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Antioxidant Activity of High Molecular Weight Chitosan and N,O-Quaternized Chitosans

Ajun Wan,*[,†] Qing Xu,[†] Yan Sun,[†] and Huili Li*[,‡]

[†]School of Chemistry and Chemical Engineering, Shanghai Jiao Tong University, 800 Dongchuan Road, Shanghai 200240, China

[‡]School of Pharmacy, Shanghai Jiao Tong University, 800 Dongchuan Road, Shanghai 200240, China

ABSTRACT: The objective of this study was to evaluate the in vitro antioxidant activity of high molecular weight chitosan based films. Three kinds of water-soluble quaternized chitosans with high molecular weight, namely *N*-(2-hydroxyl) propyl-3-trimethyl ammonium chitosan chloride (400-HTCC and 1240-HTCC), *N*-(2-hydroxyl) propyl-3-triethyl ammonium chitosan chloride (400-HTEC and 1240-HTEC), and *O*-(2-hydroxyl) propyl-3- trimethyl ammonium chitosan chloride (400-O-HTCC) were prepared from high molecular weight chitosans (400 and 1240 kDa). The in vitro antioxidant activity of a high molecular weight chitosan (1240-CS) and five quaternized chitosans was evaluated and compared as radical scavengers against 1,1-diphenyl-2-picrylhydrazyl radicals (DPPH•), hydroxyl radical (•OH), and superoxide radical ($\bullet O_2^-$) using established methods, and the effect of the molecular weight, the concentration, the newly generated hydroxyl group, the extra introduced positive charge of quaternary ammonium salt group, etc., on the antioxidant activity of these high molecular weight chitosans is discussed. The data obtained in vitro models exhibited good antioxidant potency and suggested the possibility that high molecular weight chitosan based films could be effectively employed as natural antioxidant materials for application in the field of food and medicine.

KEYWORDS: *antioxidant activity, chitosan, quaternized chitosan, high molecular weight, water-soluble*

INTRODUCTION

Currently, food security has always been a big issue worldwide and increasing attention has been paid to develop materials with film-forming capacity together with antioxidant properties which help improve food safety and shelf life. Because oxidation is a major problem affecting food quality and biological applications, antioxidant packaging is one of the most promising active packaging systems. Antioxidants, defined as substances that when present at low concentrations compared to those of oxidizable substrates significantly delay or inhibit oxidation of those substrates, can act at different levels in an oxidative sequence. This is be illustrated by considering one of the many mechanisms by which oxidative stress can cause damage by stimulating the free radical chain reaction of lipid peroxidation. Free radical chain reactions within tissue can be inhibited by antioxidant substances that retard the formation of free radicals and by competing for existing radicals and removing them from the reaction medium.[1] Many synthetic antioxidants such as butylated hydroxyanisole, butylated hydroxytoluene, *t*-butylhydroquinone, and propyl gallate are commonly used. However, the use of synthetic antioxidants is under strict regulation due to their potential health hazards. Therefore, recently, the search for effective natural antioxidants to be used in foods or medicinal materials to replace synthetic ones has attracted the most attention. Further, natural polymer based film with antioxidant activity is always one of the most effective methods of maintaining food quality.

Chitosan (CS), a linear copolymer consisting of β-(1→4)-2-amino-2-deoxy-D-glucan (glucosamine) and β-(1→4)-2-acetamido-2-deoxy-D-glucan (*N*-acetyl glucosamine), is a natural cationic polymer produced by deacetylation of chitin, which is commonly extracted from the shells of crabs, shrimp, and krill. CS has attracted much attention because of its unique biological, chemical, and physical properties, including antioxidant activity,[2−4] antitumor activity, immuno-enhancing effect, increased protective effect against infection with some pathogens, antifungal activity, and antimicrobial activity.[5,6] As one of the most promising biopolymers for a variety of potential applications, CS can be used in many fields, including food processing, pharmaceuticals, biomaterials, and agriculture.[7−12] It has useful reactive groups such as a hydroxyl group (−OH) and an amido group (NH_2). However, the poor solubility at pH > 6.5 greatly limits its applications. To overcome this drawback and expand its use, functional groups have been introduced into CS to make it water-soluble. Both hydroxyl group and amido group affect the antioxidant ability of CS, and some studies have investigated the effect of the forms of the amino group in chitosan backbone on radical scavenging activity. For example, Guo et al. systematically studied the antioxidative properties of Schiff bases of chitosan, N-substituted chitosan, and N-quaternized chitosan, and obtained chitosans with different forms of the amino group that had different •OH scavenging ability which was related to contents of the active hydroxyl group and amino group in the molecular chains, and the positive charge of the N-quaternized chitosan could strengthen its antioxidant ability.[13−15] Pasanphan et al. reported that the chitosan-conjugated gallic acid introducing extra phenolic hydroxyl groups onto the chitosan may show water-solubility and have synergistic antioxidant capacity.[16,17] Schreiber et al. found that the gallic acid grafted chitosan had 89.5% DPPH• scavenging ability and could reduce the level of thiobarbituric acid reactive substances (TBARS), peroxide, and

