Carbohydrate Polymers 76 (2009) 17–22

Contents lists available at ScienceDirect

Carbohydrate Polymers

journal homepage: www.elsevier.com/locate/carbpol

Effect of oleoyl-chitosan nanoparticles as a novel antibacterial dispersion system on viability, membrane permeability and cell morphology of *Escherichia coli* and *Staphylococcus aureus*

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ARTICLE INFO

Article history:
Received 24 July 2008
Received in revised form11 September 2008
Accepted 15 September 2008
Available online 22 September 2008

Keywords:
Oleoyl-chitosan nanoparticles
Antibacterial dispersion system
Membrane integrity and permeability

ABSTRACT

A novel chitosan antibacterial dispersion system was prepared by oleoyl-chitosan (O-chitosan) nanoparticles (OCNP) and the bactericidal activity against *Escherichia coli* and *Staphylococcus aureus* was evaluated by the enumeration of viable organisms at different incubation times. We further investigated the antimicrobial mode of OCNP using a combination of approaches, including cell integrity measurements, outer membrane (OM) and inner membrane (IM) permeabilization assays, SDS–PAGE and transmission electron microscopy (TEM). Results showed that when treated with OCNP, release of intracellular components quickly increased for both *E. coli* and *S. aureus*. OCNP also rapidly increased the 1-*N*-phenylnaphthylamine (NPN) uptake and the release of cytoplasmic β-galactosidase via increasing the permeability of OM and IM. Besides, SDS–PAGE indicated the content of cellular soluble proteins decreased significantly in OCNP-treated bacteria. TEM observations demonstrated adsorption behaviors of OCNP on bacteria and extensive cell surface alterations of OCNP-treated bacteria. OCNP has potential value in the determination of antibacterial mechanism of chitosan.

1. Introduction

Chitosan is a natural nontoxic biopolymer derived by partially deacetylated of chitin. Though having a broad range of inhibitory spectrum (Choi et al., 2001; Chung, Wang, & Chen, 2003; Liu, Guan, Yang, Li, & Yao, 2001), chitosan is only soluble in acidic media like acetic acid, which releases hydrogen ions when the acids penetrate bacteria cells, and the cells are oxidized. Because the oxidation inhibits enzyme activity, multiplication of microorganisms is contained (Jung & Beuchat, 2000; Ryu, Deng, & Beuchat, 1999; Tamblyn & Conner, 1997). This property cannot be ignored when investigated the antibacterial activity of chitosan. Besides this, the precipitation occurred 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 used to evaluate the antibacterial action of chitosan.

Until recently, the mechanism of how chitosan acted upon bacteria has not been elucidated clearly. There have been a large number of reports discussing chitosan's antibacterial mechanism. In one mechanism (Helander, Nurmiaho-Lassila, Ahvenainen, Rhoades, & Roller, 2001; Sudarshan, Hoover, & Knorr, 1992), positively charged chitosan molecules interfere with the negatively charged residues on the bacterial surface. Chitosan interacts with the membrane of the bacteria to alter cell permeability. So far, it has been shown that chitosan binds and interact with artificial membranes with different chitosan/phosphatidylcholine ratios (v/v), and is capable of increasing the permeability of the bacterial membrane (Liu, Du, Wang, & Sun, 2004). However, there is no direct evidence for such an interaction yet.

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 hydrophobic groups into chitosan and produce 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 (Li et al., 2006, 2007), 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 (Xing et al., 2008). These characteristics could make it a novel

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doi:10.1016/j.carbpol.2008.09.016

potential antibacterial dispersion system to study the antibacterial mode. Thus, it is important to investigate the possible mechanisms of action of OCNP.

In this paper, OCNP as a novel antibacterial dispersion system were prepared. *E. coli* (Gram-negative) and *S. aureus* (Gram-positive) were chosen to be models for the antibacterial assay of OCNP. The bactericidal activity of OCNP, the effect on cell membranes permeability, SDS–PAGE analysis and the morphology of *E. coli* and *S. aureus* affected by OCNP were studied to determine the mode of action of OCNP.

