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Preparation, characterization and antimicrobial activity of quaternized carboxymethyl chitosan and application as pulp-cap

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Abstract

Quaternized carboxymethyl chitosan (QCMC) was prepared from which carboxymethyl chitosan (CMC) was prepared from chitosan first, then *N*-quaternary ammonium group was introduced by the reaction of CMC with 2, 3-epoxypropyl trimethylammonium. The structures of the derivatives were characterized by FT-IR, XRD, ^{13}C NMR, ^{1}H NMR and gel permeation chromatography. In vitro antimicrobial activities of QCMC were evaluated against *Escherichia coli*, which is a Gram-negative bacterium, and *Staphylococcus aureus*, which is a Gram-positive bacterium. In compared with carboxymethyl chitosan (CMC) and quarternary chitosan (QC) of the same degree of substitusion (DS), we found that QCMC has stronger antimicrobial activity. Then we went deep into study of the relationship between their structure and antimicrobial activity, found that the DS of CMC do little effect to their antimicrobial activity, but as the increase of their DS of quaternization or the decrease of their molecular weight, the antimicrobial activity of QCMC become stronger. QCMC was complexed with calcium hydroxide as pulp-cap. Animal experiment results indicated that QCMC can strongly induce reparative dentine formation and showed a better ability in dentin inducing compared with calcium hydroxide.

Keywords: Quaternized carboxymethyl chitosan; Antimicrobial activity; Structure–function relationship

1. Introduction

Chitosan (CS), originated from chitin, the second most abundant natural biopolymer only to cellulose, is a nontoxic copolymer consisting of β-(1,4)-2-acetamido-2-deoxy-D-glucopyranosyl and β-(1, 4) -2-amino-2-deoxy-D-glucopyranosyl units [1]. CS has attracted more and more researchers due to its multiple bioactivities [2] such as antimicrobial [3–5], antitumor [6] and immune enhancing effects [7]. The antimicrobial and antifungal activities of CS have been followed with great interest. CS inhibits the growth of a wide variety of bacterial and fungi showing broad spectra of antimicrobial activity, high-killing rate and low-toxicity toward mammalian cells. CS (p*K*a = 6.8), however, exhibits its antimicrobial activity only in an acidic medium because of its poor solubility above pH 6.5. Thus, water-soluble chitosan derivatives soluble to both acidic and basic physiologic circumstances may be good candidate for the polycationic bibyeocide [1]. Because the antimicrobial activity of chitosan is very limited, various efforts have been taken to improve it. Some researchers studied the effect of the molecular weight, degree of deacetylation, solvent, pH, etc. [3,4] on the antimicrobial activity of chitosan, so as to enhance the activity by adjusting these factors. Other researchers have set out to modify chitosan to gain derivatives with higher activity such as *N*-sulfonated and *N*-sulfobenzoyl chitosan [8], carboxymethyl-chitosan [9], quaternary ammonium salt of chitosan [10], etc.

In an attempt to improve antimicrobial activity of chitosan, our papers report the preparation of quaternized carboxymethyl chitosan (QCMC) in which carboxymethyl group and quaternary ammonium group contemporaneously introduced onto chitosan molecular chain. CMC and QC which represent a family of very important derivatives of chitosan, respectively, have been found to possess many bioactivities [11–13]. Since, antimicrobial mechanism of chitosan has suggested that the trace metal cations selectively chelated by the chitosan could be necessary for the microorganism's growth and therefore could inhibit the proliferation of the microbial [14], it would be interesting to investigate if carboxymethyl chitosan, which possesses negative charges on the carboxyl groups making itself an excellent chelating host for metal cation substrate,

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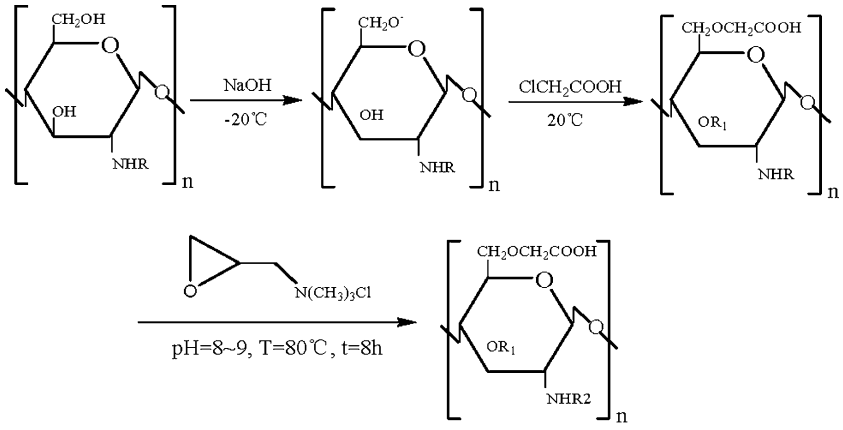

R = H, $COCH_3$
R_1 = H, CH_2COOH
R_2 = H, $COCH_3$, $CH_2CH(OH)CH_2N(CH_3)_3Cl$

Scheme 1. Synthesis of carboxymethyl chitosan and quaternized carbosymethyl chitosan.

could show any antimicrobial activities. By now almost no study has been conducted on their interaction on antimicrobial activity. Here, we wish to report in this paper the preparation of chemically modified carboxymethyl chitosan and the antimicrobial activities of these chitosan derivatives against a Gram-positive bacterium *Staphylococcus aureus* and a Gram-negative bacterium *Escherichia coli*, measured by the agar plate method. The preparation of carboxymethyl chitosan and its quaternized derivatives is presented as Scheme 1.

