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Synthesis of chitosan derivative with dual-antibacterial functional groups and its antibacterial activity

Zhihan Li, Fei Yang, Rendang Yang
State Key Laboratory of Pulp & Paper Engineering, South China University of Technology, Guangzhou 510640, China
Correspondence to: R. Yang (E-mail: rdyang@scut.edu.cn)

ABSTRACT: The O-fumaryl ester (OFTMCS) of N,N,N-trimethyl chitosan (TMCS) has been synthesized as a water-soluble chitosan (CS) derivative bearing dual-functional groups, with the aim of discovering novel CS derivatives with good water solubility and enhanced the antibacterial activity compared with unmodified CS. OFTMCS was characterized by FT-IR, ^{13}C NMR, XPS, XRD and Zeta potential analyses. The XPS results indicated that the degree of substitution (DS) on the C_2-NH_2 group of the CS was 0.78, and that the DS on its C_6-OH group was 0.31. The TGA results showed that the thermal stability of OFTMCS was lower than that of unmodified CS. The antibacterial activities of OFTMCS were investigated by assessing the mortality rates of the representative Gram-positive and Gram-negative bacteria *Staphylococcus aureus* and *Escherichia coli*, respectively. The results indicated that OFTMCS exhibited superior antibacterial activity to CS at a lower dosage. The synthesis of CS derivatives bearing dual-functional groups could therefore be used as a promising strategy to enhance the antibacterial activity of CS. The antimicrobial mechanism of action of OFTMCS was discussed. © 2015 Wiley Periodicals, Inc. J. Appl. Polym. Sci. **2015**, *132*, 42663.

KEYWORDS: biomaterials; biomedical applications; synthesis and processing

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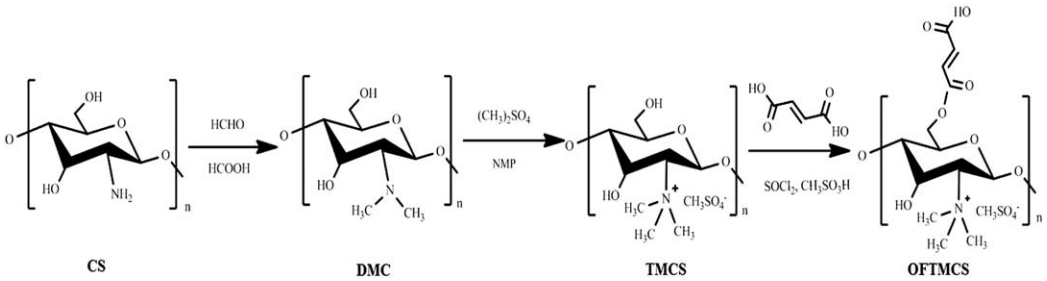
INTRODUCTION

Chitosan (CS) is the only known positively charged natural polysaccharide and is the deacetylated form of chitin. The core structure of CS is therefore composed of a linear arrangement of glucosamine units, which are more precisely known as 2-amino-2-deoxy-(1→4)-β-D-glucopyranan units.[1] CS is endowed with excellent material properties and has been applied to good effect in a variety of research areas, including medicine,[2] biotechnology,[3] textiles,[4] botany,[5] and wastewater treatment.[6] CS and its oligomers have recently attracted considerable interest because of their broad-spectrum antimicrobial activities.[7,8] The antimicrobial activities of CS towards a variety of bacteria and fungi have been well documented in the literature and attributed in all cases to its polycationic nature.[9,10] However, CS exhibits poor aqueous solubility and is also poorly soluble in most commonly used organic solvents, which has limited its utilization. Numerous modifications have been investigated with the aim of improving the solubility of CS, such as carboxymethylation,[11] alkylation[12] and sugar-modification.[13] Unfortunately, modifications of this type generally lead to CS derivatives that exhibit only moderate levels of antimicrobial activity, which has limited their application.

CS possesses reactive amino and hydroxyl groups that can be used to chemically modify CS and alter its properties via a variety of reactions, such as N-quaternization and O-esterification.[14] Among the CS derivatives reported to exhibit higher antibacterial activity than native CS, quaternized CS derivatives have been the subject of a growing level of attention because of their enhanced water solubility (WS) properties and increased overall positive charge.[15] Indeed, the quaternization of N-alkyl CS derivatives using dimethyl sulfate represents a feasible approach for the elaboration of water-soluble cationic polyelectrolytes.[16] Furthermore, the antibacterial activity of N,N,N-tri-methyl chitosan (TMCS) was evaluated against an array of common bacteria, including both Gram-positive and Gram-negative bacteria, where it exhibited good aqueous solubility over a wide pH range.[17] Although the quaternization of CS derivatives generally leads to improved levels of antimicrobial activity compared with unmodified CS, the resulting quaternized CS derivatives do not represent suitable alternatives for the substitution of existing antimicrobial agents. Li *et al.*[18] recently proposed that CS derivatives bearing dual-functional groups exhibited enhanced levels of antibacterial activity, as well as higher levels of growth suppression activity against a number of Gram-negative and Gram-positive bacteria compared with the corresponding single functional group derivatives. CS can undergo chemical reactions at its C_2-NH_2 and C_6-OH positions to give the corresponding modified derivatives. For example, CS can undergo esterification reactions at its C_6-OH group to afford the corresponding CS



Scheme 1. Synthesis of the O-fumaryl ester of TMCS.

esters, with the C_2-NH_2 group generally being converted to the corresponding quaternary ammonium group The introduction of an ester group at the C_6-OH position of CS can give rise to dual-antibacterial functionality when it is taken together with C_2 polycationic quaternary ammonium groups, which greatly enhance the antibacterial properties of CS derivatives. Fumaric acid is an excellent broad-spectrum antibacterial agent and its activity has been attributed to its unsaturated carbonyl structure and two carboxylic acid groups.[19,20] Furthermore, fumaric acid has been used as a food preservative because of its strong antimicrobial activity against food pathogenic microorganism, and was also used to improve the microbial safety of alfalfa and clover sprouts.[21,22]

