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Synthesis and anticoagulant activity of sodium alginate sulfates

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ABSTRACT

Sodium alginate sulfates prepared from sodium alginate through reaction with an uncommon sulfating agent ($N(SO_3Na)_3$) which was synthesized by sodium bisulfite and sodium nitrite in aqueous solution. The factors that could affect the degree of substitution (DS) of sodium alginate sulfates were investigated in detail. A sodium alginate sulfate with DS of 1.87 was obtained under optimal conditions. The structures of the derivatives were characterized by FTIR and ^{13}C NMR. FTIR spectra showed the characteristic absorptions of sulfate ester bonds at 1249 cm^{-1} and 873 cm^{-1}. The in vitro coagulation assay of human plasma containing the sodium alginate sulfates was determined with respect to activated partial thromboplastin time (APTT), thrombin time (TT) and prothombin time (PT). These activities strongly depended on the DS, molecular weight (M_w) and the concentration of sodium alginate sulfates. The introduction of sulfate groups to hydroxyl groups greatly prolonged the APTT and TT. Low S% and concentration sodium alginate sulfates showed little anticoagulant activity. The high DS and concentration could inhibit the activity of IIa and Xa to prolong APTT and TT. The low molecular weight resulted in higher anti-factor Xa activity to promote anticoagulant activity. Generally, the introducing of sulfate groups could not increase PT, it had little effect on coagulation factors in the extrinsic pathway.

1. Introduction

Sodium alginate, the sodium salt of alginic acid, was a water soluble anionic polymer. It was widely used to produce microsphere, beads, microcapsule and tablets for drug delivery system and it showed long time effects and reduced side-effects of the drug (Kaneko et al., 1998). Bearing in mind their gelling ability, stabilizing properties and high viscosity in aqueous, sodium alginate and their derivatives were widely used in the food, cosmetics and pharmaceutical industries (Chunmei, Mingzhu, Jun, & Xu, 2009). Sodium alginate was also used as artificial skin in healing of split-thickness skin graft donorsites (Vanstraelen, 2000).

Sulfated polysaccharides, including natural and synthesized ones, had great blood-compatibility or even anticoagulant activity (Alban, Schauerte, & Franz, 2002). The chemical modification of the carboxyl and hydroxyl groups could generate great biological activities of products (Vikhorrva et al., 2005). After sulfated modification the sodium alginate would contain sulfate and carboxyl groups, as the nearest structural analogues of the natural blood anticoagulant heparin. The heparin had been widely used for anticoagulant therapy for more than 50 years (Clarence & Eric, 1941). Heparin from animal sources had the potential to induce disease affecting mammals, such as the avian influenza virus and bovine spongiform encephalopathy (Simone et al., 2009). These reasons strongly motivated the necessity to find new anticoagulants and antithrombotics to replace heparin.

According to previous studies had shown that sulfated polysaccharides which were natural or chemically modified presented anticoagulant activities that were attributed the sulfate groups substitution of the glucosamine residue, which were key position in the glucosamine residue of heparin that were substituted with sulfate groups (Preeyanat, Warayuth, Dumrat, & Prachya, 2002). The main mechanism by non-fractionated 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 their degree of the substitution, the molecular weight and the position of sulfate groups (Jiraporn et al., 2009).

This paper reported the sodium alginate sulfates prepared from sodium alginate through reaction with an uncommon sulfating agent which was synthesized by sodium bisulfite and sodium nitrite in aqueous solution. Traditionally, sulfating agents were prepared by sulfuric acid, chlorosulfonic acid, sulfuryl chloride, sulfur trioxide and sulfamic acid. Some organic solvents had been used as reaction medium, such as pyridine, dimethyl sulfoxide and formamide (Guiseley, 1978; Schierbaum & Kordel, 1978; Tessler,

