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ORIGINAL CONTRIBUTION

Synthesis and aggregation behavior of *N*-succinyl-o-carboxymethylchitosan in aqueous solutions

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Abstract A simple and novel method was developed to successfully synthesize *N*-succinyl-*O*-carboxymethylchitosan (NSOCMCS) using *N*-acylation of chitosan with succinic anhydride. The NSOCMCS structure was characterized by Fourier transform infrared and ^{1}H nuclear magnetic resonance. The aggregation behaviors of NSOCMCS were studied using fluorescence spectroscopy, dynamic light scattering, and atomic force microscopy techniques. The critical aggregation concentration of NSOCMCS in water was determined to be 0.2–0.3 mg/ml. The apparent hydrodynamic radium of an NSOCMCS aggregate was dependent on the concentration. The aggregates demonstrated a much swollen association conformation in aqueous solution. The possible aggregation mechanisms for the NSOCMCS in water are discussed. Moreover, a driving force to form an open aggregation morphology was revealed in this study. Aggregation behaviors are important physicochemical properties of NSOCMCS, which impact the potential application in the biomedical field.

Keywords *N*-succinyl-*O*-carboxymethylchitosan · Preparation · Aggregation behaviors · Characterization

Introduction

Aggregation of polymers has been an extensive research topic attracting a great deal of attention in recent years because they are determinates of the apparent molecular weight and polymer morphology in the aqueous solutions. Both of which are closely related to polymer properties and applications [1–4]. Aggregation of amphiphilic nonionic polymers, block copolymers [2, 5, 6], and hydrophobically modified polyelectrolytes including block polyelectrolytes in organic solvents or aqueous solutions [1, 7] has been reported over the past two decades. These polymeric amphiphilic self-assemblies have found widespread applications in the field of drug delivery systems because they are capable of forming aggregates in aqueous solutions. These aggregates are composed of a hydrophobic core and a hydrophilic shell. They are good vehicles for delivering hydrophobic drugs, as the drugs are protected from possible degradation by enzymes [8–12]. The morphology of nanoparticles produced from amphiphilic polymers can be varied by changing the composition of hydrophobic and hydrophilic composites on the polymer chains. Various forms of morphologies have been reported, such as sphere, vesicles, rods, lamellas, tubes, large compound micelles, and large compound vesicles. Some of these structures are good candidates for drug delivery applications [13, 14].

Chitosan is a natural macromolecule consisting of β-1,4-linked 2-amino-2-deoxy-D-glucopyranose, which is derived by *N*-deacetylation of chitin in aqueous alkaline medium [15]. Chitosan is a well-known biocompatible, biodegradable, and low-toxicity biomaterial, and many applications of chitosan have been developed in the biomedical and tissue engineering fields [16]. Because chitosan is water-insoluble due to strong intermolecular hydrogen bonding, the synthesis and physicochemical properties of the water-soluble or amphiphilic chitosan derivatives have been investigated [17–21]. To acquire amphiphilic chitosan derivatives, two techniques have been developed. One method is achieved by grafting a hydrophilic polymer such

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as poly(ethylene glycol) (PEG) to the chitosan backbone. The chitosan-*g*-PEG forms aggregates in aqueous solution spontaneously by intermolecular hydrogen bonding [17, 18]. The other method is hydrophobical modification of chitosan. The modified chitosan self-aggregates can be used as a matrix for a plasmid DNA delivery system. The aggregation of hydrophobically modified chitosan can occur either within a single chain or among different chains. In general, intrapolymer aggregation may be favored in highly dilute aqueous solutions, but interchain aggregation will dominate with the increase in concentration [19–21].

Recently, *O*-carboxymethylchitosan (OCMCS) with a high substitution degree of 6-*O*-carboxymethyl groups has been found to be a good candidate for antitumor drug delivery [22] due to its amphiphilic properties [23]. Moreover, OCMCS has special bioactivities; for example, OCMCS can induce the fusion of dipalmitoyl-*sn*-glycero-3-phosphocholine multilamellar vesicles; and the biomaterial surface immobilized with OCMCS can mediate effectively the attachment, spreading, and organization of 3T3 fibroblasts [24]. Obviously, some of the bioactivities and applications of chitosan derivatives are closely correlated with their aggregation behaviors.