In this study, we have successfully synthesized a series of water-soluble CS derivatives bearing dual-functional groups, and the structures of these derivatives are depicted in Scheme 1. O-Fumaryl ester N,N,N-tri-methyl chitosan (OFTMCS) was synthesized by reacting TMCS free of O-methylation with fumaryl chloride. The quaternization of the amino groups of CS would lead to the formation of permanently positively charged quaternary ammonium groups which would enhance the antimicrobial activity of the CS derivatives as well as improving their aqueous solubility over the entire pH range. It was envisaged that the hydroxyl groups of the TMCS material, which was free from O-methylation at its C_6 position, would react with fumaryl chloride to give the corresponding O-fumaryl ester with concomitant conversion of the amino group at the C_2 position to the quaternary ammonium salt, which would also lead to an increase in the antimicrobial activity of the resulting CS derivative. The CS derivatives generated in this way were evaluated in terms of their antimicrobial activities against the Gram-positive and Gram-negative bacteria *Staphylococcus aureus* and *Escherichia coli*, respectively. Furthermore, we have provided a detailed discussion of the synergistic antibacterial mechanism of this new CS derivative towards *S. aureus* and *E. coli* as the theoretical basis for the combined use of antibacterial groups.

EXPERIMENTAL

Materials

Chitosan (Mw = 50 kDa, DD $\geq$ 95.0% according to the manufacturer) was purchased from Hedebei Ocean Biochemical (Shandong, China). Dimethyl sulfate (AR) was obtained from ChengDu KeLong Chemical (Sichuan, China). All of the other reagents used in this study were purchased from commercial suppliers and used without further purification. *E. coli* (ATCC8739) and *S. aureus* (ATCC6538) were provided by the Guangdong Institute of Microbiology.

Synthesis of the CS Derivatives

Synthesis of TMCS. TMCS, which was free from O-methylation, was synthesized according to a previously reported procedure with minor modifications.[16] Briefly, CS (3.0 g) was added to a solution of formic acid (15.0 g) in distilled water (100 g), and the resulting mixture was stirred at ambient temperature for 30 min, to allow for the CS to dissolve completely. Formaldehyde (12 mL) was then added to the mixture in a dropwise manner at 70°C, and the resulting mixture was stirred for 48 h at the same temperature. The pH of the mixture was then adjusted to pH 12 by the addition of a 1*N* solution of sodium hydroxide, which led to the formation of a gel. The gel was filtered and washed with ethanol to remove any unreacted formaldehyde, and the product was then extracted for 48 h in a Soxhlet extractor with diethyl ether to give pure N,N-dimethyl chitosan (DMCS). DMC (2 g) was dissolved in NMP (40 mL) with stirring overnight at 45°C. Dimethyl sulfate (8 mL) was added to the NMP solution, and the resulting mixture was stirred at 45°C for the desired time before being poured into ethanol to precipitate the production. The resulting derivatives were placed in dialysis solution bags (MWCO 14,000), and dialyzed in deionized water for 48 h by changing the water every 12 h. The resulting dialyzed material was then lyophilized to give the desired TMCS material.

Preparation of Fumaryl Chloride. Dry fumaryl acid was dissolved in thionyl chloride, and the resulting mixture was heated at reflux for 3–5 h. The mixture was then cooled to ambient temperature and distilled on a rotary evaporator to give fumaryl chloride.

Synthesis of OFTMCS

TMCS (2 g, 1.0 mmol calculated as glucosamine units) was dissolved in methanesulfonic acid (20 mL), and the resulting solution was cooled to 5°C on an ice-water bath. Fumaryl chloride (the ratios of TMCS to fumaryl chloride were adjusted to 1 : 1 eq/glucosamine unit of CS) was then added to the TMCS solution in a dropwise manner with external cooling from an ice-water bath, and the resulting mixture was stirred at 40°C for 24 h. The pH of the mixture was then adjusted to neutrality by the addition of ammonium hydroxide. The desired product was subsequently precipitated from acetone and placed in dialysis solution bags (MWCO 14000), where it was dialyzed in deionized water for 48 h by changing the water every 12 h. Lyophilization of the resulting material gave OFTMCS.

Characterization

FT-IR Spectroscopy. FT-IR spectra were recorded on a Nicolet FT-IR 5700 spectrophotometer (Bruker, Rheinstetten, Germany) using KBr pellets. The FT-IR spectra were scanned against a blank KBr pellet background at wave numbers in the range of 4000–500 cm^{-1} with a resolution of 4.0 cm^{-1} at 25°C.

NMR Spectroscopy. ^{13}C NMR spectra were recorded on a Bruker AV600 MHz spectrometer (Bruker). All of the samples for NMR analysis were dissolved in D_2O.

X-ray Photoelectron Spectroscopy (XPS). The elemental compositions (i.e., C, O, N, and S) of CS and its derivatives were determined by XPS on a Kratos Axis Ultra DLD system (UK). In this way, it was possible to calculate the degree of substitution (DS) of each sample after the percentages of particulate organic carbon and nitrogen contained in each sample had been obtained.

Crystallization Behaviors of the CS Derivatives. The crystallinity of the CS derivatives was investigated by X-ray diffraction (XRD) analysis on a D8 Advance X-ray diffractometer (Bruker) with Cu Kα radiation ($\lambda = 0.15418$ nm) at 40 kV and 50 mA. The relative intensity was recorded in the scattering range of 5–40° (2θ) at a scanning rate of 1°/min.

Zeta Potential of CS Derivatives. The zeta-potential values of the CS derivatives were determined using a 3000 HSa nanometer particle size and potential analyzer (Malvern Instruments, UK) at 25°C with a scattering angle of 173°, The sample concentration was set at 0.1% (w/v).

Thermal Gravimetric Analysis (TGA)

TGA was carried out on a SDT-Q 500 simultaneous thermal analyzer (TA) under a nitrogen atmosphere from 50–600°C at a heating rate of 10°C/min.

Solubility Test

One gram of CS or its derivatives was dispersed in distilled water (100 mL, pH 7.0), and the resulting mixture was stirred at 25°C for 12 h. The insoluble portion was then separated, washed with ethanol and dried under vacuum in an oven at 50°C for 48 h. The WS was calculated according to eq. (1).

$$\mathrm{WS} = [(1 - w)/1] \times 100 \quad (1)$$

where w is the weight of the undissolved sample (g).

