Synthesis, characterization, and the antioxidant activity of \(N,N,N\)-trimethyl chitosan salts

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**A B S T R A C T**

Chitosan, possessing excellent properties, has been drawing broad attention. For the further utilization of chitosan, chemical modification is performed in improving its water solubility and the bioactivities. In the current study, four \(N,N,N\)-trimethyl chitosan salts, including \(N,N,N\)-trimethyl chitosan citrate (TMCSCI), \(N,N\)-trimethyl chitosan ascorbate (TMCSCa), and \(N,N,N\)-trimethyl chitosan gallate (TMCSGa), were prepared via \(N,N,N\)-trimethyl chitosan iodide (TMCSI). The as-prepared products were characterized by FT-IR and \(^1\)H NMR. Meanwhile, the degree of substitution were calculated by elemental analysis results. Furthermore, scavenging activities (against DPPH radicals and superoxide radicals) test and reducing power test were selected to evaluate the antioxidant property of \(N,N,N\)-trimethyl chitosan salts in vitro. The results indicated that TMCSAs and TMCSGa displayed excellent activity, probably due to the enhancement of ascorbate and gallate in antioxidant activity. However, because of the weak antioxidant property of citrate and acetylsaliclylate, the activity was lower for TMCSCI and TMCSCa. For example, in the DPPH radicals scavenging assay, the scavenging rates of chitosan, TMCSCI, TMCSCa, TMCSAs, and TMCSGa were 25.22, 84.11, 6.90, 2.70, 94.92, and 96.75% at 0.4 mg/mL, respectively. Generally, TMCSAs and TMCSGa could be regarded as a potential source of antioxidants.

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1. Introduction

Reactive Oxygen Species (ROS), including both the oxygen radicals such as superoxide anion radicals \((O_2^-)\) and hydroxyl radicals \((\cdot OH)\), and some nonradical derivatives of oxygen such as hydrogen peroxide \((H_2O_2)\) and singlet oxygen \((^1O_2)\), are generated in various metabolic processes of normal cells which may damage cell membranes, DNA, cellular proteins, and lipids leading to heart, liver, kidney related chronic diseases such as liver damage, aging, cancer, stroke, myocardial infarction, and Alzheimer’s disease [1–4]. Antioxidant can exert health-promoting and disease-preventing by scavenging free radicals in humans and animals. However, some of synthetic antioxidants have carcinogenic effect which is harmful to human health [5]. Therefore, for this reason and for the important application of these compounds in fields of cosmetic, pharmaceutical, and food, the replacement of harmful antioxidants by natural ones has encouraged the search for new natural source.

Chitosan, composing of randomly distributed \(\beta-(1 \rightarrow 4)\) linked D-glucosamine and N-acetyl-D-glucosamine residues, is obtained by alkaline deacetylation of chitin [6, 7]. Due to its nontoxicity, good biocompatibility, and susceptibility to chemical modification, chitosan has gained significant interest for applications in biotechnology, wastewater, cosmetics, agriculture, food technology, textiles, medical and pharmaceutical research, and so on [8–11]. However, chitosan also suffers from a few disadvantages such as poor solubility in neutral and alkaline conditions and requires further development to achieve the targeted results and desired range of efficiency [12]. When the chitosan molecule is converted to its quaternary ammonium salt, the number of positive charges is increased, thereby greatly improving its water solubility, antifungal activity, as well as antioxidant property [13, 14]. In addition, the biological activity of quaternary ammonium salt of chitosan could be affected by the active groups that grafting onto chitosan according to earlier report [15]. They reported that the quaternary ammonium salt of chitosan derivatives with stronger electronegativity groups showed higher antifungal and antibacterial activities. Thus, the selection of substituted groups into quaternary ammonium salt of chitosan derivatives could be key to increasing the bioactivity of such compounds.
N,N,N-trimethyl chitosan is a positively charged chitosan derivative that can dissolve at neutral pH. It is well known as an absorption enhancer of drugs, a vehicle for gene transfer, and an antibacterial agent [16–18]. Commonly, methyl iodide is employed as a methylation agent in the preparation of N,N,N-trimethyl chitosan. However, the product-N,N,N-trimethyl chitosan iodide (TMCSI) is unsuitable for the direct application in fields of food and cosmetics. Ion exchange is a simple and efficient method to replace iodide ions with other nontoxic and bioactive ions [16]. Some hydroxyl acids, including ascorbic acid, gallic acid, and citric acid, have been reported to possess excellent antioxidant activity [19, 20]. Especially, ascorbic acid, as a natural antioxidant, may scavenge peroxyl radical and inhibit cytotoxicity induced by oxidants. In addition, gallic acid was also found to be a strong antioxidant in emulsion or lipid systems. Therefore, these active acid anions can be selected to exchange iodine ions on N,N,N-trimethyl chitosan iodide (TMCSI) to observe several N,N,N-trimethyl chitosan salts, which were expected to achieve some non-toxic and efficacious antioxidants.

