Carbohydrate Polymers 75 (2009) 385–389

Contents lists available at ScienceDirect

Carbohydrate Polymers

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

Antibacterial activity of chitosan tripolyphosphate nanoparticles loaded with various metal ions

Wen-Li Du [a], Shan-Shan Niu [b], Ying-Lei Xu [a], Zi-Rong Xu [a,*], Cheng-Li Fan [a]

[a] Institute of Feed Science, College of Animal Science, Zhejiang University, Key Laboratory of Molecular Animal Nutrition, Ministry of Education, Hangzhou 310029, China
[b] Laboratory of Fruit Molecular Physiology and Biotechnology, The State Agricultural Ministry Laboratory of Horticultural Plant Growth Development and Biotechnology, Department of Horticulture, Zhejiang University, Hangzhou 310029, China

ARTICLE INFO

Article history:
Received 19 January 2008
Received in revised form 26 July 2008
Accepted 31 July 2008
Available online 20 August 2008

Keywords:
Chitosan nanoparticles
Antibacterial activity
Particle size
Zeta potential

ABSTRACT

The aim of this study was to prepare and select chitosan nanoparticles loaded metal ions with high antibacterial activities. Chitosan nanoparticles were prepared based on ionic gelation between chitosan and sodium tripolyphosphate. Then, Ag^{+}, Cu^{2+}, Zn^{2+}, Mn^{2+}, or Fe^{2+} was individually loaded onto chitosan nanoparticles. Their particle sizes and zeta potentials were measured. Their antibacterial activities were evaluated by determination of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) against *Escherichia coli* 25922, *Salmonella choleraesuis* ATCC 50020 and *Staphylococcus aureus* 25923 *in vitro*. Results showed that antibacterial activity was significantly enhanced by the metal ions loaded, except for Fe^{2+}. Especially for chitosan nanoparticles loaded Cu^{2+}, the MIC and MBC against *E. coli* 25922, *S.choleraesuis* ATCC 50020 and *S. aureus* 25923 were 21–42 times lower than that of Cu^{2+}, respectively. Moreover, it was found that antibacterial activity was directly proportional to zeta potential.

1. Introduction

Chitosan has attracted considerable interest because of their unique combination of properties, such as biocompatibility, biodegradability, metal complexation and antibacterial activity. Therefore, chitosan has a variety of current and potential applications in various fields, for example, biotechnology (Mao et al., 2001), pharmaceutics (Illum, 1998), wastewater treatment (Ramnani & Sabharwal, 2006), cosmetics (Kumar, Muzzarelli, Muzzarelli, Sahiwa, & Domb, 2004), and food science (Chien, Seu, & Yang, 2007).

The antibacterial activity of chitosan has been widely explored (Hong, Park, Lee, & Meyers, 2002; Liu et al., 2006; Tsai, Zhang, & Shieh, 2004). A number of chitosan derivates with different modifications have been prepared to improve its antibacterial activity (Jia, Shen, & Xu, 2001; Liu, Du, Yang, & Zhu, 2004; Yang, Chou, & Li, 2005). The metal-chelating property of chitosan has been mainly used in wastewater treatment. Recently, different metal-chitosan complexes have been prepared to improve its antimicrobial activity, for example, chitosan–Ag^{+} complex (Chen, Wu, & Zeng, 2005; Yi, Wang, & Liu, 2003) and chitosan–Zn^{2+} complex (Wang, Du, & Liu, 2004). Cu^{2+} exhibits high antibacterial activity *in vitro* (Domek, LeChevallier, & McFeters, 1984). Various kinds of Cu^{2+} -organic complexes with remarkable antimicrobial activity have been synthesized (Carcelli, Mazza, Pelizzi, & Pelizzi, 1995; Jantova, Labuda, Vollek, & Zaskova, 1997).

