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Influence of functional groups on the in vitro anticoagulant activity of chitosan sulfate

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Abstract

A new method for the chemical modification of chitosan sulfate was used to prepare *N*-propanoyl-, *N*-hexanoyl- and *N*,*O*-quaternary substituted chitosan sulfate. Structural analysis by elemental analysis, FTIR, ^{13}C NMR, and ^{1}H NMR spectroscopy, and gel-permeation chromatography showed that these methods could conveniently be used for the introduction of functional groups. The influences of the acyl or quaternary groups on the anticoagulant activity of the polysaccharides were studied with respect to activated partial thromboplastin time (APTT) thrombin time (TT), and prothrombin time (PT). The propanoyl and hexanoyl groups increased the APTT activity, and the propanoyl groups also increased the TT anticoagulant activity slightly, while the *N*,*O*-quaternary chitosan sulfate showed only a slight TT coagulant activity.

Keywords: Chitosan sulfate; Anticoagulant activity; Acylation; Quaternization

Sulfated polysaccharides that are either of natural, semisynthetic, or synthetic origin, among them dextran sulfate, lacper polysaccharide sulfate, chitin and chitosan sulfate, carrageenan, and others, have shown multiple biological activities.[1–7] Anticoagulant activity, one of the most attractive activities, had been widely studied.[8,9] The structure–activity relationships showed that, in general, the presence of sulfate groups is an essential requirement for the anticoagulant activity. In some cases increasing the degree of sulfation was beneficial to the activity,[10–12] e.g., the anticoagulant activity with respect to thrombin time (TT) of pullulan sulfate dramatically increased up to a level similar to heparin when the DS of the sulfate groups ranged from 0.47–1.80 per glucose unit.[10] Other structural parameters such as the position of the sulfate groups, molecular weight, configuration and conformation of the uronic acid, the backbone structure of the polysaccharide also add to the anticoagulant activity.[10,12–14] The arrangement of the sulfate groups also interfered with the coagulation process, especially in the thrombin-mediated fibrin formation characterized by TT.[10] In case of chitosan sulfate, the existence of a C-6 sulfate group is a key requirement, as desulfation at C-6 always leads to lack of anticoagulant activity.[15]

Furthermore, chemical modification of sulfated polysaccharides has also attracted great interest because it enhances the structural similarity to that of heparin, a drug that has been used clinically for more than 50 years.[16] It is generally accepted that the anticoagulant activity of the sulfated polysaccharides partly results from the strong interaction between the negatively charged sulfate groups and some positively charged peptidic sequences.[17] In addition, negatively charged carboxyl groups have been introduced into the sulfated polysaccharide to further increase the anticoagulant activity.[15,18,19] Other chemical modifications such as hydrolysis,[20] benzylamination,[6] have also been applied to improve the anticoagulant activity.

In case of chitin and chitosan, Nishimura and coworkers have for sulfated carboxymethyl chitin demonstrated that the cooperative effect of the carboxyl and sulfate groups could increase the anticoagulant activity toward bovine fibrinogen to the level expressed by heparin.[21] *N*-Acetyl groups could also possibly improve

