Synthesis, characterization, and the antioxidant activity of the acetylated chitosan derivatives containing sulfonium salts

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Abstract

In this study, a new class of chitosan derivatives possessing sulfonium salts was synthesized, and characterized by FT-IR, \textsuperscript{1}H NMR, \textsuperscript{13}C NMR, and elemental analyses. IR spectra, \textsuperscript{1}H NMR and \textsuperscript{13}C NMR of the structural units of these polymers validated the designed chitosan derivatives were successfully synthesized. In addition, the antioxidant potential of chitosan and chitosan derivatives was assessed in vitro, screened by 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging, hydroxyl radical scavenging, and superoxide radical scavenging, respectively. Results revealed that designed chitosan derivatives could effectively scavenge DPPH radical, hydroxyl radical, and superoxide radical with inhibition rate of more than 90\% at 1.6 mg/mL, higher than chitosan. Moreover, in the cytotoxicity assay, no cytotoxicity was observed for the L929 cells with chitosan and its derivatives at all the testing concentrations. These results indicated that the acetylated chitosan derivatives containing sulfonium salts may be a promising natural antioxidant for the pharmaceutics, food, cosmetics and agriculture management.

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1. Introduction

The free radicals have been implicated in pathogenesis of various diseases in human body, including cancer, diabetes mellitus, inflammatory diseases, neurodegenerative disorders and ageing. Oxygen free radicals are products of normal cellular metabolism, the delicate balance about free radicals is a very important aspect of living organisms and is achieved by mechanisms called redox regulation. The most common basic biochemical reactions are the redox reactions which occur virtually in all living organisms. Redox (Reduction oxidation) reactions are reactions which involving electron transfer between atoms and molecules (during the redox reaction, the reducing agent is oxidized, i.e. loses electrons, which are transferred to the oxidant that is reduced, i.e. gains electrons). Free radicals contain unpaired electrons, it can inhibit their normal function by damage cellular lipids, proteins, or DNA. If the polymer that has an ability to donate one or two electrons that has a great binding affinity with the free radicals leading to successful maintenance of the structural configuration and integrity followed by protect of the cell. These cationic polymers such as chitosan and its derivatives are being widely sought as an excellent reducing agent and antioxidant [1,2].

Chitin is a common constituent of the shells of crustaceans, furthermore, it is often present in fungi, yeasts, diatoms sponges, corals, moluscs, and worms. It is insoluble in water so that it is the major source of surface pollution in coastal areas. But when chitin is fully or partially N-deacetylated, it will turn into chitosan which is composed of randomly distributed β-(1&4)-linked D-glucosamine and N-acetyl-D-glucosamine [3,4]. As a linear, natural cationic biopolymer, a great number of studies have shown that chitosan have various biological activities due to adsorption, electrostatic interactions and entrapment, such as antioxidant, antimicrobial, hypcholesterolemic, immunity-enhancing and antitumor effects, drug delivery, and accelerating Calcium and Ferrum absorption and so on [5,6]. Chitosan has received considerable attention for diverse applications, especially in pharmaceutics, food, cosmetics, agriculture, environmental protection, and wastewater management [7–10].

Because both chitosan and its oligosaccharides showed antioxidant effects, it has been reported that chitosan derivatives can be used as excellent antioxidants to control free-radical oxidation by means of chemical modification on three types of reactive functional groups including an amino/acetamido group at the C-2, as well as both primary and secondary hydroxyl groups at the C-3 and C-6 positions, respectively [11,12]. Studies on the biological activities of chitosan and its chemical modification have been increasing, as no single type of chitosan or its...
derivative exerts the most excellent biological activities. To date, a large number of chitosan derivatives have been synthesized via quaternization, carboxylation, and phosphorylation. Therefore, in order to increase the biological activity of polysaccharide, our research team has devoted many efforts on the preparation of functional polysaccharide derivatives bearing quaternization, carboxylation, and phosphorylation groups by different chemical modification pathways, but the research of chitosan derivatives containing sulfonium salts are uncommon.