All of the experiments were conducted in triplicate. To evaluate the effect of pH on the WS of derivatives, a 5 g sample of the CS derivatives was dissolved in 1% (v/v) acetic acid (100 mL). The subsequent stepwise addition of a 1*N* NaOH solution allowed for the transmittance of the solution to be recorded at a variety of pH values using a UV spectrophotometer at 600 nm.[23]

Antibacterial Test

The antibacterial activities of the CS and its derivatives were tested using a modified colony counting method on bacteria selected for their resistance.[24] In this study, *E. coli* and *S.aureus* were cultured overnight on beef extract peptone medium at 37°C. After twice successive transfers, the activated strains were inoculated into 100 mL nutrient broth and cultured at 37°C for 24 h. Bacterial cells were harvested by centrifugation at 3000 r/min for 10 min using an Eppendorf 5804R centrifuge (Hamburg, Germany), washed and resuspended in sterile normal saline (0.5%) solution. Using McFarland standards, the bacteria suspensions of *E. coli* and *S. aureus* containing 10^7 CFU/mL were prepared and used for the antibacterial test. The prepared bacterial suspension (0.1 mL) was inoculated under aseptic condition into 10 mL of nutrient broth medium. Each sample contained the CS derivatives at the concentration of 0.1, 0.25, 0.5, 0.75, 1.0, 5.0, or 10.0 mg/mL, respectively, whereas a blank without test materials was prepared for comparison. All of the samples were incubated at 37°C under constant agitation. During incubation, 0.1 mL of each suspension was taken to determine the bacteria count by serial dilution with triplicate plating on agar plates. Then the plates were taken out of the incubator, and the inhibitory activity was calculated. The data were the reported as the mean values of at least three parallel experiments with discrepancies among them of less than 5%. The inhibition ratios for CS and its derivatives were calculated using eq. (2).

$$\text{Inhibition ratio } (\%) = [(A_0 - A_1)/A_0] \times 100 \quad (2)$$

where A_0 is the initial cell number (CFU/mL) and A_1 is the cell number following the treatment process (CFU/mL).

Integrity of the Cell Membrane

The release of intracellular components that absorb at 260 nm is an indication of membrane damage, as described by Chen and Cooper.[25] *E. coli* (ATCC 8739) and *S. aureus* (ATCC 6538) were selected as model Gram-negative and Gram-positive bacteria, respectively, to investigate the antimicrobial mechanism of action of these CS derivatives. Briefly, the cultured bacteria were harvested by centrifugation at 3000 r/min for 10 min before being washed with sterile water and re-suspended in a sterile 0.5% (w/v) NaCl solution. The final concentration of the bacterial suspension was adjusted to an OD_{420} value of 0.8 to measure the OD_{260}, which represents the release of DNA and RNA from the cells. The solutions of CS, TMCS and OFTMCS were inoculated into bacterial suspensions to give a final concentration of 0.1% or 1%, respectively. Isopropanol (IPA) was used as a control run of classical antibacterial agent. The release of cell materials over time at 260 nm was recorded on a Hach DR3900 UV/vis spectrophotometer (USA).

Observation by Scanning Electron Microscopy

Bacterial strains (*E. coli* and *S. aureus*) were collected at the late logarithmic phase of growth with initial concentrations of about 10^8 CFU/mL. The cell suspensions were incubated with an OFTMCS solution (final concentration 0.1 wt %) at 37°C for 8 h. The bacterial cells were subsequently collected by centrifugation at 3000 r/min for 10 min, and the resulting pellets were washed three times with a 0.1 N solution of PBS before being fixed with a 2.5% (w/v) solution of glutaraldehyde in sterile water. The cells were then washed in the same buffered solution and were post-fixed for 30 min with osmium tetroxide in a 0.1*N* solution of cacodylate buffer (pH 7.2). The samples were dehydrated in a graded series of acetone, vacuum dried and sprayed onto a glass plate before being observed on a S-3700N scanning electron microscope (SEM) (Zeiss, Germany).

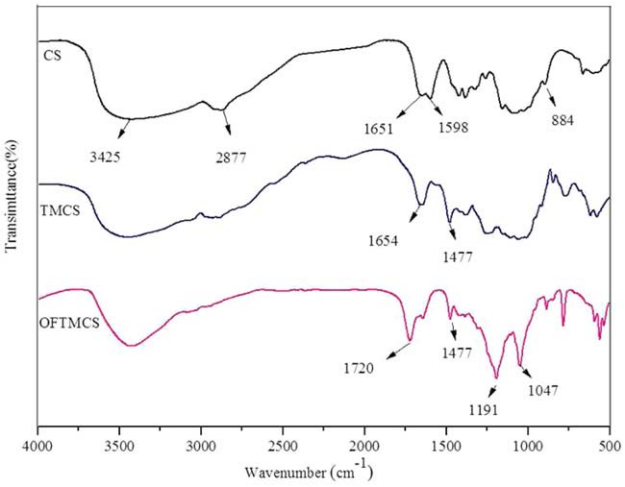


Figure 1. FTIR spectra of CS and its derivatives. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

RESULTS AND DISCUSSION

FT-IR Analysis

As shown in Figure 1, CS contained a broad band at 3425 cm^{-1}, which was attributed to the stretching vibrations of the —NH and —OH groups, as well as the inter- and extra-molecular hydrogen bonding interactions of the CS molecules. The FT-IR spectrum of CS also contained a weak peak at 2877 cm^{-1}, which was related to the stretching vibrations of the —CH groups. The characteristic bands at 1651, 1598, and 884 cm^{-1} were attributed to the one amide, one amine and three amide absorptions of CS, respectively.[26]

Compared with CS, the FT-IR spectrum of TMCS contained a new strong absorption at 1477 cm^{-1}, which was attributed to the asymmetrical stretching vibration of the C—H moieties in the methyl groups of TMCS. Furthermore, the peak at 1598 cm^{-1}, which was assigned to the primary amine of CS, disappeared in the FT-IR spectrum of TMCS, confirming that the amino group has been substituted.[16]

The FT-IR spectrum of OFTMCS contained a new absorption band at 1720 cm^{-1}, which was characteristic of a C=C—COOR group.[20] The FT-IR spectrum of OFTMCS also contained peaks at about 1191 and 1047 cm^{-1}, which were attributed to the stretching vibrations of the C—O and C—O—C groups resulting from the esterification reaction of TMCS with fumaryl chloride.[20,27] Notably, the characteristic absorption band belonging to the C—H moieties of the methyl groups of TMCS was still present at 1477 cm^{-1}.

