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Interaction between chitosan and alkyl β-D-glucopyranoside and its effect on their antimicrobial activity

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Abstract

The interactions between chitosan and nonionic surfactant–alkyl β-D-glucopyranoside (AG) in solid state and aqueous solution have been investigated by using FT-IR spectroscopy, thermal analysis, powder X-ray diffraction, viscometry and gel permeation chromatography (GPC). The FT-IR spectra demonstrated that interaction between $-NH_3^+$ and –OH groups of chitosan and –OH groups of AG occurred by electrostatic force or hydrogen bonding. Results of thermal analysis and X-ray diffraction indicated that higher orders of structure such as aggregation chain conformation etc and crystallinity of chitosan seemed changed by addition of AG. Results of viscometry and GPC showed that, in aqueous solution, AG most likely made the chitosan chains extend and disaggregate. Interestingly, antimicrobial activity of the dynamic association (mixed solution of chitosan and AG) was much higher than that of chitosan and AG separately. Minimum inhibition concentration (MIC) of dynamic association was 2–8 times lower than that of chitosan alone, and it could kill more microorganisms in a shorter time. The reasons for the enhanced antimicrobial activity of the dynamic association mixture are carefully discussed in terms of the interaction between chitosan and AG.

Keywords: Chitosan; Alkyl β-D-glucopyranoside; Interaction; Antimicrobial activity

1. Introduction

Chitosan, the partially deacetylated derivative of chitin, is a nontoxic copolymer consisting of (1 → 4)-2-acetamido-2-deoxy-β-D-glucopyranosyl and (1 → 4) -2-amino-2-deoxy-D-glucopyranosyl units. It has attracted considerable interest due to its biological activities such as antimicrobial (Choi et al., 2001; Jeon, Park, & Kim, 2001; No, Park, Lee, & Meyers, 2002; Roller & Covill, 1999), antitumor (Suzuki et al., 1986), and immune enhancing effects (Sugano, Yoshida, Ilashimoto, Enomoto, & Ilirano, 1992) etc.

As a natural antimicrobial agent, the antibacterial and antifungal activities of chitosan (Shahidi, Arachchi, & Jeon, 1999) have been reported widely. Because the antimicrobial activity of chitosan is very limited, various efforts have been taken to improve it. Some researchers studied the effect of the molecular weight, degree of deacetylation, solvent, pH, etc. (Jeon et al., 2001; No et al., 2002) on the antimicrobial activity of chitosan, so as to enhance the activity by adjusting these factors. Other researchers have set out to modify chitosan to gain derivatives with higher activity such as N-sulfonated and N-sulfobenzoyl chitosan (Chen, Liau, & Tsai, 1998), carboxymethylchitosan (Chen, Du, & Liu, 2000), quaternary ammonium salt of chitosan (Jia, Shen, & Xu, 2001), etc. In an attempt to improve antimicrobial activity of chitosan, our papers report the preparation of complexes of chitosan with some materials which also showed some antimicrobial activity, such as surfactants, essential oils, metals and some organic acids (our, to be published work). Among the complexes of chitosan with surfactants, chitosan/alkyl β-D-glucopyranoside complex showed excellent cooperative activity.

Alkyl β-D-glucopyranoside (AG) represents a nonionic surfactant class produced on a large scale. Compounds in this class are being used in a wide range of technical and consumer products, such as detergents, cleaning agents,

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cosmetic products and pesticide formulations, because of excellent behavior at interfaces (Czichocki, Fiedler, Haage, Much, & Weidner, 2002; Kim, Woo, Kim, & Kim, 2001). This new class of surfactants, made from renewable raw materials, namely glucose and fatty acid alcohols, draws interest recently due to their outstanding biodegradability, excellent dermatological properties, good performance as surfactants and synergistic effects with other surfactants (Kim et al., 2001).

The purpose of this article is to investigate the interaction between chitosan and AG, so as to make clear why AG could promote the antimicrobial activity of chitosan. Therefore, in this paper, the interactions between chitosan and AG in solid state and aqueous solution have been investigated. Antimicrobial activity of the mixed solution, compared with chitosan and AG solution, was evaluated against five different microorganisms. The relationship between the interaction of chitosan with AG and the antimicrobial activity is discussed.

