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Antibacterial activity of oleoyl-chitosan nanoparticles: A novel antibacterial dispersion system

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Abstract

A novel chitosan antibacterial dispersion system was prepared by oleoyl-chitosan (O-chitosan) nanoparticles (OCNP), and the antibacterial activity against *Escherichia coli* and *Staphylococcus aureus* was investigated. Results showed that OCNP could be well distributed in nutrient broth and had strong antibacterial activity. The minimum inhibitory concentrations (MICs) of all OCNP samples ranged from 31.25 to 125 mg/l against *E. coli.* For *S. aureus*, the MIC of all samples was 125 mg/l. OCNP of low chitosan molecular weight (MW) appeared most effective against *E. coli*. For *S. aureus*, the effect of chitosan MW on the antibacterial activity of OCNP was not pronounced. *E. coli* was most susceptible to OCNP of O-chitosan with degrees of substitution (DS) 5%, while no marked difference was found among OCNP of O-chitosan with different DS against *S. aureus.* OCNP exhibited the most pronounced antibacterial activity at pH 6.0 in the experimental range. The integrity of cell membranes was destroyed when bacterial suspensions were treated with OCNP.

Keywords: Chitosan; Oleoyl-chitosan nanoparticles; Antibacterial dispersion system

1. Introduction

Chitosan, α-(1–4)-2-amino-2-deoxy-β-D-glucan, is a natural nontoxic biopolymer derived by partially deacetylated of chitin, a major component of the crustacean shells. It has attracted considerable interest due to its unique biological activity, such as antimicrobial activity (Li et al., 2007a, 2007b; No, Park, Lee, & Meyers, 2002; Park, Je, Byun, Moon, & Kim, 2004; Rabea, Badawy, Stevens, Smagghe, & Steurbaut, 2003; Yoshihiko et al., 2003), antitumor activity (Koide, 1998; Mitra, Gaur, Ghosh, & Maitra, 2001; Qin et al., 2004; Suzuki et al., 1986), and immune enhancing effect (Jeon & Kim, 2001). The antibacterial activity of chitosan is influenced by a number of factors that included the species of bacteria (No et al., 2002), concentration (Liu et al., 2006; Zheng & Zhu, 2003), pH (Liu, Guan, Yang, Li, & Yao, 2001), the solvent, molecular weight (MW) (Liu et al., 2006) and so on. However, chitosan is only soluble in acidic media like acetic acid, which also has the antibacterial activity on bacteria. Besides this, the precipitation occurs upon addition of chitosan solution to the culture medium, which makes it difficult to investigate the antibacterial activity and antibacterial mechanism of chitosan correctly. Therefore, a novel dispersion system of chitosan is necessary to be established and be used to evaluate the antibacterial action of chitosan.

Chitosan-based nanoparticles can be easily formed through self-aggregation. There have been many reports of hydrophobic modifications of chitosan and nanoparticle formation by self-aggregation in aqueous solution (Chen, Lee, & Park, 2003; Kim et al., 2001; Liu, Desai, Chen, & Park, 2005). These modifications can introduce hydropho-

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bic groups into chitosan and produced chitosan amphiphilic polymers. Some of these chitosan amphiphilic derivatives can form nanosized self-aggregation in aqueous media (Janes, Fresneau, Marazuela, Fabra, & Alonso, 2001). In our previous study, oleoyl-chitosan (O-chitosan) nanoparticles (OCNP) were prepared using an O/W emulsification method based on O-chitosan, which were synthesized by grafting oleoyl onto the $-NH_2$ at C-2 in the chitosan molecule. Different from chitosan, nanoparticles systems of chitosan could be well distributed in aqueous solution and less affected by pH of the solution. These characteristics could make it a novel potential antibacterial dispersion system to study the antibacterial mode. However, few investigations have been focused on the antibacterial dispersion system of self-assembled chitosan nanoparticles.

In this paper, OCNP as a novel antibacterial dispersion system were prepared. *Escherichia coli* (Gram-negative) and *Staphylococcus aureus* (Gram-positive) were chosen to be models for the antibacterial assay of OCNP. The influences of chitosan MW, degrees of substitution (DS) of O-chitosan, concentration of the nanoparticles and pH of the solution on the antibacterial activity of OCNP were examined. Additionally, the integrity of the cell membranes of *E. coli* and *S. aureus* influenced by OCNP was investigated.

