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Preparation, characterization and antimicrobial activity of 6-amino-6-deoxychitosan

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ABSTRACT

6-Amino-6-deoxychitosans with molecular weights from 0.23×10^4 to 1.41×10^4 and degree of substitution from 0.85 to 0.96 were prepared via N-phthaloylation, tosylation, azidation, hydrazinolysis and reduction of azide groups. Their structures were characterized by FT-IR, ^{1}H NMR, ^{13}C NMR, gel permeation chromatography (GPC) and elemental analysis. The antimicrobial activities of 6-amino-6-deoxychitosans against *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*, and *Aspergillus niger* were investigated. The results showed that 6-amino-6-deoxychitosans had a wide spectrum of effective antimicrobial activities. Compared with chitosan, 6-amino-6-deoxychitosans had much better antimicrobial activities. Their minimum inhibitory concentrations (MICs) were between 0.025% and 0.1% (w/v) in acetic/sodium acetate solution with different pH from 5.4 to 7.5. 6-Amino-6-deoxychitosans could also inhibit growth of bacteria tested in distilled water under pH 6.6–8.45. The antimicrobial mechanism was complex and the positive charge on the amino groups was not the sole factor resulting in the antimicrobial activities.

1. Introduction

Chitosan is a 1,4-β-linked copolymer consisting of 2-acetamido-2-deoxy-β-D-glucopyranosyl and 2-amino-2-deoxy-β-D-glucopyranosyl units. It is a partially or fully deacetylated derivative of chitin, which is the second most abundant natural biopolymer and obtained from the shells of Crustacea such as crabs, shrimps, lobsters, prawns, and cell walls of some fungi such as *Mucor rouxii*, *Absidia coerulea* and *Aspergillus niger* (Cai et al., 2006; Muzzarelli, Ilari, Tarsi, Dubini, & Xia, 1994). Chitosan has received much attention as a functional biopolymer due to its biological activities. Of all bioactivities of chitosan, the antimicrobial activity is particularly interesting and has been observed against the growth of many fungi, bacteria and yeasts. Chitosan has wide spectrum of activity and high killing rate against Gram-positive and Gram-negative bacteria, but lower toxicity toward mammalian cells (Fereidoon, Janak, & Jeon, 1999). Thus chitosan is widely used as an antimicrobial agent in the fields of food industry, cosmetics, biomedicine and agriculture.

Chitosan is a weak base and can be dissolved in dilute acid, but it is insoluble in water and other organic solvents. To improve antimicrobial activity of chitosan, it is an important method to prepare chitosan derivatives for improving its solubility, the content of amino group or positive charge density of chitosan. Usually quaternary ammonium salts of chitosan such as N-[(2-hydroxy-3-trimethylammonium)propyl]chitosan chloride (Peng et al., 2010), chitosan N-betainates (Asplund, Soininen, Nevalainen, & Järvinen, 2006), *N*,*N*,*N*-trimethyl chitosan, *N*–*N*-propyl-*N*,*N*-dimethyl chitosan, *N*-furfuryl-*N*,*N*-dimethyl chitosan (Jia, shen, & Xu, 2001; Rúnarsson et al., 2007) and methylated-(4-N,N-dimethylaminocinnamyl) chitosan chloride (Sajomsang, Gonil, & Saesoo, 2009) were prepared for increasing its antimicrobial activity because these derivatives were water-soluble, possessed permanent positive charge on the polysaccharide backbone and showed higher antimicrobial activity at neutral or alkaline environments. In addition, more amino groups were incorporated into chitosan to enhance its antimicrobial activity, too. Hu et al. reported synthesis of guanidinylated chitosan derivatives by the guanidinylation reaction of chitosan with aminoiminomethanesulfonic acid, and compared with chitosan, guanidinylated chitosan had much better antibacterial activity (Hu et al., 2007). A water-soluble chitosan derivative, arginine-functionalized chitosan with more amino groups in the sugar units has been prepared, too. It was able to inhibit almost all the bacteria, and its antibacterial activity could be related to its interaction with the cell membrane, in which it

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increased membrane permeability (Tang et al., 2010; Xiao, Wan, Zhao, Liu, & Zhang, 2011).

6-Amino-6-deoxychitosan is a chitosan derivative which 6-hydroxy groups are substituted by amino groups in chitosan molecule. It has two amino groups in the partial sugar units and higher positive charge density in acid solution compared to chitosan. Satoh et al. first prepared 6-amino-6-deoxychitosan from 6-deoxy-6-halo-N-phthaloylchitosan via 6-azidation (Satoh, Nagasaki, Sakairi, & Shinkai, 2004). 6-Amino-6-deoxychitosan and its galactosylated derivatives have been investigated as a gene carrier in COS-1 cells and HepG2 cells, respectively, which exhibited enhanced gene transfer efficiency (Satoh et al., 2006, 2007). Recently, 6-amino-6-deoxychitosan was used as suitable supports for Pd nanosized particles, and the catalytic activity of the supported Pd catalysts was evaluated with Suzuki–Miyaura and Heck carbon–carbon cross-coupling reactions (Makhubela, Jardine, & Smith, 2011). Moreover, trimethylated and triethylated 6-NH_2-6-deoxy chitosan were also synthesized, and they showed higher antibacterial activity against Gram-positive *Staphylococcus aureus* bacteria (Sadeghi et al., 2008). However, there are no papers about the antimicrobial activity of 6-amino-6-deoxychitosan, yet. In this paper, an appropriate procedure for preparing 6-amino-6-deoxychitosan was reported, which was that the primary hydroxyl groups were converted into the primary amino-groups from 6-tosyl-N-phthaloylchitosan via 6-azidation, and the antimicrobial activities of 6-amino-6-deoxychitosans obtained were evaluated against four different microorganisms.