Direct pulp capping is considered a valid treatment method in today's endodontics, because successful capping can preserve tooth vitality in an exposed pulp cavity. Calcium hydroxide preparations are normally used as capping materials [15–18]. The main advantage of calcium hydroxide is its biological activity. It presents antimicrobial and anti-inflammatory activities principally due to the high pH value of the surrounding environment (around 12.5) following its dissolution. Most bacteria do not resist a pH above 9.5, and the alkalinity allows the resolution of the exudates, which maintain the inflammatory state. Calcium hydroxide acts as a chemical buffer because of this alkalinity and as a thermal buffer towards metallic materials because of its low-thermal conductivity. Finally, it does not inhibit the polymerization of composite. Calcium hydroxide also presents some drawbacks: (i) it provokes pulp necrosis during the first days, then the pulp reacts by establishing an atubular tertiary dentine bridge, but this dentine formation is made to the detriment of the pulpal volume with long-term biological consequences; (ii) when the paste is only calcium hydroxide, its application in the root canal system is easy but the low hardening and the retraction by drying do not allow tight fillings, consequently it is only used as temporary material in this indication for which hermeticity is a priority; (iii) to get round this disadvantage, i.e. to increase the crushing strength and to decrease the setting time, polymeric bases were added, but under these conditions the setting time is too short to use these materials as root canal filling. We complexed QCMC with calcium hydroxide as pulp-cap agent, in order to keep the advantages of calcium hydroxide while minimizing its drawbacks. The carboxymethyl group which has been introduced to the chitosan chain was easy to combine with Ca^{2+} which increased the combination with outer $Ca(OH)_2$ while the quaternary group makes it has much stronger antimicrobial ability. The complex which can reduce the destroy of $Ca(OH)_2$ to the pulp of a tooth, induce reparative dentine formation is an excellent biological pulp capping material worthy to study more deeply.

2. Experimental

2.1. Materials and methods

Chitosan was supplied by Yuhuan Aoxing biochemistry Co. Ltd in Zhejiang province in China, with a deacetylation degree of 87% (determined by elemental analysis [19]) and the molecular weight (M_w) calculated from the GPC method was about 4.5×10^5. Standard pullulans for GPC were purchased from Showa Denko, Tokyo, Japan; 2, 3-epoxypropyl trimethylammonium chloride was prepared in the lab. All other chemicals were of reagent grade and were used without further purification as received. A Gram-positive bacterium *S. aureus* and a Gram-negative bacterium *E. coli* were provided by China center for type culture collection (CCTCC in Wuhan University) and inoculated on a gel containing 1% peptone, 2% agar, 3% meat extract and 0.5% NaCl. Calcium hydroxide was purchased from Densply Co. Female Wistar rats, 12 weeks old, 240–260 g, were used for this experiment.

2.2. Preparation of quaternized carboxymethyl chitosan

2.2.1. Carboxymethl chitosan

Chitosan (10 g) suspended in NaOH (15 ml) was kept −20 °C overnight. The frozen alkali chitosan was transferred to 2-pripanol (100 ml) and $ClCH_2CO_2H$ was added in portions. Stirring at room temperature for 4 h and HOAC was added to the mixture to adjust the pH to 7.0. The carboxymethyl chitosan (CMC) salt was filtered and washed with EtOH. After

dialyzing against deionized water for 4 days, the product was vacuum dried at room temperature. By changing the alkali concentration, a series of CMC with various degree of substitution was prepared.

2.2.2. Quaternization of carboxymethl chitosan

The quaternization of CMC was conducted as follows. Concentrated hydrochloric acid (0.1 mol) was dropped into the solution of trimethylamine (0.1 mol) at 4 °C to avoid gasification of trimethylamine in the latter reaction. When it was stirred for about 10 min, the resulting solution was added by epoxy chloropropane (0.086 mol) at 31 °C. After homogenization, the mixing solution was heated to 51 °C, and was then trickled slowly by aqueous NaOH (0.1 mol) solution in order to maintain trimethylamine hydrochloride to slowly decompose into trimethylamine, which easily react with epoxy chloropropane. The addition of NaOH was performed within 1–1.5 h, after another 2 h of stirring, the reaction mixture was refined by vacuum distillation at 50 °C.

CMC (5 g) was dissolved in 20 ml distilled water and 2, 3-epoxypropyl trimethylammonium chloride was added with different mole ratio to glucosamine unit. The mixture was reacted at 80 °C for 8 h with stirring then dialyzed for 4 days and finally lyophilized to give QCMC as a yellow power.

2.2.3. Degradation of quaternized carboxymethyl chitosan

QCMC powder (5 g) was suspended in 250 ml deionized water, after stirring at 40 °C for 2 h, hydroperoxide of a desired volume was added for predetermined time to yield QCMC of various molecular weights.

2.3. Characterization

FT-IR spectra were recorded with KBr pellets on a Nicolet FT-IR 360 spectrophotometer. Sixteen scans at a resolution of 4 cm^{-1} were averaged and referenced against air.

X-ray diffraction patterns of the degraded chitosan fractions were measured by a Shimadzu Lab XRD-6000 diffractometer and used a Cu Kα target at 40 kV and 50 mA at 20 °C. The relative intensity was recorded in the scattering range (2θ) of 5–40°. ^{13}C and ^{1}H NMR spectra were recorded on a Varian mercury VX-300 spectrometer and chemical shifts were given by taking methanol as reference in D_2O at 323 K.

