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Preparation and in vitro anticoagulant activities of alginate sulfate and its quaterized derivatives

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Abstract

Alginate sulfate (AS) and its ***quaterized derivatives (QAS-1, QAS-2, and QAS-3) were prepared from sodium alginate. Their structure was characterized by elemental analysis, FT-IR, ^{13}C NMR, and gel permeation chromatography. The in vitro coagulation assay of human plasma containing the sulfates indicated that AS had considerably high anticoagulant activity especially to the intrinsic coagulation pathway. Its APTT reached 226 s at 16.7 μg/ml, comparing to that of heparin 125 s at 10 μg/ml. After quaterization the anticoagulant activities of QAS-1, QAS-2, and QAS-3 decreased as the degree of substitution of quaterization increased. The lowest one was given by QAS-3, which gave APTT as 260 s at 66.7 μg/ml, while AS did as 24 min at the same concentration. It could be concluded that AS could be used as a novel anticoagulant drug, and its quaterized derivatives, which contained a great many of sulfate groups but low anticoagulant activity, might have potential application in other field such as anti-HIV.
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Keywords: Alginate sulfate; Quaterization; Anticoagulant activity

1. Introduction

Sodium alginate is a polysaccharide from brown seaweed. It was widely used to produce microspheres, beads, microcapsule and tablets for drug delivery systems and it showed long time effect and reduced side-effects of the drug (Kaneko, Kanada, & Miyagi, 1998). Sodium alginate was also used as artificial skin in healing of split-thickness skin grafte donorsites (Vanstraelen, 1992).

The blood- or tissue-compatibility of sodium alginate was not able to meet the requirement in some cases. In order to increase the compatibility heparin was grafted onto sodium alginate materials (Li & Zhou, 1999). On the other hand sulfated polysaccharides, including natural and synthesized ones, had great blood-compatibility or even anticoagulant activity (Alban, Schauerte, & Franz, 2002). If alginate was sulfated it would also showed high blood compatibility because the structural similarity to that of heparin, which had been widely used for anticoagulant therapy for more than 50 years (Crafoord & Jorpes, 1941). Heparin is a linear polysaccharide with a disaccharide repeating unit containing preponderantly of α-D-glucosamine alternating with α-L-iduronic acid, it also contains sulfate and carboxyl groups (Paulo & Mariana, 1999). Alginate is the only polysaccharide that naturally contains carboxyl groups in each constituent residue (Ikeda, Takemura, & Ono, 2000) and composed of (1–4) linked β-D-mannuronic acid and α-L-guluronic acid (Kennedy, Griffiths, & Atkins, 1984). After sulfated modification it would contain sulfate and carboxyl groups and uronic units like heparin composed. But although sulfated alginate had been prepared earliest in 1962 (Scheweiger, 1967a,b) it had not been used as anticoagulant.

Sulfated polysaccharides were also applied in anti viral therapy such as anti-HIV. On the contrary in this field the anticoagulant activity often played a negative role because of the inducing of bleeding (Bagasra, Whittle, & Heins, 1991). Chemically modification such as desulfation had been used to eliminate the anticoagulant activity (Baumann, Harald, & Bernd, 1998; Nishimura, Kai, & Shinada, 1998).

This paper reported the alginate sulfates (AS) prepared from sodium alginate through reaction with $ClSO_3H$ in formamide. Its anticoagulant activity was measured by activated partial thrombosis time (APTT), thrombin time (TT) and prothrombin time (PT). The derivative greatly prolonged APTT, but seldom influenced TT and PT. In order

