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Synthesis, Characterization, and Anticoagulant Activity of Carboxymethyl Starch Sulfates

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ABSTRACT: To develop a renewable and compatible anticoagulant as potential heparin alternative, carboxymethyl starch sulfate (CMSS) was prepared by the reaction of carboxymethyl starch (CMS) and sulfating reagent [$N(SO_3Na)_3$]. The chemical structures of CMS and CMSS were characterized by Fourier transform infrared spectroscopy and ^{13}C nuclear magnetic resonance. The influences of reaction parameters, including the pH of sulfating reagent, the molar ratio of sulfating reagent to CMS, reaction time, and temperature on the degree of substitution of sulfate groups (DS) of CMSS were studied. Meantime, the DS of each CMSS was determined by barium sulfate–glutin nephelometery method. Moreover, the anticoagulant activity of CMSS was investigated by the coagulation assays of activated partial thromboplastin time, thrombin time, and prothrombin time. The results revealed that the anticoagulant activity of CMSS was closely related to the DS value and concentration. The anticoagulant activity was promoted with the increasing of the DS and concentration. The molecular weight (M_w) in measured range had little impact on anticoagulant activity in contract to the DS and concentration. In this article, the CMSS with the DS of 1.91, concentration of 75 μg/mL and the M_w of 2.61×10^4 had the best blood anticoagulant activities. © 2012 Wiley Periodicals, Inc. J. Appl. Polym. Sci. 000: 000–000, 2012

KEYWORDS: carboxymethyl starch sulfate; sulfating reagent; degree of substitution; anticoagulant activity

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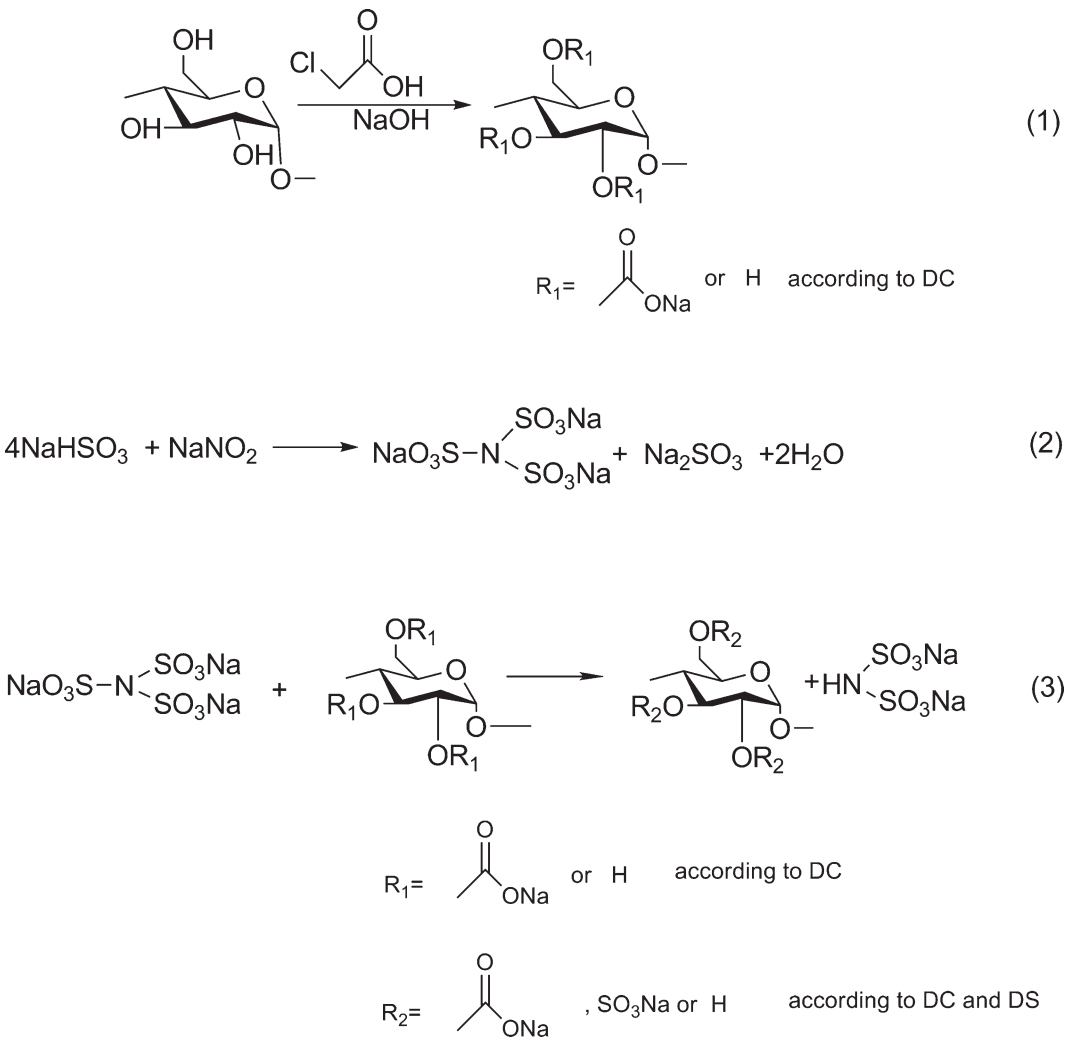
INTRODUCTION