2. Materials and methods

2.1. Materials

Chitosan (M_W = 38 kDa), degree of deacetylation 82%, was made from crab shell and obtained from Biotech (Mokpo, Korea). Sodium dodecyl sulfate (SDS), Acrylamide and *N,N′*-methylenebisacrylamide were obtained from Amresco (Beijing, China). 1-*N*-phenylnaphthylamine (NPN) and *o*-nitrophenyl-β-D-galactoside (ONPG) were purchased from Sigma Chemicals (Shanghai, China) and used without further purification.

2.2. Preparation of OCNP

OCNP were prepared according to our previous method (Li et al., 2006, 2007). In this study, the sample with a degree of substitution (DS) 5% of oleic acid was chosen to prepare nanoparticles for its superior antibacterial activity (Xing et al., 2008).

2.3. Cultivation of the microorganisms

E. 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 and placed in nutrient broth, which was then incubated at 37 °C for 12 h. By appropriately diluting with sterile nutrient broth, the cultures of *E. coli* and *S. aureus* containing $\sim 10^7$ CFU/ml were prepared and used for further study.

2.4. Bactericidal assay

The bactericidal activity of OCNP was measured by enumeration of viable organisms, as previously described (Choi et al., 2001; Liu et al., 2004; Sudarshan et al., 1992). In this experiment, the concentrations of *E. coli* and *S. aureus* were adjusted to $\sim 10^4$ CFU/ml with sterile nutrient broth, respectively. After adjusted to pH 6.0 with 10% NaOH solution, OCNP were added to reach final concentrations of 300 mg/l and 150 mg/l, respectively. Samples were removed after 5, 10, 20, 40, 80 and 160 min, respectively. Portions (50 μl) were spread on triplicate nutrient agar plates and incubated at 37 °C for 24 h, and then the numbers of colonies were counted.

2.5. Integrity of cell membranes

If the bacteria membrane is compromised, release of cytoplasmic constituents of the cell can be monitored. Through the detection of absorbance at 260 nm, one can estimate the amount of DNA and RNA released from the cytoplasm (Chen & Cooper, 2002). Bacterial cultures grown in nutrient broth were harvested by centrifugation at 11,000*g* for 10 min, washed and resuspended in 0.5% NaCl solution. The final cell suspension was adjusted to an absorbance at 420 nm of 0.7. The solutions of OCNP and 0.5% acetic acid solution (control) were adjusted to pH 6.0 with 10% NaOH solution. A 1.5-ml portion of OCNP solution or acetic acid solution was mixed with 1.5 ml of each bacterial cell suspension. The release of materials absorbing at 260 nm over time was monitored with a 1601 UV–VIS spectrophotometer (Shimadzu, Tokyo, Japan).

2.6. Outer membrane permeabilization assays

Outer membrane (OM) permeabilization activity of OCNP was determined by the NPN assay (Ibrahim, Sugimoto, & Aoki, 2000; Loh, Grant, & Hancock, 1984). *E. coli* cultures grown in nutrient broth were harvested by centrifugation at 11,000*g* for 10 min, washed and resuspended in 0.5% NaCl solution. The final cell suspension was adjusted to an absorbance at 420 nm of 1.0. The solutions of OCNP and 0.5% acetic acid solution (control) were adjusted to pH 6.0 with 10% NaOH solution. A 1.5-ml portion of OCNP solution or acetic acid solution was mixed with 20 μl of 1 mM NPN. The fluorescence was recorded with an F-4500 fluorescence spectrophotometer (Hitachi, Tokyo, Japan), with an excitation wavelength of 350 nm and an emission wavelength of 420 nm, respectively. After the addition of 1 ml of a cell suspension, an increase in fluorescence due to partitioning of NPN into the OM was recorded immediately as a function of time until there was no further increase in the emission intensity.

