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Self-aggregation and antibacterial activity of *N*-acylated chitosan

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Abstract

Chitosan was selectively *N*-acylated with acetic, propionic and hexanoic anhydrides under homogeneous condition to prepare *N*-acetyl chitosan (NACS), *N*-propionyl chitosan (NPCS) and *N*-hexanoyl chitosan (NHCS), respectively. NACSs with different *N*-acetylation degrees were obtained by controlling the degree of *N*-acetylation. The chemical structures of *N*-acylated chitosans including degree of deacetylation (DD), weight-average molecular weight (M_w), radius of gyration ($\langle S_2 \rangle_Z^{1/2}$) and crystal structure were studied by FTIR, GPC-LLS and X-ray diffraction techniques. Aggregation behavior of *N*-acylated chitosan was investigated by rheometer. Intramolecular aggregation of NPCS and NACS was stronger with NPCS stronger than NACS. The effect of concentration of polymer, concentration of salt and temperature on self-aggregation of NACS and NPCS was investigated. Hydrophobic interaction of *N*-acylated chitosan substituted with longer acyl chains was stronger. With moderate DD, intramolecular aggregation occurs predominantly. In vitro antibacterial activity test of *N*-acylated chitosans was evaluated against two Gram-positive bacteria and two Gram-negative bacteria. Relative inhibition time (RIT) of NHCS with concentration of 1 mg/ml against *Escherichia coli* and *Pseudomonas aeruginosa* was more than 2–6 times longer than that of NACS and NPCS. *N*-acylated chitosan with lower DD had inhibitory effect on the growth of bacteria than that with moderate DD. The results showed that intermolecular aggregation characteristic of *N*-acetylated chitosans with low DD may help in forming bridge to interact with bacterial cell.

Keywords: *N*-acylated chitosan; Self aggregation; Antibacterial activity

1. Introduction

Self-aggregation phenomena of amphiphilic polymers in aqueous solution are of growing interest with respect to biological importance and pharmaceutical or biotechnological applications [1]. Recently, biopolymers and various synthetic charged or nonionic polymers have been used as hydrophilic backbone to prepare amphiphilic polymers by hydrophobic substitution [2,3]. The self-association of hydrophobes covalently linked to water-soluble polymers can occur either within a single polymer chain or among different polymer chains, or both.

Chitosan, derived from chitin, is a natural nontoxic biopolymer consisting of β-(1,4)-2-acetamido-2-deoxy-D-glucose and β-(1,4)-2-amino-2-deoxy-D-glucose units. Strong intramolecular hydrogen bonding was formed in solution because of the large number of OH and acetamido groups on the chitosan molecular chains. In addition, the hydrophobic moieties in chitosan, i.e. acetyl groups and glucosidic rings, can play a significant role on aggregation and in the formation of hydrophobic interaction [4–6]. However, few studies deal with the different acyl chains on self-aggregation of chitosan.

In recent years, chitosan and its derivatives received considerable attention due to their potential beneficial biological activities, such as antitumor [7], antimicrobial [8–11], immune enhancing effects [12], etc. The antimicrobial action of chitosan is influenced by both intrinsic factors and the environmental conditions. Conformational behavior studies are important

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for a better understanding of chitosan solution behavior and biological activities of chitosan. Many papers have reported that half *N*-acetyl chitosan had better physiological activity such as antitumor activity and immune enhancing effects in vivo [13,14]. And water-soluble chitosan with DD around 50% had the highest affinity for *Escherichia coli* cells and was adsorbed in highest amounts [15]. However, special information on the correlation of aggregation and antibacterial activity of *N*-acylated chitosan is lacking, although it is necessary for understanding correctly the antimicrobial effect of chitosan.

In the present study, a series of acylated chitosans with different acyl groups were prepared and their structures were characterized in detail. In order to elucidate the intimate relationship between antibacterial activity and aggregation, self-aggregation behavior of *N*-acylated chitosans was investigated by rheometer. Antibacterial activities of *N*-acylated chitosans were evaluated against two Gram-positive bacteria and two Gram-negative bacteria.

2. Experimental

2.1. Materials

Chitosan was supplied by Yuhuan Ocean Biochemistry Co. Ltd. (Taizhou, China). The molecular weight calculated from the GPC method was about 2.1×10^5 Da. The deacetylation degree was 92%, which was determined by pH titration method [16]. Acetic anhydride and propionic anhydride were of analytical grade and hexanoic anhydride was of chemical grade. Other chemicals used were of analytical grade.

2.2. Preparation of N-acylated chitosans

The *N*-acylation reaction was generalized as follows. Chitosan (4 g) was dissolved in 100 ml of 3% acetic acid, the desired amount of acyl anhydrides (acetic anhydride, propionic anhydride and hexanoic anhydride) ethanol solution (corresponding to a molar ratio of 0.5 compared with chitosan residue) was added slowly with stirring, respectively. After a further 4 h of continuous stirring, the reaction solution was neutralized. Finally the products were washed with water and ethanol repeatedly and dried under vacuum to obtain a series of *N* selectively acylated products. Acetyl, propionyl and hexanoyl substituted chitosans are designated as NACS, NPCS and NHCS, respectively. NACS with different DDs was prepared by the same method and the number following NACS indicated the molar ratio of 0.1, 0.2, 0.3, 0.4 and 0.5 carboxylic anhydrides compared with chitosan residue. Excess amounts of anhydrides are required to get highly substituted *N*-acylated chitosan, which forms gels.

2.3. Characterization of N-acylated chitosans

FTIR spectra were recorded in powder form in KBr discs in the range of 4000–400 cm^{-1} on a Nicolet 670 FTIR spectrophotometer (Madison, USA).

The DD of *N*-acylated chitosan was determined by pH titration method: chitosan (0.1 g) was dissolved in a known excess of 0.1 M HCl acid (10 ml). From the titration of this solution with a 0.1 M NaOH solution, a curve with two inflexion points was obtained. The amount of the acid consumed between these two points was considered to correspond to the amount of the free amino groups in the solution. The titration was performed with a DELTA-320-S pH meter (Mettler-Toledo, Switzerland).