Weight-average molecular weights (M_w) of samples were measured by GPC. The GPC equipment consisted of connected columns (TSK G5000-PW and TSK G3000-PW), TSP P100 pump and RI 150 differential refractometer and Jiangshen Workstation. Each sample was dissolved in 0.1 mol/l aq NaCl which was the eluent, then filtered through 0.45 μm Millipore filters. The flow rate was maintained at 1.0 ml/min. The sample concentration was 0.4 mg/ml. the weight-average molecular weight (M_w) was calculated by the following equation:

$$\lg(M_w) = -0.4383\ \mathrm{Ve} + 8.9236 \tag{1}$$

DS of carboxymethyl group of each sample was estimated from potentiometric titration [20]. Samples were dissolved in 0.1 mol/l hydrochloric acid (50 ml) in the presence of 0.1 mol/l sodium chloride and titrated with 0.1 mol/l sodium hydroxide. The alkalimetric curves were recorded on a DELTA-320-S pH meter.

The degree of substitution of the quaternization group was determined by the potentiometry [21]. Potentiometric titration of the chloride ion on QC and QCMC was carried out with the aqueous silver nitrate, using a calomel electrode as the reference, and a silver electrode for the measurement. DS is calculated as follows:

$$\mathrm{DS} = \frac{\frac{C\times V}{1000}}{\frac{C\times V}{1000} + \frac{W - \frac{C\times V\times M_2}{1000}}{M_1}} \tag{2}$$

where C (mol l^{-1}) is the concentration of AgNO3 solution, V (ml) is the volume of $AgNO_3$ solution, W (g) is the weight of quaternary chitosan, M_1 (mol g^{-1}) is the molar mass of glucosamine and M_2 (mol g^{-1}) is the molar mass of quaternary chitosan.

2.4. Evaluation of antimicrobial activity in vitro

The agar plate method was used to determine the minimum inhibition concentration (MIC) of CMC, QC and QCMC as follows: the samples were prepared at a concentration of 1% (w/v), then they were autoclaved at 121 °C for 25 min. Duplicate two-fold serial dilutions of each sample were added to nutrient broth (beef extract 5 g, peptone 10 g to 1000 ml distilled water, pH 7.0) for final concentration of 0.1, 0.05, 0.025, 0.0125, 0.00625, and 0.00313%. Some samples were prepared and diluted by the same way except for final concentration of 0.00065 and 0.00033%. The culture of each bacterium was diluted by sterile distilled water to 105–106 CFU/ml. A loop of each suspension was inoculated on nutrient medium with sample or control added. After inoculation, the plates were incubated at 37 °C for 72 h, and the colonies were counted and the MIC values were obtained.

The minimum inhibitory concentration (MIC) was considered to be the lowest concentration that completely inhibited against on agar plates comparing, disregarding a single colony or a faint haze caused by the inoculum [22].

2.5. Animal test

Each animal was anesthetized with an intraperitoneal injection of sumianxin (0.08 ml/100 g). Electrosurgery of the gingival tissue was carried out to prepare an access to the mesial aspect of the right and left upper first molars. V-like cavities were then prepared in the cervical third of the mesial aspect of the first upper molars with a high-speed contra-angle. Two teeth per rat were prepared. The left one is controlled group, which the cavity was prepared but filled only with calcium hydroxide and compound resin. The right one is the test group, which was filled with the complex of QCMC and calcium hydroxide and the compound resin. So that after 7, 14, and 21 days, respectively, six rats per group were killed by perfusion of the fixative solution (10% neutral formaline)

through the heart. Block sections including the three molars, bone, and gingival were immersed in the fixative and kept at 4 °C for 24 h. They were demineralized either with sodium formiate or with 10% EDTA and embedded in paraplast. Serial sections of 5 mm were stained with H&E [18].

3. Results and discussion

3.1. Structure characterization of CMC and QCMC

3.1.1. FT-IR spectra

Fig. 1 presents the FT-IR spectra of CS, CMC and QCMC. The absorption bands at 1655, 1593, 1323, 1381 cm^{-1} in the spectrum of CS assigned to amides I, II, III and $–CH_3$ vibration bends [23]. Two strong peaks at 1605 and 1419 cm^{-1} (in CMC spectrum) and 1603 and 1415 cm^{-1} (in QCMC spectrum) were observed due to the asymmetrical and symmetrical stretching of COO– group. In the spectrum of CMC, the C–O stretching band at 1030 cm^{-1} corresponding to the primary hydroxyl group disappears, verifying a high carboxymethylation of OH-6. The characteristic peak of second hydroxyl group at 1080 cm^{-1} was not changed. Compared with CS, QCMC showed the disappearance of the NH_2-associate band at 1593 cm^{-1}, which can be ascribed to the characteristic peak of primary amine N–H vibration deformation and appearance of a new peak at 1480 cm^{-1}, which was attributed to the methyl groups of the ammonium. It was indicative of the formation of *N*-(2-hydroxy, 3-trimethylammonio)-propyl chitosan chloride at $–NH_2$. The IR spectrum was in agreement with the reported spectra [10,24].

3.1.2. X-ray analysis

The X-ray diffractograms of CS, CMC and QCMC are shown in Fig. 2. It could be seen that there were some differences of peak height, width and position between them.

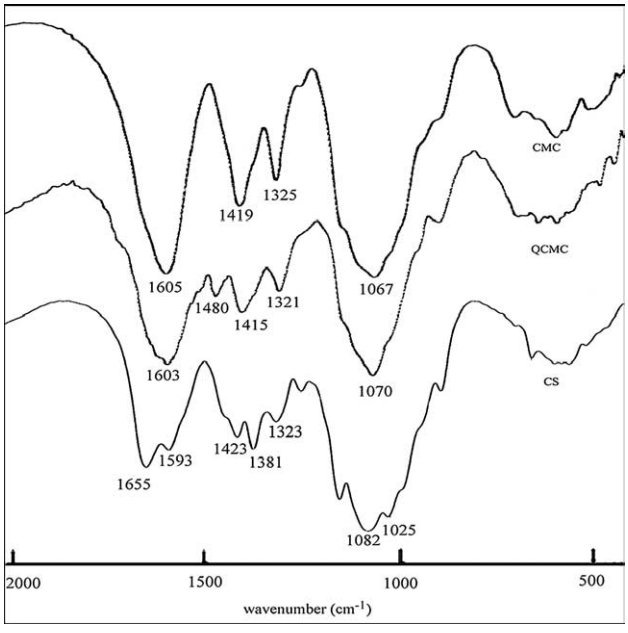

Fig. 1. FT-IR spectra of CS, CMC and QCMC.

