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Non-toxic *O*-quaternized chitosan materials with better water solubility and antimicrobial function

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ABSTRACT

Five water-soluble *O*-quaternary ammonium salt-chitosans (QAS-CS) bearing *N*-methyl-*N*-R-*N*, *N*-*bis*(2-hydroxyethyl) ammonium bromides (*R* = -benzyl (chloride, BNQAS-CS), -dodecyl (C12QAS-CS), -tetradecyl (C14QAS-CS), -hexadecyl (C16QAS-CS), -octadecyl (C18QAS-CS)) were prepared, respectively. They were characterized by FTIR, ^{1}H NMR and elemental analysis. Through chemical modification of *O*-quaternized chitosans, the water solubility of all QAS-CS was improved distinctly. Their antibacterial properties indicate good antibacterial abilities against gram-positive bacteria and bad against gram-negative bacteria, therein, C12QAS-CS and C14QAS-CS are the best. More importantly, their cytotoxicity was markedly lower than the corresponding QAS monomers by evaluating for AT2 cell line using CCK-8 assay. The strategy provides a facile way to design and develop new types of antibacterial chitosan materials with better water solubility, better antimicrobial ability and lower cytotoxicity for primary additive agent of self-owned intellectual band-aids.

1. Introduction

Chitosan (CS) is a linear polysaccharide with non-toxic, good biodegradability, biocompatibility and antimicrobial activity, which makes it potentially useful for biomedical applications, including an antimicrobial agent either alone or blended with other polymers. [1–3] The antimicrobial activity of chitosan against a variety of bacteria and fungi coming from its polycationic nature is well known. However, this activity is limited above pH 6.5, where CS starts to lose its cationic nature and become poor soluble. In order to improve the solubility, physicochemical properties and bioactivity, several chemical modifications of CS have been reported in the past decades. Almost all CS derivatives are synthesized through the structural modifications for -NH_2 [4–11] and –OH [12,13], such as pyridyl-substituted CS [14–16] or carboxymethyl CS, [17–20] quaternary ammonium salt-chitosans (QAS-CS).

Quaternized chitosan and its derivatives are successfully carried out by using iodomethane, iodoethane, dimethylsulfate, or grafting with a compound that containing the quaternary ammonium moiety itself. They have two major advantages over the parent chitosan: water-solubility and permanent positive charge [1]. The prepotent quaternization is on the primary amino group of the C-2 position in chitosan with above-mentioned reagents. After the structural modifications for -NH_2, the disappearance of the primary amino group which can be as the hydrogen bonding donors to bond biological target seriously influence the biological activity of chitosan derivatives. However, chitosan derivatives with only the structural modifications for -OH are very rare. And the quaternized chitosan for -OH is prepared by reacting chitosan with glycidyl quaternary ammonium moiety, such as *O*-(2-hydroxyl) propyl-3-trimethyl ammonium chitosan chloride (*O*-HTCC). [12,13] The results showed that the active amino groups in the chitosan chains play an important role in the antioxidant activity.

Recently, our group designed and synthesized a series of novel dihydroxy quaternary ammonium salts (QAS) with long chain alkyl bromides (BNQAS, C12QAS, C14QAS, C16QAS, and C18QAS) [21]. Their antibacterial properties indicated good antibacterial abilities against *Escherichia coli*, *Staphylococcus aureus*, *Bacillus subtilis*, and the best antifungal activities against *cytospora mandshurica*, *botryosphaeria ribis*, *physalospora piricola and glomerella cingulata*. The antibacterial activities are influenced by the alkyl chain length [22,23]. As the growth of the alkyl chain, the antibacterial activities gradually increase, until they reach a reasonable limit. The long

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chain alkyl group in QAS can change the geometric arrangement of QAS, which can help two flexible hydroxyl groups of QAS to pass through cell membrane into the cell to passivate enzyme and damage bacteria [24,25]. However, their structural features and high cytotoxicity limit their direct application for novel clinical antibacterial materials. Recently, self-owned intellectual band-aids with antibacterial and hemostatic function are studying in our group, so non-toxic and possessing antibacterial quaternized chitosans can be as essential additive agent to improve the antibacterial hemostatic function and biocompatibility.