Weight-average molecular weight (M_w) and radius of gyration ($\langle S_2 \rangle_Z^{1/2}$) of samples were also determined by gel permeation chromatography combined with laser light scattering (GPC-LLS) on a multi-angle laser photometer ($\lambda = 633$ nm, DAWNDSP, Wyatt Technology Co., USA) combined with a P100 pump equipped with TSK-GEL G5000PWXL column and differential refractive index detector (RI-150) at 25 °C. The eluent was 0.12 M CH_3COOH–0.05 M CH_3COONa at a flow rate of 0.5 ml/min. All the solutions used were first filtered with a sand filter and then with a 0.20 mm filter (Whatman, UK). Astra software was utilized for data acquisition and analysis. The refractive index increments (dn/dc) were determined by using an Optilab refractometer (OPTILAB-DSP, Wyatt Technology Co., USA) at 25 °C.

X-ray diffraction (XRD) spectra of the powder samples were performed using a D8 Advance diffractometer (Bruker, USA) with Cu target and Kα radiation ($\lambda = 0.154$ nm) at 40 kV and 50 mA. The scanning rate was 4°/min and the scanning scope of 2θ was 5°–45° in a fixed time mode with a step interval of 0.02° at room temperature.

2.4. Self-aggregation behavior of N-acylated chitosan solutions by rheological measurement

The rheological properties were performed using a strain-controlled ARES rheometer (TA Inc., New Castle, USA). The rheometer was equipped with two sensitive force transducers for torque measurements ranging from 0.004 to 100 g cm. A Couette (two concentric cylinders) cell geometry was used for monitoring the steady state shear flow of the samples' modulus. The rheometer is equipped with a thermobath with circulating water that was calibrated to give a temperature in the sample chamber within ±0.5 °C of the set value. The values of the strain amplitude were checked to ensure that all measurements were carried out within the linear viscoelastic regime, where the G' and G'' are independent of the strain amplitude according to the result of dynamic strain sweep.

The steady state viscosity measurements were carried out in the shear rate range from 0.1 to 100 s^{-1}. The solutions were investigated at various concentrations at 25 °C and in the solvent 0.3 M acetic acid (CH_3COOH) in the presence of 0.05 M sodium acetate (CH_3COONa). For the frequency sweep measurements, the sweep of the frequency was from 0.02 to 100 rad/s, while the temperature was kept at 25 °C and each frequency sweep took 900 s to be completed.

2.5. Evaluation of antibacterial activity in vitro

Staphylococcus aureus ATCC 25923, *E. coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853, used for antibacterial evaluation, were provided by the Typical Culture Collection Center in Wuhan University, China, and *Bacillus subtilis* was provided by the Biology Engineering College of Hubei University of Technology, China. The cultures, obtained by growing the bacteria overnight at 37 °C in nutrient broth, were diluted with sterile normal saline (0.9%) solution and each of the culture suspensions containing ca. 10^5–10^6 CFU/ml was used for the antibacterial test.

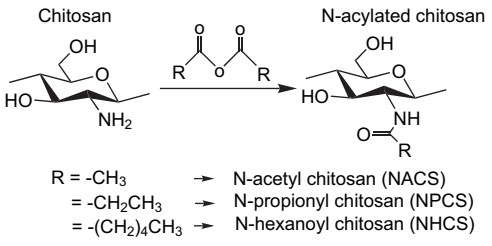
Sample solutions of 10 mg/ml in 0.2 M sodium acetate–0.2 M acetic acid buffer (pH 5.4) were autoclaved at 121 °C for 15 min, and each of these solutions (1 ml) and nutrient agar (peptone 1%, beef extract 0.5%, NaCl 0.5%, agar 2%, pH 6.9) were poured into autoclaved petri dishes, cooled, one loopful of microorganism suspension was spread on the cooled nutrient agar, and then incubated at 37 °C. Sodium acetate (0.2 M)–acetic acid (0.2 M) buffer (pH 5.4) was used as a control. Relative inhibition time (RIT) was defined and measured as the difference between the time when microbial colonies were visible in agar plates with and without test samples [17].

3. Results and discussions

3.1. Characterization of N-acylated chitosans

The addition of a series of carboxylic anhydrides to the solution of chitosan under homogeneous conditions produced a series of *N*-acylated chitosans. The reaction route and chemical structures of *N*-acylated chitosans with different acyl chain lengths are shown in Scheme 1.

The FTIR spectra of *N*-acylated chitosans exhibit many differences than that of chitosan (Fig. 1). The native chitosan was used as starting material with 92% DD. The absorption band at 1599 cm^{-1} was referenced as N–H bending vibration of $-NH_2$. For NACS0.1, a new peak at 1641 cm^{-1} assigned to the carbonyl stretching of secondary amides (amide I band) appeared. The broad peak at around 3430 cm^{-1} corresponds to the stretching vibration of $-NH_2$ group and –OH group shifted to higher frequency, indicating hydrogen bond destruction by *N*-acylation. With further acylation of NACS0.2 and NACS0.3, the vibrational band corresponding to primary amino groups at 1599 cm^{-1} gradually disappeared and



Scheme 1. *N*-acylated chitosans with different acyl chain lengths.

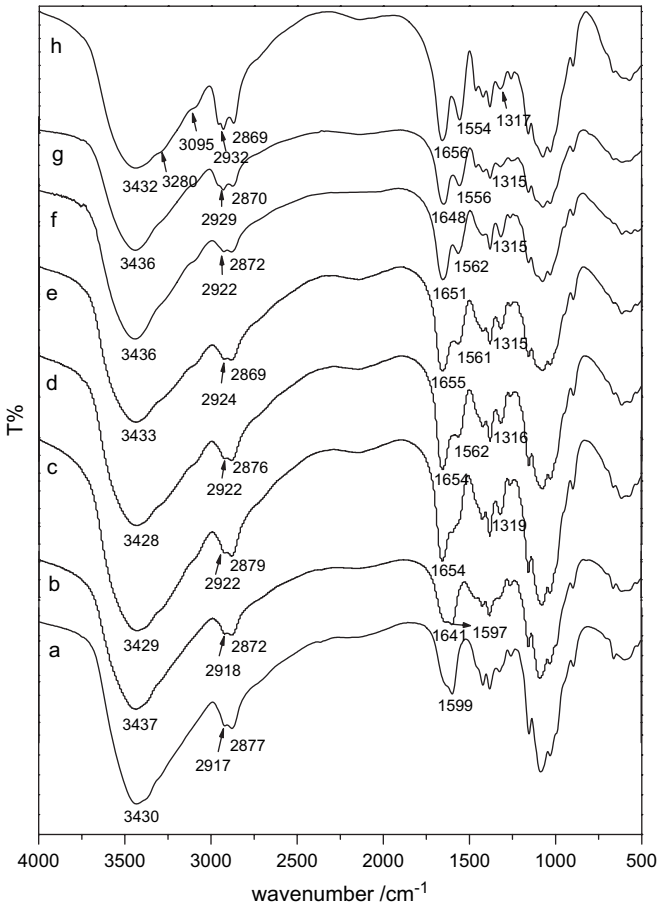


Fig. 1. FTIR spectra of chitosan (a) and *N*-acylated chitosan NACS0.1 (b), NACS0.2 (c), NACS0.3 (d), NACS0.4 (e), NACS0.5 (f); NPCS0.5 (g) and NHCS0.5 (h).