2.7. Inner membrane permeabilization assays

Inner membrane (IM) permeabilization was determined by measuring the release of cytoplasmic β-galactosidase activity from *E. coli* into the culture medium using ONPG as the substrate (Ibrahim et al., 2000; Sudarshan et al., 1992). Bacteria grown to logarithmic phase in nutrient broth containing 2% lactose were harvested by centrifugation at 11,000*g* for 10 min, washed and resuspended in 0.5% NaCl solution. The final cell suspension was adjusted to an absorbance at 420 nm of 1.2. The solutions of OCNP and 0.5% acetic acid solution (control) were adjusted to pH 6.0 with 10% NaOH solution. OCNP solutions (1.6 ml) were each mixed with bacteria suspension (1.6 ml) and 30 mM ONPG solution (150 μl). The production of *o*-nitrophenol over time was determined by monitoring the increase in A_{420} using a 1601 UV–VIS spectrophotometer (Shimadzu, Tokyo, Japan).

2.8. SDS–PAGE of whole-cell proteins

Bacteria were prepared as described above. Samples containing *E. coli* and *S. aureus* ($\sim 10^7$ CFU/ml) in nutrient broth were incubated with 300 mg/l OCNP at 37 °C, respectively. Controls were run without OCNP. Aliquots of 4 ml were withdrawn after 30, 60 and 120 min, and then centrifuged. The pellet was subjected to SDS–PAGE according to Laemmli (1970), with the aid of a vertical electrophoresis apparatus Bio-Rad (Hercules, CA, USA). The SDS–PAGE was performed with a 4% stacking gel and a 10% separating gel followed by Coomassie brilliant blue staining.

2.9. Transmission electron microscopy (TEM)

Bacteria were prepared for electron microscopy as described above. Samples containing *E. coli* and *S. aureus* ($\sim 10^7$ CFU/ml) in nutrient broth were incubated with 300 mg/l OCNP for up to 30 min, respectively. After incubation, samples were fixed in 2.5% glutaraldehyde (w/v). A drop containing the bacteria was deposited on to a carbon coated grid and negatively stained with 2% (w/v) PTA. The grids were examined using a JEOL JEM-1200EXII electron microscope (Japan Electron Optics Laboratory, Tokyo, Japan).

2.10. 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, and then compared by *t*-test. *P-value* < 0.05 was considered statistically significant. Calculations were done using the software SigmaPlot 10.0.

3. Results and discussion

3.1. Bactericidal activity of OCNP

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. Further experiment was to measure the bactericidal activity of the novel antibacterial dispersion system.

The bactericidal activity of OCNP and the native chitosan against *E. coli* and *S. aureus* is shown in Tables 1 and 2, respectively. The final population of test organisms in all controls did not change obviously. On the other hand, test organisms in nutrient broth containing OCNP or chitosan exhibited various extents of population reduction.

As shown in Tables 1 and 2, the bactericidal activity was greater in suspensions treated with 300 mg/l than with 150 mg/l OCNP. The viable cells decreased with the increased in contact time of the OCNP and cells. With *E. coli*, the surviving cell numbers in OCNP of 300 mg/l decreased 1.22 $\log_{10}$CFU/ml, while the surviving cell numbers in OCNP of 150 mg/l decreased 1.04 $\log_{10}$CFU/ml compared to the control in 5 min. With *S. aureus*, OCNP of 300 mg/l and 150 mg/l exhibited a population reduction of more than 1.7 $\log_{10}$CFU/ml compared to the control after 5 min. Thereafter the surviving cell numbers kept on decreasing dramatically. All of the bacteria were killed within 160 min for *E. coli*. In the case of *S. aureus*, no viable cells were noted in the medium after 80 min. When compared with chitosan, OCNP (300 mg/l) showed an equal bactericidal activity except against *E. coli* at 20 and 40 min. Results showed that OCNP, as a novel antibacterial dispersion system, still kept the original bactericidal activity of chitosan. It would have the potential value of further study on the antibacterial mechanism.

3.2. 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).