2. Materials and methods

2.1. Materials

Chitosan (CS) from a crab shell was supplied by Zhejiang Aoxin Biotechnology Co., Ltd. (Taizhou, China), its molecular weight was 210 kDa and deacetylation degree was 92%. The molecular weight was measured by a gel permeation chromatography, and the deacetylation degree (DD) was determined by elemental analysis. Beef extract and peptone were purchased from Shanghai Chemical Agent Co. (Shanghai, China). Sodium borohydride and sodium azide were chemically pure reagents. All other chemicals were of reagent grade.

2.2. Preparation of 6-amino-6-deoxychitosan

2.2.1. Phthaloylation of chitosan

The phthaloylation of chitosan was according to modified method described in the literature (Kurita, Ikeda, Yoshida, Shimojoh, & Harata, 2002). 15 g of chitosan was suspended in 300 ml of DMF containing 5% (v/v) water and stirred overnight. Then 45.3 g of phthalic anhydride was added to the mixture and the mixture was heated in nitrogen at 120 °C with stirring. After 8 h of reaction, the resulting mixture was cooled to room temperature and poured into ice water. The precipitate was collected on a filter, washed three times with 500 ml of distilled water and 500 ml ethanol at room temperature, and dried to give 23.9 g of the product as a pale tan powdery material. The phthaloylated chitosan was named P-CS. The degree of substitution was 0.95, as determined by the C/N ratio of elemental analysis. Anal. Calcd for $(C_{14}H_{13}NO_6)_{0.92}(C_{16}H_{17}NO_8)_{0.03}(C_8H_{13}NO_5)_{0.05}\cdot 0.9H_2O$: C, 54.30; H, 4.89; N, 4.59. Found: C, 54.34; H, 4.92; N, 4.57.

2.2.2. Tosylation of phthaloylated chitosan

14.5 g of P-CS was added into a mixture solution of 12 ml of triethylamine and 138 ml of pyridine. Then tosyl chloride was added under vigorous stirring at room temperature. Three molar ratios of tosyl chloride to sugar residue (9:1, 6:1 and 3:1) were used. After 17 h of reaction, the mixture was poured into 1500 ml of distilled water with stirring. The precipitate was collected on a filter, washed repeatedly with distilled water until neutral, then washed three times with 500 ml ethanol and dried to give a solid. These tosylated phthaloylchitosans obtained were coded as PT-CS-1, PT-CS-2 and PT-CS-3, respectively. The reaction process was also performed twice with 3:1 molar ratio of tosyl chloride to sugar residue. The obtained product was coded as PT-CS-4.

2.2.3. Azidation of tosyl chitosan derivative

A mixture of tosylated phthaloylchitosan (50 mmol) and 125 ml dry DMF was treated with NaN_3 (9.75 g) at 100 °C for 20 h. The solution was then poured into 500 ml of ice water. The precipitate was collected, washed with distilled water, ethanol and ether, and then dried in vacuo at 50 °C to give the product. The phthaloylchitosan azide derivatives obtained were coded as PA-CS-1, PA-CS-2, PA-CS-3 and PA-CS-4. PA-CS-2 was treated with NaN_3 using the above same process again. The product was coded as PA-CS-5.

2.2.4. Removal of N-phthaloyl groups and reduction of azide group

Phthaloylchitosan azide derivative (20 mmol) was suspended in 131 ml of distilled water. Then 66 ml of hydrazine monohydrate was added. After 10 h of reaction at 70 °C, the solution was concentrated under reduced pressure until dry. The process of concentration was repeated after addition of 100 ml H_2O. The resulting solid was washed with 200 ml of distilled water, 200 ml of ethanol and 50 ml of ether, and dried to give 6-azide-6-deoxy chitosan (A-CS-1, A-CS-2 A-CS-3, A-CS-4 and A-CS-5).

6-Azide-6-deoxy chitosan (10 mmol) was suspended in 160 ml of DMSO, and stirred for 60 min at 60 °C, then $NaBH_4$ (8.08 g) was added. 50 ml of distilled water was added after 70 h of reaction. The solution was adjusted pH to 5.5 with acetic acid. The mixture was dialyzed against pH 9.0 of dilute NaOH solution for 24 h, and then adjusted pH to 6.5 with acetic acid, and dialyzed against distilled water for 48 h. The dialysate was concentrated under reduced pressure below 45 °C, and lyophilized to obtain 6-amino-6-deoxychtosan (N-CS-1, N-CS-2, N-CS-3, N-CS-4 and N-CS-5).

2.3. Characterization

Weight average molecular weight (Mw) and degree of polydispersity (DP) of sample were measured by a gel permeation chromatography (GPC). GPC system incorporated in a TSP P100 instrument (USA). The TSK G3000-pw column (Japan) was used. The eluent was 0.2 M HAc/0.1 M NaAc buffer with a flow rate of 1.0 ml/min. The temperature of the column was maintained at 30 °C. The eluent was monitored with RI 150 refractive index detector. The sample concentration was 5 mg/ml. The standards used to calibrate the column were TOSOH pullulan of defined Mw ranging from 2.7 to 788 kDa. All data provided by the GPC system were collected and analyzed using the Jiangshen Workstation software package.

C, H, N elemental analysis was performed using a Carlo-Erba 1106 elemental analyzer.