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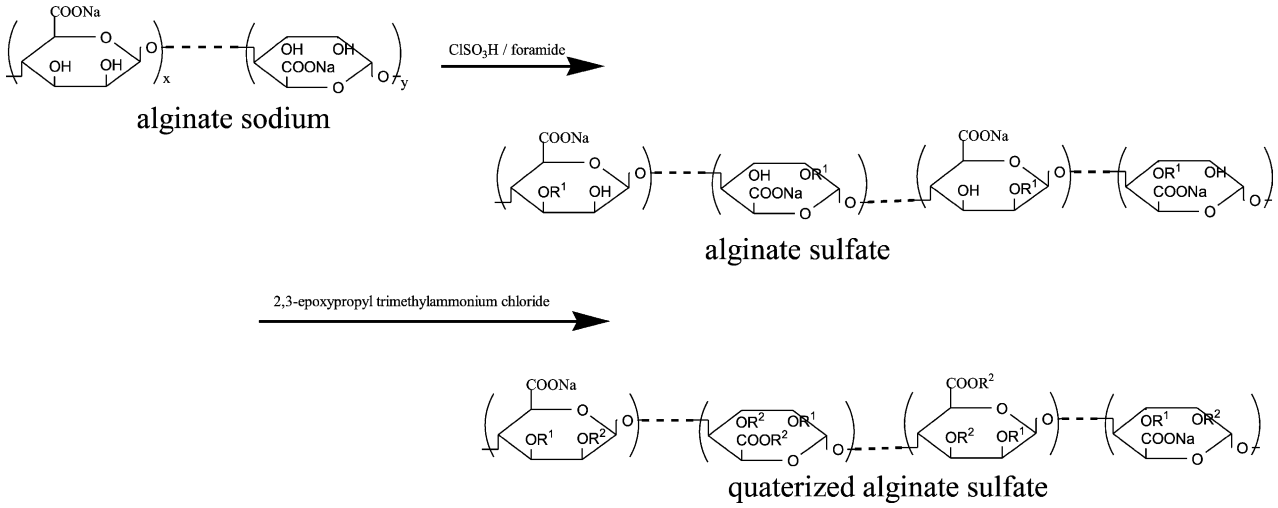

Scheme 1. The synthesis of alginate sulfate and its quaterized derivatives, in which R^1 was SO_3Na and R^2 was $CH_2CH(OH)CH_2N(CH_3)_3Cl$.

to decrease the anticoagulant activity for other application AS was quaterized by 2,3-epoxypropyl trimethylammonium chloride. The results showed that APTT, TT, and PT was successfully decreased comparing to that of AS.

The synthesis of the samples was presented as Scheme 1. The structures of alginate sulfate and its quaterized derivatives were confirmed by elemental analysis, FT-IR, ^{13}C NMR, and gel permeation chromatography (GPC).

2. Experimental

2.1. Materials

Sodium alginate was commercial reagent with M_w 3.2×10^5; 2,3-epoxypropyl trimethylammonium chloride was prepared in the lab; activated partial thromboplastin, prothrombin (ISI 1.22) and thrombin were applied by Shanghai Sun Bio. Corp. Human plasma was bought from Wuhan blood center; $ClSO_3H$, formamide and other reagent were all commercial reagents at analysis grade and used without further purification; dialyzer was equipped with dialysis bag from sigma corp. The permeable M_w was <3000.

2.2. Methods

FT-IR Spectra were measured by FT-IR-8201PC Spectra meter (Shimadzu) with KBr disk; ^{13}C NMR spectra were recorded on a Mercury Vx-300 (Varian, 300 MHz) spectrometer, the solvent was D_2O. Sulfur content (S%) of the samples was measured by SC-132 sulfur meter (LECO), while C%, N%, and H% was measured by Elemental Analyzer-MOD 1106 (Carlo Erba Strumentazione). The average molecular weight (M_w) of samples was measured by GPC. GPC system incorporated a TSP P100 instrument, a TSK G3000-PW column was used, the eluent was 0.1 mol/l NaCl. The flow rate was maintained at 1.0 ml/min, the temperature of the columns was maintained at 30 °C, the eluent was monitored by a RI150 refractive index detector, the sample concentration was ca. 0.4% (w/v). The standards used to calibrate the column were TOSOH pullulan. All data provided by the GPC system were collected and analyzed using the Jiangshen Workstation software package.