The active amino and carboxyl groups on the OCMCS chains provide us great opportunities to design instrumentally the novel amphiphilic biomacromolecules with tailored physicochemical and biophysical properties. In this study, a simple and novel method was developed to synthesize the *N*-succinyl-*O*-carboxymethylchitosan (NSOCMCS) using *N*-acylation of chitosan with succinic anhydride. It is supposed that NSOCMCS should have amphiphilic properties.

Experimental

Materials

Chitosan powder was supplied by Lianyungang Biologicals, China, which has a deacetylation degree of 90% and viscosity average molecular weight of 20,000 Da. All commercially available solvents and reagents were used without further purification.

OCMCS synthesis was performed according to our previous work

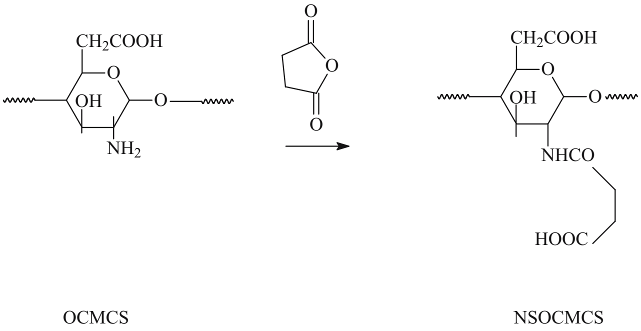
Two-gram chitosan was immersed into 25-ml 50-wt.% NaOH solution to swell and alkalize for 24 h [23]. The alkalized chitosan was crushed into filtration cakes and then transferred into a flask. Monochloroacetic acid (5 g) was dissolved in isopropanol (25 ml) and added into the flask drop-wise for 20 min and reacted for 8 h at room temperature. The reaction mixture was filtered to remove the solvent. The filtrate was dissolved in 100-ml water, and then 2.5-M HCl was added to adjust the pH to 7. With centrifugation of the above solution for removal of the precipitate and then addition of 150-ml anhydrous ethanol to the super-solution, the product precipitated from the solution. The solid was filtered and rinsed with anhydrous ethanol three times and vacuum dried at room temperature. The molar fraction of carboxymethylated groups and amino groups on OCMCS molecules was found to be approximately 100 and 75%, respectively.

NSOCMCS synthesis

One-gram OCMCS was dissolved into 200-ml distilled water and then transferred into a flask. Succinic anhydride (0.2 g) was dissolved in acetone (20 ml) and added into the flask by drop-wise for 30 min at room temperature, and then the reaction was allowed for 4 h at 40 °C. The reaction mixture was cooled to room temperature. The mixture precipitated in an excess of ethanol, was filtered to remove the solvent, and then washed with 70, 80, 100% ethanol, respectively. Finally, the product was dried at 40 °C under vacuum for 24 h. The obtained white powder NSOCMCS was 1.1 g. The NSOCMCS solutions were prepared with double deionized water from the Millipore purifier, and the pH of the NSOCMCS aqueous solution was determined to be approximately 7.0 (Scheme 1).

Characterization

The Fourier transform infrared (FTIR) spectrum of NSOCMCS was recorded using a Biorad FTS 6000 spectrometer at room temperature using a KBr pallet. The ^{1}H nuclear magnetic resonance (NMR) spectra were determined on a Bruker ACF 300 spectrometer using D_2O as solvent. Chemical shifts (δ) were given in ppm using tetramethylsilane as an internal reference.



Scheme 1 Synthesis of NSOCMCS

Steady-state fluorescence spectroscopy

Steady-state fluorescence spectra were recorded on a Shimadzu RF 5301 spectrometer equipped with a quartz fluorescence cell. Pyrene, from an acetone stock solution of 1×10^{-3} M, was used as a micropolarity-sensitive probe in a final concentration of 6.0×10^{-7} M in water. The excitation wavelength was set to 335 nm. The changes in the intensity ratio of the first and the third vibronic bands (I_1/I_3), namely, 373 and 384 nm (I_1 and I_3, respectively) in the emission spectra were used to detect the shift of the hydrophobic microdomain.