prominent bands at 1654 and 1562 cm^{-1} were observed. The peak at 1562 cm^{-1} to the N–H bending vibrations of the amide II band and the peak at 1316 cm^{-1} to the amide III band. The results indicated that NACS0.3, NACS0.4 and NACS0.5 had lower DD as well as NPCS0.5 and NHCS0.5 [18]. With increase of degree of acylation, the broad peak at around 3437 cm^{-1}, corresponding to the stretching vibration of N–H and O–H bond and the peaks at 1655 cm^{-1} assigned to stretching of the C=O group hydrogen bonded to N–H of neighboring intra-sheet chain shifted to lower frequency. It suggests that hydrogen bonds caused by acetamido or amino and hydroxy promote intramolecular or intermolecular aggregation. The intensities of the peaks at 2924 cm^{-1} (ν_{as} CH_2) and 2869 cm^{-1} (ν_s CH_2) increased with the increase of the acyl chain length in the order of NACS0.5 ≈ NPCS0.5 < NHCS0.5. Furthermore, in the spectrum of NHCS0.5, there was an increase of peak intensity at around 3280 and 3095 cm^{-1}, indicating lower intermolecular associations involving C(2)NH···O=C(7) and C(6)OH···O=C(7) hydrogen bonds [19]. In addition, there was no *O*-acyl characteristic peak (1750 cm^{-1}, 1240 cm^{-1}). These results clearly confirmed that the chitosan was *N* selectively substituted by acyl group.

Table 1
Molecular properties of *N*-acylated chitosans

	DD[a] (%)	$M_w \times 10^4$ [b] (Da)	$M_n \times 10^4$ [b] (Da)	M_w/M_n [b]	$\langle S_2\rangle_Z^{1/2}$ [b] (nm)
NACS0.1	87.7	7.5	4.0	1.88	33.8
NACS0.2	64.8	7.2	4.0	1.79	33.7
NACS0.3	56.5	5.4	2.9	1.88	30.7
NACS0.4	45.0	6.1	4.5	1.36	28.9
NACS0.5	38.2	9.4	3.7	2.50	41.5
NPCS0.5	39.2	8.2	4.1	1.99	34.9
NHCS0.5	41.4	9.0	4.7	1.91	46.9

[a] Measured by titrimetric method.
[b] Determined by GPC-LLS.

The DDs of chitosan derivatives are shown in Table 1. With the increase of molar ratio of acyl anhydrides to glucosamine unit, DDs of NACS gradually decreased, which coincided well with the information of FTIR. With the addition of 0.5 mol carboxylic anhydrides per glucosamine residue, DDs of three *N*-acylated chitosans were about 40%. It indicated that chitosan could be substituted using this mild and convenient acylation. It is a very simple method to prepare chitosan derivatives with different acyl chain lengths and easy to control the degree of deacetylation.

Meanwhile, the dimensions of chitosan chains and their related hydrodynamic volume depend on the semi-rigid character of the polysaccharide chains. Table 1 shows the M_w, M_w distribution and radius of gyration ($\langle S_2\rangle_Z^{1/2}$) of *N*-acylated chitosans. The M_ws of *N*-acylated chitosans were in the range of $5.4-9.0 \times 10^4$ Da. With decrease of DD, the M_w of NACS0.5 was higher than that of NACS0.1, NACS0.2, NACS0.3 and NACS0.4 because the rigidity of chain increased with the decrease of DD [20]. Similarly, the radius of gyration of NACS0.5 was higher than that of *N*-acetylated chitosan with higher DD, also indicating an increase of the chain stiffness with lower DD. But NPCS0.5 had lower M_w and value of $\langle S_2\rangle_Z^{1/2}$ than NACS0.5 and NHCS0.5. It suggests that the tendency of intramolecular aggregation was stronger for NPCS0.5. With the increase of the acyl chain, M_w and the value of $\langle S_2\rangle_Z^{1/2}$ of NHCS0.5 increased than those of NACS0.5 and NHCS0.5, indicating NHCS0.5 chain was more expanded.

The XRD patterns of chitosan and various *N*-acylated chitosans are shown in Fig. 2. The patterns of chitosan exhibited its characteristic peaks at $2\theta = 10.4°$, 19.8°, which coincided with the pattern of the "tendon hydrate polymorph" of chitosan reported previously [21]. The crystalline structure of chitosan derivatives was gradually altered with decreasing DD and increasing acyl chain length. NACS0.1 and NACS0.2 with high DD had similar patterns which displayed a wide peak at $2\theta = 19.8°$ without the appearance of peak at $2\theta = 10.4°$. It suggested that the crystallinity of chitosan was almost lost; probably due to the loss of hydrogen bonding that coincided well with the information of FTIR spectra. With the decrease of DD, new sharper peaks appeared at $2\theta = 8.07°$ and the intensity increased, indicating that the GlcNAc residues in NACS were distributed so as to form a highly ordered structure through hydrogen bonding. NACS0.5, NPCS0.5 and NHCS0.5 exhibited diffraction peaks in the lower-angle region at $2\theta = 8.07°$, 7.91° and 5.57°, respectively. The *d*-spacings of the three *N*-acylated chitosans calculated by the Bragg's equation were 1.073, 1.150 and 1.582 nm, respectively. The *d*-spacings increased linearly with increasing the chain length of the acyl substituents, which coincides with the previous reports by Wu et al. [19]. These major changes suggested a more crystalline and possibly more stable organization than for other forms of chitosan.