2. Experimental

2.1. Materials

Chitosan from a shrimp shell was purchased from Yuhuan Ocean Biochemical Co (Zhejiang, China), molecular weight was 781 kDa (measured by GPC) and deacetylation degree was 85.5% (determined by elemental analysis). Glucopon 600 CS UP, AG in aqueous dispersion with a concentration of >20% and R-phases of 36/38, was obtained from Cognis Deutschland GmbH. The general chemical structure of AG (Kim et al., 2001) is shown in Fig. 1. All other chemicals were of reagent grade.

2.2. Preparation of chitosan/AG complex and solution

Chitosan was dissolved into 0.2 M acetic acid/0.1 M sodium buffer, pH 4.8, and the final concentration was adjusted to 1% (w/v) AG was diluted to 1% aqueous solution. Chitosan solution was dropped into AG solution. The resultant solution was gently stirred for 2 h, and then stored for about 2 days at room temperature to allow equilibration. This solution was directly used for assays of antimicrobial activity. Films of the chitosan/alkyl β-D-glucopyranoside complexes (CS/AG) were cast from the above solution, after 3 days of dialysis against distilled water on a glass plate at room temperature for 3 days and finally dried to constant mass under vacuum at 50 °C for 2 days. Pure chitosan acetate (CSAC) and AG were films also prepared under the same conditions. The films were cut into powdery pieces before characterization.

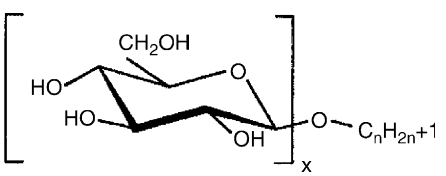

Fig. 1. Chemical structure of AG. x is degree of polymerization in head group and n alkyl chain length.

2.3. Characterization of chitosan/AG complex

IR spectra were taken in KBr pellets on a 170SX FT-IR spectrophotometer (America Nicolet company) by the method of transmission. Thermogravimetry (TG) and differential thermogravimetry (DTG) curves of samples were performed by a Setsys 16 TG/DAT/DSC (Setaram, Caluire, France) under nitrogen atmosphere of 0.15 MPa and argon atmosphere of 0.10 MPa from 20 to 600 °C at a heating rate of 10 °C/min. X-ray diffraction patterns were measured by a D_{max}-ray diffractrometer and used a Cu Kα target at 40 kV and 50 mA.

2.4. Interaction of chitosan and AG in solution

The viscosity of chitosan in 0.2 M HAc/0.1 M NaAc aqueous solution and 0.2 M HAc/0.1 M NaAc/0.5% (w/v) AG solution were measured with an Ubbelohde capillary viscometer at 30 °C. The specific viscosity (η_{sp}), reduced viscosity (η_{sp}/c) ad intrinsic viscosity ([η]) were calculated according to the reference book (Zhang, Xue, Mo, & Jin, 2003). The intrinsic viscosity was estimated using the Shulz–Blaschke formula: $\eta_{sp}/c = [\eta] + k''[\eta]\eta_{sp}$.

A GPC system incorporating a TSP P100 instrument and a TSK G5000-PW column ($7.5 \times 300\ mm^2$) was used to study the effect of AG on the aggregation behavior of chitosan. The eluent was 0.2 M HAc/0.1 M NaAc buffer, with a flow rate of 1.0 ml/min. The temperature of the columns was maintained at 30 °C. The eluent was monitored through an RI 150 refractive index detector. The concentration of chitosan was 0.05% (w/v), and 0.01% AG was added to give the AG–chitosan solution. All data provided by the GPC system were collected and analyzed using the Jiangshen Workstation software package (DaLian Jiangshen, DaLian, China).

2.5. Assays for antimicrobial activity

Escherichia coli ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, *Staphylococcus aureus* ATCC 25923 for antimicrobial assay were provided by the Typical Cultural Collection Center in Wuhan University, China *Staphlococcus epidermidis* and *Candida albicans* were donated by the People's Hospital of HuBei province in China. Bacteria were incubated on nutrient agar (peptone 1%, beef extract 0.5%, NaCl 0.5%, agar 2%, pH 6) at 37 °C for 1 day, while *C. albicans* was incubated at 28 °C for 2 days.