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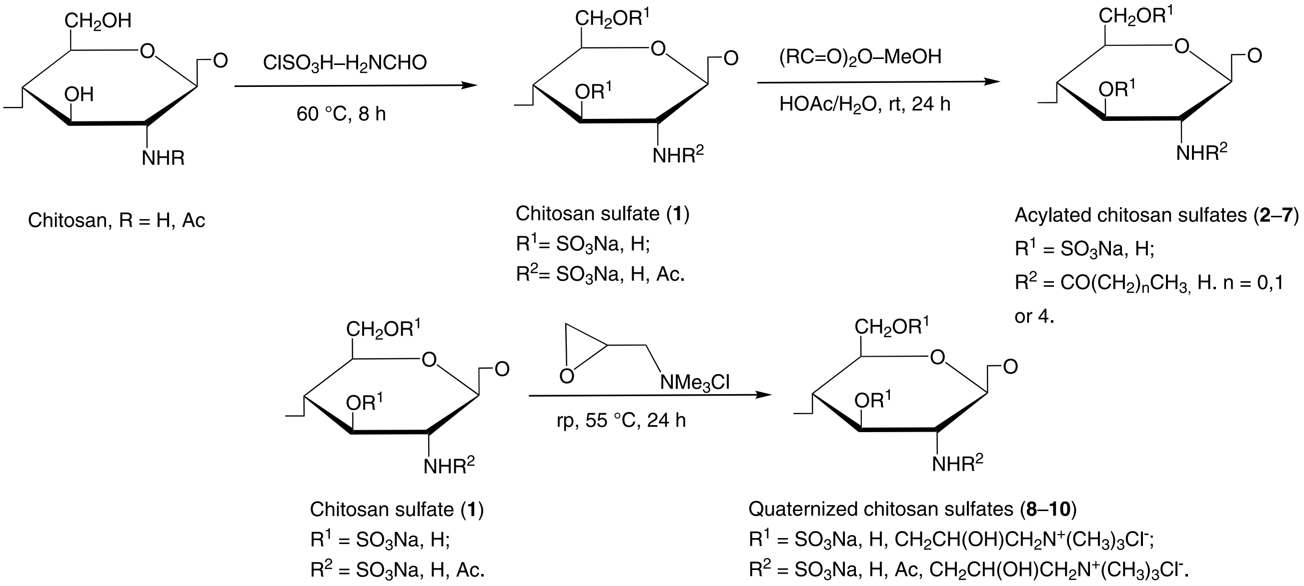
the anticoagulant activity with respect to activated partial thrombinplastin time (APTT) and TT, but the effects of the *N*-acetyl content have not been carefully studied.[22] Therefore, in this paper two types of hydrophobic groups were introduced onto the amino groups of the chitosan sulfate (**1**) to obtain *N*-propanoyl chitosan sulfate (**2**, **3** and **4**) and *N*-hexanoyl chitosan sulfate (**5**, **6**, and **7**). Another reason for the introduction of acyl groups was to improve the hydrophobicity of the chitosan sulfate, which had been confirmed to enhance anticoagulant activity in dextran sulfate.[6] In addition, quaternary chitosan sulfates (**8**, **9**, and **10**) were also studied to verify that the anticoagulant activity of the sulfates was largely dependent on the density of negative groups.[17] The synthesis of chitosan sulfate and its derivatives is presented in Scheme 1.

The structure of the derivatives was fully characterized by elemental analysis, FTIR, ^{13}C, and ^{1}H NMR spectroscopy, and gel-permeation chromatography (GPC). As is shown in Table 1, the degrees of substitution of the acylation (N-hexanoylation and N-propanoylation) or quaternization could be favorably



Scheme 1.

Table 1
Elemental analysis, degree of substitution, and elution volume of chitosan sulfate (**1**) and its derivatives

Sample	Molar ratio [a]	Elemental analysis (wt%)				Degree of substitution [b]	M_v ($\times 10^3$ g mol^{-1}) [c]
		S%	C%	N%	H%		
1		10.90	32.20	4.50	5.93		6.87
2	0.5:2	10.33	32.24	5.67	4.65	0.21	5.21
3	1.5:2	9.60	34.79	5.22	4.91	0.59	5.31
4	3:2	9.13	36.08	4.99	4.71	0.81	5.95
5	0.5:2	10.15	33.63	5.54	4.90	0.18	5.89
6	1.5:2	8.95	38.40	4.88	5.52	0.53	5.35
7	2.5:2	8.30	40.83	4.54	5.85	0.75	6.57
8	0.5:1	9.20	32.74	6.37	5.77	0.22	7.55
9	2:1	8.02	35.21	6.85	5.89	0.57	7.63
10	3:1	7.44	36.01	7.00	6.03	0.72	7.90

[a] Molar ratio of functional reagents (propanoic anhydride, caproic anhydride or 2,3-epoxypropyltrimethyl ammonium chloride) to glucosamine unit.

[b] Substitution degree of N-hexanoylation, N-propanoylation, or quaternization calculated according to the C/N wt% from the elemental analysis.

[c] The M_v of standard pullulan samples for calibration were 4.73×10^4, 2.28×10^4, 1.8×10^4, 0.59×10^4. The Mark–Houwink equation of pullulan[23] and the samples[4] for universal calibration were $[\eta]=0.0185M_v^{0.67}$ and $[\eta]=1.75\times10^{-5}M_v^{0.98}$, respectively.