The potential industrial applications of starch are limited by certain undesirable characteristics such as water-insolubility, nonexpandability, and nonscalability of viscosity.[1] Therefore, starch is usually modified physically or chemically to achieve a particular property for meeting the requirements of applications.[2,3] Carboxymethyl starch (CMS) is an important class of product within the modified starch products. Because of its water solubility and excellent properties in emulsification, dispersion, adhesion, and film-forming, CMS enjoys a wide range of applications in various industrial fields and is recognized as a safe, high-quality, and economical chemical fertilizer in the world.[4–6] Starch derivatives have attracted much attention in the burgeoning biological macromolecular industry, because they are cheap, nontoxic, renewable, and compatible with many other materials for industrial applications.[7] In recent years, it was found that sulfate polysaccharides shown good blood-compatibility or even anticoagulant activity.[8] After sulfation, the CMS would contain sulfate and carboxyl groups, as the nearest structural analogs of the natural blood anticoagulant heparin. The heparin had been widely used for anticoagulant therapy for more than 50 years. Heparin from animal sources had the potential to induce diseases affecting mammals, such as the avian influenza virus and bovine spongiform encephalopathy.[9] These reasons strongly motivated the necessity to find new anticoagulants and antithrombotics to replace heparin.

Previous studies had shown that natural or chemically modified sulfated polysaccharides presented anticoagulant activities. This was attributed to the sulfate group substitution of the glucosamine residue, which was key position in the glucosamine residue of heparin.[10] The main mechanism by nonfractionated heparin exerted its anticoagulant effects was by accelerating the plasma serine proteinase inhibitor, such as thrombin (IIa factor) and Xa factor. The anticoagulant activities of sulfated polysaccharides were influenced by the degree of the substitution, the molecular weight, and the position of sulfate groups.[11]

Many papers reported the way to prepare starch sulfate and CMS, and the biologic activities which were antitumor, anticoagulant, and other activities.[12–14] However, there was no any literature study about carboxymethyl starch sulfates (CMSS).



Scheme 1. The synthesis of CMS (1), sulfating reagent (2), and CMSSs (3).

Moreover, traditionally, the esterifying agent is strong acid such as oleum,[15] chlorosulfonic,[16] and sulfur trioxide,[17] and so forth. To minimize the degradation of starch chain in the process of synthesis, the organic solvent usually is used such as pyridine, dimethyl sulfoxide, triethylamine, toluene, and dichloroethane.[18–22] Although the use of these organic solvents can reduce the degradation of starch chain and have a higher degree of esterification, all these solutions have some shortcomings such as high toxicity, serious environmental pollution, higher cost, and difficulties in experimental operation and postprocessing. For that reason, the approximately neutral sulfating reagent [$N(SO_3Na)_3$] is used to modify CMS in aqueous solution for preparing CMSS with a high substitution degree.

In previous work, we synthesized the sodium alginate sulfate (SAS),[23] but many researches reported that the anticoagulant activities of sulfate polysaccharide were not only related to the sulfate group percent, concentration, and molecular weight[24,25] but also to the structure of polysaccharide including the sulfation position, type of sugar, and glycosidic branching.[16,26] Although starch and sodium alginate belong to nature polysaccharide, their structure are different, even if using the same sulfating reagent, the properties of their modified products are not identical. Thus, it is necessary to synthesize starch sulfate, we aimed to prepare CMSS by the reaction of CMS and an uncommon sulfating reagent which was synthesized by acting the sodium bisulfite with sodium nitrite in aqueous solution and study its anticoagulant activity like heparin.

MATERIALS AND METHODS

Materials

Corn starch, food grade quality, was kindly supplied by Tengsheng Agricultural Products Group (Gansu, China). All the other chemicals were of analytical grade. Sodium bisulfite, sodium nitrite, glutin, and barium chloride were produced by Tianjin No. 2 Chemical Factory (Tianjin, China). Activated partial thromboplastin, prothrombin, and thrombin were commercial reagents from Shanghai Sun Bio. Corp. (Shanghai, China). Human plasma was supplied by Blood Center of Wuhan (Hubei, China).

Synthesis of Carboxymethyl Starch

The synthesis of CMS was performed in two steps as shown in Scheme 1. Alkalization was performed by mixing starch (25 g), anhydrous alcohol (100 mL), and NaOH (6.25 g) in distilled

water (18.75 mL) at 35°C. The mixture was stirred for 1 h. Then, monochloro acetic acid (17.5 g) was added. After that, the mixture was heated to 60°C and stirred for 3 h.

After neutralizing with hydrochloric acid aqueous solution and washing out the generated sodium chloride with an alcohol/water mixture, the product was dried in an oven at 45°C. The obtained CMS was used for the synthesis of CMSS.[27]

Preparation of Sulfating Reagent

The sulfating reagent was prepared as shown in Scheme 1 according to Cui et al.'s[28] method. The molar ratio of sodium bisulfite to sodium nitrite is 4.25. The preparation was performed in a 500-mL three-necked round-bottom flask equipped with a dropping funnel, condenser, and mechanical stirrer. After sodium bisulfite (48.7 g) was dissolved in distilled water (100 mL) in the flask, the saturated sodium nitrite (7.6 g) solution was added dropwise to the three-necked round-bottom flask under mechanical stirrer at 90°C for 90 min. In this way, the sulfating reagent [$N(NaSO_3)_3$] was produced.

