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Aggregation of Hydrophobically Modified Chitosan in Solution and at the Air–Water Interface

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ABSTRACT: Oleoyl-chitosans (O-chitosans), with three degrees of substitution (DS), were synthesized by reacting chitosan with oleoyl chloride. The chemical structures of these polymers were characterized by ^{1}H NMR and FTIR. The results suggested the formation of an amide linkage between amino groups of chitosan and carboxyl groups of oleic acid. These O-chitosans exhibited poor solubility in aqueous acidic solution. The solubility of O-chitosans decreased as the DS values increased. The transmittance of O-chitosans (2 g/L) with DS 5%, 11%, 27% in 1% (v/v) HCl solution were 69.5%, 62.7%, 48.6%, respectively. These O-chitosans were not soluble at neutral or alkali pH. Formation of self-aggregation was observed using pyrene as a fluorescent probe in the O-chitosans aqueous solution. The increase of DS of O-chitosans resulted in significant decrease of critical aggregation concentration (CAC). The CAC of the O-chitosans with DS 5%, 11%, 27% were 79.43, 31.6, 10 mg/L, respectively. The surface tension of solution could be reduced slightly by all of the O-chitosans. The surface tension of O-chitosans solution decreased with the increase of DS values. © 2006 Wiley Periodicals, Inc. J Appl Polym Sci 102: 1968–1973, 2006

Key words: oleoyl-chitosan; solution properties; fluorescence; surface tension; self-aggregation

INTRODUCTION

Polyelectrolytes bearing a bulky hydrophobic group show a strong tendency of intra- or intermolecular aggregation in polar solvent.[1,2] The aggregation of amphiphilic polymers is of growing interest with respect to biological importance and pharmaceutical or biotechnological applications.[3] The polymeric micelles formed from the molecular assemblage have the ability to take water insoluble drugs or low molecular weight organic compounds and disperse them in the aqueous solution. Longer hydrophilic chains and bigger hydrophobic groups help stabilize the micelle structure and protect drug compounds from the environment.[4] Since the hydrophobic core and hydrophilic shell should be biodegradable and nontoxic, many investigations of hydrophobic polymers are focused on the natural biomaterials.[5]

Chitosan, α-(1-4)-2-amino-2-deoxy-β-D-glucan, is a deacetylated form of chitin, an abundant natural polysaccharide present in crustacean shells. Its unique characteristics such as positive charge, biodegradability, biocompatibility, nontoxicity, and rigid linear molecular structure make this macromolecule ideal as a drug carrier and delivery material.[6,7] Chitosan is soluble in aqueous solutions of various acids, but chitosan molecules have no amphiphilic property and cannot form micelles in water. Thus, there are many reports on hydrophobic modifications of chitosan, for example, palmitoyl glycol chitosan,[8] deoxycholic acid-modified chitosan,[9] poly(*N*-isopropylacrylamide)–chitosan,[10] linoleic acid-modified chitosan,[11] linolenic acid-modified chitosan,[12] *N*-alkyl-*O*-sulfate chitosan,[13] chitosan-polylactide graft copolymer,[14] *N*-acetylchitosan, *N*-propionylchitosan, and *N*-butyrylchitosan,[15] butanoyl-chitosan, hexanoy-chitosan, benzoyl-chitosan,[16] etc.

These modifications can introduce hydrophobic group into chitosan and form amphiphilic chitosan polymers. Some of these amphiphilic chitosan polymers can form nano-sized self-aggregation in aqueous media.[17] Long chain fatty acyl derivatives of chitosan is a novel hydrophobic modification method that is used to form nanoparticles. In our previous work, we have prepared linoleic acid-modified chitosan nanomicelles[11] and linolenic acid-modified chitosan nanoparticles.[12] But most of these researches have studied the self-aggregation of product with only one degree of hydrophobic substitution (DS). For example, the DS of linoleic acid-modified chitosan was 5.1%,[11] and the DS of linolenic acid-modified chitosan was 1.8%.[12] However, the low degree of hydrophobic substitution may result in weakening stability of the aggregation. Thus, it was expected that the DS of hydrophobic-modified chitosan would effect on the aggregation behavior in the aqueous solution. Herein, we investi-

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gated in detail the effect of DS of hydrophobic-modified chitosans on the physicochemical characteristics and self-aggregation behavior in aqueous media.