Inhibitory effect: to prepare microorganism suspensions, colonies of microorganisms on agar plates were transferred into sterile saline (0.9%w/v) solution, then diluted to obtain a suspension containing 10^5–10^6 cells/ml. Sample solutions were autoclaved at 121 °C for 15 min. Sample solutions (1 ml) and autoclaved nutrient agar (9 ml) were poured into autoclaved petri-dishes, cooled, one platinum loop of microorganism suspension was drawn as a stripe on the cooled nutrient agar, then the plate incubated at 37 °C for bacteria, 28 °C for *C. albicans*. Observations were made to see whether colonies were visible with the naked eye after incubation for 19 and 38 h. All treatments were triplicate.

The Minimum inhibitory concentration (MIC) was tested as follows: sample solutions were two-fold diluted serially, then treated as above. The MIC was defined as the lowest concentration of the tested sample at which the microorganism colonies were not visible to the naked eye within 19–38 h.

Biocidal activity: microorganism cell suspension was diluted to $\sim 10^4$ cell/ml. Cell suspension (1 ml) was added to sample (4 ml) solutions that had been autoclaved at 121 °C for 15 min. Samples were removed after 5, 30, 60, 120, 180 min. Aliquots (50 μl) were spread on nutrient agar plates in triplicate and then incubated at 37 °C for 1–2 days, and the colony forming unit (CFU) was counted.

3. Results

3.1. Characterization of chitosan/AG complex in solid state and aqueous solution

3.1.1. IR spectra

As is well known, FT-IR is a very useful tool for detecting the interaction in polymer blends, so in this paper, FT-IR was employed to examine the possible interactions between the blend components. Fig. 2 shows the FT-IR spectra of CSAC, AG and CS/AG at different ratios. In these spectra, C-N stretching vibration of CSAC at 1334 cm^{-1} (Williams & Fleming, 1980) shifted to 1330 cm^{-1} (curve 2) and 1323 cm^{-1} (curve 3) in the complexes. Also absorption bands between 1000 and 1200 cm^{-1} which come from free hydroxyl groups (Williams & Fleming, 1980) shifted slightly. The band at 1083 cm^{-1} of CSAC shifted to 1080 cm^{-1} (curves 2 and 3), and band at 1027 cm^{-1} of AG shifted to 1031 cm^{-1} (curve 4). These indicated the interactions between $-NH_3^+$ groups of chitosan and –OH hydroxyl groups of AG, as well as between –OH hydroxyl groups of chitosan and AG, possibly occurred. In addition, the weak absorption band at 2800–2000 cm^{-1} was attributed to the NH_3^+ overtones (Williams & Fleming, 1980). Compared with the spectra of CSAC and AG, the band of CS/AG (curve 2) had a relatively stronger absorbent, which indicated that a hydrogen bond was possibly formed between CSAC and AG.

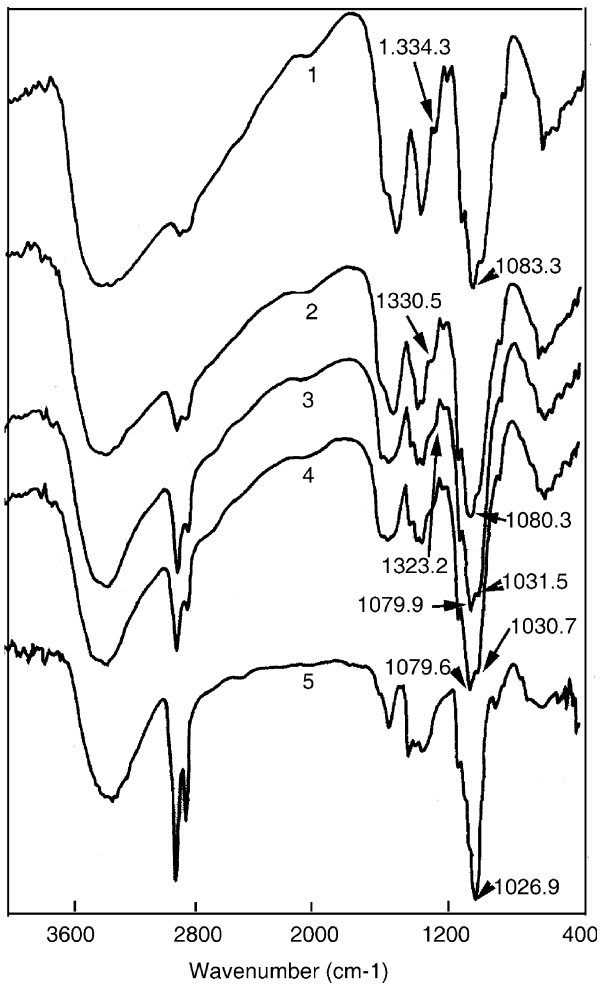

Fig. 2. IR spectra of CSAC (1), AG (5) and CS/AG with different ratio (w/w) of chitosan to AG (4:1 (2), 1:1 (3), 1:4 (4)).