3.2. Self-aggregation behavior of N-acylated chitosans

The half *N*-acetylated chitosan derivatives strongly suggest that the *N*-acetyl groups randomly distribute and the deviation from randomness is very small [22]. *N*-acylated chitosans had similar random sequence. Random sequences of acyl have a tendency for intrapolymer association. Furthermore, amide spacer bonds between hydrophobes and the polymer main chain seem to be a structural factor that favors intrapolymer hydrophobe associations [23]. Viscometric properties of polymer solutions were used extensively to gain insight into the structure and conformation of polymers in solution [24]. Polymers with a strong tendency for interpolymer associations could lead to a large increase in solution viscosity upon an increase in the polymer concentration, which may be followed by gelation upon further increasing the polymer concentration. In contrast, polymers with a strong propensity for intrapolymer self-aggregation could lead to the formation of unimolecular micelles (unimer micelles [25]) independent of polymer concentration, yielding much less viscous aqueous solutions even at very high polymer concentrations [26].

The rheological curves (variation of the viscosity as a function of the shear rate) of *N*-acylated chitosans at high polymer concentrations are given in Fig. 3. The shear-thinning behavior observed for NHCS0.5 was pronounced at concentration of 20 mg/ml and the viscosity is higher than that of others. The result revealed that intermolecular hydrophobic interaction was predominant. NPCS0.5 and NACS0.5 samples had curves below the chitosan one at same concentration of 30 mg/ml. This is consistent with the study of Desbieres [27], revealing the presence of intramolecular interactions. And NPCS0.5 had curve below the NACS0.5, indicating that the intramolecular interactions of NPCS0.5 are stronger than NACS0.5, which was consistent with M_w and radius of gyration. It seems that these hydrophobic interactions can enhance the stability and participate in a self-assembled network organization. The solutions of NACS0.5 and NPCS0.5 showed indistinct shear-thinning behavior as shear rate is less than 1 s^{-1}, while chitosan solution had a Newtonian behavior. NACS0.5 and NPCS0.5 at the high concentration, interpolymer aggregates formed by many bridges (Fig. 8). But the intermolecular interactions can be easily disrupted under shear at a short time because of weak energy of the intermolecular interactions.

Zero-shear viscosities of *N*-acylated chitosan solutions with concentration of 10 mg/ml are shown in Table 2. Viscosity of NHCS0.5 is much larger than that of NPCS0.5 and NACS0.5. It showed that hydrophobic association of *N*-acylated chitosan

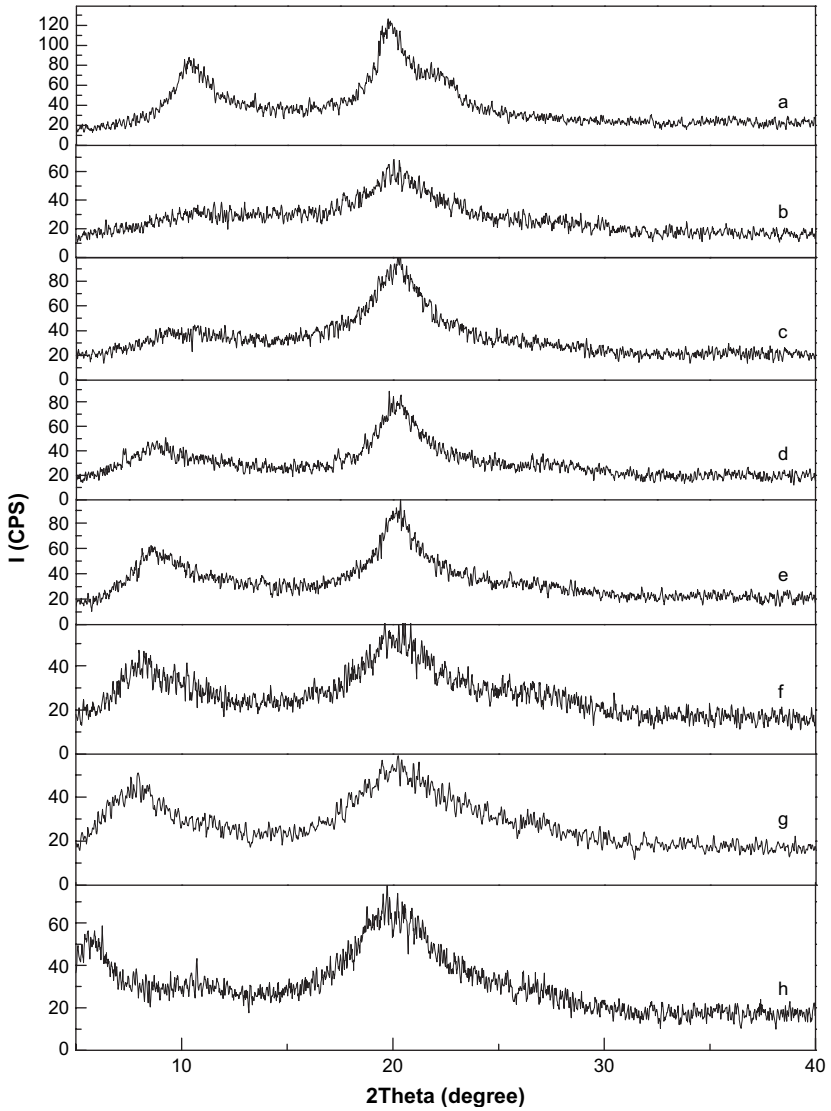


Fig. 2. X-ray diffraction patterns of chitosan (a) and *N*-acylated chitosan NACS0.1 (b), NACS0.2 (c), NACS0.3 (d), NACS0.4 (e), NACS0.5 (f); NPCS0.5 (g) and NHCS0.5 (h).

increased with the increase of acyl chain length. But the viscosity of NACS0.1, NACS0.4 and NACS0.5 is higher than that of NACS with medium DD. It may be hypothesized that two effects increased the viscosity in two ways: first, with the decrease of DD, the increase of acetyl groups increased steric hindrance for close molecules together and second, with the increase of acetamido groups, the intramolecular hydrogen bonds increased and intermolecular hydrogen bonds decreased, and the presence of intermolecular hydrogen bonds, molecular weight and viscosity increased [20].