The rule, the thioether linkage within bioactive molecules was believed to improve medicinal properties which can cure tumor cells [13,14]. A polymer that mentioned combination of the antioxidant activity of chitosan and the biological activity of thioether with superior antioxidant activity was obtained, it is necessary to attach polar functional groups (ex. thioether compounds) to the chitosan backbone chain. It is worth mentioning that chitosan derivatives containing cationic sulfonium salts investigated herein turned out to have excellent antioxidant activity against DPPH-radical, hydroxyl-radical, and superoxide-radical. In this study, as a testing intermediate, the Br-CS was first synthesized by the reaction between chitosan and bromoacetyl chloride [15–17]. Subsequently, the thioether groups were introduced into Br-CS through the reaction of alkylate to obtain chitosan derivatives containing cationic sulfonium salts [18]. The chemical structure of the derivatives was characterized by FT-IR, elemental analysis, 1H NMR, and 13C NMR spectra. Moreover, the antioxidant activity and cytotoxicity of chitosan which contained sulfonium salts of different chain length have also been evaluated.

2. Experimental

2.1. Material

Chitosan with degree of deacetylation of 0.735 and molecular weight of 5–8 kDa was purchased from Golden–Shell Pharmaceutical Co. Ltd. (Zhejiang, China). Dimethyl sulfoxide, Ethyl Sulfide, Propyl sulfide, Butyl sulfide, and bromoacetyl chloride were purchased from Sigma-Aldrich Chemical Corp (Shanghai, China). Ethanol, acetone, DMF (N,N-Dimethylformamide), and DMSO (Dimethyl sulfoxide) were all analytical grade and used without further purification, were all purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China).

The extent of functionalization (degree of substitution) in chitosan derivatives was performed using the elemental analyses by combustion. The detection of elemental carbon, hydrogen, nitrogen and sulfur in chitosan derivatives were performed on a Vario EL III (Elementar, Germany). The samples should be completely wrapped with the foil before analysis. The degrees of substitution (DS) of chitosan derivatives were evaluated on basis of the ratios of carbon and nitrogen or carbon and sulfur according to the following equations:

\[ DS_1 = \frac{n_1 \times M_C - M_N \times W_{C/N}}{n_2 \times M_C} \]  
\[ DS_2 = \frac{M_N \times W_{C/N} + n_2 \times M_C \times DS_1 - n_1 \times M_C}{M_C} \]  
\[ DS_3 = \frac{(n_1 - n_2 \times DS_1 + n_2 \times DS_2) \times M_C}{M_{C/S}} \times m_1 \times M_S - n_4 \times M_C \]  
\[ DS_4 = \frac{n_1 - n_2 \times DS_1 + n_2 \times DS_2 \times M_C}{M_{C/S}} \times m_1 \times M_S - n_5 \times M_C \]  
\[ DS_5 = \frac{n_1 - n_2 \times DS_1 + n_2 \times DS_2 \times M_C}{M_{C/S}} \times m_1 \times M_S - n_6 \times M_C \]  
\[ DS_6 = \frac{n_1 - n_2 \times DS_1 + n_2 \times DS_2 \times M_C}{M_{C/S}} \times m_1 \times M_S - n_7 \times M_C \]

where DS1, and DS2 represent the deacetylation degree of chitosan, the acetylated degree of chitosan derivative, DS3, DS4, DS5, and DS6 represent the degree of substitution of thioether groups in chitosan derivatives. MC, MN, and MS are the molar mass of carbon, nitrogen and sulfur, MC = 12, MN = 14, MS = 32; n1, n2, n3, n4, n5, n6, and n7 are the number of carbon of chitin, a acetyl groups, two acetyl groups, two thioether groups (Dimethyl sulfide, Ethyl Sulfide, Propyl sulfide, Butyl sulfide), n1 = 8, n2 = 2, n3 = 4, n4 = 4, n5 = 8, n6 = 12, n7 = 16, WC/N represents the mass ratio between carbon and nitrogen, and WC/S represents the mass ratio between carbon and sulfur.

The chemical structure of both all chitosan and its modified products were characterized by Jasco-4100 Fourier Transform Infrared Spectrometer (Japan, provided by JASCO Co., Ltd. Shanghai, China). All the spectrum were collected in transmission mode (T) and samples were prepared with KBr powder as a pellet and scanned against a blank KBr pellet as a background at wave number range 4000–400 cm−1 with a resolution of 4 cm−1 in 16 scans at 25 °C.

Nuclear magnetic resonance spectra (1H NMR and 13C NMR) were carried out on a Bruker AVIII-500 Spectrometer (Switzerland, provided by Bruker Tech. and Serv. Co., Ltd. Beijing, China), using tetramethylsilane (TMS) as an internal standard and D2O as solvent at 25 °C.

