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壳聚糖金属配合物的抑菌特性及机理研究

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摘　要:制备了壳聚糖(CS)与金属离子 Zn (II)、Ni (II)和 Co (II)的配合物,通过红外光谱和紫外-可见吸收光谱进行了结构性能的表征。体外抑菌法研究了壳聚糖金属配合物对细菌 *S. aureu* 和 *E. coli* 的抑菌活性。结果表明:壳聚糖金属配合物的抑菌活性较壳聚糖增强,且与所含的金属离子种类有关,其中 CS-Zn 体现出更强的抑菌活性。因此,选 CS-Zn 为代表通过测定细胞内溶物的 OD_{260nm} 判断细胞膜的完整性、荧光探针 1-N-苯萘胺(NPN)的荧光变化来判断细胞外膜的渗透性,以研究其对大肠杆菌(*E. coli.*)的抑菌机理。透射电镜(TEM)结果表明 CS-Zn 能够破坏细菌细胞膜,使细胞内溶物溢出。

关键词:壳聚糖金属配合物;抑菌活性;机理

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Antibacterial Characteristics and Mechanism of Chitosan-metal Complexes

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Abstract: Complexes of chitosan (CS) with Zn (II), Ni (II) and Co (II) were prepared, characterized by FT-IR and UV-vis spectra. The antibacterial activities of these complexes against *Staphylococcus aureus* and *Escherichia coli* were investigated *in vitro*. The results showed the antibacterial activities were dependent on the property of metal ions, and CS-Zn exhibited the best antibacterial activity. Hence, CS-Zn was selected to evaluate the antibacterial characteristic against *E. coli*. The integrity of the cell membrane and the permeability of the outer membrane (OM) were studied respectively, by determining the release from cells of material that absorb at 260 nm and changes in the fluorescence of cells treated with the fluorescent probe 1-N-phenylnaphthylamine (NPN). Transmission electron microscopy (TEM) analysis results indicated that CS-Zn disrupted bacterial cell membranes with the release of cellular content.

Key words: Chitosan-metal complexes; antibacterial activity; mechanism

Introduction

Chitosan (CS) is one of the most promising biomaterials in the world, not only because of its abundance but also due to its various properties. CS is known for its antimicrobial properties, higher killing rate and lower toxicity toward mammalian cells[1]. Many attempts have been taken up to improve the antibacterial activity of CS, such as structural modification and forming complexes with other antimicrobial materials[2]. CS is a powerful chelating agent, which is easy to form complexes with transition metals and heavy metals. Most researches of CS-metal complexes focused on their applications in the removal of metal ions, dyeing, catalysis, water treatment and many other industrial processes[3]. However, few researches pay attention to their biological activities and antibacterial mechanism. In this paper, three metal ions were used to prepare CS-metal complexes, and the antibacterial activities of the complexes against *S. aureus* and *E. coli* were studied *in vitro*. In addition, to explain the antibacterial mechanism of CS-Zn, the integrity of the cell membranes, permeability of OM and morphologies of *E. coli* treated with CS-Zn were investigated.

Materials and Methods

Materials

CS (50 kDa, degree of acetylation was 80%) was pur-

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chased from Yuhuan Ocean Biochemical (Zhejiang, China); 1-N-phenylnaphthyl-amine (NPN) was obtained from Sigma-Aldrich China (Shanghai, China). Strains of *S. aureus* (ATCC 26113) and *E. coli* (ATCC 35218) were supplied by Chinese medicine hospital of Tianshui, Gansu, China. Fresh inoculants for the experimental assessment were prepared on nutrient agar at 37 ℃ for 24 h.

Preparation of CS-metal complexes

CS samples were dissolved in 1% (*v/v*) acetic acid to obtain a solution with concentration of 1%. By stirring, desired amount of metal ions (1 mol metal ions per 1 mol amine group of CS) were added into the solution. The pH value was adjusted to 6.0 by NaOH. After agitating for 3 h, the mixture was poured into acetone, and precipitates were collected by filtering. Repeatedly washed with ethanol and finally dried under vacuum[4].