2.3. Preparation of alginate derivatives

2.3.1. Alginate sulfate

The sulfation of the sodium alginate was according to the method applied for chitosan (Fang & Jiang, 1998) a simple procedure was summarized as follows: 10 g sodium alginate was added to the sulfating reagent contained 80 ml formamide and 20 ml $ClSO_3H$, the mixture was preserved at 60 °C for 4 h to give a brown solution. 200 ml acetone was added to precipitate the solution, the precipitate was redissolved in distilled water and its pH was adjusted to 10–11 by 0.1 mol/l NaOH, then the solution was dialyzed for 72 h and concentrated to give AS 15 g with S% as 13.21%. C% and H% of AS were 21.05 and 1.93, respectively. The degree of substitution of the sulfate groups was 1.41 per uronic acid calculated according to the ratio of S% to C% given that the carboxyl and sulfate groups formed sodium salt completely.

2.3.2. Quaterization of sodium alginate sulfate

The quaterization of AS was according to the prevailing method (Xu, Lu, & Ding, 1997). 2 g AS was dissolved in 20 ml distilled water and different quantity of 2,3-epoxypropyl trimethylammonium chloride was added. The mixture was maintained at 40 °C for 24 h with stirring, then dialyzed for 72 h, and finally lyophilized to give quaterized alginate sulfate (QAS-1, QAS-2, and QAS-3).

2.4. In vitro coagulation assay

The APTT, PT, TT of AS, QAS-1,QAS-2, and QAS-3 were measured as reported (Lu, Yoshida, & Nakashima, 2000). Before test, different concentrations of samples were added to test system and APTT, TT or PT were recorded. The value of control assay for APTT, TT and PT were 58, 13.88, and 16.13 s, respectively. All the data were the mean ($d = 4$).

3. Results and discussion

3.1. Structural characterization

3.1.1. Elemental analysis of AS and its quaterized derivatives

Table 1 gave the elemental analysis of the samples. When the usage of the epoxy increased, the C%, N% increased clearly. Although the epoxy was not completely reacted with AS, the DS was closely depended on the usage of the epoxy. For this, the content of the quaternary groups could be favorably controlled by the mole ratio of the additional of 2,3-epoxypropyl trimethyl ammonium chloride in the reaction.

3.1.2. FT-IR spectra of the quaterized sodium alginate sulfate

Fig. 1 presents the FT-IR spectra of sodium alginate, AS, QAS-1, QAS-2 and QAS-3. The frequencies at 946, 890 and 820 cm^{-1} of the sodium alginate indicated that the soidum alginate contained guluronic acids and mannuronic acids, respectively (Chandia, Matsuhiro, & Vasquez, 2001). The acid environment in the sulfation would lead to hydrolysis of the sodium alginate (Ikeda et al., 2000). After sulfation these frequencies changed little. According to this it could be concluded that the hydrolysis showed no selectivity on the two different acids. AS, QAS-1, QAS-2, and QAS-3 presented two new peaks at ~1250 and 794 cm^{-1}, which characterized the existence of the sulfate groups (Fang & Jiang, 1998). The degree of sulfation was considerably high, so the two

Table 1
Relation between DS and the usage of epoxy

Sample	Usage of epoxy (g)		S%	C%	N%	H%	DS[b]
	Weight (g)	Mol ratio[a]					
AS	0	0	13.21	21.05	0	1.93	0
QAS-1	1.40	1	11.45	25.22	1.35	3.12	0.38
QAS-2	2.80	2	10.32	27.84	2.21	4.20	0.69
QAS-3	4.20	3	9.77	33.20	2.64	4.56	0.87

[a] Mole ration of 2,3-epoxypropyl trimethylammonium chloride added to the uronic acid unit.
[b] The degree of substitution of of quaternary groups per uronic acid calculated according N%/C%.

