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Synthesis, characterization and properties of carboxymethyl kappa carrageenan

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ABSTRACT

In order to develop a wound healing material possessing anticoagulant activity, antimicrobial activity and moisture absorbability and moisture-retention capacity, carboxymethyl κ-carrageenan (CMKC) was synthesized by the reaction of κ-carrageenan with monochloroacetic acid. The products were characterized by FT-IR, ^{13}C NMR, degree of substitution (DS) and molecular weight. Anticoagulant activity of CMKC was investigated by APTT, TT and PT assays. The results showed that CMKC with a low DS promoted anticoagulant activity in comparison with κ-carrageenan, but as the DS further increased from 0.42 to 1.09, the activity decreased. Antibacterial activity was evaluated and we found that the introduction of carboxymethyl groups conferred antibacterial activity onto κ-carrageenan. Results indicated that CMKC exhibited good antimicrobial properties against *Escherichia coli* and *Staphylococcus aureus*, and the antibacterial activity of CMKC enhanced as the DS increased. CMKC displayed better moisture-absorption and water-retention ability than κ-carrageenan and as the DS increased, these properties of CMKC increased.

1. Introduction

Carrageenans are a family of linear, sulfated galactans, extracted from a certain species of marine red algae (Knutsen, Myladobodski, Larsen, & Usov, 1994). Due to their excellent physical functional properties and biological activities, carrageenans are extensively utilized in the food, cosmetics, textile and pharmaceutical industries (Campo, Kawano, da Silva, & Carvalho, 2009). To extend the use of κ-carrageenan, the industrial and scientific interests in the carboxymethylation of κ-carrageenan have increased significantly in recent years. Products such as metal adsorbent, magnetic nanospheres and encapsulant for oral delivery have been developed from carboxymethyl carrageenan (Aranilla, 2008; Leong et al., 2011; Yagi et al., 2010). However, the properties of carboxymethyl κ-carrageenan, especially the anticoagulant activity, antibacterial activity and moisture absorbability and moisture-retention capacity, to our knowledge, have not been studied.

Some sulfated polysaccharides, such as the sulfates of chitin, chitosan and alginate (Huang, Mendis, & Kim, 2005; Nishimura et al., 1998), which exhibit potent biological activities including anticoagulant, antithrombotic, antiinflammatory and antitumor activities, present advantages as an alternative source for heparin (Alban, Schauerte, & Franz, 2002; Fareed, Hoppensteadt, & Bick, 2000). However, the traditional methods of preparing sulfated polysaccharides have provided problems, e.g., high cost, strict preparation conditions, and toxic waste, owing to the use of strongly hydrolytic sulfating agents, such as chlorosulfonic acid, together with organic solvents such as pyridine (Bajdik et al., 2009; Cirelli & Covian, 1989; Guiseley, 1978; Wolfrom & Juliano, 1960). Kappa carrageenan, as a prominent natural marine sulfated polysaccharide, exhibits potent anticoagulant, antithrombotic and anti-inflammatory activities (Mourão, 2004; Silva et al., 2010; Yamada et al., 1997). Compared to other synthesized substitutes of heparin, synthesis of carboxymethyl κ-carrageenan can avoid the problems of the synthesis of sulfated polysaccharides which we have mentioned above, and also provide great advantages such as technical simplicity, low cost and environmental protection. Moreover, after carboxymethylation, the carboxymethyl κ-carrageenan would contain sulfate and carboxyl groups, as the nearest structural analogues of heparin. However, there are no reports on the anticoagulant activity of carboxymethyl κ-carrageenan.

The usual wound healing materials with antimicrobial effects against infection have been developed by incorporating antimicrobial agents, including iodine, silver ions and antibiotics (Murphy, Lee, & Herndon, 2004). Despite frequent use, there is growing evidence that silver is highly toxic to keratinocytes and fibroblasts,

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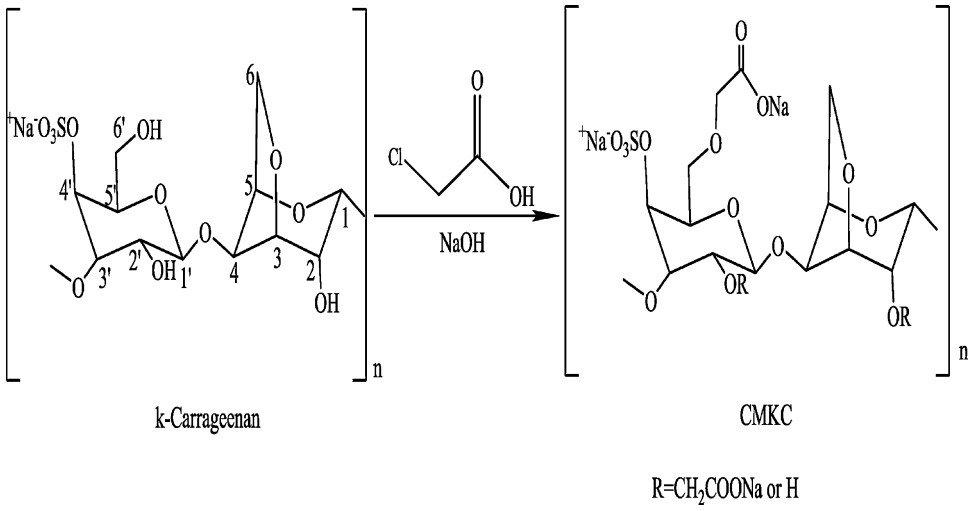