Characterization

IR spectra of samples were taken on KBr pellets with a Spectrum One FT-IR 360 spectrophotometer (Perkin Elmere). UV-vis spectra of samples in 0.3% (v/v) hydrochloric acid were collected using a UV-2450 spectrophotometer.

Antibacterial test

Antibacterial activities of CS and CS-metal complexes were evaluated by optical density method described as follows: By appropriate diluting with sterile distilled water, each culture containing about 10^6-10^7 CFU/mL was prepared for the antimicrobial test. The tested samples were dissolved in 0.3% (v/v) hydrochloric acid at a concentration of 1% (w/v), and then autoclaved at 121 ℃ for 15 min, each sample was added to nutrient broth to the final concentration of 1000 μg/mL, 1 mL of the cultured bacteria solution was inoculated on nutrient medium with sample or control. The plates were incubated on the rotary shaker (128 rpm) at 37 ℃. The inhibitory effects were estimated periodically by measuring OD_{610nm} values. Hydrochloric acid (0.3%) was used as a control instead of samples. For determination of the minimum inhibitory concentration (MIC) of CS and complexes, samples solution was added to nutrient agar and made the final concentrations range 1000 μg/mL from 31.25 μg/mL. The MIC was considered to be the lowest concentration that completely inhibited against on agar plates comparing with control, disregarding a single colony or a faint haze caused by the inoculum[5].

Integrity of cell membrane

Cell membrane integrity was examined by determination of the release of material absorption value at 260 nm[6]. The cultured bacterial were harvested, washed and suspended in sterile physiological saline. The final cell suspension was adjusted to an optical density absorbance at 630 nm (OD_{630nm}) of 0.6 to measure OD_{260nm}. The CS-Zn complexes solution of 1.0 mg/mL was mixed with cell suspension to the ratio of 1∶1 (*v/v*), and the release over time of materials absorbing at 260 nm was recorded periodically with an UV spectrophotometer (Rayleigh, UV-9200, Beijing, China).

OM permeability assays

OM permeability was determined by the NPN assay described by Ibrahim[7]. *E. coli* suspension was adjusted to obtain an OD_{420nm} of 1.0. One milliliter of CS-Zn solution or 0.3% HCl was mixed with 20 μL of 1 mM NPN. Fluorescence, with an excitation wavelength of 350 nm and an emission wavelength of 420 nm, was recorded with a RF-5301PC fluorescence spectrophotometer, from immediately after the addition of 1.0 mL of a cell suspension until there was no further increase in the emission intensity.

TEM

E. coli were prepared for electron microscopy as previously described[8]. *E. coli* was grown in nutrient broth to obtain a culture with the concentration was 10^8 CFU/mL.