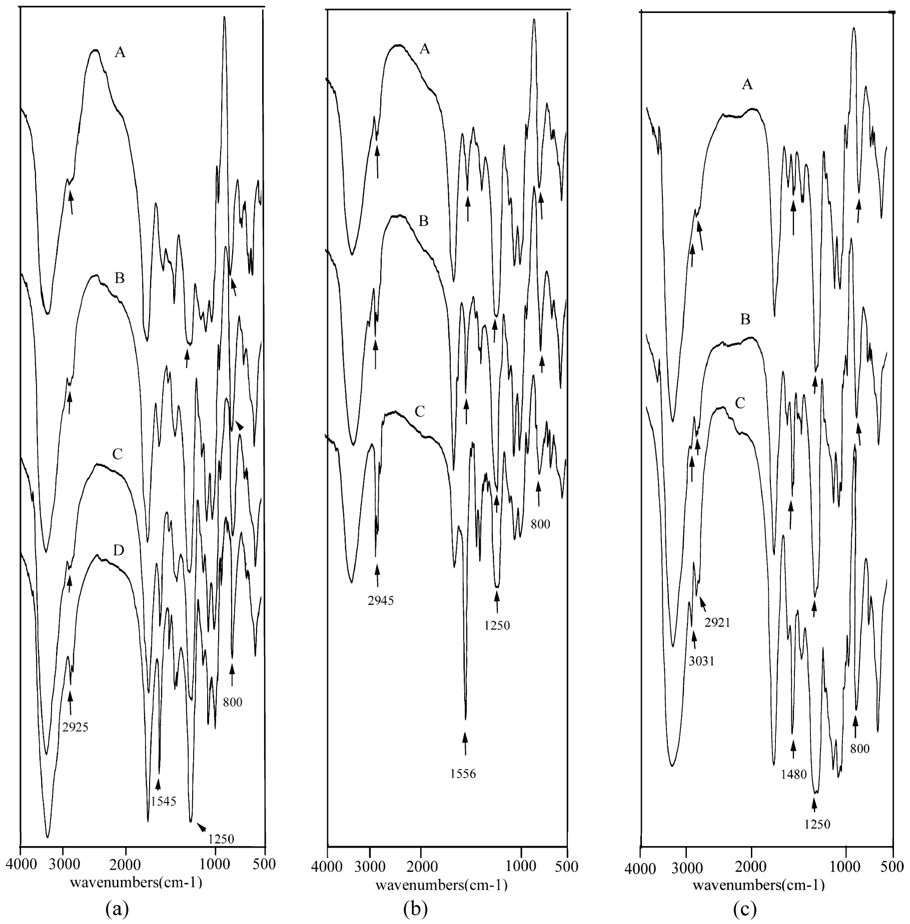


Fig. 1. FTIR spectra of chitosan sulfate and its derivatives. (a) chitosan sulfate **1**, A; and *N*-propanoylchitosan sulfate **2**, B; **3**, C; **4**, D. (b) *N*-hexanoylchitosan sulfate with substitution degree **5**, A; **6**, B; **7**, C. (c) Quaternized chitosan sulfate with substitution degree **8**, A; **9**, B; **10**, C.

controlled by the molar ratio of additional functional reagents such as the acid anhydride and 2,3-epoxypropyl trimethylammonium chloride relative to the glucosamine units. Although the reagents did not react completely with the chitosan sulfates, a higher molar ratio always led to a higher degree of substitution. The prevailing method of introducing functional groups to chitosan sulfate was carried out by: (1) modification of chitosan; and (2) sulfation.[21,22] The disadvantage of it was that functional groups might be removed in the strongly acidic medium of the sulfation reaction as observed in our experiments. Therefore, we adopted a different method for introducing functional groups, which was: (1) synthesis of chitosan sulfate; and (2) acylation or quaternization in homogeneous systems. The results indicated that the synthetic process was a nearly ideal and convenient method for introducing functional groups into chitosan sulfate. Although the S wt% decreased when the degree of substitution increased, the numbers of sulfate groups did not change because all of the acylated or quaternized derivatives originated from the chitosan sulfate (**1**). The elemental analysis data of N/S wt% also confirmed this (data not shown).