In the current study, we selected citrate, acetylsalicylate, ascorbate, and gallate acid anions as counterions to replace iodide ions to obtain several novel N,N,N-trimethyl chitosan salts (TMCSi, TMCSAc, TMCSAs, and TMCSGa). Meanwhile, their synthesis and antioxidant property were also reported. Firstly, the C2-NH2 of chitosan was modified as N,N,N-trimethyl chitosan iodide (TMCSI). And then, trimethyl chitosan cation and acid anions were combined using ion exchange method and the new chitosan derivatives-N,N,N-trimethyl chitosan salts were formed in this way. The chemical structures of the derivatives were characterized by FT-IR, 1H NMR, and elemental analysis. Meanwhile, the antioxidant activity was also investigated systematically by the assessment of superoxide radicals’ scavenging activity, DPPH radicals’ scavenging activity, and reducing power. Quaternized chitosan with more positive charges could cause the enhancement of antioxidant activity. In addition, some active acid anions are also helpful for the antioxidant activity of chitosan derivatives. Therefore, several effective chitosan antioxidants are expected to be achieved through ion exchange method.

2. Materials and methods

2.1. Materials

Chitosan was purchased from Qingdao Yunzhou Biochemistry Co., LTD (China). The degree of deacetylation is 81% and the viscosity average-molecular weight is 7.8 × 103. Iodomethane, citric acid, acetylsalicylic acid, ascorbic acid, and citric acid were purchased from Sigma-Aldrich Chemical Corp. (Shanghai, China). The other reagents were of analytical grade and used without further purification.

2.2. Analytical methods

2.2.1. Fourier transform infrared (FT-IR) spectroscopy

FT-IR spectrometers, in the range of 4000–400 cm−1 with resolution of 4.0 cm−1, were recorded on a Jasco-4100 (Tokyo, Japan, provided by JASCO China (Shanghai) Co. Ltd., Shanghai, China). All samples were mixed with KBr disks for testing.

2.2.2. Nuclear magnetic resonance (NMR) spectroscopy

1H Nuclear Magnetic Resonance (1H NMR) spectra was measured using a Bruker AVIII-500 Spectrometer (500 MHz, Switzerland, provided by Bruker Tech. and Serv. Co., Ltd., Beijing, China) at 25 °C and using 99.9% Deuterium Oxide (D2O) as solvents.

2.2.3. Elemental analysis

The elemental analyses by combustion were used to evaluate the degrees of substitution in chitosan derivatives. The analyses of elemental carbon, hydrogen and nitrogen in chitosan derivatives were performed on a Vario Micro Elemental Analyzer (Elementar, Germany). The degrees of substitution (DS) of chitosan derivatives were calculated on the basis of the percentages of carbon and nitrogen according to the following equations [21]:

\[ DS_1 = \frac{n_1 \times M_C - M_N \times W_{C/N}}{n_2 \times M_C} \]
\[ DS_2 = \frac{M_N \times W_{C/N} + n_2 \times M_C \times DS_1 - n_1 \times M_C}{n_3 \times M_C} \]
\[ DS_3 = \frac{M_N \times W_{C/N} + n_2 \times M_C \times DS_1 - n_1 \times M_C - n_3 \times M_C \times DS_2}{n_4 \times M_C} \]

