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Chitosan- metal complexes as antimicrobial agent: Synthesis, characterization and Structure-activity study

Xiaohui Wang, Yumin Du(✉), Lihong Fan, Hui Liu and Ying Hu

Wuhan University, College of Resource and Environmental Science, Wuhan 430072, China
E-mail: duyumin@whu.edu.cn; Fax: 86+027+68778501

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Summary

Chitosan (CS) metal complexes with bivalent metal ions, including Cu(II), Zn(II), Fe(II) were prepared, and characterized by FT-IR, XRD, AAS and elemental analysis. The crystalline and structural properties of chitosan-metal complexes were different from those of chitosan, and the $-NH_2$, -OH groups in chitosan molecule were considered as the dominating reactive sites. In vitro antimicrobial activities of the obtained chitosan-metal complexes, which were found to be much better than free chitosan and metal salts, were examined against two gram-positive bacteria (*S. aureus* and *S. epidermidis*), two gram-negative bacteria (*E. coli* and *P. aeruginosa*) and two fungi (*C. albicans* and *C. parapsilosis*). Results indicatd that the inhibitory effects of chitosan-metal complexes were dependent on the property of metal ions, the molecular weight and degree of deacetylation of chitosan and environmental pH values. Electro microscopy confirmed that the exposure of S. auresus to the chitosan-Cu(II) complex resulted in the disruption of cell envelop. Based on the discussion upon the antimicrobial mechanism of chitosan-metal complexes and their molecular structures, the structure-activity correlation for the antimicrobial activities was elucidated. All the results show that chitosan-metal complexes are a promising candidate for novel antimicrobial agents that can be used in cosmetic, food, textile et al.

Introduction

Chitosan, as one of the most abundant natural polymers, is non-toxic, biodegradable and biocompatible. Different from most other natural polymers, chitosan has high reactivity and processability for its specific molecular structure and polycationic nature [1-2]. Recent years, chitosan and its derivatives received considerable attention due to their potential beneficial biological activities, such as antitumor, antiulcer, immunostimulatory, anticoagulant, antimicrobial et al [3-10]. The antimicrobial activity of chitosan was observed against a wide variety of microorganisms including fungi, algae, and some bacteria [11]. chitosan has several advantages over other type of disinfectants because it possesses a higher antibacterial activity, a broader spectrum of activity, a higher killing rate, and a lower toxicity toward mammalian cells.
The antimicrobial action of chitosan is influenced by both intrinsic factors and the environmental conditions, such as the type of chitosan and microorganisms, the degree of polymerization, the degree of deacetylation, and the pH of medium [11].

Many attempts have been taken up to improve the antimicrobial activity of chitosan, such as structural modification, adjustment of molecular factors, and forming complexes with other antimicrobial materials. We have ever reported the preparation and antimicrobial effect of chitosan derivatives, e.g. carboxymethyl chitosan, sulfate chitosan, quaternized chitosan, and chitosan complexes with surfactant, organic acids, essential oils and zinc ions [8, 12-17].

It is well known that metal ions, such as Ag^{+}, Cu^{2+}, Zn^{2+} et al, form an important group of antimicrobial agents, which have different active target from most bacteriostatic polymers. Chitosan is a powerful chelating agent, which is easy to form complexes with transition metals and heavy metals. Most researches of chitosan-metal complexes focused on their applications in the sequestration or removal of metal ions, dyeing, catalysis, water treatment, and many other industrial processes [18]. However, few researches pay attention to their biological activities. In the previous work [16], we have found that chitosan-Zn complex had wide spectrum antimicrobial activity, that was affected by the chelate ratio. But the influence of the property of metal ions, molecular parameter of chitosan and environmental factors on the antimicrobial activity of chitosan-metal complexes, which is important for their future application, were not clear. Thus further work is required to make clear their relationship and search out optimized chitosan-metal antimicrobial agent.

In this study, the antimicrobial activity of chitosan(CS)-metal complexes against Gram-positive bacteria, Gram-negative bacteria and fungi was systemically studied. Three metal ions were used to prepare CS-metal complexes, whose structures were comprehensively characterized and discussed. The antimicrobial activities of the CS-metal complexes were investigated in compare with free chitosan and metal salts. And the effects of chitosan's molecular parameters e.g. the degree of deacetylation, the molecular weight and pH of medium on the antimicrobial activities of CS-metal complexes were discussed.

Experimental

Materials

Chitosan (CS) from the shrimp shell was obtained from Yuhuan Ocean Biochemical Co. (Zhejiang, China), the degree of deacetylation (DD) was 94% and average molecular weight (MW) was 788kDa. Beef extract and peptone were purchased from Shanghai Chemical Agent Co. (Shanghai, China). The other chemicals used were of analytical grade.