2. Materials and methods

2.1. Materials

Chitosan (MW = 1100 kDa), degree of deacetylation 82%, was made from crab shell and obtained from Biotech Co. Oleoyl chloride, pyridine, chloroform, methylene chloride, acetic acid, and sodium tripolyphosphate (STPP) were purchased from Sigma Chemicals and used without further purification.

2.2. Preparation of OCNP

Chitosan was degraded by the method of acetic acid hydrolysis, and OCNP with different chitosan MW and DS of O-chitosan were prepared in our pervious study (Li et al., 2006, 2007a, 2007b). Different OCNP samples used in this paper are shown in Table 1.

Table 1
OCNP with Different MW of chitosan and DS of O-chitosan

series	OCNP samples	MW of chitosan (kDa)	DS of O-chitosan (%)
I	A	38	2.5
	B	38	5
	C	38	11
II	B	38	5
	D	300	5
	E	1100	5

2.3. Dispersion of OCNP in the culture medium

In order to explain the possible interaction between OCNP and the culture medium when mixed together, sample B was selected to test its dispersion in nutrient broth at different concentrations. The solution of chitosan (chitosan MW = 38 kDa), O-chitosan (chitosan MW = 38 kDa, O-chitosan with DS 5%) and sample B were first prepared with 0.1 M acetic acid. Different concentrations of chitosan, O-chitosan and sample B were prepared with two-fold serial broth dilution (Qi, Xu, Jiang, Hu, & Zou, 2004). A number of test bottles each containing 10 ml of sterile nutrient broth were prepared. To the first bottle, 10 ml of samples (1000 mg/l) was added. After mixing, 10 ml of the mixture was transferred to the second bottle, and similar transformations were repeated. Hence, each bottle contained a test sample solution with half of the concentration of the previous one. The final pH of solutions was adjusted to 5.0 or 6.0 with 10% NaOH solution. The controls were 0.1 M acetic acid solution of chitosan, O-chitosan and sample B (1000 mg/l), respectively. The transmittance of the solution was recorded on a 1601 UV–vis spectrophotometer (Shimadzu, Tokyo, Japan), using a quartz cell with an optical path length of 1 cm at 610 nm.

2.4. Cultivation of microorganisms

Escherichia coli (ATCC 25992) and *S. aureus* (ATCC 25923) were used as the test organisms. A representative bacteria colony was picked off with a wire loop, placed in nutrient broth, and then incubated at 37 °C for 12 h. By appropriately diluting with sterile distilled water, the cultures of *E. coli* and *S. aureus* containing $\sim 10^7$ CFU/ml were prepared and used for the antibacterial test.

2.5. Evaluation of the antibacterial activity in vitro

The solution of OCNP (1000 mg/l) was first prepared with 0.1 M acetic acid then adjusted to pH 6.0 with 10% NaOH solution. The MIC of OCNP was determined by two-fold serial broth dilution (Qi et al., 2004) described above and optical density method. The control only contained nutrient broth and 0.1 M acetic acid without OCNP. After adjusting to pH 6.0 with 10% NaOH solution, all of the samples were inoculated under aseptic conditions with 50 μl of the inoculums of bacteria and incubated at 37 °C for 24 h, then mensurated the turbidity of the cultured medium at 610 nm. The lowest concentration of OCNP that inhibited the growth of bacteria was considered as the MIC.

To determine the effect of pH, the solution of OCNP (1000 mg/l) was adjusted to pH 4.0, 4.5, 5.0, 5.5 and 6.0 with 10% NaOH solution. In this experiment, the concentrations of *E. coli* and *S. aureus* were adjusted to $\sim 10^4$ CFU/ml with sterile distilled water, respectively. 4 ml OCNP solution was added to 1 ml of the cell suspensions. The same volume of sterile distilled water was added

to the control group. Samples were blended fully and removed after 5 min. Portions (50 μl) were spread on triplicate nutrient agar plates and incubated at 37 °C for 24 h, then the numbers of colonies were counted (Choi et al., 2001; Sudarshan, Hoover, & Knorr, 1992).