The molecular weight of the chitosan sulfate plays an important role in the anticoagulant activity.[21,22] As the universally calibrated M_vs[4,23]of the acylated and quaternized derivatives were similar to that of the original chitosan sulfate (Table 1), the comparison of their anticoagulant activity would be little affected by molecular weight[21,22] because the M_vs of all samples were between 5×10^3 and 8×10^3.

The sulfate groups attached to the glucosamine unit could be favorably confirmed by FTIR spectra (~ 1250 cm^{-1} (ν(S=O)) and ~ 810 cm^{-1} (ν(C–O–S))[21,22,24,25] shown in Fig. 1(a)). After acylation, new peaks at ~ 1560 cm^{-1} ($\delta(NH_2)$, amide II[24]), 1465 cm^{-1}, 1416 cm^{-1} ($\delta(CH_2)$) and 2920–2970 cm^{-1} (ν(CH))[26]could be easily observed (Fig. 1(a and b). From the increasing intensities it could be concluded that the acyl groups were successfully introduced with different degrees of substitution. The acylation reaction occurred selectively on the *N*-position because no stretching vibration for ester groups ($\sim 1700-1800$ cm^{-1})[24,26] could be observed. *N*-hexanoyl chitosan sulfates resulted in more carbons in the acyl groups, so its ~2920–2980 cm^{-1} (ν(CH))[26] peaks were sharper and stronger. The appearance of new peaks at 1490 cm^{-1} (δ(C–H) of $-NCH_3$) and 3030 cm^{-1} (ν(C–H) of $-NCH_3$) in the FTIR spectra of the quaternized chitosan sulfates also confirmed the existence of the ammonium groups Fig. 1(c).[27]

The ^{13}C NMR spectra indicated that the C-6 and C-2 hydroxyl groups were both partly sulfated because of the existence of sulfated and unsulfated signals (C-6, δ 66 and 61; C-2, δ 53 and 54)[24,25] (Fig. 2). The signal of sulfated C-3 (δ 77) was not obvious, but the signal for unsulfated C-3 (δ 72)[24,25] had considerable intensity (Fig. 2). So the degree of sulfation on the C-3 hydroxyl groups should be very low, assuming that the sulfation had occurred on this position. After acylation, the signals of the carbonyl groups of the amide groups (δ 184), and that of the saturated carbons of the acyl groups were all significantly detected as was shown in (Fig. 2).[24,26] After quaternization, the signals assigned to the ammonium groups (δ 54, 62 and 67)[27] were also detected. In contrast to the acylated chitosan sulfate, no signals assigned to the carbonyl groups appeared in quaternized chitosan sulfate. The introduction of the functional groups could also be confirmed by ^{1}H NMR spectroscopy (Fig. 3) with $CHCl_3$ as standard. In comparison with chitosan sulfate, the acylated derivatives showed signals corresponding to the *N*-propanoyl groups (δ 1.0 and 2.1), and the *N*-hexanoyl groups (δ 0.7, 1.2, 1.4 and 2.0).[26] The quaternized derivatives also showed the signals corresponding to $-N(CH_3)_3$[27] (δ 3.1).