3.1.2. Thermal analysis

TG (a) and DTG (b) curves of CSAC, AG and their complex are shown in Fig. 3. From these curves it can be seen that CSAC became degraded in three stages. It has been reported that TG curves of chitosan show two stages (Qin, Du, & Xiao, 2002). The first stage in the TG curve of chitosan was explained as the loss of water. The second stage was due to the degradation of the chitosan, and the greatest weight loss point was at 298 °C (Qin et al., 2002) or 334 °C with weight loss of 49% (Tirkistani, 1998). In our study, the first and third stages of CSAC are very similar to the two reported stages for chitosan. The difference between chitosan and CSAC is that acetic acid has been introduced into CSAC. So it is likely that the second stage of CSAC is attributable to the loss of acetic acid.

The TG and DTG curves of CS/AG were very different from those of CSAC and AG, and showed three stages. The first water-loss stage reached a maximum at 83 °C, with a weight loss of ~6%. T_{max} (the temperature when weight loss reaches a maximum) of the first stage was higher than that of CSAC (70 °C). The second stage was also attributed

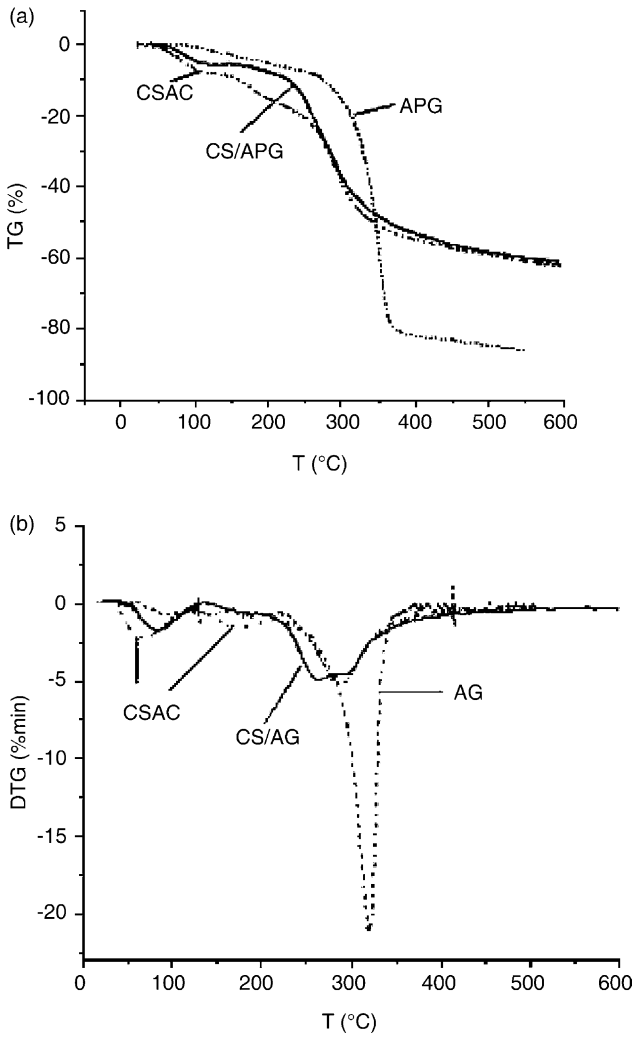

Fig. 3. TG (a) and DTG (b) curves of CSAC, AG, CS/AG with ratio of 1:1 (w/w).

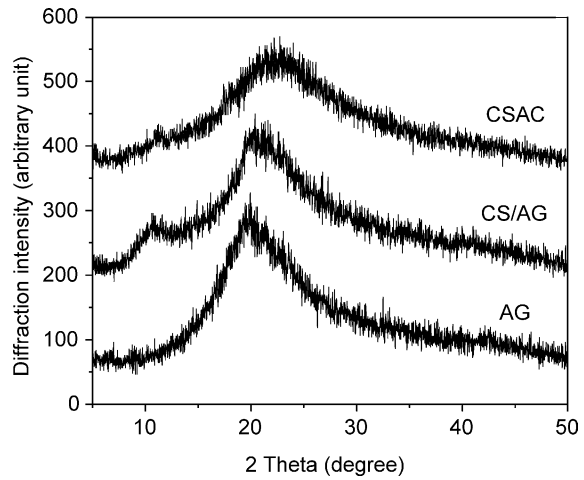

Fig. 4. X-ray diffraction patterns of CSAC, AG and CS/AG with ratio of 1:1 (w/w).