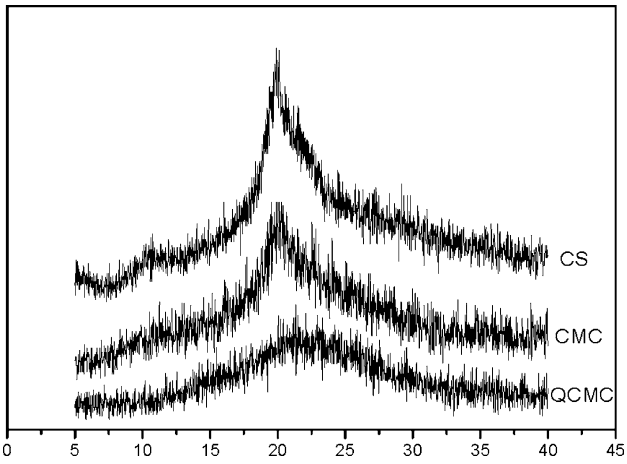

Fig. 2. X-ray diffraction patterns of CS, CMC and QCMC.

CS consisted of two major peaks at 2θ 12 and 21, while CMC exhibited two characteristic peaks at 2θ 11 and 20. The X-ray diffraction pattern of CS and CMC coincided with the pattern of the L-2 polymorph of shrimp chitosan reported by Sato and Kim [25,26]. Compared with CS, which showed relatively, narrow peak at 2θ 20, CMC had a relatively broader peak at 2θ 20 and the peak at 2θ 12 significantly weaken. In diffraction spectrum of QCMC the peak at 2θ 12 was even disappeared and the peak at 2θ 20 became even broader and it became amorphous. It is well-known that the width of X-ray diffraction peak is related to the size of crystallite, the broadened peak usually results from small crystallites [27]. Hence, in this reaction, the carboxymethylation reaction first took place preferentially in the amorphous region and then proceeded very moderately from the edge to the inside of the crystalline region [28] and with further quaterization, the crystalline structure was destroyed and the crystallinity disappeared.

3.1.3. ^{13}C NMR and ^{1}H NMR

NMR method is the most effective technique concurrently to determine the structure of chitosan derivatives [21,29]. Fig. 3 depicts the ^{13}C NMR spectrum of QCMC. The peak at $\delta =$ 54.3 ppm was attributed to the carbons of the trimethylammonium group (C-d). The signal for –COOH substituted on –OH is obvious at 178 ppm. The peaks at $\delta = 101.5$, 62.6, 72.4, 77.9, 74.9 and 60.8 ppm were attributed to the C-1, C-2, C-3, C-4, C-5 and C-6, respectively. The peaks at $\delta = 51.7$ and 68.7 ppm were attributed to C-*a* and C-*c*, respectively. The peak appearing at 64.4 ppm was attributed to C-*b*. The peaks at $\delta = 22.5$ ppm was attributed to the carbons of the residual CH_3 acetyl. The results were in agreement with Qin et al. [24].

Fig. 4 shows the ^{1}H NMR spectrum of QCMC. The peak at $\delta = 1.83$ ppm was assigned to the proton of residual CH_3 acetyl. The most intensive signal at $\delta = 3.04$ ppm was attributed to the protons of the methyl groups of the quaternary ammonium salt. The peaks at $\delta = 4.58$, 2.66, 3.52, 3.71, 3.59 and 3.74 ppm were attributed to H-1, H-2, H-3, H-4, H-5 and H-6, respectively. The peak at $\delta = 4.37$ ppm was attributed to the CH_2 at carboxymethyl group. The peaks at $\delta = 2.42$, 4.14 and

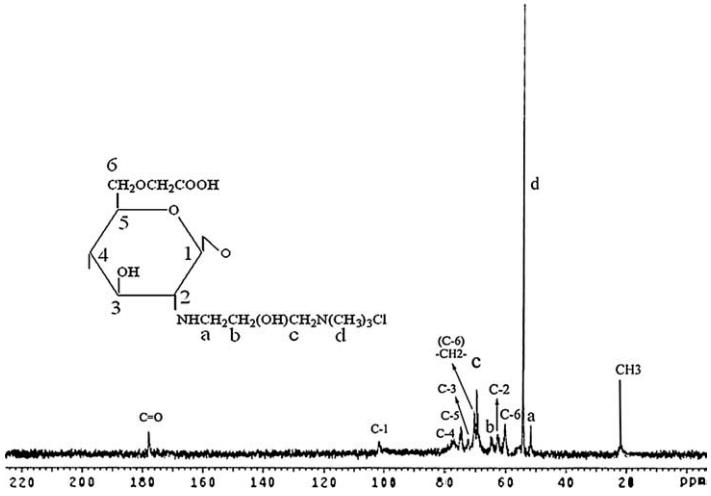

Fig. 3. ^{13}C NMR spectrum of QCMC.

3.23 ppm were attributed to the H-*a*, H-*b* and H-*c*, respectively. The results were consistent with the reported spectra [24].

