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Preparation of new crosslinked chitosan with crown ether and their adsorption for silver ion for antibacterial activities

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Abstract

New di-Schiff base type crown ethers crosslinked chitosan (CCTS-1) was synthesized by the reaction of 4,4′-diformyldibenzo-18-c-6 crown ether with crosslinked chitosan. New di-secondary amine type crown ethers crosslinked chitosan (CCTS-2) was prepared by the reaction between CCTS-1 and sodium borohydride. Their structures were confirmed by Fourier transform infrared spectral analysis, X-ray powder diffraction analysis and elemental analysis. The adsorption rates by CCTS-2 for Ag^+ for 1 h were 96% at pH 6.0, and Ag^+ initial concentration 0.5 mmol/l. The complexes of CCTS [Bull. Chem. Soc. Jpn 60 (1987) 444], CCTS-2 and silver ion against three bacteria were studied. The bacteriostasis zone diameters of the complex of CCTS-2 and Ag^+ (CCTS-2−Ag^+) containing 0.00355 mmol Ag^+ against *Staphylococcus Aureus*, *Escherichia coli* and *Pseudomonas aeruginona* are 11, 10 and 7.5 mm, respectively, while those of the complex of CCTS and Ag^+ (CCTS−Ag^+) under similar conditions were 11, 10 and 6.0 mm, respectively. This research will be useful for designing crosslinked-chitosan-based adsorption for preconcentration of Ag^+ for medical bacteriostasis.

Keywords: New crosslinked chitosan; Adsorption for Ag^+; Bacteriostasis

1. Introduction

The biopolymers including cellulosics, alginates, proteins, chitin, and chitin derivatives are some of the best chelation ion-exchange materials (Deans & Dixon, 1992; Muzzarelli, 1971; Kurita, Sannan, & Iwakura, 1979). For example, chitosan (CTS) is a deacetylation derivative of chitin and can adsorb metals owing to its amino and hydroxyl groups. However, CTS can be dissolved in acid media. Crosslinked chitosans synthesized by the reaction of CTS with crosslinking agents can overcome the disadvantage of CTS and still keep good adsorption properties for many metal ions. Allan, Kendra and Uchida discovered that chitosan and its salts showed antimicrobial activity. Many researchers are engaged in this field (Allan & Hadwiger, 1979; Kendra & Hadwiger, 1984; Walker-Simmons & Ryan, 1984). The mechanism of antibacterial activities can be demonstrated as follows. (1) The cationic nature of chitosan binds with sialic acid in phospholids, consequently, restraining the movement of microbiological substances (Seo, 1993). (2) Oligomeric chitosan penetrates into the cells of microorganism and prevents the growth of cells by prohibiting the transforming DNA into RNA (Muzzarelli, Jeunianx, & Gooday, 1986). (3) Chitosan can bind some metal ions to restrain the growth of microbe (Cuero, Osuji, & Washington, 1991; Jackson & Health, 1993). For visible physiological activity and antitumour effect, CTS finds application in biochemistry and pharmaceutics. $AgNO_3$ is usually used in disinfection, its high concentration is applied in corroding increased granulation tissues and its low concentration in conjunctivitis (Zhao, Quan, & Li, 2000). Ag^+ with concentration 0.03 ~ 1 μ g/ml can kill bacteria. Generally, $AgNO_3$ is not stable, and can be easily reduced as Ag, which limits its medical application. The coordination of chitosan with Ag^+ is prepared and it has bacteriostasis which provides experimental basis for medical application (Zhan, Xiong, Liu, & Xie, 2002).

Because crown ethers have particular molecular structures, they have good and different complex selectivity for many metal ions. But they are not recycled easily after being used; therefore, their applications were limited. If crown ethers were crosslinked to chitosan chains to

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give crown ethers crosslinked chitosan containing double structures and properties of chitosan and crown ethers, these novel chitosan derivatives would have stronger complex with and better selectivity for metal ions than corresponding crown ethers and crosslinked chitosan. These novel crown ethers crosslinked chitosan have space net structures with crown ethers embedded in it and each mesh has a certain space volume. The silver ion that has the proper volume can be adsorbed by the novel chitosan derivative. As mentioned earlier, it can be predicted that the complex of novel polymer and silver ion would have application prospects for medical application of bacteriostasis.