In this paper, oleoyl-chitosans (O-chitosans) with different DS were prepared. The molecule structures of O-chitosans were analyzed by FTIR and ^{1}H NMR. Oleic acid was chosen as a hydrophobic group that made O-chitosans strongly amphipathic. The effect of the DS on the aggregation behavior of these O-chitosans in aqueous medium and at the air–water interface was investigated by fluorescence spectroscopy and surface tension.

EXPERIMENTAL

Materials

Chitosan, degree of deacetylation 82%, molecular weight 35 kDa, was made from crab shell and obtained from Biotech (Mokpo, Korea). Pyrene, oleoyl chloride, pyridine, chloroform, and methylene chloride were purchased from Sigma Chemicals. Pyridine was dried over KOH for 48 h and then distilled. Chloroform was dried over $CaCl_2$ for 48 h and then distilled.[18]

Synthesis of O-chitosans

Chitosan (1.0 g) was soaked in pyridine for 72 h, and the pyridine was evaporated under reduced pressure. Chitosan was then soaked again in a mixture of pyridine (30 mL) and chloroform (15 mL) for 1 day. The mixture was cooled to -10 to -5°C in an ice–salt bath, and oleoyl chloride dissolved in chloroform (5 mL) was added dropwise for 1 h. The mixture was then stirred for 2 h at room temperature and further refluxed for 10 h. The product was poured into methanol (100 mL), and the precipitated product was filtered with filter paper, extracted in a Soxhlet extractor with methanol for 8 h, and dried in vacuum for 24 h. The degree of N-acylation, which can be defined as the number of oleic acid groups per 100 anhydroglucose units of chitosan, was evaluated by FTIR method.[19]

FTIR and ^{1}H NMR spectroscopy

The IR spectra of chitosan and O-chitosans were recorded on an Avater-360 FTIR spectrometer (Nicolet) at 20°C following the method of Shigemasa et al.[20] For the IR spectroscopic analysis, 2 mg of the samples was mixed with 100 mg of KBr and made into pellets.

^{1}H NMR spectra of samples were recorded on a JNM-ECP600 spectrometer at 25°C. The samples were dissolved in 1% CD_3OOD of D_2O solution (v/v) to give the concentration of 30 mg/mL. The measurement conditions were as follows: a spectral window of 500 Hz, 32k data points, a pulse angle of 30°, an acquisition time of 2.03 s, and 32 scans with a delay of 1 s between each scan.[21]

Solubility test of O-chitosans

Solubility of the O-chitosans was evaluated by the turbidity.[22] After being pulverized gently, the samples (10 mg) was dissolved in 1% (v/v) aqueous HCl (5 mL), and the transmittance of the solution was recorded on a UV–vis spectrophotometer (UV-160, Shimadzu), using a quartz cell with an optical path length of 1 cm at 600 nm. The pH dependence of the water solubility of the chitosan and O-chitosans was also estimated from measurement of transmittance of the solution.[23] Briefly, O-chitosans (10 mg) was dissolved in 2% (v/v) HCl solution (5 mL), the pH of the solution was adjusted by the addition of 10% NaOH solution and the transmittance of the solution at 600 nm as a function of pH value was recorded.

Measurement of fluorescence spectroscopy

Pyrene, used as a hydrophobic probe, was purified by repeated recrystallization from ethanol and vacuum dried at 20°C. Purified pyrene was dissolved in ethanol at the concentration of 0.4 mg/mL. About 10 μL of this solution was pipetted into a test tube, and the ethanol was driven off by under a stream of nitrogen gas. About 10 mL of O-chitosans solution was added to the test tube, bringing the final concentration of pyrene to 2 μM. The mixture was incubated for 3 h in a water bath at 65°C and shaken in a BS-10 skaking water bath overnight at 20°C. Pyrene emission spectra were obtained using a Shimadzu RF-5301PC fluorescence spectrophotometer (Shimadzu Co., Kyoto, Japan). The probe was excited at 343 nm,[24] and the emission spectra was obtained in the range of 360–500 nm at an integration time of 1.0 s. The excitation and emission slit opening were 15 and 1.5 nm, respectively.