The microorganisms tested were provided by China Center for Typical Culture Collection, Wuhan University, except Cand. Albicans that was original isolated from patient was provided by the people's hospital of Huber province in China.

Instrumentals

FT-IR spectra of CS and CS-Metal complexes were recorded with KBr discs in the range of 4000 – 400cm^{-1} on Nicolet-170 SX Fourier transfer infrared spectrometer. X-ray diffraction was recorded by a Rigaku Kmax-r AX diffractometer with scaning scope of 5^{o}-40^{o} scanning speed of 4^{o}/min, using Cu Kα radiation. C, H, N elemental analysis was performed using a Carlo-Erba 1106 elemental analyzer. Metal ions content was measured by a Hitachi 180-80 atomic absorption spectrometry. The

molecular weights were measured by a GPC equipment which consisted of the connected column (TSK G5000-PW and TSK G3000-PW), TSP P100 pump, and RI 150 refractive index detector. All data provided by the GPC system were collected and analyzed using the Jiangshen Workstation software package.

Preparation of Chitosan sample

Acetylation of Chitosan
Chitosan with a degree of deacetylation (DD) of 50% was produced by acetylation method [19]. The DD of chitosan was measured by potentiometric titration method.

Depolymerization if Chitosan
Chitosan suspension in deionized water after stirred at 65 °C for 1hr was hydrolyzed with hydrogen peroxide solution [20]. By varying the amount of hydrogen peroxide and the reaction time, various molecule weight products were obtained. At the end of predetermined time, the mixture was subjected to vacuum filtration. The solid was then washed with deionized water, finally was collected by drying at 30°C. The supernatant solution was poured into acetone and the precipitate was collected by solvent evaporation and drying. The molecular weights of products were calculated using GPC.

Preparation of CS-metal complexes

Chitosan sample was dissolved in diluted 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 chitosan) were added into the solution. The pH value was increased to 6.0 by dropping NaOH solution. After agitating for 3hr, the mixture was poured into excess of acetone. The precipitates collected by filtering was repeatedly washed with ethanol and finally dried under vacuum to constant weight.

Antimicrobial assessment

The antimicrobial activities of chitosan-metal complexes and starting materials (chitosan, metal salts) were evaluated using the agar plate method as described in the following [7-9]. A loopful of each culture was spread to give single colonies on nutrient agar (agar 15g, peptone 10g, beef extract 3g, NaCl 3g in distilled water 1L; pH 7.0) and incubated at 37 °C for 24hr. A representative colony was picked off with a wire loop and place in nutrient broth (peptone 10g, beef extract 3g, NaCl 3g in distilled water 1L; pH 7.0), which was then incubated at 37 °C overnight. By appropriated diluting with sterile distilled water, each culture containing ca. 10^6-10^7 CFU/ml was prepared which was used 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 they were autoclaved at 121 °C for 25min. Two fold serial dilutions of each sample were added to nutrient broth for final concentration of 1000μg/ml to 31.3μg/ml. Hydrochloric acid (0.3%) was used as a control instead of samples. A loop of each suspension was inoculated on nutrient medium with sample or control added. After inoculation, the plates were incubated at 37 °C for 48hr, and the colonies were counted and the MIC values were obtained. All the experiments were applied in triplicate test at least.

The minimum inhibitory concentration (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 [21].

Results and discussion

Compositional and structure study of chitosan-metal complexes

Chitosan was found to be very easy to form complexes with Cu(II), Fe(II), Zn(II). The formula of CS-Cu, CS-Fe and CS-Zn complexes were shown in Table 1. The calculated concentrations of C, H, N and metal were shown in bracket below the found concentration, which were in good agreement with the analytical data. The content of metal in the complexes increased in the order of Cu>Zn>Fe, which consisted with the famous Irving-williams Series.

Table 1. Composition of Chitosan-metal Complexes.

