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Research Article

Antimicrobial activity of Fe-loaded chitosan nanoparticles

To increase the antimicrobial activities of chitosan, chitosan nanoparticles loaded with Fe^{2+} or Fe^{3+} were prepared by surfactant-assisted chitosan chelating Fe^{2+} , Fe^{3+} and ionic gelation chitosan. Their chelating rates were determined by spectrophotometry. The particle sizes and zeta potentials of chitosan nanoparticles loaded with Fe^{2+} or Fe^{3+} were measured by size and zeta potential analysis. The nanoparticles antimicrobial activities were evaluated by different concentration against *Escherichia coli*, *Staphylococcus aureus*, *Candida albicans* in vitro. Results showed that the mean diameter of chitosan nanoparticles loaded with Fe^{2+} or Fe^{3+} were 206.4 and 195.2 nm, respectively. Their zeta potentials were +28.82 and +28.26 mV, respectively. The chelating rate of chitosan nanoparticles loaded with Fe^{2+} was greatly higher than with Fe^{3+} . Their antimicrobial activity was showed greatly higher at lower concentrations compared to chitosan, and the antibacterial effect of chitosan nanoparticles loaded with Fe^{2+} or Fe^{3+} was preliminary observed.

Keywords: Antimicrobial activity / Characterization methods / Chitosan / Iron / Nanoparticles

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1 Introduction

Chitosan [β -(1 $\rightarrow$ 4)-2-amino-2-deoxy-d-glucose] is a unique cationic polysaccharide and is generally represented as a homopolymer. Chitosan is the second most plentiful natural biopolymer and is relatively cheap [1]. It has attracted considerable interest due to its biological properties, such as antibacterial activity [2–5], antitumor activity [6,7], immune enhancing effect [8], and good edible security. Chitosan is a cationic polysaccharide (pK_a 6.5) [9]. Therefore, chitosan is only soluble in acidic media like acetic acid, which exhibits its antimicrobial activity on bacteria and epiphyte. Besides this, the antimicrobial activity of chitosan is influenced by a number of factors, including the species of bacteria, concentration, pH, solvent, molecular weight [10] etc.

There have been many reports on the antibacterial activity of chitosan nanoparticles [11,12]. Recently, chitosan nanoparticles loaded with various metal ions have been prepared to improve its antibacterial activity, for example, chitosan nanoparticles loaded Ag^+ [13–16] and chitosan nanoparticles loaded Cu^{2+} [17,18]. However, both Ag^+ and Cu^{2+} belong to the heavy metal ions; they will affect the health of human easily. Moreover, Ag-loaded chitosan nanoparticles suffer from a change in color as result of

the reaction between silver ions and oxygen or sulfur, which may seriously limited their application.

To increase the antimicrobial activities of chitosan, in this study, a using Tween-80 surfactant-assisted ionic gelation method was investigated for synthesizing uniformly distributed chitosan nanoparticles loaded with Fe^{2+} or Fe^{3+} . Their particle sizes and zeta potentials were measured by size and zeta potential analysis. Their chelating rates were determined by Spectrophotometry. Their antimicrobial activities were evaluated by different concentration against *Escherichia coli*, *Staphylococcus aureus*, *Candida albicans* in vitro.

2 Materials and methods

2.1 Materials

Chitosan was provided by Jinke Biochemical Co. Ltd (Zhejiang, China), the molecular weight and deacetylation degree were 13.76 kd and 91.02%, respectively. Sodium tripolyphosphate (TPP) is abbreviated from sodium polyphosphate was purchased from Dongsheng Chemical Reagent Factory (Zhejiang, China). Tween-80 from Longyou Chemical Reagent Factory (Zhejiang, China). And ferrous sulfate, ferric sulfate were analytically pure. All the other chemicals were analytical grade and no further purification was required. Nutrient agar and Sabouraud's agar were both obtained from Hangzhou Microbial reagents Co. Ltd.

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Escherichia coli, *Staphylococcus aureus*, *Candida albicans* were provided by the Biotechnology Research Institute of Zhejiang University of Technology, and stored at 4°C.

2.2 Preparation of nanoparticles

Chitosan was dissolved in acetic acid (1%, v/v) obtaining a 2.03 mg/mL (w/v) chitosan solution. Sodium polyphosphate was dissolved in deionized water to a concentration of 1.12 mg/mL. Tween 80 (0.02%, w/v) was precisely weighed before being mixed with chitosan solution and then raised to pH 4.6 with sodium hydroxide. Fe^{2+} and Fe^{3+} were added dropwise to chitosan solution respectively to reach final concentration of 240 $\mu\text{g/mL}$ to form metal ions-chelating chitosan. Then 50 mL of TPP solution was added dropwise to 150 mL of mixture under stirring for 8 min at room temperature. The chitosan nanoparticles loaded with Fe^{2+} , Fe^{3+} were precipitated by centrifugation (12 000 rpm for 30 min) at 4°C, and then washed twice with deionized water. The precipitate was suspended in deionized water for future analysis.