where DS1, DS2, and DS3 represent the deacetylation degree of chitosan, the degrees of substitution of N,N,N-trimethyl chitosan iodide, and degrees of substitution of N,N,N-trimethyl chitosan salts (TMCSI, TMCSAc, TMCSAs, and TMCSGa); MC and MN are the molar mass of carbon and nitrogen, MC = 12, MN = 14; n1, n2, n3, and n4 are the number of carbon of chitin, acetamido group, trimethyl, and acid group, n1 = 8, n2 = 2, n3 = 3, TMCSI: n4 = 6, TMCSAc: n4 = 9, TMCSAs: n4 = 6, TMCSGa: n4 = 7; W_{C/N} represents the mass ratio between carbon and nitrogen in chitosan derivatives.

2.3. Synthesis of chitosan derivatives

2.3.1. Synthesis of N,N,N-trimethyl chitosan iodide (TMCSI)

TMCSI was prepared according to earlier method [11]: Chitosan (10 mmol) was dispersed in 80 mL of N-methyl-2-pyrrolidone (NMP) and stirred at room temperature for 1 h. Then, NaI (4.5 g), 15% NaOH aqueous solution (15 mL), and CH3I (15 mL) were added, subsequently. The mixture was refluxed for an additional 2 h at 60 °C. After reflux reaction, the solution was poured into ethanol to afford some flavescent precipitate and TMCSI collected by filtration was obtained by freeze-drying overnight in vacuum.

2.3.2. Synthesis of N,N,N-trimethyl chitosan salts (TMCSI, TMCSAc, TMCSAs, and TMCSGa)

TMCSI was then dissolved in 15% of sodium citrate solution, sodium acetylsalicylate solution, sodium ascorbate solution, and sodium gallate solution, respectively, in order to replace the iodide ions with acid anions. The solutions were dialyzed with distilled water for 3 days, and N,N,N-trimethyl chitosan salts including TMCSI, TMCSAc, TMCSAs, and TMCSGa were obtained after freeze-dried (Scheme 1).

2.4. Antioxidant assays

2.4.1. DPPH-radical scavenging activity assay

The DPPH scavenging properties of the products were evaluated by the following method [22]: different concentrations of testing samples and 2 mL of DPPH ethanol solution (180 μmol/L) were incubated for 30 min at room temperature. Then, the absorbance of the remained control (DPPH was substituted with ethanol), and A_{blank} 517 nm is the absorbance of the blank (samples were substituted with distilled water). Vitamin C was used as a positive control.

2.4.2. Superoxide-radical scavenging activity assay

The superoxide-radical scavenging ability was assessed following the model of Tan’s methods [23]. The reaction mixture, a total volume
3 mL, involving testing samples of chitosan or chitosan derivatives (5 mg/mL, 0.06, 0.12, 0.24, 0.48, and 0.96 mL), phenazine methosulfate (PMS, 30 μM), nicotinamide adenine dinucleotide reduced (NADH, 338 μM), and nitro blue tetrazolium (NBT, 72 μM) in Tris–HCl buffer (16 mM, pH 8.0), was incubated at 25 °C for 5 min. The absorbance was read at 560 nm against a blank. Three replicates for each sample were tested and the superoxide-radical scavenging effect was calculated according to the following equation:

\[
\text{Scavenging effect} \% = \left(1 - \frac{A_{\text{sample} \, 560 \, \text{nm}} - A_{\text{control} \, 560 \, \text{nm}}}{A_{\text{blank} \, 560 \, \text{nm}}} \right) \times 100
\]

where \(A_{\text{sample} \, 560 \, \text{nm}}\) is the absorbance of the samples, \(A_{\text{control} \, 560 \, \text{nm}}\) is the absorbance of the control (NADH was substituted with distilled water), and \(A_{\text{blank} \, 560 \, \text{nm}}\) is the absorbance of the blank (samples were substituted with distilled water). Vitamin C was used as a positive control.