2.2. Synthesis

2.2.1. Synthesis of acetylated chitosan (Br-CS) [19]  
Br-CS was synthesized as follows: 1.62 g (10 mmol) of chitosan was dissolved in 35 mL of DMF (N,N-dimethylformamide) at room temperature, the reaction system is now neutral. After that 30 mmol of bromoacetyl chloride was added dropwise. The solution was stirred for 24 h at 30 °C, and then was continued stirring for 6 h at 60 °C, the reaction system is acidic. When the system was cooled to room temperature, excess absolute ethyl alcohol was added to the flask to precipitate the concentrated solution, and the precipitate was filtered. The products were washed with ethyl alcohol for three times and freeze dried overnight in vacuum, yield: 34.53% (Scheme 1).

2.2.2. Synthesis of the acetylated chitosan derivatives containing sulfonium salts (MACS/EACS/PACS/BACS) [20]  
A solution of Br-CS (10 mmol) and dimethyl sulfide (30 mmol), ethyl Sulfide (30 mmol), propyl sulfide (30 mmol), butyl sulfide (30 mmol) in 50 mL of DMSO (dimethyl sulfoxide) was refluxed at 60 °C for 24 h, respectively. When the temperature returns to room temperature, the solutions were precipitated with excess acetone, filtered, washed carefully with ethyl alcohol. The product was freeze dried overnight in vacuum, yield: MACS 58.62%, EACS 55.95%, PACS 50.53%, BACS 46.87% (Scheme 1).

2.3. Antioxidant activity assay

2.3.1. DPPH radicals’ scavenging ability assay  
The test of DPPH-radical scavenging ability was evaluated according to some researchers’ methods with some modifications [21]. The method is as follows: the stock solution was prepared by dissolving 35.49 mg DPPH with 500 mL ethyl alcohol and then stored in a dark place, which was away from light. Then 2 mL of freshly prepared DPPH as the source of free radicals was added into 1 mL of chitosan and its derivatives solution containing various concentrations (0.1, 0.2, 0.4, 0.8, and 1.6 mg/mL) at 25 °C for 20 min in dark place. Control group: 2 mL DPPH was took place of the 2 mL ethanol. Vc (Vitamin C) was used as a positive control and the absorbance was determined at 517 nm.

Scavenging index (%) = \[ \frac{1 - \frac{A_{\text{Sample} \ 517\text{nm}}}{A_{\text{control} \ 517\text{nm}}}}{A_{\text{blank} \ 517\text{nm}}} \times 100 \]  

The DPPH radical scavenging activity was calculated by the following Formula (7).
where $A_{\text{sample} \ 517 \text{nm}}$ represents the absorbance of the sample with ethanol solution of DPPH at 517 nm after incubation, $A_{\text{control} \ 517 \text{nm}}$ represents the absorbance of sample with ethanol solution at 517 nm after incubation, $A_{\text{blank} \ 517 \text{nm}}$ represents the absorbance of deionized water and ethanol solution of DPPH at 517 nm after incubation.

### 2.3.2 Hydroxyl-radical scavenging activity assay

The formation of OH from Fenton reagents was quantified with H$_2$O$_2$ oxidation. We measured the hydroxyl radical scavenging activity according to a previously reported method with some modifications [22]. The method is as follows: the phosphate-buffered saline (pH = 7.4) was prepared first. Then a solution of H$_2$O$_2$ (30%) and safiranine T (360 μg mL$^{-1}$) were prepared in phosphate-buffered saline (pH = 7.4). A solution of EDTA–Fe$^{2+}$ (2 mM) was prepared in water. 1 mL sample solution with concentration of 0.1, 0.2, 0.4, 0.8, and 1.6 mg/mL, was incubated with EDTA–Fe$^{2+}$ solution (0.5 mL), phosphate-buffered saline (pH = 7.4, 1 mL), safiranine T (1 mL), and H$_2$O$_2$ (1 mL). Control group: 1 mL H$_2$O$_2$ was took place of the 1 mL phosphate-buffered saline. After incubating at 37 °C for 30 min, the absorbance of the samples was measured at 520 nm using Vc as a positive control. Three replicates for each sample were tested and the Hydroxyl radical’s scavenging ability was calculated by the following Formula (8):

$$ \text{Scavenging effect} \% = \frac{A_{\text{control} \ 520 \text{nm}} - A_{\text{blank} \ 520 \text{nm}}}{A_{\text{control} \ 520 \text{nm}}} \times 100 $$

where $A_{\text{sample} \ 520 \text{nm}}$ represents the absorbance of the sample with configured test solution at 520 nm after incubation, $A_{\text{control} \ 520 \text{nm}}$ represents the deionized water with configured test solution without hydrogen peroxide solution at 520 nm after incubation, $A_{\text{blank} \ 520 \text{nm}}$ represents the absorbance of deionized water and configured test solution at 520 nm after incubation.