Scheme 1. The synthesis of carboxymethyl κ-carrageenan.

and the use of antibiotics may lead to bacterial resistance (Burd et al., 2007; Poon & Burd, 2004). For these reasons there was strong motivation to find new wound healing materials with inherent antibacterial activity. Heparin, the most sulfated and acidic glycosaminoglycan, has been widely utilized in treating wounds for more than 40 years (Saliba, 1967). However, there are some potential adverse effects with heparin use including thrombocytopenia, allergy and inducing diseases such as bovine spongiform encephalopathy (Michael, 2001). As we have mentioned above, carboxymethyl κ-carrageenan is the nearest structural analogue to heparin. Thus, the carboxymethylation of κ-carrageenan offers possibilities to produce excellent wound healing materials with antibacterial activity.

In addition, some reports reveal that when wounds are retained in a moist but not wet condition, the migration of epithelial cells from the edge of the wound to the wounded area is faster than when wounds are kept in a dry state (Chen, Wang, Chen, Ho, & Sheu, 2006). The aim of modern "moist healing" wound dressings is to create the moist conditions that can facilitate optimum healing (Qin, 2008). Carboxymethyl groups are strongly hydrophilic, and the introduction of carboxymethyl groups into κ-carrageenan is a convenient and effective method to make this polysaccharide have moisture-absorption and moisture-retention abilities.

In this study, carboxymethyl κ-carrageenan was synthesized (Scheme 1), and the products were characterized by FT-IR, ^{13}C NMR, degree of substitution (DS) and molecular weight (M_w). The properties including anticoagulant activity, antibacterial activity and moisture absorbability and moisture-retention capacity of carboxymethyl κ-carrageenan were evaluated, and the influences of carboxymethylation on these properties of κ-carrageenan were investigated.

2. Materials and methods

2.1. Materials

Kappa-carrageenan was purchased from Dehui Ocean Biotechnological Co. Ltd. (Qingdao, China). Monochloroacetic acid, sodium hydroxide and other reagents were kindly supplied by Sinopharm Group Chemical Reagent Corp. And all the reagents were of analytical grade and were used without further purification. Activated partial thromboplastin, prothrombin and thrombin were applied by Shanghai Sun Bio. Corp. Human plasma was bought from Blood Center of Wuhan.

2.2. Carboxymethylation of κ-carrageenan

Kappa-carrageenan (5 g) was suspended in 100 ml 80% EtOH–H_2O solution, and then 10 ml 20% NaOH solution was added dropwise over a period of 15 min. The reaction mixture was kept at 35 °C for 1 h with vigorous stirring. $ClCH_2CO_2H$ was added in NaOH solution, after stirring at room temperature for 30 min, the mixture was added to the reaction mixture which was then heated to 55 °C for 4 h. Next, CH_3COOH was added to the mixture to adjust the pH to 7.0. The carboxymethyl κ-carrageenan salt was obtained through vacuum filtration and washed three times with 80% ethanol solution. The product was dried in oven at 50 °C. By changing the molar ratios of monochloroacetic acid (MCA) to κ-carrageenan, a series of CM-κ-carrageenan with various DSs were prepared.

2.3. FT-IR measurements

IR spectra of samples were performed with a Nicolet 170SX Fourier transform infrared spectrometer. The test specimens were prepared by the KBr-disk method.

2.4. ^{13}C NMR spectra of the carboxymethyl κ-carrageenan

^{13}C NMR spectra were recorded on a Bruker AMX-500 NMR spectrometer at an ambient temperature. The samples were dissolved in D_2O. Tetramethylsilane (TMS) was used as internal standard.

2.5. Preparation of CMKC with different molecular weights

Carboxymethyl κ-carrageenan (CMKC) was degraded by an oxidative method involving hydrogen peroxide (H_2O_2) (Murinov, Romanko, Kuramshina, Kabalnova, & Murinov, 2007). CMKC (10 g) was added to 400 ml distilled water, then heated to 80 °C, and stirred until a homogeneous solution was formed. H_2O_2 solution (30%, w/w) of a desired volume was dropped into the CMKC solution within 30 min. After degradation for 4 h, 3 volumes of ethanol were added to the CMKC solution to cause a precipitate. The precipitate was centrifuged at 3000 rpm for 10 min, collected, and then refined three times by the dissolution-precipitation process. Finally, the precipitate was collected and dried in vacuum at room temperature to obtain CMKC with different molecular weights.

2.6. Measurement of the degree of substitution

The DS value of each sample was estimated from potentiometric titration. Accurate 0.2 g samples were dissolved in 0.1 mol/L hydrochloric acid (50 ml) and titrated with 0.1 mol/L sodium hydroxide. The pH values were measured with a DELTA-320-S pH meter as incremental volumes of NaOH solution were added (Muzzarelli et al., 1984). The DS value was calculated by the following equation:

$$\mathrm{DS} = \frac{385A}{1000 - 80A}, \quad A = (V_2 - V_1)_{\mathrm{NaOH}} \cdot C_{\mathrm{NaOH}}/m_{\mathrm{CMKC}}$$

where A is the general amount of $-CH_2COOH$ and $-CH_2COONa$ per gram sample.