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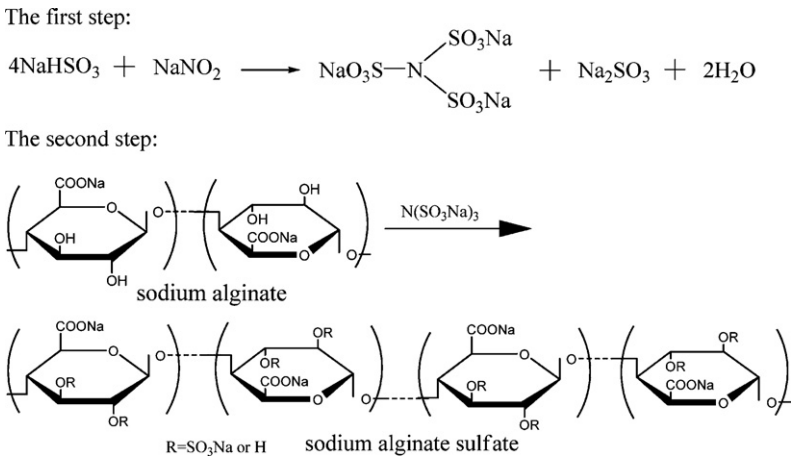


Scheme 1. The synthesis of sodium alginate sulfates.

1978). These agents not only could result in extreme hydrolytic or degradation of sodium alginate chain during reaction, but also lead to serious pollution problems (János et al., 2009). Compared with traditional methods, the whole reaction was carried out in aqueous solution. The sulfating agent used in this work was non-toxic, low cost and little pollution.

The anticoagulant activities of sodium alginate sulfates were measured by activated partial thrombosis time (APTT), thrombin time (TT) and prothrombin time (PT). The APTT was greatly prolonged by the derivatives, but TT and PT were seldom influenced. The synthesis of the samples was presented as Scheme 1.

2. Experiment

2.1. Materials

Sodium alginate was commercial reagent; sodium bisulfite, sodium nitrite and other reagent used in this investigation were of analytical grade and without further purification. They were kindly supplied by Sinopharm Group Chemical Reagent Corp. Activated partial thromboplastin, prothrombin and thrombin were applied by Shanghai Sun Bio. Corp. Human plasma was bought from Blood Center of Wuhan.

2.2. Preparation of sulfating agent ($N(SO_3Na)_3$)

The preparation of sulfating agent was carried out in a three-necked round-bottom flask equipped with a dropping funnel, condenser and magnetic. The entire device was placed in oil bath. A predetermined amount of sodium bisulfite was dissolved in 40 ml distilled water in the flask. Then the sodium nitrite previously dissolved in 10 ml distilled water was added dropwise to the reaction vessel under magnetic stirring at 90 °C and reacted for 1.5 h. In this way, the sulfating agent ($N(SO_3Na)_3$) was obtained.

2.3. Synthesis of sodium alginate sulfates

Sodium alginate sulfates were synthesized as follows: first of all, the obtained sulfating agent solution was adjusted to a suitable pH using sodium hydroxide. Then, 5 g sodium alginate was added to the above solution under magnetic stirring, the reaction was allowed to proceed for a certain time at preset sulfation temperature. Then the solution was dialyzed for 72 h and distilled by rotary evaporator. Afterwards, the sodium alginate sulfate was dried at 40 °C for 2 days.

2.4. FTIR 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.

2.5. ^{13}C NMR spectra of the sodium alginate sulfates

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

2.6. Measurement of degree of substitution

In this work, the barium sulfate nephelometry method was used to measure the DS of sodium alginate sulfates (Dodgson & Price, 1962). 0.03 g of sodium alginate sulfate was hydrolyzed using 10 ml of HCl for 8 h at 100 °C. Then the solution was evaporated to dryness and the residue was dissolved in 10 ml of distilled water. After that 0.5 ml of the hydrolysis solution, 2.0 ml of distilled water, 1.25 ml of glutin–barium chloride solution (prepared by dissolution of 0.5 g barium chloride and 0.5 g glutin in 100 ml distilled water in the 60–70 °C) and 0.7 ml 8%of trichloroacetic acid were added. The contents were stirred for 1 min and allowed to set for 15–20 min. In the end, the absorbency of barium sulfate was measured with a spectrophotometer at 360 nm. A standard curve was recording with different concentration of potassium sulfate instead of hydrolysis solution plus the other agents in the same conditions given above. And blank test was 0.5 ml distilled water instead of potassium sulfate solution, other conditions remained unchanged. The DS of the sodium alginate sulfates was determined by comparison with the standard curve. The DS, which designated the average number of sulfate groups on each anhydroglucose unit, was established from the sulfur content using the following formulae (Lim, Whang, Yoon, & Ko, 2001):

$$DS = \frac{198[S]}{(3200 - 102[S])}$$

where [S] was the sulfur content (%) of sodium alginate sulfate obtained from the above calculation.