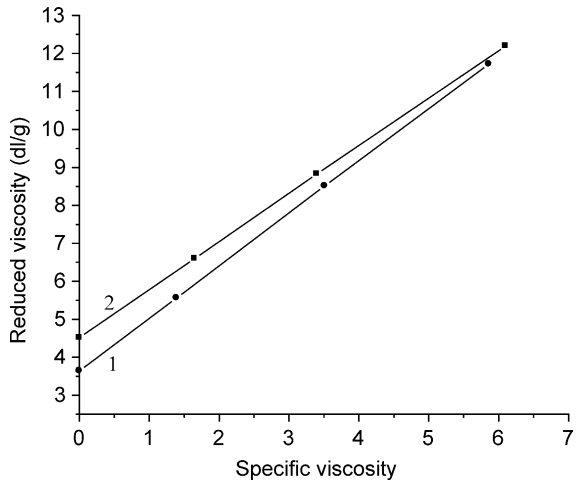

Fig. 5. Reduced viscosity versus specific viscosity of chitosan in 0.2 M HAc/0.1 M NaAc solution (1) and in 0.2 M HAc/0.1 M NaAc/0.05%AG solution (2).

to the loss of acetic acid. The third stage was the degradation stage of CS/AG reaching two maxima at ~264 and 296 °C, with weight loss of ~51%. T_{max} (264 °C) for the complex was therefore lower than CSAC (290 °C) and AG (319 °C). These results demonstrated that it is easier for the complex to degrade and more difficult to lose water. These data indicate that AG most likely changed the higher degrees of structure order of the chitosan, such as aggregation behavior or chain conformation.

3.1.3. X-ray diffraction study

The X-ray diffractograms of CS/AG, CSAC and AG are shown in Fig. 4. It could be seen that CSAC showed a relatively broader peak at 23° (2θ), with height of 225. AG had a relatively narrow peak at 19.7° (2θ), with height of 280. CS/AG showed two peaks at 20.2–21.7° (2θ), with height of 254–260 and 10.8° (2θ). Hence, CS/AG formed new crystallite.

From above results, it can be seen that complex of CSAC with AG was formed, most likely due to the electrostatic

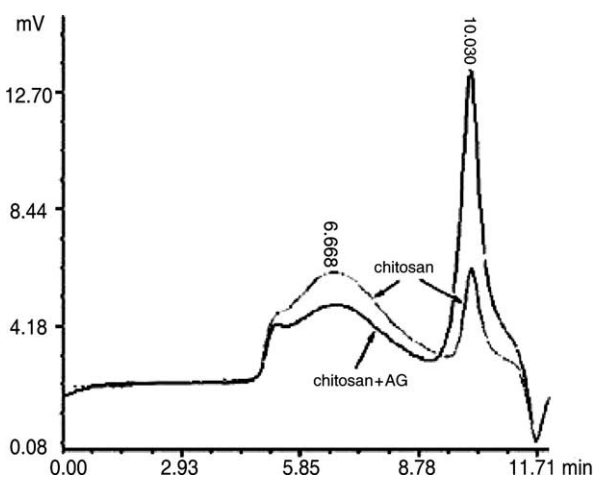

Fig. 6. GPC curves of chitosan and chitosan adding of AG (chitosan + AG).

Table 1
MICs of chitosan, AG and the CS/AG dynamic association mixture against different microorganisms (%)

Samples	*S. aureus* G^+ bact.	*S. epidermidis* G^+ bact.	*E. coli* G^- bact.	*P. aeruginosa* G^- bact.	*C. albicans* fungi
Acetate buffer					
(HAc/NaAc)	(0.02 M/0.01 M)[a]	(0.02 M/0.01 M)	(0.02 M/0.01 M)	0.01 M/0.005 M	(0.02 M/0.01 M)
Chitosan	0.05	0.05	0.025	0.025	(0.1)
AG	0.006	(0.1)	(0.1)	(0.1)	0.05
CS/AG	0.006/0.003	0.025/0.013	0.013/0.006	0.006/0.003	0.05/0.025

[a] No antimicrobial activity at the concentration in bracket.

interaction or hydrogen bonding between $-NH_3^+$, $-OH$ groups of chitosan and $-OH$ groups of AG. The difference in crystallinity between combined CS/AG and separate CSAC and separate AG suggested complex formation and that higher orders of the structure of chitosan became changed by the addition of AG, which in turn likely led to the comparatively lower degradation temperature of CS/AG.