### 2.4.3. Reducing power assay

The reducing power was determined according to the method of Xing with minor modification [24]. 1 mL of different concentrations of testing samples in phosphate buffer (200 μmol/L, pH 6.6) were mixed with 1 mL of 1% potassium ferricyanide, and the mixture was incubated at 50 °C for 20 min. Then, the reaction was terminated by trichloroacetic acid (10%, w/v). After centrifuged at 3000 rpm for 10 min, the upper layer of solution was mixed with 3 mL of distilled water and 0.6 mL of ferric chloride (0.1%, w/v), and the absorbance was recorded at 700 nm. Higher absorbance of the mixture indicated higher reducing power.

### 2.5. Statistical analysis

All the experiments were performed in triplicate and the data were expressed as means ± the standard deviation (SD, n = 3). Significant difference analysis was determined using Scheffe’s multiple range test. A level of P < 0.05 was considered statistically significant.

### 3. Results and discussion

#### 3.1. Structure of chitosan, N,N,N-trimethyl chitosan iodide, and N,N,N-trimethyl chitosan salts

Each step of the synthesis was followed by FT-IR spectra (Fig. 1) and \(^1\)H NMR spectra (Fig. 2). The elemental analyses, yields, and the degrees of substitution of N,N,N-trimethyl chitosan salts are shown in Table 1.

![Scheme 1. Synthesis routes for N,N,N-trimethyl chitosan salts.](image)

![Fig. 1. FT-IR spectra of chitosan, N,N,N-trimethyl chitosan iodide, and N,N,N-trimethyl chitosan salts.](image)
1747 cm\(^{-1}\) for the C=O stretching and at 1582 cm\(^{-1}\), 798 cm\(^{-1}\), and 763 cm\(^{-1}\) for the characteristic bands of benzene ring [29]. FT-IR of TMCSAs shows the peak absorptions at 1720 cm\(^{-1}\), 894 cm\(^{-1}\), and 755 cm\(^{-1}\), which are attributed to the absorption of ascorbate anions [30]. For the spectrum of TMCSGa, absorption peaks at 1550 cm\(^{-1}\), 794 cm\(^{-1}\), and 736 cm\(^{-1}\) refer to the C–C stretching vibration of phenyl of gallate. Meanwhile, absorption band at 883 cm\(^{-1}\) refers to the C–H stretching of gallate [31]. Above-mentioned results preliminarily demonstrated that \(N,N,N\)-trimethyl chitosan salts were obtained and their structures will be further demonstrated by \(^1\)H NMR.

As shown in the \(^1\)H NMR spectrum of chitosan, the chemical shifts of hydrogen protons on C-2, C-3 to C-6, and C-1 are exhibited at about 3.2 ppm, 3.6–3.9 ppm, and 4.6 ppm, respectively [7]. Besides all the characteristic proton signals of chitosan, the \(^1\)H NMR spectrum of TMCSI shows the prominent peak of \(-N^+(\text{CH}_3)_3\) at 3.1 ppm [11]. As to the spectra of \(N,N,N\)-trimethyl chitosan salts, the chemical shift of \(-N^+(\text{CH}_3)_3\) still exists and it moves to lower field because of the graft of acid anions. Besides, the specific positions of the hydrogen protons of acid anions are marked in Fig. 2. (TMCSI: New peaks locating at about 2.59 ppm and 2.64 ppm are related to the resonances of citric acid anion [28]. TMCSAc: New peaks locating at about 2.30 ppm and 6.90–7.80 ppm (benzene ring) are attributed to the chemical shifts of acetylsalicylic acid anion [29]. TMCSAs: New peaks locating at about 4.47 ppm, 3.98 ppm, and 3.72 ppm are assigned to the resonances of ascorbic acid anion [30]. TMCSGa: New peak locating at about 7.02 ppm is related to the resonance of gallic acid anion.) Hence, these data adequately indicate the successfully synthesis of \(N,N,N\)-trimethyl chitosan iodide and \(N,N,N\)-trimethyl chitosan salts.