The zero-shear viscosity as a function of the polymer concentration, the viscosity rose with increasing polymer concentration (Fig. 4). The transition between dilute and semi-dilute solutions can be studied with the Utracki and Simha representation which is the bi-logarithmic plot of the zero-shear specific viscosity as a function of polymer concentration to permit the determination of the critical overlapping concentration (C^*) which corresponds to the observed break in the plot. C^* of NACS0.5 and NPCS0.5 was 9.8 and 10.3 mg/ml and that of NHCS0.5 was 4 mg/ml. The longer the acyl hydrophobic chain, the lower the C^* and the more pronounced the sharpness of the viscosity curves above C^*. These results are typical for associating polymers. However, the C^* of NPCS0.5 is more than that of NACS0.5, Tien et al. reported that the longer side chain and higher degree of substitution on the *N*-acyl chitosan were able to enhance the stability of substituted chitosan via ‘‘hydrophobic self-assembly’’ [28].

In the dilute regime, the viscosity is essentially controlled by intramolecular interactions. In the semi-dilute unentangled regime, the viscosity is dominated by intermolecular hydrophobic associations, and in the semi-dilute entangled regime, the viscoelastic behavior can be described by a sticky reputation mechanism [29].

The influence of concentration of salt and temperature on the aggregation behavior of *N*-acylated chitosan was investigated. Fig. 5 shows the influence of different concentrations

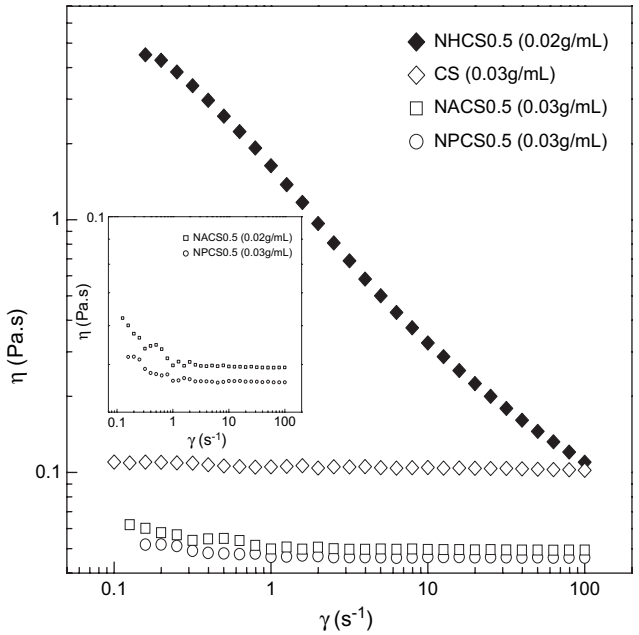


Fig. 3. Comparison of rheological behaviors of different *N*-acylated chitosans (solvent: 0.3 M HAc−0.05 M NaAc; $T = 25$ °C).

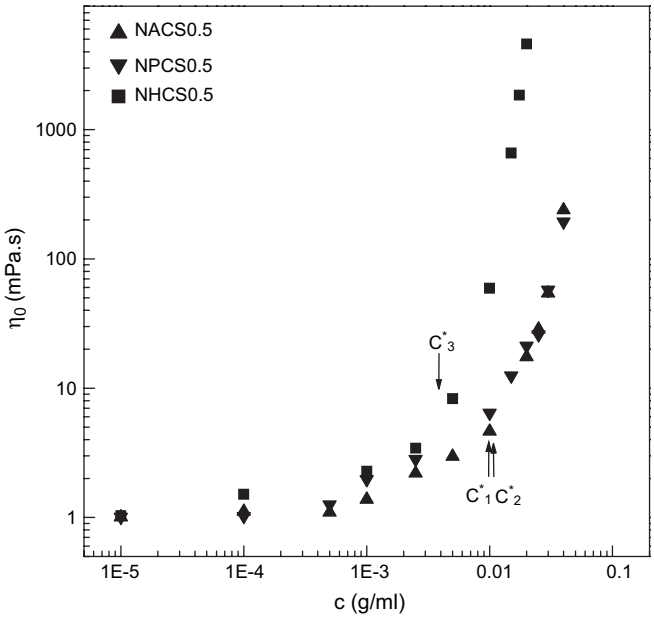


Fig. 4. Variation of the zero-shear solution viscosity with *N*-acylated chitosan concentration (solvent: 0.3 M CH_3COOH−0.05 M CH_3COONa; $T = 25$ °C).

of salt on the viscosities of NACS0.5 and NPCS0.5. The viscosities of NACS0.5 and NPCS0.5 in 0.3 M acetic acid without sodium acetate had a Newtonian behavior because of conceivable electrostatic repulsions. With increasing sodium acetate concentration to 0.3 M, the viscosities of NACS0.5 and NPCS0.5 increased and shear-thinning behavior was observed distinctly. This is usually observed and normally attributed to the enhancement of intermolecular hydrophobic associations. The two behaviors caused by salt have been ascribed to the balance between two effects: screening of the electrostatic repulsions, which lead to chain contraction and thus to a viscosity decrease, and salting out effect of salt, which leads to enhanced hydrophobic association. The second effect favors either a viscosity increase if the polymer concentration is high enough to allow intermolecular association or a viscosity decrease if intrachain interactions prevail [30].

The effect of temperature on the aggregation of *N*-acylated chitosan is shown in Fig. 6. When the temperature is 40 °C, Newtonian behavior was observed in NACS0.5 and NPCS0.5, the reason is the destabilization of intermolecular association caused by hydrogen bonds as temperature increases.

As the association effect increased with polymer concentration it may be expected to observe a gelation concentration. Increasing the concentration will allow to observe the transition from the rheological behavior of a solution (the storage modulus $G' <$ the loss modulus G'') to that of a gel ($G' > G''$). This evolution is demonstrated in Fig. 7. For 0.02 g/ml NHCS0.5 sample, with the increase of frequency, the samples became fluid-like, $G'' > G'$ over the entire frequency range studied. Increasing the concentration to 0.025 g/ml, at lower frequency, G' was slightly higher than G'', meaning that gel had been formed. With further increase of concentration to 0.03 g/ml, the originally viscous fluids exhibited distinctly gel-like characteristics, with G' greater than G''. However, the further increase in frequency caused a significant decrease in storage modulus, which was indicative of structure break by mechanical shear. The storage modulus became lower than the loss modulus and the system behaved like a solution. The rheological behavior was typical of a ''weak gel'', which had the frequency dependence. The entanglements among long acyl chains were heightened leading to the formation of gel. It was responsible for the hydrophobic interactions of hexanoyl chain. However, 0.03 g/ml of NACS0.5 and NPCS0.5 did not have weak gel behavior. The data also showed that hydrophobic interaction of *N*-acylated chitosan increased with the increase of acyl chain length.