Preparation of Carboxymethyl Starch Sulfate

CMSS was synthesized according to the following steps as shown in Scheme 1. After the pH value of the sulfating reagent solution was adjusted to 10 using hydrochloric acid or sodium hydroxide, CMS (10 g, DC 0.67) was added to this solution under mechanical stirring. The reaction proceeded at 60°C for 6 h, and the molar ratio of $NaNO_2$ to the anhydroglucose unit (AGU) of CMS is 2.5. Then, the sodium CMSSs was acidified with hydrochloric acid to prepare the CMSS. Afterward, the mixture was dialyzed against distilled water for three days. After reduced pressure distillation, the product was dried in an oven at 45°C.

FTIR Spectra

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

^{13}C NMR Spectra

The ^{13}C nuclear magnetic resonance (^{13}C NMR) spectra were obtained with use of a 400 MHz a Bruker AMX-500 NMR spectrometer (Bruker Optics, Germany) at 30°C. The samples were dissolved in D_2O and the concentration of the sample solution was about 5%.

Measurement of Degree of Substitution of Carboxymethyl Groups

The method of Cu salt precipitation sodium-CMS (Na-CMS) was used, and Na-CMS was obtained by neutralizing the CMS with sodium hydroxide solution. The necessary amount of sodium hydroxide for the neutralization was calculated from the results obtained by back titration. After neutralization, the Na-CMS was precipitated by addition of ethanol, and the precipitate was dried in an oven at 60°C and was then ground. A weighed sample of Na-CMS was dissolved in water and precipitated by adding a copper sulfate ($CuSO_4$) solution in excess. After the precipitation, the excess of $CuSO_4$ was determined by titration with ethylenediaminetetraacetic acid (EDTA) in the presence of Murexide as an indicator. The blank was also titrated. From the difference of the volume of EDTA used for the titration of the Na-CMS, V_s, and that used for the blank, V_b, the amount of bound Cu^{2+} was determined. The procedure was repeated three times for each sample and the average value of the EDTA volume difference was used for the calculation of the amount of —CH_2COONa groups, n_{-CH_2COONa}, in the following way:

$$n_{-CH_2COONa} = (V_s - V_b) \times c \times 2 \times 2.5$$

where c is the concentration of the EDTA solution (0.5×10^{-4} mol/L); 2 is the ratio of the number of moles of —CH_2COONa and number of moles of EDTA used for the titration of Cu in the solution, and 2.5 is the ratio of the solution volume (250 mL) and the volume taken for titration (100 mL). The degree of substitution of carboxymethyl groups (DC) is calculated from an equation similar to Equation:[5]

$$DC = \frac{162 \times n_{-CH_2COONa}}{m_{ds} - 80 \times n_{-CH_2COONa}}$$

Where 162 g/mol is the molar mass of an AGU, 80 g/mol is the net increase in the mass of the AGU for each —CH_2COONa group substituted and m_{ds} (g) is the mass of dry sample calculated from known sample mass.

Measurement of Degree of Substitution of the Sulfate Groups

The degree of substitution of the sulfate groups (DS) of CMSS was measured by the barium sulfate nephelometry method.[23] CMSS (0.03 g) was hydrolyzed with hydrochloric acid (10 mL) at 100°C for 8 h to ensure that all the sulfate groups were split off from the starch backbone. Next, the solution was dried by reduced pressure distillation and distilled water (10 mL) was added to the leftover. Subsequently, the hydrolysis solution (0.5 mL), distilled water (2.0 mL), and glutin–barium chloride (1.25 mL) were added to a quartz cuvette and allowed to react for 15 min. The absorbency of barium sulfate was then determined with an ultraviolet spectrophotometer at 360 nm. A standard curve was recorded with 0.5 mL of potassium sulfate solution (0.01 mol/mL) instead of sample solution added with the other agents in the concentrations given above. Distilled water (0.5 mL) without CMSS plus the other agents used as blank, the DS of the CMSS was determined by comparison with the standard curve.

Degradation of Carboxymethyl Starch Sulfates

To study the molecular weight on properties of CMSS, oxidation degradation method was used to degrade the products to different molecular weights of CMSS. At first, 10 g of CMSS were accurately weighed for three times, 100 mL of distilled water used to dissolve them, respectively. Afterward, 30, 20, and 10 mL of hydrogen peroxide were added, respectively, to the above three solutions. The reaction was maintained at 70°C for 4 h.

Light Scattering Measurements

The weight-average molecular weight (M_w) of CMSS was determined with static light scattering. The light-scattering spectrometer (ALV/SP-125, ALV, Germany) equipped with an ALV-5000/E multi-τ digital time correlator and a He–Ne laser ($\lambda = 632.8$ nm) in an angular range from 30 to 150° at 10° intervals at 25°C. The test CMSS solutions were prepared in 0.1 mol/L NaCl aqueous solution and made optically clean by filtration through 0.22-μm Millipore filters. The specific refractive-index increments (dn/dc) of CMSS in 0.1 mol/L NaCl aqueous solution were measured on an Optilab refractometer (Wyatt Technology) at 632.8 nm and 25°C and were found to be 0.140 cm^3/g.

