Asian Journal of Chemistry; Vol. 24, No. 9 (2012), 4022-4024

ASIAN JOURNAL OF CHEMISTRY

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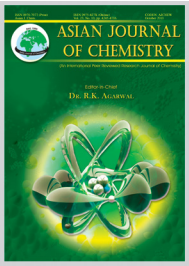

Synthesis and Antimicrobial Activities of a Quaternary Ammonium Salt of Chitosan†

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AJC-11303

A novel quaternary ammonium salt of chitosan (QAS-CTS) was prepared through a nucleophilic substitution reaction between chitosan (CTS) and a quaternary ammonium salt (QAS), N,N-dimethyl epoxypropyl octadecyl ammonium chloride was synthesized *via* a quaternization reaction between N,N-dimethyl-octadecylamine (ODA) and epichlorohydrin. FTIR was employed to characterize and confirm the structures of each product. Antimicrobial tests show that QAS-CTS has higher antimicrobial activity than both CTS and QAS against either *E. coli* or *B. subtilis*. The minimum inhibitory concentrations (MIC) of QAS-CTS against *E. coli* and *B. subtilis* are 0.6 and 0.4 mg/mL, respectively.

Key Words: Antibacterial agent, Quaternary ammonium salt, Chitosan, Antimicrobial activity.

INTRODUCTION

Chitin, a natural renewable polysaccharide widely found in shells of shrimps, crabs, insects and algae cell walls, is second largest biopolymer only to cellulose. Chitosan (CTS), a derivative of deacetylation of chitin, has apparent antibacterial activity against bacteria, fungi, even algae owing to its special molecular structure and polycationic nature[1-4]. However, chitosan is only soluble in acids or acidic solutions, which limits its scope of application and its antimicrobial ability is not high enough for finishing textiles. Therefore, solubility and antimicrobial modification of chitosan is needed. Different from many other antimicrobial agents, chitosan has high processability and reactivity, making it easier to be modified by common ways. Quaternary ammonium salts (QAS), a type of amphiphilic molecules, has some antimicrobial activity themselves. The combination of chitosan and QAS have improved chitosan in both its solubility and antimicrobial activity[5-7].

Here, we report a facile two-step method for preparation of a novel antimicrobial quaternary ammonium salt of chitosan (QAS-CTS). A quaternization reaction between N,N-dimethyl-octadecylamine (ODA) and epichlorohydrin was employed to synthesize a quaternary ammonium salt (QAS), followed by a nucleophilic substitution reaction between CTS and QAS. The antimicrobial activities of CTS, QAS and QAS-CTS were evaluated and compared.

EXPERIMENTAL

Chitosan (CTS) with 85 % degree of deacetylation was obtained from Zhejiang Jinqiao Biochemical Co. N,N-dimethyl-octadecylamine (ODA), epichlorohydrin, agar, peptone and beef extract were purchased from Sinopharm Chemical Reagent Co. All other chemicals involved were of analytical grade and used without further purification. The microorganisms tested (E. coli and *B. subtilis*) were provided by the Key Lab of Biotechnology of Hefei University.

Synthesis of N,N-dimethyl epoxypropyl octadecyl ammonium chloride (QAS): 40 mL epichlorohydrin and 200 mL isopropanol are mixed together, followed by dropwise addition of N,N-dimethyl-octadecylamine in a molar ratio of N,N-dimethyl-octadecylamine: epichlorohydrin = 1:1.2. The mixture was agitated for 4 h at 70 ºC. The conversion rate of reaction was detected every 1 h. After cooling down, part of isopropanol was evaporated out and acetone was added. Quaternary ammonium salt powder was filtered out, rinsed repeatedly with acetone and dried under vacuum below 40 ºC to give white powder.