Complex	Formula	C%	H%	N%	M%[1]
CS-Cu	$Cu(C_6H_6O_4N)_{1.7}\bullet SO_4\bullet 4H_2O$	23.93	5.47	4.49	12.32
		(23.8)	(5.2)	(4.6)	(12.6)
CS-Fe	$Fe(C_6H_6O_4N)_3\bullet SO_4\bullet 6\ H_2O$	25.03	5.28	4.88	6.39
		(25.2)	(5.3)	(4.9)	(6.5)
CS-Zn	$Zn(C_6H_6O_4N)_2\bullet SO_4\bullet 2\ H_2O$	27.94	5.18	5.68	12.17
		(27.3)	(4.9)	(5.3)	(12.3)

[1]M% corresponds to the concentration of metal ions

The great intramolecular hydrogen bond leads to chitosan's crystalline character that distinguished it from most carbohydrate polymers [1]. As shown in Fig 1, the XRD pattern of chitosan exhibited its characteristic crystalline peak at 10.4^o and 19.8^o. In the XRD pattern of chitosan-metal complexes, the characteristic peaks of chitosan were weakened and disappeared, while many new diffraction peaks appeared. It indicated the formation of a new regular crystalline phase [22], in which, the diffraction peaks of chitosan disappeared because the hydrogen bond within chitosan were destroyed by metal ions that chelate with $-NH_2$, -OH. And the decrease of hydrogen bonds prefers to

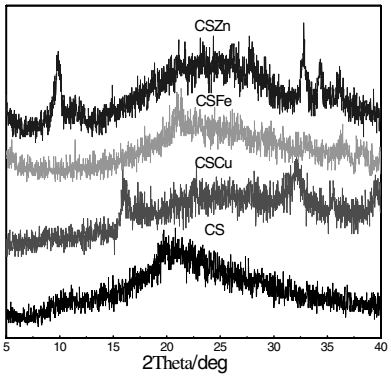

Figure 1. XRD spectra of chitosan (CS) and chitosan-metal complexes.

make aggregated chitosan chain extend and disaggregate. Thus more positive charge carrier can be exhibited that will be good to antimicrobial actions.

Figure 2 shows the FT-IR spectra of CS, CS-Cu, CS-Zn and CS-Fe. The wide peak at 3437cm^{-1} corresponding to the stretching vibration of $-NH_2$ group and –OH group [23] shifted to lower frequency in the complexes, indicating $-NH_2$ or –OH groups take part in complexation. Absorption bands at 1660cm^{-1} assigned to acetamide group and 1598 cm^{-1} assigned to "free" amine group [24] disappeared in the complexes. Instead, a new absorption band at 1620-1630cm^{-1} appeared, that was considered as characteristic peak of the association of chitosan and metal [25]. It suggested that the amine or the acetamide group at C2 interacted with metal. The peak at 1420 cm^{-1} attribute to bending vibration of –OH gradually disappeared in the CS-metal complexes, indicating –OH take part in chelation. The band at 1110cm^{-1}, 619 cm^{-1} assigned to ionic SO_4^{2-} [26] was found in all complexes' spectra, which indicated that SO_4^{2-} existed in the complexes in the ionic form.

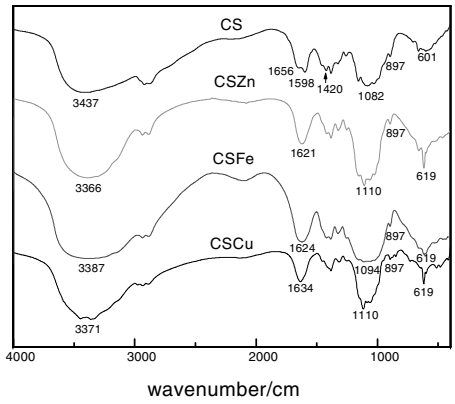

Figure 2. FT-IR spectra of chitosan (CS) and chitosan-metal complexes.

Based on the analysis above, we speculate the molecular structure of chitosan-metal complex as follow:

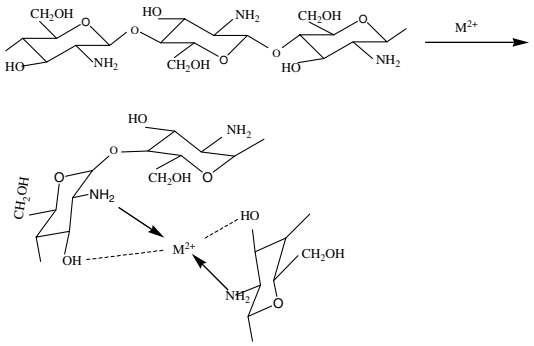

Figure 3. Possible molecule structure of chitosan-metal complexes.

As shown, metal ion like a bridge connected one or more chains of chitosan through interacting with –OH and $-NH_2$.

Antimicrobial assessment

Chitosan displays antimicrobial activities only in acid environment [11]. In low pH medium, chitosan possess a lot of poly-cationic amines that interact readily with negatively charged substances at the cell surface of bacteria, such as proteins, phospholipids, fatty acid, and subsequently inhibit the growth of microorganisms [27]. The complex reaction between chitosan and metal ions may be described according to the Lewis acid-base theory [28]. Metal ions, acting as acceptor of electrons, showed stronger activity than H^+. Thereby metal ions are also called as "super acid". After chelating with metal ions, the positive charge density of chitosan increases, leading to an enhanced adsorption of polycation onto the negatively charged cell surface. Reasonably, the antimicrobial activities of chitosan-metal complexes should be higher than chitosan itself.