In this article, we first synthesized 4,4′-diformyldibenzo-18-c-6 crown ether (Wada, Hirayama, Namiki, Kikukawa, & Matsuda, 1980). Then, it was reacted with CTS, reacted with epichlorohydrin again to give *N*-Schiff base type crosslinked chitosan dibenzo-18-c-6 (CCTS-1). And the *N*-secondary amine type crosslinked chitosan dibenzo-18-c-6 (CCTS-2) was synthesized by the reaction of CCTS-1 with sodium borohydride. The preparation of crosslinked chitosan CCTS was according to Ohga et al. (1987). Their structures were confirmed by FT-IR spectra analysis, elemental analysis, and X-ray powder diffraction analysis. The adsorption of CCTS-2 for Ag^+ were studied and CCTS-2–Ag^+ was discussed about its bacteriostasis compared with CCTS, CCTS-2, CCTS–Ag^+.

2. Experimental

2.1. Materials

Chitosan (with 80% deacetylation) was purchased from Yuhuan Organisms Co. Ltd., Hangzhou, Zhejiang Province, China. 4,4′-Diformyldibenzo-18-c-6 crown ether was prepared according to the procedure reported previously (Wada et al., 1980). The preparation of CCTS was according to Ohga et al. (1987). Metal ion solutions containing the desired concentrations of Ag^+ were prepared from analytical grade stock solutions (1000 mg/l). Working standard solutions of lower concentration were prepared after serial dilution of the stock solutions daily. The concentrations of silver (Ag^+) were 54 mg/l.

Three bacteria tested for antimicrobial activity of CCTS-2, CCTS, CCTS-2–Ag^+ and CCTS–Ag^+ included two gram-negative bacteria, (*Escherichia coli*, *Pseudomonas aeruginona*), one gram-positive bacteria (*Staphylococcus Aureus*), which were provided by the Center for Typical Culture Collection, Wuhan University, China. The cultivation medium for the aforementioned microorganism was broth peptone culture medium, which contained peptone 10 g, beef extract 5 g, agar 17–20 g, water 1000 ml, pH was adjusted to 7 with 1 M HCl and 1 M NaOH.

2.2. Preparation of CCTS-1

Powdered chitosan (1.0 g) was dissolved in 60 ml of 1% (wt) acetic acid and diluted with ethanol. Then, 4,4′-diformyldibenzo-18-c-6 crown ether, which was dissolved in chloroform and ethanol, was slowly dropped into the above solution under nitrogen. After 2 ml of epichlorohydrin was slowly added, 20 ml of 5% (w/v) NaOH was added in drops. The mixture was refluxed with good agitation for 24 h, filtered, washed with deionized water, and extracted with chloroform in a Soxhlet's extractor for 4 h to remove any unreacted 4,4′-diformyldibenzo-18-c-6. Precipitates were dried to give a light brown CCTS-1, as shown in Fig. 1. Panmilled and sieved (200 mesh), it was ready for use in the experiments.

2.3. Preparation of CCTS-2

Following the procedure of CCTS-1, a sticky solution was obtained and 1.0 g sodium borohydride dissolved in 20 mL ethanol was slowly dropped in the solution. The mixture was refluxed for another 6 h and dried to obtain a faint yellow solid. The product was washed by Soxhlet's extraction with ethanol to remove any unreacted 4,4′-diformyldibenzo-18-c-6 and sodium borohydride, and dried to give CCTS-2, as shown in Fig. 1. It was used after passage through a 200 mesh sieve.

2.4. Apparatus

Infrared spectra were measured on a NICOLET 5DX Fourier transform infrared spectrophotometer. Wide-angle X-ray diffraction patterns were obtained with a flat-film camera using nickel-filtered Cu Kα radiation produced by a Rigakn (D/MAX, 111A) diffractometer. The pH values were measured with a Model DF-180 PH/MV meter. The concentrations of the metal ions were analyzed using automatic absorption spectrophotometry (AAS, Hitachi 180-80).