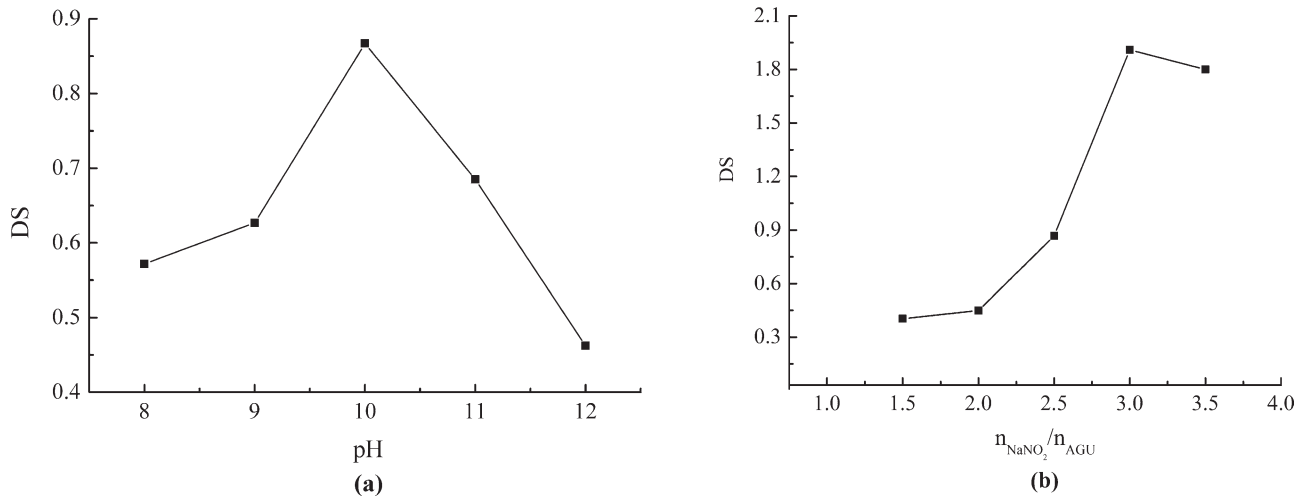


Figure 1. (a) Influence of the pH of sulfating reagent on the DS of CMSS and (b) influence of different molar ratios of sulfating reagent to CMS on the DS of CMSS.

Anticoagulant Activity Assays

The anticoagulant activities of all the samples were investigated by the classical coagulation assays of activated partial thromboplastin time (APTT), prothrombin time (PT), and thrombin time (TT) using heparin as reference compound. APTT experiment was performed in the following way: citrated normal human plasma was mixed with the solution of samples and APTT assay reagent, and then the mixture was incubated for 5 min at 37°C. Afterward, $CaCl_2$ (0.025 mol/L) was added and the clotting time was recorded. For PT experiment, citrated normal human plasma was mixed with the solution of sample and incubated for 5 min. Then, the PT reagent was preincubated for 5 min at 37°C. After that, the PT reagent was added and clotting time was recorded. The TT assay reagent preincubated for 5 min at 37°C was added and its clotting time was also recorded. All the samples were dissolved in physiological saline solution.[29]

RESULTS AND DISCUSSION

Optimization of Reaction Conditions of Synthesizing Carboxymethyl Starch Sulfates

The DS of CMSS was not only related to the type of sulfating reagent but also to the reaction conditions. To prepare different DSs and the highest DS of CMSS for further use, four reaction conditions were discussed in this step.

Influence of the pH Value of Sulfating Reagent on the DS. The influence of the pH value of sulfating reagent on the DS of CMSS was shown in Figure 1(a). As observed obviously, with the increase of the pH value from 8 to 10, the DS of CMSS increased from 0.58 to 0.86, and further increase of pH value, the DS decreased. Thus, the maximal DS (0.86) of CMSS was obtained at pH 10. The results were attributed to two reasons. On the one hand, during the sulfation reaction, the alkaline environment facilitated diffusion and penetration of the sulfating reagent into the CMS granular structures. On the other hand, further increasing pH value inactivated the sulfating reagent and made it consumed more by the side reactions. Therefore, we came to the conclusion that pH 10 was the optimal pH value.

Influence of Different Molar Ratios of Sulfating Reagent to Carboxymethyl Starch on the DS. The influence of different molar ratios of sulfating reagent to CMS on the DS was presented in Figure 1(b). The ratio of sulfating reagent to CMS can be described by the molar ratio of sodium nitrite added in the Scheme 1 to CMS added in the Scheme 1. According to Figure 1(b), the DS increased from 0.41 to 1.86 with the increase in the molar ratio of sodium nitrite to CMS AGUs from 1.5 to 3.0. Although due to the stereohindrance effect and electrostatic repulsion, at higher ratio of 3.5 the DS decreased. As a result, the optimal molar ratio of sulfating reagent to CMS AGUs was 3.0.