Table 2. Antimicrobial activities of chitosan and chitosan metal complexes evaluated by MIC (μg/ml).

Microorganisms	CS	CS-Cu	CS-Zn	CS-Fe
S. aureus	250	31.3	62.5	125
S.epidermidis	500	62.5	62.5	250
E.coli	500	31.5	31.5	125
P.aeruginosa	1000	62.5	62.5	125
C.albicans	>1000	>1000	>1000	>1000
C.parapsilosis	>1000	1000	1000	1000

Table 2 shows the minimum inhibitory concentration (MIC) of CS, CS-Cu, CS-Fe and CS-Zn against two gram-positive bacteria: *S. aureus* and *S. epidermidis*; two gram-negative bacteria: *E. coli.* and *P. aeruginosa*; two fungi: *C. albicans* and C. *Parapsilosis.* Chitosan-Metal complexes showed considerable wide spectrum antimicrobial activities, which were obviously stronger than that of chitosan. The antimicrobial activities of CS-Cu and CS-Zn were comparably better than that of CS-Fe. This order is also in agreement with Irving-williams Series. As proved by elemental analysis data above, with more extra-nuclear electrons and smaller radius cupric ions have stronger interaction with chitosan than zinc ions and then ferrous ions. As a result, cupric complexes with higher charge intensity are easier to associate with cell surface and show higher antimicrobial activity. Zinc can also interact with chitosan easily, and after the formation of complex, its "Lewis acid" activity will be greatly improved [26]. Therefore zinc complexes also show great antimicrobial activity. In compare, the antimicrobial activity of Fe-chitosan complexes was lower

Table 3. Antimicrobial activity chitosan-metal complexes and corresponding metal salt evaluated by MIC(μg/ml).

	C (Zn/complexes)	C (Zn/metal salt)
S. aureus	4.4	18
S. epidermidis	4.4	18
E. coli	2.2	18
P. aeruginosa	4.4	36
C. albicans	18	18
C. parapsilosis	18	>72

than the other two complexes. Because the distribution of negative charge on the cell surfaces of microorganisms was quite different, the inhibitory effects are not same against different microorganisms.

The antimicrobial activity of chitosan-metal complex and that of metal were compared in table 3. Obviously, the antimicrobial activity of chitosan-metal complex was much better than corresponding metal ions. It indicated that the complex exhibited the cooperated function of chitosan and metal. As an antimicrobial agent, chitosan-metal complex have advantage of higher activity than chitosan and lower toxicity than metal.

Table 4. The antimicrobial activity of chitosan-Cu with different molecule weight evaluated by MIC((μg/ml).

Microorganisms	2.6kDa	8.7kDa	18.4kDa	38.8kDa
S. aureus	62.5	31.3	31.3	31.3
S. epidermidis	125	125	62.5	62.5
E. coli.	500	250	125	125
P. aeruginosa	>1000	1000	500	125
C. albicans	>1000	>1000	>1000	>1000
C. parapsilosis	500	1000	>1000	>1000

To investigate the relationship between antimicrobial activity and chain length, hydrolyzed chitosan with varying molecular weight (Mw) were prepared. They were made to react with cupric ions to prepare CS-Cu complexes with varying Mw, whose antimicrobial activities against the six microorganisms were evaluated as shown in table 4. The result showed that in the Mw range of 26kDa to 388kDa, the antimicrobial activities of CS-Cu enhanced with increasing molecule weight. Cupric complex of chitosan oligomer with Mw less than 10kDa was also found to inhibit growth of S. aureus weakly, however, the inhibit effect were much more limited than those of polysaccharide-Cu complex. Higher antibacterial activity of chitosan compared with chitosan oligomers had been reported by several workers [29, 9]. To make clear the antibacterial mechanism action of chitosan-metal complexes with differed Mw, we investigated the *S. aureus* cells treated with chitosan-Cu and chiooligosaccharide-Cu complexes using TEM. Through TEM observation, the integrity of most *S. aureus* cells was disrupted after exposure to chitosan-Cu complex for a short time. (d) ~ (g) in figure 4 are the representative morphology of *S. aureus* cells after treatment of chitosan-Cu complex. They presented a procedure of morphological changes of incubated cells (g), partially disruption of out membrane (f), disruption of the cytoplasmic membrane (e) and release cytoplasmic constituents (d). In particular, the cell in fission in (e) showed a totally disruption of out membrane while the intramembrane was kept integral, which indicate the target site of chitosan-Cu complex is the envelope of cells. At the same time, no obvious disruption of cell membrane was observed after treatment of chitooligosaccharide-Cu. As to chitooligosaccharide-Cu, there may be other antimicrobial mechanisms. It was reported by Liu XF et al. that the FITC-labeled chitosan oligomers were observed at the inside of the E. coli cell, which were suggested to block the transcription from DNA [30]. Probably, chitooligosaccharide-Cu has similar mechanism as chitosan oligomers, that can not be confirmed here through TEM.