2.5. Adsorption procedure

To 25 ml of an aqueous solution of metal ion (1.25×10^{-1} mmol, $AgNO_3$) were added 25 mg of CCTS-2 samples. After shaking for 0, 0.5, 1, 2, 4, 6 h at room temperature, respectively, the mixture was centrifuged and filtered. The metal ion concentration in the filtrate was determined by AAS, and the adsorption capacities of CCTS-2 (mg Ag^+/g adsorbent) and the adsorption rate of the metal ions were calculated by the method reported previously (Wang, Cheng, Zhu, Tang, & Feng, 1998).

All the experiments were carried out in triplicate.

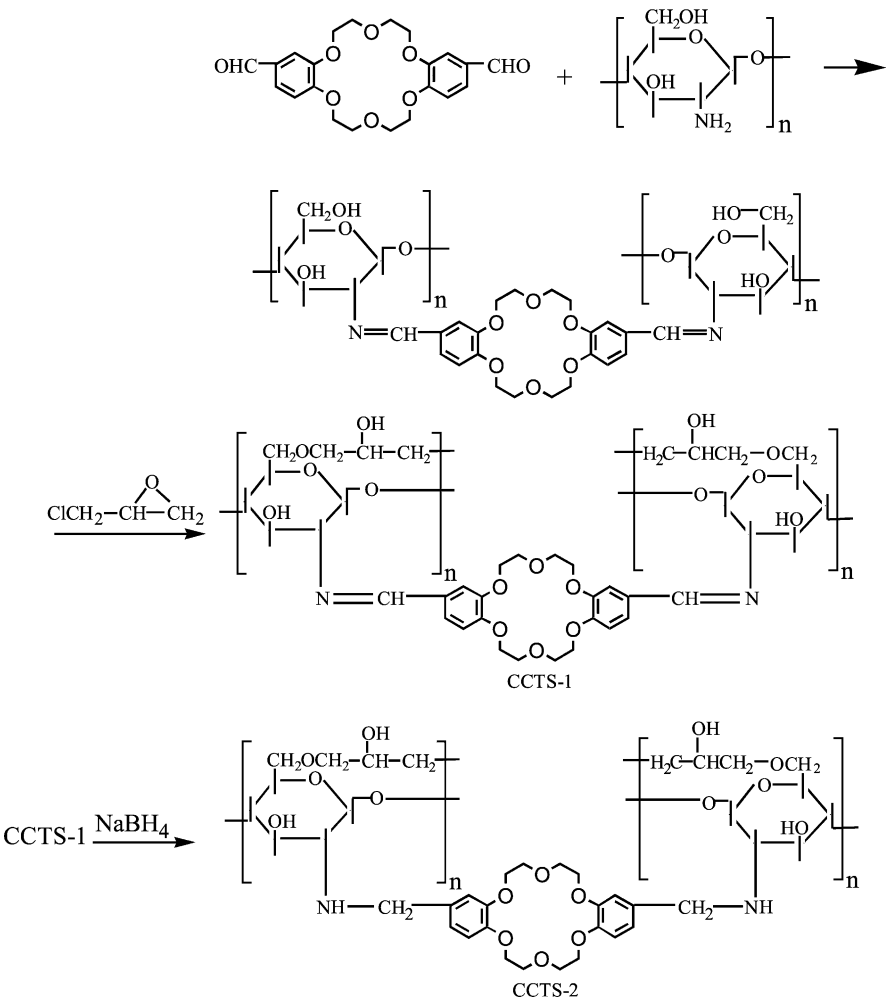

Fig. 1. Reaction scheme for the synthesis of CCTS, CCTS-1, CCTS-2.

2.6. Preparation of the complexes of CCTS, CCTS-2 and Ag^+ and their bacteriostasis

For example, in order to obtain a sample of the complex of CCTS-2–Ag^+, 25 mg of CCTS-2 were placed in a 100 ml flask with 25 ml $AgNO_3$ solution (0.5 mmol/l). The pH was adjusted to 6 using 1% dilute nitric acid (Section 3.4). After shaking for 3 h at room temperature, the product was filtered, washed with distilled water repeatedly and dried to obtain about 25 mg solid powder. Through the measurement of AAS, Ag^+ adsorption capacities of CCTS–Ag^+ and CCTS-2–Ag^+ were 43 and 38 mg Ag^+/g adsorbent.