Measurement of surface tension

The aggregation behavior of the O-chitosans at the air–water interface was also determined from the measurements of surface tension. The surface tension measurements of chitosan and O-chitosans solutions at various concentrations were carried out on a Krüss K12 Processor Tensionmeter with plate method of Wilbelmy at 30 ± 0.1°C. All of the solutions were kept at room temperature for 1 day before surface tension measurement.[25]

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TABLE I
The Degree of Substitution and Yield of O-chitosans

Initial monomolar ratio (oleoyl chloride/NH_2)	DS (%)	Yield (%)[a]
4 : 1	5	98.0
2 : 1	11	96.32
1 : 2	27	93.25

DS = number of oleic acid groups per 100 anhydroglucose units of chitosan (%).
[a] Yield = O-chitosan (g)/chitosan (g) × 100%.

Statistical analyses

The assays were performed at least in triplicate on separate occasions. The data collected in this study were expressed as the mean value ± standard deviation.

RESULTS AND DISCUSSION

Preparation and characterization of O-chitosan

O-chitosans with different DS were prepared using chitosan and oleoyl chloride and the DS of the O-chitosans are shown in Table I. The synthetic procedures for the coupling oleoyl chloride and chitosan are represented in Figure 1.

The infrared spectra of chitosan and O-chitosans are shown in Figure 2. The presence of both 2-aminoglucose and 2-acetamidoglucose repeat units was confirmed by bands at 1655 cm^{-1} (υ C=O), 1570 cm^{-1} (δ N—H of primary amino), 1555 cm^{-1} (δ N—H of amide II) and 3000–4000 cm^{-1} (OH, NH_2) [Figure 2(a)]. The spectra of O-chitosans [Figure 2(b–d)] exhibited many alterations: the absorption at 3000–4000 cm^{-1} (OH, NH_2) decreased, the band at 1570 cm^{-1} (δ N—H of amide) decreased, while prominent bands at 1655 cm^{-1} (υ C=O) and 1555 cm^{-1} (δ N—H of amide II) were observed. The peaks at 2924 cm^{-1} (υ_{as} CH_2), 2854 cm^{-1} (υ_s CH_2), 1464 cm^{-1} (δ CH_2),[18] 1182 cm^{-1} (twisting vibration of CH_2) were stronger and shaper in the latter, their intensity was proportional to the substitution. These results confirmed that the chitosan was substituted with oleoyl group.[19]

The ^1H NMR spectra of the original chitosan and the O-chitosan (DS 5%) were compared in Figure 3. The proton assignment of chitosan [Figure 3(a)]:

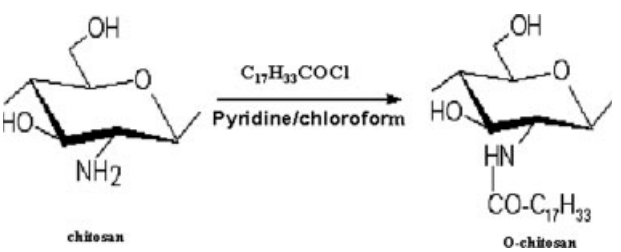

Figure 1 Synthetic procedure of O-chitosans.