3.1.4. DS and M_w

A series of carboxymethylation and quaternization reactions were conducted in this paper to get samples with various degree of substitution of carboxymethyl group and quaternary group. As shown in Table 1, alkali concentration was the most important factors to regulate carboxymethylation of chitosan. Work by Tokura and co-workers demonstrated that the DS value of CMC-chitosan increased with NaOH concentration changing from 20 to 40% [30]. And in the present work, when NaOH concentration increased from 40 to 60%, the DS value increased from 0.56 to 0.86. At lower NaOH concentration, the rigid crystalline structure of chitosan was difficult to disrupt to ensure penetration of the $ClCH_2CO_2H$ into the interlocking polymer chains.

In order to obtain quaternary chitosan derivatives with different DS, several conditions were tried. It was found that the DS was affected by the ratio of 2,3-epoxypropyltrimethyl ammonium chloride to chitosan. This result was consistent with Jia et al. [10]. Under optimal conditions, Q and QCMC with different DS were obtained. The characteristics of them are listed in Table 1.

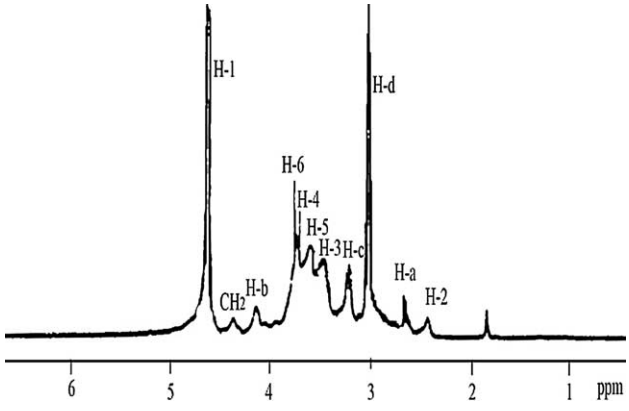

Fig. 4. ^{1}H NMR spectrum of QCMC.

Weight average molecular weights of degraded samples determined by GPC are shown in Table 2, together with reaction conditions. The molecular weight of samples studied varies from 4.72×10^5 to 1.1×10^4. All samples obtained by degradation are white free-flowing powders.

3.2. Antimicrobial activity of CMC and QCMC

The antimicrobial activities of CMC, QC and QCMC are shown in Tables 3 and 4. It was found that these samples showed effectively antimicrobial activities against not only *E. coli* but also *S. aureus* which were used in the test, although differences existed among them. Generally, the samples had more effective inhibition on *S. aureus* than *E. coli*. The fact may be attributed to their different cell walls. *S. aureus*, a typical Gram-positive bacterium, its cell wall is fully composed of peptide polyglycogen. The peptidoglycan layer is composed of networks with plenty of pores, which allow foreign molecules to come into the cell without difficulty. But *E. coli*, a typical Gram-negative bacterium, the cell wall of which is made up of a thin membrane of peptide polyglycogen

Table 1
Carboxymethyl chitosan (CMC), quatery chitosan (QC), QCMC of different degree of substitution

Sample no.	NaOH conc. (%)	Mole ratio of functional reagent to glucosamine units	DS of CMC	DS of QC
CMC_3	50	–	0.73	–
CMC_1	30	–	0.45	–
CMC_2	40	–	0.56	–
CMC_4	60	–	0.86	–
QC_1	–	3:1	–	0.78
QC_2	–	2:1	–	0.59
QC_3	–	1:1	–	0.32
Q_1CMC_3	50	3:1	0.73	0.78
Q_2CMC_3	50	2:1	0.73	0.59
Q_2CMC_3	50	1:1	0.73	0.32
Q_2CMC_1	30	2:1	0.45	0.59
Q_2CMC_2	40	2:1	0.56	0.59
Q_2CMC_4	60	2:1	0.86	0.59

Table 2
Reaction conditions and molecular weights of degraded QCMC

Sample no.	Reagent	Conc. (%)	Volume (ml)	Time (h)	T (°C)	M_w ($\times 10^5$)
Q_2CMC_3-1	untreated					4.72
Q_2CMC_3-2	H_2O_2	30	1	0.5	40	2.28
Q_2CMC_3-3	H_2O_2	30	2	1.5	50	0.45
Q_2CMC_3-4	H_2O_2	30	3	6	50	0.11

and an outer membrane constituted of lipopolysaccharide, lipoprotein and phospholipids. Because of the bilayer structure, the outer membrane is a potential barrier against foreign molecules.

Compared with CMC, QC and QCMC in Table 3, QCMC had much better antimicrobial activities, whose MIC values were 4–8 times lower than those of CMC and 2–4 times lower than those of QC. It was noticed that the introduction of carboxymethyl group and quaternized group to the chitosan chain greatly enhanced the antimicrobial activity of the QCMC. We can deduce that carboxymethyl group and quaternary ammonium group are in synergistic effect.

As shown in Table 4, compared with Q_2CMC_1, Q_2CMC_2, Q_2CMC_3 and Q_2CMC_4, which have same degree of substitution of quaternized group, we found that no clear effect of DS value of carboxymethyl group on antimicrobial activity. Compared with Q_1CMC_3, Q_2CMC_3 and Q_3CMC_3, which have same degree of substitution of carboxymethyl group, it can be observed that the antimicrobial activities of them enhanced with increasing of the DS of the. Compared with Q_2CMC_3-1, Q_2CMC_3-2, Q_2CMC_3-3 and Q_2CMC_3-4, which have same degree of substitution of both carboxymethyl group and quaternized group, the results demonstrated that antimicrobial activity of them was affected by its molecular weight remarkably. Lower molecular weight resulted in better antimicrobial ability, and when molecular weight was below 1×10^4, the antimicrobial activity of QCMC was strong and the MIC values reached to 0.00313%.