2.7. Determination of viscosity-average molecular weight

The viscosity-average molecular weight (M_w) was obtained from viscometry and extrapolation. Each sample was dissolved in 0.1 mol/L aq. NaCl, which was the eluent. Viscosity measurements were done at 25 ± 0.1 °C in Ubbelohde viscometers. From efflux time of polymer solution (t) and that of solvent i.e., 0.1 M NaCl (t_0), relative viscosity $\eta_{rel} = t/t_0$ was obtained. Specific viscosity was calculated from the relationship $\eta_{sp} = \eta_{rel} - 1$. Reduced viscosity for a set of polymer solutions was calculated having concentrations in (g/ml). Intrinsic viscosity was then obtained from common ordinate intercept on extrapolation of plots of reduced viscosity versus concentration (Pal, Sen, Mishra, Dey, & Jha, 2008). The M_w was calculated according to the Mark–Houwink equation for carboxymethyl κ-carrageenan in 0.1 M NaCl aqueous solution at 25 °C:

$$[\eta] = K \cdot M_w^{\alpha}$$

According to the report of Vreeman, Snoeren, and Payens (1980), $K = 8.84 \times 10^{-3}$ and $\alpha = 0.86$.

2.8. Clotting assays

The anticoagulant activity of the carboxymethyl κ-carrageenan (CMKC) was investigated by the classical coagulant assays activated partial thrombosis time (APTT), thrombin time (TT) and prothrombin time (PT) (Lu et al., 2000). For APTT assay, citrated normal human plasma was mixed with a solution of sample and incubated for 1 min at 37 °C, and then APTT assay reagent (100 μl) was added to the mixture and incubated for 5 min at 37 °C. Thereafter, Prewarmed (37 °C) 0.025 mol/L $CaCl_2$ (100 μl) was added and clotting time was recorded. For PT assay, the plasma mixed with CMKC solution was incubated for 3 min at 37 °C. Then PT assay reagent 0.2 ml, pre-incubated for 3 min at 37 °C, was added to the mixture and clotting time was recorded. For TT assay, the plasma mixed with CMKC solution was incubated for 3 min at 37 °C. Then TT assay reagent 0.2 ml was added to the mixture and recorded the clotting time.

2.9. Evaluation of antibacterial activity

Antibacterial tests were carried out by the agar diffusion method using filter paper disks (Oliveira, França, Kuster, Teixeira, & Rocha, 2010). Two bacterial species (*Staphylococcus aureus* and *Escherichia coli*) maintained at 4 °C were used for the antimicrobial tests. Petri dishes were inoculated with 0.1 ml test bacteria solutions of *S. aureus* and *E. coli*, respectively and then activated at 37 °C for 24 h. Paper discs (6 mm in diameter), previously impregnated and saturated with κ-carrageenan and carboxymethyl κ-carrageenan (CMKC) were placed on the agar plates. The agar plates were incubated at 37 °C for 24 h. Antimicrobial activity was evaluated by measuring the zone of inhibition against the test organisms. All tests were performed in triplicate for κ-carrageenan and CMKC.

2.10. Moisture absorption and retention test

The moisture-absorption and retention ability of the samples was measured according to the method reported by Matsumura, Cheng, Minami, Yoshikawa, and Kariyone (1989). Before the moisture-absorption test, the samples were dried over P_2O_5 in vacuum for 24 h. The water absorption ability was evaluated by the percentage of weight increase of dry sample (R_a):

$$R_\alpha(\%) = 100 \times \frac{M_n - M_0}{M_0}$$

where M_0 and M_n are the weights of sample before and after putting in the saturated $(NH_4)_2SO_4$ desiccator (81% relative humidity) and the saturated $CaCl_2$ desiccator (32% relative humidity) at 20 °C after 48 h of the test.

Wet samples were prepared by adding 10% water to each sample and then put in the silica gel desiccator for another 48 h at 20 °C for the moisture-retention test. The moisture-retention ability was evaluated by the percentage of residual water of wet sample (R_h):

$$R_h(\%) = 100 \times \frac{H_n}{H_0}$$

where H_0 and H_n are the weights of water in the sample before and after putting in the silica gel at 20 °C after 48 h of the test.

3. Results and discussion

3.1. Characterization of carboxymethyl κ-carrageenan by FT/IR and ^{13}C NMR

The IR spectra (Fig. 1) and ^{13}C NMR spectra in D_2O (Fig. 2) of carboxymethyl κ-carrageenan and κ-carrageenan showed the successful synthesis of carboxymethyl κ-carrageenan (CMKC).