Atomic force microscopy

Atomic force microscopy (AFM) topographs of NSOCMCS aggregates were obtained using a Digital Instruments Multimode IIIa atomic force microscope equipped with an E-scanner. Tapping mode silicon nitride cantilevers TESP with nominal spring constants of 20–100 N/m and nominal resonance frequencies of 200–400 kHz were employed. A piece of freshly cleaved mica (about 5×5 mm) was dipped into the fresh filtered solution for 1 to 2 min and then dried in a stream of N_2 followed by vacuum-drying at a pressure of 1.3×10^{-4} Pa overnight. To minimize possible contamination of the surface by ambient air, each sample was freshly prepared just before the AFM experiments.

Dynamic light scattering

The frequency of scattered light fluctuates around the incident light due to the constant motion of the polymer molecules. Dynamic light scattering (DLS) measures the intensity fluctuations with time and correlates these fluctuations to the properties of the scattering objects. In general, the terms of correlation functions of dynamic variables are always used to describe the response of the scattering molecules to the incident light. From the expression

$$\Gamma = Dq^2, \tag{1}$$

the translational diffusion coefficients, D, can be determined. Γ is the decay rate, which is the inverse of the relaxation time, τ; q is the scattering vector $(q = (4\pi n \sin(\theta/2))/\lambda)$ where θ is the scattering angle, n is the refractive index of the solvent, and λ is the wavelength of the incident light. If the Stokes–Einstein equation is used, the apparent hydrodynamic radius R_h can be calculated using the following equation:

$$R_h = \frac{kT}{6\pi\eta D}$$

where k is the Boltzmann constant, T is the absolute temperature, and η is the solvent viscosity. A modified commercial laser light scattering (LLS) spectrometer (ALV/SP-125, Germany) was used, which equipped a solid-state laser (Coherent DPSS) with an output power of 400 mW at λ_0 532.0 nm and an ALV-5000 multi-τ digital time correlator, to perform the dynamic light scattering experiments. The CONTIN Laplace inversion program was used to analyze the time correlation function, and the probability of reject was set to 0.5. All the dynamic LLS measurements were done at 25 °C.

Results and discussion

The synthesis and characterization of NSOCMCS

Figure 1 shows the FTIR spectra of (a) OCMCS and (b) NSOCMCS. From the OCMCS spectrum, it was found that distinctive absorption bands appear at 1,600 and 1,418 cm^{-1} due to asymmetric and symmetric stretching vibration of COO^{-}, indicating a successful substitution of carboxymethyl groups. Because the amino group characteristic at 1,599 cm^{-1} is overlapped with that of carboxyl groups, the peak at 1,600 cm^{-1} shows strong. The absorption band at 1,069 cm^{-1} (skeletal vibration involving the C–O stretching) is the characteristic of its saccharine structure. Compared with that of OCMCS, the strong, broad bands in the NSOCMCS spectrum at 3,392 and 3,274 cm^{-1} are assigned to the hydrogen bonded –OH and –NH bands [23], and the peak at 1,600 cm^{-1} shifts to 1,574 cm^{-1} [vibration involving the amide (–NHCO–) stretching]. These results indicate that a successful acylation took place on the amino position of OCMCS.