Preparation of quaternary ammonium salt of chitosan (QAS-CTS): Chitosan and isopropanol are mixed together in a mass/volume ratio of chitosan: isopropanol = 1:40 (g/mL), followed by agitating at 40 ºC for 3 h for the sufficient swelling of chitosan. 15 % quaternary ammonium salt aqueous solution was added in a molar ratio of CTS:QAS = 1:4. The mixture

†Presented to The 5th Korea-China International Conference on Multi-Functional Materials and Application.

was agitated at 80 ºC for 12 h. After cooling down, acetone was added in to give slight yellow powder, which was subsequently rinsed repeatedly with acetone/ethanol (4:1, v/v) and dried under vacuum below 40 ºC. FTIR spectra were measured with a spectral resolution of 1 cm^{-1} on a Nicolet NEXUS 870 FTIR spectrophotometer using KBr powder at room temperature.

Antibacterial assessment: The antibacterial activities of CTS, QAS and QAS-CTS were evaluated using the agar plate method. Typically, a loopful of each culture was spread to give single colonies on nutrient agar (agar 15 g, peptone 10 g, beef extract 3 g, NaCl 3 g in 1 L water; pH 7.0) and incubated at 37 ºC for 24 h. A representative colony was picked out with a wire loop and place in nutrient broth (peptone 10 g, beef extract 3 g, NaCl 3 g in 1 L water; pH 7.0), which was then incubated at 37 ºC overnight. By appropriated diluting with sterilized distilled water, each culture containing *ca.* 10^5-10^6 CFU/mL was prepared which was used for the antibacterial test. Samples were dissolved in distilled water at various concentrations from 0.01-1.0 mg/mL and then were autoclaved at 121 ºC for 20 min. A loop of each suspension was inoculated on each nutrient medium with sample or control added. Then, the inoculated plates were incubated at 37 ºC for 48 h and the colonies were counted and the minimal inhibitory concentrations (MIC) values were obtained. Each test was repeated three times at least.

RESULTS AND DISCUSSION

FTIR characterization of QAS and QAS-CTS: Fig. 1 shows the FTIR spectra of CTS, QAS and QAS-CTS. In FTIR spectrum of QAS, peaks at 2925 and 2850 cm^{-1} are assigned to the asymmetric and symmetric stretching vibration of C-H; 1473 and 720 cm^{-1} are ascribed to the asymmetric and symmetric bending vibration of -CH_2; 955 cm^{-1} is designated to the epoxy ring vibration. In FTIR spectrum of CTS, the broad band around 3430 cm^{-1} is attributed to O-H and -NH_2 overlapped stretching vibration; band at 1650 cm^{-1} is assigned to amide bond vibration; 1606 cm^{-1} is ascribed to -NH_2 bending vibration and 1067 cm^{-1} is ascribed to O-C stretching vibration. QAS-CTS has a FTIR spectrum similar to the superimposed spectra of both CTS and QAS, also showing bands around 3430, 2925, 2850, 1650, 1473 and 720 cm^{-1}, but no -NH_2 bending vibration at 1067 cm^{-1} and epoxy ring vibration at 955 cm^{-1}, implying that -NH_2 on CTS and epoxy ring on QAS have undergone the nucleophilic substitution reaction to form QAS-CTS.

Comparison of antibacterial performance between CTS, QAS and QAS-CTS: CTS, QAS and QAS-CTS solutions with same concentration (0.6 mg/mL) were employed to test antibacterial performance against the common gram-negative bacterium (*E. coli*) and gram-positive bacteria (*B. subtilis*). Their inhibitory rates were calculated by the colony counting method and the results were summarized in Table-1.