2.6. Integrity of cell membranes

If the bacteria membrane is compromised, release of cytoplasmic constituents of the cell can be monitored. Bacterial cell membrane integrity was examined by determination of the release of materials absorbing at 260 nm (Chen & Cooper, 2002). Bacterial cultures grown as above were harvested, washed and resuspended in 0.5% NaCl solution. The final cell suspensions were adjusted to an absorbance at 420 nm of 0.7. The solutions of sample B and acetic acid solution (control) were adjusted to pH 6.0 with 10% NaOH solution, respectively. A 1.5-ml portion of OCNP solution or acetic acid solution was mixed with 1.5 ml of each bacterial cell suspensions, and the release over time of materials absorbing at 260 nm was monitored with a 1601 UV–vis spectrophotometer (Shimadzu, Tokyo, Japan).

2.7. Statistical analyses

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

3. Results and discussion

3.1. Dispersion of OCNP in the culture medium

In the present study, OCNP were prepared using an O/W emulsification method based on O-chitosan, which were synthesized by grafting oleoyl onto the $-NH_2$ at C-2 in the chitosan molecule. The nanoparticle formulation had a spherical shape and it was well dispersed in acetic acid solution without any aggregation, these properties were different from its raw material chitosan. The OCNP were mixed with *E. coli* suspensions and spread portions on nutrient agar plates, and the result showed that most of the bacteria were killed within a few minutes. This phenomenon evidenced that OCNP had a strong antibacterial activity, and this property was similar to its raw material chitosan. According to the results, the OCNP had the properties to be as a dispersion system to replace its raw material chitosan in the next step of antibacterial experiments.

Further experiment was to measure the transmittance of the culture medium with the addition of OCNP to check the dispersion of nanoparticles in it. The transmittance of chitosan, O-chitosan, OCNP (sample B) with different concentrations and pH are shown in Figs. 1 and 2. At pH 6.0, the transmittance of 0.1 M acetic acid solution of chitosan, O-chitosan, sample B were 92.67%, 92.70%, 66.17%, respectively. When added to the culture medium, the mixture of sample B and nutrient broth showed higher transmittance than that of chitosan and O-chitosan at every concentration. As the concentrations ranged from 1.95 to 250 mg/l, the transmittance decreased, respectively. When the concentration achieved 500 mg/l, the transmittance increased slightly. At pH 5.0, the transmittance of 0.1 M acetic acid solution of chitosan, O-chitosan, sample B were 96.03%, 93.60%, 84.90%, respectively. When added to the culture medium, the mixture of sample B and nutrient broth still showed higher transmittance than that of chitosan and O-chitosan at every concentration. It indicated that OCNP could be well distributed in nutrient broth for a nice dispersion in the tested concentration range. It would be a novel antibacterial dispersion system with potential value of further study on the antibacterial activity and antibacterial mechanism.

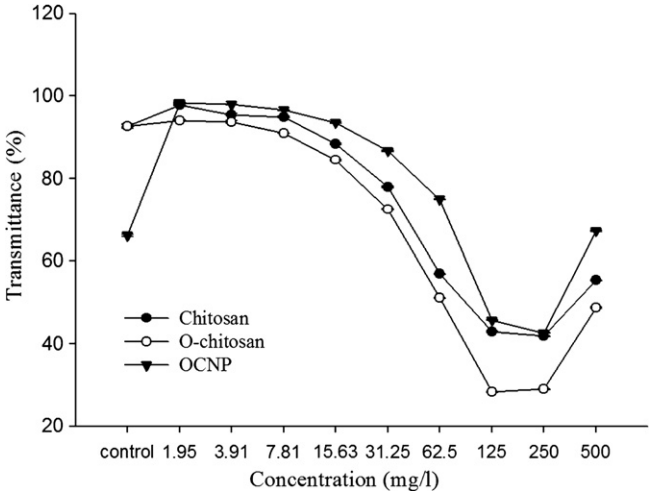

Fig. 1. The transmittance of mixture of chitosan (-●-), O-chitosan (-○-), OCNP (-▼-) and nutrient broth at pH 6.0.