Fig. 1 showed the infrared spectra of κ-carrageenan (A) and carboxymethyl κ-carrageenan (B). Fig. 1A showed the basic characteristics of κ-carrageenan. As reported previously, the bands at 1260 cm^{-1}, 850 cm^{-1} were assigned respectively to O=S=O symmetric vibration and C_4–O–S stretching vibration. They stood for total $-SO_4$ and C_4–O–S of β-D-galactose respectively. The band at 930 cm^{-1} proved the existence of C–O–C of 3,6-anhydro-D-galactose (Silva et al., 2010). It also displayed an absorbance

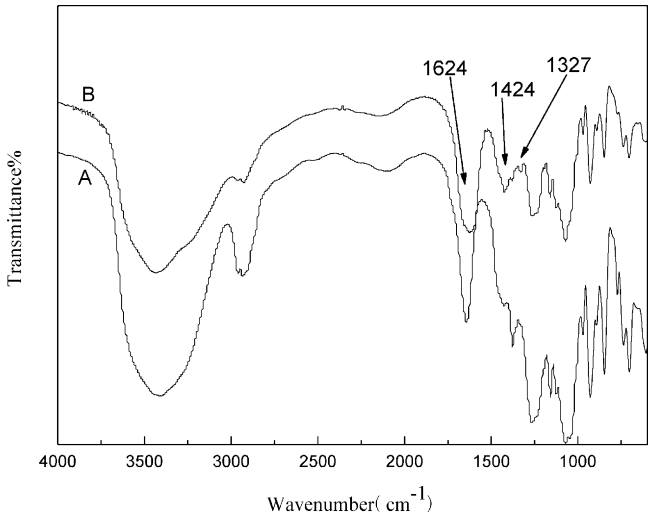

Fig. 1. FT/IR spectra of κ-carrageenan (A) and carboxymethyl κ-carrageenan (B).

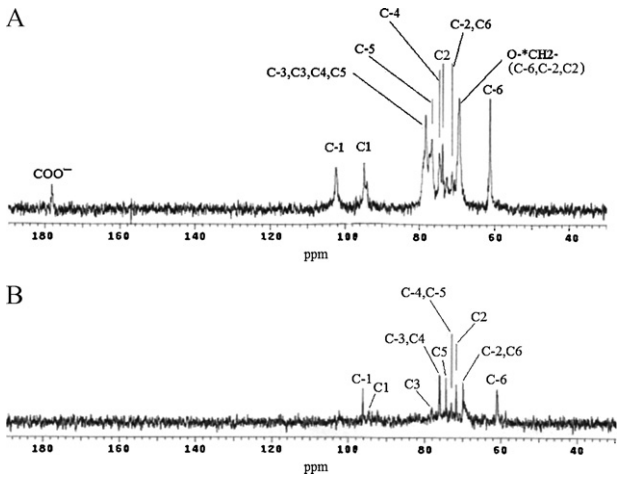

Fig. 2. ^{13}C NMR spectra of κ-carrageenan (A) and carboxymethyl κ-carrageenan (B) (C-1,C-2,C-3,C-4,C-5,C-6 belong to G4S and C1,C2,C3,C4,C5,C6 belong to ADG).

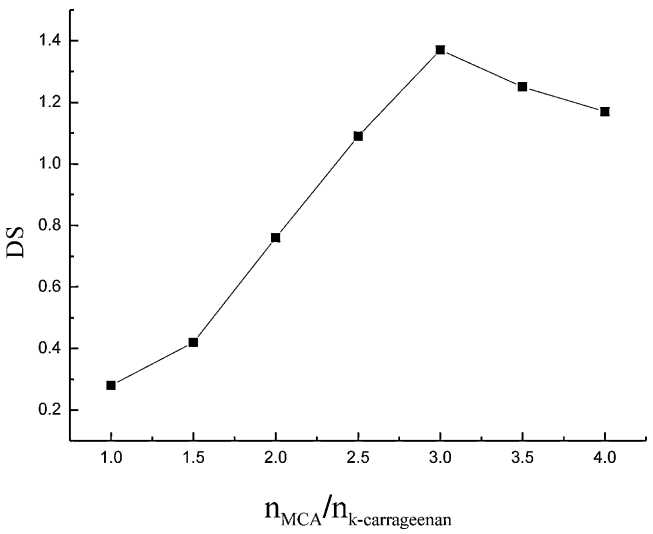

Fig. 3. Effect of various molar ratios of MCA to κ-carrageenan on the DS.

band at 1159 cm^{-1} due to bridge –O stretch. And band around 1070 cm^{-1}, 2933 cm^{-1},and 3411 cm^{-1} was reported to be related to C–O stretch, C–H stretch and O–H stretch respectively (Chen & Park, 2003). Fig. 1B was the IR spectrum of CMKC. The intensity of each absorption band standing for quantity of functional group had changed greatly. Compared with Fig. 1A, the peak intensity at around 1325 cm^{-1} of Fig. 1B increased obviously, which proved the quantity of C–O stretch on the galactose increased. The typical absorption band around 1624 cm^{-1} and 1424 cm^{-1}, corresponding to the asymmetric vibration and symmetric vibration of –COO$^-$ (Xu, Xin, Li, Huang, & Zhou, 2010), indicated the introduction of carboxymethyl groups.