In our previous study (Xing et al., 2008), we found that treatment of *E. coli* and *S. aureus* with OCNP (300 mg/l and 150 mg/l) resulted in a gradual release of intracellular components. In detail, A_{260} increased as soon as OCNP mixed with *S. aureus*. In case of *E. coli*, there was a lag of about 5 min before intracellular components were detected at 260 nm. In addition, A_{260} of *S. aureus* increased faster than that of *E. coli* before 30 min. This was probably due to the differences in cell wall structure and composition. The

Table 1
Effect of chitosan and OCNP on the numbers of *E. coli* in cell suspensions incubated at room temperature

Samples	Incubation time (min)									
	5		10		20		40		80	
	Final population (cfu/ml)	Population reduction[a] (log cfu/ml)	Final population (cfu/ml)	Population reduction (log cfu/ml)	Final population (cfu/ml)	Population reduction (log cfu/ml)	Final population (cfu/ml)	Population reduction (log cfu/ml)	Final population (cfu/ml)	Population reduction (log cfu/ml)
Control[b]	9.13×10^4		8.97×10^4		8.67×104		9.27×10^4		9.20×10^4	
Chitosan (300 mg/l)	5.46×10^3	1.22 ± 0.03A[c]	1.59×10^3	1.75 ± 0.02A	3.11×10^2	2.45 ± 0.01A	1.85×10^2	2.70 ± 0.01A	_[d]	4.96 ± 0.01A
OCNP (300 mg/l)	5.63×10^3	1.22 ± 0.02A	1.98×10^3	1.62 ± 0.06A	3.51×10^2	2.39 ± 0.01B	2.00×10^2	2.66 ± 0.01B	_	4.96 ± 0.01A
OCNP (150 mg/l)	8.07×10^3	1.04 ± 0.01B	5.87×10^3	1.20 ± 0.03B	8.93×10^2	1.99 ± 0.01C	3.48×10^2	2.42 ± 0.01C	6.67×10^0	4.14 ± 0.03B

[a] Population reduction = log(final population in control/final population in test sample).
[b] Acetic acid (pH 6.0).
[c] Values in the same column with different letters are significantly different ($p < 0.05$) according to *t*-test.
[d] No viable cell was detected.

Table 2
Effect of chitosan and OCNP on the numbers of *S. aureus* in cell suspensions incubated at room temperature

Samples	Incubation time (min)							
	5		10		20		40	
	Final population (cfu/ml)	Population reduction[a] (log cfu/ml)	Final population (cfu/ml)	Population reduction (log cfu/ml)	Final population (cfu/ml)	Population reduction (log cfu/ml)	Final population (cfu/ml)	Population reduction (log cfu/ml)
Control[b]	8.23×10^4		8.36×104		8.30×10^4		8.47×10^4	
Chitosan (300 mg/l)	1.26×10^3	1.82 ± 0.02B[c]	1.22×10^2	2.84 ± 0.05A	1.60×10^1	3.72 ± 0.02A	3.33×10^0	4.51 ± 0.35A
OCNP (300 mg/l)	1.09×10^3	1.88 ± 0.02A	1.14×10^2	2.87 ± 0.05A	1.47×10^1	3.75 ± 0.04A	_[d]	4.93 ± 0.00A
OCNP (150 mg/l)	1.57×10^3	1.72 ± 0.02C	2.90×10^2	2.46 ± 0.01B	4.17×10^1	3.30 ± 0.03B	_	4.47 ± 0.50A

[a] Population reduction = log(final population in control/final population in test sample).
[b] Acetic acid (pH 6.0).
[c] Values in the same column with different letters are significantly different ($p < 0.05$) according to *t*-test.
[d] No viable cell was detected.

cell wall of *E. coli* is made up of a thin membrane of peptidoglycan and an outer membrane composed of lipopolysaccharide, lipoprotein and phospholipids. But the peptidoglycan layer of the cell wall of *S. aureus* is composed of networks with plenty of pores, so foreign molecules could enter the cell without difficulty. Since *S. aureus* does not have the OM to prevent the influx of foreign molecules, it was not surprising to see that *S. aureus* is more sensitive than *E. coli* to exogenous agents. Moreover, the release rate of intracellular components induced by OCNP was concentration-dependent.

Liu et al. (2004) reported the antibacterial activity of chitosan as related to membranes permeability. They found that release of 260 nm absorbing materials quickly increased, and the damage of cell membranes was concentration-dependent. Our results evidenced that OCNP could destroy the integrity of bacterial cell membranes and induce the release of intracellular component; this was similar to the raw material chitosan.