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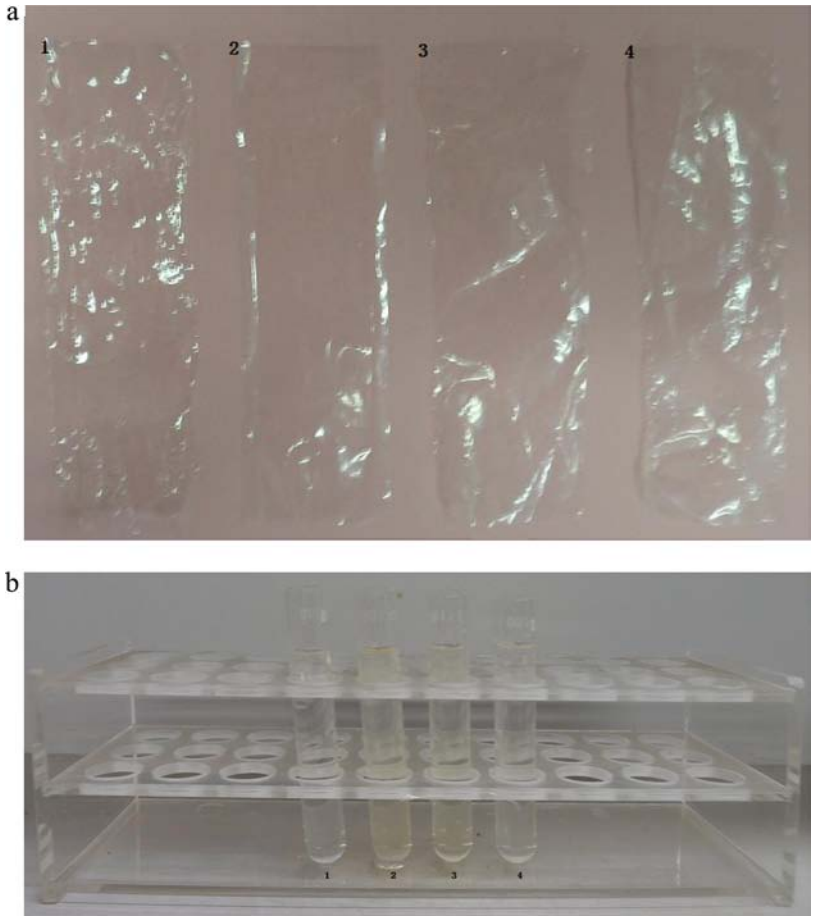

Figure 1. (a) high molecular weight chitosan based films: (1) unmodified chitosan film (CS); (2) HTCC film; (3) HTEC film; (4) O-HTCC film. (b) Aqueous solution of degraded chitosans from the films.

conjugated trienes formation as compared to polyethylene bags.[18] A good review article has been published summarizing the preparation methods and the applications of a wide variety of chitosan based antimicrobial films and coatings as biopackaging for food preservation.[19] However, few attempts have been made to evaluate the antioxidant activity of high molecular weight (HMW) CSs and quaternized chitosans (QCSs) modified in different sites of chitosan backbone for use in food packaging materials. Our laboratory fabricated HMW chitosan and quaternized chitosan films with properties of water solubility and free radical scavenging (Figure 1).

In his paper, by directly introducing quaternized glycidyl trimethylammonium chloride (GTMAC) and glycidyl triethylammonium chloride (GTEAC) groups on different sites of HMW CS (400 and 1240 kDa), three new kinds of water-soluble quaternized chitosans with the addition of hydroxyl groups and quaternary ammonium salt groups, namely *N*-(2-hydroxyl) propyl-3-trimethyl ammonium chitosan chloride (400-HTCC and 1240-HTCC), *N*-(2-hydroxyl) propyl-3-triethyl ammonium chitosan chloride (400-HTEC and 1240-HTEC), and *O*-(2-hydroxyl) propyl-3-trimethyl ammonium chitosan chloride (400-O-HTCC) were obtained. Their in vitro antioxidant activity was evaluated and compared as radical scavengers against 1,1-diphenyl-2-picrylhydrazyl radicals (DPPH•), hydroxyl radical (•OH), and superoxide radical ($\bullet O_2^-$) using established methods, and the possible effect, such as the molecular weight (MW), the concentration, the hydroxyl group including the one in chitosan backbone and the one newly generated, the amino group, the extra introduced positive charge of quaternary ammonium salt group, the steric hindrance, etc., on the antioxidant activity of these HMW chitosans iss discussed. This work is aimed at making use of a renewable and sustainable material, chitosan, to produce new materials with water solubility together with antioxidant activity. The data obtained in the in vitro model exhibited good antioxidant potency of these HMW chitosans and suggested the possibility that these chitosans could be effectively employed as natural antioxidant materials for use in food and medicine fields.

MATERIALS AND METHODS

Materials. CS (MW 400 and 1240 kDa, all with 97% deacetylation) was obtained from Shanghai Bo'ao Biotechnology Co. (Shanghai, China). H_2O_2 was purchased from Aldrich Chemical Co. (USA). Ethylene diamine tetra-acetate (EDTA) was purchased from Fluka (Buchs, SG1, Switzerland). Nitro blue tetrazolium (NBT) was purchased from Sigma (St. Louis, MO, USA). Riboflavin and methionine were purchased from Sinopharm Group Chemical Reagent Co. Ltd. (China). Thiobarbituric acid (TBA), trichloroacetic acid (TCA), deoxyribose (DR), potassium ferricyanide, and ferric chloride ($FeCl_3$) were purchased from Sigma Chemicals Co. GTMAC was purchased from Fluka Chemical Co. All other chemicals and reagents used in this experiment were obtained from commercial sources and were of the highest purity available. Freshly deionized and redistilled water was prepared in our laboratory.

Methods. The MW of CS employed as the starting material for synthesis of the QCS was further determined by the capillary viscometry method using an Ubbelohde viscometer (Shanghai Meter