Influence of Different Reaction Temperatures on the DS. The influence of different reaction temperature on DS of CMSS was shown in Figure 2(a). As can be seen, the DS value increased with the increase of sulfation reaction temperature from 40 to 70°C. This may result from the increased reaction rate at higher temperatures. Increasing the temperature raised the proportion of molecules with higher energy than the activation energy. Consequently, the rate of reaction increased and thus DS of CMSS increased. However, when the temperature further increased to 80°C, the DS of CMSS decreased. It is reasonable to believe that the CMS formed gel easily, particularly in alkaline medium. Thereby, CMS was difficult to react with sulfating reagent. Therefore, it could be concluded that the optimal reaction temperature was 70°C.

Influence of Reaction Time on the DS. The effect of reaction time on DS was presented in Figure 2(b). As shown, the DS increased sharply as the reaction time increased from 2 to 6 h. The longer duration of reaction enhanced dissolution and diffusion of the reagents, and esterification was enhanced. Moreover, swelling of starch molecules improved with the increase of reaction time, which reinforced the absorption of reagent by the starch molecules. Obviously, the increase in the DS was not remarkable after 6 h. The reasons might be that the reaction was completed into dynamic balance due to the influence of stereohindrance effect and electrostatic repulsion. Therefore, it may be

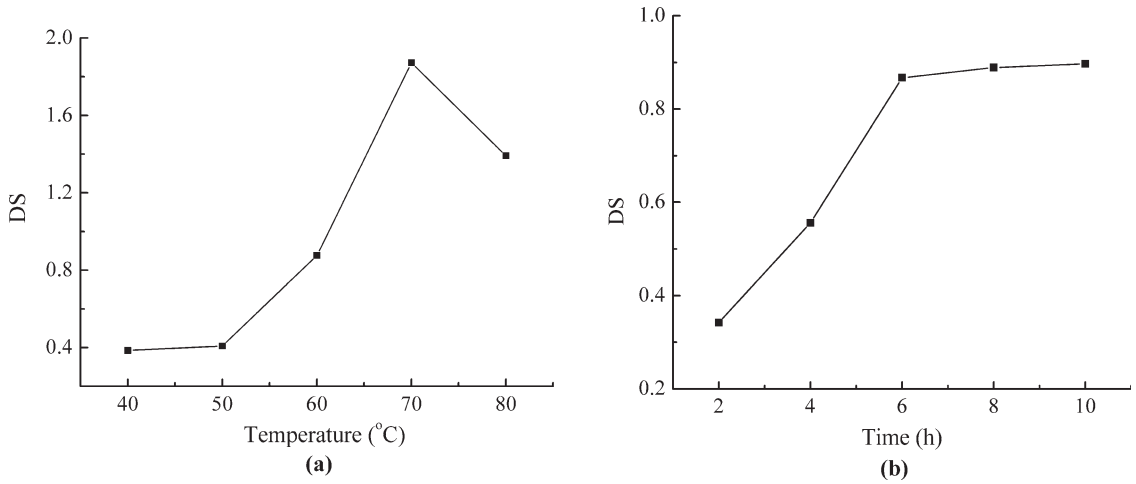


Figure 2. (a) Influence of different reaction temperatures on the DS of CMSS and (b) influence of reaction time on the DS of CMSS.

reasonable to keep the reaction for 6 h to save time and the cost of production.

The Optimal Reaction Conditions. According to the above results, the optimal reaction conditions for synthesizing CMSS were found to be as flow: the pH of the reaction medium was 10. The reaction temperature was 70°C. The reaction time was 6 h. The ratio of sodium nitrite to CMS was 3.0. Under these optimal conditions, a maximum DS of 1.91 was obtained. Compared with our previous work of SAS,[23] the optimal condition were pH 9, the ratio of sodium nitrite to the weight of sodium alginate 2/198 (mol/g), temperature 40°C and time 4 h. The maximum DS was 1.87. Although the optimal conditions of CMSS were similar to SAS, some changes were found. First, the CMSS require higher alkalic condition, higher temperature, and less time. This may be due to the differences between CMS and sodium alginate in chemical structure. Second, the DS of CMSS was higher than SAS, it was suggested that this sulfate reaction was more suitable for preparing CMSS.

Characterization of Carboxymethyl Starch Sulfate by FTIR Spectra

The FTIR spectra of the native corn starch, CMS and the CMSS were shown in Figure 3. The band stretch around 3500 cm^{-1} is attributed to the hydrogen-bonded hydroxyls on the starch molecules. The band at 2926 cm^{-1} is attributed to CH_2 symmetrical stretching vibrations. In the corn starch as shown in Figure 3(a), the band at 1642 cm^{-1} is assigned to scissoring of two O—H bonds of absorbed water molecules. In the spectrum of CMS as shown in Figure 3(b), There were two peaks obtained for CMS at 1612 cm^{-1} and 1412 cm^{-1}, which were corresponded to the carboxylate C=O asymmetric stretching and the C=O symmetric stretching. The results indicated that CMS was synthesized successfully.[4] In comparison with CMS, the most striking differences were two absorption bands observed in the CMSS spectrum as shown in Figure 3(c). One was at 1234 cm^{-1} describing an asymmetrical S=O stretching vibration and the other was at 867 cm^{-1} indicating a symmetrical C—O—S vibration associated to a C—O—SO_3 group.[30] Therefore, FTIR indicated that sulfated reaction had actually occurred between CMS and sulfating reagent.