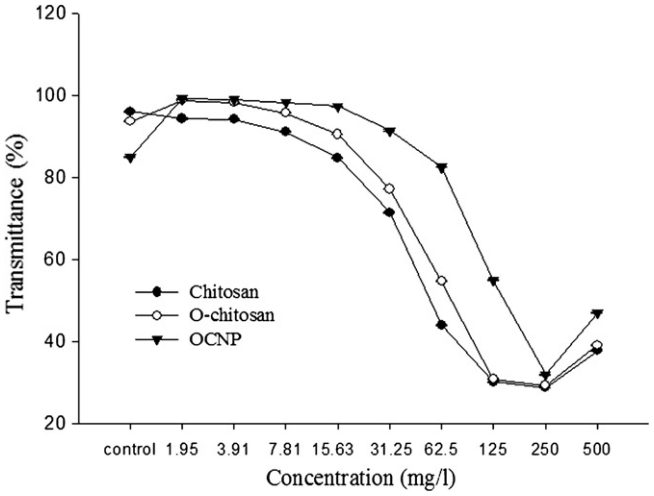

Fig. 2. The transmittance of mixture of chitosan (-●-), O-chitosan (-○-), OCNP (-▼-) and nutrient broth at pH 5.0.

3.2. Effect of concentration on the antibacterial activity of OCNP

The effect of concentration on the antibacterial activity of OCNP against *E. coli* and *S. aureus* is shown in Figs. 3 and 4. As the concentrations ranged from 1.95 to 7.81 mg/l, the optical density (OD) values were no difference between the experiment groups and control group for both bacteria. For *E. coli*, with increase of the concentration of OCNP in the medium, which varied from 7.81 to 31.25 mg/l (samples A, B, C), from 15.63 to 62.5 mg/l (sample D) and from 31.25 to 125 mg/l (sample E), their OD values decreased, respectively. So the MIC values of all OCNP samples were 31.25 mg/l (samples A, B, C), 62.5 mg/l (sample D) and 125 mg/l (sample E) against *E. coli*. For *S. aureus*, when the concentration was higher than 31.25 mg/l, all OCNP samples had shown their antibacterial activity obviously. When the concentration achieved 125 mg/l, almost all bacteria were killed. Therefore, the MIC of all OCNP samples was 125 mg/l against *S. aureus*. The results indicated that the antibacterial activity increased as the concentration of OCNP increased, which was similar to its raw material chitosan (Liu et al., 2006).