Preparation of microorganism suspension: Take one or several colonies of microorganism in agar plates with sterile tampon into sterile saline (0.9%) solution, then diluted to 10^7–10^8 CFU/ml. Pepton broth agar cultural medium sterilized in a flask and cooled to 45–50 °C were distributed to sterilized petri dishes with a diameter of 9 cm. Spread bacteria suspension on solid agar plates. Sample powders containing 0.00355 mmol Ag^+ (9 mg CCTS–Ag^+, 10 mg CCTS-2–Ag^+, 0.6 mg $AgNO_3$) and 10 mg CCTS, 10 mg CCTS-2 were placed in assigned spots on agar plate, respectively. Then incubated at 37 °C for 24 h. At the end of the period, observe and record whether colonies were visible and inhibition zones were evaluated in millimeters. Studies were performed in triplicate.

3. Results and discussion

3.1. Elemental analysis

The elemental analysis results of CCTS, CCTS-1 and CCTS-2 are shown in Table 1. It could be seen that

Table 1
Elemental analysis results of CCTS, CCTS-1 and CCTS-2

	C% (wt)	H% (wt)	N% (wt)
CCTS	38.70	6.51	5.41
CCTS-1	37.52	6.50	5.31
CCTS-2	36.91	6.58	5.28

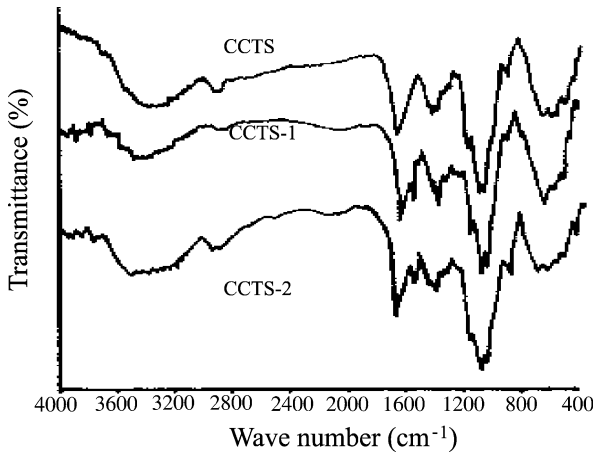

Fig. 2. Fourier transform infrared spectra of CCTS, CCTS-1 and CCTS-2.

the contents of nitrogen in CCTS-1 and CCTS-2 were a little lower than that in CCTS. It was thought that the decrease was due to the presence of the Schiff base and secondary amine types dibenzo-crown ethers produced in the reactions of CCTS-NH_2 with 4,4′-diformyldibenzo-18-c-6 crown ethers.

3.2. Infrared spectra analysis

Fig. 2 showed the infrared spectra of CCTS, CCTS-1 and CCTS-2. For CCTS-1 and CCTS-2, the intensity of the N–H and O–H stretching vibrations in the region of 3150–3200 cm^{-1} decreased, the characteristic peaks of aromatic backbone vibration appeared at 1560 and 1461 cm^{-1}, respectively, and the characteristic peaks of aromatic ether appeared at 1256 and 1069 cm^{-1}. For CCTS-1, the characteristic peak of C=N stretch vibration appeared at 1642 cm^{-1}, but it disappeared in the spectra of CCTS-2. It was also seen that CCTS, CCTS-1 and CCTS-2 had the characteristic peak of pyranoside vibration

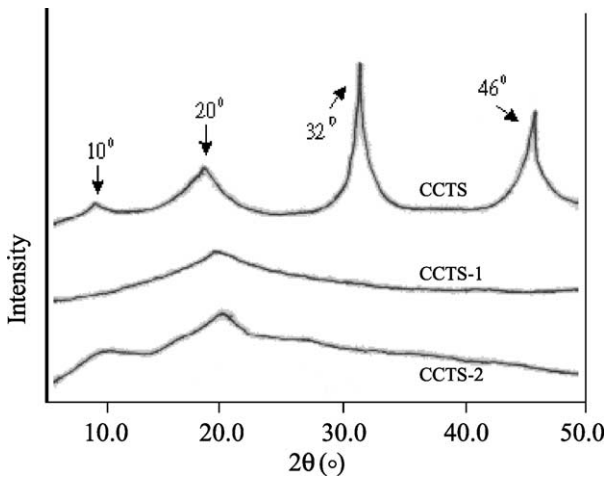

Fig. 3. X-ray diffraction patterns of CCTS, CCTS-1 and CCTS-2.