2.7. Degradation of sodium alginate sulfates

In order to study the molecular weight on properties of sodium alginate sulfates, oxidation degradation method was used to

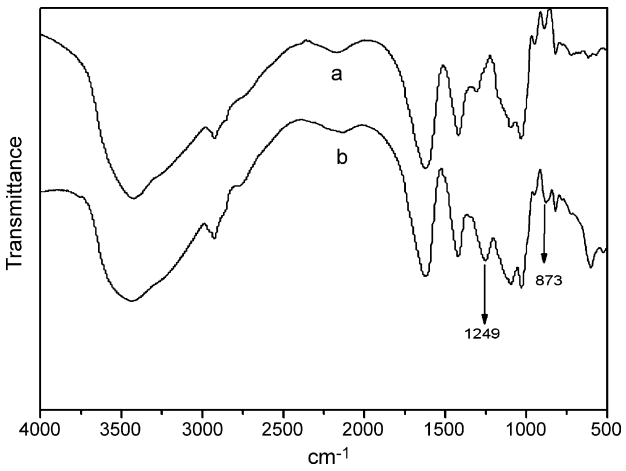


Fig. 1. IR spectra of sodium alginate (a) and sodium alginate sulfates (b).

degrade the products with different molecular weights while the other parameters were the same for the research (Chen et al., 2002). At first, 0.5 g sodium alginate sulfates were accurately weighed for three times, 50 ml distilled water used to dissolve them, respectively. After that added 4.53 g, 3.0 g and 1.8 g hydrogen peroxide to the above three solutions. The reaction was maintained at room temperature for 4 h.

2.8. Light scattering measurements

The weight-average molecular weight (M_w) of sodium alginate sulfate was determined with static light scattering. The light-scattering intensities were measured with a modified commercial light scattering spectrometer (ALV/SP-125, ALV, Germany) equipped with an ALV-5000/E multi-τ digital time correlator and a He–Ne laser (λ = 632.8 nm) in an angular range from 30 to 150° at 10° intervals at 25 °C. The test sodium alginate sulfate solutions were prepared in 0.1 M NaCl aqueous solution, and made optically clean by filtration through 0.22 μm Millipore filters. The specific refractive-index increments (dn/dc) of sodium alginate sulfate in 0.1 M 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.

2.9. In vitro coagulation assay

The anticoagulant activities of the sulfated sodium alginates were investigated by the classical coagulation assays PT, APTT and TT using unfractionated heparin as reference compounds. Before test, different DS, molecular weight and concentration of samples were prepared, respectively.

The assays were carried out according to the instructions of the manufactures. APTT assay was summaries as follows: citrated normal human plasma was mixed with a solution of sodium alginate sulfates and incubated for 3 min at 37 °C, then APTT assay reagent 0.1 ml, pre-incubated for 3 min at 37 °C, was added to the mixture and incubated for 5 min at 37 °C. After that, 0.025 mol/l $CaCl_2$ 0.1 ml, pre-incubated for 3 min at 37 °C, was added and recorded the clotting time. For PT assay, citrated normal plasma was mixed with a solution of sodium alginate sulfates and 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 recorded the clotting time. For TT assay, citrated normal plasma was mixed with a solution of sodium alginate sulfates and incubated for 3 min at 37 °C. Then TT assay reagent 0.2 ml was added to the mixture and recorded the clotting time. TT assay reagent didn't need incubate at 15–25 °C (Jianhong, Yumin, Ronghua, Yunyang, & Tianyu, 2002).

3. Results and discussion

3.1. Structural characterization

3.1.1. FT-IR spectra of the sodium alginate sulfates

Fig. 1 presents the FT-IR spectra of sodium alginate and sodium alginate sulfate with DS = 1.4. The spectra showed two characteristic absorption bands, one is 1249 cm^{-1} describing an asymmetrical S=O stretching vibration and the other at 873 cm^{-1} indicating a symmetrical C–O–S vibration associated to a C–O–SO_3 group (Jianhong et al., 2002). However, these two frequencies were absent in the spectrum of sodium alginate, which proved that the product was sodium alginate sulfate. The degree of substitution was considerably high, so the two peaks (1250, 873 cm^{-1}) were very strong.