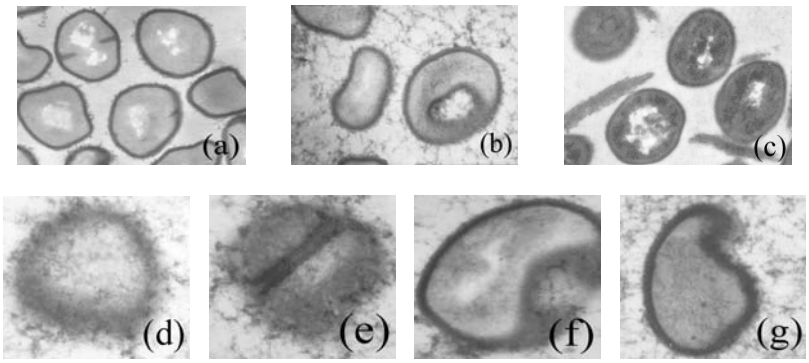

Figure 4. Transmission electron photograph of intact *S. Aureus* cells (a) and *S. Aureus* cells after treatment with chitosan-Cu complex (b), chitooligosaccharide-Cu complex (c). (d)~(g) are the representative morphology of *S. Aureus* cells exposure to chitosan-Cu.

The effect of deacetylation degree (DD) on the antimicrobial activity of CS-Metal complex was studied. As shown in Figure 4 (a), under idental experimental conditions, the antimicrobial activities of CS-metal complexes were enhanced with increasing DD. This may be due to the more amine groups in the complexes, the greater cationic charge density could be obtained. For the same reason, the CS-metal complexes with greater DD were easier to absorb on the outer membrane of microorganism and inhibit its growth.

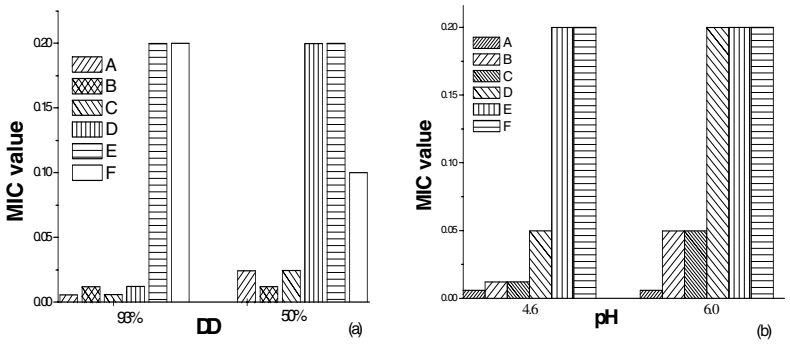

Figure 5. Antimicrobial activity of chitosan-Cu complexes with different Mw, DD and pH values, in which A, B, C, D, E, F respectively corresponds to microorganisms: *S. aureus , S. epidermidis, E. Coli*, *P. aeruginosa , C. Albicans and C. Parapsilosis.*

The correlation of antimicrobial activity of CS-metal complexes with pH of growth medium was studied by varying pH of medium. Figure 4 (b) showed the effect of environmental pH on the antimicrobial activity of CS-metal complex. It can be found that CS-metal complexes showed greater activity at lower pH. It may be explained as lower pH favored the complexes form cationic polyelectrolytes.

Conclusion

CS-metal complexes with differed bivalent chelating metal ions, Mw and DD of chitosan were prepared and characterized with XRD, FT-IR, elemental analysis and

AAS. The complexes showed wide spectra antimicrobial activities, which were much higher than free chitosan and metal salts. Through the discussion upon the antimicrobial mechanism and molecular structure of chitosan-metal complexes, it is concluded that the chitosan-metal complexes showed higher antimicrobial activities because of the stronger positive charge after complexation. More chelated metal ions, stronger bonding, higher molecular weight and degree of deacetylation of chitosan and lower pH value of environment help to obtain higher positive charge, thus resulted in better antimicrobial acitivities.

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