Fig. 2 depicts the ^{13}C NMR spectra of κ-carrageenan (A) and carboxymethyl κ-carrageenan (B) in D_2O (chemical shift ranging between δ = 48–200 ppm). The chemical shifts of the carbon peaks of κ-carrageenan in the NMR spectra were published (Van de Velde & Rollema, 2006). In Fig. 2B, downfield displacements of the signals for anomeric carbon of β-D-galactopyranose-4-sulfate (G4S) to 102.4 ppm, anomeric carbon of 3, 6-anhydro-α-D-galactopyranose (ADG) to 94.8 ppm, as compared with their positions in κ-carrageenan at 96.1 ppm, 94.3 ppm in Fig. 2A respectively, revealed the substitution in the sulfated polysaccharides. In Fig. 2B, a shoulder peak at 178.2 ppm, which is the characteristic signal of –CH_2*COO^- group, is observed (Aguir & M'Henni, 2005). Further evidence of carboxymethylation having taken place on the C-6 hydroxyl group of the β-D-galactopyranose-4-sulfate unit (G4S-unit) was the appearance of a shoulder peak (δ = 61.2 ppm) on the C-6 peak (δ = 61.0 ppm). This suggested two different environments for C-6, depending on whether or not carboxymethylation has occurred on the C-6 hydroxyl group. In addition, obvious chemical shifts at around 69.3 ppm corresponding to –*CH_2COO^- in the carboxymethyl substituents were found (Rinaudo, Desbrières, Le Dung, Thuy Binh, & Dong, 2001), indicating that the hydroxyl groups in κ-carrageenan were carboxymethylated successfully. The chemical shifts of the carbon peaks in the ^{13}C NMR spectra were approximately consistent with published values (Leong et al., 2011).

3.2. Influence of various molar ratios of MCA to κ-carrageenan on the DS

The main reaction parameters that are expected to affect the process of the carboxymethylation were reaction temperature, ratio of organic solvent to water, reaction time, and molar ratio of sodium hydroxide and monochloroacetic acid (MCA) to κ-carrageenan (Lawal, Lechner, Hartmann, & Kulicke, 2007). To improve the introduction of carboxylmethyl groups in κ-carrageenan while minimizing the undesired side reaction, the optimization of the carboxymethylation process was studied by Leong et al. (2011). It should be noted that our investigation has examined only the influence of various molar ratios of MCA to κ-carrageenan (n_{MCA}/n_{KC}) on the DS, while other reagents were held constant at 5 g NaOH, 100 ml of 80% ethanol, 55 °C reaction temperature and 4 h reaction time. The influence of n_{MCA}/n_{KC} on DS is presented in Fig. 3. The DS varied from 0.28 to 1.37 as the ratio varying from 1.0 to 4.0. As observed, the DS increased with increasing n_{MCA}/n_{KC}, suggesting that formation of sodium carboxymethyl κ-carrageenan is favored when the n_{MCA}/n_{KC} was in the range of 3.0. However at higher n_{MCA}/n_{KC} (>3.0), the DS decreased. This is because during the carboxymethyation process, the NaOH provides the alkaline environment for the reaction as well as serves as the swelling agent to facilitate diffusion and penetration of the etherifying agent to the κ-carrageenan particles. So the increasing n_{MCA}/n_{KC} could lead to usage of NaOH, while under the reaction condition, the NaOH dosage is changeless (Volkert, Loth, Lazik, & Engelhardt, 2004). The more the MCA is, the less the NaOH can react with KC, so higher MCA/KC ratio leads to lower DS. In a previous report, the degree of carboxymethylation of κ-carrageenan was calculated from the ^{1}H NMR spectrum using the integral of the proton peaks between the chemical shifts of 3.4–5.2 ppm by Leong et al. (2011). In our investigation, the DS value of each sample was estimated from potentiometric titration. The result was in concordance to previous report of Leong et al.

3.3. Average molecular weight of the CMKC

In order to study the molecular weight on properties of carboxymethyl κ-carrageenan (CMKC), we applied oxidative degradation method involving H_2O_2 to degrade the products to different molecular weights while the DS was the same. The average molecular weight (M_w) was determined via viscometry and extrapolation. The M_w of the κ-carrageenan and CMKC polysaccharides were estimated to be 3.85×10^5 and 2.99×10^5, respectively. The M_w of low-molecular-weight CMKC were measured to be 1.26×10^4, 2.29×10^4, 4.57×10^4 and 6.06×10^4. Compared with κ-carrageenan polysaccharides, we found that the solubility of low-molecular-weight CMKC increased considerably and the color changed from transparent to yellow.