### 2.3.3. Superoxide-radical scavenging activity assay

We measured the superoxide radical scavenging activity based on some previous methods of research with some modifications [23]. Briefly, the Tris–HCl buffer (16 mM, pH 8.0) were prepared first and then the solution of NADH (365.7 μg/mL), nitro blue tetrazolium (NBT 245.3 μg/mL), and PMS (18.38 μg/mL) was prepared in Tris–HCl buffer (pH = 8.0). 1 mL of sample solution at various concentrations (0.1, 0.2, 0.4, 0.8, and 1.6 mg/mL) was mixed with Tris–HCl buffer (0.5 mL), NADH (0.5 mL), NBT (0.5 mL), and PMS (0.5 mL), the mixture was shaken vigorously and reacted at 25 °C for 30 min in dark place. Control group: 0.5 mL NADH was took place of the 0.5 mL Tris–HCl buffer. The absorbance of samples was determined at 560 nm using Vc as a positive control. Three replicates for each sample were tested and the superoxide radical scavenging ability was calculated by the following Formula (9):

$$ \text{Scavenging effect} \% = \frac{1 - A_{\text{sample} \ 560 \text{nm}} - A_{\text{control} \ 560 \text{nm}}}{A_{\text{blank} \ 560 \text{nm}}} \times 100 $$(9)

where $A_{\text{sample} \ 560 \text{nm}}$ represents the absorbance of the sample with configured test solution at 560 nm after incubation, $A_{\text{control} \ 560 \text{nm}}$ represents the sample with configured test solution that the nicotinamide adenine dinucleotide reduced (NADH) solution was replaced the Tris–HCl buffer at 560 nm after incubation, $A_{\text{blank} \ 560 \text{nm}}$ represents the absorbance of deionized water and configured test solution at 560 nm after incubation.

The microplate reader absorbance of the tested mixture was measured with a DNM-9602G microplate reader (China, Pulang New Technology Co. LTD., Beijing, China). Data analysis was processed by computer programs Excel (Microsoft, Redmond, WA, DC, USA) and Origin 8 (Origin Lab, Northampton, MA, USA) and the data were expressed as mean ± SD. Values of P < 0.05 were considered to be statistically significant.

### 2.4. Cytotoxicity assay

The cells are the mouse fibroblasts that also calls “Mouse connective tissue L cell line 929 clone”, were cultured in RPMI medium supplemented with 1% penicillin and streptomycin and 10% fetal bovine serum at 37 °C and maintained under 5% CO$_2$ air atmosphere. The cell viability of all sample’s derivatives was evaluated by CCK-8 assay in vitro with minor modification [24]. L929 cell lines were cultured in a 96-well plate and incubated for 24 h at 37 °C in a 5% CO$_2$ humidified atmosphere, and increasing concentrations of the samples (0–1000 μg/mL) were added and incubated for 24 h. After 24 h, the absorbance was read using Elx808 microplate reader (Biotek, America) at a wavelength of 450 nm. The cells treated with culture medium alone considered as a control, reported as percentage of control (as 100%), and recording cell viability using the following equation [10]:

$$ \text{Cell viability} \% = \frac{A_{\text{sample} \ 450 \text{nm}} - A_{\text{blank} \ 450 \text{nm}}}{A_{\text{negative} \ 450 \text{nm}} - A_{\text{blank} \ 450 \text{nm}}} \times 100 $$

where $A_{\text{sample} \ 450 \text{nm}}$ is the absorbance of the samples (containing cells, CCK-8 solution, and sample solution) at 450 nm, $A_{\text{blank} \ 450 \text{nm}}$ is the absorbance of the blank (containing RPMI medium and CCK-8 solution) at 450 nm, $A_{\text{negative} \ 450 \text{nm}}$ is the absorbance of the negative (containing cells and CCK-8 solution) at 450 nm.