Based on these results, it can be concluded that OFTMCS had been successfully synthesized (Figure 1).

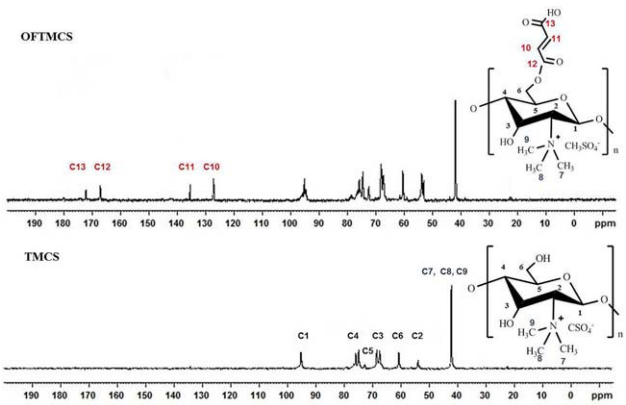


Figure 2. ^{13}C-NMR spectra of TMCS and the O-fumaryl ester of OFTMCS. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

NMR Analysis

The structures of the CS derivatives were further confirmed by ^{13}C NMR analysis, as shown in Figure 2. The ^{13}C NMR spectrum of TMCS contained peaks at 53.95(C2), 60.58(C6), 68.34(C3), 72.18(C5), 74.90(C4), and 95.8(C1) ppm, which were attributed to the CS backbone. The peak at 42.9 ppm was consistent with the presence of a $—N(CH_3)_3$ group in the molecule. These spectral data for CS coincided with those reported previously by other researchers.[16] The ^{13}C NMR spectrum of OFTMCS contained signals 167.2 and 172.3 ppm, which were attributed to the carbonyl carbon atoms of the =CH—COOH and =CH—COOR fumaryl group, respectively. Several other signals were also observed at 135.5 and 131.3 ppm, which were attributed to the alkenyl carbon atoms of the =HC—COOR and =HC—COOH fumaryl groups, respectively.[20,27] These data therefore provided further confirmation that the CS derivatives had been successfully synthesized (Figure 2).

XPS Analysis and Zeta Potential

XPS is a very useful tool for the identification and analysis of material elements. With this in mind, XPS was used in the current study to provide further confirmation that TMCS and OFTMCS had been successfully synthesized based on their elemental analysis data. The DS of the CS derivatives prepared in the current study were estimated based on the POC and PON values, as shown in Table I. The DS value of the single substitution product containing carbon and nitrogen of the ligands to $-NH_2$ group on CS could be calculated by eq. (3), which was modified for base according to the model described by Jiang *et al.*[28,29]

$$DS = \frac{R}{X}\left(\frac{W_{c1}}{W_{n1}} - \frac{W_{c0}}{W_{n1}}\right) \quad (3)$$

where R is the ratio of the relative atomic masses of nitrogen and carbon, W_{c1} and W_{n1} are the atomic concentrations of C and N in the CS derivatives, Wc_0 and Wn_1 are the atomic concentrations of C and N in the unmodified CS, and X is the atomicity of C atom of the substitutional group. The DS of the $C_2—NH_2$ group was calculated to be 77.90%, whereas the DS of the $C_6—OH$ group was determined to be 31.02%.

Figure 3(a) shows the XPS spectra of CS and the CS derivatives. The resolved N_{1s} spectra of unmodified CS was shown in Figure 3(b). The peak at 397.5 eV was assigned to $—NH_2$ and N—C=O chemical bindings, while the peak at 399.7 eV was assigned to amino groups in the ammonium form (NH_3^+). In comparison of Figure 3(b,c), the atomic percentage of carbon increased while that of nitrogen decreased, which is due to the inclusion of the quaternary amine [R–$N(CH_3)_3$] group in

Table I. The Results of XPS Analysis and Zeta Potential of CS Derivatives

	Atomic %				Degree of substitution (%)		
Sample	C %	N %	O %	S %	DS of O	DS of N	*Zeta potential*
CS	63.56	6.87	24.00	0	0	0	+37.4
TMCS	64.72	5.43	27.69	2.15	77.90	0	+49.1
OFTMCS	*64.69*	*5.03*	*29.52*	*0.76*	*77.90*	*31.02*	*+36.3*

TMCS. As compared to natural CS, the resolved N_{1s} spectrum of TMCS revealed obvious changes: the significant increase of the peak at 400.8 eV was assigned to the insertion of N—C type nitrogen from the R— $N^+(CH_3)_3CH_3SO_4^-$ group. Consequently, the decrease of the peak relative intensity at 397.9 eV due to the partial disappearance of $C—NH_2$ type carbons after quaternization.[30]

The high resolution spectrum of C_{1s} for unmodified CS was in agreement with data reported by several other researchers.[31,32] The C_{1s} spectrum of unmodified CS [Figure 3(d)] was fitted based on the following contributions: 282.4 eV for C=C species (sp^2, peak 1) and 282.5 eV for C—C species (sp^3, peak 1), 284.0 eV for C—O or C—N species (peak 2) and 285.9 eV for C=O or O—C—O species (peak 3). Compared with natural CS, the carbon peak at around 286.3 eV was higher, which indicated that the atomic concentration (%) of C–N increased following the trimethyl quaternization [i.e., the formation of the $R—N(CH_3)_3$ group], as shown in Figure 3(e). After the O-fumaryl esterification reaction, there was an increase in the intensity of the peak at 282.4 eV, which was attributed to the insertion of C=C and C—C type carbon atoms and bonds from the fumaric acid group, as shown in Figure 3(f). Furthermore, the peak at 286.4 eV increased in intensity because of the incorporation of C=O and O—C—O type carbon atoms following the O-esterification reaction.