As is indicated in Table-1, both CTS and QAS have certain antibacterial activities against ether *E. coli* or *B. subtilis*, according with previous reports. As predicted, QAS-CTS displays reasonably higher antibacterial activity than ether CTS or QAS, which may be explained as follows. Under normal circumstances, when contacting with bacteria, chitosan tends

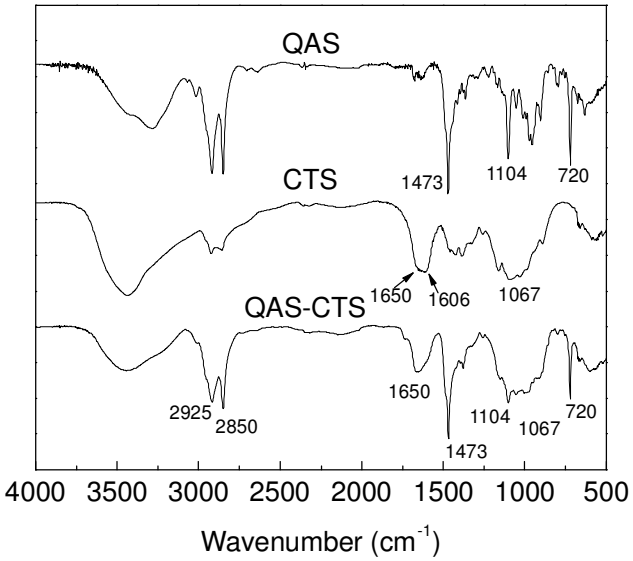

Fig. 1. FTIR spectra of CTS, QAS and QAS-CTS

TABLE-1
COMPARISON OF ANTIBACTERIAL RATES BETWEEN CTS, QAS AND QAS-CTS

Microorganisms		Sample			
		Control	CTS	QAS	QAS-CTS
E. coli	Colony No.	172	114	103	0
	Inhibitory rate (%)	–	34	41	100
B. subtilis	Colony No.	153	95	83	0
	Inhibitory rate (%)	–	38	46	100

to adsorb onto the surfaces of cells to form a layer of polymer film, which prevents nutrient transport into cells and thus obstructs the growth of bacteria. As for QAS, it may first take advantage of its long hydrophobic alkyl chains on one end to penetrate into the cells and then make use of the other positively-charged end to adsorb onto the cytoplasm anions, leading to flocculation inside cells, disrupting the normal physiological activities of cells, finally killing bacteria. Apparently, QAS-CTS possesses the structural features of both CTS and QAS and for this reason that QAS-CTS displays two antibacterial ways, one like CTS, the other like QAS, leading to the higher antibacterial activity of QAS-CTS than either CTS or QAS.

Antibacterial assessment of QAS-CTS: Figs. 2 and 3 display the culture media in which the tested microorganisms, *E. coli* and *B. subtilis*, have already been inoculated and incubated. As is seen, QAS-CTS shows significant antibacterial activities against both *E. coli* and *B. subtilis*. Comparing with the control group (Fig. 2a), QAS-CTS with 0.1 mg/mL of concentration (Fig. 2b) exhibits 52 % inhibitory rate and, its antibacterial effect improves apparently with the increase of concentration. 0.6 mg/mL QAS-CTS has completely inhibited the growth of *E. coli*. As shown in Fig. 3, it seems that QAS-CTS shows stronger antibacterial activity against Gram-positive bacteria. QAS-CTS with 0.1 mg/mL concentration (Fig. 3b) exhibits 77 % inhibitory rate against *B. subtilis* and, no growth is observed till the concentration was increased to 0.4 mg/mL.

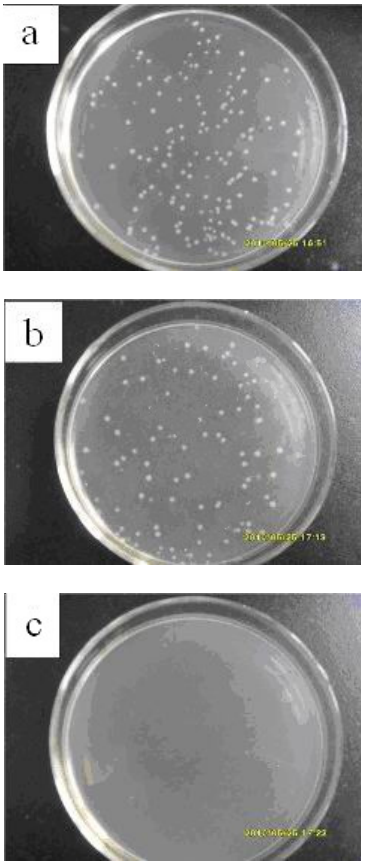

Fig. 2. Antibacterial effect of QAS-CTS in different concentrations on *E. coli*. (a) Control; (b) 0.1 mg/mL QAS-CTS; (c) 0.6 mg/mL QAS-CTS