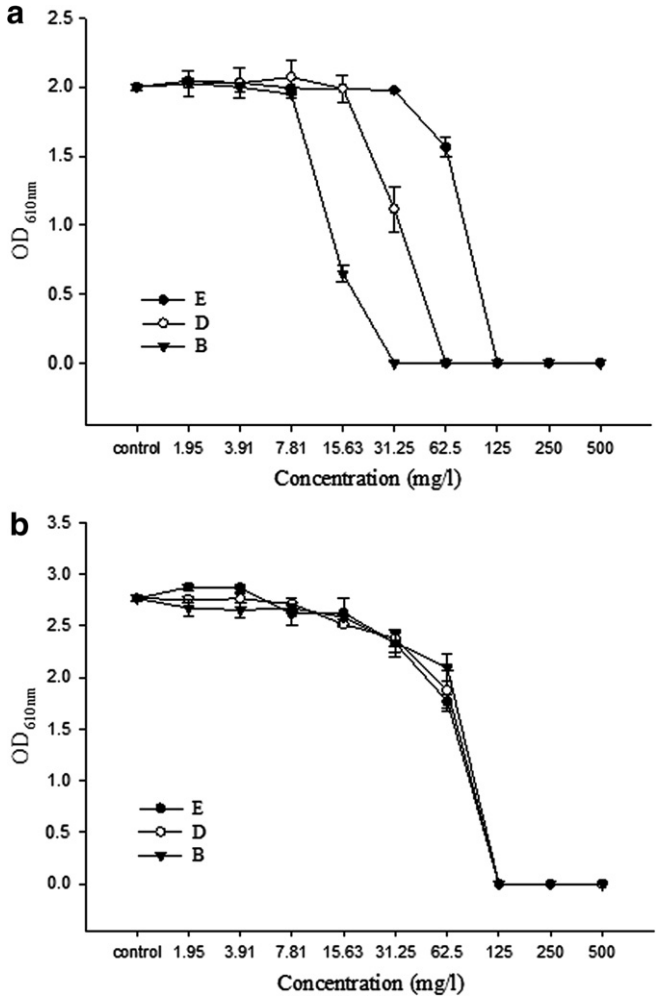

Fig. 3. Effect of concentration to the antibacterial activity of OCNP with different chitosan MW against (a) *E. coli* or (b) *S. aureus*.

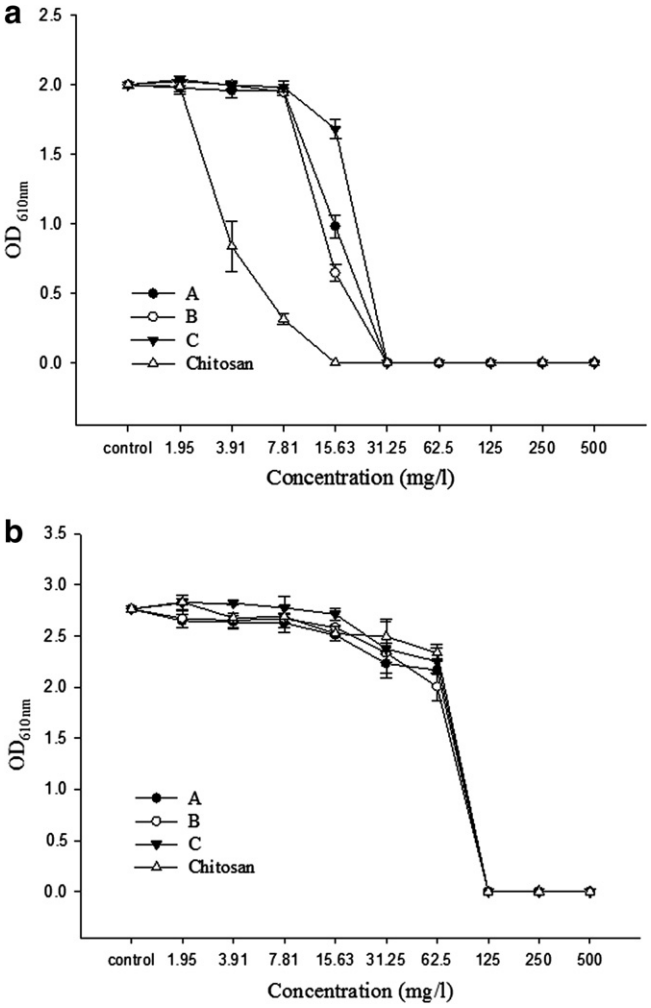

Fig. 4. Effect of concentration to the antibacterial activity of chitosan and OCNP with different DS of O-chitosan against (a) *E. coli* or (b) *S. aureus*.

3.3. Effect of chitosan MW on the antibacterial activity of OCNP

OD values versus the concentration for the OCNP with different chitosan MW against *E. coli* and *S. aureus* are shown in Fig. 3. As the concentrations ranged from 1.95 to 7.81 mg/l, the OD values were no difference between the experiment groups and control group for both bacteria. With Gram-negative bacterium, OCNP of low chitosan MW of 38 kDa (sample B) appeared most effective against *E. coli* (Fig. 3a). With the chitosan MW varying from 38 to 300 kDa, the MIC values of OCNP increased from 31.25 to 62.5 mg/l. When the chitosan MW achieved 1100 kDa, the MIC was 125 mg/l. It meant the antibacterial activity of OCNP decreased while chitosan MW increased against *E. coli*. However, this trend was not observed with Gram-positive bacterium. In contrast to the response of

E. coli, growth of *S. aureus* was almost suppressed by OCNP with different chitosan MW at 125 mg/l (Fig. 3b). Therefore, the effect of chitosan MW on the antibacterial activity of OCNP against *S. aureus* was not as pronounced as that observed with *E. coli*.