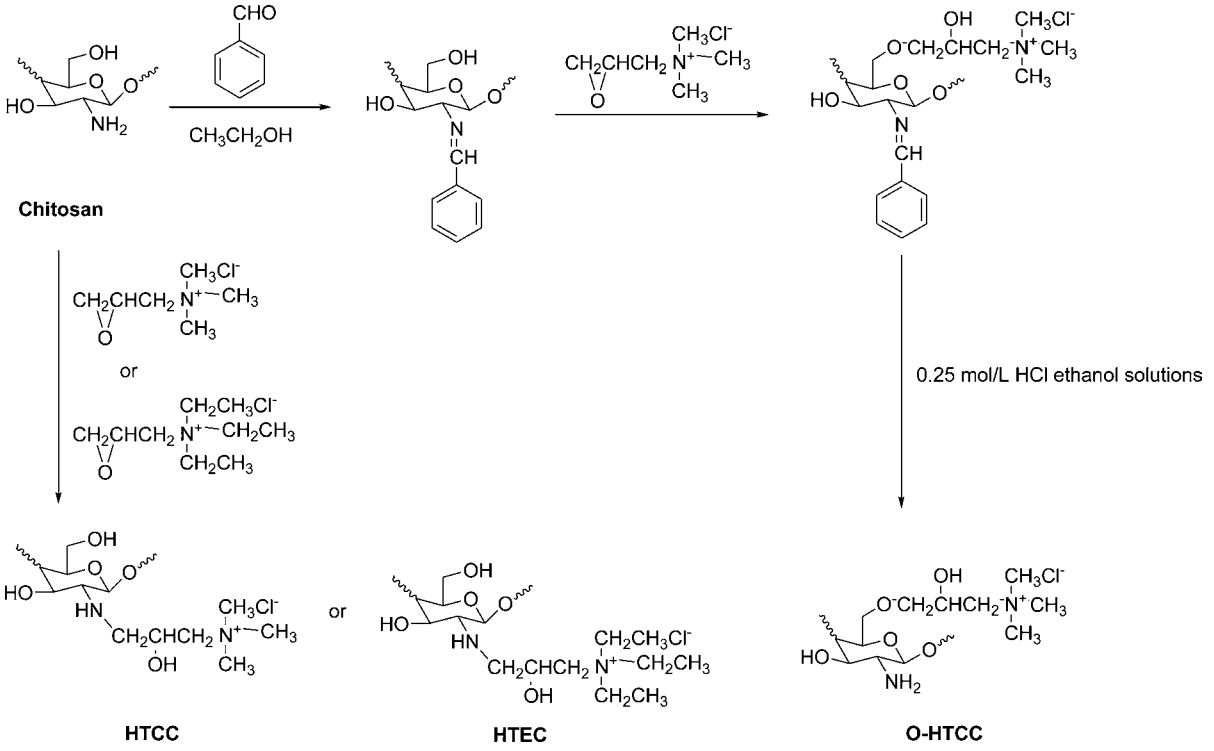

Figure 2. Synthetic pathway of N,O-quaternized chitosans.

Experimental Factory). In a solution of 0.1 mol/L CH_3COOH–0.2 mol/L NaCl at 25 °C, the viscosity average molecular weight (M_v) was calculated using the Mark–Houwink equation: $[\eta] = K_m M_v^{\alpha}$, where $K_m = 1.81 \times 10^{-3}$, $\alpha = 0.93$.[20] The degree of deacetylation of CS was further determined by FTIR analysis using a method described in detail elsewhere by Miya et al.[21] Elemental analyses were recorded on a Vario EL III CHN analyzer. FT-IR spectra were obtained with a Spectrum 100 (Perkin-Elmer, USA) using 16 scans at a resolution of 4 cm^{-1}. ^1H NMR spectra were obtained on a Mercury 400 spectrometer (400 MHz for ^1H) in D_2O containing a small amount of CD_3COOD at 25 °C. The free radical scavenging ability was assessed on an SH-G biochemistry chemiluminescence meter (BCM) (Shanghai Measurement Equipment Factory, Shanghai, China). The BCM is composed of three parts: an automatic rotating sample support, in which 12 sample cells (glass tubes, diameter = 10 mm, height = 20 mm) can be placed, a chemiluminescence monitor, and a data processor. Each sample cell can rotate and cross the monitor at a set time interval according to a self-set program. When testing, the chemiluminescence intensity of a reaction system can be recorded in the data processor at the set time interval.

Synthesis of Quaternized Chitosans. N-Quaternized chitosan derivatives were synthesized using an established method that is outlined in Figure 2.[22] Chitosan (3.25 g, 20.0 mmol) was dispersed in distilled water (33 mL) at 85 °C. GTMAC (11.7 mL, 60.9 mmol) was added in three portions (3.9 mL each) at 2 h intervals. After 10 h of reaction, the clear and yellowish reaction solution was poured into cold acetone (110 mL) while stirring and kept in the refrigerator overnight. The next day, acetone was decanted and the remaining gel-like product was dissolved in methanol (55 mL). The solution was precipitated in 4:1 mixture of acetone and ethanol (135 mL). The white product was collected by filtration and further purified by washing with hot ethanol using a Soxhlet extractor for 24 h. The final product was dried at 70 °C overnight to obtain HTCC. With the same method but replacing GTMAC with GTEAC, HTEC was obtained (Figure 2).

O-HTCC was obtained according to a method used by our group.[23] Briefly, chitosan (3.25 g, 20.0 mmol) was dispersed into 95% ethanol (100 mL), and benzaldehyde (6.36 g, 60.0 mmol) in 50 mL of ethanol was added. The solution was stirred for 1 h at room temperature and then placed for 20 h in vacuum (55–60 °C). The resulting solution was adjusted to 7.0 with 1 mol/L NaOH solution and filtered. The precipitate was washed with methanol several times and added to GTMAC (6.40 mL, 33.3 mmol) in 50 mL of isopropyl alcohol. The resulting mixture was stirred at 70 °C for 16 h. The precipitate was washed with methanol and acetone in turn and added to 100 mL of 0.25 mol/L HCl ethanol solutions. The mixture was stirred at room temperature for 24 h. After most of the ethanol was removed, 30 mL of H_2O was added to the mixture. The mixture was precipitated in acetone and filtered. The obtained precipitate was dissolved in some distilled water once more and dialyzed against distilled water for 3 days, concentrated, precipitated in acetone, and dried under vacuum at 80 °C for 48 h to obtain O-HTCC (Figure 2).

QCSs were prepared from CS with the MW of 400 and 1240 kDa, which were labeled 400-HTCC, 1240-HTCC, 400-HTEC, 1240-HTEC, and O-400-HTCC, respectively.

Antioxidant Assays. The antioxidant activity of CS and QCS was evaluated by three antioxidant assays: DPPH•, •OH, and $\bullet O_2^-$.

DPPH• Scavenging Activity. The DPPH• scavenging activity of CS and QCS was measured using the method of Hatano et al.[24] CS or QCS (2.0 mL in distilled water) was added to a methanolic solution of DPPH (2.0×10^{-4} M, 2.0 mL) and 5 mL of methanol. The mixture was vigorously shaken for 10 s and left to stand at room temperature for 30 min. The scavenging activity of CS or QCS on DPPH• was determined by the absorbance at 517 nm, and the percent scavenging activity was calculated according to the following equation:

$$\%\text{scavenging activity} = (\text{Abs}_{\text{control}} - \text{Abs}_{\text{sample}})/\text{Abs}_{\text{control}} \times 100$$

where $\text{Abs}_{\text{control}}$ is the absorbance of the control (without the test sample) and $\text{Abs}_{\text{sample}}$ is the absorbance of the sample (with the test sample).