3.3. OM permeabilization of E. coli

Gram-negative bacteria such as *E. coli* have two cell envelope membranes. We examined the ability of OCNP to interact with both the outer and inner membranes. NPN, which is a hydrophobic probe, is normally excluded by an intact out membrane of Gram-negative bacteria. Increased fluorescence uptake of NPN occurs in bacterial suspensions containing cells whose OM is damaged and functionally invalid, since the quantum yield of NPN is greatly increased in a glycerophospholipid milieu as opposed to an aqueous one (Helander & Mattila-Sandholm, 2000; Loh et al., 1984; Träuble & Overath, 1973).

Based on this principle, the NPN uptake by OCNP-treated *E. coli* is shown in Fig. 1. The addition of OCNP to *E. coli* suspensions in the presence of NPN caused a time-dependent increase in fluorescence. As soon as OCNP mixed with *E. coli* suspensions, there was immediate uptake of NPN with the amount released being maximal after about 6 min. Thereafter the NPN uptake was almost unchanged until 10 min. The fluorescence increase was dose-dependent so that the maximum fluorescence was greater with the higher than the lower concentration of OCNP, which was in accordance with the cell membranes integrity results. In control suspensions, there was almost no NPN uptake in 10 min. Helander et al. (2001) reported that relatively high concentrations of chitosan (250 ppm) were required to obtain significantly increased NPN uptake. Increase of the uptake of the hydrophobic probe NPN further suggested the permeabilizing action of OCNP, which was similar to its raw material chitosan.

3.4. IM permeabilization of E. coli

When the IM became compromised, β-galactosidase, a normally endoenzyme, could permeate the cytoplasmic membrane. The ability of OCNP to permeate the *E. coli* IM was evaluated the production of *o*-nitrophenol (ONP) as a function of cytoplasmic β-galactosidase release, with bacteria grown in lactose containing medium. When *E. coli* cells were treated with OCNP (Fig. 2), the OCNP caused considerable release of the enzyme into the medium within 90 min. There was immediate release of cytoplasmic β-galactosidase followed by a progressive release for up to 70 min to reach a steady state, and then at a decreasing rate up to 90 min. In control suspensions, the release of β-galactosidase increased at a decreasing rate up to 120 min after a lag of about 40 min. As shown in Fig. 2, the release of cytoplasmic β-galactosidase caused by OCNP was concentration-dependent (after about 50 min), which was agreeable with the assay of integrity of bacterial cell membranes and OM permeabilization of *E. coli*. Other workers (Liu et al., 2004) have reported similar results using chitosan. Results indicated that OCNP rapidly increase the permeability of the IM.

3.5. SDS–PAGE

When *E. coli* and *S. aureus* were treated with OCNP, followed by analyses of protein contents in the cell-free supernatant, there was about 2.5-fold increase compared with control culture supernatants (data not shown), further suggested the permeabilizing action of nanoparticles. To confirm this, both *E. coli* and *S. aureus* were analyzed by SDS–PAGE for their soluble proteins. Protein electrophoresis bands of OCNP-treated *E. coli* and *S. aureus* were significantly different from that of normal bacteria. As shown in Fig. 3, bands of all-molecular-weight proteins were obviously shallow in the test group (Fig. 3A and B, lanes 2–4). The content of protein reduced even more obviously as the incubation time extended. In the case of *S. aureus*, the protein contents reduced markedly even disappeared after 120 min (Fig. 3B, lane 2). Considering the result that protein contents in the cell-free supernatant increased, it was suggested that OCNP decreased the content of cellular soluble proteins by permeating and disrupting cell membranes.