The data analysis was processed by computer programs Excel (Microsoft, Redmond, WA, DC, USA) and Origin 8 (Origin Lab, Northampton, MA, USA). P < 0.05 was determined as significant. Data are the means ± standard deviation (SD) of five independent studies done each in quintuplicate.
3. Results and discussion

3.1. Structure characterization

The synthesized chitosan derivatives were characterized by elemental analysis (Table 1), FTIR (Fig. 1), $^1$H NMR (Fig. 2), and $^{13}$C NMR (Fig. 3).

3.1.1. Degree of substitution

Table 1 shows the yields and the substitution degrees of chitosan and chitosan derivatives. The changes in $W_{C:N}$ or $W_{C:S}$ indicate the target functional groups grafting on chitosan successfully. The data in the table are shown acetylated chitosan has perfect degree of substitution and yield. The substitution degrees of the acetylated chitosan derivatives containing sulfonium salts are different, which is about 10%. The data can preliminarily prove that the thioether groups are incorporated into the chitosan structure, but further confirmation is needed.

3.1.2. FTIR spectroscopy

Fig. 1 depicts the FT-IR spectrum of initial chitosan, acetylated chitosan, and acetylated chitosan derivatives containing sulfonium salts. Differences are found in the chitosan spectra because of changing acetyl groups and thioether groups. In spectrum of initial chitosan, an intense broad band with the wide band appearing at 3374 cm$^{-1}$ that is associated with the hydrogen bonding can be assigned to the hydrogen bonding involving the N—H, O—H stretching. The weak band at 2281 cm$^{-1}$ corresponded to the characteristic absorbance peak of C—H. The resulting bands at 1600 cm$^{-1}$ was due to C—O vibration, N—H bending mode [25]. The C—O vibrations produce a group of peak in the region around 1027 cm$^{-1}$. In spectrum of acetylated chitosan, it was worthwhile to note that the band at around 1735 cm$^{-1}$ and 1522 cm$^{-1}$ appeared after acetylation, which was assigned to the absorbance peak of COO and NH—C=O reported by prior studies on acetylation of chitosan. Therefore, in our conditions, the acetylation occurred on the free amino group and hydroxyl group, the acetylated product was an N,O-acetylatedchitosan [26,27]. The absorption peaks of the -S-C- groups were located at about 951 cm$^{-1}$, and new peaks appear at around 710 cm$^{-1}$ in the spectra of chitosan derivatives can be attributed to methylation in the face of incorruptible swaying [28–30]. The appearance of these new peaks can preliminarily prove that the sulfonium salt groups are incorporated into the chitosan structure, but further confirmation is needed.

3.1.3. NMR spectroscopy

From the $^1$H NMR spectra, the structures and physicochemical features of the initial chitosan, acetylated chitosan, and acetylated chitosan derivatives containing sulfonium salts were further confirmed with TMS as an internal standard. For example, as shown in Fig. 2, the peak of solvent (D$_2$O) appeared at 4.8 ppm. The singlet from 3.5 ppm to 4.1 ppm were owed to H$_5$, H$_4$, H$_3$, and H$_1$ protons of chitosan backbone, respectively [31]. The signal from 3.5 ppm to 4.1 ppm was owed to H$_2$, H$_6$. In contrast, new signals belonging to Br-CS appear at 4.4 ppm which are related to the proton of -CH$_2$Br [19]. The shift appears at 0.8–2.7 ppm for the -CH$_2$ and -CH$_3$ groups of sulfonium salt groups, these protons of -CH$_2$ and -CH$_3$ in the -CH$_2$-CH$_3$ group of chitosan derivatives which linked to the sulfur atom, respectively, exhibited resonances at this chemical displacement interval [30,32,33]. $^1$H NMR spectrum of the synthesized polymer suggested that the polymerization of thioether groups had already happened. The target products have been obtained.