Intramolecular and intermolecular aggregations of *N*-acylated chitosans may be explained: intramolecular aggregation of NPCS and NACS was stronger even at high concentration, with NPCS stronger than NACS. For longer acyl chain and a higher degree of substitution, the hydrophobic interaction enhanced the stability and participated in the self-assembled network

Table 2
Zero-shear solution viscosity of *N*-acylated chitosan solution at concentration of 10 mg/ml

Sample	η_0 (mPa s)
NACS0.1	4.749
NACS0.2	4.416
NACS0.3	2.756
NACS0.4	4.822
NACS0.5	4.621
NPCS0.5	6.427
NHCS0.5	59.233

Solvent: 0.3 M HAc−0.05 M NaAc; $T = 25$ °C.

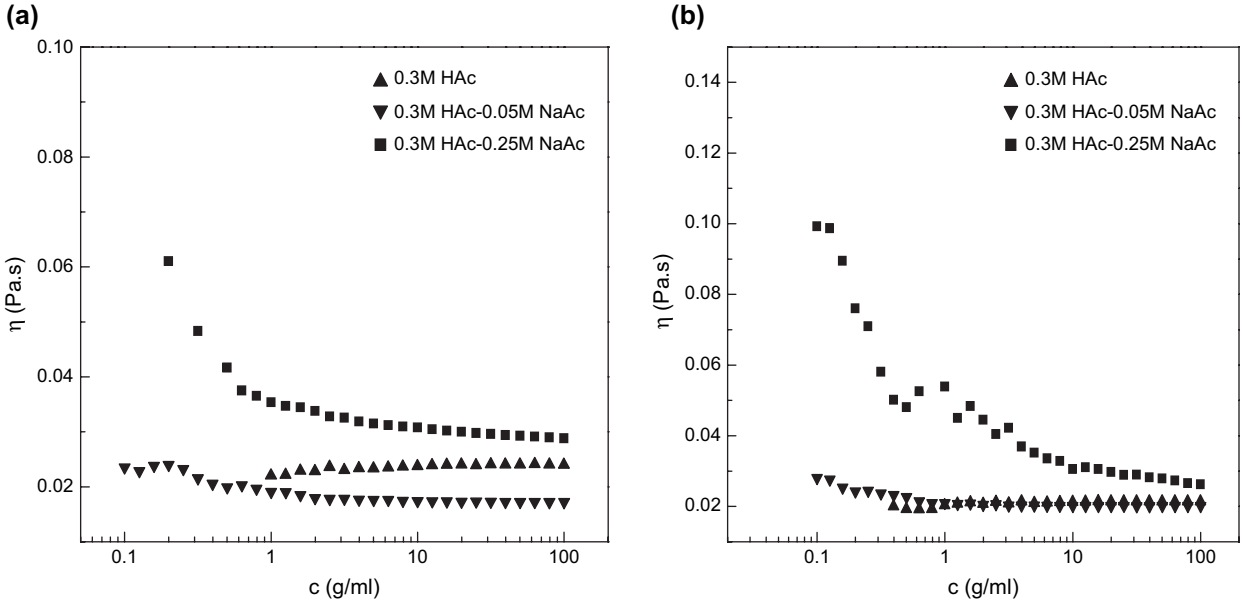


Fig. 5. Comparison of rheological behaviors of NACS0.5 (a) and NPCS0.5 (b) with concentration of 0.02 g/ml in different concentrations of CH_3COONa in the presence of 0.3 M CH_3COOH ($T = 25$ °C).

organization due to the destabilization of the local conformation by intrachain hydrogen bonds [31]. Hydrophobic interaction of *N*-acylated chitosan substituted with longer acyl chains was stronger.

The influence of DD (85–40%) on self-aggregation of *N*-acylated chitosan may be explained: for NACS with high DD, loss of hydrogen bonding and strong electrostatic repulsions of high charge density disfavored intra aggregate. For NACS with moderate DD, intramolecular aggregate prevailed because of semi-flexible chain with apparent charge density decreasing. C═O group hydrogen bond to O–H of neighboring intra-sheet chain and hydrophobic characteristic of increasing content of acyl. For NACS with low DD, intramolecular aggregates have strong tendency and intermolecular aggregates formed because of increase of the chain stiffness, which may be explained in Fig. 8. With the increase of concentration, loops of the intramolecular closed aggregation dissociate and have more chance to form an open association with many bridges. These interpolymer aggregates are markedly different from *N*-acylated chitosan with high DD.

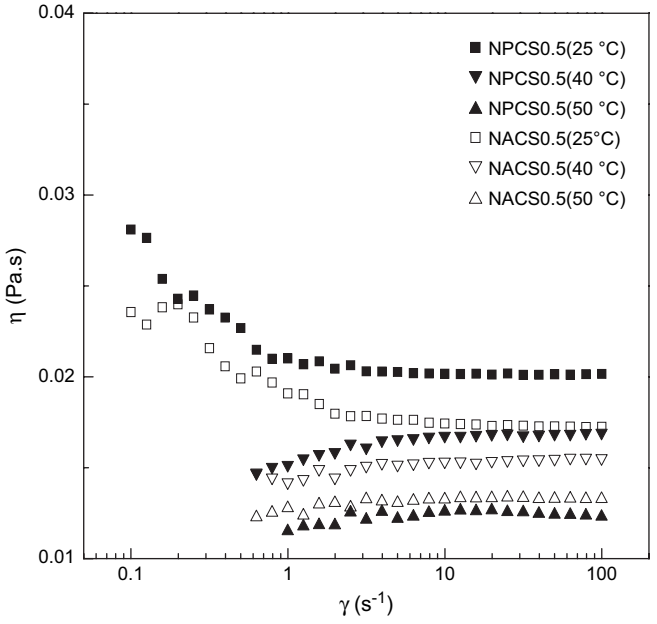


Fig. 6. Comparison of rheological behaviors of NACS0.5 and NPCS0.5 in different temperatures with concentration of 0.02 g/ml (solvent: 0.3 M CH_3COOH–0.05 M CH_3COONa; $T = 25$ °C).