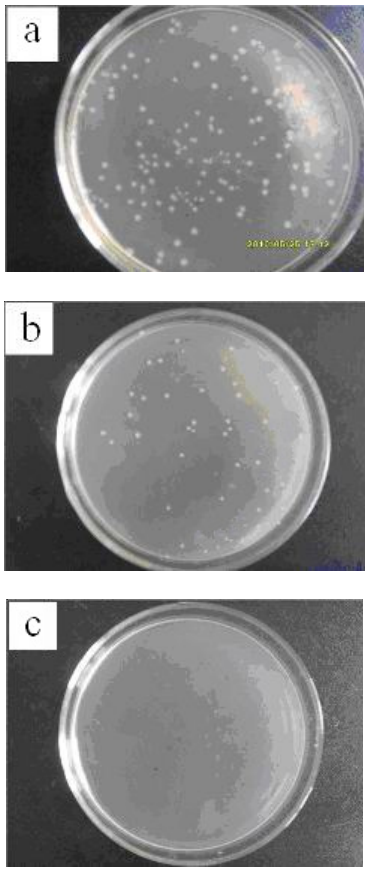

Fig. 3. Antibacterial effect of QAS-CTS in different concentrations on *B. subtilis*. (a) Control; (b) 0.1 mg/mL QAS-CTS; (c) 0.6 mg/mL QAS-CTS

Table-2 displays the detailed results expressing the antibacterial activities of QAS-CTS against *E. coli* and *B. subtilis*, from which the minimum inhibitory concentration (MIC) can be obtained. Herein, MIC is defined as the lowest concentration of compounds at which the microorganisms tested do not show visible growth. Therefore, MICs for *E. coli* and *B. subtilis* are 0.6 and 0.4 mg/mL, respectively, indicating that QAS-CTS has better antibacterial activity against *B. subtilis* than *E. coli*.

TABLE-2
ANTIBACTERIAL ASSESSMENT OF QAS-CTS IN DIFFERENT CONCENTRATIONS

Microorganisms		Concentration (mg/mL)						
		0.05	0.1	0.2	0.4	0.6	0.8	1.0
E. coli	No. (s)[a]	106	82	20	8	0	0	0
	No. (c)[b]				172			
	R (%)[c]	38	52	88	95	100	100	100
B. subtilis	No. (s)	91	35	5	0	0	0	0
	No. (c)				153			
	R (%)	41	77	97	100	100	100	100

[a]No. (s): The colony number in sample nutrient medium. [b]No. (c): The colony number in control nutrient medium. [c]R: Inhibitory rate, equaling to No./s divided by No./c.

Conclusion

A quaternary ammonium salt, N,N-dimethyl epoxypropyl octadecyl ammonium chloride was synthesized *via* a quaternization reaction between N,N-dimethyl-octadecylamine and epichlorohydrin with mild conditions. A novel quaternary ammonium salt of chitosan (QAS-CTS) was prepared through a nucleophilic substitution reaction between chitosan and the quaternary ammonium salt. FTIR analysis confirmed that QAS-CTS was successfully obtained with high yield. QAS-CTS displays much higher antibacterial activity than both CTS and QAS against either *E. coli* or *B. subtilis*. And it seems that QAS-CTS has better antibacterial activity against gram-positive bacteria than gram-negative bacteria.

ACKNOWLEDGEMENTS

This work was financially supported by the Natural Science Foundation of Anhui Educational Department (No. KJ2011A247) and the Natural Science Foundation of Anhui Province (No. 1208085MB24).

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