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Antimicrobial Preservative Coating Prepared with Quaternized Carboxymethyl Chitosan

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Quaternized carboxymethyl chitosan (QCMC) was prepared from chitosan by introducing carboxymethyl group and quaternary ammonium group, respectively. With these two groups, quaternized carboxymethyl chitosan, a chitosan derivative, had excellent antimicrobial activity, good water solubility and good prospect to prepare antimicrobial preservative coating for fruits and vegetables. To decrease water vapor permeating rate, glycerol was added to form dense films, which was confirmed by the results of SEM.

Keywords: Chitosan derivative, Edible coating, Antimicrobial activity, Water vapor permeability.

INTRODUCTION

In these years, toxic plasticizers in food have drawn many people's attention. Consumers interest in fruits, vegetables and other food containing content of health-promoting compounds with antioxidant properties[1,2]. Developing materials with film-forming capacity and antibacterial activity, which improve the food safety and extend shelf-time, has been areas of research focus. Many works have been demonstrated that a large amount of natural edible materials possess these properties and natural degradation ability. So now, the petroleum-based nondegradable plastics are being replaced by these natural materials. Polysaccharides, proteins, and lipids are the three main polymeric ingredients used to produce edible films[3].

Chitosan (Ch), the second-most abundant natural biological polysaccharide after cellulose, has many applications in food protections due to its excellent biocompatibility and biodegradability[4,5]. Chitosan film has antimicrobial activity and a selective permeability to gasses (CO_2 and O_2) and excellent water-resisting capacity[6]. So, it can be used to keep foods fresh and extend food's shelf-time.

On the other hand, poor solubility of chitosan limits its applications. To improve its solubility, a lot of work has been done and many good experiences have been obtained in this field. O-Carboxymethyl chitosan and N,O-carboxymethyl chitosan are developed to increase chitosan's solubility[7,8]. Moreover, it has been proved that quaternized chitosan has better solubility than pure chitosan[9,10]. Introducing carboxymethyl group and quaternization into chitosan, therefore, are two effective methods to improve chitosan's solubility.

Chitosan and its derivatives resulting in the disruption of microorganisms membrane is one, if not the only, mechanisms for their owning antimicrobial activity. The antimicrobial activity should be attributed to the amino groups on chitosan chains, which can unite with proton to form $-NH_3^+$ in acidic medium and inhibit the microorganisms moving and growing [11,12]. However, the antimicrobial activity of $-NH_3^+$ is just moderate. To further enhance chitosan's antimicrobial activity, Li *et al.*[13] attempted to load chlorine into chitosan bone.

In fact, films prepared with hydrophilic substance (such as chlorine-contained chitosan) is poor at water-resisting, stronger water solubility[13]. To overcome this problem, a lot of work has been done. Some experts added hydrophobic substance to decrease the water vapor permission rate[14,15]. Glycerol and citric acid, with large quantities of hydroxyl groups, can be used to fill the gaps between chitosan and its derivative chains by forming hydrogen bonds to decrease the water vapor permission rate of films prepared with chitosan and its derivative[16].

In this work, quaternized carboxymethyl chitosan (QCMC) was taken to form edible coating to prevent food from contamination and spoiling. Quaternized carboxymethyl chitosan was synthesized by the method reported by Sun *et al.*[9]. The structure of QCMC was characterized by FT-IR and ^1H NMR. *in vitro* antimicrobial activities were evaluated against *Escherichia coli*. To enhance the water barrier properties of QCMC, glycerol was mixed with QCMC to form coating solution. And then, the water barrier properties of chitosan film, QCMC film and QCMC/GLY film was determined by the ATSM E96-95.

EXPERIMENTAL

Chitosan was purchased from Sinopharm Chemical Reagent Co., Ltd, with a deacetylation degree of 80-90 % and viscosity of 50-800 mPa s. Monochloroacetic and glycerol were also purchased from Sinopharm Chemical Reagent Co., Ltd. All other chemicals were of reagent grade and used without further purification as received. *Escherichia coli* was provided by Biological Experiment Center of Yancheng Institute of Technology.

