

Antibacterial activity of chitosan tripolyphosphate nanoparticles loaded with various metal ions

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ABSTRACT

The aim of this study was to prepare and select chitosan nanoparticles loaded metal ions with high anti-

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 12,000g 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 $\mu\text{g}/\text{ml}$ 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^- .

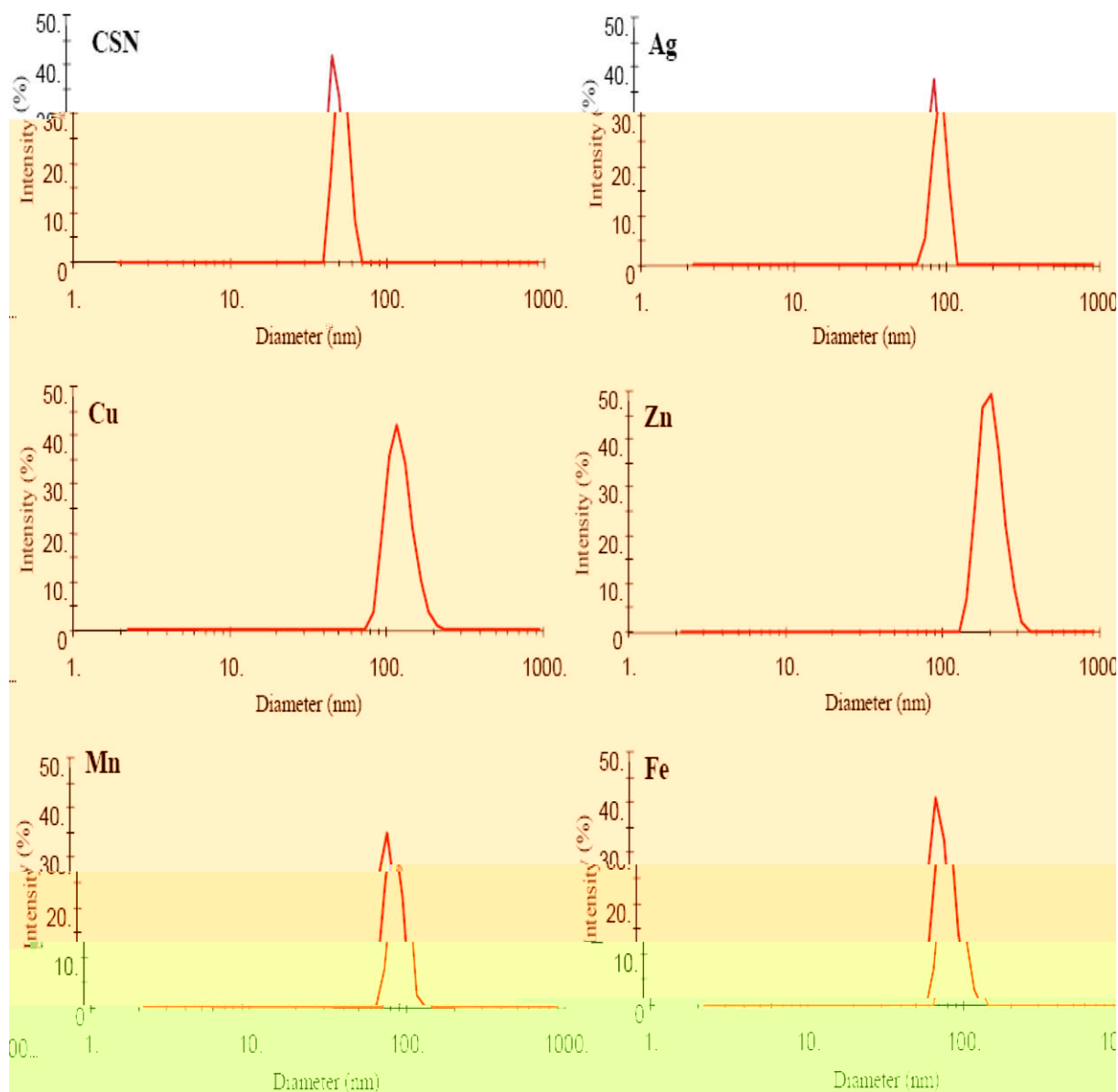


Fig. 1. Size distribution. (A) Chitosan nanoparticles; (B) chitosan–Ag nanoparticles; (C) chitosan–Cu nanoparticles; (D) chitosan–Zn nanoparticles; (E) chitosan–Mn nanoparticles; (F) chitosan–Fe nanoparticles.