2.3 Particle characterization

Nanoparticles size and zeta potential were determined with a Beckman Coulter DelsaNano C particle size and zeta potential analyzer. For this, a sample of 5 mL was placed in the analyzer chamber with colorimetric cell and measured immediately.

2.4 Determination of chelating rate between metal ions and chitosan

The chelating rate between metal ions and chitosan was determined by Spectrophotometry. Briefly, phenanthroline (0.2%, w/v) was precisely weighed and dissolved in deionized water. Phenanthroline was added to samples and raised to pH 4.0–6.0 staying for 30 min, then the absorbance value of Fe^{2+} was determined by Spectrophotometry at 510 nm and Fe^{3+} was at 360 nm. Relationships between the concentration of iron ion and the corresponding OD value show the standard curve equation $y = 0.0941x - 0.0008(\text{Fe}^{2+})$ and $y = 0.0096x + 0.0091(\text{Fe}^{3+})$. The results show that the fitting curve of the linear correlation coefficient are 0.9998(Fe^{2+}) and 0.9974(Fe^{3+}) separately, which means the linear equations are reliable. The concentration of Fe^{2+} and Fe^{3+} were calculated according to the standard curve. Then the chelating rate can be calculated by the following equation:

$$\text{Chelating rate} = [C_0 - C_1] / C_0 \times 100\%$$

where C_0 is the initial concentration of Fe^{2+} (or Fe^{3+}) and C_1 is the residual concentration of dissociative Fe^{2+} (or Fe^{3+}).

2.5 Evaluation of antimicrobial activity in vitro

We use nutrient agar which containing 0.5% beef extract, 1% peptone, 0.5% NaCl (pH 7.5) as bacteria plate medium, and Sabouraud's agar which containing 1% peptone, 4% glucose as

fungi plate medium. They were all autoclaved for 20 min at 121°C.

Bacterial and fungal activated by transfer twice via the culture medium, a loopful from each of those tubes are inoculated in the broth. The seed culture was grown in a 250 mL shake flask containing 50 mL of liquid medium and incubated at 30°C on a rotary shaker (200 rpm) for 18 h. Bacteria and epiphyte containing 8.96×10^6 – 1.24×10^7 CFU/mL were prepared and used for the antimicrobial activity test. The concentration of the Chitosan [contained 1% (v/v) acetic acid], chitosan nanoparticles loaded with Fe^{2+} or Fe^{3+} (pH was controlled on 6.0, all autoclaved for 20 min at 121°C), ferrous sulfate, ferric sulfate were range from 3 to 12 $\mu\text{g/mL}$. The seed cultures with drug sample were serially diluted (1:10) in sterile deionized water for bacteriological analysis. Samples (0.1 mL) of the serial dilutions were spread on the surface of the appropriate dry medium in petri dishes to determine the total viable count (TVC) on Plate Count Agar and incubated at 30°C for 48 h. The results of the antimicrobial activity were read after 24 h of incubation at 30°C. The test was performed triplicate for each sample.

Then the sterilization rate can be calculated by the following equation:

$$\text{sterilization rate} = (N_0 - N/N_0) \times 100\%$$

where N_0 is the viable count of untreated bacteria liquid and N is the viable count of treated bacteria liquid. Each sterilization test made three parallel, and the results averaged.

2.6 Minimum inhibitory concentration testing

The turbidometric and micro-dilution methods were applied to examine MIC [19]. 0.5 mL different concentrations of each sample were added to 5 mL of liquid medium. A 50 μL aliquot of newly prepared bacterial suspension (10^5 – 10^6 cfu/mL) was added into each tube. The tubes were incubated at 37°C with shaking at 180 rpm for 24 h in a constant-temperature incubator. The minimum inhibitory concentration (MIC) was read by the equivalent to the concentration of the tube without visible growth with naked eye or microscope. The sample was used as prepared and tested at final concentrations of 0.001, 0.005, 0.01, 0.02, 0.04, 0.06, 0.08 and 0.1%.

3 Results

3.1 Determination of chelating rate between metal ions and chitosan

The chelating rate between metal ions and chitosan was determined by ultraviolet-visible spectrophotometry. As shown in Fig. 1, the chelating rate of chitosan- Fe^{2+} was greatly higher than that of chitosan- Fe^{3+} . When the concentration of Fe^{2+} achieved 240 $\mu\text{g/mL}$, the chelating rate, which reached a maximum, between Fe^{2+} and chitosan was 99.45%. While the chelating rate between Fe^{3+} and chitosan was 49.44% at that concentration. Both of the chelating rate decreased with further increase to 360 $\mu\text{g/mL}$. Anyhow, the chelation between Fe^{2+} and chitosan was much easier, compared to Fe^{3+} and chitosan.