3.1.2. ^{13}C NMR spectra of the sodium alginate sulfate

Fig. 2 shows the ^{13}C NMR spectra of sodium alginate and sodium alginate sulfate. From the figure, we can see the signal of carbon atom of sodium alginate derivatives was very complicated, but much useful information could still be found from its ^{13}C NMR. The chemical shift of C-1 and C-6 were at 100 and 176 ppm, respectively, that of C-2–C-5 were between 60 and 80 ppm. The chemical shifts of sodium alginate were complicate, after being sulfates, the ^{13}C NMR spectra became more complicated because the carbons C-2 and C-3 which directly attaching to electronegative sulfate ester groups would shift to lower field position, while C-4 and C-5 were indirectly attaching to sulfate ester groups would shift to higher field position (Gamazade et al., 1997). More than this, the incompletely sulfation of the hydroxyl groups at C-3, C-2 would also make the chemical shift different. So the sodium alginate sulfates had many different types of carbons.

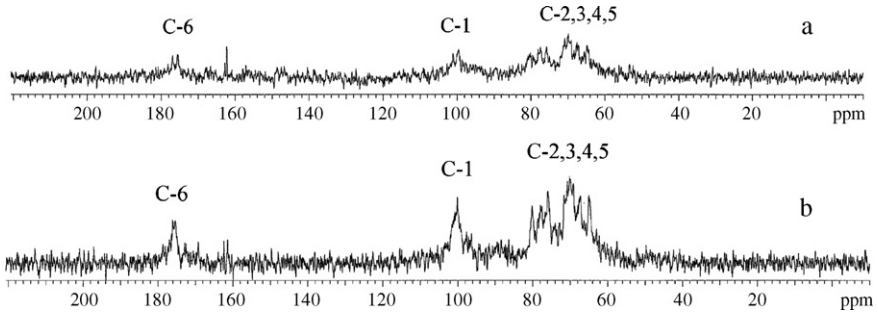


Fig. 2. ^{13}C NMR spectra of sodium alginate (a) and sodium alginate sulfates (b).

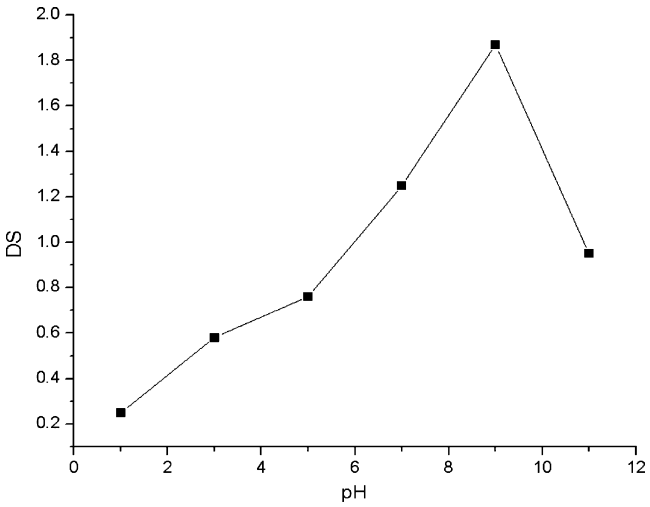


Fig. 3. Effect of pH of synthesizing sodium alginate sulfates on DS.

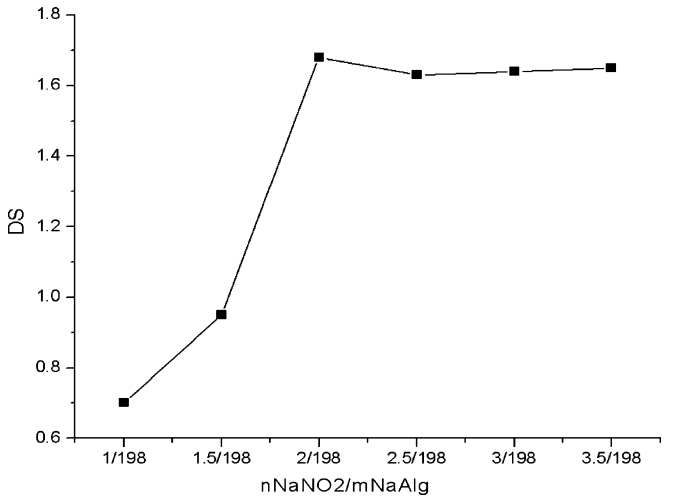
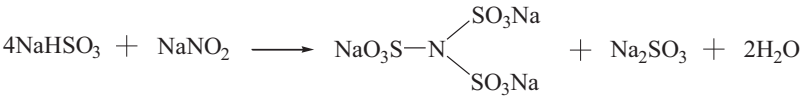


Fig. 4. Effect of the reagent/sodium alginate ratio of synthesizing sodium alginate sulfates on DS.