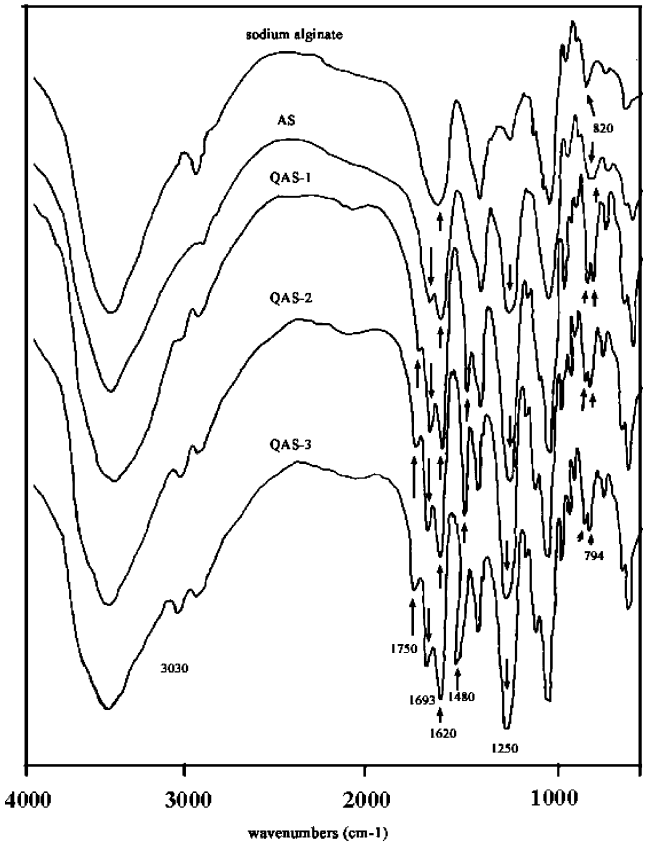

Fig. 1. FT-IR spectra of sodium alginate and its derivatives.

peaks (794, 1250 cm^{-1}) were very strong. After sulfation another new peak at 1694 cm^{-1} also appeared. It might be assigned to the carbonyl groups of –COOH. In the dialysis the final pH was about 6–7, some –COOH might formed. This was very different from that of sodium alginate, in which the carboxyl groups were completely deionized and existed as COO^- so that no stretching vibration of carbonyl groups of COOH could be observed.

The FT-IR spectra of QAS-1, QAS-2 and QAS-3 showed that after quaterization the new peaks at 1489 and 3037 cm^{-1} could be clearly observed. This two frequency could be owing to the stretching vibration and C–H deformation of the methyl groups attached to the N atomic (Xu et al., 1997). The hydroxyl groups and the carboxyl groups of the uronic acid could both attack the epoxy groups, so the quarternized derivatives of AS showed a carbonyl absorption of the ester groups COOR at 1751 cm^{-1}. The existence of the asymmetrical and symmetrical stretching vibration of COO^- (1620 and 1412 cm^{-1}) (Peniche-Covas, Anguelles-Monal, & Davidenko, 1999) and the stretching vibration of COOH (1694 cm^{-1}) showed that some COO^- groups and COOH still existed. It could be seen that when the degree of quaterization increased the strength of 3030, 1751 and 1489 cm^{-1} increased as the FT-IR spectra of QAS-1, QAS-2 and QAS-3 showed.