Synthesis of quaternized carboxymethyl chitosan

Synthesis of carboxymethyl chitosan (CMC): Chitosan (10 g) was mixed with NaOH solution (60 wt. %, 15 mL) and kept at -20 °C overnight. The frozen alkali chitosan was transferred into isopropanol (100 mL). Then, $ClCH_2COOH$ (30 g) was added into the isopropanol. After stirred at room temperature for 4 h, acetate acid was prepared to adjust the pH between 7-8. The obtained solid was filtered and washed with acetone. After dialyzing against ultrapure water for 3 days, the product, the carboxymethyl chitosan (CMC), was obtained by vacuum-dry

Synthesis of quaternized carboxymethyl chitosan (QCMC): Carboxymethyl chitosan (5 g) was dissolved in 20 mL deionized water and 2,3-epoxypropyl-trimethylammonium chloride was added. The mixture was reacted at 80 °C for 8 h with stirring, then dialyzed for 4 days. Finally, QCMC, with yellow appearance, was obtained after vacuum-dry (**Scheme-I**).

FT-IR spectra were recorded with KBr pellets on a NEXUS-670 spectrophotometer (NICOLET, USA). ^1H NMR spectra were recorded on AVANCE III 300M and chemical shift were given by taking methanol as reference in D_2O at 293.4 K.

Evaluation of antimicrobial activity *in vitro*: The antimicrobial activity of chitosan and QCMC was tested by the inhibition zone method. For Gram-negative activity, *Escherichia coli* was used to investigate the antimicrobial activity of chitosan and QCMC. A nutrition agar containing about 10^5 colony-forming units (CFU)/mL bacteria was poured onto a Petri dish and then solidified for 1 h at 5 °C. A well (diameter 8 mm) was made onto each bacterial-inoculated ager plate and then different sample solution (50 µL) were dropped a clear inhibition zone around the sample-loaded well after incubating at 37 °C for 24 h. The inhibition zone diameter was measured with vernier caliper. Area of inhibition zone was used to assess the antimicrobial activity of each substance.

Native chitosan didn't dissolve in pure water, so acetic acid (1 %, v/v) was added. To remove the effect of acetic acid, the antimicrobial activity of acetic acid was assessed. Additionally, the antimicrobial activity of penicillin was assessed to learn the antimicrobial activity as an standard.

Water vapor permeability (WVP): Water vapor permeability was investigated by gravimetry following standard ASTM E96-95. Film with an exposed area of 50 cm^2 were tested at 90 % relative humidity in a desiccator. Weight loss graphs were plotted with respect to time and the linear least-square method was used to calculate water vapor transmission rate (WVTR) using the equation:

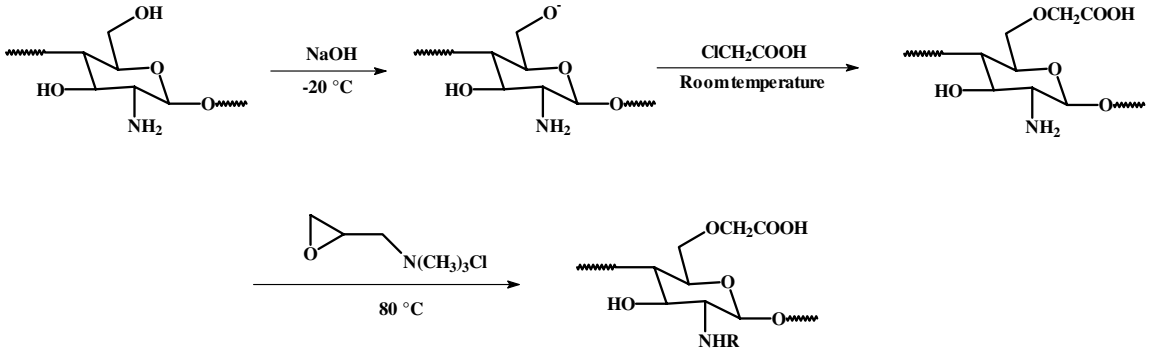
$$WVP = \frac{\Delta m}{A \cdot \Delta p \cdot \Delta t} l$$

where Δm is the mass of the water vapor lost through the films, l is the thickness of film, Δp is the partial water vapor pressure difference (Pa) across the two sides of the film, Δt is the interval time (h). Water vapor permeability measurement was replicated once per hour.