Nanoparticles are made of natural or artificial polymers ranging in size from 10–1000 nm (Kreuter, 2001). Nanoparticles display unique physical and chemical features because of effects such as the quantum size effect, mini size effect, surface effect and macro-quantum tunnel effect. Chitosan tripolyphosphate nanoparticles have been synthesized and mainly used as drug carrier as reported in previous studies (De Campos, Sánchez, & Alonso, 2001; Janes, Fresneau, Marazuela, Fabra, & Alonso, 2001; Xu & Du, 2003).

In our previous study, chitosan nanoparticles had been prepared, characterized, and used to adsorb eosin Y from aqueous solutions (Du, Xu, Han, Xu, & Miao, 2008). Here, Ag^{+}, Cu^{2+}, Zn^{2+}, Mn^{2+} or Fe^{2+} was separately loaded onto chitosan nanoparticles. Then, particle size and zeta potential of the chitosan nanoparticle loaded with different metal ions were measured. In order to evaluate and compare their antibacterial activities, *Escherichia coli* 25922, *Salmonella choleraesuis* ATCC 50020 and *Staphylococcus aureus* 25923 were chosen as tested bacteria. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of the materials against these bacteria were determined *in vitro*.

2. Materials and methods

2.1. Materials

Chitosan, originated from shrimp shell, was purchased from Yuhuan Ocean Biochemical Co. (Zhejiang Yuhuan, China). The degree of deacetylation and the molecular weight were about 90%

* Corresponding author. Tel.: +86 571 86022925; fax: +86 571 86994963.
E-mail address: wldu2008@yahoo.com (Z.-R. Xu).

0144-8617/$ - see front matter © 2008 Published by Elsevier Ltd.
doi:10.1016/j.carbpol.2008.07.039

and 150 kDa as determined by elemental analysis and viscometric method, respectively. Sodium tripolyphosphate (TPP) and chlortetracycline were obtained from Sigma Chemical Co., USA. The water used throughout this work was the reagent-grade water produced by Milli-Q SP ultra-pure-water system of Nihon Millipore Ltd., Tokyo. The other chemicals used were analytical grade reagents commercially available and used without further purification.

2.2. Preparation of the nanoparticles

Chitosan nanoparticles were prepared based on the ionotropic gelation between chitosan and sodium tripolyphosphate. Briefly, chitosan was dissolved in 1% (v/v) acetic acid to obtain a 0.3% (w/v) chitosan solution. TPP was dissolved in water to a concentration of 1%. Under magnetic stirring at room temperature, 1 ml of tripolyphosphate solution was added into 25 ml of chitosan solution. The mixture was stirred for 20 min, then, treated with sonication at 1.5 kW for 30 min. The suspension was subsequently centrifuged at 12,000*g* for 10 min. The precipitate was suspended in water, centrifuged again, then freeze dried. The freeze-dried chitosan nanoparticles were suspended in water for characterization or directly used for other experiments. Chitosan nanoparticles loaded Ag^{+}, Cu^{2+}, Zn^{2+}, Mn^{2+}, and Fe^{2+} were obtained by adding metal ion solutions into the chitosan nanosuspensions (0.3%, w/v) to reach a final concentration of 120 μg/ml and stirring for 12 h at room temperature. Chitosan nanoparticles loaded with metal ions were further purified as described above.

2.3. Characterizations

Particle size and zeta potential were measured using a Zetasizer Nano-ZS-90 (Malvern Instruments). The analysis was performed at a scattering angle of 90° under 25 °C. For zeta potential measurements, samples were dispersed in 0.1 mM KCl and measured under the automatic mode. For chitosan nanoparticles loaded Ag^{+}, 0.1 mM KNO_3 was used to replace of KCl in order to avoid the reaction between Ag^{+} and Cl^{-}.