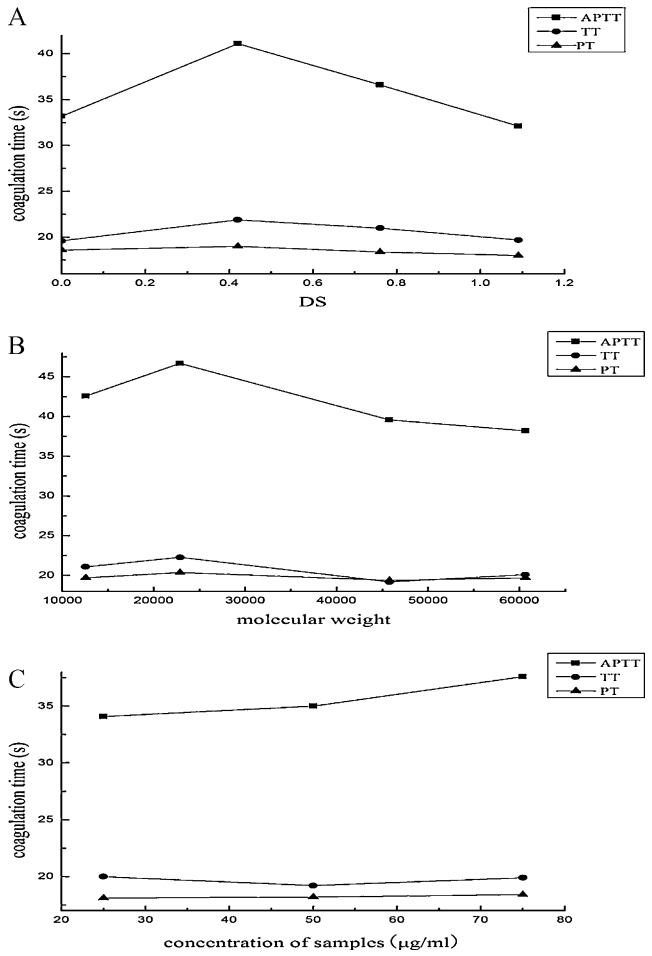

Fig. 4. Effect of DS (A), molecular weight (B) and concentration (C) of carboxymethyl κ-carrageenan on coagulation time.

3.4. Anticoagulant activity

3.4.1. Effect of DS on anticoagulant activity

Anticoagulant activity of carboxymethyl κ-carrageenan (CMKC) with different DSs was evaluated by the classical coagulation assays of activated partial thrombosis time (APTT), thrombin time (TT) and prothrombin time (PT), using physiological saline as a reference standard. In this work, the DS of 0.42, 0.76 and 1.09 samples were adopted as an anticoagulant, respectively. The significant and interesting results are given in Fig. 4A. The physiological saline showed APTT as 25.4 s, PT as 18.5 s and TT as 17.5 s, which was the normal. Compared with physiological saline, all the κ-carrageenan and CMKC polysaccharides of 75 μg/ml significantly prolonged APTT. And CMKC with the lowest DS of 0.42 displayed the highest anticoagulant activity. The clotting time for APTT at 75 μg/ml reached 41.1 s. It could be seen that as the content of the carboxymethyl groups increased (from 0.42 to 1.09) the activities of the CMKC did not increase accordingly. On the contrary, it decreased. Even CMKC with the highest DS of 1.09 showed weaker anticoagulant activity than κ-carrageenan.

The result indicated that the introduction of a few carboxymethyl groups into a sulfated polysaccharide promoted the anticoagulant activity (Yang, Du, Huang, Wan, & Wen, 2005). It could be in sulfated polysaccharides, carboxymethyl groups and sulfate groups had the synergistic action (Olson, Bjork, & Bock, 2002). After incorporation of carboxymethyl groups into the lacquer polysaccharide, sulfated polysaccharide did have higher anticoagulant activity. While as the degree of carboxymethylation increased from 0.42 to 1.09, APTT of CMKC decreased. This interesting phenomenon had been reported by Yang et al. (2005). It could be attributed to the fact that, on the one hand, incorporation of carboxymethyl groups increased the charge density, on the other hand, carboxymethyl groups competed with sulfate groups to interact with the basic sites of protein molecules. Since the affinity between carboxymethyl groups and proteins was less than that between sulfates and proteins, the carboxymethyl groups which replaced the sulfates would result in low anticoagulant activity (Yang et al., 2005).

From the image, carboxymethyl κ-carrageenan prolonged APTT and TT, but it hardly prolonged PT. It is generally accepted that activated partial thrombinplastin was an intrinsic coagulation pathway factor, while prothrombin was the extrinsic coagulation pathway factor (Lu et al., 2000). From above it could be concluded that CMKC had great influence on the intrinsic coagulation pathway, but little influence on the extrinsic coagulation pathway. This conclusion was consistent with a previous literature report by Silva et al. (2010).

3.4.2. Effect of molecular weight on anticoagulant activity

The molecular weight (M_w) of polysaccharides is one of the most important structural factors that affect the anticoagulant activity (Alban et al., 2002). Generally, the appropriate molecular weight was 2.6×10^4 (Yang et al., 2005). In this work, samples with molecular weights of 1.26×10^4, 2.29×10^4, 4.57×10^4 and 6.06×10^4 were used in the anticoagulant assay. The influence of molecular weight of carboxymethyl κ-carrageenan on anticoagulant activity is depicted in Fig. 4B. The clotting time for APTT and TT increased as the molecular weight rose from 1.26×10^4 to 2.29×10^4 and decreased slowly with further increase in molecular weight. It was speculated that because of high molecular weights, high apparent viscosity and poor water solubility, polysaccharides of higher M_w could prevent them from passing through the cell membrane, entering the interior of the cell and interacting with coagulation cofactors and their target proteases (Alban & Franz, 1994). In general, for sulfated polysaccharides, the increase of anticoagulant activity with decreasing molecular mass was a trend, and low molecular weight promoted anticoagulant activities.