Determining the thickness of the films: Thickness of the film was determined using a screw micrometer with the nearest 0.001 mm. Informed value was an average of at least five random locations of the film sheets. The measurement was carried out after the measurement of WVP and was used in it.

RESULTS AND DISCUSSION

FT-IR spectra: In order to determine the structure of the chitosan derivatives, FT-IR were carried out (Fig. 1). The absorption bands at 1030 and 1080 cm^{-1} in chitosan spectrum assigned to the primary hydroxyl group and second hydroxyl group is not charged, the C-O stretching band at 1030 cm^{-1} corresponding to the primary hydroxyl group disappears. Two strong peaks at 1633 and 1253 cm^{-1} are observed in QCMC spectrum due to the asymmetrical and symmetrical stretching of COO^- group. The absorption bonds at 1662, 1600, 1313 cm^{-1} in the spectrum of chitosan assign to amide I, II, III vibration bends. Compared with chitosan, QCMC shows the disappearance of the characteristic peak of primary amine N-H vibration deformation and appearance of a new peak at 1050 cm^{-1}, which is attributed to the methyl group of the ammonium. It indicates the formation of N-(2-hydroxy,3-trimehylammonio)-propyl chitosan chloride at $-NH_2$. The results of IR spectrum are in agreement with the result of QCMC.



R = H, $CH_2CH(OH)CH_2N(CH_3)_3Cl$

Scheme-I: Synthesis of quaternized carboxymethyl chitosan

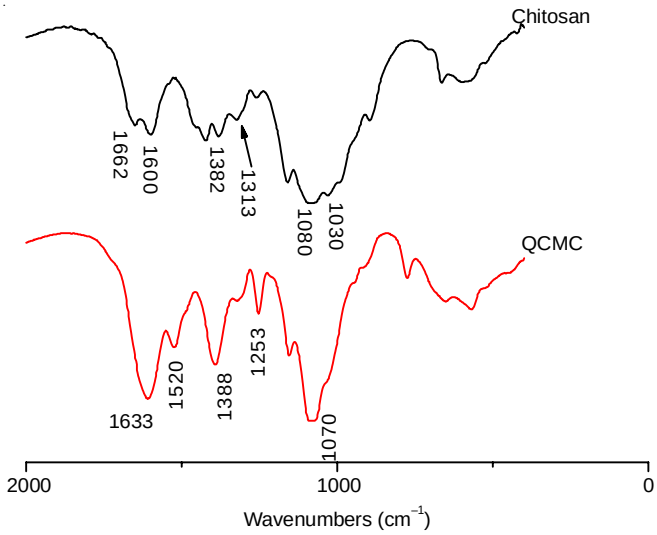


Fig. 1. FT-IR spectra of chitosan and QCMC

^{1}H NMR: Fig. 2 depicts the ^{1}H NMR spectrum of QCMC. The peak at δ = 1.83 ppm was assigned to the proton of residual CH_3 acetyl. The most intensive signal at δ = 3.04 ppm is attributed to the protons of the methyl groups of the quaternary ammonium salt. The peaks at δ = 4.58, 2.66, 3.52, 3.71, 3.59 and 3.74 ppm are attributed to H-1, H-2, H-3, H-4, H-5 and H-6, respectively. The peak at δ = 4.37 ppm is attributed to the CH_2 at carboxymethyl group, H-7. The peaks at δ = 2.42, 4.14 and 3.23 ppm are attributed to H-8, H-9 and H-10, respectively. The results are consistent with the reported spectra of QCMC.