| Compounds | Yields (%) | Elemental analyses (%) | Degrees of substitution | Deacetylation |
|-----------|------------|------------------------|-------------------------|--------------|
|           | C         | N         | H         | C/N         |             |
| Chitosan  | 40.851    | 7.474     | 6.879     | 5.47        | 0.81        |
| TMCSI     | 87.6      | 29.211    | 4.076     | 5.485       | 7.17        |
| TMCSGa    | 75.3      | 42.685    | 4.644     | 9.800       | 9.19        |
| TMCSAc    | 72.8      | 50.361    | 4.690     | 9.723       | 10.7        |
| TMCSAs    | 76.5      | 42.005    | 4.656     | 6.988       | 9.02        |
| TMCSGa    | 68.7      | 44.932    | 4.483     | 9.391       | 10.0        |
The degrees of substitution (DS) for chitosan derivatives were calculated on the basis of the percentages of carbon and nitrogen and the results are shown in Table 1. As is shown, the intermediate TMCSI presents the highest degree of substitution. As to the four final products (TMCSi, TMCSAc, TMCSAs, and TMCSGa), the DS of TMCSAc and TMCSGa are 0.46 and 0.48, respectively, whereas the DS of TMCSi and TMCSAs are 0.39 and 0.36.

### 3.2. Antioxidant activity

Two common free radicals (DPPH radicals and superoxide radicals) and reducing power are selected to evaluate the antioxidant property of chitosan, N,N,N-trimethyl chitosan iodide, and N,N,N-trimethyl chitosan salts. The results are shown in Figs. 3-5.

The results of DPPH radical scavenging effect of chitosan, N,N,N-trimethyl chitosan iodide, and N,N,N-trimethyl chitosan salts are given in Fig. 3. Obviously, TMCSAs and TMCSGa show the best antioxidant activity in scavenging DPPH radical. Even at the minimum test concentration, TMCSAs and TMCSGa can achieve a scavenging rate of 95%. The scavenging activity of TMCSI is also relatively good. However, at low test concentrations, the DPPH-radical scavenging effect of TMCSI is inferior to TMCSAs and TMCSGa. For example, at 0.1 mg/mL, the scavenging rates of TMCSI, TMCSAs, and TMCSGa are 64.03, 94.84, and 96.84%, respectively. As to TMCSI and TMCSAc, their DPPH-radical scavenging ability remains significantly low in comparison with TMCSAs and TMCSGa and it even lower than chitosan. As a result, TMCSAs and TMCSGa can be an excellent antioxidant for DPPH free radicals, but TMCSI and TMCSAc are unsuitable.

Fig. 4 shows the superoxide-radical scavenging activity of chitosan, N,N,N-trimethyl chitosan iodide, and N,N,N-trimethyl chitosan salts. After ion exchange, the enhanced antioxidant activity of TMCSI, TMCSAs, and TMCSGa is obvious compared with TMCSI. Meanwhile, TMCSAs and TMCSGa still show the best antioxidant effect and their scavenging rates can reach to 100% when the concentration is 0.4 mg/mL. In addition, TMCSI exhibits scavenging activity with IC50 value of 0.5 mg/mL while TMCSI with IC50 value of 0.75 mg/mL. Hence, TMCSI can also be considered as a better antioxidant for superoxide radicals than TMCSI. As to TMCSAc, although its antioxidant activity and water solubility are better than chitosan, it still cannot meet the commercial demand for active values.

The antioxidant activity of chitosan, N,N,N-trimethyl chitosan iodide, and N,N,N-trimethyl chitosan salts was also evaluated using reducing power assay and the results are presented in Fig. 5. When the iodide ion was replaced with ascorbate and gallic acid anions, the reducing power of chitosan derivatives is significantly increased. TMCSGa is the best reducing agent and its absorbing ability remains about 4 A at different test concentrations. At low concentrations, the reduction ability of TMCSAs is lower than TMCSGa, but when the concentration is higher than 0.4 mg/mL, its absorbance can reach to 4 A. For chitosan, TMCSI, TMCSAc, and TMCSGa, their absorbance is under 1A and they have little reduction capability.