To further confirm the successful modification of samples, the structures of chitosan and its derivatives were further characterized by $^{13}$C NMR. $^{13}$C NMR results, obtained for prepared compounds at ambient temperature in D$_2$O, are presented in Fig. 3. The signals between 55 ppm and 100 ppm were assigned to the carbons of chitosan [31]. After reacting with bromoacetyl chloride, new signals appear at about 170 ppm (-COO-) and 42.5 ppm (-CH$_2$Br) [30]. In this spectrum, for MACS, EACS, PACS, and BACS, carbon resonances for -S-CH$_3$ and -S-CH$_2$ are at 25–40 ppm [34–37], respectively. Meanwhile, the signals of ester group (-COO-) move to around 175 ppm and the chemical shifts of -CH$_2$Br are move to around 37 ppm. $^{13}$C NMR spectra of acetyl groups and thioether groups indicate that these compounds were synthesized.

3.2. Solubility and antioxidant activity

Fig. 4 shows the aqueous solution of the chitosan, Br-CS, MACS, EACS, PACS, and BACS at 2.0 mg/mL. The raw material chitosan and the synthesized products showed favorable water solubility. And the antioxidant activity of chitosan, Br-CS, MACS, EACS, PACS, and BACS against three common radicals was determined by calculate the scavenging effect of DPPH radical, hydroxyl radicals, superoxide radicals.

The model of DPPH radical-scavenging was commonly used to evaluate free radical scavenging capacity. DPPH is a stable radical with nitrogen at its center and it has a strong absorption peak at 517 nm in ethanol solution. When the radical scavenger was added, the lone electrons of DPPH molecules were paired and its scavenging principle of DPPH radical is illustrated in Scheme 2 [38].

As shown in Fig. 5, the scavenging activities of all samples both dose dependently increased with concentrations from 0.1 to 1.6 mg/mL, suggesting that both CS and chitosan derivatives had a certain scavenging activity on the DPPH radical. Noteworthy, the DPPH radical scavenging capacity of the acetylated chitosan derivatives containing sulfonium salts was significantly higher than that of CS and acetylated chitosan.
At 1.6 mg/mL, the scavenging activity of the acetylated chitosan derivatives containing sulfonium salts is as excellent as Vc, up to 100%. Since the acetylated chitosan derivatives contained sulfonium salts with cation that would combine with nitrogen atoms of DPPH, the pairing of lone electrons occurred to eliminate the DPPH radical.

Among the active oxygen radicals, the hydroxyl radical is one of the strongest oxidative free radicals and most likely to react with biological molecules (such as lipid, protein and DNA), leading to cytotoxicity, mutagenesis, carcinogenesis and other diseases. The hydroxyl radical scavenging activity was based on Fentons reaction [38]:

\[
\text{Fe}^{2+} + \text{H}_2\text{O}_2 \rightarrow \text{OH} + \text{OH}^- + \text{Fe}^{3+}
\]

\[
\text{Fe}^{2+} + \cdot \text{OH} \rightarrow \text{Fe}^{3+} + \text{OH}^-
\]

\[
\text{Fe}^{3+} + \text{H}_2\text{O}_2 \rightarrow \text{Fe}^{2+} + \text{OH}_2^- + \text{OH}^-
\]
When the chromogenic saffron is added to the reaction system, it reacts with the light radical. RH stands for the chromogenic saffron. The reaction process is as follows:

\[
\text{RH} + \cdot \text{OH} \rightarrow \text{R} + \text{H}_2\text{O}
\]

\[
\text{R}^+ + \text{Fe}^{3+} \rightarrow \text{R}^+ + \text{Fe}^{2+}
\]

\[
\text{OH}^\cdot + \text{H}_2\text{O}_2 \rightarrow \cdot \text{OH} + \text{O}_2 + \text{H}_2\text{O}
\]
After sample was added into the solution, it could react with light radical, which hindered the reaction between chromogenic saffron and radical. The absorbance of chromogenic saffron decreased, which could characterize the reaction between sample and free radical. The results are showed in Fig. 6. After successful synthesis, the antioxidant activity of all the acetylated chitosan derivatives containing sulfonium salts was significantly (P < 0.05) higher than that of CS and Br-CS. Meanwhile, with the increase of concentration, the scavenging effect of chitosan and its derivatives was increased. The strongest scavenging activity of MACS and EACS was 100% at a concentration of 1.6 mg/mL.

Similarly, the superoxide radical scavenging effect of Vc is rapidly up to 100% in the range of test concentration. When the concentration is 1.6 mg/mL, scavenging indices were listed as follows: Vitamin C 100%, Chitosan 59.89%, Br-CS 69.23%, MACS 97.14%, EACS 93.62%, PACS 91.94%, and BACS 97.31%. The highest scavenging rate was observed at 97.94% for BACS that was more than 1.6-fold of that (59.89%) for CS, but still lower than that of Vc (100%) at the same dose.