The antimicrobial mechanisms of these derivatives suggested to being on one hand, the positive charge of the group at C-2 resulted in a polycationic structure which can be expected to interacted with the predominantly anionic components (lipopolysaccharides, proteins) of the microorganisms' surface [31]. The interaction resulted in great alteration of the structure of outer membrane which caused release of major proportion of proteinaceous material from the cells [32] when the quaternized group was introduced onto the molecular chain, the positive charge was strengthened. On the other hand, when carboxymethyl group was introduced along the molecular chain, the presence of a molecular with hydrophilic ends and forming weak interaction between hydrophilic ends and chitosan enhances the antimicrobial activity. More work is needed to confirm this hypothesis.

The exact mechanism of the antimicrobial action of chitosan and its derivatives is still unknown, but different mechanisms have been proposed. As chitosan and derivatives with large molecular weight, they cannot directly access to the intracellular parts of the cells and can be expected to interact with cell surface. While chitosan and derivatives with low-molecular weight can enter into the intracellular parts of the cells, combine with DNA, restrain mRNA from combining with protein, and as a result destroy the transcribe of DNA [33].

Table 3
The antimicrobial activity of chitosan (CS), carboxymethyl chitosan (CMC), quarternized chitosan (QC) and quarternized carboxymethyl chitosan (QCMC)

Samples	DS of CMC	DS of QC	M_w ($\times 10^5$)	*Escherichia coli*	*Staphylococcus aureus*
CMC	0.46	–	4.30	0.05	0.1
QC	–	0.60	3.89	0.0125	0.025
QCMC	0.45	0.59	4.51	0.00625	0.0125

Table 4
The antimicrobial activity of QCMC with different molecular structure factor

Samples	DS of CMC	DS of QC	M_w ($\times 10^5$)	*Escherichia coli*	*Staphylococcus aureus*
Q_2CMC_1	0.45	0.59	4.51	0.00625	0.0125
Q_2CMC_2	0.56	0.59	4.64	0.00625	0.0125
Q_2CMC_3	0.73	0.59	4.72	0.00625	0.00625
Q_2CMC_4	0.86	0.59	4.66	0.00625	0.0125
Q_1CMC_3	0.73	0.78	4.21	0.0125	0.0125
Q_3CMC_3	0.73	0.32	4.83	<0.00625	0.00625
Q_2CMC_3-1	0.73	0.59	4.72	0.00625	0.0125
Q_2CMC_3-2	0.73	0.59	2.28	0.00625	0.00625
Q_2CMC_3-3	0.73	0.59	0.45	0.00313	0.00313
Q_2CMC_3-4	0.73	0.59	0.11	<0.00313	0.00313

3.3. Animal pulp-cap experiment

The normal pulp tissue structure of the Wistar rat was shown in Fig. 5(a) and (b).

After 7 days, in the test group (Fig. 5(c,) (e) and (g)), pulp cells were grouped near the perforation area, Unidentified inflammatory cells and hyperplasia cell differentiation were observed. The pulp tissue was normal far from the perforation area. Dentin debris was pushed by the probe inside the pulp. In the controlled group, the capping induced an inflammatory reaction at the surface of the pulp. At the border between implanted material and pulp, continuous or discontinuous fibrous structures began to form, underlined by an odontoblast-like palisade. (Fig. 5(d,f,h)).

After 14 days, in the test group (Fig. 6(a), (c), and (e)), there was already some tendency to self-repair some distance away from the wound, mostly in the cervical junction between the pulp chamber and root canal. This reparative dentine formed