Until now, there have been many investigations of the relationship between MW and the antibacterial activity of chitosan. However, little information about relationships of chitosan MW and the antibacterial activity by chitosan nanoparticles has been reported. Liu et al. (2006) reported that the antibacterial activity of low MW chitosan is stronger than that of the high MW chitosan against *E. coli*. Effect of chitosan MW on the antibacterial activity of OCNP was similar to that of chitosan. No et al. (2002) reported that among one series of chitosan with MW ranging from 28 to 1671 kDa, chitosan of 470 kDa appeared most effective against *S. aureus*. It was found that OCNP with high chitosan MW and low chitosan MW showed an equal antibacterial activity for *S. aureus*. It meant that the effect of chitosan MW on the antibacterial activity of OCNP was not as pronounced as chitosan against *S. aureus*.

3.4. Effect of DS of O-chitosan on the antibacterial activity of OCNP

The effect of DS of O-chitosan on the antibacterial activity of OCNP against *E. coli* and *S. aureus* is shown in Fig. 4. No marked difference was found among three OCNP samples tested in relation to *S. aureus*, while sample B formed by O-chitosan with DS 5% exhibited the most pronounced antibacterial activity against *E. coli*. It was found that OCNP and chitosan (chitosan MW = 38 kDa) exhibited an equal antibacterial activity against *S. aureus*, whereas OCNP presented inferior inhibitory activity than chitosan (chitosan MW = 38 kDa) against *E. coli*. It was interesting to find that the antibacterial activity of OCNP increased upon increasing the DS of O-chitosan from 2.5% to 5% and decreased with further increase to 11%. In our previous study, Li et al. (2007a, 2007b) reported that the increase of DS might facilitate OCNP self-aggregation, but in the high DS sample with high viscosity and low solubility nanoparticles were not readily formed. Solubility of O-chitosan was decreased as the DS increased from 5% to 11%, therefore, it could be suggested that the effective concentration of OCNP (sample C) with DS 11% might be a little lower than the concentration set in this paper. The decreased antibacterial activity observed with the OCNP (sample C) may be attributed to the decrease in solubility of O-chitosan with DS 11%.

3.5. Effect of pH on the antibacterial activity of OCNP

The effect of pH on the antibacterial activity of OCNP against *E. coli* and *S. aureus* is shown in Fig. 5. The antibacterial activity increased as the pH increased from 4.0 to 6.0 and reached a maximum at pH 6.0 for both *E. coli*