•OH Scavenging Activity. The •OH scavenging activity of CS and QCS was measured using the method of Halliwell et al.[25] The reaction mixtures in a final volume of 1.0 mL, each of which contained CS or QCS (0.11–1.10 mg mL^{-1}), were incubated with DR (37.5 mM), H_2O_2 (1 mM), $FeCl_3$ (100 μM), EDTA (100 μM), and ascorbic acid (100 μM) in potassium phosphate buffer (20 mM, pH 7.4) for 60 min at 37 °C. Then 1 mL of TBA (1% W/V) and 1 mL of TCA (2% W/V)

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were added to terminate the reaction. Then the mixtures were heated in a boiling water bath for 15 min and cooled down to measure their absorbance at 535 nm against reagent blank.

$\bullet O_2^-$ Scavenging Activity. The $\bullet O_2^-$ scavenging activity of CS and QCS was performed by using the method of photoreduction of NBT with some modifications.[26] Each of the several reaction mixtures, in a volume of 4.0 mL, contained CS or QCS at various concentrations, along with the following regents: 13 mM methionine, 10 mM riboflavin, 70 μM NBT, 100 mM EDTA, and 0.05 M phosphate buffer (pH 7.8). The color was developed by illumination of the mixtures at 4000 Lx for 30 min at a constant temperature of 35 °C, and then, the absorbance was measured at 560 nm.

Statistical Analysis. All experiments are repeated a minimum of three times and measured in triplicate. Results reported are means ± SD unless otherwise noted. Statistical significance is analyzed using Student's *t* test. Differences between experimental groups are considered significant when the *p*-value is less than 0.05.

RESULTS AND DISCUSSION

Synthesis and Characterization of Quaternized Chitosans. *HTCC and HTEC.* The synthetic pathway of HTCC and HTEC were illustrated in Figure 2. In a neutral aqueous solution, CS (400 or 1240 kDa with 97% deacetylation) was coupled with GTMAC or GTEAC to give the water-soluble QCS (HTCC or HTEC), in which the hydroxyl groups of chitosan are not sufficiently nucleophilic to induce ring-opening of GTMAC or GTEAC, whereas the amino group of chitosan is nucleophilic enough to do that.[22]

O-HTCC. The water-soluble O-HTCC was prepared using our previously reported method.[23] As shown in Figure 2, first, CS was modified by the chemical conjugation of benzaldehyde in ethanol solution in order to protect the amino group of CS. Then GTMAC was introduced onto the N-protected CS and then the hydroxyl groups of CS became the nucleophile to induce ring-opening of GTMAC. Because the hydroxyl group on C-3 position (secondary alcohol) of the N-protected chitosan was in a relatively bulky environment, also, due to the GTMAC with a large bulk. The reaction between GTMAC and hydroxyl group on C-6 position (primary alcohol) of N-protected chitosan was predominant. After alcoholysis under acidic conditions, the N-protected CS was deprotected to obtain O-HTCC.

Table 1 shows the elemental analysis results and degree of quaternization of CSs. As shown in Table 1, with the increase of MW of CSs, the degree of quaternization of CSs was reduced (entry 2 vs 3, entry 4 vs 5). Because the higher the MW, the stronger the intramolecular and intermolecular hydrogen bonding will be, the reaction is not easy; while the higher the MW, under the same conditions, the greater the viscosity of the polymer solution will be, thus the quaternary ammonium salt and CS could not fully contact to react, and in the case of the same MW CS modified with different quaternary ammonium salt, the substitution degree of GTMAC was higher than GTEAC (entry 2 vs 4, entry 3 vs 5), and it is likely because the GTEAC has a larger steric hindrance, which led to less sufficient reaction with CS. In addition, the degree of substitution of the quaternary ammonium salt on C-6 position of CS (entry 6) was the smallest among all the QCSs, which is due to the low nucleophilicity of the hydroxyl group in neutral aqueous solution, and at the same time because the steric hindrance of CS is further increased with the protection of the C-2 position.

Table 1. The Elemental Analysis Results and Degree of Quaternization of Chitosans

		elemental analyses (%)			
entry	compd	C	N	H	degree of quaternization of chitosan (%)
1	chitosan (MW: 400 or 1240 kDa)	44.80	8.62	6.81	
2	HTCC (MW: 400 kDa)	45.87	8.92	8.17	90.1
3	HTCC (MW: 1240 kDa)	45.81	8.89	8.17	84.6
4	HTEC (MW: 400 kDa)	49.92	8.03	8.75	80.2
5	HTEC (MW: 1240 kDa)	48.36	7.71	8.41	74.7
6	O-HTCC (MW: 400 kDa)	42.23	8.17	7.44	39.8

Figure 3 shows the IR spectra of CS and QCS. By comparing the difference of IR spectra, it could be drawn out that

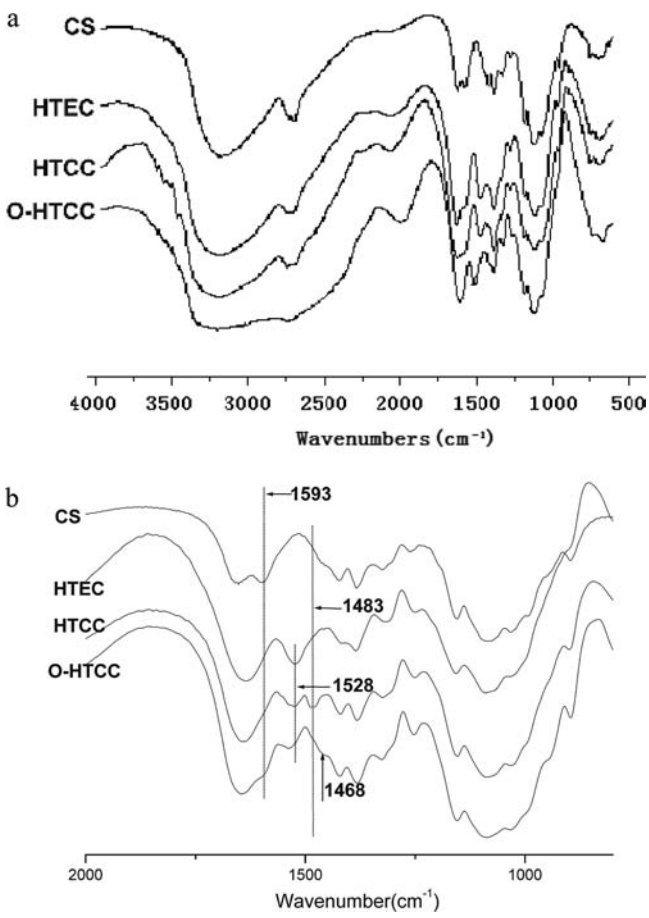

Figure 3. IR spectra data of chitosan and quaternized chitosans.