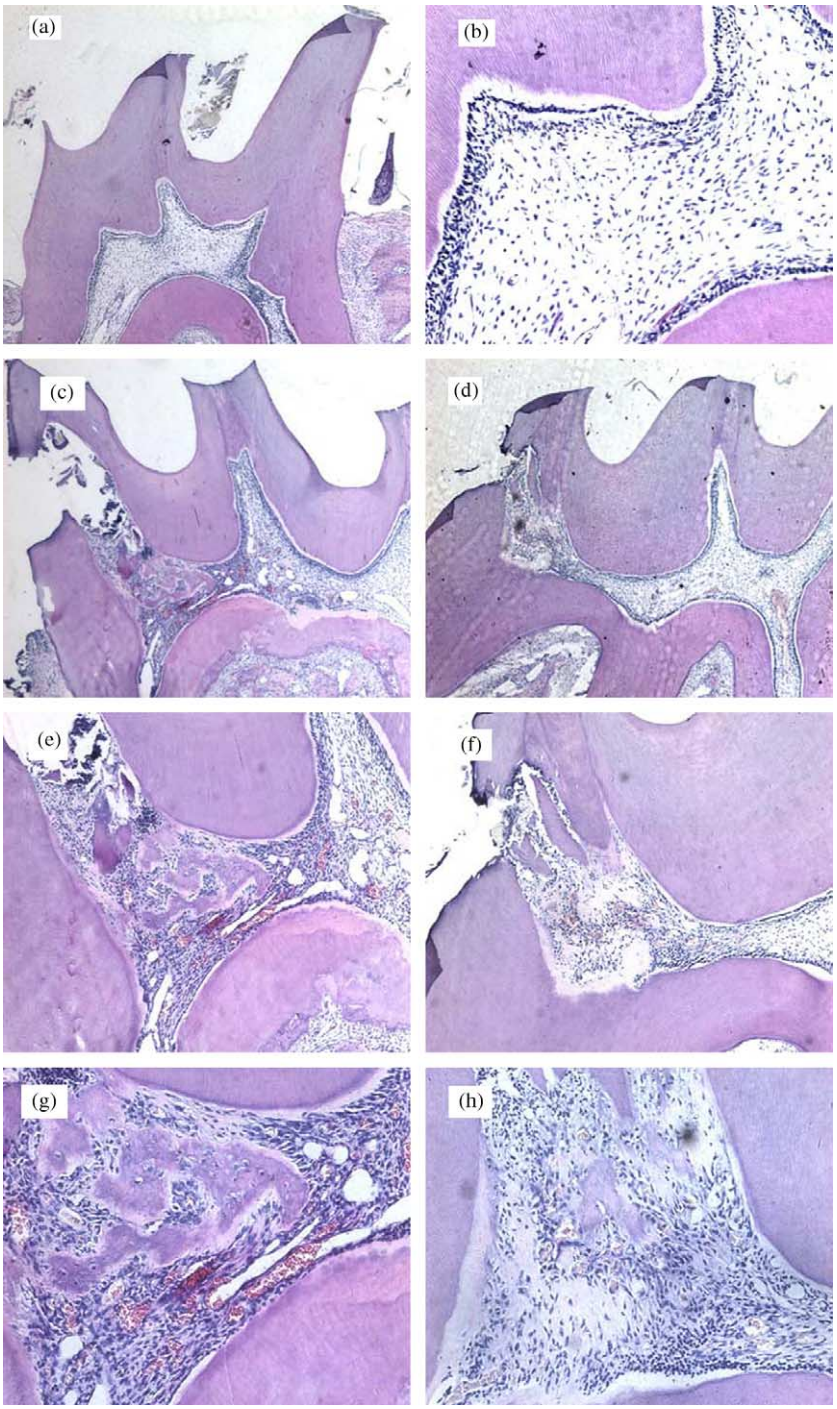

Fig. 5. (a) and (b) are normal pulp tissue; (c)–(h) are 7 days after operation; (c), (e) and (g) are test group (d), (f) and (h) are calcium hydroxide group; (c) and (d) (magnification: 5×), (e) and (f) (magnification: 10×), (g) and (h) (magnification: 20×).

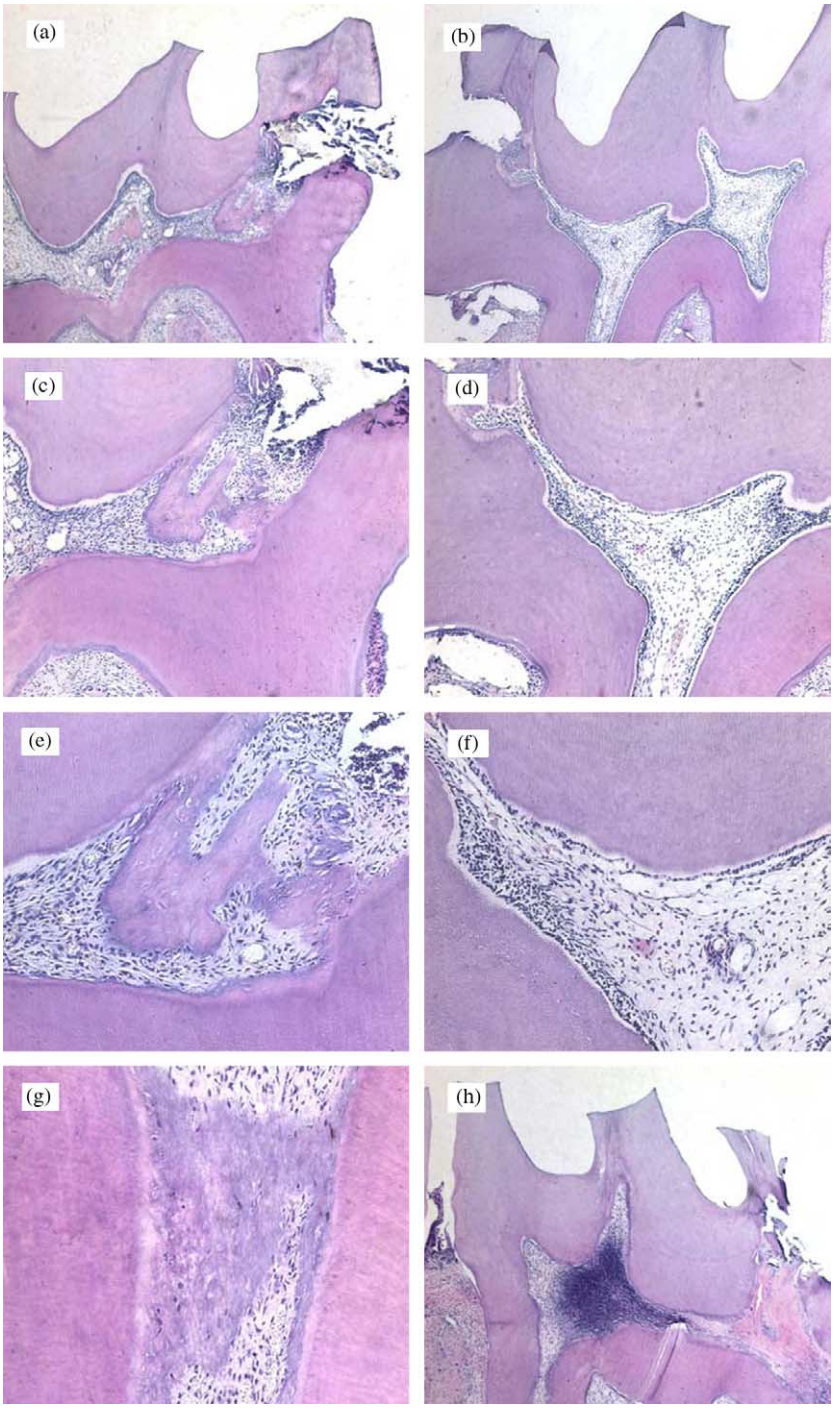

Fig. 6. (a)–and (f) are 14 days after operation; (g) and (h) are 21 days latter; (a), (c), (e) and (g) are test group; (b), (d), (f) and (h) are calcium hydroxide; (a) and (b) (magnification: 5×), (c) and (d) (magnification: 10×), (e)–(h) (magnification: 20×).

integrated barrier structure, which separated the inflammatory tissues to the normal pulp tissues. Above the barrier, inflammatory cells were also observed near the perforation area while under the barrier there was no evidence inflammation, and the hyperplasia and differentiation of the cells were active which approach to the normal tissues. In the controlled group (Fig. 6(b), (d) and (f)), a certain degree formation of reparative dentine was also detected near the perforation, but the barrier structure was not formed.