As can be seen from Table I, TMCS exhibited a greater zeta potential than unmodified CS. This result indicated that the quaternization approach was particularly efficient in terms of enhancing the overall positive charge of CS. The zeta potential of OFTMCS was +36.3 mV, and therefore lower than those of CS and TMCS (Table I).

XRD and Solubility of the CS Derivatives

The X-ray diffractograms of CS, TMCS and OFTMCS are shown in Figure 4(a). It is clear from these results that there were several differences in the heights, widths and positions of the peaks across the three samples. The X-ray diffractograms for CS contained two broad peaks at 11.7° and 20.3°, which indicated that CS possessed a high degree of crystallinity because of the extensive networks of inter- and extra-molecular hydrogen bonds between the CS molecules, as previously reported.[12] Compared with unmodified CS, the X-ray patterns of TMCS and OFTMCS revealed several changes in terms of their diffraction angles and peak intensities. The intensity of the peak originally recorded at 20.3° for unmodified CS was significantly reduced in the X-ray patterns of the TMCS and OFTMCS materials. Furthermore, another peak had shifted to a higher 2θ value of around 10.21°, which indicated that the crystal forms had been destroyed in these materials. It has been previously reported that there is a close relationship between the crystallinity of a polymer and its solubility, and that polymers with low crystallinities tend to dissolve more readily than those with higher crystallinities.[33] As shown in Figure 4(b), all of the samples tested in the current study gave transmittance values close to 100% at pH<6. This result indicated that the CS, TMCS and OFTMCS molecules have good solubility under acidic conditions. The transmittance of the CS solution rapidly decreased, and the solution was easily transferred to floc as the pH increased from 6.0 to 9.0. In contrast, the transmittance values of the TMCS and OFTMCS solutions slowly decreased as the pH was increased from 6.0 to 9.0. Furthermore, the results show that OFTMCS exhibited the best WS of all of the samples tested in the current study. The reason for OFTMCS exhibiting the highest solubility is probably related to the monomethyl fumarate and cationic trimethyl ammonium groups being strongly hydrophilic and capable of forming numerous hydrogen bonding interactions to water. It was clear from these results that the introduction of a group with a good hydration capacity greatly decreased the number of intermolecular and intramolecular hydrogen bonds of CS.[34]

TG Analysis

The thermal properties of CS and its derivatives were analyzed by TGA, and the results are shown in Figure 5. The TGA curves of CS and its derivatives indicated that CS degraded in three stages. For unmodified CS, the first stage of the degradation process occurred in the temperature range of 60–210°C, with a weight loss of about 2%, which was attributed to the loss of physically absorbed water. The second stage of the degradation process for unmodified CS started at a temperature of 240°C and finished at a temperature of 310°C. This degradation step was accompanied by a weight loss of 33%, corresponding to the pyrogenic decomposition and ablation of the molecular chains of CS, and occurred quite rapidly. The third stage in the degradation of CS occurred immediately after the second stage as a gradual change, and was attributed to the thermal decomposition of CS together with the oxidation of the existing decomposition products to CO_2. The CS derivatives prepared in the current study also degraded over three stages. TMCS and OFTMCS exhibited greater weight losses during the first stage of their degradation compared with unmodified CS, which was attributed to TMCS and OFTMCS being more hydrophilic than unmodified CS following the introduction of the quaternary

