactant, 2150 deg cm^2 dmol^{-1} for CCMC-6.9−BSA interactant and 1500 deg cm^2 dmol^{-1} for CCMC-9.8−BSA interactant (Fig. 10). It was obviously showed that the unfolding of BSA induced by urea was remarkably suppressed upon interaction with CCMC self-aggregated nanoparticles. Furthermore, CCMC self-aggregated nanoparticles with the higher DS of cholesterol moiety more effectively suppressed the unfolding of BSA. Therefore, we deduced that the resistance of BSA against the denaturant increased upon interaction with CCMC self-aggregated nanoparticles.

4. Conclusion

CCMC self-aggregated nanoparticles could interact with BSA, and this interaction was significantly affected by DS of cholesterol moiety of CCMC. The higher order structure of BSA changed upon interaction with CCMC self-aggregated nanoparticles and its stability against a denaturant such as urea remarkably improved. In this sense, CCMC self-aggregated nanoparticle system was hoped to be a novel carrier for the proteins, and the further investigations are in progress now.

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