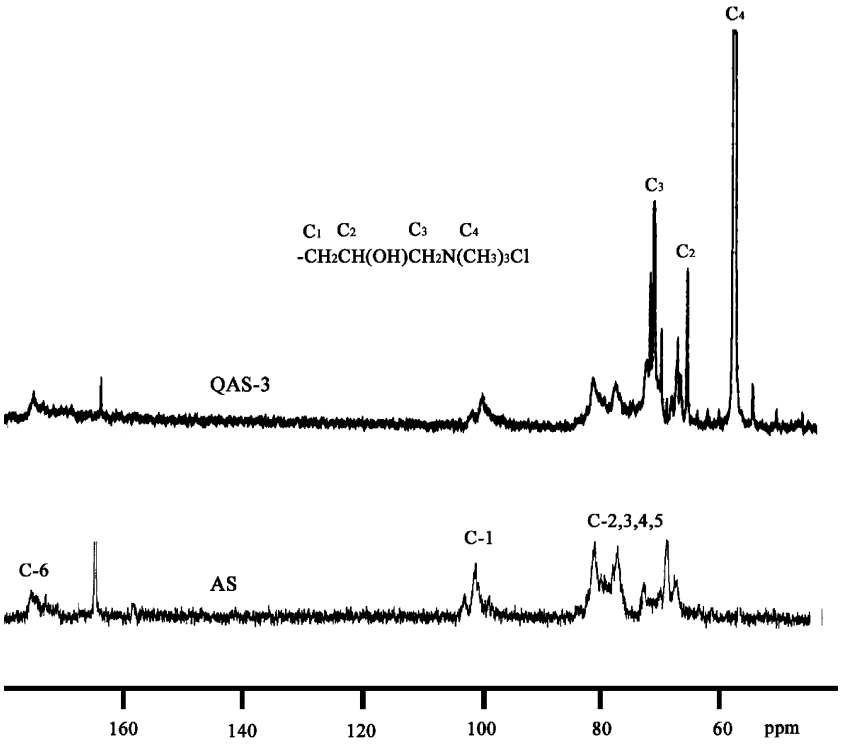

Fig. 2. ^{13}C-NMR spectra of AS and its quaterized derivative QAS-3.

3.1.3. ^{13}C NMR spectra of the sodium alginate sulfate and its quaterized derivatives

Fig. 2 shows the ^{13}C NMR spectra of AS and AAS-3. The chemical shift of C-1 and C-6 were at ~100 and 180 ppm, respectively, that of C-2–C-5 were between 60 and 80 ppm (Ikeda et al., 2000). The carbons in the mannuronic acid and guluronic acid of the sodium alginate had different chemical shifts, their chemical shifts were complicate. After being sulfated, the ^{13}C NMR spectra became more complicated because the carbons directly attaching to electronegative sulfate ester groups, would shift to lower field position, while C-4 and C-5 that were indirectly attaching to sulfate ester groups would shift to higher field position (Gamazade, Sklyar, Nasibov, & Sushkov, 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 sulfate had a great many of types of carbons. Fig. 1 just simply characterized the chemical shift of the uronic acid according to that reported (Ikeda et al., 2000). But the chemical shift of C-6 did not change; it just showed a single peak at 175 ppm. This might mean that the sulfation had not occurred at C-6, which was a carboxyl groups.

The ^{13}C NMR spectra of QAS-3 showed that after quaternization, three new peaks at 54, 65, 68 ppm, could be observed. They could be assigned to carbons of the quaterized groups, respectively, C_2, C_3, and C_4, respectively. The chemical shift of C_1 could not be observed clearly, that of C4 might overlap it (Xu et al., 1997). There are three methyl carbons so 54 ppm peak was much stronger than the other three.

3.1.4. Molecular weight of the derivatives

Table 2 presents the GPC elution volume of the samples. Because the Mark-Houwink equation of pullulan could not be used for alginate sulfate, the M_w of the samples were not given. But it could be seen that the elution volume of AS, QAS-1, QAS-2, and QAS-3 had similar elution volume at about 7.2–7.7 min. This means that in the quaterization procedure no depolymerization occurred. On the contrary after quaterized the M_w increased slightly because the elution time of QAS-1, QAS-2, and QAS-3 were lower than that of AS. This could be attribute to the quaternary groups attached to the uronic acids. The anticoagulant activity of the sulfated polysaccharide was greatly influenced by molecular weight of the samples. From above it could also be concluded that comparison of the anticoagulant activity of the samples would not be affected by M_w because their M_w was similar.

Table 2
M_w of the samples characterized by GPC elution volume

M_w of pullulan ($\times 10^4$)	Elution volume (min)	Samples	Elution volume (min)
4.73	6.02	AS	7.71
2.28	6.53	QAS-1	7.27
1.8	6. 92	QAS-2	7.31
0.59	7.26	QAS-3	7.35

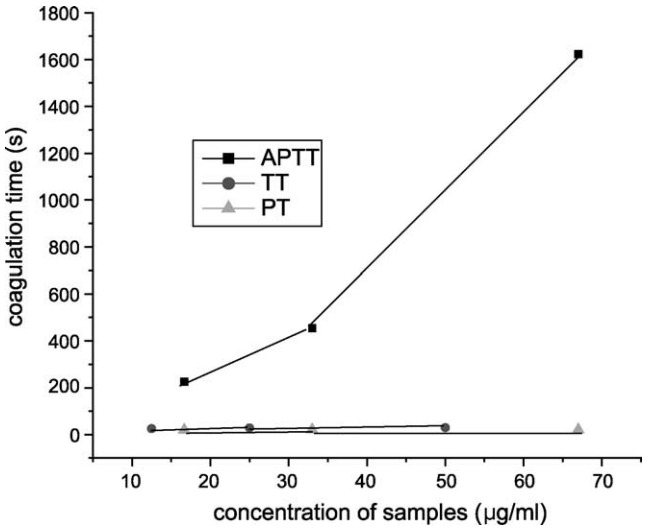

Fig. 3. Anticoagulant activity of AS with respect to APTT, TT and PT.