After 21 days, in the test group (Fig. 6(g)), a mixture of large areas of reparative dentine and osteodentin aggrades along the pulp wall and forms reparative barrier structure near or beneath the pulp chamber and there was also some weak inflammation. In the controlled group, reparative dentine was also formed and there was no evidence inflammation, but the putrescence of the pulp was detected which indicated that the putrescence and collapse region was formed partly (Fig. 6(h)).

Calcium hydroxide has been used in dentistry as a major capping material having the capacity to induce the formation of a mineralized dentin bridge, but it has no direct inducing effect to the pulp cells. QCMC can stimulate the formation of a thick reparative dentin layer, strongly induce reparative dentine formation and plays an important role in the formation of reparative dentin after implantation into the pulp. This is the first time that such a novel bioactive property showing stimulation of reparative dentin formation was reported, opening new perspectives for future therapy bone-inductive capacity.

4. Conclusions

From these studies, we successfully prepared a series of QCMC. We demonstrate that antimicrobial activities of QCMC were affected by the DS of quaternary group and the molecular weight while no clear effect of DS of carboxymethyl group on the antimicrobial activity was observed. When QCMC was complexed with calcium hydroxide as pulp-cap, animal experiment results indicated that QCMC can strongly induce reparative dentine formation and showed a better ability in dentin inducing compared to calcium hydroxide.

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References

[1] Li Z, Zhuang XP, Liu XF, Guan YL, Yao KD. Polymer 2002;43:1541–7.
[2] Wu CS. Polymer 2005;46:147–55.
[3] Yoon JP, Yong ML, Si NP, Seung YS, Chong PC, Seung JL. Biomaterials 2000;21:153–9.
[4] Jeon YJ, Park PJ, Kim SK. Carbohydr Polym 2001;44(1):71–6.
[5] No HK, Park NY, Lee SH. Int J Food Microbiol 2002;74:65–72.
[6] Suzuki K, Mikami T, Okawa Y. Carbohydr Res 1986;151:403–8.
[7] Sugano M, Yoshida K, Hashimoto M. Advances in chit and chitosan. London: Elsevier; 1992 p. 472–8.
[8] Chen C, Liau W, Tsai G. J Food Prot 1998;61:1124–8.
[9] Chen LY, Du YM, Liu Y. J Wuhan Univ (Nat Sci Ed) 2000;46:191–4.
[10] Jia ZS, Shen DF, Xu WL. Carbohydr Res 2001;333:1–6.
[11] Chen LY, Du YM. Biomaterials 2004;25(11):3725–32.
[12] Sun LP, Du YM. Acta Polym Sinica 2004;2:191–5.
[13] Xu YM, Du YM. Biomaterials 2003;24(27):5015–22.
[14] Hagiwara K, Kuribayashi Y, Iwai H. Carbohydr Polym 1999;39:245–8.
[15] Siham S, Pierre M, Jacques M, Bruce B, Philippe B. J Mater Sci: Mater Med 2002;13:125–31.
[16] Ranly DM, Godoy FG. J Dentistry 2000;28:153–61.
[17] Souza CCA, Nascimento AB, Teixeira HM, Fontana UF. Dent Mater 2001;17:230–40.
[18] Six N, Decup F, Lasfargues JJ. J Mater Sci: Mat Med 2002;13:225–32.
[19] Xu J, Mccarthy SP, Gross RA. Macromolecules 1996;29:3436–40.
[20] Muzzarelli RAA, Tanfani F, Emanuelli M. Carbohydr Res 1984;126: 225–31.
[21] Domard A, Rinaudo M, Int. J Biol Macromol 1986;8:105–7.
[22] Speciale A, Musumeci R, Blandino G, Milazzo I, Caccamo F, Nicoletti G. Int J Antimicrob Agents 2002;19:111–8.
[23] Zong Z, Kimmura Y, Takahashi M. Polymer 2000;41:899–906.
[24] Qin CQ, Xiao L, Du YM, Shi XW, Chen JW. React Funct Polym 2002;50: 165–71.
[25] Sato H, Tabeta R. Macromolecules 1987;20:2424–30.
[26] Kim JH, Lee YM. Polymer 1993;34:1952–7.
[27] Gao QW, Shao ZZ, Sun YY, Lin H. Polym J 2000;32:269–74.
[28] Qin CQ, Du YM, Xiao L. J Polym Degrad Stab 2002;76:211–8.
[29] Lebouc F, Dez I, Madec PJ. Polymer 2005;46:319–25.
[30] Nishimura S, Ikeuchi Y, Tokura S. Carbohydr Res 1984;134:305–12.
[31] Helander IM, Nurmiaho E, Ahvenainen R, Rhoades J, Roller S. Int J Food Microbiol 2002;71:235–44.
[32] Helander IM, Latva KK, Lounatmaa K. Microbiology 1998;144:385–90.
[33] Sudharshan NR, Hoover DG, Knorr D. Food Biotechnol 1992;6:257–72.