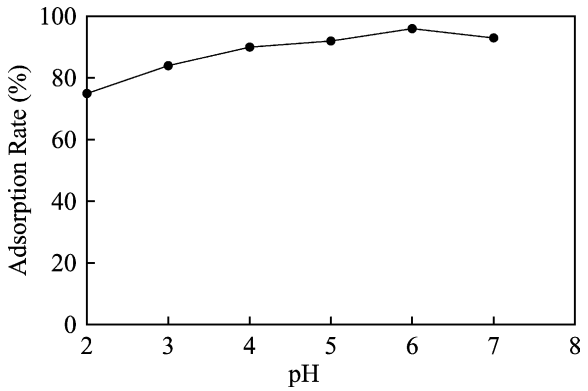

Fig. 4. Effect of pH on the adsorption efficiency of CCTS-2.

at 900 cm^{-1}. All of the evidence supported the introduction of crown ether groups in the crosslinked chitosan.

3.3. X-ray diffraction analysis

Fig. 3 showed the wide-angle X-ray diffraction (WAXD) patterns of CCTS, CCTS-1 and CCTS-2. The WAXD pattern of the showed the characteristic peaks at $2\theta = 10°$, 20°, 32° and 46°. For CCTS-1 the peaks at $2\theta = 10°$, 32° and 46° disappeared, and the characteristic peak at $2\theta = 20°$ decreased. For CCTS-2 the peaks at $2\theta = 32°$ and 46° disappeared, and the characteristic peaks at $2\theta = 10°$ and 20° decreased.

It can be thought that the decrease in crystallinity of chitosan derivatives was attributed to the deformation of the strong hydrogen bond in CCTS as the amino groups reacted with active formyl in 4,4′-diformyldibenzo-18-c-6 crown ethers. Both derivatives gave a low crystallinity, indicating that they were considerably more amorphous than chitosan.

3.4. Adsorption of CCTS-2 for Ag^+

According to the procedure mentioned earlier for 3 h, the adsorption of Ag^+ by chitosan derivatives was observed at different pH values. From Fig. 4, it could be seen that the adsorption of metal ion by CCTS-2 was pH sensitive.

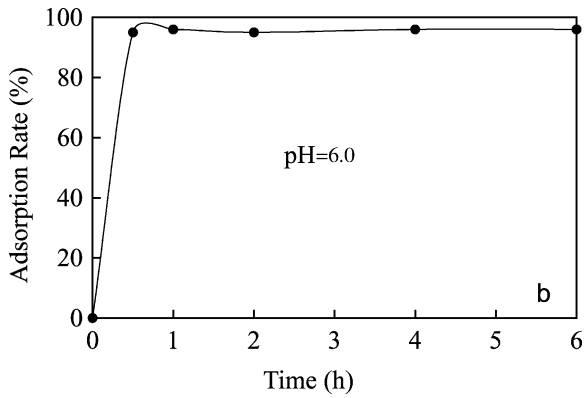

Fig. 5. The adsorption kinetic curve of CCTS-2 for Ag^+.

Table 2
The average diameters of inhibition zones against different bacteria

Sample	Weight (mg)	Ag^+ (mmol)	*Staphylococcus aureus* (mm)	*Escherichia coli* (mm)	*Pseudomonas aeruginona* (mm)
CCTS-2	10	0	0	0	0
CCTS-2–Ag^+	10	0.00355	11	10	7.5
CCTS–Ag^+	9	0.00355	11	10	6.0
CCTS	10	0	0	0	0
$AgNO_3$	0.6	0.00355	11	10	6.0

The uptake of metal ions by chitosan derivative showed a rise as the pH increased from 2 to 6. This could be due to the greater availability of free amino groups at higher pH values. The reduced adsorption of metal ions at acidic pH values could be attributed to the fact that at a lower pH the metal ions that would coordinate with the lone pair of nitrogen would have to compete with H_3O^+ for an active site (Jha, Iyengar, & Prabhakara Rao, 1988).