Based on the analyses of the antioxidant effect of the products, we could gain some conclusions as follows: firstly, TMCSAs and TMCSGa have better ability of scavenging DPPH radicals, scavenging superoxide radicals, and reducing power compared with TMCSI and chitosan. Ascorbic acid is a water-soluble vitamin that can act in the body as a cofactor for several enzymes and an antioxidant which protects low density lipoproteins from oxidation, reduces harmful oxidants in the stomach, and promotes iron absorption as well as other physiological functions of animals through interacting directly with Reactive Oxygen Species (ROS) [32]. Studies have shown that it is because ascorbic acid is an electron donor, suggesting a direct role for protection against oxidative damage [33]. In addition, many studies have also shown that gallic acid, a phenolic compound, can exhibit remarkable antioxidant activities by donating H-atoms from phenol groups to radicals, inhibiting interaction of radicals to health benefit compounds [34]. Hence, the introduction of ascorbic acid anions and gallic acid anions improves the antioxidation activity of N,N,N-trimethyl chitosan. Secondly, TMCSI possesses good ability of scavenging superoxide radicals while it is very poor in scavenging DPPH free radicals ability and reduction ability. This result illustrates that TMCSI has certain antioxidant activity, but this activity is targeted. Thirdly, in all antioxidant property assay, the
activity of TMCSAs is consistently lower than TMCSI. Acetylsalicylic acid, well known under its trade name of aspirin, is usually reported as an an- 
gealcs to relieve minor pains, an antipyretic to reduce fever, and an anti-inflammatory medication and its antioxidant capacity is far lower than some hydroxyl acids [29]. Therefore, the introduction of acetylsa-
licylate anions cannot further enhance the antioxidant activity of N,N-
trimethyl chitosan. In summary, we have screened two kinds of chito-
san derivatives with excellent antioxidant activity. These chitosan anti-
oxidants are safe and non-toxic. They can be developed for human con-
sumption in many fields.

4. Conclusions

Chitosan, derived from chitin, is the only readily available basic amino-polysaccharide in the nature and its nontoxicity has various applicability. Furthermore, quaternized chitosan with positive charge shows excellent bioactivity, such as antioxidant and anti-
fungal activity, and it has caused a lot of research attention. Inspired it, we have designed a simple synthesis method to graft antioxidative groups onto N,N,N-trimethyl chitosan in order to obtain several novel chitosan derivatives that possess better antioxidant property. In this paper, the successful synthesis of N,N,N-trimethyl chitosan salts containing citrate (TMCSI), acetylsalicylate (TMCSAc), ascor-
bate (TMCSAs), and galate (TMCSGa) using ion exchange method was reported. Their structures were confirmed by FT-IR, 1H NMR, and elemental analysis. Meanwhile, the antioxidant activity of N,N,N-
trimethyl chitosan salts was also explored according to earlier methods. The results indicated that TMCSAs and TMCSGa, which contain trimethyl chitosan cation and active acid anions, showed better antioxidant property than TMCSI and chitosan. Maybe the combination of trimethyl chitosan cation and acid anion is synergis-
tic and can attract the single electron of free radicals to inhibit the free radical chain reaction. Hence, TMCSAs and TMCSGa deserve fur-
ther study for the development of effective antioxidant that can be applied in fields of food, cosmetic industry, as well as medical. Nevertheless, due to the poor antioxidant activity of citrate and ace-
ylsalicylic acid anions, the activity of TMCSI and TMCSAc is re-
duced compared with TMCSI. In summary, these results may pave the way to develop chitosan antioxidant and further comprehensive study to confirm this hypothesis on mechanism of structure and an-
tioxidant activity will be carried out.

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Conflict of interest statement

The authors have declared no conflicts of interest.

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