In this paper, five QAS, BNQAS, C12QAS, C14QAS, C16QAS and C18QAS was successfully grafted into the free -OH of chitosan and generated five water-soluble *O* -quaternary ammonium salt-chitosan derivatives (Scheme 1), BNQAS-CS, C12QAS-CS, C14QAS-CS, C16QAS-CS, C18QAS-CS. They were characterized by FTIR, ^{1}H NMR and elemental analysis. Their antibacterial properties against gram-positive bacteria: *S. aureus*, *α-H-tococcus*, *β-H-tococcus*, gram-negative bacteria: *E. coli*, *Pseudomonas aeruginosa*, *Proteus vulgaris*, and two funguses: *Aspergillus*, *Canidia albicans* were tested by the methods of qualitative test (inhibition zone method) and quantitative test (oscillation method). In addition, their cytotoxicity for AT2 cell line was evaluated using CCK-8 assay.

2. Materials and methods

2.1. Materials

CS (MW 3 kDa, all with 85% deacetylation, elemental analysis (%) found: C 45.19, H 6.80, N 8.36.) was obtained from Shandong Haidebei Biotechnology Co., Ltd. (Shandong, China). Sodium carboxymethyl glucan biological amino colloid (Shuerta, 500 ppm, 50 mL) was purchased from Shanxi Pierfu Biotechnology Co., Ltd. (Shanxi, China). *N*-methyldiethanolamine (MDEA), dimethylaminopyridine (DMAP), 4-toluene sulfonyl chloride (TsCl) and *p*-toluenesulfonyl chloride were purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China) and were used as obtained without further purification. BNQAS, C12QAS, C14QAS, C16QAS, and C18QAS were prepared according to a literature [21]. *S. aureus* (ATCC6538), *α-Hemolyticstrep-tococcus* (α-*H-tococcus*, CMCC(B) 31005), β-Hemolyticstrep-tococcus (β-*H-tococcus*, ATCC21059), *E. coli* (ATCC25922), *P. aeruginosa* (ATCC9027), *P. vulgaris* (CMCC(B) 49027) and fungus (*Aspergillus* (CMCC(F) 98003), *Canidia Albicans* (ATCC10231)) were purchased from American Type Culture Collection, Manassas, USA.

2.2. Methods

Infrared (IR) samples were prepared as KBr pellets, and spectra were obtained in the 400–4000 cm^{-1} range using a Nicolet MAGNA-IR 550 FTIR spectrometer. Elemental analyses were performed on a Perkin–Elmer Model 2400 analyzer. ^{1}H NMR data were collected using a Bruker Avance 400 spectrometer. Chemical shifts were reported in δ relative to TMS. The viscosity was determined by the capillary viscometry method using an 1835-Ubbelohde viscometer (Shanghai Meter Experimental Factory).

2.3. Synthesis

2.3.1. Synthesis of QAS p-toluenesulfonate

A 50 mL chloroform solution with QAS (0.02 mol, BNQAS, C12QAS, C14QAS, C16QAS, C18QAS, respectively), DMAP (0.001 mol) and triethylamine (0.015 mol) was cooled to 0 °C. 150 mL Chloroform with TsCl (1.9 g, 0.01 mol) was added dropwise under magnetic stirring over a 30-min period and the temperature did not exceed 5 °C. When the addition was completed, the mixture was allowed to warm slowly to room temperature with stirring for 2 h (monitored by TLC). After added 100 mL diethyl ether, the mixture was kept at −20 °C about 4 h, and then filtrated. The oil crude products were purified on silica gel by column using petroleum ether/EtOAc (5:1, v/v) as the eluent to afford QAS *p*-toluenesulfonate.

2.3.2. BNQAS p-toluenesulfonate

Yield: 45%. ^{1}H NMR (400 MHz, D_2O) δ 7.64, 7.26(d, 4H, -$C_6H_4CH_3$), 7.37, 7.30(m, 5 H, -C_6H_5), 4.37(s, 2 H, -$CH_2C_6H_5$), 3.86–3.35(m, 8H, -NCH_2CH_2O-), 2.83(s, 3H, -NCH_3), 2.27(s, 3H, -$C_6H_4CH_3$). IR (KBr Pellet cm^{-1}): 3395(br), 2952(s), 2884(s), 2602(s), 2494(s), 1772(s), 1723(s), 1458(s), 1365(s), 1177(s), 1036(s), 992(s), 922(s), 852(s), 760(m), 709(m).