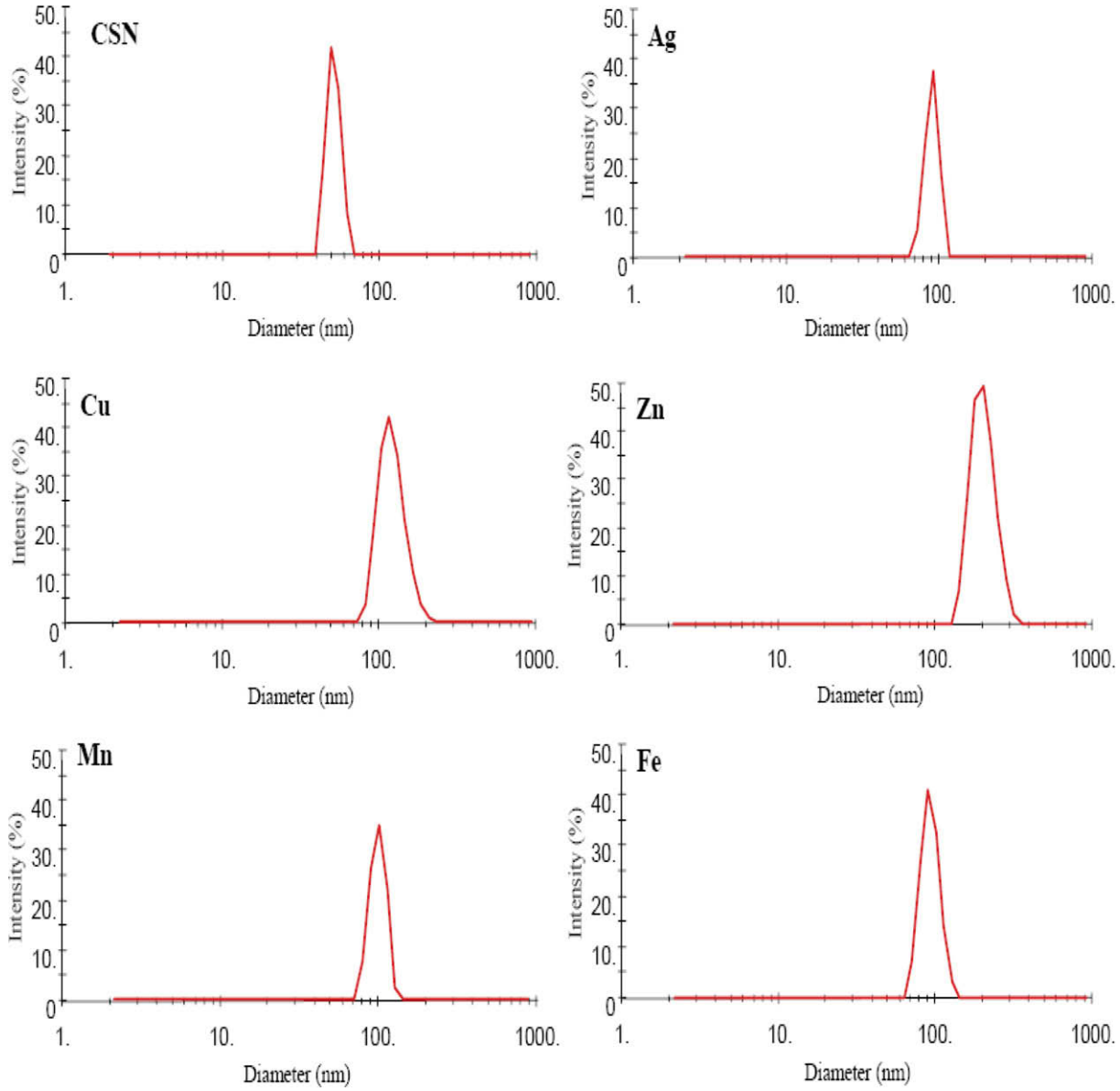

Fig. 1. Size distribution. (A) Chitosan nanoparticles; (B) chitosan–Ag nanoparticles; (C) chitosan–Cu nanoparticles; (D) chitosan–Zn nanoparticles; (E) chitosan–Mn nanoparticles; (F) chitosan–Fe nanoparticles.