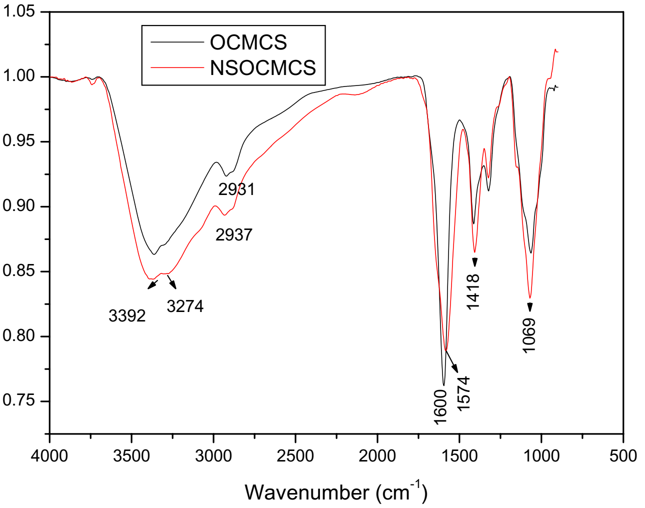


Fig. 1 FTIR spectra of OCMCS and NSOCMCS

The ^{1}H-NMR spectrum of the NSOCMCS is given in Fig. 2. The ^{1}H-NMR assignments of NSOCMCS were as follows: ^{1}H-NMR (D_2O) δ=2.56 (H_*); δ=3.67–4.02 (H_1, H_2, H_3, H_4, H_5); δ=3.11–3.22 (H_6; labeled in Fig. 2). According to the ratio of the integral peak of H_* and H_6, it can be known that the molar ratio of carboxymethylated groups and *N*-succinyl groups on NSOCMCS molecules is about 1.0:0.72, respectively. Because the molar fraction of carboxymethylated groups and amino groups on OCMCS molecules was 100 and 75%, respectively, it can be concluded that most of the amino groups had changed into amide groups through *N*-acylation of OCMCS with succinic anhydride.

Fluorescence spectroscopy

The aggregation behavior of NSOCMCS in water was examined by steady-state fluorescence spectroscopy using pyrene as a fluorescence probe. Figure 3 shows the fluorescence emission spectra of pyrene incorporated into NSOCMCS aggregates in water at 25 °C. The excitation wavelength was 335 nm. If the hydrophobic microdomains are formed in aqueous solution, the hydrophobic pyrene preferably lies in these hydrophobic microdomains to increase its solubility in solution. In the fluorescence spectra, the intensity ratio of the first and the third vibrational bands is sensitive to the circumstance of the pyrene molecules. When the pyrene is dissolved into the NSOCMCS aggregates, the total emission intensity increases, which indicates an increase in the pyrene concentration in solution with addition of NSOCMCS (Fig. 3). In addition, the value of I_1/I_3 drastically decreases. The dependence of I_1/I_3 on polymer concentration is a measure of the critical aggregation concentration (cac). The transition indicates the threshold concentration for the aggregation of polymeric amphiphiles. The change in I_1/I_3 with concentration is shown in Fig. 4. At low concentrations, the I_1/I_3 values are close to a value of 1.72 for pyrene in water, followed by a sharp decrease at a concentration of 0.2–0.3 mg/ml. The cac for NSOCMCS is 0.2–0.3, which is much higher than that of OCMCS (0.05 mg/ml) [23]; the reason for this will be discussed shortly.