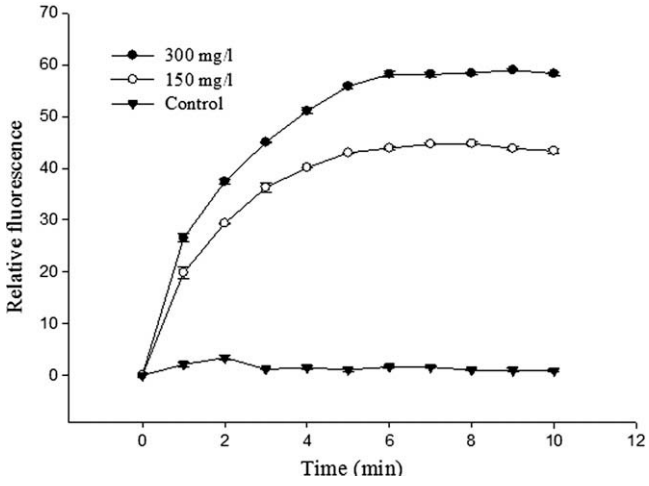

Fig. 1. The uptake of 1-*N*-phenylnaphthylamine (measured as fluorescence intensity in arbitrary units) by *E. coli* with addition of 300 mg/l (●), 150 mg/l (○) of OCNP and pH 6.0 acetic acid solution (▼).

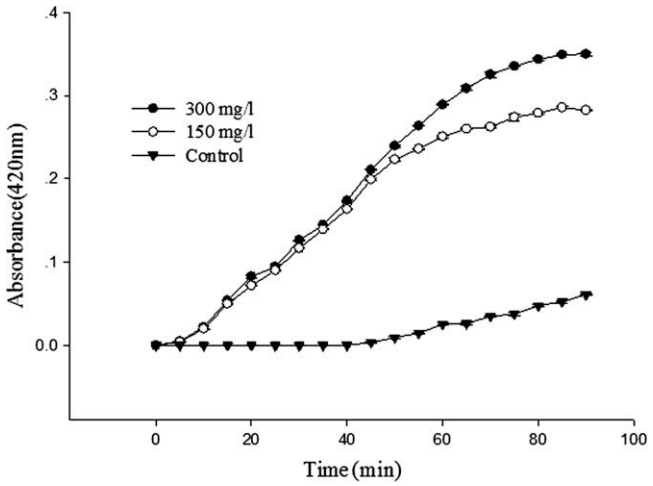

Fig. 2. Release of cytoplasmic β-galactosidase activity (measured by absorbance at 420 nm) of *E. coli* cells treated with 300 mg/l (●), 150 mg/l (○) of OCNP and pH 6.0 acetic acid solution (▼).

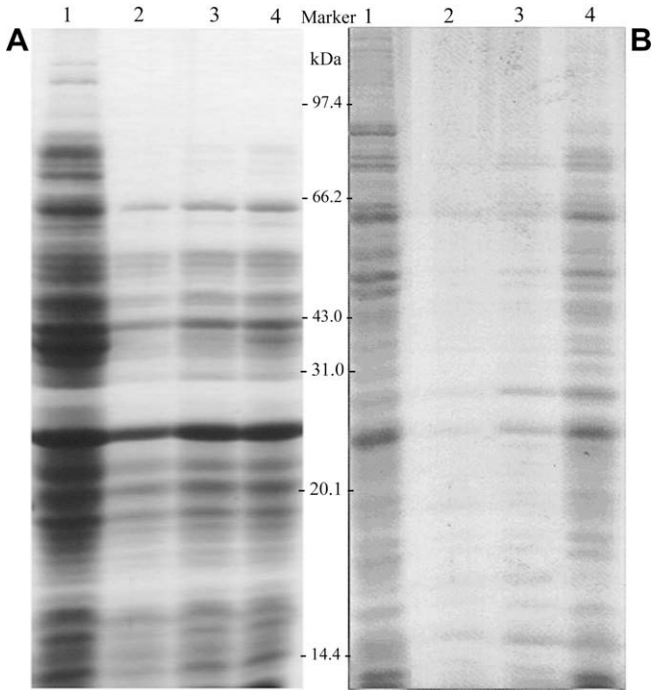

Fig. 3. (A) SDS–PAGE of *E. coli* cells treated with 300 mg/l OCNP. Lane 1: untreated *E. coli*; lane 2: OCNP-treated *E. coli* for 120 min; lane 3: OCNP-treated *E. coli* for 60 min; lane 4: OCNP-treated *E. coli* for 30 min. (B) SDS–PAGE of *S. aureus* cells treated with 300 mg/l OCNP. Lane 1: untreated *S. aureus*; lane 2: OCNP-treated *S. aureus* for 120 min; lane 3: OCNP-treated *S. aureus* for 60 min; lane 4: OCNP-treated *S. aureus* for 30 min.