Results and Discussion

Characterization of CS-metal complexes

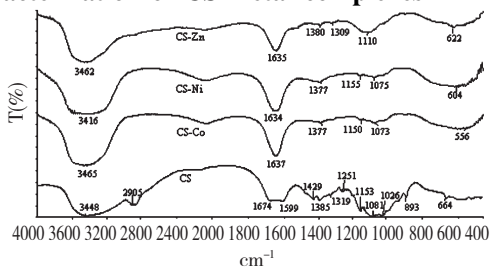

Fig. 1 IR spectra of CS and CS-metal complexes

Fig. 1 showed the IR spectra of CS and CS-metal complexes. As for CS, the wide peak at 3400-3500 cm^{-1} corresponding to the vibration of $-NH_2$ group and -OH group. Peaks at 1152.71 cm^{-1}, 1080.96 cm^{-1} and 893.39 cm^{-1} assigned to the saccharide structure. The peak at 1673.53 cm^{-1} is the amide I band, and the absorption at 1598.60 cm^{-1} disappeared in the complexes is attributed to $-NH_2$ stretching band. A new absorption band at 1634-1637 cm^{-1} appeared, which suggested that the amine or the acetamide group at C2 interacted with metal ions. The bending vibration peak of -OH at 1421.15 cm^{-1} gradually disappeared in CS-metal complexes also indicated -OH have taken part in chelation. The maximal absorption bands of CS is at 191 nm, but for CS-Zn, CS-Co and CS-Ni, which shifted to 201, 217 and 209 nm each, owing to needing more energy of electron transition. Furthermore, there is a new absorption band of CS-Zn, CS-Co and CS-Ni appeared at 255, 246 and 240 nm, it is possible that the lone-pair electrons of nitrogen or oxygenic occurred $n \rightarrow \sigma^*$ transition, resulted in the changing of electronic spectrum. The results demonstrated that valence electron transition was existed between metal ions and CS, and formed compounds among them.

Antibacterial test

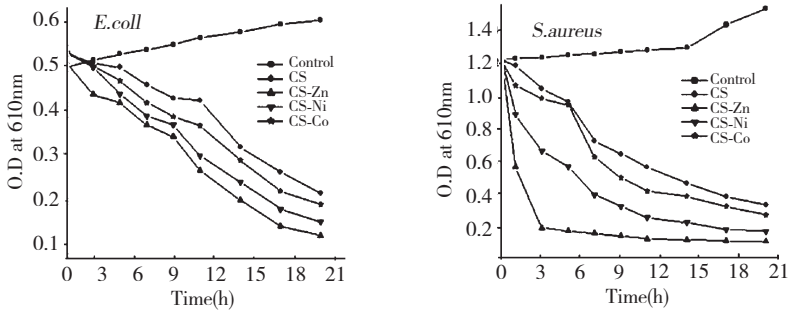

Fig. 2 Inhibitory effect of CS and complexes on the growth of *E. coli* and *S. aureus*

The complex reaction between CS and metal ions may be described according to the Lewis acid-base theory. Metal ions, acting as acceptor of electrons, showed stronger activity than H^+. Thereby metal ions are also called as "super acid"[9]. After chelating with metal ions, the positive charge density of CS increased, leading to an enhanced adsorption of polycation onto the negatively charged cell surface. Reasonably, the antimicrobial activities of CS-metal complexes should be higher than CS itself. Antibacterial activity of CS and CS-metal complexes were explored by optical density method. *E. coli* and *S. aureus* were selected as test cells because they are the most frequent bacteria in wound infection and representative Gram-negative and Gram-positive bacteria. Fig. 2 shows OD_{610nm} of CS and CS-metal complexes towards *E. coli* and *S. aureus*. The OD values of CS-metal complexes to *E. coli* and *S. aureus* are less than control and CS, and are stable with the increasing of time, which shows that CS-metal complexes has a higher rate of killing cells and higher antibacterial activity than CS. Also, the antibacterial activity of CS-Zn was strongest than that of CS-Ni and CS-Co. This order is in agreement with Irving-williams Series. With more extranuclear electrons and smaller radius zinc ions have stronger interaction with CS than Ni ions and Co ions. As a result, zinc complexes with higher charge intensity are easier to associate with cell surface and show higher antimicrobial activity. After the formation of complex, its "Lewis acid" activity will be greatly improved.

The MIC values of CS-metal complexes, ranged from 500 μg/mL to 31.25 μg/mL, were slightly different from CS. For *E. coli*, the MIC values of CS, CS-Zn, CS-Ni and CS-Co was 500, 250, 250 μg/mL and 250 μg/mL, respectively. For *S. aureus*, the MIC values of CS, CS-Zn, CS-Ni and CS-Co was 500, 31.25, 125 μg/mL and 250 μg/mL, respectively, which indicated the antibacterial activity of CS-Zn was improved by 2-8 times than that of CS. Hence, CS-Zn was selected to investigate the antibacterial mechanism against *E. coli*.