Figure 1. Determination of chelating rate between metal ions and chitosan.

Figure 2. Size distribution by intensity of chitosan nanoparticles loaded with (A) Fe²⁺ and (B) Fe³⁺.

3.2 Particle characterization

Nanoparticles size and zeta potential were determined with a Delsa Nano C (Beckman Coulter). The resulting mean diameter of chitosan nanoparticles loaded with Fe²⁺, Fe³⁺ were 206.4 and 195.2 nm, respectively. Their zeta potentials were +28.82 and +28.26 mV, respectively. The size distribution by intensity was shown in Fig. 2.

3.3 Antimicrobial activity

The effect of concentration on the antimicrobial activity of samples against (A) *Escherichia coli*, (B) *Staphylococcus aureus* and (C) *Candida albicans* was shown in Fig. 3. The antimicrobial ac-

Figure 3. Effect of concentration on the antimicrobial activity of samples against (A) *Escherichia coli*, (B) *Staphylococcus aureus* and (C) *Candida albicans* under pH 6.0.

tivity increased as the concentrations of sample ranged from 3.0 to 12.0 µg/mL, except for Fe²⁺ and Fe³⁺. Fe²⁺, Fe³⁺ were almost no effective on microorganisms. Chitosan nanoparticles and chitosan nanoparticles loaded with Fe²⁺, Fe³⁺ showed significantly higher antimicrobial activity than chitosan and related metal ions. Chitosan nanoparticles loaded with Fe²⁺ showed higher antimicrobial activity than chitosan nanoparticles loaded with Fe³⁺, while their zeta potentials were similar to each other.

Table 1. Effect of concentration on the antimicrobial activity of samples toward *Escherichia coli*, *Staphylococcus aureus* and *Candida albicans*

Sample	Minimum inhibition concentrations ($\mu\text{g}/\text{mL}^{-1}$)		
	<i>Escherichia coli</i>	<i>Staphylococcus aureus</i>	<i>Candida albicans</i>
Chitosan- Fe^{2+} nanoparticle	20	10	40

Therefore, their antimicrobial activity exhibited much higher than chitosan may due to their quantum size effect, small size effect and large surface area.

It is worth mentioning that particle size and potentiometric analyzer shows that the zeta potential of chitosan is $+45.88$ mV, the zeta potential of chitosan nanoparticles loaded with Fe^{2+} and chitosan nanoparticles loaded with Fe^{3+} are $+28.82$, $+28.26$ mV, respectively. According the above data as shown in Fig. 3, the zeta potential of chitosan is the highest, but its bactericidal performance is the poorest.

Chitosan has strong affinity towards metal ions because of the presence of numerous amine and hydroxyl groups [20, 21], thus it can chelate with many metal ions. Fe-loaded chitosan nanoparticles show slight lower antibacterial activity than the Ag-loaded chitosan nanoparticles [22]. The sensitivities of *Escherichia coli*, *Staphylococcus aureus* and *Candida albicans* to chitosan load Fe^{2+} nanoparticle determined by the MIC values, and the results are presented in Table 1. The MIC values of chitosan load Fe^{2+} nanoparticle against *Escherichia coli*, *Staphylococcus aureus* were 10 and 20 mg/mL, respectively. Chitosan generally showed stronger bactericidal effects for gram-positive bacteria than gram-negative bacteria. The minimum inhibitory concentration (MIC) of chitosan also differed with individual bacteria and molecular weights of chitosan. Differences in MIC values obtained by other workers are probably due to differences in experimental materials and conditions such as methods used, characteristics of chitosan applied, or the pH of the medium.

4 Discussion

We can observe the $12 \mu\text{g}/\text{mL}$ Fe^{2+} -loaded chitosan nanoparticle and chitosan treated cells of *Candida albicans* through optical microscopy. The effect of different treating time on cellular morphology was shown in Fig. 4.