^{13}C NMR Characterization

The ^{13}C NRM spectra of CMS and CMSS were shown in Figure 4. In the spectrum of CMS, The prominent peak at 175.9 ppm was assigned to the —COO^- carbon (C-8) of the carboxymethyl group. The resonances at 77.9 and 70.5 ppm were attributed to C4 and C3. The peaks at 94.0 and 90.2 ppm were assigned to C-1 and C1′. As observed, two peaks appeared here because of the carboxymethylation status of C-2. Unsubstituted C2 and C3 were assigned at 68.3 and 70.5 ppm, whereas the substituted C2′ and C3′ appeared at 71.2 and 72.3 ppm, indicating that the carboxymethylation caused a downfield shift of 2–3 ppm. The signal at 58.1 ppm was assigned to C6 while the resolved signal for substituted C6′ appeared at 66.7 ppm, indicating a downfield shift in the signal of C6 after substitution. The signals at 67.0 ppm were assigned to the —CH_2— carbon (C7) of methylene groups in the carboxylate substituents.[7] These results

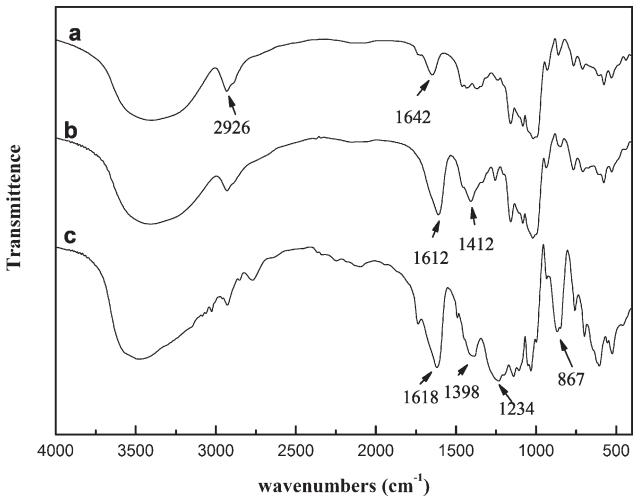


Figure 3. FTIR spectra of corn starch (a), CMS (b), with DC of 0.67 and CMSS (c) with DS of 1.87.