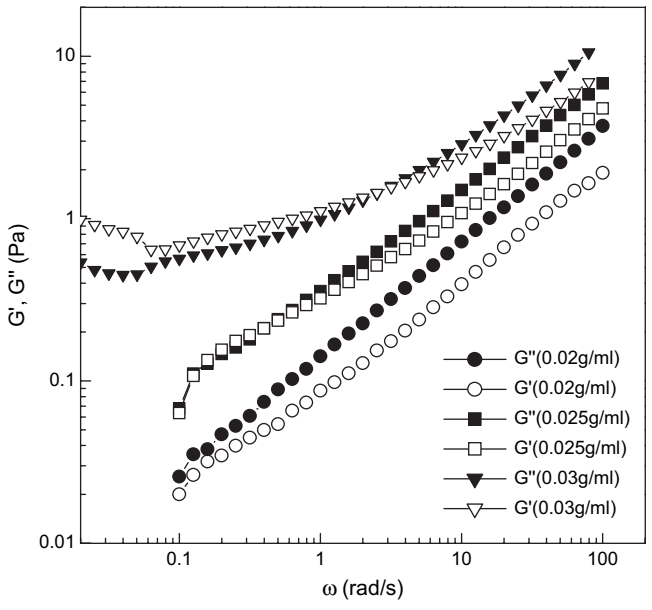


Fig. 7. Influence of concentration and frequency on the dynamic moduli G' and G'' of NHCS0.5 solutions (solvent: 0.3 M CH_3COOH–0.05 M CH_3COONa; $T = 25$ °C).

Table 3
RITs (h) of 1 mg/ml *N*-acylated chitosan in 0.02 M acetic buffer (pH 5.4)

Sample	G^+ bacteria		G^- bacteria	
	Staphylococcus aureus	*Bacillus subtilis*	*Escherichia coli*	*Pseudomonas aeruginosa*
Chitosan	>85	>85	>85	>85
NACS0.1	30	>85	>85	>85
NACS0.2	20	15	24	45
NACS0.3	15	5	5	5
NACS0.4	5	15	15	15
NACS0.5	5	5	24	8
NPCS0.5	8	8	24	32
NHCS0.5	15	20	53	53

3.3. Antibacterial activity of N-acylated chitosans

Relative inhibition times (RITs) of chitosan and *N*-acylated derivatives with concentration of 1 mg/ml in 0.02 M acetic buffer (pH 5.4) against two Gram-positive bacteria *S. aureus* and *Bacillus subtilis* and two Gram-negative bacteria *E. coli* and *P. aeruginosa* are shown in Table 3. Chitosan shows better inhibitory activity than *N*-acylated chitosan at concentration of 1 mg/ml. NHCS0.5 showed better inhibitory activity than NACS0.5 and NPCS0.5 especially against Gram-negative bacteria. RITs of NHCS0.5 against *E. coli* and *P. aeruginosa* were more than 2–3 times longer than that of NACS0.5 and NPCS0.5.

It has been postulated that the antibacterial action of chitosan occurs as a result of several mechanisms. Chitosan exhibited antibacterial activity only in an acidic medium, which could be due to the positively charged amino groups at C-2 in the chitosan molecule in solution below its pK_a (6.3), as they could interact with the predominantly anionic molecules at the cell surface. This interaction could change the permeability of the cell membrane of the microorganisms, resulting in a leakage of intercellular components, and then caused the death of the cell [32]. Inhibitory activity of chitosan and NACS0.1 with high DD was better than the others with low DD. With the decrease of DD by the *N*-acylation reaction, the amino group was partly converted to amide group, the positive charge density of the *N*-acylated chitosan decreased. But intramolecular aggregation formed and its nanosize increased larger specific surface area of smaller particles was obtained and more bacteria were adsorbed and immobilization on the surface of aggregate. Hydrophobic part of hydrophobic polymer aggregate in the inner and the hydrophobic part including NH_3^+ was exposed to outer (Fig. 8), the positively charged amino groups increased in the surface of the aggregate. So, NACS0.3 and NPCS0.5 showed inhibitory effect against *S. aureus*. NACS0.5, NPCS0.5 and NHCS0.5 with low DD had better inhibitory effect especially against Gram-negative bacteria. Aggregation characteristic of *N*-acetylated chitosans with low DD may help in forming bridge to interact with bacterial cell. Strand et al. reported that *E. coli* cells flocculated most efficiently with highly acetylated chitosan. The presence of *N*-acetylglucosamine (GlcNAc) residues had clear beneficial effect on flocculation of Gram-negative bacteria and bridging as the dominating flocculation mechanism [15]. Meanwhile, hydrophobic characteristic of *N*-acylated chitosan also help for the interaction of polymer molecule and bacterial cell. Hydrophobic characteristic of NHCS0.5 is likely to be a contributing factor for its better inhibitory effect. Further study should be done to explain the fact.

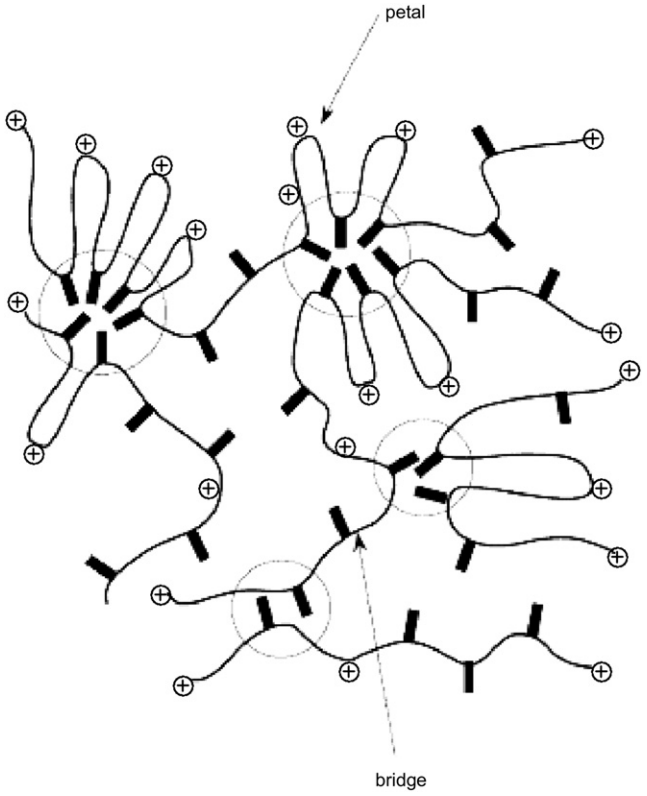


Fig. 8. Simultaneous formation of intramolecular aggregates and intermolecular cross-links of *N*-acylated chitosans with desired amount of hydrophobic acyl chains (a small loop in a micelle is called "petal" and a subchain connecting two junctions on different chains is called "bridge").