Effect of concentration on the antibacterial activity

of CS-Zn

The effect of concentration to the antibacterial activity of CS-Zn against *E. coli* and *S. aureus* is shown in Table 1. For *E. Coli.*, with the increasing of the CS-Zn concentration, the viable population of *E. Coli.* observably decreased from 62.5 to 7.81 μg/mL, and the MIC value was 250 μg/mL against *E. coli.* For *S. aureus*, CS-Zn sample showed obvious antibacterial activity above the concentration of 31.25 μg/mL. While the concentration achieved to 62.5 μg/mL, almost all bacteria were killed, so the MIC of CS-Zn was 62.5 μg/mL against *S. aureus*. However, the MIC of CS was 0.5 mg/mL against *E. coli* and *S. aureus*, which indicated the antibacterial activity of CS-Zn was improved by 2-8 times than that of CS.

Table 1 Antibacterial activity of CS-Zn with different concentrations

Organism	Viable population ($\times 10^{10}$ cfu/mL)				
E. coli	Control	62.50	31.25	15.63	7.81
E. coli	12.05 ± 0.04^{a}	0 ± 0^{b}	1.5 ± 0.01^{c}	2.08 ± 0.00^{d}	4.65 ± 0.02^{e}
S. aureus	Control	250	125	62.50	31.25
S. aureus	15.14 ± 0.04^{a}	0 ± 0^{b}	5.25 ± 0.01^{c}	10.48 ± 0.01^{d}	13.21 ± 0.01^{d}

* LSD test, P = 0.05, a→e Mean ± standard deviation (n = 3). Means in same column with different superscript letters were significantly different.

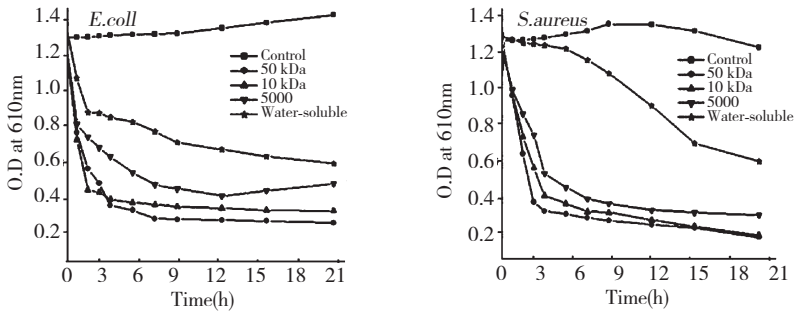

Fig. 3 Antibacterial activity of CS-Zn with different molecular weights against *E. coli* and *S. aureus*

Effect of CS molecular weight on the antibacterial activity of CS-Zn

OD values versus the time for the CS-Zn with different CS molecular weight against *E. coli* and *S. aureus* were shown in Fig. 3. As the final concentration of CS-Zn was 1.0 mg/mL, the OD values were obviously different from the experimental groups and control group. The results illustrated that the antibacterial activity of CS-Zn enhanced with the increasing of molecule weight from 5 kDa to 50 kDa, the molecular weight of 50 kDa appeared the most effective antibacterial activity against *E. coli* and *S. aureus*. The effect of CS molecular weight on the antibacterial activity of CS-Zn was similar to that of CS.

Integrity of cell membrane

The cytoplasm and membrane of bacterial cell undoubtedly is the target for many inhibition agents. After the inhibition agents interacted with bacterial membranes, they cause functional changes in bacterial membrane. When bacterial membranes become compromised by interaction with inhibition agents, first, low molecular mass species such as K^+ and PO_4^{3-} tend to leach out, followed by DNA, RNA and other materials. These intracellular components are easily detected by the absorption at 260 nm as an indication of membrane damage[6]. In other words, the release of 260 nm absorbing material corresponds to the antibacterial activity of CS. The amount of DNA released from the *E. coli* suspension treated with CS-Zn was shown in Fig. 4. The ratio of OD for *E. coli* suspension treated with and without CS-Zn was plotted versus time. Upon addition of the CS-Zn, the OD of the *E. coli* suspension at 260 nm quickly increased within 6 0min, thereafter the ratio was almost unchanged. However, the amount of 260 nm absorbing material tended to decrease with time extension. This quick release of 260 nm absorbing materials