FT-IR spectra were recorded with KBr pellets on a Nicolet FT-IR 5700 spectrophotometer (USA). 32 scans at a resolution of 4 cm^{-1} were averaged and referenced against air.

^1H and ^{13}C NMR spectra were recorded on Varian NMR-400 NMR spectrometer (USA) at ambient temperature. The samples were dissolved in D_2O.

2.4. Microorganisms and culture conditions

Staphylococcus aureus ATCC 25923, *Escherichia coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853 were provided by the

Typical Culture Collection Center in Wuhan University, China. *A. niger* was provided by the Biology Engineering College of Hubei University of Technology, China. The cultures obtained by growing the bacteria overnight at 37 °C in nutrient broth were diluted with sterile normal saline (0.9%) solution, and each of the culture suspensions containing ca. 10^6–10^7 CFU/ml was used for the antibacterial test.

2.5. Evaluation of antibacterial activity in vitro

CS in 1% (w/v) acetic acid was adjusted to pH 5.4 by the addition of 2 mol/l NaOH aqueous solution. The 6-amino-6-deoxychitosans were dissolved in 0.1% (w/v) aqueous acetic acid solution, and the different pH values of these solutions were adjusted to 5.4, 6.5 and 7.5 by addition of 0.5 mol/l NaOH aqueous solution. The concentration of sample solutions was 1% (w/v). All sample solutions were autoclaved at 121 °C for 15 min. The above sample solutions were diluted 2-fold serially, and each of these solutions (1 ml) and nutrient agar (peptone 1%, beef extract 0.5%, NaCl 0.5%, agar 2%, 9 ml) were mixed and poured into autoclaved petri-dishes. The solvents with different pH values were used as controls instead of samples. A loop of microorganism suspension was inoculated on cooled nutrient agar with sample or control added. After inoculation, the petri-dishes were incubated at 37 °C. Observed and recorded whether colonies were visible with naked eye after regular incubation time. All the experiments were triplicate. Minimum inhibitory concentration (MIC) was defined as the lowest concentration of the tested sample at which the microorganism colonies were not visible with naked eye within 16–38 h.

3. Results and discussion

3.1. Preparation of 6-amino-6-deoxychitosan and characterization

3.1.1. Phthaloylation of chitosan and characterization

Protection by phthaloyl groups was chosen for the amino groups of chitosan. Phthaloylation of chitosan was performed in DMF/water (v/v, 95/5) with excess phthalic anhydride at 120 °C for 8 h using Kurita's method (Kurita et al., 2002). FT-IR spectra of chitosan and N-phthaloyl chitosan were shown in Fig. 1. The FT-IR spectra of chitosan derivatives showed two strong absorptions at 1777 cm^{-1} and 1712 cm^{-1} assigned to the stretching vibration of C=O. The twin absorptions are characteristic of imide. This suggests the cyclic phthalimide was formed. The strong absorption at 721 cm^{-1} was due to the bending vibration of C–H in the aromatic ring, and indicated there were 1,2-bissubstitution in the aromatic ring. The bands at 1290 and 1260 cm^{-1} assigned to C–O stretching vibration of ester did not appear. Degree of substitution calculated from the C/N value of elemental analysis was 0.95. These indicated N-phthaloylation was complete, and only very few ester groups could be formed because esterification was inhibited by water produced in the N-phthaloylation process and in the mixed solvent (Kurita et al., 2002).

3.1.2. Tosylation of phthaloylated chitosan and characterization

In order to obtain high degree of substitution, molar ratios of tosyl chloride to sugar residue used were 3, 6 and 9. FT-IR spectra of tosyl chitosans were shown in Fig. 1. The spectra showed two characteristic absorption bands, one at 1176 cm^{-1} describing an S=O stretching vibration and the other at 814 cm^{-1} indicating a symmetrical C–O–S vibration associated to a C–O–SO_2 group. From the spectra of PT-CS-1, PT-CS-2 and PT-CS-3, the absorption intensities of these bands increased gradually with molar ratios of tosyl chloride to sugar residue. It suggested the degree of substitution increased gradually, too. PT-CS-4 was the product obtained after tosylation was performed twice with 3:1 molar ratio of tosyl chloride to sugar residue. Its absorption intensities of these bands were strongest. It showed higher DS could be reached with small amount of tosyl chloride and increase of reaction times. In addition, the bands at ~1025 cm^{-1} and ~1068 cm^{-1} were related to C–O stretching vibration of primary hydroxyl group and second hydroxyl group, respectively (Chen, Du, Wu, & Xiao, 2002). In the spectra, the bands at 1025 cm^{-1} disappeared, and the absorption intensity at 1176 cm^{-1} and 814 cm^{-1} was very strong, indicating the primary hydroxyl group was substituted highly. The absorption intensity at ~1068 cm^{-1} reduced, suggested partial tosylation occurred in second hydroxyl group.