quaternary ammonium salt group was introduced into the chitosan backbone. As shown in Figure 3b, all the samples had absorption peaks at 895 and 1155 cm^{-1}, which assigned to the saccharine structure. The absorption peak at 1656 cm^{-1} in CS was assigned for the C═O stretch of the secondary amide (3.0% of repeat units of the CS), and the peak at 1593 cm^{-1} was ascribed to the N−H bending of the primary amine. Compared to HTCC and HTEC, the existed absorption peak at 1593 cm^{-1} showed that alkylation in chitosan occurred at

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−OH group and obtained O-HTCC. The peak at 1528 cm^{-1} in HTCC and HTEC was ascribed for the N−H bending in the secondary amine. HTCC and O-HTCC produced bands at 1483 and 1468 cm^{-1}, the C−H bending of trimethylammonium group. In addition, the spectrum showed a broad band at around 3400 cm^{-1} due to the increased number of hydroxyl groups (Figure 3a).[22,23]

To further confirm the success of the reaction, ^{1}H NMR analysis of the pure unmodified CS and QCSs was performed in CD_3COOD−D_2O solution. The peak positions of their functional group are shown in Figure 4. From the obtained ^{1}H NMR spectra, peaks at about 1.9 ppm (−$COCH_3$ from chitin) and 3.5−4.0 ppm (C-3, 4, 5, 6) were observed for both unmodified CS and QCSs. The peak at around 3.1 ppm was found for HTCC and O-HTCC, which represented the −$^+N(CH_3)_3$ (Figure 4b,d) groups in GTMAC.[22] The H-a peak at about 2.9 ppm shifted to 3.3 ppm following the chemical structure of HTCC and O-HTCC.[23] However, the peak at around 1.25 ppm was only found for HTEC, which represented the −CH_3 (labeled as H-e in Figure 4c) groups in GTEAC. These distinct differences between the ^{1}H NMR spectra for these chitosans further confirmed the synthesis of QCSs.

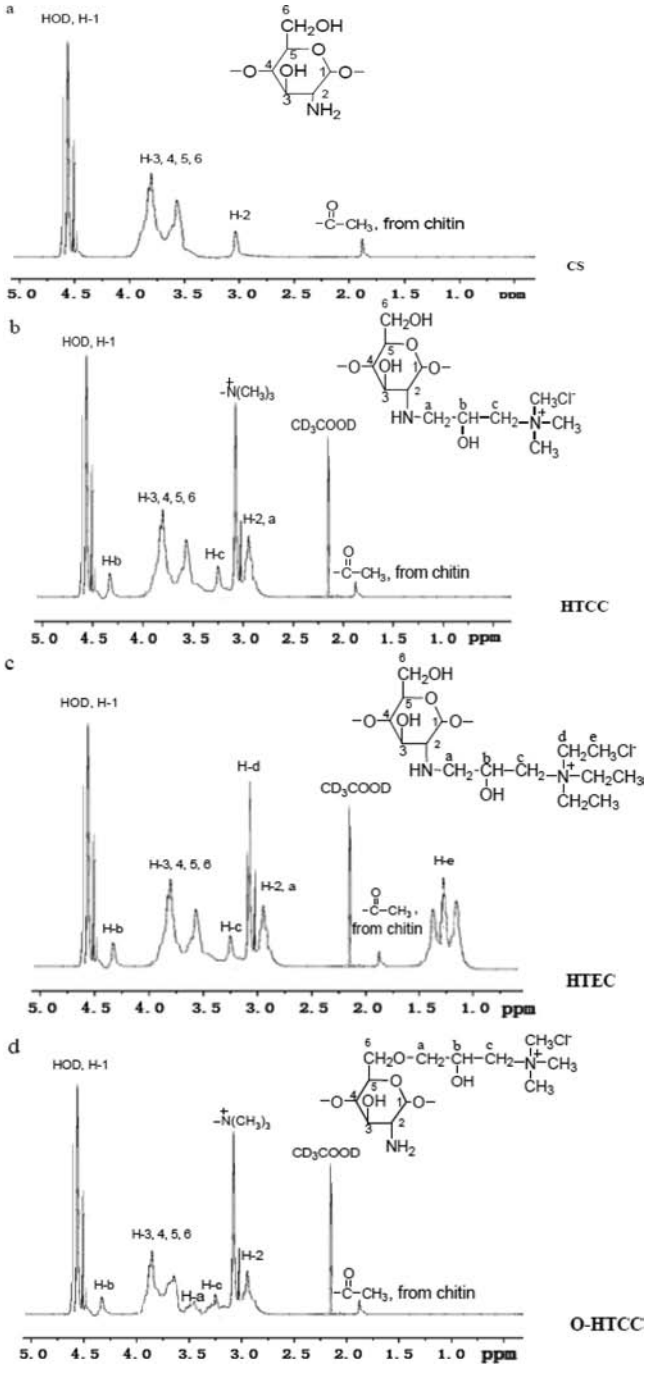

Figure 4. ^{1}H NMR spectra data of high molecular weight chitosan and quaternized chitosans: (a) CS, (b) HTCC, (c) HTEC, (d) O-HTCC.