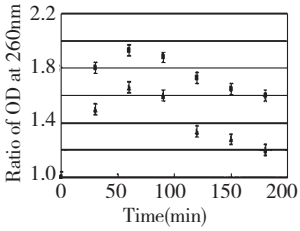

Fig. 4 Release of 260 nm absorbing material from *E. coli* with addition of 1.0 mg/mL (▲) or 2.5 mg/mL (■) of CS-Zn

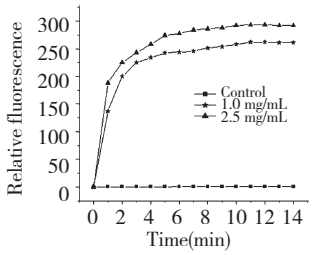

Fig. 5 The uptake of NPN (measured as fluorescence intensity in arbitrary units) by *E. coli* with addition of 0.3% (v/v) hydrochloric acid or 1.0 mg/mL or 2.5 mg/mL of CS-Zn

was in good agreement with fast killing kinetics of the CS-Zn.

The release of 260 nm absorbing material from *E. coli* with addition of CS-Zn indicated that CS-Zn had the ability to damage the cell membrane of *E. coli*, and made the nucleic acid released. Antibacterial activity of CS as related to membranes permeability had been evaluated in literature[10]. They studied on the integrity of cell membranes using CS against *E. coli* and *S. aureus*. Their results showed that the release of 260 nm absorbing materials quickly increased, and the damage of cell membranes was concentration-dependent. Our results evidenced that CS-Zn induced the release of intracellular component by destroying the integrity of bacterial cell membranes, which was similar to CS.

Permeabilization of the OM

As illustrated in Fig. 5, the addition of CS-Zn to *E. coli* suspensions at the presence of NPN caused a time-dependent increase in fluorescence, the relative fluorescence increased to a maximum in 10 min. The maximum fluorescence of CS-Zn at the higher concentration was greater than the lower. Similar results were obtained by Helander[8], who reported that CS at pH 5.3 caused a significant increase in NPN uptake, and that relatively high concentrations of CS (250 ppm) were required to obtain significant increase NPN uptake.

TEM

In electron micrographs, the control cells showed an intact and apparent cell membrane as an electron dense line (Fig. 6a). However, *E. coli* of CS-Zn treated showed badly disrupted and altered of cell membrane after 24 h, as depicted in Fig. 6b and Fig. 6c. Extra- and intracellular changes compared with the non-treated cells including a separation of the cytoplasmic membrane from the cell envelope and coagulation of the cytosolic components, and disruption of the outer membrane structure with membrane sloughing and breaching, even disappeared. There were irregularities on the *E. coli* cell surface, irregularly shaped and without membranes or cell wall on one side, and pores formed on the cell surface. Morphology of the bacteria were multiplicity, and the cells were markedly degraded from bacilliform to spherical shape (Fig. 6b) and irregularly condensed masses with bleb-like structures. The profile of bacterial became faint, the surface appeared burs, their structure turned dim and hollow, even perforated and crashed. TEM studies showed that pore formation on the bacterial cell surface, indicated that it was lytic

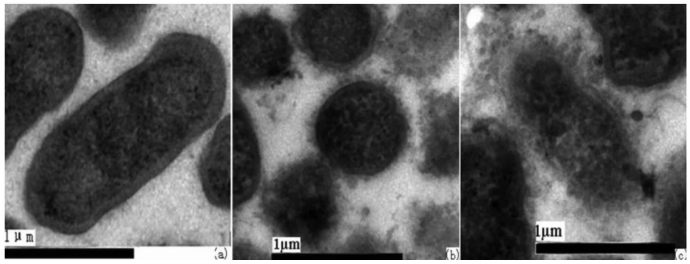

Fig. 6 TEM diagram of *E. coli* treated with buffer (a) and CS-Zn (b, c) (amplified 25000)

rather than static activity for CS-Zn toward *E. coli*. The cell membranes appeared to be damaged. Thus, membrane damage was the inhibition mechanism of CS-Zn against *E. coli*.