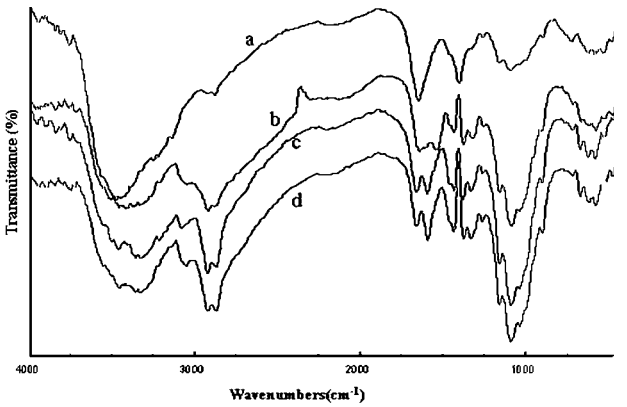

Figure 2 The FTIR of chitosan and O-chitosans (a) chitosan, (b) O-chitosan (DS 5%), (c) O-chitosan (DS 11%), and (d) O-chitosan (DS 27%).

$\delta_{2.0}$ = CH_3 (acetyl group of chitosan); $\delta_{2.95}$ = CH (carbon 2 of chitosan); $\delta_{3.3–3.7}$ = CH (carbon 3–6 of chitosan); $\delta_{4.2–4.5}$ = CH (carbon 1 of chitosan); the proton assignment of O-chitosan [Figure 3(b)]: $\delta_{1.2}$ = CH_3 (methyl group of oleoyl); $\delta_{2.0}$ = CH_3 (acetyl group of chitosan); $\delta_{2.95}$ = CH (carbon 2 of chitosan); $\delta_{3.15}$ = CH_2 (oleoyl protons); $\delta_{4.2–4.5}$ = CH (carbon 1 of chitosan). The ^1H NMR spectrum confirmed the presence of major functional groups linked to chitosan on O-chitosan as previously reported. A new peak at 1.20 ppm, which originated from the ^1H NMR spectra of O-chitosan, was mainly due to characteristic methyl protons of the reacted chitosan with oleic acid.[26]

The solubility of chitosan and O-chitosans in aqueous solution are shown in Figure 4 O-chitosan and O-chitosans were soluble in aqueous acidic solution. As the DS of O-chitosans increased, the solubility tended to decrease. The transmittance of 1% (v/v) HCl solution of the O-chitosans with DS 5%, 11%, 27% were 69.5%, 62.7%, 48.6%, respectively. The solubility of chitosan and O-chitosans was due to the protonation of primary amino groups. This indicates that a significant amount of D-glucosamine residues still remained for the unique solubility of O-chitosans.[27] The solubility of chitosan and O-chitosans in aqueous

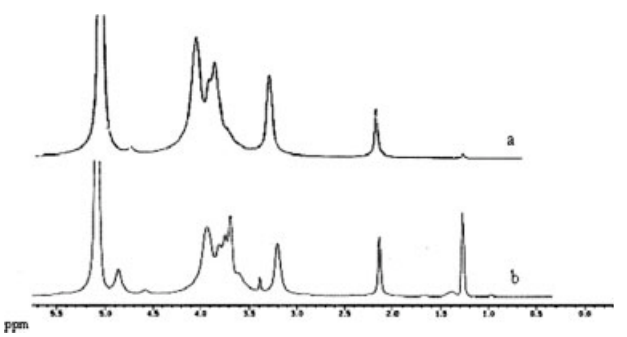

Figure 3 ^1H NMR spectra of (a) chitosan and (b) O-chitosan (DS 5%).

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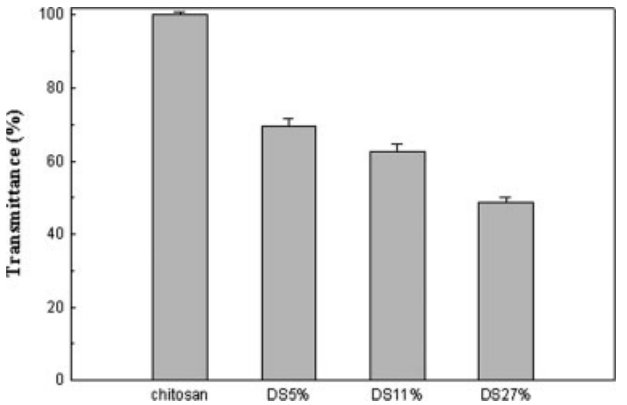