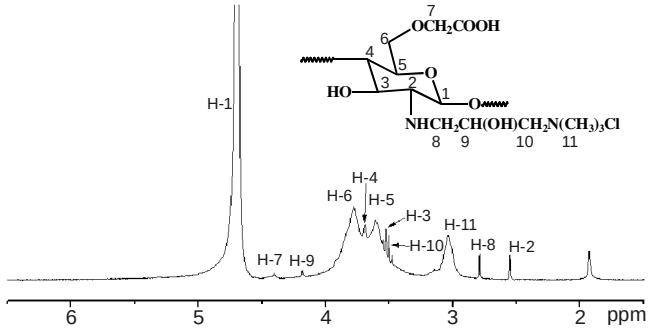


Fig. 2. ^{1}H NMR spectrum of quaternized carboxymethyl chitosan

Antimicrobial activity: Fig. 3 shows the inhibition zones of acetic acid, chitosan, QCMC and penicillin. Table-1 shows the sizes of each inhibition zone. The disk diffusion and agar dilution methods results in a semi-quantitative antimicrobial activity between chitosan and QCMC.

TABLE-1
SIZES OF EACH INHIBITION ZONE

	Concentration	Size of inhibition zone
Penicillin	2000 μ/mL	7.065 cm^2
QCMC/Acetic acid	0.01/0.01 g/mL	1.5386 cm^2
Chitosan/Acetic acid	0.01/0.01 g/mL	0.9499 cm^2
Acetic acid	0.01 g/mL	None

Quaternized carboxymethyl chitosan has the larger inhibition zone against *Escherichia coli*. The antimicrobial of QCMC is half as strong against as native chitosan. Due to the introduction of quaternary ammonium groups, QCMC chain has more charges, which will be more helpful for chains to adhere to membrane and kill microorganisms.

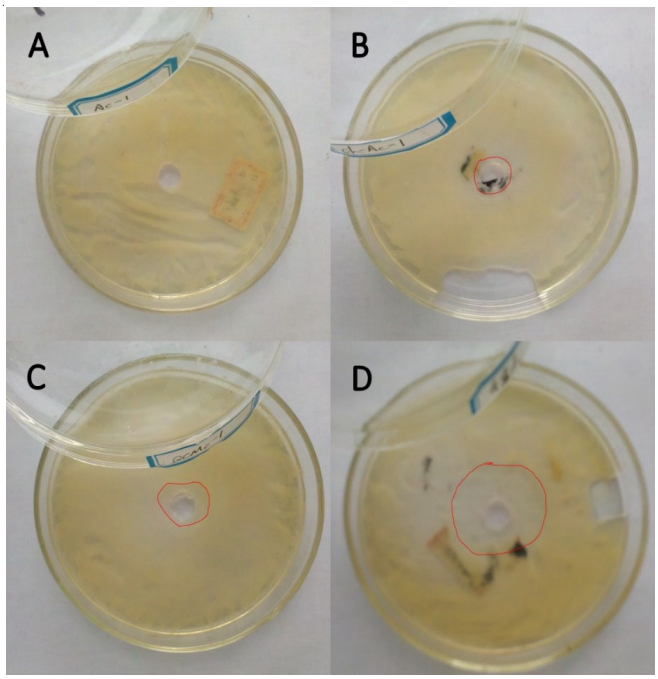


Fig. 3. Photographs of inhibition zones. Acetic acid (A), chitosan (B), QCMC (C), penicillin (D)

Water vapor permeability (WVP): WVPs of chitosan film, QCMC film and QCMC/GLY film were investigated with ASTM 95-96. Fig. 4 shows water vapor permeability rate (WVPR). The water vapor permeability rate of QCMC film is fast as half against as WVPR of QCMC/GLY film. However, WVPR of QCMC/GLY film is similar with chitosan film. This maybe is due to glycerol could connect QCMC chains with hydrogen bonds so that glycerol and QCMC could form dense film without obvious gaps. Therefore, WVPR of QCMC/GLY film is closed to that of chitosan film, which owns well-WVP.