3.4.3. Effect of concentration of carboxymethyl κ-carrageenan on anticoagulant activity

Besides DS and molecular weight, the concentration of carboxymethyl κ-carrageenan was another factor influencing anticoagulant activity. In this work, the concentration of CMKC was 25 μg/ml, 50 μg/ml and 75 μg/ml, respectively. Fig. 4C shows that the anticoagulant activity increased with increasing concentration. The sulfate polysaccharides generated anticoagulant activity via accelerating the plasma serine proteinase inhibitor, such as thrombin (IIa factor) and Xa factor (Roberta, Maria, & Daniela, 1993). The high concentration could increase the density of negative charge to inhibit the activity of thrombin and Xa factor, and thus led to higher anticoagulant activity.

3.5. Antibacterial activity

Paper disc diffusion method is one of the simple, rapid and sensitive methods to characterize antibacterial activity qualitatively. Antimicrobial activity was evaluated by measuring the zone of inhibition against the test organisms (Emmanuel et al., 2010). This investigation was carried out under the condition of simulating human physiological pH value (7.2–7.6), using two species, *E. coli* and *S. aureus*. In our work, antibacterial effect of κ-carrageenan and carboxymethyl κ-carrageenan (CMKC) with the DS of 0.28, 0.42, 0.76 and 1.09 were investigated. Table 1 shows antimicrobial activities with zone of inhibition between the edge of the filter paper

Table 1
Zone of inhibition (IZ, mm)[a] of KC and CMKC with different DSs against *E. coli* and *S. aureus*.

Sample no.	DS	Microorganisms	
		E. coli	*S. aureus*
κ-Carrageenan		0	0
CMKC-1	0.28	1.3	1.1
CMKC-2	0.42	2.0	1.9
CMKC-3	0.76	3.2	3.0
CMKC-4	1.09	4.1	3.7
RA[b]		7	10

[a] Between the edge of the filter paper and the edge of the inhibition area.
[b] Reference antibiotics (ampicillin).

and the edge of the inhibition area (IZ), From the results of Table 1, the IZ of κ-carrageenan could hardly been seen. While all CMKC with different DSs showed antimicrobial properties with regard to their IZ, ranging from 1.1 to 4.1 mm. CMKC exhibited strong antimicrobial properties against both *E. coli* and *S. aureus* in comparison with κ-carrageenan. The results can obviously be observed in Fig. 5. The antibacterial activity of CMKC increased as the degree of carboxymethylation increased, proving that the carboxymethyl groups contributed to the antibacterial activity.

The results clearly indicated that κ-carrageenan had no antibacterial activity, which was in concordance to previous report (Campo et al., 2009). But after carboxymethylation, the κ-carrageenan polysaccharide exhibited effective antibacterial activity. This result was significantly different from carboxymethyl chitosan in pervious report (Xu et al., 2010). Xu et al. believed that carboxymethyl groups did not enhance the antibacterial activity directly. The mechanism for the antibacterial activity of carboxymethyl chitosan can be attributed to the polycationic nature based on the amino groups present in chitosan molecules (Liu, Guan, Yang, Li, & Yao, 2001). In contrast to carboxymethyl chitosan, the $-SO_3$ and $-CH_2COO^-$ are both anionic, hence there is no positive charge in CMKC polymer chains. So the mechanism for antibacterial activity of CMKC should be considered, and further study is still required. According to the experimental results, it could be concluded that the introduction of carboxymethyl groups made κ-carrageenan generate antibacterial activity.

3.6. Moisture-absorption and retention properties

The moisture-absorption and moisture-retention properties of dry and wet carboxymethyl κ-carrageenan (CMKC) samples were examined and compared with those of hyaluronic acid (HA). As shown in Table 2, all CMKC products displayed better R_a and R_h than κ-carrageenan, indicating that the introduction of carboxymethyl groups is a convenient and effective method to make κ-carrageenan have moisture-absorption and moisture-retention ability. With the increase in DS, the moisture-absorption and retention ability of CMKC increased accordingly. It seemed that under high relative humidity conditions (RH 81% and 32%), water molecules were accessible to the surface of the galactosyl residues and readily developed hydrogen-bond interactions with the CMKC molecular chains. As a result, they intervened between chains or chain sheets in to give an expanded network (Okuyama, Noguchi, Miyazawa, Yui, & Ogawa, 1997). All the wet samples of CMKC released water slowly in the case of dry conditions (in silica gel). Most molecular chains