The adsorption kinetics of CCTS-2 for Ag^+ are shown in Fig. 5. The experimental results demonstrated that chitosan derivatives exhibited a considerable uptake of Ag^+. The increase in the adsorbed metal ion contents leveled off after exposure to chitosan derivatives and the adsorption rate was kept almost to constant until 6 h, indicating the attainment of adsorption equilibrium and forming stable Ag^+ complex. Longer contact time (>3 h) between chitosan derivatives and metal solutions have already been reported not to result in an increase in metal ion uptake (Deans & Dixon, 1992; Eiden, Jewell, & Wightman, 1980; Peniche-Covas, Alvarez, & Arguelles-Monal, 1992). It could be seen that the adsorption balance time was about 1 h for Ag^+ at pH 6, initial ion concentration 0.5 mmol/L. The adsorption rate of CCTS-2 for Ag^+ was 96% (1 h).

3.5. The bacteriostasis of complexes of CCTS, CCTS-2 and Ag^+

Table 2 presented the bacteriostasis of CCTS, CCTS-2, CCTS–Ag^+, CCTS-2–Ag^+ and $AgNO_3$ against three different bacteria. The results showed that the diameters of inhibition zone against *S. Aureus* and *E. coli* of CCTS-2–Ag^+ containing 0.00355 mmol Ag^+ were the same as those of CCTS–Ag^+ and $AgNO_3$ containing same mmol Ag^+, 11 and 10 mm, respectively. The bacteriostasis difference between CCTS–Ag^+ and $AgNO_3$ was little, but $AgNO_3$ decomposed easily while CCTS-Ag^+ and CCTS-2–Ag^+ was more stable than the former in experiment. In this experiment, the diameters of inhibition zone of CCTS, CCTS-2 toward these three bacteria were zero, which showed that CCTS, CCTS-2 cannot restrain these three bacteria in this experimental condition. This proved that the bacteriostasis in the same condition was derived from Ag^+ structure. CCTS-2–Ag^+ showed selective antimicrobial activity against *P. aeruginona*, whose diameter was the largest one, 7.5 mm while CCTS–Ag^+, $AgNO_3$ were 6.6 mm, respectively. It possibly resulted from the different affinity between the cell wall and CCTS–Ag^+, CCTS-2–Ag^+ and $AgNO_3$, which may be derived from their different structures.

4. Conclusion

By the reactions of CTS with epichlorohydrin and 4,4′-diformyldibenzo-18-c-6 crown ether, novel chitosan derivatives CCTS-1, CCTS-2 were prepared. Their adsorption and selectivity properties were studied. This study showed that the adsorption of silver ion by CCTS-2 by the chitosan derivative was optimum at pH 6.0 for Ag^+ with ion concentration 0.5 mmol/l and was very rapid intially within 1 h. The increase in the adsorbed metal ion contents that leveled off 6 h after exposure to chitosan derivatives indicated the attainment of adsorption equilibrium and formation of stable Ag^+ complex. The adsorption rate by CCTS-2 was 96% for Ag^+ for 1 h. As indicated by the experiment, the bacteriostasis zone diameters of CCTS-2–Ag^+ containing 0.00355 mmol Ag^+ against *S. Aureus*, *E. coli* and *P. aeruginona* are 11, 10, and 7.5 mm, respectively, while those of CCTS–Ag^+ at the same condition are 11, 10, and 6.0 mm. CCTS-2–Ag^+ compared with CCTS–Ag^+ and $AgNO_3$ has selective antibacterial effect towards *P. aeruginona* with good stability avoiding unstable lone Ag^+. This research will be useful for designing crosslinked-chitosan-based adsorption for medical bacteriostasis.

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