3.2. Optimization of reaction conditions of preparing sulfating agent

The preparation of the sulfating agent by the reaction of sodium bisulfite and sodium nitrite:



As can be seen from the reaction equation, theoretical molar ratio of sodium bisulfite and sodium nitrite was 4:1, and the reaction was exothermic, when the temperature in the system was higher than 60 °C, the reaction would be very violent. So in order to control the reaction rate, sodium nitrite should be slowly dropping. However, sodium bisulfite would decompose to be sulfur dioxide gas in the heat circumstances. Thus, in the reaction parts of sodium bisulfite which had not reacted with sodium nitrite was decomposed, so the volume of sodium bisulfite will be need more. So the best molar ratio was 4.25:1. And this reaction required a higher temperature to trigger. Considered these two factors, the optimal reaction temperature was 90 °C and the reaction time was for 1.5 h (Dapeng, Mingzhu, Rui, & Yinghui, 2007).

3.3. Optimization of reaction condition of preparing sodium alginate sulfates

3.3.1. Effect of pH of synthesizing sodium alginate sulfate on DS

A maximum DS was obtained at pH 9.0. The relationship between pH and DS of the sodium alginate sulfates was showing in Fig. 3. DS increased from 0.76 to 1.87 when pH rose from 5 to 9; then the DS reduced with a further increase in pH. The maximum DS was obtained at pH 9. There were two factors can be explained for the result. First, the hydroxyl groups in the anhydroglucose units of sodium alginate under alkaline conditions would be activated. The anion at high pH was favorable for sodium alginate to react with the sulfating agent and would obtained high DS. Second, when the pH of sulfation medium was too high or too low, sodium alginate sulfate may be hydrolyzed.

3.3.2. Effect of the reagent/sodium alginate ratio of synthesizing sodium alginate sulfates on DS

The reagent/sodium alginate ratio had a significant influence on the DS of sodium alginate sulfates. The reagent/sodium alginate ratio could be described by the mole ratio of sodium nitrite to the mass of sodium alginate. According to Fig. 4, the DS increased when the ratio rose from 1/198 to 2/198. At higher ratios the DS became constant. From the sulfating reaction equation, the stoichometric proportion of sulfating agent (N $(SO_3Na)_3$) to the hydroxyl groups in the anhydroglucose units was 1:2 and each anhydroglucose unit in sodium alginate contained two hydroxyl groups. Thus, according to the stoichiometric proportion, each molecule of the sulfating agent will react with one anhydroglucose unit. As the molecular weight of anhydroglucose units in sodium alginate was 198, so the theoretic value of n_{NaNO2}/m_{NaAlg} (mol/g) was 1/198 could lead the reaction to completion. However, the sodium nitrate prepared for sulfating agent was less than 1.0 mol. Moreover, the chemical activity of the sulfating agent was moderate, and the activity of N $(SO_3Na)_3$ and HN $(SO_3Na)_2$ would lower with the reducing of $-SO_3Na$ groups. So that HN $(SO_3Na)_2$ would not react completely, only until a dynamic chemical equilibrium was formed. Therefore, the required amount of sodium nitrite would be higher than the theoretical value. However, on the other hand, because of the steric effects and electrostatic repulsion between the sulfate substituents and hydroxyl groups in the sodium alginate sulfates, it was impossible that not reacted hydroxyl could be further sulfation, which led to the sulfating agent dosage reduce (Dapeng et al., 2007). As a combination of both effects, the best condition was 2:198 mol/g.