3.1.4. Viscometry study

Chitosan showed different values of intrinsic viscosity in 0.2 M HAc/0.1 M NaHAc solution ($[\eta] = 3.64 \pm 0.2$ dl/g) and in 0.2 M HAc/0.1 M NaHAc/0.5% AG solution ($[\eta] = 4.52 \pm 0.3$ dl/g) (Fig. 5). Since intrinsic viscosity is an indirect measure of the size and shape of polymeric chains (Kurata & Tsunashima, 1989), the comparison of $[\eta]$ values obtained for the same polymer hydrated in different media provides information about the changes in the polymer conformation occurring in those media. The higher $[\eta]$ value of chitosan in 0.2 M HAc/0.1 M NaHAc/0.5% AG solution indicated a more extended polymer conformation, which maybe due to the dispersion of the hydrophobic long-chain alkyl group.

3.1.5. GPC measurements

GPC was used to determine whether the aggregation behavior of chitosan was affected by adding of AG Polysaccharides easily form aggregates by the hydrogen bond between $-OH$ groups. Like other polysaccharides such as 3,6-O-CM-chitin (Chen & Du, 2002) and A-gum (Xu & Zhang, 1999), chitosan showed two main peaks in the GPC curve (Fig. 6). The peak at 6.668 min was generally considered to correspond to the aggregates with high weight-average molecular weight, and the peak at 10.038 min was considered to correspond to single chains with low weight-average molecular weight. Compared with the curve for chitosan, the first peak of chitosan + AG was relatively lower and the second peak relatively higher. That means that the addition of AG caused aggregated chitosan chains tend to disaggregate to single chains.

From the above results, it can be seen that the aggregated chitosan chains tend to a more extended chain conformation and disaggregate to single chains. This result can partly explain the lower degradation temperature and new crystallinity of chitosan/AG, because extended or even single chains should be easier to be degraded and form new crystallites.

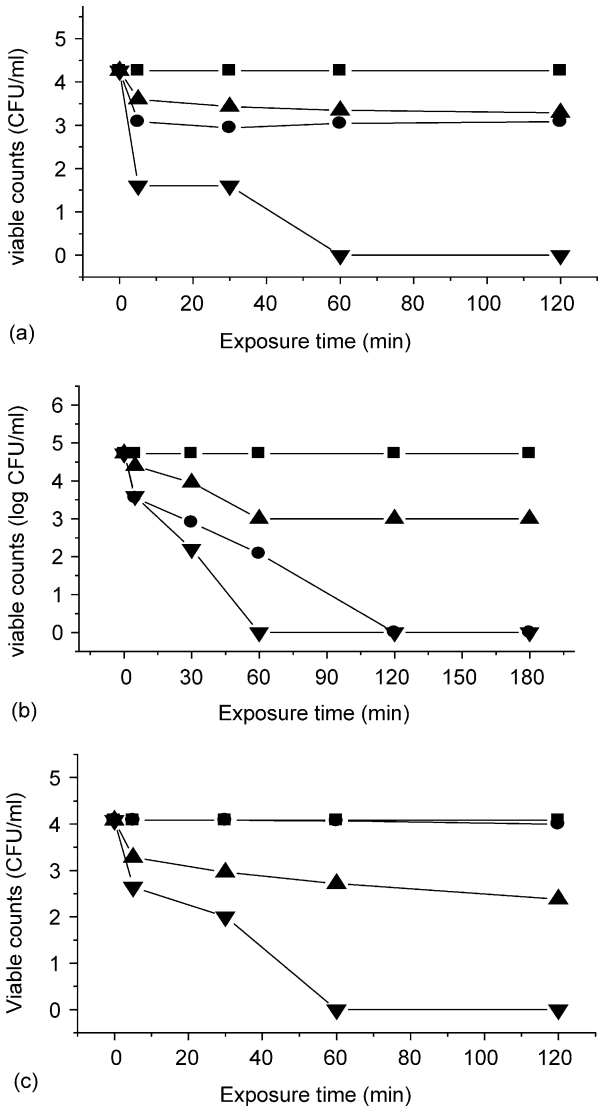

Fig. 7. Biocidal activity of (a) *S. aureus*, (b) *E. coli*, (c) *C. albicans* treated with acetate buffer, pH 4.8 (■), 0.4% chitosan acetate solution (●), 0.4% AG (▲), and the dynamic association mixture (0.4% chitosan + 0.4% AG) (▼). Results are represented as means ± SD of three experiments.