Figure 4 Substitution dependence of water solubility of O-chitosans.

solution at various pHs are shown in Figure 5. Chitosan and O-chitosans were soluble in an aqueous acidic solution below pH 6.5.[27] However, these derivatives were not soluble at neutral or alkaline pH. The solubility of chitosan and O-chitosans decreased with increase in pH values. This may be due to two reasons: (1) The hydrophobic oleoyl groups were introduced to hydrophilic $—NH_2$ groups to the decrease in concentration of ionized $—NH_3^+$ and (2) at higher substitution ratios, O-chitosans samples assumed crystalline structure again unlike intact chitosan because of the hydrophobicity of substituted oleoyl chains, which seemed to decrease the solubility capacity. Hydrophobic *N*-acyl groups were present in the inner part of chain clusters, and hydrophilic amino groups are present at the outer part. The solubility was influenced by the proportion of the hydrophobic and the hydrophilic group.[28]

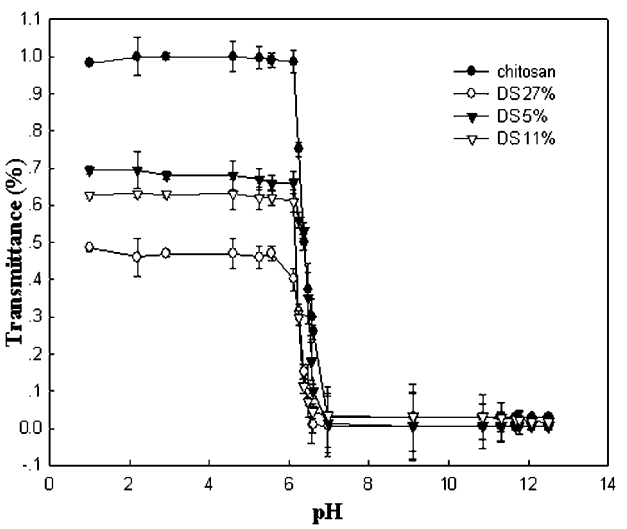

Figure 5 pH dependence of water solubility of O-chitosans and chitosan.

Aggregation behavior of O-chitosan

Self-aggregates of O-chitosan in solution

Figure 6(a) showed the fluorescence spectra of pyrene in chitosan solution in 1% (v/v) acetic acid. Each spectrum corresponded to chitosan concentration of 0.0001–5 g/L. The fluorescence spectra of pyrene at various concentrations of the O-chitosans are shown in Figure 6(b). At the lower concentrations of O-chitosan solution (0.0001–0.01 g/L), the spectra were similar to those of chitosan. At the higher concentrations (0.1–5 g/L), the spectra were drastically different from those of chitosan. This revealed that an apolar local microenvironment of the pyrene in the O-chitosan solution.

The variation of I_3/I_1 ratio with the logarithm of concentration of O-chitosans/chitosan is shown in Figure 7. The peak height I (I_1) at 372 nm is indicative of the sensitivity of pyrene to hydrophobic environment while the peak III (I_3) at 384 nm is relatively unaffected. The peak I_3/I_1 ratio can therefore be used to determine the reactivity or aggregation properties of amphipathic molecules to the change in environment hydrophobicity in the aqueous system. The critical aggregation concentration (CAC) of samples was determined from the change of the quotient of vibrational band intensities in fluorescence emission spectrum of pyrene in a conventional way.[29] At low poly-