Conclusion

In this study, CS-metal complexes with different chelating metal ions showed much higher antibacterial activities than free CS. Furthermore, CS-Zn showed the strongest antibacterial activity against *E. coli* and *S. aureus*. To make clear the antibacterial mechanism of CS-metal complexes, the *E. coli* cells treated with CS-Zn was investigated. When *E. coli* was treated by CS-Zn, the release of intracellular component increased via destroying the integrity of bacterial cell membranes. The OM permeability results demonstrated CS-Zn increased the permeability of the OM. Since bacteria were negatively charged and CS-Zn had a high positive charge density under acid condition, electrostatic interactions quickly bring them into contact with each other. On the basis of the above experimental results, it was suggested that CS-Zn complexes firstly disrupted the OM of bacteria. While more CS-Zn was introduced, without the protection of the OM, the relatively high concentration influenced the conformation of membrane protein by interacting with proteins on the cell membrane or cell wall, denatured membrane proteins, and lead to a complete disintegration of the bacterial membrane with the releasing of intracellular contents, and the ultimate lysis of cell. All the results showed that CS-metal complexes were a promising candidate for novel antimicrobial agents and provided further insight into the pharmaceutical application of CS and supported the possible usage of CS as a template for the development of new antibiotics.

References

1 Liu XF, Guan YL, Yang DZ, *et al*. Antibacterial action of chitosan and carboxymethylated chitosan. *J Appl Polym Sci*, 2008, 79: 1324-1335.

2 Sanpui P, Murugadoss A, Prasad PVD, *et al*. The antibacterial properties of a novel chitosan-Ag-nanoparticle composite. *Inter J Food Microb*, 2008, 124: 142-146.

3 Chen AH, Liu SC, Chen CY, *et al*. Comparative adsorption of Cu(II), Zn(II), and Pb(II) ions in aqueous solution on the crosslinked chitosan with epichlorohydrin, *J Hazard Mater*, 2008, 154: 184-191.

4 Wang XH, Du YM, Fan LH, *et al*. Chitosan-metal complexes as antimicrobial agent: synthesis, characterization and structure activity study. *Polym Bull*, 2005, 55: 105-111.

5 Farag RS, Daw ZY, Hewedi FM, *et al*. Antimicrobial activity of some egyptian spice essential oils. *J Food Prot*, 1989, 52: 665-667.

6 Chen CZ, Cooper SL. Interactions between dendrimer biocides and bacterial membranes. *Biomaterials*, 2002, 23: 3359-3368.

7 Ibrahim HR, Sugimoto Y, Aoki T. Ovotransferrin antimicrobial peptide (OTAT-92) kills bacteria through a membrane damage mechanism. *Biochim Biophys Acta*, 2000, 1523: 196-205.

8 Helander IM, Nurmiaho-Lassila EL, Ahvenainen R, *et al*. Chitosan disrupts the barrier properties of the outer membrane of gram-negative bacteria. *Inter J Food Microb*, 2001, 71: 235-244.

9 Wang XH, Du YM. Chitosan-metal complexes as antimicrobial agent: synthesis, characterization and structure activity study. *Polym Bull*, 2005, 55: 105-113.

10 Liu H, Du YM, Wang XH, *et al*. Chitosan kills bacteria through cell membrane damage, *Inter J Food Microb*, 2004, 95: 147-155.