3.3.3. Effect of the sulfating temperature of synthesizing sodium alginate sulfate on DS

From Fig. 5, the DS increased as the reaction temperature rose from 25 °C to 40 °C and decreased slowly with future increase in temperature. There were two significant effects in this process. First, with the rising of the reaction temperature, the crystalline regions of sodium alginate granules would be destroyed by the swelling behavior, which would become amorphous. At the same time, active reaction centers formed. So increase of the temperature could promote the reaction. On the other hand, the high temperature hampered the reaction because that the sulfation of sodium alginate was an exothermic reaction, which led the chemical equilibrium go to the opposite direction (Dapeng et al., 2007). When the temperature was below 40 °C, the first one was a dominant factor,

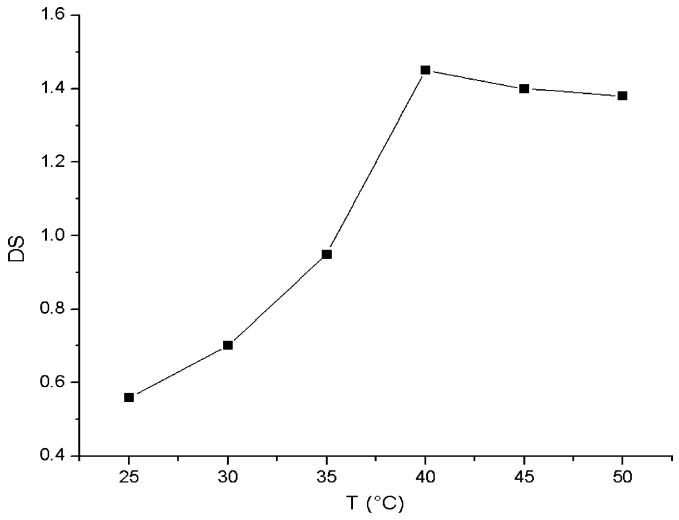


Fig. 5. Effect of the sulfating temperature of synthesizing sodium alginate sulfates on DS.

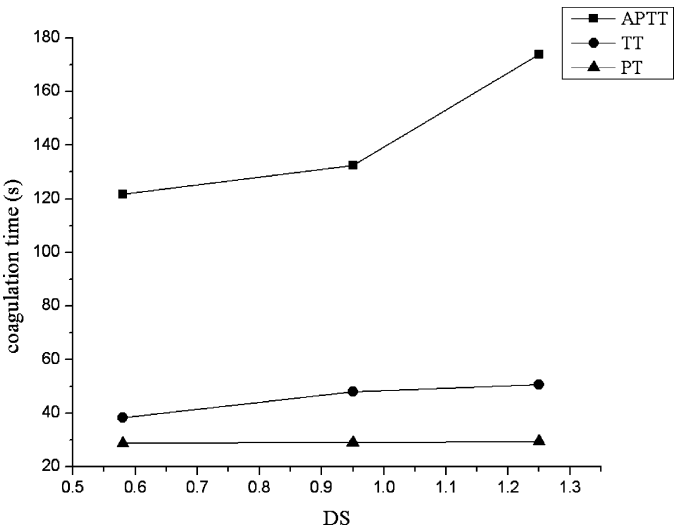


Fig. 7. The curve of DS effect coagulation time.

while the second factor was decisive as the sulfation temperature increases. Therefore, it was reasonable to conclude that optimum temperature was 40 °C.

3.3.4. Effect of the sulfating time of synthesizing sodium alginate sulfate on DS

As can be seen, Fig. 6 shows that the DS increased with reaction time rising from 1 h to 4 h, the DS became constant with a further increase in reaction time. During the sulfating reaction, the crystal regions of sodium alginate particles were destroyed to be amorphous state by high temperature, then the active centers were formed to trigger the reaction took place. However, all these steps required a long time. And because of the low activity of the sulfating agent, the sulfation reaction itself required longer time. However, if the time was too long, the by-products may increase. Therefore, it can be concluded that 4 h was the optimal sulfation time.

3.4. Calculation of molecular weight

In order to study the molecular weight on properties of sodium alginate sulfates, we used oxidation method to degrade the products with different molecular weights while the other parameters were the same for the research. The molecular weight determined by static light scattering. Molecular weights measured were 1.49×10^4, 2.46×10^4 and 3.53×10^4.