2.4. Bacteria growth conditions

Escherichia coli 25922, *S.choleraesuis* ATCC 50020 and *S. aureus* 25923, provided by the Center for Typical Culture Collection of China, were used to evaluate the antibacterial activity. Muller–Hinton (MH) broth and MH agar (Difco, USA) were used as growth media.

Bacteria were incubated at 37 °C shaken in a thermostat. At the exponential phase, bacteria were harvested by centrifuge at 4000*g* for 10 min under 4 °C, then, washed twice with 10 mM phosphate buffer saline (PBS, pH 7.2). The bacteria were suspended in PBS and adjusted to $\sim 1 \times 10^7$ CFU/ml for further use.

2.5. Evaluation of antibacterial activity in vitro

The minimum inhibitory concentration was determined by a broth dilution method, recommended by the NCCLS (NCCLS, 2000). The chitosan, chitosan nanoparticles, chitosan nanoparticles loaded different metal ions, silver nitrate, copper sulfate, zinc sulfate, manganese sulfate, ferric sulfate and chlortetracycline were gradually diluted in MH broth. Chitosan was also diluted in MH broth, which contained 0.25% (v/v) acetic acid due to its insolubility. Bacteria were inoculated to achieve a bacterial concentration of 1∼2 × 105 CFU/ml. MIC was read after 24 h of incubation at 37 °C equivalent to the concentration of the tube without visible growth. To evaluate MBC, a sample of 100 μl was transferred from each tube without visible growth to a MH agar plate and incubated at 37 °C for another 24 h The MBC was read as the concentration of the tube without bacterial growth. The test was performed triplicate for each bacterium. Value that agreed on two or more occasions was adopted as the MIC or MBC of the strain.

3. Results and discussion

3.1. Particle size and zeta potential

Size (including size distribution) and zeta potential are essential characteristic parameters for nanosuspensions (Müller, Jacobs, & Kayser, 2001). Fig. 1 showed size distribution profiles of the chito-