3.6. Transmission electron microscopy (TEM)

To search for clues to possible alternative mechanisms of action of OCNP on *E. coli* and *S. aureus*, TEM was performed on bacteria that had been treated with OCNP for up to 30 min. The electron micrographs showed a remarkable modification of bacterial cell shape after a period of exposure to 300 mg/l OCNP. Although large numbers of cells were examined, only representative cells have been presented in Figs. 4 and 5.

For *E. coli and S. aureus*, untreated bacteria displayed a smooth and compact surface, without release of intracellular components and notable ruptures or pores on the cell surface (Figs. 4A and 5A). In contrast, OCNP-exposed bacteria exhibited a wide range of significant abnormalities. When bacterial suspensions were exposed to OCNP after 5 min, there were some OCNP with intact spherical structure adhering to the surface of *E. coli*. A little different from that of *E. coli*, the adhering of OCNP to *S. aureus* was fewer in amount and occurring rarely. The arrows pointed to spherical OCNP attached to the cell surface (Figs. 4B and 5B). Deep roughening and collapse of the cell surface was found after 15 min. There was still some OCNP attached to the cell surface, but the shape of nanoparticles was irregular. As the arrows pointed to, some nanoparticles were even blurry in the reaction process (Figs. 4C and 5C). This phenomenon would be attributed to two reasons. One was the characteristic of chitosan and the pH of bacterial cultures. Chitosan is only soluble in acid solution but bacterial cultures were alkaline. Therefore, the precipitation occurred upon addition of chitosan solution to the culture medium. The other was due to the electrostatic reaction of OCNP and bacteria. Both reasons might affect the stability of nanoparticles. When bacterial suspensions were exposed to OCNP after 30 min (Figs. 4D and 5D), a ghost-like appearance was observed in which the *E. coli* cells seemed transparent and looking empty. Besides, there was serious damage on the bacterial walls and an apparent hole at one of the

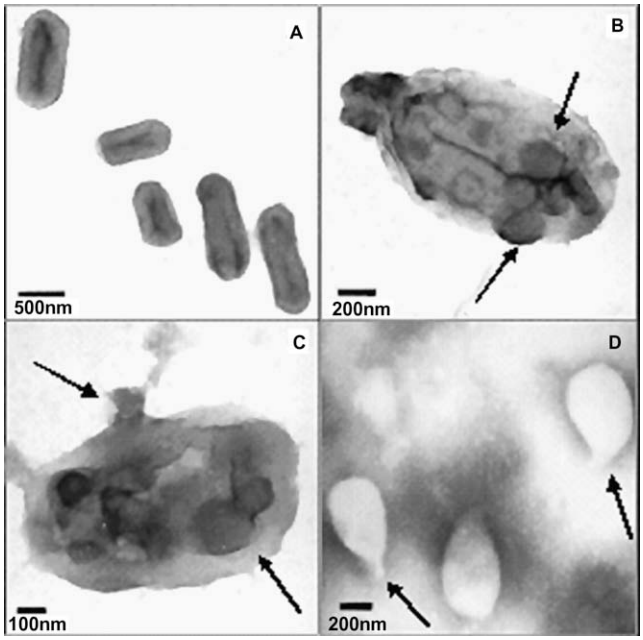

Fig. 4. TEM of *E. coli* cells treated with 300 mg/l OCNP for up to 30 min. (A) untreated *E. coli*; (B) OCNP-treated *E. coli* for 5 min; (C) OCNP-treated *E. coli* for 15 min; (D) OCNP-treated *E. coli* for 30 min.