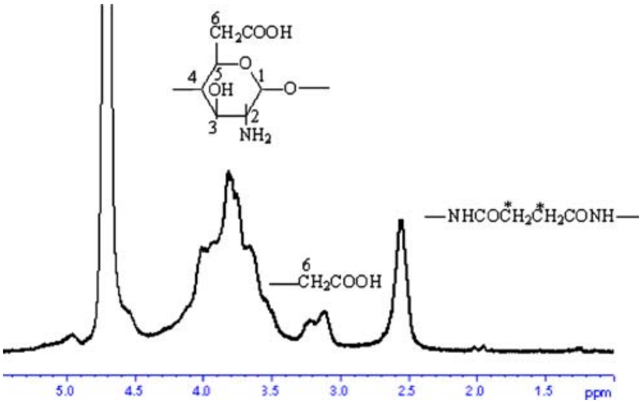


Fig. 2 ^{1}H-NMR spectrum of NSOCMCS

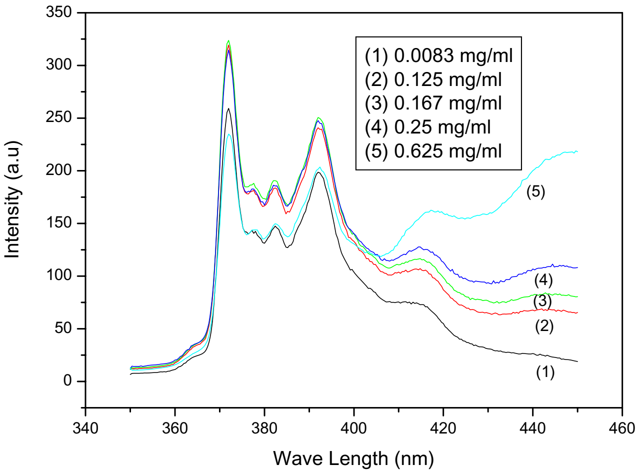


Fig. 3 Effect of polymer concentration on the fluorescence emission spectra of pyrene (6.0×10^{-7} M) in water *(1)* 0.0083, *(2)* 0.125, *(3)* 0.167, *(4)* 0.25, *(5)* 0.625 mg/ml of NSOCMCS. The excitation wavelength was 335 nm

Dynamic light scattering

The dynamics of NSOCMCS in aqueous solution was examined by DLS. Figure 5 shows the relaxation time distribution functions of 0.06 mg/ml of NSOCMCS in water at different scattering angles. The relaxation times of the slow decay shifted to lower values with increasing angles. The decay rate Γ (the reciprocal of peak relaxation time) of the slow decay was found to be proportional to the

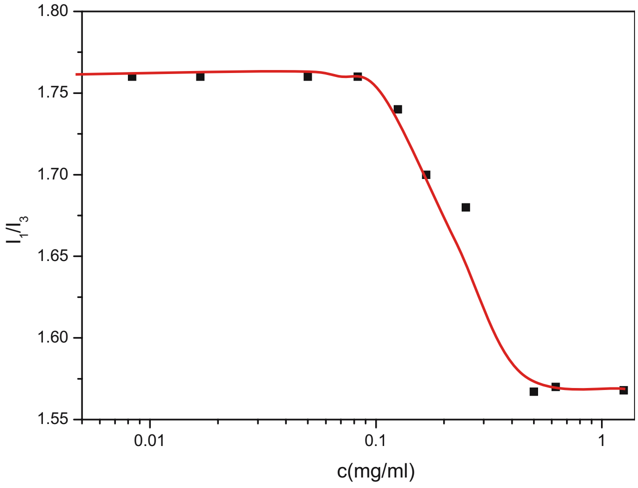


Fig. 4 The concentration dependence of the intensity ratio I_1/I_3 of pyrene in NSOCMCS aqueous solutions

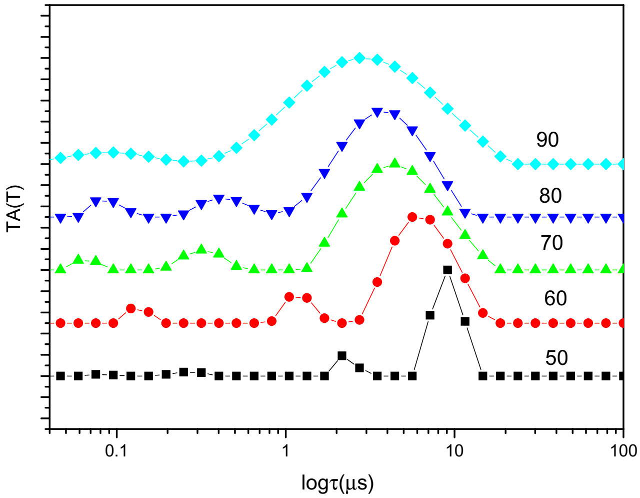


Fig. 5 Relaxation time distribution functions of 0.06 mg/ml NSOCMCS in water at different scattering angles

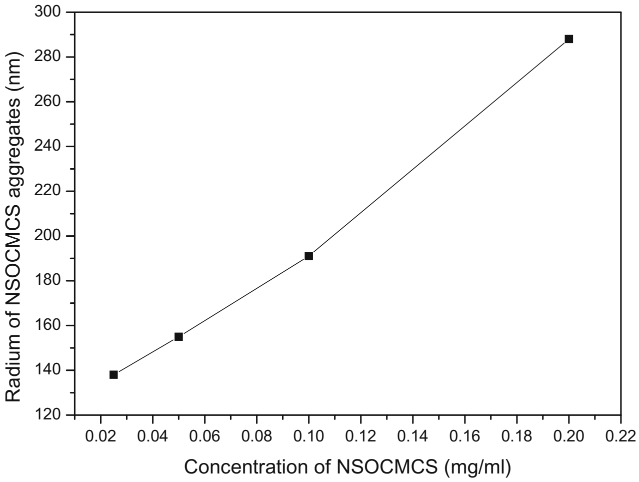


Fig. 7 The hydrodynamic radium of aggregates dependence of NSOCMCS concentrations