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 4000g 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 $1\sim 2 \times 10^5$ 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 μ l 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-

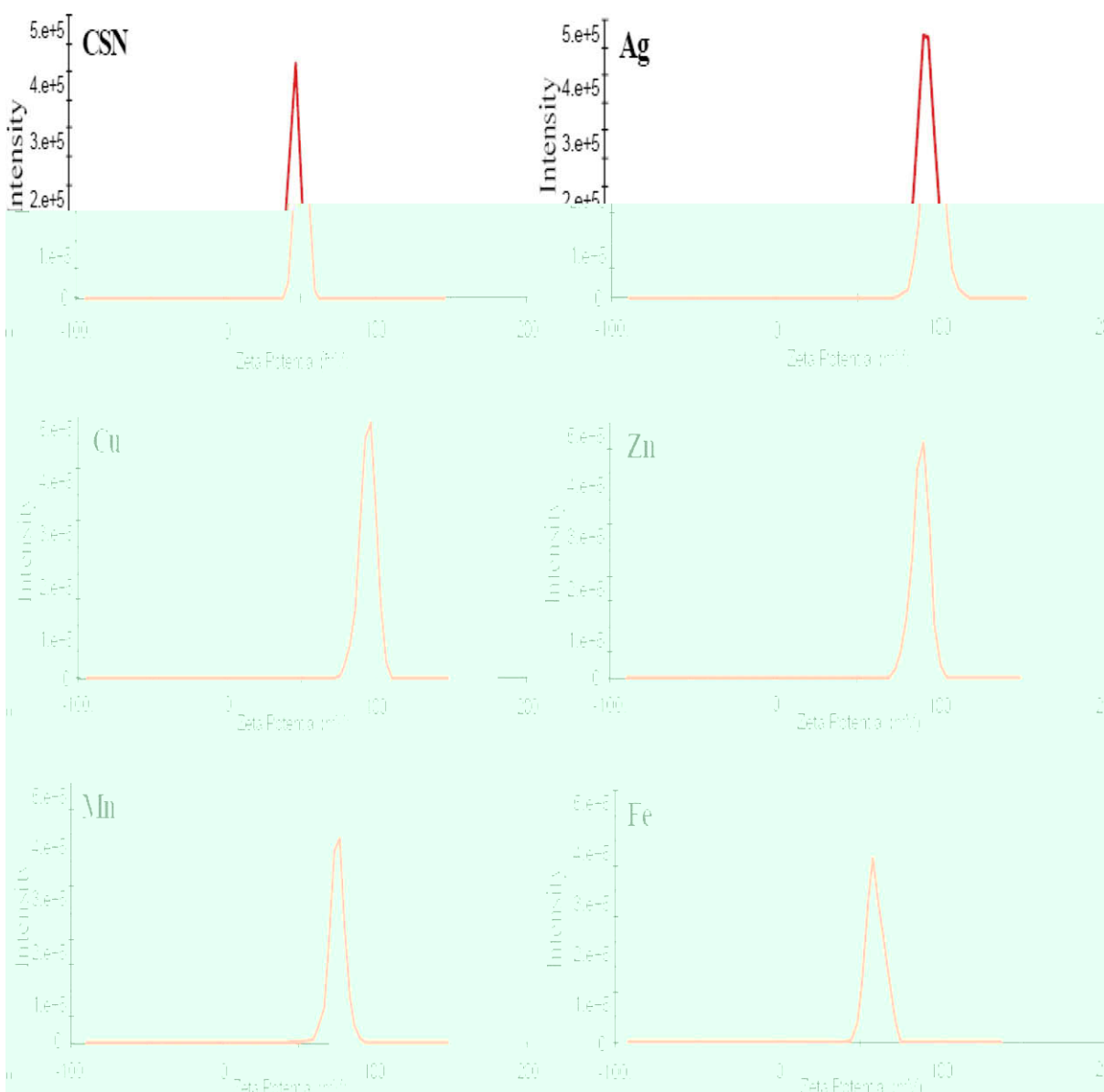


Fig. 2. Zeta potential distribution. (A) Chitosan nanoparticles; (B) chitosan–Ag nanoparticles; (C) chitosan–Cu nanoparticles; (D) chitosan–Zn nanoparticles; (E) chitosan–Mn nanoparticles; (F) chitosan–Fe nanoparticles.

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⁺, Cu²⁺, Zn²⁺, Mn²⁺, or Fe²⁺, 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 Cu²⁺ loaded, which was +88.69 mV. The zeta potentials of nanoparticles loaded Zn²⁺ and Mn²⁺ were +86.65 and +75.74 mV, respectively. The nanoparticles loaded Fe²⁺ 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 chitosan dissolved in MH broth containing 0.25% (v/v) acetic acid, were determined and shown in Table 1. Except Fe²⁺ 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 Cu²⁺ 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 Cu²⁺, 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 –NH₂ 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	<i>E. coli</i>		<i>S. choleraesuis</i>		<i>S. aureus</i>	
	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 Cu²⁺, and Zn²⁺, compared to those of chitosan nanoparticles 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.

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