3.2. Antimicrobial activity

MIC values in Table 1 showed that chitosan could inhibit the growth of all the bacteria tested, but not the fungus *C albicans*. AG could only inhibit *S. aureus* among the bacteria, but it showed antifungal activity on *C. albicans* at concentrations tested. The mixed chitosan and AG solution which is a dynamic association mixture leading to the formation of CS/AG complex when dried exhibited antimicrobial activity against both bacteria and fungi, so it had a broader inhibition range. In addition, it had lower MICs (evaluated by the chitosan concentration in the dynamic association mixture) on bacteria than did chitosan alone. MICs of dynamic association on the bacteria tested were 2–8 times lower than for the individual alone. For example, MIC of the dynamic association mixture on *S. aureus* (0.006% chitosan) was eight times lower than that of chitosan alone (0.05%). So the dynamic association mixture showed stronger antimicrobial activity and broader inhibition range.

The biocidal activity of chitosan, AG and dynamic association mixture were assessed using *E.coli*, *S. aureus*, *C. albicans*. The dynamic association mixture killed up to 2.65 log CFU/ml *S. aureus* after 5 min exposure, and killed all after 60 min exposure (Fig. 7a). Whereas chitosan and AG killed 1.2 log CFU/ml and 0.6 log CFU/ml *S. aureus*, respectively, in 5 min, but after this, there were little change in the amount of killed cells. And the dynamic association mixture killed approximately 2.52 log CFU/ml of *E. coli* after 30 min exposure, and killed all after 60 min exposure (Fig. 7b). Whereas chitosan killed all *E. coli* after 120 min, and AG only killed 1.72 log CFU/ml even after 180 min. *C. albicans* cells were all killed after an exposure to dynamic association for 60 min (Fig. 7c). Whereas AG could only killed 1.69 log CFU/ml after 120 min, and chitosan almost showed no biocidal activity on *C. albicans*. So the dynamic association mixture showed obviously stronger biocidal activity than chitosan and AG, respectively.

4. Discussion and conclusions

4.1. Interaction between chitosan and AG in solid state and aqueous solution

In this work, it has been found that complex formation between chitosan and AG, formed by forming of an