2.3.3. C12QAS p-toluenesulfonate

Yield: 33%. ^{1}H NMR (400 MHz, D_2O) δ 7.64–6.93(d, 4H, -$C_6H_4CH_3$), 4.40–3.36(m, 8H, -NCH_2CH_2O-), 3.00(s, 3H, -NCH_3), 2.12(s, 3H, -$C_6H_4CH_3$), 1.36, 1.08, 0.68(m, 25H, -$C_{12}H_{25}$). IR (KBr Pellet cm^{-1}): 3300(br), 2923(s), 2853(s), 2675(m), 2491(m), 1731(m), 1645(m), 1598(m), 1466(s), 1375(m), 1217(s), 1177(s), 1121(s), 1084(s), 1034(s), 1010(s), 848(m), 682(m).

2.3.4. C14QAS p-toluenesulfonate

Yield: 31%. ^{1}H NMR (400 MHz, D_2O) δ 7.61–7.15(d, 4H, -$C_6H_4CH_3$), 3.85–3.35(m, 8H, -NCH_2CH_2O-), 2.99(s, 3H, -NCH_3), 2.16(s, 3H, -$C_6H_4CH_3$), 1.25, 1.08, 0.73 (m, 25H, -$C_{14}H_{29}$). IR (KBr Pellet cm^{-1}): 3317(br), 2923(s), 2853(s), 1597(m), 1465(s), 1365(s), 1217(s), 1177(s), 1121(m), 1096(m), 1033(s), 1010(s), 921(m), 815(s), 762(m), 721(m).

2.3.5. C16QAS p-toluenesulfonate

Yield: 29%. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.76–7.13(d, 4H, -$C_6H_4CH_3$), 4.01–3.36(m, 8H, -NCH_2CH_2O-), 3.26(s, 3H, -NCH_3), 2.31(s, 3H, -$C_6H_4CH_3$), 1.64, 1.21, 0.85(m, 25H, -$C_{16}H_{33}$). IR (KBr Pellet cm^{-1}): 3418(br), 2921(s), 2851(s), 1735(m), 1629(s), 1467(s), 1401(s), 1384(s), 1260(m), 1088(s), 1052(s), 721(m).

2.3.6. C18QAS p-toluenesulfonate

Yield: 18%. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.80–7.40(d, 4H, -$C_6H_4CH_3$), 4.50(s, 1H, -OH), 4.11–3.46(m, 8H, -NCH_2CH_2O-), 3.35(s, 3H, -NCH_3), 2.46(s, 3H, -$C_6H_4CH_3$), 1.72, 1.23, 0.84(m, 25H, -$C_{18}H_{37}$). IR (KBr Pellet cm^{-1}): 3415(br), 2922(s), 2851(s), 1723(m), 1632(s), 1465(s), 1385(s), 1255(m), 1090(m), 1033(s), 921(m), 721(m).

2.4. Synthesis of CS-BA

CS (4.0 g, 24 mmol) was dissolved in 60 mL acetic acid solution (10%), and 60 mL ethyl alcohol was added into this solution. Under stirring, 40 mL ethyl alcohol with benzaldehyde (BA, 12.7 g, 120 mmol) was added dropwise. The reaction system was maintained at 70 °C for 4 h. The mixture was adjusted pH into neutral with 40% sodium hydroxide solution. The crude product was collected by filtration and further purified in ethyl alcohol. Upon Soxhlet extraction by anhydrous ethanol more than 24 h, the light yellow solids of benzal chitosan (CS-BA) were obtained. Yield: 85%. ^{1}H NMR (400 MHz, CF_3COOD) δ 8.61(s, 1H, -CH=N-), 7.82(d, 3H, -C_6H_5), 7.50(s, 2H, -C_6H_5), 5.4–3.4(m, 9.38H, CS), 2.04 (s, 1H, Acetyl-).

2.4.1. Synthesis of QAS-CS

CSBA (3.8 g, 15 mmol) and QAS *p*-toluenesulfonate (100 mmol) was dissolved in 100 mL isopropanol and 15 mL 40% NaOH solution. The mixture was stirred for 24 h at 80 °C. After decanted 50 mL acetone and 50 mL ethanol, yellow solids were obtained by filtration. The yellow solids were dissolved in 150 mL 0.25 M HCl and ethanol