As the phenomenon in Fig. 4 combined with the experimental results in Fig. 3, we can considered the basic sterilized function of chitosan nanoparticles load iron is to destroy the bacterial cell wall. Because of the large surface area of the Fe-loaded chitosan nanoparticles, nanoparticles could be tightly adsorbed onto the surface of bacteria cells so as to disrupt the membrane, which lead to the leakage of intracellular components, thus killing the bacteria cells. Moreover, the outer membrane of Gram negative bacteria such as *Escherichia coli* consists of lipopolysaccharides (LPS) containing phosphate and pyrophosphate groups which render the cell surface negatively charged [23]. As the Fe-loaded chitosan nanoparticle is a cationic polymer, it can attach to the *E. coli* cell wall by electrostatic interaction. Gram-negative bacteria is more sensitive than Gram-positive bacteria and epiphyte, that's why the Fe-loaded chitosan nanoparticles exhibits higher antibacterial activity against Gram-negative bacteria than

Figure 4. Treated cells of *Candida albicans* under optical microscopy with (A) Fe^{2+} -loaded chitosan nanoparticle and (B) chitosan.

Gram-positive bacteria, and both are higher than the antibacterial activity against epiphyte.

To sum up, chitosan loaded metal particulates possess high bactericidal performance. It may benefit from the natural bactericidal functional components of chitosan. Beyond that, the particles have some unique factors such as small size effect, quantum size effect, large surface area.

The nanoparticles obtained in the present study have small particle size and positive surface charges, which may improve their stability in the presence of biological cations and improve for their antibacterial activities due to the interaction with

negatively charged biological membranes and site-specific targeting in vivo [24]. These studies show that chitosan metal-loaded nanoparticles could inhibit the growth of various microorganisms markedly and exhibit higher antibacterial activity than chitosan itself.

When the concentration of Fe^{2+} achieved 240 $\mu\text{g/mL}$, the chelating rate, which reached a maximum, between Fe^{2+} and chitosan was 99.45%, such high chelating rate can make the composite stabilized. As the experimental results showed in Fig. 1, the higher concentration of Fe^{2+} cannot improve the chelating rate. At the same time, such high chelating rate can protect Fe^{2+} to be oxidated, and it is relatively stable in food system. Thereafter, zeta potential, that is, surface charge, could greatly influence particle stability in suspension through the electrostatic repulsion between particles. It can also determine nanoparticle interaction in vivo with the cell membrane of bacteria, which is usually negatively membrane of bacteria. For a physically stable nanosuspension solely stabilized by electrostatic repulsion, a zeta potential of ± 30 mV is required [25]. And the data suggested that chitosan nanoparticles and chitosan nanoparticles loaded metal ions prepared here were relatively stable.

According to relevant literatures, the Ag-loaded chitosan nanoparticles have outstanding antimicrobial activity, which was more efficient than either silver nanoparticles (AgNPs) or chitosan alone for inactivating bacteria, possibly due to synergistic effect of both the AgNPs and chitosan in the composite.

Copper has been shown to have antibacterial properties in studies in vivo [26,27]. In another literature, copper-loaded chitosan nanoparticles exhibit greatly higher antibacterial activity than chitosan itself, and slightly higher than chitosan nanoparticles without copper.

In comparison, the Cu-loaded chitosan nanoparticles exhibit the lowest antibacterial activity, the Ag-loaded chitosan nanoparticles show the highest. It seems that Fe-loaded chitosan nanoparticles didn't performance as good as Ag-loaded chitosan nanoparticles did in antimicrobial activity. While Ag and Cu are both heavy metal, their edible safety is unproved, the Ag-loaded and Cu-loaded chitosan nanoparticles couldn't be used as food preservative.

Due to its good security, Fe-loaded chitosan nanoparticles are expected to be discovered as food preservative. The high chelating rate between Fe^{2+} and chitosan can lead to the Fe-loaded chitosan nanoparticles to be in a relatively stable state in liquid food, and it can keep sterilization effect for a long time. It can completely dispersed and stabled in liquid food due to its few hundred nanometers of the particle size. Therefore, the Fe-loaded chitosan nanoparticles can widely used in food industry, especially in liquid food such as milk and juice.

5 Concluding remarks

The Fe-loaded chitosan nanoparticles were prepared by a surfactant-assisted chitosan chelating Fe^{2+} , Fe^{3+} and ionic gelation method. Chitosan nanoparticles loaded with Fe^{2+} , Fe^{3+} showed significantly higher antimicrobial activity than chitosan and related metal ions. Compared with the previously reported literatures, The Fe-loaded chitosan nanoparticles exhibit higher antibacterial activity than Cu-loaded chitosan nanoparticles,

slightly lower than Ag-loaded chitosan nanoparticles, while iron ion have obviously more secure than silver ion and copper ion in human body. Therefore, the Fe-loaded chitosan nanoparticles used as a food preservative will have a better application prospect in the future.

Practical application

Chitosan nanoparticles loaded with Fe^{2+} or Fe^{3+} can disperse completely and stable in liquid food and keep sterilization effect for a long time. It can widely be used in food industry, especially in liquid food such as milk and juice, and also expected to be used in many fields because of their no-toxicity, excellent antimicrobial activity and potential advantages.

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6 References

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