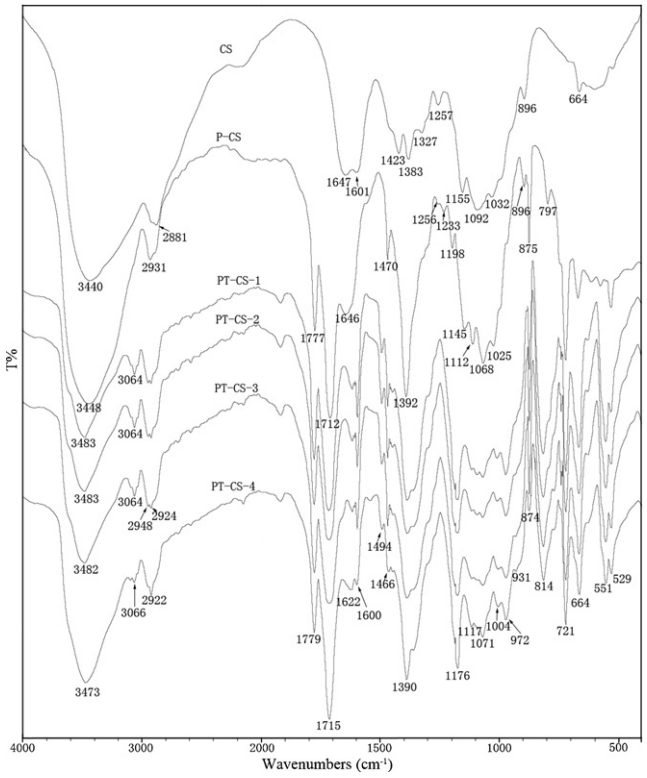

Fig. 1. FT-IR spectra of chitosan (CS), phthaloylchitosan (P-CS), and tosylated phthaloylchitosan derivatives (PT-CS-1, PT-CS-2, PT-CS-3 and PT-CS-4).

3.1.3. Preparation of azidochitosan derivatives and characterization

Azidation of the tosyl chitosan derivatives with sodium azide in DMF was carried out at 100 °C. FT-IR spectra of azido derivatives obtained were shown in Fig. 2. The strong absorption band at 2108 cm^{-1} was due to stretching vibration of azide group. The band at 1178 cm^{-1} due to S=O stretching vibration still existed, and the band at 814 cm^{-1} originating from C–O–SO_2 group was shifted to 820 cm^{-1}, but the absorption intensity became weak, indicating substitution of azide group for tosyl group was incomplete. The absorption intensities of the bands at 1178 cm^{-1} and 820 cm^{-1} were in the order of PA-CS-1 > PA-CS-2 > PA-CS-3. It could be because substitution of azide groups for tosyl groups of C-3 in the sugar residue was difficult due to the steric hindrance. For PA-CS-4, the absorption intensities of the bands were weaker than those of PA-CS-1. It could be because there were more tosyl groups at C-6 in the PT-CS-4. PA-CS-5 was the product which PA-CS-2 was treated with NaN_3. Compared with PA-CS-2, their absorption intensities of the bands at 1178 cm^{-1} and 820 cm^{-1} almost were the same. It indicated further that the tosyl groups unsubstituted could be at the C-3 position of chitosan.

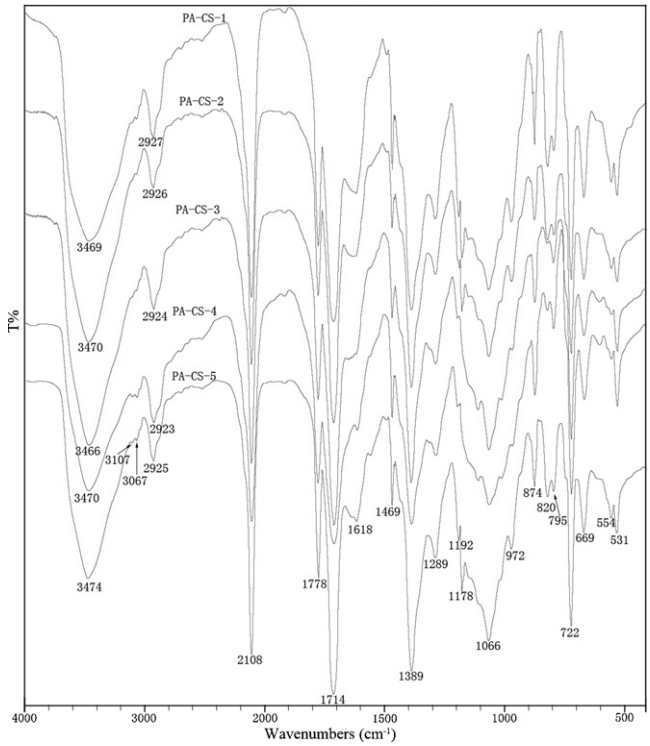

Fig. 2. FT-IR spectra of phthaloylchitosan azide derivatives (PA-CS-1, PA-CS-2, PA-CS-3, PA-CS-4 and PA-CS-5).

3.1.4. Removal of N-phthaloyl groups and reduction of azide group

Removal of N-phthaloyl groups was preformed by basic hydrolysis with hydrazine monohydrate. FT-IR spectra of 6-azide-6-deoxychitosan obtained after removal of N-phthaloyl groups were shown in Fig. 3. Compared with the FT-IR spectra of N-phthaloyl chitosan, two strong absorptions at ~1777 cm^{-1} and ~1712 cm^{-1} assigned to the C=O stretching vibration of N-phthaloyl groups and the characteristic band at ~721 cm^{-1} due to the C–H bending vibration in the aromatic ring disappeared. The band at 1589 cm^{-1} due to the bending vibration of amino groups appeared. These showed that N-phthaloyl groups were