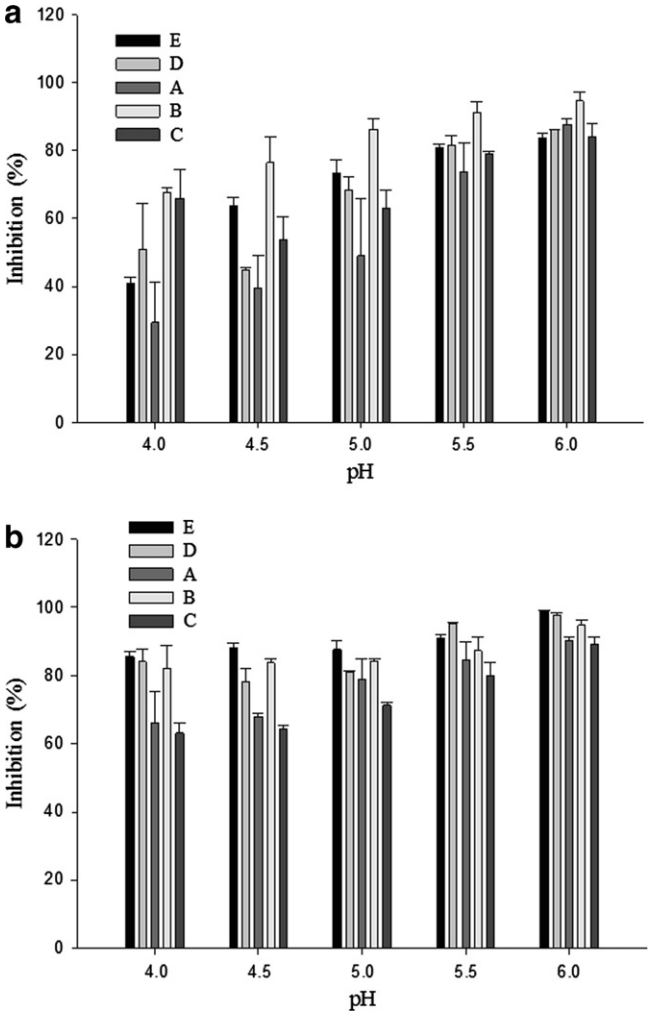

Fig. 5. Effect of pH on the antibacterial activity of OCNP against (a) *E. coli* or (b) *S. aureus*.

and *S. aureus*. OCNP could be well distributed in acetic acid solution as the pH varied from 4.0 to 6.0. We attempted to test the antibacterial activity of OCNP in neutral and alkaline pH but were frustrated in the poor stability under such conditions (data not shown). Therefore, the upper pH value studied was limited to 6.0 to make sure that OCNP could keep their nice dispersivity. There are many studies regarding the effect of pH on the antibacterial activity of chitosan. Several workers (Fujimoto, Tsuchiya, Terao, Nakamura, & Yamamoto, 2006; No et al., 2002) reported that acidic pH increased the antibacterial effect of chitosan. While Liu et al. (2001) evidenced that the antibacterial activity of chitosan decreased gradually with pH varying from 6.3 to 4.0. Effect of pH on the antibacterial activity of OCNP was similar to that of chitosan reported by Liu et al. (2001). Chitosan cannot dissolve in water but in acetic acid solution that also had the antibacterial activity (Liu et al. 2006). This property cannot be ignored. OCNP with strong antibacterial activity at pH 6.0 could reduce the effect of acetic acid on the bacterial growth greatly.

3.6. Integrity of bacterial cell membranes

The cytoplasmic cell membrane is a structural component, which may become damaged and functionally invalid when bacterial suspensions are exposed to antimicrobial agents. If bacterial membrane became compromised, small ions such as K^+ and PO_4^{3-} tend to leach out first, and followed by large molecules such as DNA, RNA and other materials. The release of these intracellular components with strong UV absorption at 260 nm is an indication of membrane damage (Chen & Cooper, 2002).

According to the results above, OCNP could exhibit pronounced antibacterial activity when the concentrations were higher than the MIC values at pH 6.0. Sample B was selected for the assay of cell membranes integrity because of its stronger antibacterial activity. The release of intracellular components upon addition of OCNP to *S. aureus* suspensions is shown in Fig. 6. When *S. aureus* suspensions were treated with OCNP, the absorbance of the suspensions at 260 nm dramatically increased up to 60 min then at a decreasing rate up to 120 min. Since *S. aureus* did not have the outer membrane (OM) to prevent the influx of foreign molecules, it was not surprising to see A_{260} increased as soon as OCNP mixed with bacterial cell suspensions. In the case of *E. coli* (Fig. 7), there was a lag of about 5 min before intracellular components were detected at 260 nm because *E. coli* had the OM to prevent the influx of foreign molecules. Then the A_{260} increased rapidly up to 80 min. Thereafter the absorbance was almost unchanged in suspensions treated with 300 mg/l OCNP, while the absorbance increased at a decreasing rate up to 120 min in suspensions treated with 150 mg/l OCNP. OCNP induced a significant amount of release of 260 nm absorbing material from these Gram-negative bacteria. Furthermore, A_{260} values were greater in suspensions treated with 300 mg/l than with 150 mg/l OCNP. Therefore, the release rate of intracellular components caused by