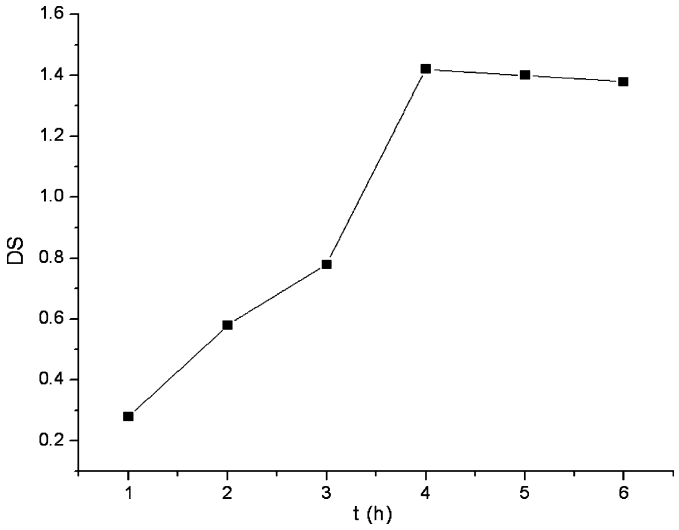


Fig. 6. Effect of the sulfating time of synthesizing sodium alginate sulfates on DS.

3.5. Anticoagulant activity

3.5.1. The effect of DS to sodium alginate sulfates anticoagulant activity

Sulfate group took an important role in anticoagulant activity. DS of sodium alginate sulfates was an important parameter influencing anticoagulant activity. In this work, the DS of 0.58, 0.95 and 1.25 samples were adopted as an anticoagulant, respectively. The experiment results were in Fig. 7.

3.5.2. The effect of molecular weight to sodium alginate sulfates anticoagulant activity

Besides DS, the molecular weight of sodium alginate sulfates was another important parameter influencing anticoagulant activity. Generally, the suitable molecular weight was 2.6×10^4 (Jianhong et al., 2002). In this work, the molecular weight of 1.49×10^4, 2.46×10^4 and 3.53×10^4 samples were adopted as an anticoagulant, respectively. The experiment results were in Fig. 8.

3.5.3. The effect of concentration to sodium alginate sulfates anticoagulant activity

The concentration of the sodium alginate sulfates could have an effect on the blood anticoagulant activitives. In this work, the concentration of 25 μg/ml, 50 μg/ml and 75 μg/ml samples were adopted as an anticoagulant, respectively. The experiment results were in Fig. 9.

3.5.4. The factors of sodium alginate sulfates to anticoagulant activity

In positive 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 (Huang, Du, & Yang, 2003). These data reflected that the anticoagulant activities of the sodium alginate sulfates were weaker than that of heparin. The sodium alginate showed APTT as 27 s at 75 μg/ml, TT as 18 s at 75 μg/ml and PT as 15 s at 75 μg/ml. The normal range of PT was 10–14 s, TT was 10–16 s, APTT was 22–38 s. The clotting time of the plasma added sodium alginate sulfates was more obviously improved. This showed that sodium alginate sulfates had anticoagulant activities.

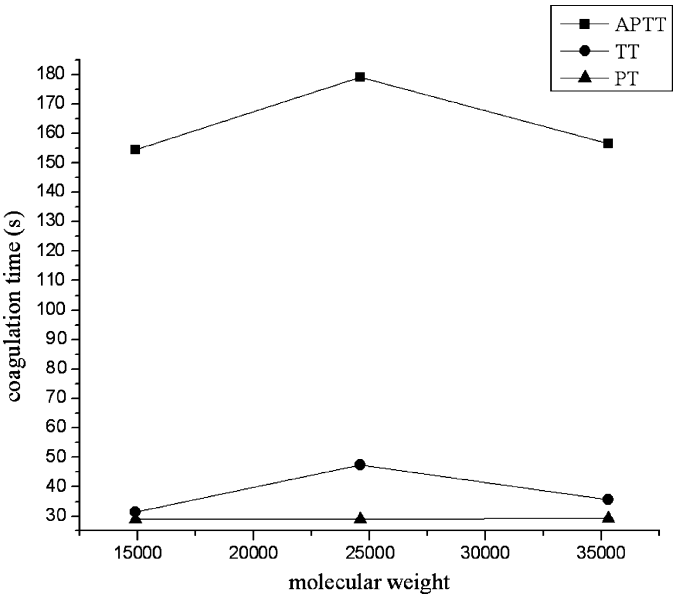


Fig. 8. The curve of molecular weight effect coagulation time.