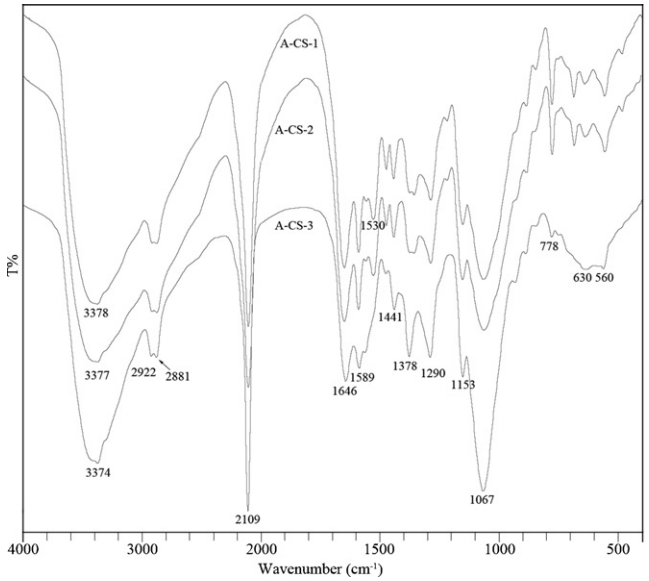

Fig. 3. FT-IR spectra of azidochitosan derivatives (A-CS-1, A-CS-2 and A-CS-3).

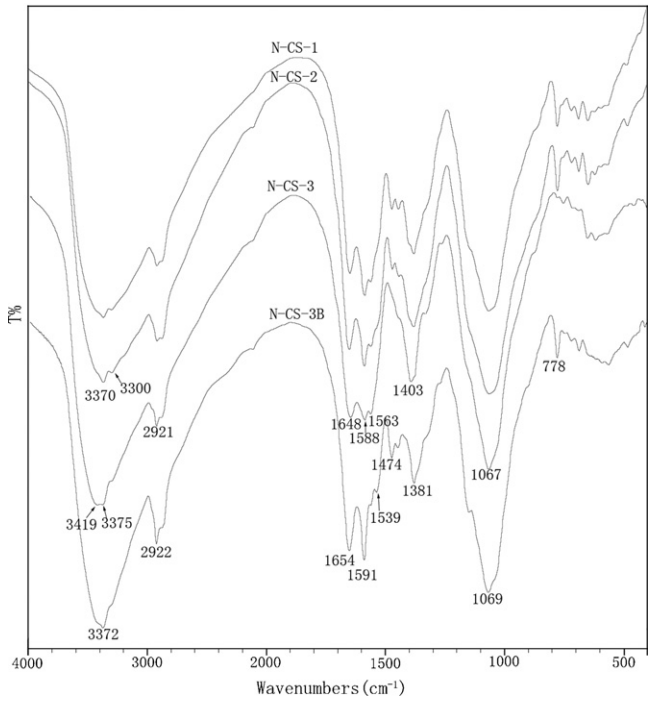

Fig. 4. FT-IR spectra of 6-amino-6-deoxy chitosans (N-CS-1, N-CS-2, N-CS-3) and 6-amino-6-deoxy chitosan treated by NaOH (N-CS-3B).

removed. However, the band at ~1646 cm^{-1} assigned to amides I vibration became very strong in the spectra. This suggested that partial N-phthaloyl groups still existed. It could be due to incomplete cyclization at the amino group in the phthaloylation reaction process and/or hydrolytic cleavage of imide ring in the hydrazinolysis process. In addition, the band at ~820 cm^{-1} originating from C–O–SO_2 group disappeared, too. It indicated that tosyl group was removed in the hydrazinolysis process.

Converting azide groups into amino groups based on reacting azido derivatives of chitosan with sodium borohydride. FT-IR spectra of 6-amino-6-deoxychitosans obtained were shown in Fig. 4. Compared with the FT-IR spectra of azido derivatives of chitosan, the absorption band of azide groups at 2109 cm^{-1} disappeared. The absorption band at ~1588 cm^{-1} was attributed to the bending vibration of amino group. Compared with the FT-IR spectrum of CS, the absorbance of these bands increased, which indicated that the content of amino group increased. These suggested azido groups were converted into amino groups. The C–O stretching band at 1032 cm^{-1} related to the primary hydroxyl group disappeared in the spectra of 6-amino-6-deoxychitosan, verifying a high amination of 6-OH. The strong absorption band at 1560 cm^{-1} was due to asymmetrical COO^- stretching vibration, and the strong absorption band at 1404 cm^{-1} was due to symmetrical COO^- stretching vibration. It indicated that partial amino groups existed as amine salt. N-CS-3B was the product obtained that N-CS-3 was treated with dilute NaOH aqueous solution to pH 9.0 and after dialysed, the dialysate was concentrated and lyophilized. In the FT-IR spectrum of N-CS-3B, the two strong absorption bands at 1560 cm^{-1} and 1404 cm^{-1} disappeared, and the absorption band at 1591 cm^{-1} due to the bend vibration of $–NH_2$ became stronger. This showed acetate was removed and the amino groups in N-CS-3B molecule were almost uncharged. At the same time, it showed further that the amino groups were partially protonated in the 6-amino-6-deoxychitosan such as N-CS-1, N-CS-2, N-CS-3, N-CS-4 and N-CS-5, too.