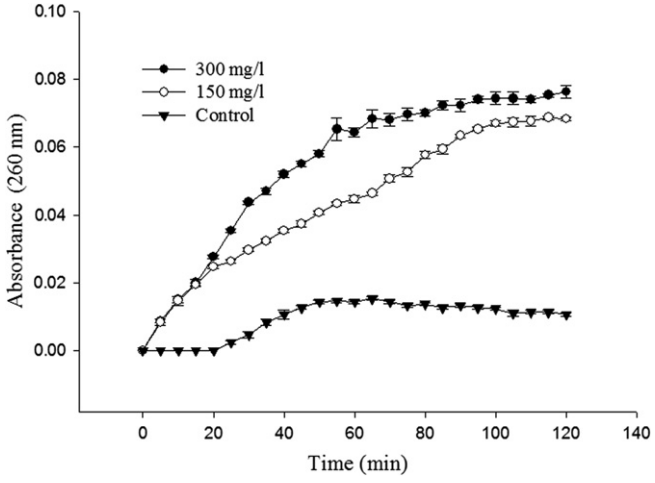

Fig. 6. Release of 260 nm absorbing material from *S. aureus* suspensions treated with 300 mg/l (-●-), 150 mg/l (-○-) of OCNP (sample B) and pH 6.0 acetic acid solution(-▼-).

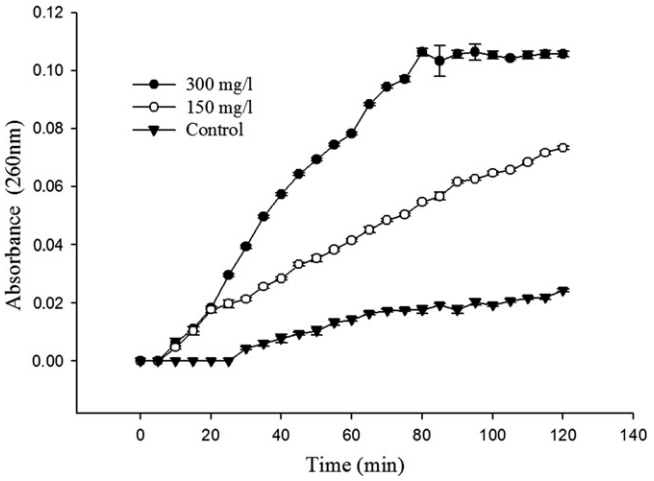

Fig. 7. Release of 260 nm absorbing material from *E. coli* suspensions treated with 300 mg/l (-●-), 150 mg/l (-○-) of OCNP (sample B) and pH 6.0 acetic acid solution (-▼-).

OCNP was concentration-dependent, which was also agreeable with the previous findings for antibacterial activity. In control suspensions, a lag of about 20 min was followed by a relatively slowly release of intracellular components up to 120 min.

Antibacterial activity of chitosan as related to membranes permeability has been evaluated in the literature (Liu, Du, Wang, & Sun, 2004). They studied on the integrity of cell membranes using chitosan against *E. coli* and *S. aureus*. Their results showed that release of 260 nm absorbing materials quickly increased, and the damage of cell membranes was concentration-dependent. Our results evidenced that OCNP could induce the release of intracellular component via destroying the integrity of bacterial cell membranes, which was similar to its raw material chitosan.

4. Conclusions

In this study, OCNP as a novel antibacterial dispersion system were prepared, which could be well distributed in nutrient broth for a nice dispersion. OCNP showed strong antibacterial activity against *E. coli* and *S. aureus*. The concentration of the nanoparticles, MW of chitosan, DS of O-chitosan and pH of the solution affected the antibacterial activity of OCNP. For *E. coli*, the MIC values of OCNP ranged from 31.25 to 125 mg/l as chitosan MW increased from 38 to 1100 kDa. In case of *S. aureus*, the MIC of all OCNP samples was 125 mg/l. OCNP of low chitosan MW of 38 kDa appeared most effective against *E. coli*. It was also found that OCNP of O-chitosan with DS 5% exhibited the most pronounced antibacterial activity against *E. coli*. While the effect of chitosan MW and DS of O-chitosan on the antibacterial activity of OCNP against *S. aureus* was not so pronounced. Furthermore, OCNP showed increased antibacterial activity when pH increased from 4.0 to 6.0 and reached a maximum at pH 6.0. When *E. coli* and *S. aureus* suspensions treated with

sample B, the release of intracellular component increased via destroying the integrity of bacterial cell membranes. OCNP, as a novel antibacterial dispersion system, still keep the original antibacterial activity of chitosan and have the potential value in the determination of the exact antibacterial mechanism of chitosan.

Acknowledgements

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