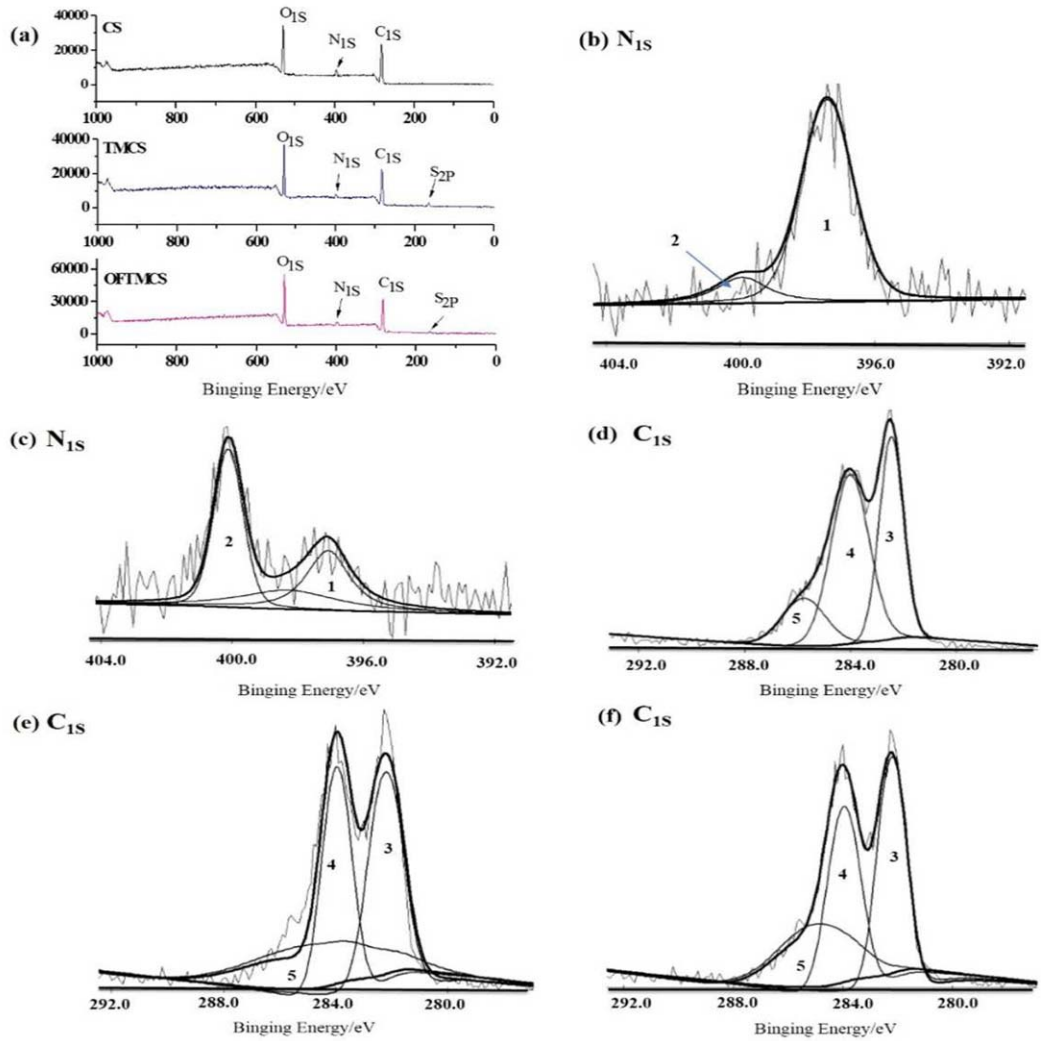


Figure 3. XPS spectra of CS, TMCS, and OFTMCS (a), N_{1s} high resolution spectra for CS (b) and TMCS (c), C_{1S} high resolution spectra for CS (d), TMCS (e) and OFTMCS (f). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

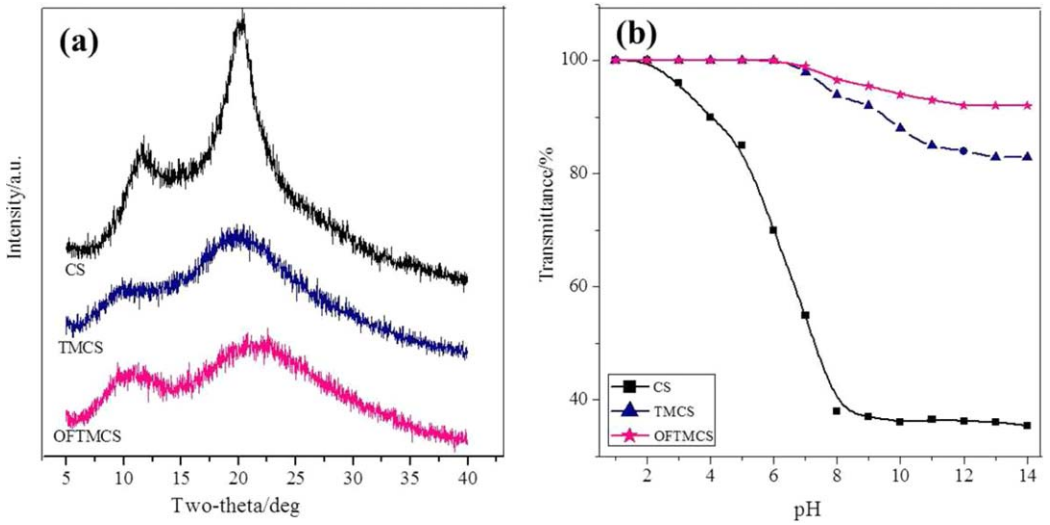


Figure 4. XRD data (a) and pH dependence on the water solubilities (b) of CS and its derivatives. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

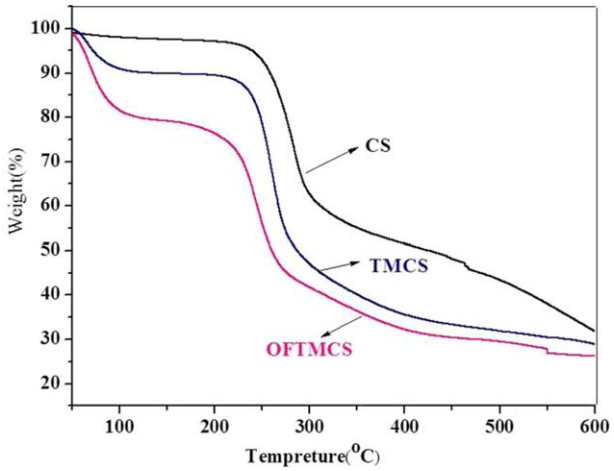


Figure 5. TGA thermograms of CS, TMCS, and OFTMCS. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

ammonium and monomethyl fumarate groups. The second degradation stage occurred at temperature of 210°C of TMCS and OFTMCS and reached a maximum at 283°C with weight loss of 35 and 29%, respectively. The third degradation stages for TMCS and OFTMCS were similar to that of CS. These results revealed that the chemical modification of CS led to a decrease in its thermal stability, with the thermal stability following the order OFTMCS < TMCS < CS. This reduction in the thermal stability of CS was attributed to the disruption of its crystal structure following the introduction of the new functional groups, which would have broken several key hydrogen bonding interactions.[29]

Antibacterial Assays

The date on antibacterial activities of CS and its derivatives against *S. aureus* and *E. coli* are shown in Figure 6. As can be seen from Figure 6(a), CS inhibits the growth of *S. aureus* and the inhibitory index is 33.8% at 0.5 mg/mL and it does not exhibit obvious inhibiting effect at lower concentrations. TMCS exhibited much higher antibacterial activity than CS, which supported the idea that the introduction of an additional positive charge through the tri-methyl quaternization process could be used as an effective strategy to improve the antibacterial activity of CS.[26,35] The O-esterification of TMCS led to the formation of OFTMCS, which exhibited stronger antibacterial activity.