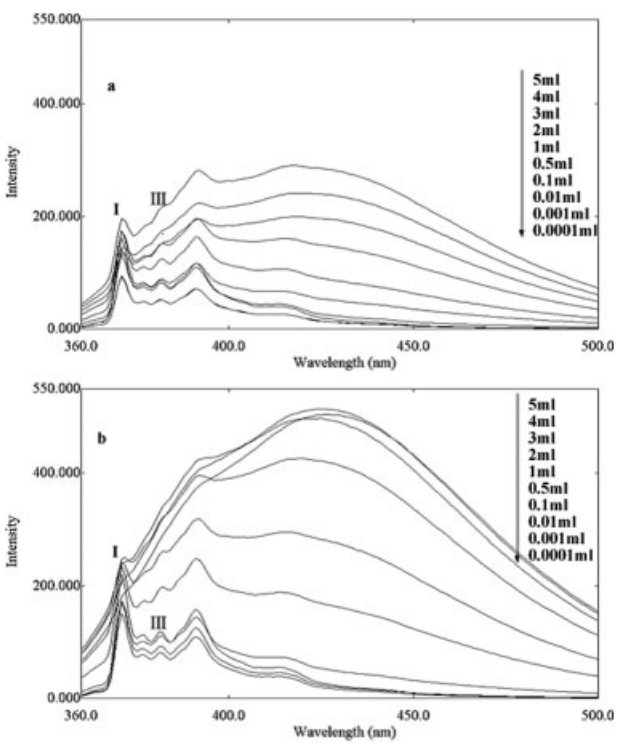

Figure 6 Fluorescence spectra of pyrene in (a) chitosan solution and (b) O-chitosan (DS 11%) solution. The concentrations of O-chitosans were from 0.0001 to 5.0 g/L in 1% acetic acid with 2.0 μM pyrene.

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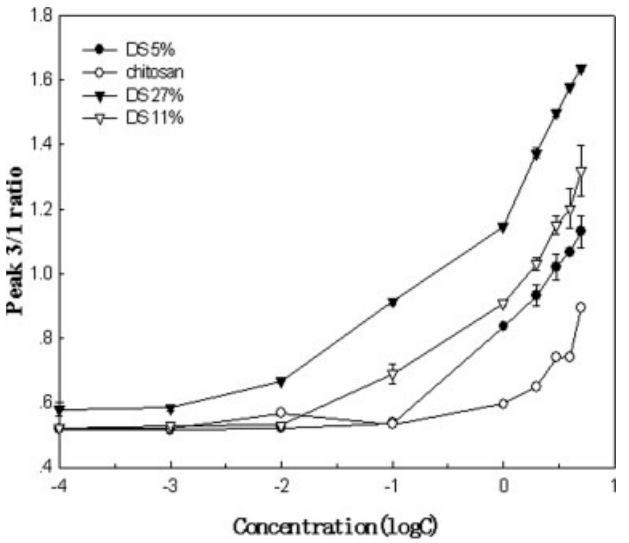

Figure 7 Peak III/I ratio of pyrene fluorescence as a functional of O-chitosans and chitosan concentration in 1% (v/v) acetic acid.

mer concentrations, the I_3/I_1 ratio was about 0.5, a value similar to that measured in an aqueous environment. As polymer concentration increased, an increase in I_3/I_1 was observed, revealing an increasingly apolar local microenvironment of the pyrene. This phenomenon suggested that hydrophobic intra- and/or intermolecular interactions between the oleoyl groups induce the formation of hydrophobic microdomains in which pyrene was solubilized.

Figure 7 also shows the effect of the DS on the I_3/I_1 ratio. It was found that high DS sample had a more pronounced hydrophobic character than the sample with low DS had. Poor ability to form micelle aggregates was observed with the low DS O-chitosan (DS 5%). The CAC values determined in acidic aqueous media were 79.43 mg/L. In contrast to this, the CAC value of samples with DS 11 and 27% were equal to 31.6 and 10 mg/L. The increase in DS (5-27%) resulted in the significant decrease in CAC value. Higher degree of hydrophobic substitutions in the macromolecule of chitosan may facilitate its self-aggregation, favoring hydrophobic interactions, and thus, the formation of dense polymer aggregates. The higher I_3/I_1 ratio observed may indicate that there were more hydrophobic cores in the high DS samples and the cores were more compact than that of the low DS samples. This could be due to water penetration into these aggregates extending up their cores in the low DS samples. This result was typical of hydrophobically modified polymers: the higher the number of hydrophobic groups grafted to a macromolecule, the greater its ability to self-aggregation in solution.