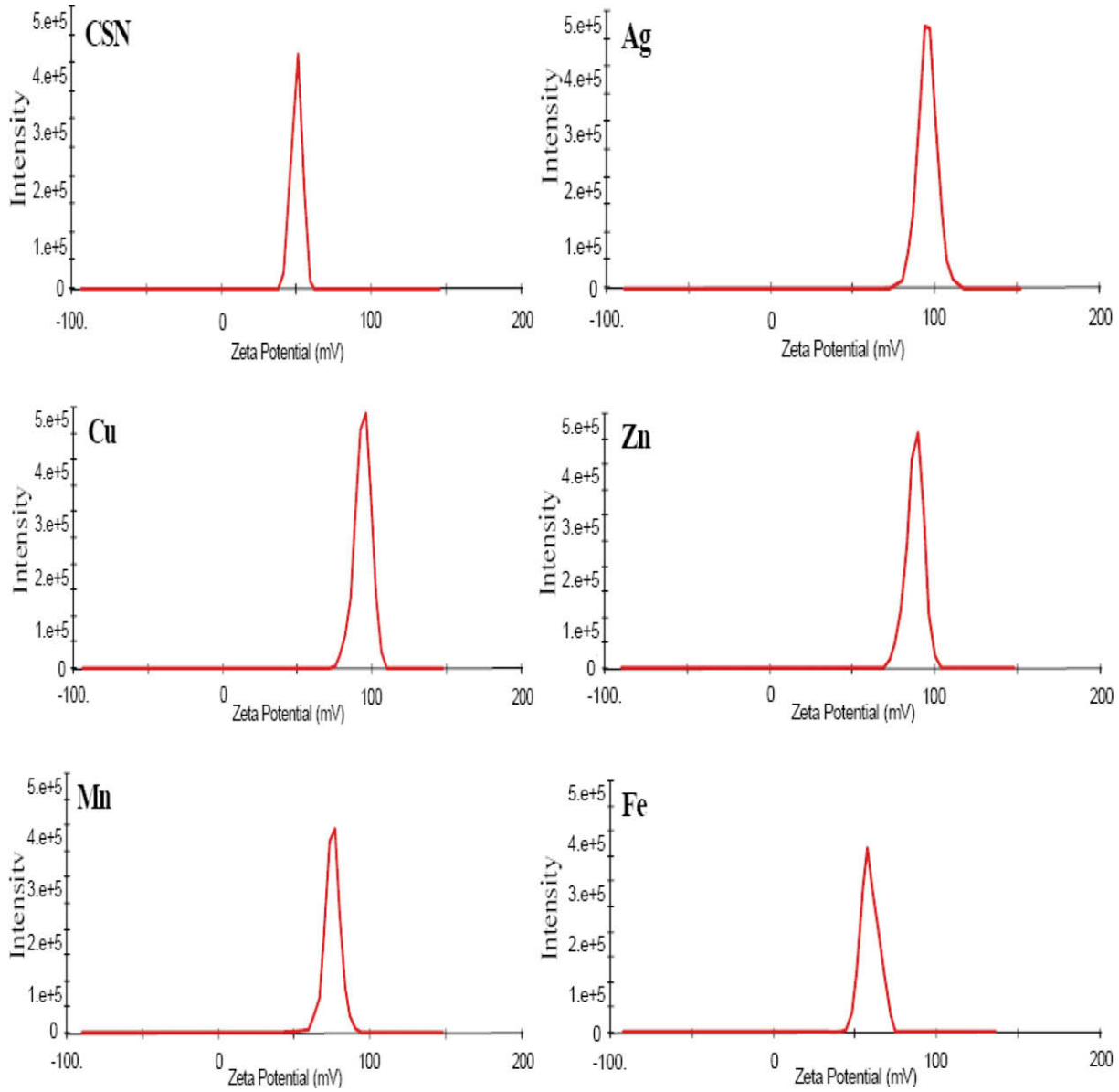

Fig. 2. Zeta potential distribution. (A) Chitosan nanoparticles; (B) chitosan–Ag nanoparticles; (C) chitosan–Cu nanoparticles; (D) chitosan–Zn nanoparticles; (E) chitosan–Mn nanoparticles; (F) chitosan–Fe nanoparticles.

san nanoparticles and nanoparticles loaded different metal ions. Chitosan nanoparticles had a mean diameter of 53.99 nm with a narrow size distribution (Width: 5.359 nm; Polydispersity index: 1.000) as shown in Fig. 1A. Both the mean diameter and the size distribution increased when metal ions were loaded (Fig. 1B–F). The mean diameters of chitosan nanoparticles, loaded Ag^{+}, Cu^{2+}, Zn^{2+}, Mn^{2+}, or Fe^{2+}, were 90.29, 121.9, 210.9, 102.3, and 95.81 nm, respectively.

As shown in Fig. 2, the chitosan nanoparticles had a zeta potential of +51.37 mV (Fig. 2A). The zeta potentials were enhanced significantly due to the loading of metal ions. The reason probably resulted from the positive charge carried by metal ions, which were loaded onto chitosan nanoparticles. The zeta potential of the nanoparticles loaded Ag^{+}, with a +92.05 mV, was the highest, following by Cu^{2+} loaded, which was +88.69 mV. The zeta potentials of nanoparticles loaded Zn^{2+} and Mn^{2+} were +86.65 and +75.74 mV, respectively. The nanoparticles loaded Fe^{2+} had the lowest zeta potential of +71.42 mV, but still higher than that of the chitosan nanoparticles. Zeta potential is a crucial parameter for stability in aqueous nanosuspensions. For a physically stable nanosuspension solely stabilized by electrostatic repulsion, a zeta potential of ±30 mV is required as a minimum (Müller et al., 2001). All these data suggested that chitosan nanoparticles and chitosan nanoparticles loaded metal ions prepared here were stable.