The antibacterial activities of the CS derivatives were found to vary with the type of microorganism. The data shown in Figure 6(b) indicate that CS is ineffective against the gram negative bacterium *E. coli* as the inhibitory index is 21.6% at 0.5 mg/mL. OFTMCS displayed the highest antibacterial activity followed by TMCS against *E. coil* at the same concentration. This result can be interpreted that the unsaturated α, β carbonyl structure of fumaric acid behave as an efficient antibacterial functional group. The carbonyl oxygen and α-carbon atoms of this functional group form an electronic relay system, which is separated in molecules. The conjugation of the π electrons of the carbonyl group with the adjacent π electrons of the olefinic bond results in an electron resonance effect, which has a strong electronic buffering capacity. This system could therefore accept an electron from the migration of the ATP and NADH cofactors involved in microbial metabolism, which would destroy the integrity of the electron transport chain of the bacteria, weaken the dynamic barrier function of the membrane, and eventually produce cell autolysis.[36,37]

The aim of the current study was to increase the total number of antibacterial groups on CS via the quaternization of its C_2—NH_2 group and the esterification of its C_6—OH group to obtain CS derivatives with dual-antibacterial functional groups. The results revealed that the antimicrobial activities of OFTMCS were much greater than those of CS and TMCS, which indicated that the quaternary ammonium group and the monomethyl fumarate group of OFTMCS could be act in synergistically. These results therefore demonstrate that the introduction of additional antibacterial groups represents an effective strategy for enhancing the antibacterial properties of CS.

Integrity of Bacterial Cell Membranes

The bacterial membrane serves as a structural component that can be compromised when bacterial suspensions are exposed to antibacterial agents.[38] Notably, the leakage of the cytoplasmic contents of the bacterial cell can be detected by absorption at 260 nm, and used as an indication of membrane damage.[25]

The release of intracellular components upon addition of CS derivatives to *S. aureus* suspension is shown in Figure 7(a). When *S. aureus* suspensions were treated with CS and TMCS,

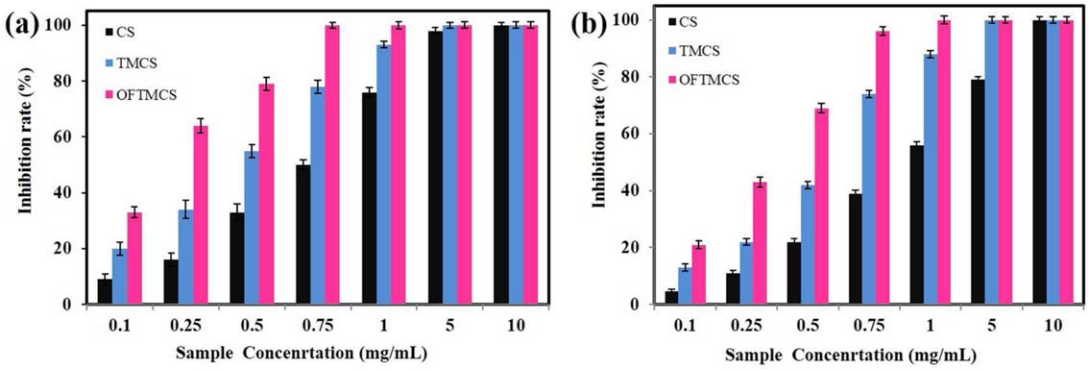


Figure 6. Inhibitory activities of CS and its derivatives towards the growth of *S. aureus* (a) and *E. coli* (b). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

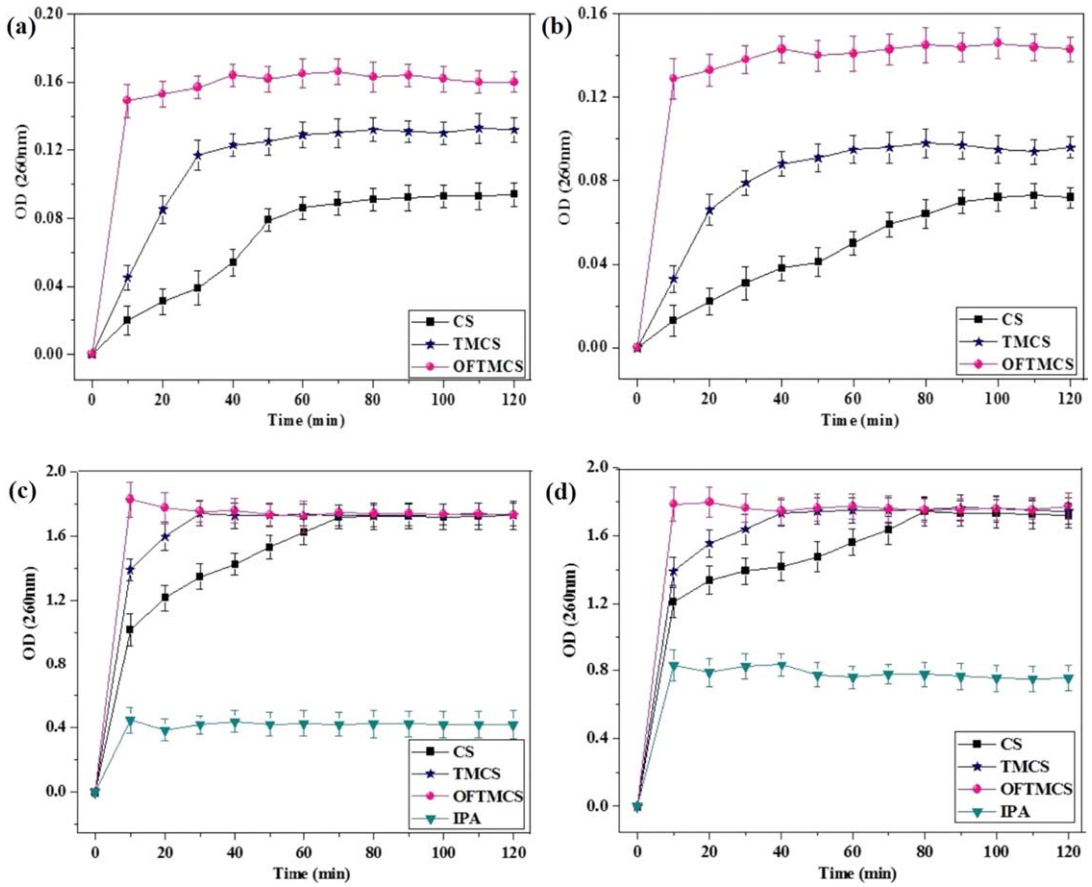


Figure 7. Effects of 0.1 wt % CS and its derivatives on the UV absorption at 260 nm of *S. aureus* (a) and *E. coli* (b); 1 wt % CS and its derivatives on the UV absorption at 260 nm of *S. aureus* (c) and *E. coli* (d). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

the absorbance of the suspensions at 260 nm gradually increased up to 80 min. Thereafter the absorbance was almost unchanged. But *S. aureus* suspension was treated with OFTMCS, the A260 increased rapidly at first and then progressively slowed down up to 20 min later. Similar to the Figure 7(a), all the CS derivatives tested in the current study showed that they had the ability to damage the cell membrane of *E. coli*, leading to the release of nucleic acids in Figure 7(b). In addition, the leaking of the nucleic acid content induced by OFTMCS was markedly higher than that of CS and TMCS at the same concentration. Moreover, Liu *et al.*[10] reported that the antibacterial activity of CS is well related to membrane permeability. In this study, OFTMCS appeared most effective leading to the release of intracellular materials, which is also consistent with the results of the inhibition activity.