square of the scattering vector (q^2), confirming the translational diffusion of scattering objects in solution. On the basis of the Stokes–Einstein relationship, the hydrodynamic radium was determined to be 152–190 nm, which should be related to the NSOCMCS aggregates. The fast decay is not q^2 dependent, which might be caused by the ill-inverse Laplace transform.

The relaxation time distribution functions of NSOCMCS in aqueous solutions with different concentrations at a scattering angle of 90° are shown in Fig. 6. The relaxation time peak increases with an increase in NSOCMCS concentration, which indicates that the aggregate size increases marginally with increasing NSOCMCS concentrations. The hydrodynamic radium R_h of the micelles determined from Eq. 1 for 0.2, 0.1, 0.05, and 0.025 mg/ml of NSOCMCS are 288, 191, 155, and 138 nm, respectively (Fig. 7). In general, if the aggregation mechanism of the amphiphilic polymers corresponds to the closed association model, the particle size of the aggregate is independent of polymer concentration [25]. In the present system, due to the polyelectrolyte property of NSOCMCS, the zeta potential for the aggregates was determined to be −25 mV from the zeta potential measurements (IS 94H). Therefore, there are electrostatic repulsive interactions among the dissociated carboxylic acid groups on the NSOCMCS chains. Moreover,

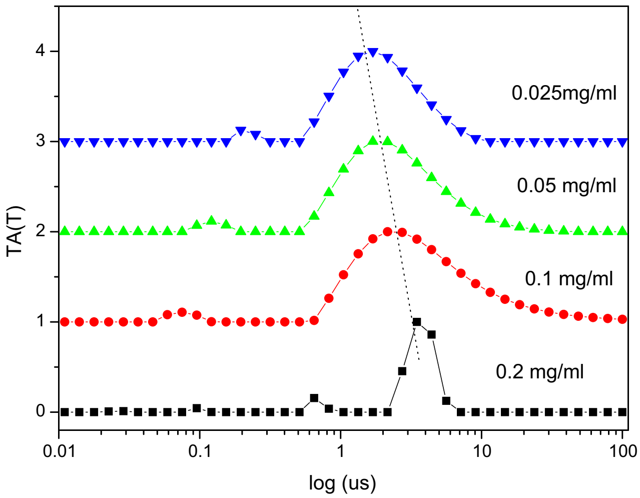


Fig. 6 Relaxation time distribution functions of NSOCMCS in aqueous solutions with different concentrations at a scattering angle of 90°

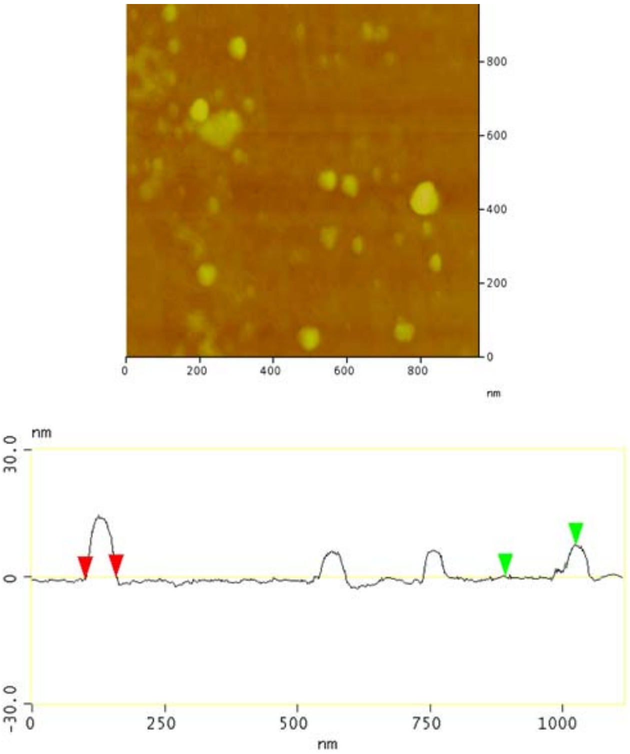


Fig. 8 AFM micrograph of aggregates (dried from 0.2 mg/ml NSOCMCS solution)