3.2. Antibacterial activity

The MIC and MBC of chitosan, chitosan nanoparticles, chitosan nanoparticles loaded different metal ions, silver nitrate, copper sulfate, zinc sulfate, manganese sulfate, ferric sulfate and chlortetracycline, also the MIC of chitoan dissolved in MH broth containing 0.25% (v/v) acetic acid, were determined and shown in Table 1. Except Fe^{2+} loaded, chitosan nanoparticles loaded metal ions showed better antibacterial activity than chitosan, chitosan nanoparticles and related metal ions. It was also noticed that chitosan nanoparticles loaded Ag^{+} exhibited the highest antibacterial activity with a MIC of 3 and 6 μg/ml against *E.coli* 25922 and *S.aureus*, respectively. While, the MIC and MBC of chitosan nanoparticles loaded Cu^{2+} against Gram-negative and Gram-positive bacteria tested here were 9, 21 and 12 μg/ml, 24 μg/ml, which was 21–42 times lower than that of Cu^{2+}, respectively.

According to previous studies (Jia et al., 2001; Shahidi, Arachchi, & Jeon, 1999; Yi et al., 2003) the antibacterial activity of chitosan under acidic environment may result from its polycationic structure due to the protonation of $-NH_2$ on the C-2 position of the D-glucosamine repeat unit. Positively charged chitosan can bind to bacterial cell surface which is negatively charged and disrupt the normal functions of the membrane, e.g. by promoting the leakage of intracellular components or by inhibiting the transport of nutrients into cells (Helander, Nurmiaho-Lassila, Ahvenainen, Rhoades, & Roller, 2001; Sudarshan, Hoover, & Knorr, 1992; Xue, Yang, Zhang, & He, 2006). It was clear that the order of antibacterial activity was the same as that of the zeta potential of chitosan nanoparticles loaded different metal ions. Thus, zeta potentials could be easily associated to the antibacterial activity. It could be seen that antibacterial activity of chitosan nanoparticles loaded metal ions was directly proportional to zeta potential. Moreover, results showed that Gram-negative bacteria were more sensitive to chitosan nanoparticles loaded metal ions. It was probably resulted from the different characteristics of the cell surfaces. It has been reported that the negative charge on the cell surface of Gram-negative bacteria was higher than on Gram-positive bacteria (Chung et al., 2004). Due to a higher negative charge on cell surface, the interaction between Gram-negative bacteria and nanoparticles was definitely stronger than that of Gram-positive bacteria.

Table 1
MIC and MBC values against *E. coli*, *S. choleraesuis*, and *S. aureus* (μg/ml)

Sample	*E. coli*		*S. choleraesuis*		*S. aureus*	
	MIC	MBC	MIC	MBC	MIC	MBC
Chitosan[a]	–[b]	–	–	–	–	–
Chitosan[c]	468	750	468	750	656	750
Chitosan nanoparticles	117	187	117	187	234	281
Chitosan nanoparticles loaded Ag^{+}	3	6	3	6	6	12
Chitosan nanoparticles loaded Cu^{2+}	9	12	9	12	21	24
Chitosan nanoparticles loaded Zn^{2+}	18	24	18	24	36	48
Chitosan nanoparticles loaded Mn^{2+}	73	97	73	97	85	97
Chitosan nanoparticles loaded Fe^{2+}	121	195	121	195	146	195
Ag^{+}	4	8	4	8	8	16
Cu^{2+}	256	512	256	512	448	512
Zn^{2+}	768	1024	768	1024	768	1024
Mn^{2+}	1472	1536	1472	1536	1600	1664
Fe^{2+}	1728	1856	1728	1856	1792	1856
Chlortetracycline	1	2	1	2	2	4

[a] Chitosan dispersed in MH broth.
[b] '–' means no antibacterial effect.
[c] Chitosan dissolved in MH broth containing 0.25% acetic acid (v/v).