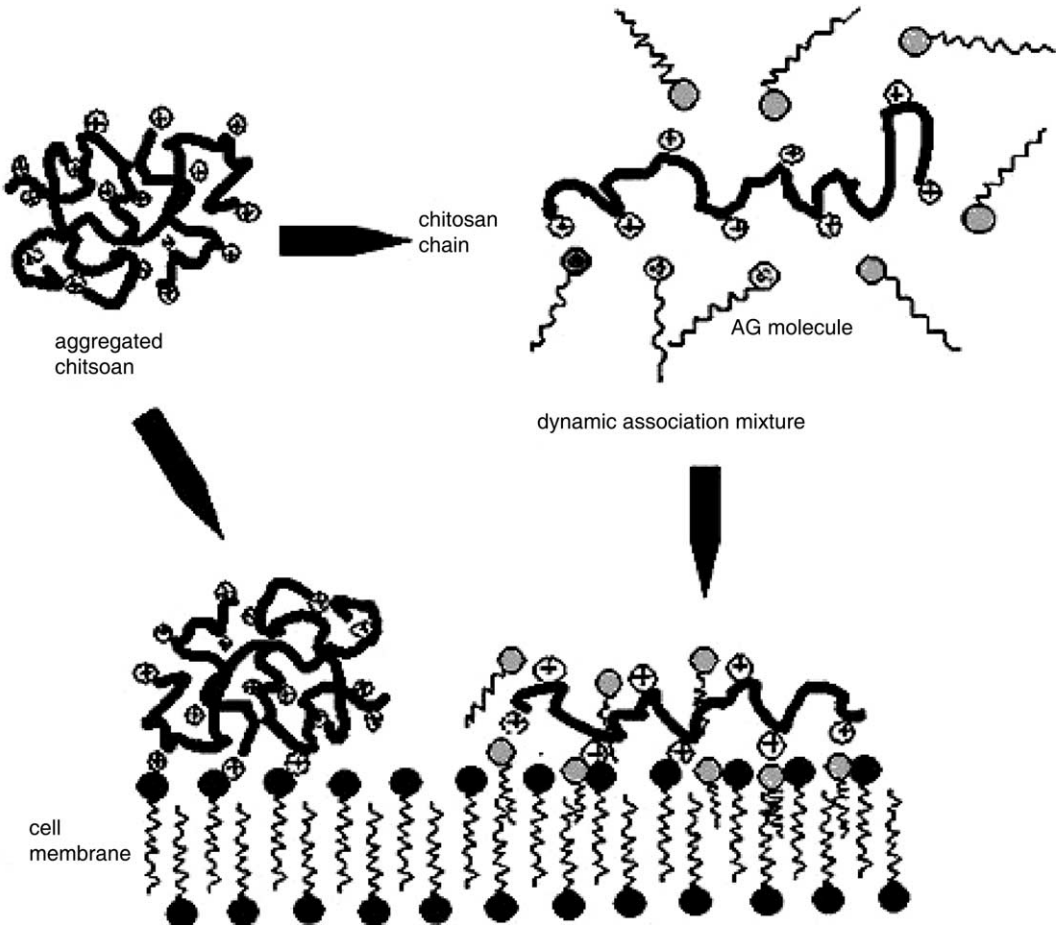

Fig. 8. Schematic interaction of dynamic association between chitosan and AG with cell membrane.

electrostatic force between $-NH_3^+$ groups of chitosan and –OH hydroxyl groups of AG and hydrogen bonding between –OH hydroxyl groups of chitosan and AG. CS/AG showed a lower degradation temperature and new crystallinity, which were likely due to the extended chain conformation and the disaggregation of chitosan to single chains.

In solution, the dynamic association mixture leading to the formation of the CS/AG complex will be formed as in Fig. 8. The antimicrobial activity of the dynamic association mixture was stronger than chitosan and AG alone. MIC of the dynamic association mixture was 2–8 times lower than those of chitosan alone. And it could kill more microorganisms in a shorter time.

4.2. Relationship between the interaction of chitosan with AG and antimicrobial activity

The exact mechanism of the antimicrobial activity of chitosan and its derivatives are still unknown. But different mechanisms have been proposed. General consideration has been put into the positive charge on the C-2 of the glucosamine monomer at below pH 6.3, which can interact with negatively charged microbial cell membranes and lead to the leakage of intracellular constituents (Helander, Nurmiaho-Lassila, Ahvenainen, Rhoades, & Roller, 2001; No et al., 2002; Papineau, Hoover, Knorr, & Farkas, 1991). Our previous study (no results showed here) demonstrated that $-NH_3^+$ in chitosan solution could interact vigorously with phosphoryl groups of phospholipids in cell membrane, so membrane was damaged. In this way, the antimicrobial activity will be strengthened if the number of $-NH_3^+$ groups in chitosan solution is increased or the interaction between chitosan and phospholipid is promoted.

As shown in Fig. 8, chitosan in acetate solution is generally aggregated. When AG is added $-NH_3^+$ and hydroxyl groups of chitosan will interact with the glycone of AG by electrostatic force or hydrogen bonding. The hydrophobicity of the long-chain alkyl in AG likely makes aggregated chitosan chain tend to extend and disaggregate to single chains. In this way, more $-NH_3^+$ in chitosan solution will be accessible to cell membrane than aggregated chitosan, though the total number of $-NH_3^+$ in chitosan solution are the same. On the other hand, the long-chain alkyl aglycone of AG tends to penetrate into the hydrophobic cell membrane, so AG will drag chitosan near to the phosphoryl groups of phospholipid. And then, chitosan will interact with the phosphoryl groups of phospholipid by a stronger ionic bond. In this way, interaction between chitosan and phospholipid is promoted. In addition, the penetration of alkyl group of AG will also interfere with normal function of cell membrane. For the above reasons, cell membrane is easier to be damaged. So the antimicrobial activity of the dynamic association increased.

In conclusion therefore, the presence of a molecule with hydrophilic and hydrophobic ends and forming weak interaction between hydrophilic ends and chitosan enhances the antimicrobial activity of chitosan.

Acknowledgements

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