4. Conclusions

In the present work, *N*-acylated chitosans with different acyl chain lengths had been successfully synthesized to obtain randomly distributed substituents in a controlled amount along the chitosan chain. The result indicated that chain stiffness increased for *N*-acylated chitosans with around 40% DD. Self-aggregation behavior of *N*-acylated chitosan was investigated by rheometer. C^* of NACS0.5 and NPCS0.5 was 9.8 and 10.3 mg/ml and that of NHCS0.5 was 4 mg/ml. In the dilute regime, the viscosity is essentially controlled by intramolecular interactions. In the semi-dilute entangled regime, the intermolecular hydrophobic associations enhanced with increase of salt concentration. And intermolecular association caused by hydrogen bonds destabilized when temperature is 40 °C. Hydrophobic characteristic of NHCS0.5 substituted with longer acyl chains was stronger. And with moderate DD, intramolecular aggregation occurs predominantly. With low DD, *N*-acylated chitosan chain forms interpolymer aggregates, forming an association with many bridges.

In vitro inhibitory activity test of *N*-acylated chitosans was evaluated against two Gram-positive bacteria and two Gram-negative bacteria. Relative inhibition times (RITs) of NHCS0.5 with concentration of 1 mg/ml against *E. coli* and *P. aeruginosa* were 2–6 times longer than that of NACS0.5 and NPCS0.5. Aggregation characteristic of *N*-acetylated chitosans with around 40% DD may help in forming bridge to interact with bacterial cell.

References

[1] Jones M, Leroux J. European Journal of Pharmaceutics and Biopharmaceutics 1999;48:101.
[2] Ogino K, Onoe Y, Abe M, Ono H, Bessho K. Langmuir 1990;6:1330.
[3] Winnik FM. Macromolecules 1989;22:734–42.
[4] Amiji MM. Carbohydrate Polymers 1995;26:211–3.
[5] Ottoy MH, Varum KM, Christansen BE, Anthonsen MW, Smidsrod O. Carbohydrate Polymers 1996;31:253–61.
[6] Schatz C, Viton C, Delair T, Pichot C, Domard A. Biomacromolecules 2003;4:641–8.
[7] Suzuki K, Mikami T, Okawa Y, Tokoro A, Suzuki S, Suzuki M. Carbohydrate Polymers 1986;151:403–8.
[8] Choi BK, Kim KY, Yoo YJ, Oh SJ, Chio JH, Kim CY. International Journal of Antimicrobial Agents 2001;18:553–7.
[9] No HK, Park NY, Lee SH, Meyers SP. International Journal of Food Microbiology 2002;74:65–72.
[10] Yoshihiko O, Mayumi S, Takahito A, Hiroyuki S, Yoshihiro S, Ichiro N, et al. Biocontrol Science 2003;8:25–30.
[11] Hu Y, Du YM, Yang JH, Kennedy JF, Wang XH, Wang LS. Carbohydrate Polymers 2007;67:66–72.
[12] Sugano M, Yoshida K, Hashimoto M, Enomoto K, Hirano S. In: Brine CJ, Sandford PA, Zikakis JP, editors. Advances in chitin and chitosan. London: Elsevier; 1992. p. 472–8.
[13] Jeon YJ, Kim SK. Journal of Microbiology and Biotechnology 2002;12:503–7.
[14] Qin CQ, Du YM, Xiao L, Zhang L, Gao XH. Chinese Chemical Letters 2002;13:111–7.
[15] Strand SP, Nordengen T, Otgaard K. Water Research 2002;36:4745–52.
[16] Tolaimate A, Desbrieres J, Rhazi M, Alagui A, Vincendon M, Vottero P. Polymer 2000;41:2463–9.
[17] Liu H, Du YM, Wang XH, Hu Y, Kennedy JF. Carbohydrate Polymers 2004;56:291–7.
[18] Dong YM, Xu ZY, Wang JW. Science in China Series B 2000;31:153–60.
[19] Wu Y, Seo T, Sasaki T, Irie S, Sakurai K. Carbohydrate Polymers 2006;26:493–9.
[20] Wang W, Bo S, Li S, Qin W. International Journal of Biological Macromolecules 1991;13:281–5.
[21] Belamie E, Domard A, Giraud-Guille MM. Journal of Polymer Science, Part A 1997;35:3181–91.
[22] Kubota N, Tatsumoto N, Sano T, Toya K. Carbohydrate Research 2000;324:268–74.
[23] Hiroshi Y, Morishima Y. Macromolecules 1999;32:7469–75.
[24] Feng YJ, Billon L, Grassl B, Bastiat G, Borisov O, Francois J. Polymer 2005;46:9283–95.
[25] Morishima YH. Macromolecules 1999;32:7469–75.
[26] Esquenet C, Terech P, Boue F, Buhler E. Langmuir 2004;20:3583–92.
[27] Desbieres J. Polymer 2004;45:3285–95.
[28] Tien L, Lacroix C, Ispas-Szabo P, Mateescu MA. Journal of Controlled Release 2003;93:1–13.
[29] Caputo MR, Selb J, Candau F. Polymer 2004;45:231–40.
[30] Choi CY, Kim SB, Pak PK, Yoo DI, Chung YS. Carbohydrate Polymers 2007;68:122–7.
[31] Kjoniksen AL, Nystrom B, Iversen C, Nakken T, Palmgren O, Tande T. Langmuir 1997;13:4948–52.
[32] Wang XY, Du YM, Yang JH, Wang XH, Shi XW, Hu Y. Polymer 2006;47:6738–44.