3.2. Anticoagulant activity

3.2.1. Anticoagulant activity of AS

Coagulation assay gave the result that AS showed a considerably high anticoagulant activity especially that characterized by APTT (Fig. 3). Its APTT reached the level of that of heparin reported (Hirano, Tanaka, & Hasegawa, 1985). It had been reported that heparin showed APTT as 125 s at 10 μg/ml. In this paper AS showed APTT as 226 s at ~17 μg/ml. When the concentration increased to 33 and 67 μg/ml, the APTT of AS reached 455 s and ~24 min. AS could also increase the activity of thrombin with respect to TT. When the concentration increased, the TT also increased from 20 (12.5 μg/ml) to 28 (25 μg/ml) and 30 s (50 μg/ml). But the anticoagulant activity with respect to TT was much lower than that of heparin (110 s at 5 μg/ml), and even lower than that of chitosan sulfate (~80 s at 10 μg/ml). Similar to most of sulfated polysaccharides and differently from heparin, AS could hardly prolong PT. The PT at different concentrations of AS had little improvement. At 12.5, 25 and 50 μg/ml the PT was around 20 s, no increase could be observed comparing to that of the control assay (18 s). Activated partial thrombinplastin was usually an intrinsic coagulation pathway factor, while prothrombin the external coagulation pathway factor (Hirano et al., 1985). From above it could be concluded that AS had great influence on the intrinsic coagulation pathway, and no influence on external coagulation pathway.

3.2.2. Reduction of anticoagulant activity by quaternary ammonium groups

The anticoagulant activity of the sulfated polysaccharides was not a merit in some cases, too high activity was unsuitable for a anticoagulant drug for the side-effect of bleeding (Bergvist, Nillson, & Hendner, 1985). And if used as anti-HIV drug, their anticoagulant activities must be decreased (Bagasra et al., 1991).

In this paper quaternary ammonium groups was introduced into AS to decrease the anticoagulant activity. The activities of QAS-1, QAS-2, and QAS-3 characterized by TT were lower than that of AS (Fig. 4). AS could slightly limited the activity of thrombin with respect to TT as discussed earlier. At every concentration TT of QAS-1, QAS-2, and QAS-3 decreased as the degree of quaterization increased. QAS-3 could hardly show anticoagulant activity, its TT at 12.5, 25 and 50 μg/ml were 18.74, 20 and 19.44 s, respectively (Fig. 4(a)). It could be seen that as the content of the quaternary group increased the activities of the samples decreased obviously. AS showed little activity with respect to PT. QAS-1, QAS-2, and QAS-3 were similar to AS. The increasing concentration of the samples could