Antioxidant activity is an important parameter to evaluate the biological activity of samples. In this study, the antioxidant capacity of samples was determined by DPPH radical scavenging assay, hydroxyl radical scavenging assay, superoxide radical scavenging assay, and the antioxidant activity of the acetylated chitosan derivatives containing sulfonium salts is higher than other polysaccharides through consulting literature materials, such as the oligo-maltose fraction from Polygonum...
Cillinerve, different phenolic acids grafted onto chitosan and a new polysaccharide from *Bletilla striata* fibrous roots (Please refer to Supporting Information) [39–41]. The results are showed in Figs. 5–7, the antioxidant rates of all tested samples increase with the rise of concentration. Secondly, the acetylated chitosan derivatives containing sulfonium salts exhibit higher scavenging effect against radicals compared with pristine chitosan. The enhanced antioxidant action is mainly ascribed to the function of sulfonium salts and the unique structural features of sulfonium salts mainly reflect in the large dipole, and lone pair electrons of sulfur atoms, which give rise to excellent electron donors and electron delocalization of sulfonium salts. The substituted groups in sulfonium salts of synthesized products which acted as electron may donor to quench free radicals by providing electron, or attack on the free radicals by an electron. In order to provide help for stabilization of the free radicals’ form, more reactive free radicals were quenched by the stronger electron-donating groups that tend to donate more electrons [23,42]. The acetylated chitosan derivatives containing sulfonium salts might act as electron donor to convert free radicals into more stable products to terminate the free radical chain reactions. Meanwhile, the results showed that BACS exhibited the stronger antioxidant properties than MACS, EACS and PACS which should be attributed to the appearance of butyl sulfonium salts group as a stronger electron donor than dimethyl sulfonium salts group, ethyl sulfonium salts group, and propyl sulfonium salts group. In this sense, the role of antioxidants on human health has been described to promote several benefits in diseases, the acetylated chitosan derivatives containing sulfonium salts propose a feasibility strategy for the development of a new generation of chitosan-based antioxidant materials.

Fig. 6. Hydroxyl radical scavenging ability of CS, Br-CS, and the acetylated chitosan derivatives containing sulfonium salts.

![Scheme 3](image-url)  
Scheme 3. The scavenging principle of superoxide radical.
3.3. Cytotoxicity analysis

L929 cells treated by the chitosan and chitosan derivatives are illustrated in Fig. 8, the results of the impact of samples on cell line showed that most tested samples showed positive results in the growth percentages on the cell lines. When the tested concentration was \(500\ \mu\text{g/mL}\), the cell viability of chitosan was greater than 85%. When the tested concentration was \(1000\ \mu\text{g/mL}\), the cell viability of MACS was only greater than 69.62%, other cell viabilities could reach to more than 93%. Additionally, as shown as Fig. 9, the morphology of L929 cells treated by the chitosan and chitosan derivatives is spindle or oval, it looks like a normal cell [24]. Our results indicated that these derivatives had no cytotoxicity at these concentrations.

4. Conclusion

In this study, a series of the acetylated chitosan derivatives containing sulfonium salts were synthesized successfully and the cytotoxicity and antioxidant activities of samples by in vitro and ex vivo assays. The increase of sulfonium salts could be led to an enhanced antioxidant activity. The acetylated chitosan derivatives containing sulfonium salts have a potential antioxidant with no toxicity in the range of tested concentration. In conclusion, these designed chitosan derivatives showed higher inhibition of ROS which suggests the chitosan derivatives scavenging efficiency of radicals through increment of excellent electron donors reduced risk of free radicals on plants and human and may be a promising natural antioxidant for the pharmaceutics, food, cosmetics and agriculture management.
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CRediT authorship contribution statement

Xueqi Sun: Conceptualization, Methodology, Software, Formal analysis, Data curation, Writing – original draft, Jingzhong Zhang: Validation, Investigation. Yingqi Mi: Methodology, Software. Yuan Chen: Validation, Investigation. Wengiang Tan: Supervision, Project administration. Qing Li: Supervision, Project administration. Fang Dong: Writing – review & editing, Supervision, Project administration. Zhanrong Guo: Writing – review & editing, Supervision, Project administration.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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