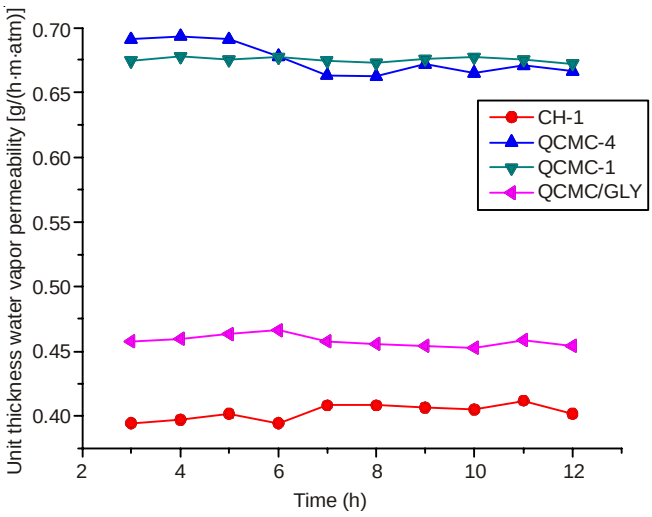


Fig. 4. Unit thickness water vapor permeability

Scanning electron microscope (SEM): Fig. 5 displays SEM images of native chitosan film, pure QCMC film and QCMC/GLY film. Many obvious gaps are observed in the

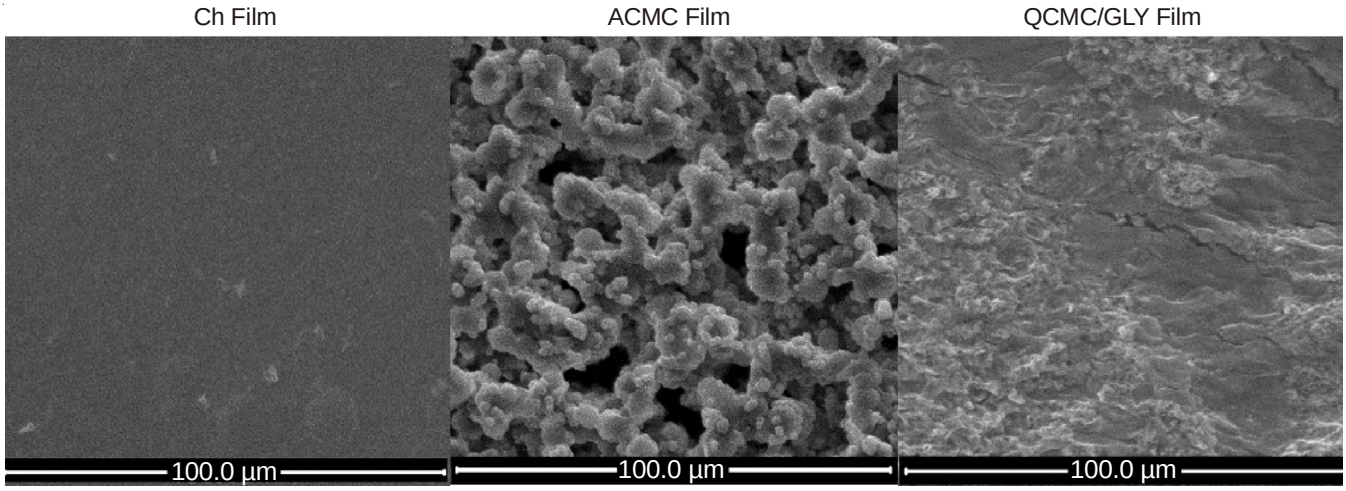


Fig. 5. SEM images of chitosan film, QCMC film and QCMC/GLY Film

QCMC film. However there was no obvious gap on QCMC/GLY film and native chitosan film. This phenomenon indicates the well-WVP of QCMC is attributed that the interaction of the hydrogen bonds among chitosan chains and the hydrogen bonds between QCMC chains and glycerol molecules. WVP tests agreed with this phenomenon.

Conclusion

With carboxymethyl and quaternary ammonium group, QCMC had stronger antimicrobial activity than native chitosan and high water solubility so that it could be using to prepare antimicrobial preservative coating. Additionally, this chemical modification could increase chitosan's durability. Mixed with glycerol, QCMC could form dense films, which was helpful for fruits to retain water.

ACKNOWLEDGEMENTS

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