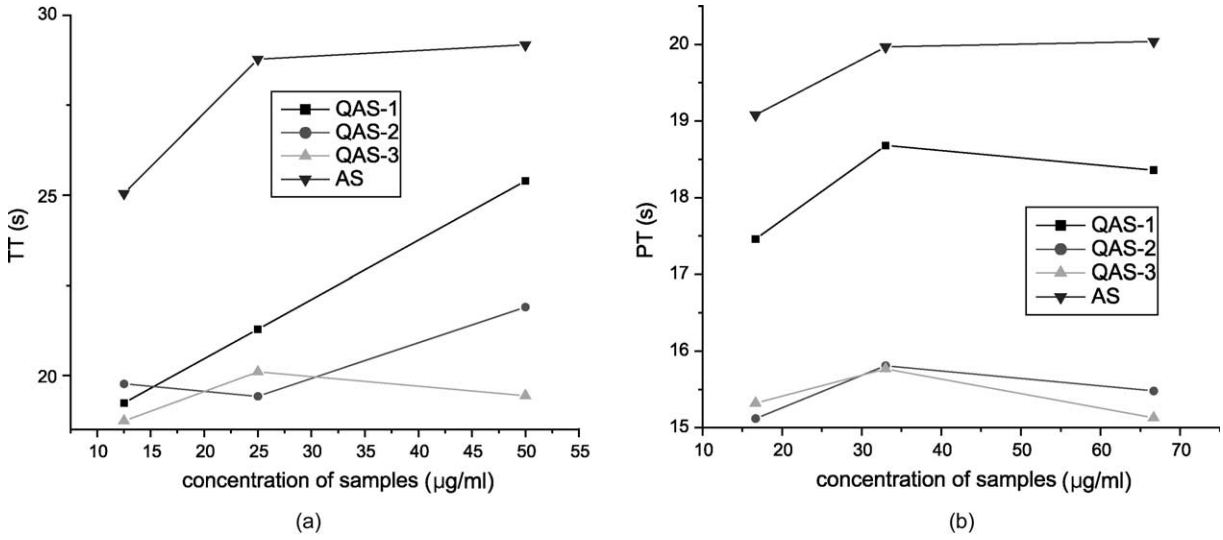

Fig. 4. Anticoagulant activities of the samples with respect to TT (a) and PT (b).

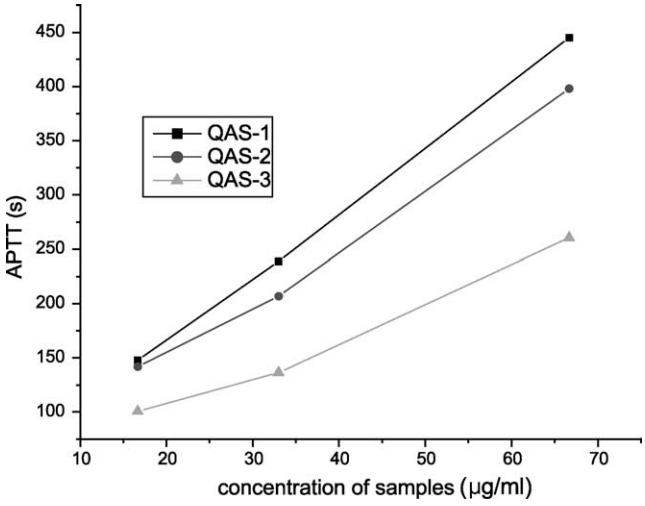

Fig. 5. APTT of quaterized derivatives of AS.

not lead to the increasing PT (Fig. 4(b)). More obvious phenomenon could be observed at the APTT of samples comparing to that of AS. The APTT of QAS-1 at 67 μg/ml (445 s) greatly decreased (AS, 25 min), just near to that of AS at 33 μg/ml, which means its activity was about 50% of AS. QAS-2 and QAS-3 gave APTT as 398 and 260 s at 67 μg/ml, respectively (Fig. 5).

This means that the introducing of the quaternary groups seriously hurt the anticoagulant activity of AS and the hurt was closely dependent on the content of the quaternary groups. The anticoagulant activity of the sulfated polysaccharides was closely dependant on the sulfate groups, the removal of the sulfate groups always deprived of the anticoagulant activity (Nishimura, Nishi, & Tokura, 1986). According the elemental analysis of the samples, the S% of the derivatives decreased as the DS of the quaternary groups increased, so the decrease of the anticoagulant activity was reasonable. It was also reported that the carboxyl groups could augment the anticoagulant activity of the sulfated polysaccharides (Nishimura et al., 1986). The introducing of quaternary groups could form complexes with the carboxyl, so the anticoagulant activity was decreased. A more important factor is that during the quaterization the content of carboxyl groups was reduced because some carboxyl groups reacted with epoxy (discussed by FT-IR spectra), this also decrease the anticoagulant activity.

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References

Alban, S., Schauerte, A., & Franz, G. (2002). Anticoagulant activity polysaccharides: Part I. Synthesis and structure–activity relationships of new pullulan sulfates. *Carbohydrate Polymer*, *47*, 267–276.

Bagasra, O., Whittle, P., Heins, B., et al. (1991). *Journal of Infection Diseases*, *164*, 1082–1090.