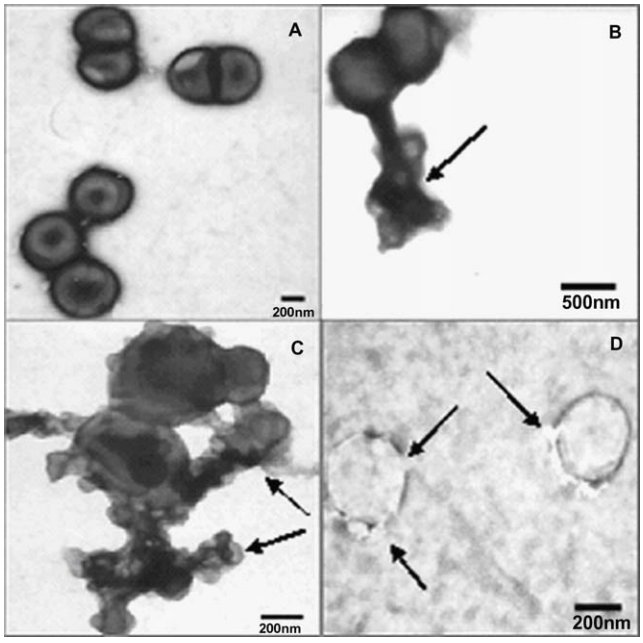

Fig. 5. TEM of *S. aureus* cells treated with 300 mg/l OCNP for up to 30 min. (A) untreated *S. aureus*; (B) OCNP-treated *S. aureus* for 5 min; (C) OCNP-treated *S. aureus* for 15 min; (D) OCNP-treated *S. aureus* for 30 min.

cell poles with loss of cell contents. For *S. aureus*, the cells seemed to have condensed and lost cytoplasmic materials, although the overall cell shape was still recognizable. Different from that of *E. coli*, OCNP could form more than one pore on membranes of some *S. aureus* cells. Lysed bacteria, surrounded by dark floccules instead of spherical OCNP, were found at this moment. This phenomenon would be attributed to reasons described above.

Electron microscopy observations are powerful tools for researchers to better understand the impact of a stressor such as chitosan on bacterial cells (Helander et al., 2001; Liu et al., 2004).

They reported that chitosan-treated bacteria exhibited extensive cell surface alterations. However, chitosan solution cannot be directly observed in electron micrographs, which makes it difficult to investigate the mode of action of chitosan on bacteria. This study employed OCNP, as a novel dispersion system, combined with microscopical visualization of the antibacterial interaction to explore the effect of chitosan-based nanoparticles on bacteria. In electron micrographs, OCNP with intact spherical structure adhered to the surface of *E. coli* and *S. aureus*. These micrographs showed that OCNP, as a novel dispersion system, had advantages over chitosan solution to confirm the binding and interacting with the bacterial surface.

Our data indicated that binding of OCNP to the highly negatively charged cell surface polymers was the first step. Then the interfacial contacting inhibitory effect might occur on the cell surface. This was followed by the pore formation or membrane disintegration. However, on the basis of our findings and the supporting literature (Raafat, von Bargen, Haas, & Sahl, 2008), we believe that the mode of action of OCNP is probably more complex than assumed above, involving a series of events, that may ultimately lead to a killing process. Our future work should aim at clarifying the actual target molecule on the cell surface or other intracellular targets.

4. Conclusions

In this study, we prepared OCNP as a novel antibacterial dispersion system. OCNP showed strong bactericidal activity against *E. coli* and *S. aureus*. When treated with OCNP, release of intracellular components quickly increased via destroying the integrity of bacterial cell membranes for both *E. coli* and *S. aureus*. OCNP also rapidly increased NPN uptake and the release of cytoplasmic β-galactosidase via increasing the permeability of OM and IM. SDS–PAGE indicated the content of cellular soluble proteins decreased significantly in OCNP-treated bacteria. Electron microscopy clearly demonstrated OCNP adhered to the surface of *E. coli* and *S. aureus* and showed extensive cell surface alterations of OCNP-treated bacteria. OCNP, as a novel antibacterial dispersion system, still keeps the original bactericidal activity of chitosan and has the potential value in the determination of the exact antibacterial mechanism of chitosan.

Acknowledgements

This work was supported by Grants from NSFC (30770582), IST-CP (2006DFA33150) and the Ph.D. Programs Foundation of Ministry of Education of China (20070423013).

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