The CAC value of O-chitosans was much lower than that of a typical monomer surfactant SDS (sodium dodecyl sulfate) that had the same hydrophobic residues (2.1×10^3 mg/L)[30] and almost equivalent to those CAC of dodecyl-disaccharides-based surfactants (86.7 and 31.6 mg/L).[31,32] The results were consistent with an earlier study by Lee et al.[33] and Ngimhuang et al.[34]

Interfacial behavior of the O-chitosans

Because of their amphiphilic structure, O-chitosans also had potential surface and interfacial properties. Surface tension studies had been done using various concentrations at the air–water interface depending on the DS of these polymers. Table II showed the surface tension of O-chitosans with different DS and various concentrations. It was seen that the surface tension decreased with increasing polymer concentration and this was slightly more pronounced for high DS samples. The surface tension value of O-chitosan of DS 27% with concentration 10, 5, 1 g/L were 67.1, 61.34, 61.01 mN/m, respectively. The result demonstrated that the introduction of hydrophobic substituents made the derivatives become amphiphilic polymers, which can decrease the surface tension. These modified O-chitosans adopted an extended conformation at the interface in which its sugar moieties were immersed in the aqueous phase, whereas the hydrophobic oleoyl groups were exposed to the air phase, to reduce surface tension. As the concentration increased, more macromolecules unfold at the interface and may adsorb irreversibly by ''anchoring'' their multiple hydrophobic functional groups into the nonpolar phase.[35]

It was also shown in Table II that the surface tension of O-chitosans tended to decrease with the DS increased, especially the samples with high concentration. The surface tensions of three samples of 10 g/L with DS 5%, 11%, 27% were 67.19, 64.4, 61.01 mN/m, respectively. More amphiphilic polymers could get to air–water interface in the solution of high DS O-chitosan, so the surface tension of O-chitosans solution decreased with the increase of DS values. These phenomena were confirmed by the fluorescence measurement. The product with high DS had more hydrophobic group and had more possibility of the adsorbed

TABLE II
Surface Tension of Chitosan and O-chitosans at Various Concentrations (g/L)

	Surface tension (mN/m)		
Samples	1 g/L	5 g/L	10 g/L
Chitosan	71.6 ± 0.02	68.525 ± 0.03	67.23 ± 0.01
DS 5%	71.1 ± 0.01	68.35 ± 0.01	67.19 ± 0.03
DS 11%	69.1 ± 0.02	66.38 ± 0.04	64.4 ± 0.02
DS 27%	67.1 ± 0.05	61.34 ± 0.02	61.01 ± 0.03

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alkyl side chains forming dense packing at the interface. But different from the low molecule surfactants, the O-chitosans showed only a slightly ability to decrease the surface tension.[36] The reason may be that the hydrophobic chains of the O-chitosans molecules as an amphiphilic polymer could associate to form aggregation in the solution while they concentrated on the surface before the surface tension got to the lowest value. And the stiffness of the backbones and the charge density for polyelectrolytes of O-chitosans also affected the adsorption at the air–water interface.

CONCLUSIONS

Oleoyl-chitosans (O-chitosans), with three DS, were synthesized by reacting chitosan with oleoyl chloride. The FTIR and ^{1}H NMR spectra confirmed the formation of an amide linkage between amino groups of chitosan and carboxyl groups of oleic acid. These O-chitosans exhibited poor solubility in aqueous acidic solution than chitosan. The transmittance of 1% (v/v) aqueous solution of the O-chitosans with DS 5%, 11%, 27% were 69.5%, 62.7%, 48.6%, respectively. These O-chitosans were not soluble at neutral or alkaline pH. The fluorescence spectroscopy showed that self-aggregates were formed in the O-chitosans solution. The increase in DS of O-chitosans resulted in the significant decrease in CAC value. The CAC of the O-chitosans with DS 5%, 11%, 27% were 10, 31.6, 79.43 mg/L, respectively. The CAC values suggested that polymer micelle was highly depended on the hydrophobic substituents. The surface tension of solution could be reduced slightly by all of the O-chitosans. The surface tension of O-chitosans solution decreased as the DS values increased.

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