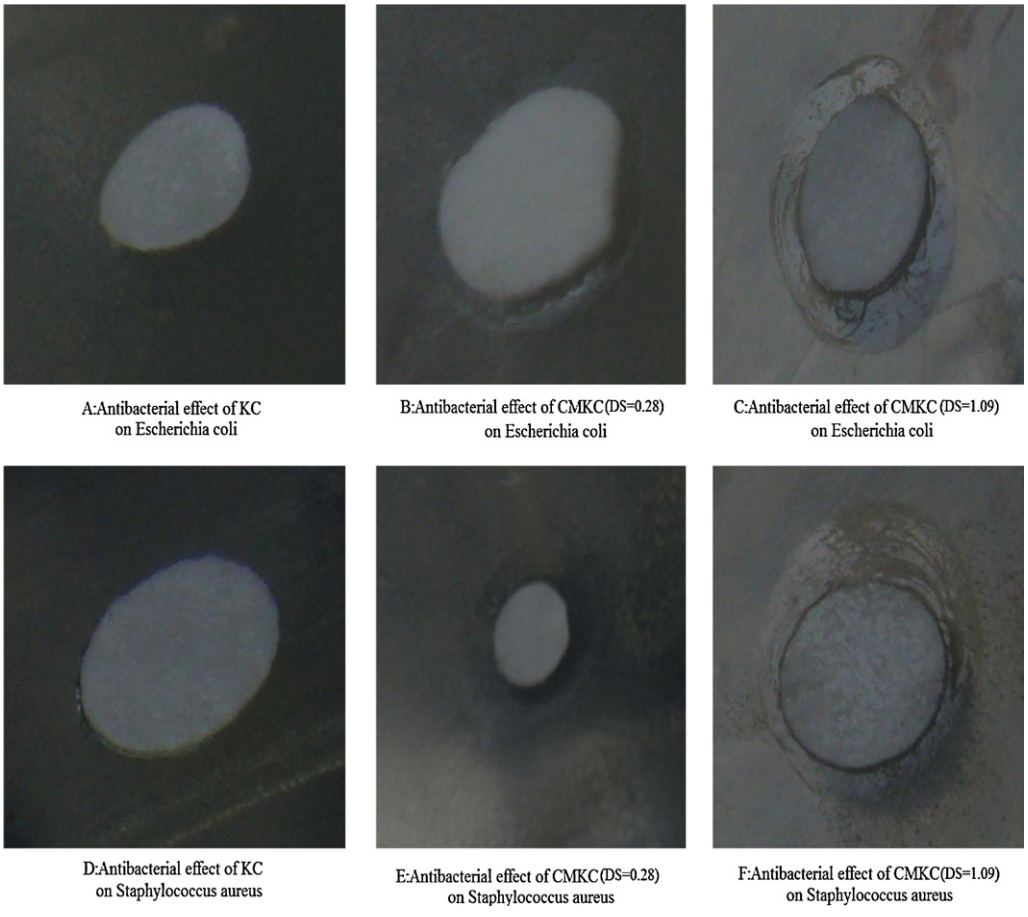

Fig. 5. Antibacterial activity of KC and CMKC with different DSs against *E. coli* and *S. aureus* .In this illustration, antibacterial activity of KC (A), CMKC (DS = 0.28) (B) and CMKC (DS = 1.09) (C) against *E. coli*, and antibacterial activity of KC (D), CMKC (DS = 0.28) (E) and CMKC (DS = 1.09) (F) against *S. aureus*.

Table 2
Moisture-absorption and retention ability of CM-κ-carrageenan with different DS values.

Sample no.	DS	R_a/% (dry sample)		R_h/% (wet sample) silica gel
		RH 81%[c]	RH 32%	
KC[a]	–	8.71	3.43	15.7
CMKC-1[b]	0.28	10.6	4.37	20.4
CMKC-2	0.42	19.2	7.08	26.9
CMKC-3	0.76	30.1	11.3	33.0
CMKC-4	1.09	45.3	17.1	44.8
CMKC-5	1.17	43.9	15.8	50.1
CMKC-6	1.25	42.5	15.0	55.7
[d]HA	–	56.2	24.8	72.8

[a] KC = κ-carrageenan.
[b] CMKC = carboxymethyl κ-carrageenan.
[c] RH = relative humidity.
[d] HA = hyaluronic acid.

bonded together by intermolecular hydrogen bonds to form this network structure, thus, water molecules were kept inside more easily, and moisture in the polymer network was difficult to diffuse to the environment (Chen, Du, & Zeng, 2003).

When the DS value increased from 0.42 to 1.09, R_a and R_h values increased rapidly. This can be explained by the ideas that with increasing carboxymethyl groups, on the one hand, hydrogen bonds between water and –COOH groups increased, while on the other hand, the intermolecular hydrogen bonds between CMKC molecular chains increased at the same time, which helps to bind large amounts of water and retain them in a the network (Chen et al., 2003). While the increasing rate of the R_a and R_h value slowed down, and even some decrease in R_a and R_h values were observed when the DS value was above 1.17. These findings were consistent with previous reports on carboxymethyl chitosan. Although the R_a and R_h values of CMKC were a little weaker than that of HA, this carboxymethylated derivative has potential to use as moisture-retention ingredient, e.g., in the wound management industry and cosmetics.

4. Conclusion

In this study carboxymethyl κ-carrageenan (CMKC) has been successfully prepared. It was demonstrated that CMKC exhibited good anticoagulant activity, antibacterial activity and moisture absorbability and moisture-retention capacity. The results indicated that the introduction of a few carboxymethyl groups into κ-carrageenan promoted the anticoagulant activity, but as the degree of carboxymethylation further increased from 0.42 to 1.09, the anticoagulant activity of CMKC decreased. The incorporation of carboxymethyl groups made κ-carrageenan generate antibacterial activity, and the activity of CMKC increased as the DS increased. Due to lack of direct evidences for antibacterial mechanism, further investigation on the mechanism for CMKC is still required. All CMKC displayed better R_a and R_h than κ-carrageenan, and with the increasing DS, the moisture-absorption and retention ability of CMKC also increased. Carboxymethyl κ-carrageenan was a promising substitute for heparin, as well as an excellent wound healing material with unique properties.

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