The ^1H NMR and DEPT 135 ^{13}C NMR spectra of 6-amino-6-deoxychitosan N-CS-2 were shown in Fig. 5. In the ^1H NMR

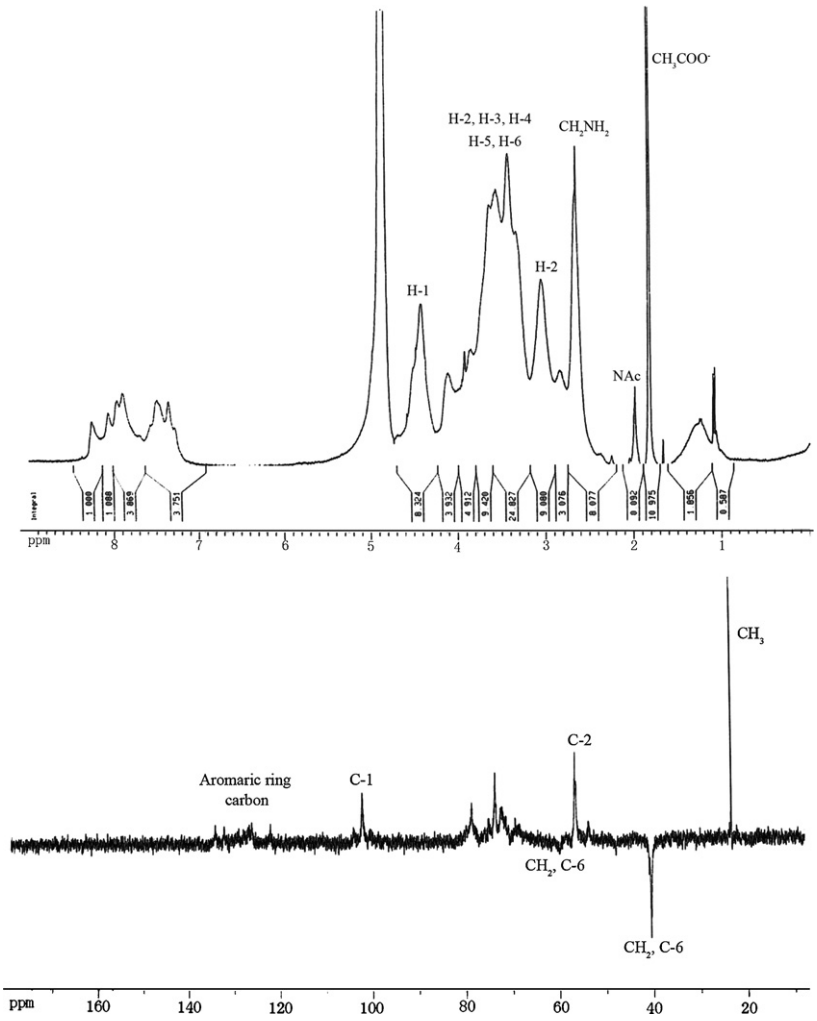

Fig. 5. ^{1}H NMR and DEPT 135 ^{13}C NMR spectra of 6-amino-6-deoxy chitosan (N-CS-2).

spectrum, the peaks at 1.83 ppm were assigned to the ^{1}H signals of CH_3COO^-, which indicated that amino groups partially existed as $-NH_3^+$. The peaks at 1.99 ppm were assigned to the proton signals of acetyl groups. The peak at 2.65 ppm was assigned to the proton signals of $-CH_2NH_2$ at C-6, which suggested the amino groups were incorporated into the C-6 position of chitosan. The peak at 3.05 ppm was assigned to the proton signals of $-CHNH_2$ at C-2. The peaks at 3.10–4.10 ppm were assigned to the ^{1}H signals at C-2–C-6. The peaks at 4.40–4.60 ppm were assigned to the ^{1}H signals at anomeric carbon. The peaks at 7.30–8.20 ppm were assigned to the proton signals at aromatic ring, which indicated the removal of the N-phthaloyl groups was incomplete. In the DEPT 135 ^{13}C NMR spectrum N-CS-2, the signals at 122–134 ppm were attributed to the aromatic ring, which indicated further the removal of the N-phthaloyl groups was incomplete. The signals at 102 ppm were attributed to anomeric carbon. The signals at 69–79 ppm were attributed to C-3, C-4 and C-5 at sugar ring; the signals at 53–57 ppm were attributed to C-2. The reverse peak at 40 ppm was attributed to C-6. It was shifted to higher field compared with ~61 ppm signal of C-6 at chitosan, which indicated this carbon was linked to amino group. In the spectrum, the peak at ~61 ppm about $-CH_2OH$ was very weak, which suggested most hydroxyl groups had been substituted with amino groups.

Weight average molecular weights of 6-amino-6-deoxychitosans measured by GPC were shown in Table 1. The Mws of 6-amino-6-deoxychitosans ranged from 0.23×10^4 to 1.41×10^4 and were very low. This suggested that chitosan was degraded severely in the reaction process. N-CS-5 was the products obtained via twice reactions with NaN_3. Compared with N-CS-2, its Mw was lower. It indicated that degradation was companied in the reaction process, which could be due to the β-elimination in an alkaline condition (Whistler & BeMiller, 1958). From N-CS-1 to N-CS-3, their Mws gradually increased. It was because the rate of degradation reaction became slow as tosyl chloride used decrease gradually. N-CS-4 was obtained via twice tosylation reactions with 3:1 molar ratio of tosyl chloride to sugar unit. Compared with N-CS-3, its Mw decreased, which could be caused by acid degradation in the tosylation process. Therefore, degradation of chitosan in the reaction process likely involved both alkaline degradation and acid degradation. The substitution degrees of 6-amino-6-deoxychitosan with different molecular weights were calculated from the elemental analysis and ^{1}H NMR data. As can be seen from Table 1, the degree of substitution of 6-amino-6-deoxychitosan was high, which ranged from 0.85 to 0.96.

3.2. Antimicrobial activity of 6-amino-6-deoxychitosan

The minimum inhibitory concentrations (MICs) of chitosan and its derivatives 6-amino-6-deoxychitosan against a gram-positive bacterium: *S. aureus*, two gram-negative bacteria: *E. coli* and *P. aeruginosa*, and a fungus: *A. niger* were shown in Table 2. In

Table 1
Molecular structure parameters of 6-amino-6-deoxychitosan.