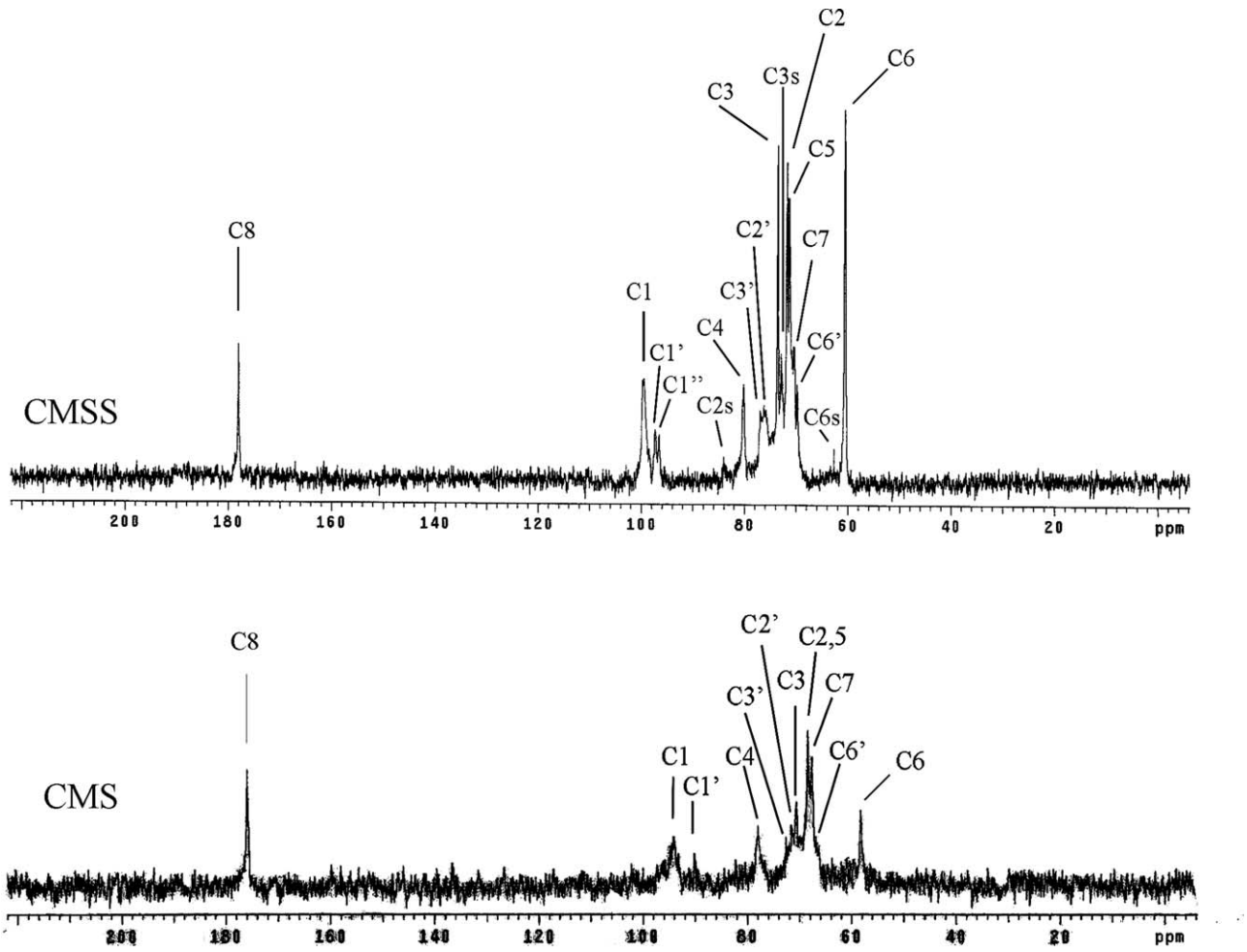


Figure 4. ^{13}C NMR spectra of CMS with DC of 0.67 and CMSS with DS of 1.87.

indicated that carboxymethyl substitution occurred in three different positions in C-2, C-3, and C-6.

In comparison with CMS, there were four new peaks appearing in the CMSS spectrum, namely C1″ (96.6 ppm), C-2s (83.8 ppm), C-3s (72.9 ppm), and C6s (62.7 ppm). These resulted from sulfation reaction of the hydroxyl groups at positions C-2, C-3 and C-6. The new peak that appeared at 62.7 ppm in the CMSS spectrum was assigned to the substituted C6 by the sulfate group. Similarly, the new peaks at 83.8 and 72.9 ppm for CMSS were assigned to C-2 and C-3, which had been partially substituted by sulfo groups. Furthermore, a new peak at 96.6 ppm could be assigned to C1″ because C-10 because C-2 were substituted by sulfate groups to influence the chemical shift of the adjacent C-1, leading to the splitting of the C-1 carbon signal.[30] From the results of ^{13}C NMR, it is reasonable to conclude that the sulfation of CMS has occurred with the substitution of C-2, C-3, and C-6.

Effect of the CMSS on the Anticoagulant Activity

In positive of control, heparin showed APTT as 125 s at 10 μg/mL, PT as 20 s at 12.5 μg/mL, and TT as 110 s at 5 μg/mL.[25] The normal range of APTT was 22–38 s, PT 10–14 s, and TT 10–16 s.[23] In our work, the blank control group (without CMSS) of APTT was 25 s, PT 11 s, and TT 14 s as shown in Figure 6.

According to Figures 5–7, we found that the CMSS with different DSs (0.221, 0.445, 0.892, and 1.910), molecular weight (1.52×10^4 g/mol, 2.61×10^4 g/mol, and 3.76×10^4 g/mol) and concentration (25, 50, and 75 μg/mL) could prolong APTT obviously, but it hardly prolonged PT and TT. As shown in Figures 5 and 6, with the increase of DS from 0.221 to 1.910 and concentration from 25 to 75 μg/mL, the clotting time increased. As can be seen in Figure 7, the clotting time increased as the molecular weight rose from 1.52×10^4 to 2.61×10^4 g/mol, while decreased slowly with future increase to 3.76×10^4 g/mol. The results indicated sulfate group, molecular weight and concentration play roles in anticoagulant activities. Compare these data with heparin and blank control group, these results also shown that CMSS exhibited good anticoagulant activity but weaker than heparin. In addition, compared with SAS which were prepared in our previous work, we found that the most of clotting time of CMSS were less than SAS, which suggested that that the anticoagulant activity of CMSS was weaker than SAS. In addition, the anticoagulant activity of sulfate polysaccharide also depended on its structure. In the measured range (M_w from 1.52×10^4 g/mol to 3.76×10^4 g/mol, DS from 0.22 to

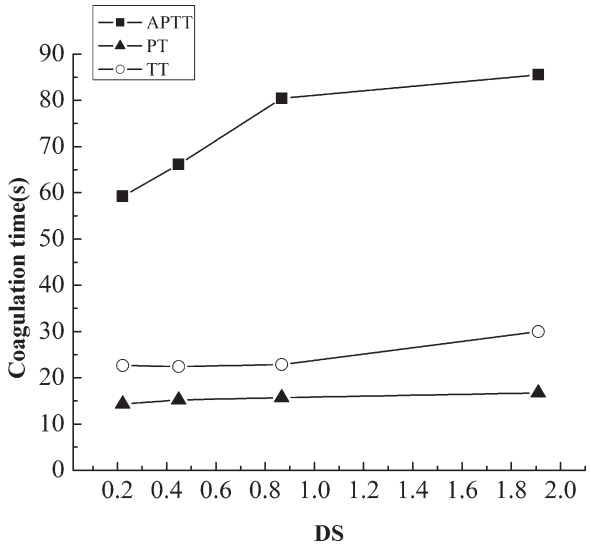


Figure 5. The effect of DS on the anticoagulant activity of CMSS (M_w = 2.61 × 10^4 g/mol, concentration = 75 μg/mL).