there are amide groups on the NSOCMCS molecules, which lead to hydrogen bonding interactions, and thus, the solvation between water and NSOCMCS. Therefore, it could be speculated that NSOCMCS aggregates should demonstrate a swollen association morphology in dilute aqueous solution. In comparison with OCMCS, there are not only hydrophobic moieties in succinic groups but also in carboxyl groups, which have been introduced into NSOCMCS. As a result, there are more dissociated carboxylic acid groups produced in water solution, which makes NSOCMCS much less hydrophobic than OCMCS. This is a possible reason why the cac value for NSOCMCS is much higher than that of OCMCS.

Atomic force microscopy

AFM has been developed as a routine technique to image nonconductive biological macromolecules such as the structures of individual biopolymers or supramolecular assemblies with a high resolution [26–28]. In this work, the formation of aggregates has been examined using dynamic light scattering and a fluorescence probe. Furthermore, AFM was used to investigate the morphology of NSOCMCS aggregates in a dried state. The AFM images are shown in Fig. 8; the aggregates made from 0.2 mg/ml NSOCMCS show a compact and spherical morphology 60–70 nm in diameter. The hydrodynamic radium R_h of NSOCMCS aggregates measured by DLS (288 nm) was almost nine times that measured by AFM. The dried particle sizes measured by AFM are much smaller than the hydrodynamic radius measured by DLS due to shrinkage associated with the drying process. In the present system, the outstanding difference in size can possibly be correlated with the strong solvation of NSOCMCS in water and the interchain electrostatic repulsive interaction. In water, the aggregates demonstrate a much swollen association conformation.

Aggregation mechanism

In the chitosan-based aqueous system, there are electrostatic interactions, hydrophobic interactions, and H bonding, which determine the solubility or assembly behaviors of chitosan derivatives [23]. In the present system, most of the amino groups are transformed into amide groups. The carboxylic acid groups, amide groups, and hydrophobic moieties ($–CH_2CH_2–$) are introduced on the chains with the *N*-acylation of OCMCS with succinic anhydride. In the NSCOCMCS water solution, the driving forces to form aggregates are the intermolecular association of the hydrophobic groups on the polymer chains and the intermolecular hydrogen bonding. Intermolecular association is driven by the hydrophobic interaction caused by the hydrophobic moieties in succinic groups, the acetyl groups, and the glucosidic rings in chitosan. Intermolecular hydrogen bonding is formed due to the amide and hydroxyl groups on NSOCMCS chains. However, the solvation and the interchain electrostatic repulsive interaction inhibit formation of aggregates. When the balance of all these factors has been reached, NSOCMCS aggregates demonstrate the stable and much swollen association morphology in water. However, in the dried state, NSOCMCS aggregates show a dense and spherical conformation due to the short solvation and interchain static repulsive interactions. This is a possible reason for the poor correlation between DLS and AFM for the particle sizes.

Conclusions

NSOCMCS was successfully synthesized using *N*-acylation of chitosan with succinic anhydride. The amphiphilic properties of NSOCMCS were examined by fluorescence spectroscopy, DLS, and AFM techniques. The cac was determined to be in the range of 0.2 to 0.3 mg/ml. The open aggregation morphology was caused by the solvation of NSOCMCS in an aqueous solution and electrostatic repulsive interactions. The main driving forces to form NSOCMCS aggregate in an aqueous solution are the intermolecular association of the hydrophobic groups on the NSOCMCS chains and the intermolecular interaction of hydrogen bonding. The glucose backbone and the hydrophobic groups on NSOCMCS molecules form the hydrophobic regions, and the dissociated carboxylic acid and hydrophilic groups around the hydrophobic domain form the hydrophilic domains. All these aggregation behaviors are of importance for understanding of the physicochemical properties of NSOCMCS and developing its potential applications in the biomedical fields.

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