Sample	Elemental analysis			Mw × 10^{-4}	DP	N-phthaloyl group[a]	NH_3^+ group[b]	DS[c]
	C (%)	N (%)	H (%)					
N-CS-1	45.03	10.90	6.76	0.23	3.12	0.30	0.73	0.94
N-CS-2	45.04	10.75	6.32	0.65	3.65	0.29	0.73	0.90
N-CS-3	43.65	10.26	6.73	1.41	1.84	0.26	0.77	0.85
N-CS-4	44.07	11.24	6.84	0.95	1.93	0.20	0.84	0.96
N-CS-5	43.63	10.41	6.59	0.40	2.86	0.28	0.74	0.89

[a] Molar rate of the group to sugar residue, based on ^{1}H NMR.
[b] Molar rate of the group to sugar residue, based on N-phthaloyl group and CH_3COO^- from ^{1}H NMR.
[c] DS of 6-OH, based on the C/N ratio of elemental analysis.

pH 5.4 of acetic acid–sodium acetate solution, chitosan and 6-amino-6-deoxychitosan showed effective antimicrobial activity against gram-positive bacteria and gram-negative bacteria, and MIC values of chitosan were 0.1% (w/v), but those of 6-amino-6-deoxychitosans were 0.025–0.1% (w/v). The minimum MIC value was only one quarter of chitosan's. 6-amino-6-deoxychitosan also showed effective antifungal activity against *A. niger* at 0.1% (w/v) concentration, but chitosan could not. In general, chitosan displays antibacterial activity only in an acid environment. It has been suggested the antimicrobial activity of chitosan in an acid environment was strongly dependent on the positive charge at C-2 (Helander, Nurmiaho-Lassila, Ahvenainen, Rhosdes, & Roller, 2001; Rabea, Badawy, Stevens, Smagghe, & Steurbaut, 2003). 6-Amino-6-deoxychitosans showed higher antibacterial activity than chitosan. This could be because 6-amino-6-deoxychitosans possessed higher positive charge densities in an acid solution.

The effect of pH on the antimicrobial activity of 6-amino-6-deoxychitosan was studied, too. The results were shown in Table 2. Chitosan did not show antibacterial activity at pH > 6.5, because of its poor solubility and also because it does contain less positive charge on the amino groups (Holappa et al., 2006; Liu, Guan, Yang, Li, & Yao, 2001). However, in the MIC data of 6-amino-6-deoxychitosans against bacteria and fungus tested (Table 2), it was found that 6-amino-6-deoxychitosan showed antimicrobial activity at pH 6.5 and 7.5. At pH 7.5, the MICs of 6-amino-6-deoxychitosans against bacteria and fungus could still reach 0.05% or 0.1% (w/v). For N-CS-1 and N-CS-4, their MICs against *S. aureus* and *E. coli* were basically smaller at lower pH. It was not surprising, since the charge density on the polysaccharide backbone was greatly influenced by pH. When pH increased, their charge density reduced. But, for N-CS-2, N-CS-3 and N-CS-5, when pH increased from 5.4 to 7.5, their MICs against *E. coli* remained unchanged, and were 0.1%, 0.05% and 0.1% (w/v), respectively. Likewise, the MICs of N-CS-1, N-CS-3 and N-CS-5 against *A. niger* were 0.1% (w/v) at the whole pH range investigated. These indicated that the positive charge density was not the sole factor resulting in the antimicrobial activity of 6-amino-6-deoxychitosan.

Furthermore, the antimicrobial activity of 6-amino-6-deoxychitosan dissolved in distilled water was investigated, too (Table 3). N-CS-1 and N-CS-4 were acetate salt of 6-amino-6-deoxychitosan, and pH values of their aqueous solutions were 6.6 and 6.28, respectively. Their MICs were consistent with those in pH 6.5 of acetic acid–sodium acetate solution. Although it has been observed that adsorption of chitosan with high deacetylation degree to *E. coli* increased with ionic strength (Strand, Vårum, & Østgaard, 2003), the antimicrobial activities of 6-amino-6-deoxychitosan seem not to be affected by ionic strength of solution. This could be because their positive charge densities in two solvents were almost equal, and when 6-amino-6-deoxychitosan had high positive charge densities, the positive charge density could be a more important factor for the antimicrobial activity than ionic strength.

N-CS-2B, N-CS-3B and N-CS-5B were the products obtained after N-CS-2, N-CS-3 and N-CS-5 were treated with different amount dilute NaOH aqueous solution, dialyzed, concentrated and lyophilized. Their pH values in distilled water were 7.51, 8.45 and 7.28, respectively (Table 3). For N-CS-3B, it did not almost contain the positive charge (see Fig. 4), but its MIC in the distilled water against *S. aureus* was 0.05% (w/v) (Table 3), and the same as that in pH 5.4 of acetic acid–sodium acetate solution. In basic condition, its antimicrobial action might be that it acted as a chelating agent rendering metals, trace elements, or essential nutrients unavailable

Table 2
MICs of 6-amino-6-deoxychitosan in acetic acid/sodium acetate solution with different pH.