Zhang, Jiang, Ding, Malcolm, and David (2007) reported that the antibacterial activity of ZnO nanoparticles increased with decreasing particle size. But, our data showed that the zeta potential influence antibacterial activity of the nanoparticles much more, while, particle size threw little effect on antibacterial activity, if any.

4. Conclusions

Generally, antibacterial activity was significantly enhanced by metal ion loaded, especially for Cu^{2+}, and Zn^{2+}, compared to those of chitosan nanopartilces and related metal ions. It was found that antibacterial activity was directly proportional to zeta potential. Moreover, Gram-negative bacteria were more sensitive than Gram-positive bacteria.

References

Carcelli, M., Mazza, P., Pelizzi, C., & Pelizzi, G. (1995). Antimicrobial and genotoxic activity of 2,6-diacetylpyridinebis (acylhydrazones) and their complexes with some first transition series metal ions. X-ray crystal structure of a dinuclear copper(II) complex. *Journal of Inorganic Biochemistry, 57*, 43–62.
Chen, S. P., Wu, G. Z., & Zeng, H. Y. (2005). Preparation of high antimicrobial activity thiourea chitosan–Ag^{+} complex. *Carbohydrate Polymer, 60*, 33–38.
Chien, P. J., Seu, F., & Yang, F. H. (2007). Effect of edible chitosan coating on quality and shelf life of sliced mango fruit. *Journal of Food Engineering, 78*, 225–229.
Chung, Y. C., Su, Y. P., Chen, C. C., Jia, G., Wang, H. L., Wu, J. C. G., et al. (2004). Relationship between antibacterial activity of chitosan and surface characteristics of cell wall. *Acta Pharmacologica Sinica, 25*, 932–936.
De Campos, A. M., Sánchez, A., & Alonso, M. J. (2001). Chitosan nanoparticles: A new vehicle for the improvement of the delivery of drugs to the ocular surface. Application to cyclosporine A. *International Journal of Pharmaceutics, 224*, 159–168.
Domek, M. J., LeChevallier, M. W., & McFeters, G. A. (1984). Evidence for the role of copper in the injury process of coliforms in drinking water. *Applied and Environmental Microbiology, 48*, 289–293.
Du, W. L., Xu, Z. R., Han, X. Y., Xu, Y. L., & Miao, Z. G. (2008). Preparation, characterization and adsorption properties of chitosan nanoparticles for eosin Y as a model anionic dye. *Journal of Hazardous Material, 153*, 152–156.
Helander, I. M., Nurmiaho-Lassila, E. L., Ahvenainen, R., Rhoades, J., & Roller, S. (2001). Chitosan disrupts the barrier properties of the outer membrane of Gram-negative bacteria. *International Journal of Food Microbiology, 71*, 235–244.
Hong, K. N., Park, N. Y., Lee, S. H., & Meyers, S. P. (2002). Antibacterial activity of chitosans and chitosan oligomers with different molecular weights. *International Journal of Food Microbiology, 74*, 65–72.
Illum, L. (1998). Chitosan and its use as a pharmaceutical excipient. *Pharmaceutical Research, 15*, 1326–1331.
Janes, K. A., Fresneau, M. P., Marazuela, A., Fabra, A., & Alonso, M. J. (2001). Chitosan nanoparticles as delivery systems for doxorubicin. *Journal of Controlled Release, 73*, 255–267.
Jantova, S., Labuda, J., Vollek, V., & Zaskova, M. (1997). Antimicrobial effects of the macrocyclic Cu(II)-tetraanhydroanimobenzaldehyde complex. *Folia. Microbiol. Prague., 42*, 324–326.