Results in Figure 7(c,d) indicated that the higher concentration of CS derivatives caused an increase in the A260 values. Notably, the addition of OFTMCS to the *S. aureus and E. coli* suspension led to a maximum A260 value in 10 min, which was the highest value achieved in the current study. These results indicated that OFTMCS had almost completely disrupted the cell membrane of bacteria, resulting in the release of almost all of its intracellular proteins. Furthermore, OFTMCS could destroy the integrity of bacterial cell membranes and induce the release of intracellular component in about 10 min, it suggested that OFTMCS quickly killed bacteria (Figure 7).

SEM Characterization of *E. coli* and *S. aureus* Cells

SEM was used to elucidate the exact mechanisms of action of OFTMCS towards both *S. aureus* and *E. coli* using bacteria that had been treated with a 0.1 wt % solution of OFTMCS for up to 1 h. As shown in Figure 8(a,c), the cell walls of the untreated *S. aureus* and *E. coli* were smooth and intact. The normal cells were surrounded by cell membranes with compact surfaces. Furthermore, there was no evidence to suggest the release of any intracellular components or notable ruptures in the surfaces of the normal cells. The damaging impact of OFTMCS on the bacterial cell walls of *S. aureus* and *E. coli* was observed as a change in their morphology, as shown in Figure 8(b,d). The treated samples showed several obvious changes, including cell wall damage, cell membrane lysis, leakage of the cytoplasmic content and cell distortion. The lipophilic properties of the fumaryl ester may have enhanced the permeability of OFTMCS. Furthermore, polycationic amino polysaccharides have been reported to rupture bacterial cell plasmalemma under stressful conditions.[39]

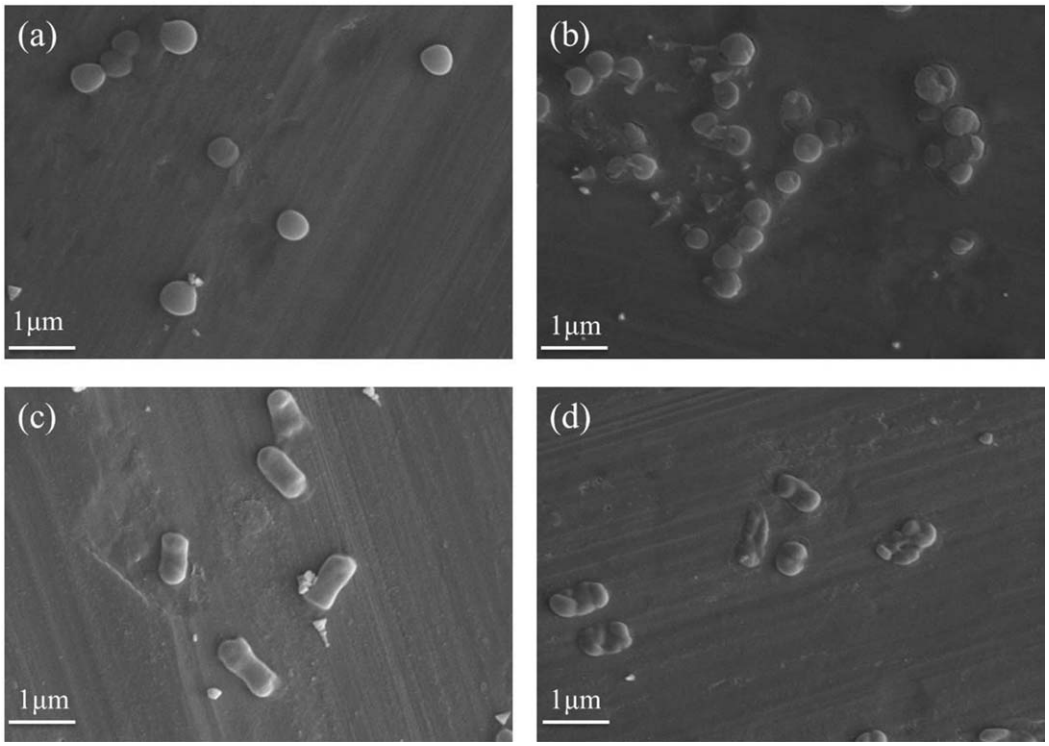


Figure 8. SEM images of the cells of *S. aureus* (a) and *E. coli* (c); cells after being treated with OFTMCS for *S. aureus* (b) and *E. coli* (d).

CONCLUSIONS

A water-soluble CS derivative (OFTMCS) has been prepared, which bears double functional groups by the reaction of TMCS with fumaric chloride. The structure of the modified CS derivative was characterized based on FT-IR, ^{13}C NMR, XRD, and XPS techniques. The introduction of two different functional groups to CS led to a decrease in the crystallinity and thermal stability of CS, which consequently led to an improvement in the WS and electropositivity of the material compared with unmodified CS. The antibacterial activity of OFTMCS was evaluated against *S. aureus* and *E. coli*, and found to exhibit higher antibacterial activities than unmodified CS and TMCS were observed in both cases. These results, therefore, demonstrate that the introduction of additional antibacterial groups represents an effective strategy for enhancing the antibacterial properties of CS. These results also suggest that OFTMCS has the potential to be used as an effective antibacterial agent in food products and biomedical science.

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