Sample	pH	*S. aureus*	*E. coli*	*P. aeruginosa*	*A. niger*
Chitosan	5.4	0.1%	0.1%	0.1%	>0.1%
N-CS-1	5.4	0.05%	0.05%	0.025%	0.1%
	6.5	0.1%	0.05%	0.05%	0.1%
	7.5	0.1%	0.1%	0.05%	0.1%
N-CS-2	5.4	0.05%	0.1%	0.025%	0.05%
	6.5	0.05%	0.1%	0.05%	0.1%
	7.5	0.1%	0.1%	0.1%	0.1%
N-CS-3	5.4	0.05%	0.05%	0.025%	0.1%
	6.5	0.1%	0.05%	0.025%	0.1%
	7.5	0.1%	0.05%	0.05%	0.1%
N-CS-4	5.4	0.05%	0.05%	0.05%	0.1%
	6.5	0.05%	0.05%	0.05%	0.1%
	7.5	0.1%	0.1%	0.1%	>0.1%
N-CS-5	5.4	0.05%	0.1%	0.05%	0.1%
	6.5	0.1%	0.1%	0.05%	0.1%
	7.5	0.1%	0.1%	0.05%	0.1%
Solvent	5.4	(0.1%)[a]	(0.1%)	(0.1%)	(0.1%)
	6.5	(0.1%)	(0.1%)	(0.1%)	(0.1%)
	7.5	(0.1%)	(0.1%)	(0.1%)	(0.1%)

[a] No antimicrobial activity at the concentration in bracket.

Table 3
MICs of 6-amino-6-deoxychitosan dissolved in distilled water.

Sample	pH	*S. aureus*	*E. coli*	*P. aeruginosa*	*A. niger*
N-CS-1	6.6	0.1%	0.05%	0.05%	0.1%
N-CS-2B[a]	7.51	0.05%	0.1%	0.05%	0.1%
N-CS-3B[a]	8.45	0.05%	>0.1%	>0.1%	0.1%
N-CS-4	6.28	0.05%	0.05%	0.05%	0.1%
N-CS-5B[a]	7.28	0.05%	0.05%	0.05%	0.1%

[a] 6-Amino-6-deoxychitosan was treated with dilute NaOH aqueous solution, and after dialysed, the dialyzate was concentrated and lyophilized.

for the organism to grow at a normal rate (Jung, Kim, Choi, Lee, & Kim, 1999; Roller & Covill, 1999) because it possessed both $-NH_2$ and $-COO^-$. In addition, Strand et al. (2003) found that the chitosan adsorption of chitosan to *E. coli* cells increased strongly with pH. Thus, it was also likely that it could interact and form polyelectrolyte complexes with acidic polymers produced at the bacterial cell surface (e.g., lipopolysaccharide, teichoic and teichuronic acids, or capsular polysaccharide) (Muzzarelli et al., 1990). However, the antimicrobial activity of N-CS-3B against gram-negative bacteria decreased in the distilled water and its MIC was >0.1% (w/v). It has been proposed that chitosan and its derivatives with low molecular weight can penetrate to the nuclei of the microorganisms, bind with DNA, and interfere with the synthesis of mRNA and proteins (Sudarshan, Hoover, & Knorr, 1992). Thus, the antimicrobial activity of 6-amino-6-deoxychitosans could be much more related to the mechanism under the alkaline condition. Tokura, Ueno, Miyazaki, and Nishi (1997) reported chitosan with Mw < 5000 can penetrate into *E. coli* cells. Mw of N-CS-3 was 1.41×10^4. So it was possible that N-CS-3B could not enter into *P. aeruginosa* cells and *E. coli* cells, but could penetrate into *S. aureus* cells. This could be because cell membrane structures of gram-negative bacteria were different from gram-positive bacteria' (Kong, Chen, Xing, & Park, 2010). At the same time, the MICs of 6-amino-6-deoxychitosans remained unchanged or decrease slightly when pH increase from 5.4 to 7.5 (Table 2). It could be another important factor that 6-amino-6-deoxychitosans with low Mws penetrate into the microbial cells besides the affect of the positive charges. In addition, the MICs of N-CS-2B against *S. aureus* and *P. aeruginosa* and N-CS-5B against *S. aureus* and *E. coli* in distilled water were 0.05% (w/v), and lower than those of N-CS-2 and N-CS-5 in pH 7.5 of acetic acid-sodium acetate solution (Tables 2 and 3), respectively. It could also be related to ionic strength of solution besides the structure of polysaccharide and microbial cell membrane, and acetic acid and sodium acetate in the solution could inhibit the penetration of 6-amino-6-deoxychitosans into the microbial cells in the alkaline solution.

4. Conclusion

In this study, the synthesis of 6-amino-6-deoxychitosan was achieved by a five-step procedure, but molecular degradation was very severe in the reaction process, and Mws of 6-amino-6-deoxychitosans obtained only ranged from 0.23×10^4 to1.41×10^4. Investigation on the antimicrobial activity of 6-amino-6-deoxychitosan against *S. aureus*, *E. coli*, *P. aeruginosa* and *A. niger* showed it had wide spectrum of antimicrobial activity and better activity than chitosan at pH 5.4. Different from chitosan, 6-amino-6-deoxychitosan still had the antimicrobial activity not only in acetic/sodium acetate solution with pH 6.5 and 7.5 but also in distilled water under pH 6.6–8.45. The antimicrobial mechanism of 6-amino-6-deoxychitosan was complex. In the acidic solution, the positive charge density in the backbone could be a main factor influencing the antimicrobial activity, but in the alkaline condition, penetration of 6-amino-6-deoxychitosans with low Mws into microbial cell to inhibit the transcription from DNA could take a more important role, and the penetration could be inhibited by ions in the solution, too. More detailed studies should be done to further make clear the mode of antimicrobial action.

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