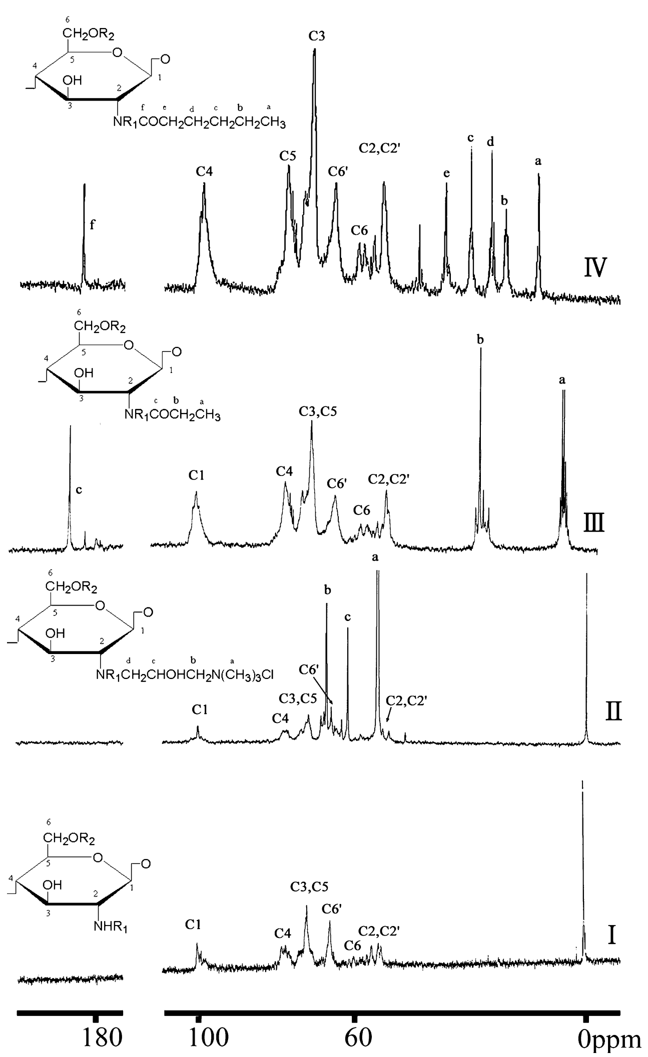


Fig. 2. ^{13}C NMR spectra of chitosan sulfate and its derivatives. I chitosan sulfate (**1**); II quaternized chitosan sulfate (**10**); III *N*-propanoyl chitosan sulfate (**4**); IV *N*-hexanoyl chitosan sulfate **7**. C-2′, C-3′ and C-6′ were the sulfated carbons, compared with the unsulfated carbons, C-2, C-3, and C-6.

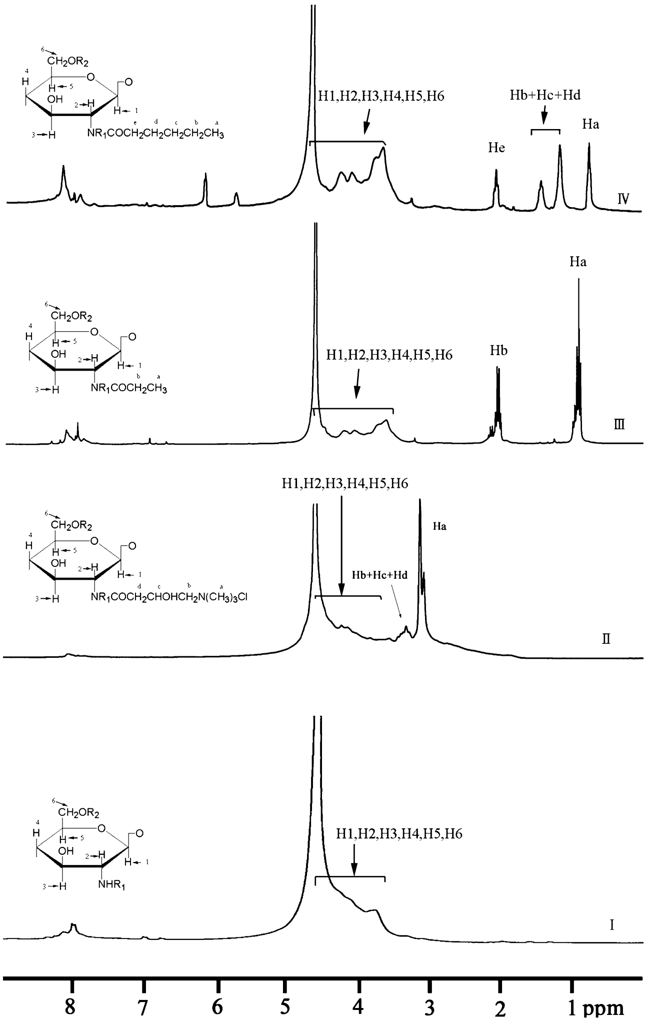


Fig. 3. ^{1}H NMR spectra of chitosan sulfate and its derivatives. I chitosan sulfate (**1**); II quaternized chitosan sulfate (**10**); III *N*-propanoylchitosan sulfate (**4**); IV *N*-hexanoylchitosan sulfate **7**.