Antioxidant Activity. Figure 5a shows the antioxidant activity of a HMW CS and five QCSs on three antioxidant assays: DPPH•, •OH, and •O_2^- at 0.8 mg/mL concentration. All of the chitosans examined were found to possess antioxidant activity. 1240-CS and 400-O-HTCC displayed the same radical scavenging ability on the order of •O_2^- > •OH > DPPH•. In the case of the N-quaternized chitosan derivatives (THCC and HTEC), low molecular weight chitosan derivatives exhibited higher DPPH• scavenging activity but lower •O_2^- scavenging activity.

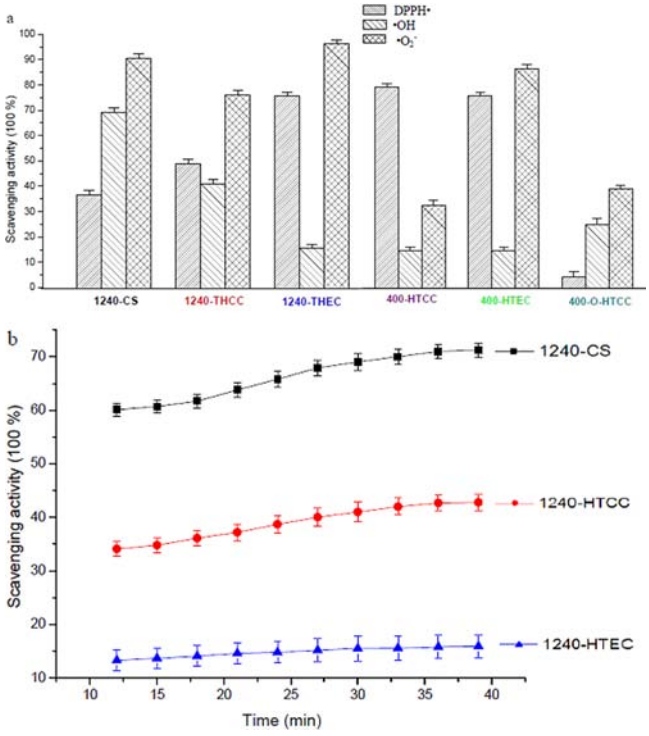

Figure 5. (a) DPPH•, •OH, and •O_2^- scavenging activity of high molecular weight chitosan and quaternized chitosans at 0.8 mg/mL. (b) Time effect of 1240-CS, 1240-HTCC, and 1240-HTEC on the •OH scavenging activity at 0.8 mg/mL.

Among various reactive oxygen species, the •OH is one of the strongest oxidizers. The effect of time on •OH scavenging ability of different type of chitosan, such as 1240-CS, 1240-HTCC, and 1240-HTEC at 0.8 mg/mL, was investigated. As shown in Figure 5b, the •OH scavenging efficiency curves were overall close to S-shape, and the S-shape gradually became weak with the increase of the molecular bulk, which is in good agreement with the recently experimental results reported by our groups.[27] In the case of 1240-CS, the scavenging efficiency slowly increased at the beginning, and then increased rapidly, to

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the slightly increase. In the case of 1240-HTEC, the scavenging efficiency increased gradually and then reached a plateau, showing a flatter curve. For 1240-HTCC, the change of free radical scavenging efficiency was between 1240-CS and 1240-HTEC.

To better evaluate the antioxidant activity of HMW CS and QCSs, the effect of concentration on three antioxidant assays was investigated in detail by plotting the concentrations of antioxidant against the percent of free radical scavenging capacity.

DPPH● Scavenging Activity. The scavenging mechanism of DPPH● is based on the reduction of methanolic DPPH solution in the presence of a hydrogen donating antioxidant due to the formation of the nonradical form DPPH-H by the reaction. Figure 6 shows the DPPH● scavenging ability of

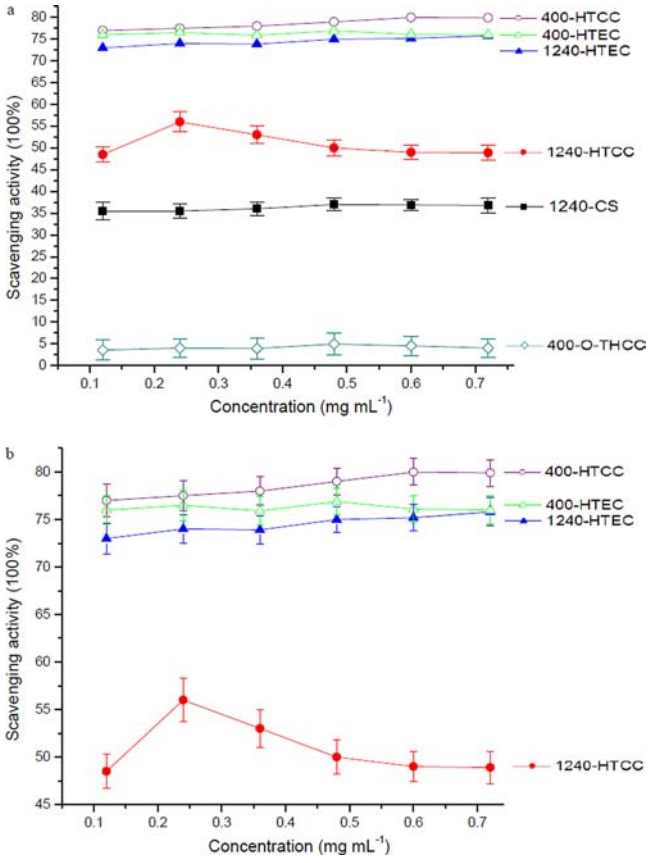

Figure 6. DPPH● scavenging activity of high molecular weight chitosan and quaternized chitosans at various concentrations.

HMW CS and QCSs. As shown in Figure 6, all the samples exhibited DPPH● scavenging ability, and the concentration of sample had little effect on the percentage of DPPH● scavenging, except for 1240-HTCC, which had a relative maximum at 0.24 mg/mL. At a concentration of 0.12−0.72 mg/mL, the percentage scavenging effect was up to 80% for 400-HTCC but not more than 5% for 400-O-HTCC. Overall, the active hydroxyl and amino in the chitosan chains play important role in the DPPH● scavenging. The differences between each sample may be explained in detail as follows.