The anticoagulant activities of sodium alginate sulfates related to the degree of substitution, molecular weight and the concentration. From the three images, anticoagulant activities promoted with the increasing of the DS and concentration. Sodium alginate sulfates can prolong APTT and TT, but it hardly prolonged PT. The PT at different DS, molecular weight and concentration of sodium alginate sulfates had little improvement.

The APTT assay measured the coagulation factors in the intrinsic pathway. The TT assay measured the formation time of fibrin from fibrinogen after the addition of plasma sample, and the PT assay measured the activity of the extrinsic pathway (Kiminori et al., 2001). The sulfate polysaccharide inhibited thrombin (IIa factor) and Xa factor to generate the anticoagulant activity. In this work, the plasma added sodium alginate sulfate decreased the activity of IIa and Xa, so the samples could prolong APTT and TT. APTT and TT were two basic indicator of coagulation activities. The value of APTT and TT showed the anticoagulant activities of sodium alginate sulfates were strong or weak. Summarizing the above analysis, sulfate group took an important role in anticoagulant activities. The same as other heparin, sodium alginate sulfates mainly through

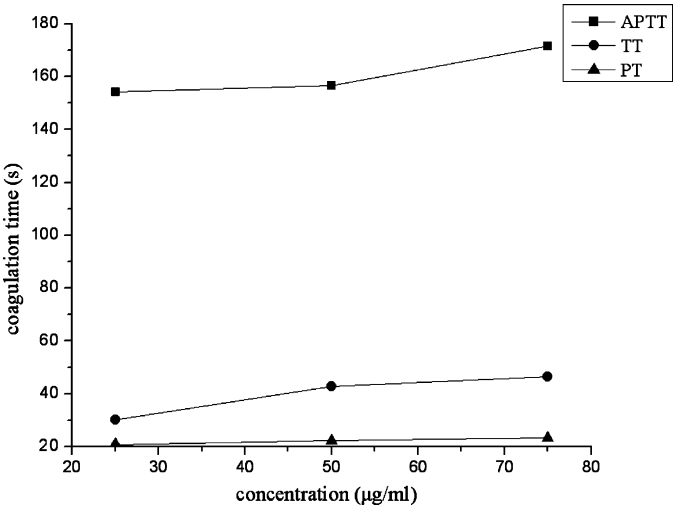


Fig. 9. The curve of concentration effect coagulation time.

the negatively charge of sulfate groups to neutralize the positively charged amino acid residues which in the anti-thrombin to improve the anticoagulant activities. The high DS and concentration could increase the density of negatively to inhibit the activity of IIa and Xa (Roberta et al., 1993). Thus, anticoagulant activity promoted with the increasing of the DS and concentration. From Fig. 8, the clotting time increased as the molecular weight rose from 1.49×10^4 to 2.46×10^4 and decreased slowly with future increase in molecular weight. Vikhovera et al., showed that decreasing molecular weight could result in higher anti-factor Xa activity (Vikhorrva et al., 2005). It may be attributed to what their pharmacokinetic of properties were improved compared to high molecular weight. In general, for sulfate polysaccharide contained sodium alginate sulfate samples, the increase of anticoagulant activities at decreasing the molecular mass was trend, the low molecular weight promoted anticoagulant activities.

4. Conclusion

In this work, sodium alginate sulfates with high DS were prepared in aqueous solution using a similar neutral sulfating agent ($N(SO_3Na)_3$). Their structure was characterized by FTIR and ^{13}C NMR. FTIR spectra showed the characteristic absorptions of sulfate ester bonds at 1249 cm^{-1} and 873 cm^{-1}. This result presented that the hydroxyl groups had been successfully replaced by sulfate groups. The DS of sodium alginate sulfate was measured by the barium sulfate nephelometry method. The molecular weight determined by static light scattering. The anticoagulant activities of sodium alginate sulfates were measured by activated partial thrombosis time (APTT), thrombin time (TT) and prothrombin time (PT). It was showed that the introducing of sulfate groups to hydroxyl groups increased the APTT and TT. The anticoagulant activity related to the degree of substitution, molecular weight and concentration. The high DS and concentration could inhibit the activity of IIa and Xa to prolong APTT and TT. The low molecular weight resulted in higher anti-factor Xa activity to promote anticoagulant activity. Generally, the introducing of sulfate groups could not increase PT, it had little effect on coagulation factors in the extrinsic pathway.

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