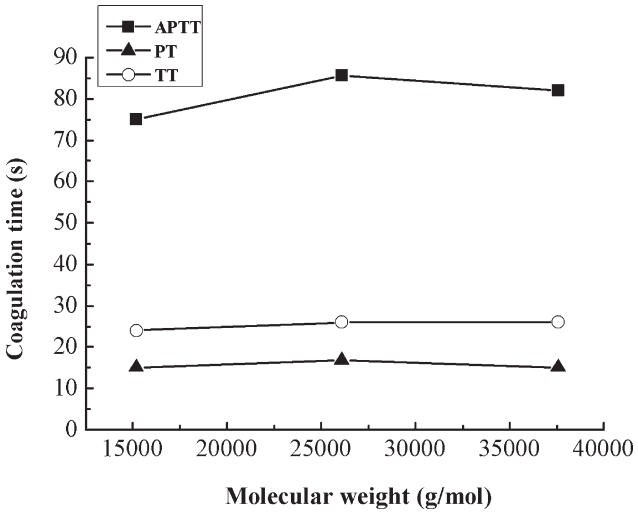


Figure 7. The effect of molecular weight on the anticoagulant activity of CMSSs (DS = 1.91, concentration = 75 μg/mL).

1.91, concentration from 25 to 75 μg/mL), the clotting time were changed slightly in Figure 7 suggested that molecular weight had little impact on the anticoagulant activity in contract with DS and concentration.

As regards to the anticoagulant mechanism,[31] the plasma added CMSS prolonged the APTT value suggested inhibition of the intrinsic coagulation pathway, whereas prolongation of TT indicated inhibition of thrombin-mediated fibrin formation. More importantly, CMSS as the same as heparin took advantage of the negative charge of sulfate group to neutralize the positive charge amino acid residues which were in the antithrombin so as to improve the anticoagulant activities.[32] As the DS increase, the sulfate groups on the CMSS structure were getting more and more and the density of negatively increased. So, the higher DS has better anticoagulant activities. In the same way, as the concentration increase, the density of sulfate groups of anticoagulant solution was also increased. So, the anticoagulant activities were improved. However, in the case of molecular weight, CMSS with M_w 2.61 × 10^4 g/mol has the best anticoagulant activity comparing with 1.52 × 10^4 and 3.76 × 10^4 g/mol. Vikhoreva et al.,[33] showed that decreasing molecular weight could result in higher antifactor Xa. It may be attributed to what their pharmacokinetic of properties were improved compared to high molecular weight. The CMSS may be lack of biological activities when the M_w was too low. So, CMSS with M_w 2.61 × 10^4 g/mol had the best anticoagulant activity in measured molecular weight range.

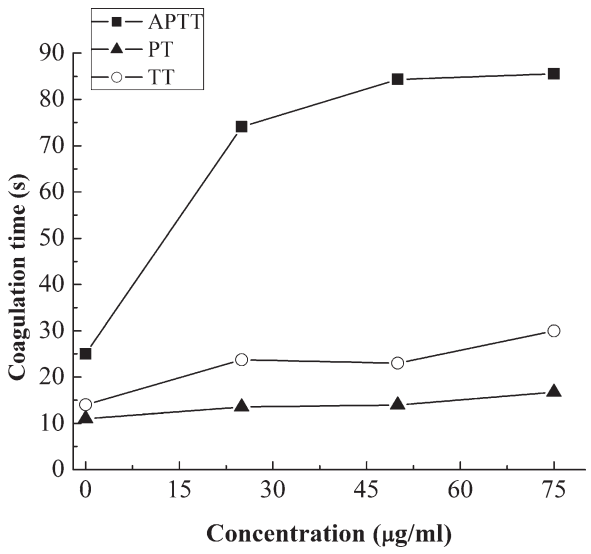


Figure 6. The effect of concentration on the anticoagulant activity of CMSSs (DS = 1.91, M_w = 2.61 × 10^4 g/mol).

CONCLUSIONS

A renewable and compatible anticoagulant CMSS was synthesized successfully by reacting CMS with the approximately neutral sulfating reagent [$N(SO_3Na)_3$]. Moreover, the parameters (the pH of the reaction medium, the reaction temperature, the reaction time, and the ratio of sodium nitrite to CMS) which influenced the DS of CMSS were studied to obtain the highest DS, and the optimal conditions were found to be as follows: the pH of the reaction medium was 10. The reaction temperature was 70°C. The reaction time was 6 h. The ratio of sodium nitrite to CMS was 3.0. CMSS got the maximum DS (1.91) under the optimal conditions. Furthermore, the CMSS was evaluated in terms of anticoagulant activity. The different effect of anticoagulant activity could be obtained by adjusting the DS, molecular weight (M_w), and the concentration of CMSS. The results showed that with the increase of the DS and concentration, the anticoagulant activity of CMSS improved. The M_w in measured range had little impact on anticoagulant activities in contract to the DS and concentration. In addition, CMSS with M_w of 2.61 × 10^4 g/mol exhibited better anticoagulant activity. Therefore, the introducing of sulfate group to hydroxyl groups could indeed enhance the anticoagulant activity.

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