Compared to unmodified 1240-CS, all of the N-quaternized chitosan derivatives (THCC and HTEC) exhibited higher DPPH● scavenging ability and O-quaternized chitosan (400-O-HTCC) exhibited lower ability. This means that the active hydroxyl in the chitosan backbone plays a more important role in the DPPH● scavenging than the amino of it, and the newly generated hydroxyl group and the extra introduced positive charge may also increase DPPH● scavenging activity.

In the case of the same kind of quaternary ammonium salt group modified CSs, DPPH● scavenging capacity decreased with the increase of the MW of QCS (Figure 6b). This may be the effect of intramolecular hydrogen bonds. It is well-known that CS with HMW has compact structure and high viscosity in solution, whose intramolecular hydrogen bonds are stronger than that of CS with LMW. The strong effect of hydrogen bonds weakens the activities of the hydroxyl and amino.[27] QCS can destroy part of the hydrogen bonds, but at the same time, new hydrogen bonds, such as the extra introduced hydroxyl and the unreacted chitosan's hydroxyl, were formed. At the same time, due to the stable DPPH● with a big bulk, it is also difficult to react with HMW QCS.

In the case of the different quaternary ammonium salt groups modified CSs, 400-HTCC and 400-THEC on DPPH● scavenging showed little difference. However, 1240-HTCC and 1240-HTEC on DPPH● scavenging had obvious difference; the latter was superior to the former (Figure 6b). This may be the effect of steric hindrance, and the effect will be more significant for the CS with higher MW: namely that the reactivity of CS can be improved by introduction large groups.[16] On the other hand, the larger steric hindrance of HTEC than that of HTCC made the intramolecular and intermolecular hydrogen bonds of 1240-HTEC less than that of 1240-HTCC. Meanwhile, the quaternized degree of 1240-HTEC was less than that of 1240-HTCC. These mean that the active hydroxyl and amino groups of 1240-HTEC are more than that of 1240-HTCC. And the effect of steric hindrance would also an important reason for the QCS with HMW on DPPH● scavenging .

●OH Scavenging Activity. The ●OH, generated by the Fenton reaction in this system, was scavenged by a 1240-CS and five QCSs. Compared to the absorbance of the control (without the test sample), decreased absorbance at 535 nm of the reaction mixture (with the test sample) indicated decreased oxidation of DR. As shown in Figure 7, the ●OH scavenging activity increased with the concentration of samples. The scavenging activity of chitosans against ●OH may be derived from following mechanisms:[28] (1) The hydroxyl groups in the polysaccharide unit can react with ●OH by the typical H-abstraction reaction. (2) ●OH can react with the residual free amino groups ($-NH_2$) to form stable macromolecule radicals. (3) The NH_2 groups can form ammonium groups (NH_3^+) with hydrion from solution and then react with ●OH through addition reaction. According to the mechanisms, the more the hydroxyl groups and the amino groups exist in a scavenger, the higher the scavenging rate will be. Therefore the high concentration of chitosans increases the scavenging rate.

At a concentration of 0.11−1.1 mg/mL, the percentage scavenging effect of 1240-CS ranged from 12−83%, which was higher than that of all the QCSs, except for 1240-THCC at the concentration below 0.50 mg/mL. It proved that the N-substituted CSs had weak ●OH scavenging ability, which is consistent with the reported.[14,15] However, at the concentration below 0.50 mg/mL, 1240-THCC had slightly higher hydroxyl radical scavenging ability than unmodified 1240-CS. This may be explained that after modification by GTMAC, some of the intramolecular hydrogen bonds of CS are destroyed and the reactivity is improved in the low

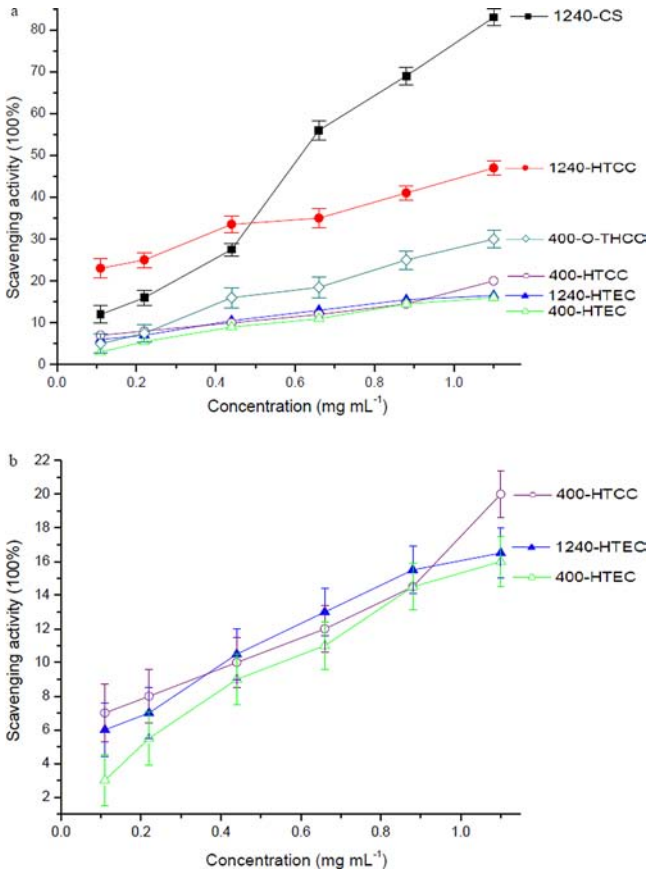

Figure 7. •OH scavenging activity of high molecular weight chitosan and quaternized chitosans at various concentrations.

concentration range. When the concentration up 0.50 mg/mL, this effect became weak.

Compared to 400-HTCC, 400-O-HTCC had higher hydroxyl radical scavenging ability. Meanwhile, 1240-HTCC and 1240-HTEC had lower hydroxyl radical scavenging ability than that of 1240-CS. These may illustrate that, for the same molecular weight of chitosans, their C-2 amino group in •OH scavenging is more important than C-6 hydroxy group, and like the scavenging ability of DPPH•, 400-HTCC and 400-THEC on •OH scavenging showed little difference; 1240-HTCC and 1240-HTEC on •OH scavenging had obvious differences. This may be the effect of steric hindrance, and for the same reason that differences in activity were caused on account of the active hydroxyl and amino. Here, the •OH scavenging ability of 1240-HTCC was superior to that of 1240-HTEC. It may be the antioxidant activity of positive charge.[14,15] Compared to 1240-HTEC with 74.7% degree of quaternization, 1240-HTCC with 84.6% degree of quaternization had more positive charge.