Baumann, H., Harald, S., & Bernd, H. (1998). Novel region- and stereoselectives O-6-desulfation of the glucosamine moiety of heparin with *N*-methylpyrrolidinone–water or *N*,*N*-dimethylformamide–water mixtures. *Carbohydrate Research*, *308*, 381–388.

Bergvist, D., Nillson, B., Hendner, U., et al. (1985). *Thrombosis Research*, *38*, 589–601.

Chandia, N. P., Matsuhiro, B., & Vasquez, A. E. (2001). Alginic acids in lessonia trabeculate, characterization by formic acid hydrolysis and FT-IR spectroscopy. *Carbohydrate Polymer*, *46*, 81–87.

Crafoord, C., & Jorpes, E. (1941). Heparin as a prophylactic against thrombosis. *JAMA*, *116*, 2831.

Fang, B., & Jiang, T. (1998). Study on the preparation of hydroxy ethyl chitosan sulfate. *Chinese Journal of Biochemical Pharmaceutics*, *19*, 163–166.

Gamazade, A., Sklyar, A., Nasibov, S., Sushkov, I., et al. (1997). Structural features of sulfated chitosrs. *Carbohydrate Research*, *34*, 113–116.

Hirano, S., Tanaka, Y., Hasegawa, M., et al. (1985). Effect of sulfated derivatives of chitosan on some blood coagulant factors. *Carbohydrate Research*, *137*, 205–215.

Ikeda, A., Takemura, A., & Ono, H. (2000). Preparation of low-molecular weight algimic acid by acid hydrolysis. *Carbohydrate Polymer*, *42*, 421–425.

Kaneko, K., Kanada, K., Miyagi, M., et al. (1998). Formation of water-insoluble gel in drly coaled tablets for the controlled release of the ophylline [J]. *Chemical and Pharmaceutical Bulletin Tokyo*, *46*(2), 728.

Kennedy, J. F., Griffiths, A. J., & Atkins, D. P. (1984). In G. O. Phillips, D. A. Wedlock, & P. D. Williams (Eds.), (*Vol. 2*) (pp. 422*Gums and stabilizers for the food industry*, Oxford: Pergamon Press.

Li, Q., & Zhou, H. (1999). Study on preparation of heparin-immobilized polycvinyl-alcohol alginate and its blood compatibility. *Journal of Jinan University (Natural Science)*, *20*, 73–77.

Lu, R., Yoshida, T., Nakashima, H., et al. (2000). Specific biological activities of Chinese lacquer polysaccharides. *Cardohydrate Polymer*, *43*, 47–54.

Nishimura, S., Kai, H., Shinada, K., et al. (1998). Regioselective synthesis of sulfated polysaccharides: Specific anti-HIV-1 activity of novel chitin sulfates. *Carbohydrate Research*, *306*, 427–433.

Nishimura, S., Nishi, N., & Tokura, S. (1986). Inhibition of the hydrolytic activity of thrombin by chitin heparinoids. *Carbohydrate Research*, *156*, 286–292.

Paulo, A. S. M., & Mariana, S. P. (1999). Searching for alternatives heparin sulfated fucans from marine invertebrates. *TCM*, *9*(8), 227–232.

Peniche-Covas, C., Anguelles-Monal, W., Davidenko, N., et al. (1999). Self-curing membranes of chitosa/PAA IPNs obtained by radical polymerization: Characterization and interpolymer complexation. *Biomaterials*, *20*, 1869–1878.

Scheweiger, R. G. (1967a). Acetylation of alginic acid. ?. Preparation and viscosities of algin acetates. *Journal of Organic Chemistry*, *27*, 1786.

Scheweiger, R. G. (1967b). Acetylation of alginic acid. ??. Reaction of algin acetates with calcium and other divalent ions. *Journal of Organic Chemistry*, *27*, 1789.

Vanstraelen, P. (1992). Comparison of calcium sodium alginate and porcine venograft in the healing of split-thickness skin graft donorsites. *Journal of Burns*, *18*, 145.

Xu, C., Lu, C., & Ding, M. (1997). Synthesis and structural characterization of chitosan derivatives soluble in alcohol. *Techology of Water Treatment (Chinese)*, *23*(5), 266–270.