Jia, Z. S., Shen, D. F., & Xu, W. L. (2001). Synthesis and antibacterial activities of quaternary ammonium salt of chitosan. *Carbohydrate Research, 333*, 1–6.

Kreuter, J. (2001). Nanoparticulate systems for brain delivery of drugs. *Advanced Drug Delivery Reviews, 47*, 65–81.

Kumar, M. N. V. R., Muzzarelli, R. A. A., Muzzarelli, C., Sahiwa, H., & Domb, A. J. (2004). Chitosan chemistry and pharmaceutical perspectives. *Chemical Reviews, 104*, 6017–6084.

Liu, N., Chen, X. G., Park, H. J., Liu, C. G., Liu, C. S., Meng, X. H., et al. (2006). Effect of MW and concentration of chitosan on antibacterial activity of *Escherichia coli*. *Carbohydrate Polymer, 64*, 60–65.

Liu, H., Du, Y. M., Yang, J. H., & Zhu, H. Y. (2004). Structural characterization and antimicrobial activity of chitosan/betaine derivative complex. *Carbohydrate Polymer, 55*, 291–297.

Mao, H. Q., Roy, K., Troung-Le, V. L., Janes, K. A., Lin, K. Y., Wang, Y., et al. (2001). Chitosan-DNA nanoparticles as gene carriers: synthesis, characterization and transfection efficiency. *Journal of Control Release, 70*, 399–421.

Müller, R. H., Jacobs, C., & Kayser, O. (2001). Nanosuspensions as particulate drug formulations in therapy rationale for development and what we can expect for the future. *Advanced Drug Delivery Reviews, 47*, 3–19.

NCCLS, 2000. methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically: Approved standard, 5th ed., NCCLS document M7-A5(ISBN 1-56238-394-9). NCCLS, Pennsylvania, USA.

Ramnani, S. P., & Sabharwal, S. (2006). Adsroption behavior of Cr(VI) onto radiation crosslinked chitosan and its possible application for the treatment of wastewater containing Cr(VI). *Reactive and Functional Polymers, 66*, 902–909.

Shahidi, F., Arachchi, J. K. V., & Jeon, Y. J. (1999). Food applications of chitin and chitosan. *Trends in Food Science and Technology, 10*, 37–51.

Sudarshan, N. R., Hoover, D. G., & Knorr, D. (1992). Antibacterial action of chitosan. *Food Biotechnology, 6*, 257–272.

Tsai, G. J., Zhang, S. L., & Shieh, P. L. (2004). Antimicrobial activity of a low-molecular weight chitosan obtained from cellulase digestion of chitosan. *Journal of Food Protection, 67*, 396–398.

Wang, X. H., Du, Y. M., & Liu, H. (2004). Preparation, characterization and antimicrobial activity of chitosan–Zn complex. *Carbohydrate Polymer, 56*, 21–26.

Xu, Y., & Du, Y. (2003). Effect of molecular structure of chitosan on protein delivery properties of chitosan nanoparticles. *International Journal of Pharmaceutics, 250*, 215–226.

Xue, Z. X., Yang, G. P., Zhang, Z. P., & He, B. L. (2006). Application of chitosan microspheres as carriers of LH-RH anologue TX46. *Reactive and Functional Polymer, 66*, 893–901.

Yang, T. C., Chou, C. C., & Li, C. F. (2005). Antibacterial activity of N-alkylated disaccharide chitosan derivatves. *International Journal of Food Microbiology, 97*, 237–245.

Yi, Y., Wang, Y. T., & Liu, H. (2003). Preparation of new crosslinked chitosan with crown ether and their adsorption for silver ion for antibacterial activities. *Carbohydrate Polymer, 53*, 425–430.

Zhang, L., Jiang, Y., Ding, Y., Malcolm, P., & David, Y. (2007). Investigation into the antibacterial behavior of suspensions of ZnO nanoparticles (ZnO nanofluids). *Journal of Nanoparticle Research, 9*, 479–489.