•O_2^- Scavenging Activity. The •O_2^- scavenging activity of HMW CS and QCSs was measured by the riboflavin−NBT−light system in vitro. Photochemical reduction of flavins generates •O_2^-, which reduces NBT, resulting in the formation of blue formazan. The assay is based on the capacity of the test sample to inhibit formazan formation by scavenging the •O_2^- generated in a riboflavin−NBT−light system. The •O_2^- scavenging ability of these samples is shown in Figure 8. All the samples exhibited effective scavenging activity against •O_2^- and showed an upward trend with the increase of sample concentration. Within the experimental concentration range, the •O_2^- scavenging ability of 1240-HTEC and 1240-CS was

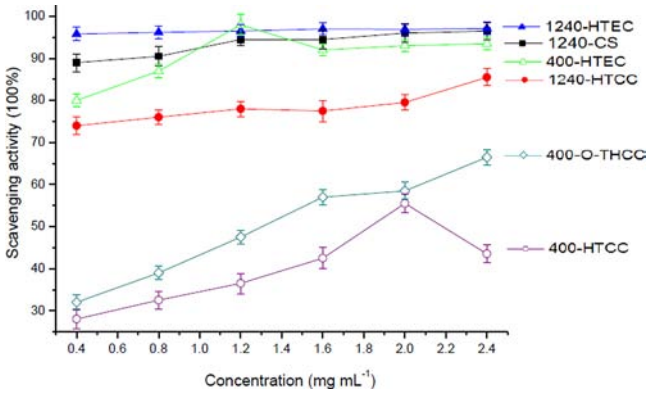

Figure 8. •O_2^- scavenging activity of high molecular weight chitosan and quaternized chitosans at various concentrations.

better than that of others, and the scavenging activity was 97.1% and 96.5%, respectively, at maximum concentration of 2.4 mg/mL. For 400-HTEC and 400-HTCC, they had a relative maximum at 1.2 and 2.0 mg/mL, respectively. Polysaccharides with the scavenging effect on •O_2^- have the same structural feature in that they all have one or more alcohol or phenolic hydroxyl groups, and the scavenging effect was related to the number of active hydroxyl groups in the molecule. On the other hand, according to the free-radical theory, the amino groups in chitosans can react with free radicals to form more stable macroradicals. Therefore, the active hydroxyl and amino groups in the polymer chains are the origin of the scavenging ability of chitosans.[29]

As shown in Figure 8, to QCSs with the same quaternary ammonium group, the scavenging ability of QCS with HMW was higher than that of QCS with LMW (1240-HTEC vs 400-HTEC, 1240-HTCC vs 400-HTCC), and to QCSs with different quaternary ammonium groups, the scavenging ability of QCS with big quaternary ammonium group was higher than that with small quaternary ammonium group (1240-HTEC vs 1240-HTCC, 400-HTEC vs 400-HTCC). These may be explained as follows: For QCS with HMW and QCS with a big quaternary ammonium group, they all had lower degree of quaternization than QCS with LMW and QCS with a small quaternary ammonium group (Table 1, entry 3 vs 2, entry 5 vs 4, and entry 4 vs 2, entry 5 vs 3), and with low degree of quaternization, the copolymer has relatively high content of hydroxyl and amino groups.[13]

Compared to 400-HTCC with amino substituted, 400-O-HTCC with hydroxyl substituted had moderately improved •O_2^- scavenging ability. This means that, like the scavenging ability of •OH scavenging, the C-2 amino group of CS in •O_2^- scavenging are more important than C-6 hydroxyl.

In this work, novel QCSs, i.e., O-HTCC, HTCC, and HTEC with HMW (400 and 1240 kDa), were prepared with the addition of hydroxyl groups and quaternary ammonium salt groups, and the in vitro antioxidant activity of a HMW CS (1240 kDa) and five QCSs was evaluated as free radical scavengers against DPPH•, •OH, and •O_2^-. The results showed that 1240-CS with 97% deacetylation displayed good antioxidant activity and different QCSs had obviously different free radical scavenging activity due to the various free radical scavenging mechanis, attributed to their different molecular weights, different contents of the active hydroxyl and amino groups, the positive charge, and also steric effect, etc. The active hydroxyl and amino groups in the chitosan chains play an

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important role in the free radical scavenging. This study was useful in assaying the antioxidant capacities of chitosans with HMW, which have promising applications in food and medicine fields. The various antioxidant mechanisms and in vitro antioxidant activity need investigate further.

AUTHOR INFORMATION

Corresponding Author

*Phone: +86 21 34201245. Fax: +86 21 54741297. E-mail: wanajun@sjtu.edu.cn (A.W.); lihl@sjtu.edu.cn (H.L.).

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Notes

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ABBREVIATIONS USED

CS, chitosan; QCS, quaternized chitosan; MW, molecular weight; HMW, high molecular weight; LMW, low molecular weight; DPPH•, 1,1-diphenyl-2-picrylhydrazyl radical; •OH, hydroxyl radical; $\bullet O_2^-$, superoxide radical; H_2O_2, hydrogen peroxide; GTMAC, glycidyl trimethylammonium chloride; GTEAC, glycidyl triethylammonium chloride; HTCC, *N*-(2-hydroxyl) propyl-3-trimethyl ammonium chitosan chloride; HTEC, *N*-(2-hydroxyl) propyl-3-triethyl ammonium chitosan chloride; O-HTCC, *O*-(2-hydroxyl) propyl-3-trimethyl ammonium chitosan chloride; EDTA, ethylene diamine tetra-acetate; NBT, nitro blue tetrazolium; TBA, thiobarbituric acid; TCA, trichloroacetic acid; DR, deoxyribose; $FeCl_3$, ferric chloride; BCM, biochemistry chemiluminescence meter

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