The anticoagulant activities of **1**–**10** were determined by in vitro activated APTT, TT, and prothrombin time

(PT) assays that characterize different stages of the coagulation process. Chitosan sulfate usually interferes with the intrinsic coagulation process and the last coagulation step, not with the extrinsic process, which is represented by APTT, TT and PT.[21,22] In this paper **1** prolonged the APTT to ~100 s, but TT was seldom prolonged. (Table 2) at 66.7 μg/mL. The S wt% of the chitosan sulfate closely related to the anticoagulant activity. As Hirano and co-workers have reported that chitosan sulfate (S% 18.7%) could increase both APTT and TT,[22] the small increase of TT might be attributed to the low S wt% (10.90%) of **1**. PT was seldom influenced by chitosan sulfate, which agreed with the published data.[21,22]

The acyl groups could significantly increase anticoagulant activity, which was especially characterized by APTT (Table 2). When the degree of acylation increased, APTT increased up to 150.65 s (**4**), while the APTT of the **1** was just 99.61 s at the same concentration, 66.7 μg/mL. The enhancement in anticoagulant activity might be due to the fact that the introduction of acyl groups decreased the positive charge density of the amino groups. The anticoagulant activity of chitosan sulfate largely results from its high level of negative charge density produced by the sulfate groups.[6] The attachment of carbonyl groups via the acyl groups could neutralize the amino groups electronically, which are positively charged at about pH 7.0, so the anticoagulant activity is increased. In contrast to APTT, the TT of *N*-hexanoyl and *N*-propanoyl chitosan sulfates showed some discrepancies in that the TT of *N*-propanoyl chitosan sulfate reached up to 26.99 s (**7**), while that of *N*-hexanoyl chitosan sulfates was just 17.83 s at the same concentration 66.7 μg/mL. This might be due to the arrangement of the sulfate groups. The orderly arrangement of the sulfate groups was more beneficial to TT anticoagulant activity of the sulfated polysaccharides in comparison to that of APTT.[4] The hexanoyl groups would seriously disrupt the arrangement of the molecules.[26] Because the propanoyl group has fewer carbon atoms, its disruption would be less serious when introduced into chitosan sulfate. Similarly to chitosan sulfate, the acylated derivatives showed no PT anticoagulant activity as presented in Table 2, it indicated that the mechanism of anticoagulant activity of the acylated derivatives was similar to that of chitosan sulfate.

Although quaternized chitosan sulfates showed APTT activities similar to chitosan sulfates, it showed slight coagulant activity with respect to TT (Table 2). When compared to acylation, the quaternization did not increase the APTT anticoagulant activity but decreased the TT anticoagulant activity. This effect might be attributed to the positive charge of the quaternary ammonium groups that could decrease the density of negative charge.[6] The appearance of TT coagulant activity of quarternized chitosan sulfate (especially that of **10**) means that TT activity was more sensitive to the structure of the polymers as reported by Alban and co-workers.[14]

It could be concluded that the anticoagulant activity of the chitosan sulfate is indeed improved by the *N*-acyl groups, especially in APTT activities. TT activities could be also enhanced if the acyl groups did not seriously disturb the regular arrangement of the polysaccharides. As anticipated, the quaternary groups decrease the anticoagulant activity. The influences of these groups might be attributed to the change in the

Table 2
APTT, TT, and PT of normal human platelet-poor plasma containing chitosan sulfate (**1**) and its acylated derivatives

Samples	APTT (s) at different concentrations (μg mL^{-1})			TT (s) at different concentrations (μg mL^{-1})			PT (INR) at different concentrations (μg mL^{-1})		
	16.67	33	66.7	16.67	33	66.7	16.67	33	66.7
1	59.75	75.96	99.61	14.58	13.41	14.56	1.08	1.00	0.97
2	91.91	93.87	106.99	14.07	17.99	18.08	0.97	0.88	0.96
3	98.99	111.06	128.73	15.63	17.96	20.39	0.95	0.98	0.90
4	96.27	123.29	138.99	15.63	18.09	26.99	1.02	1.00	0.94
5	81.37	91.40	100	16.18	15.57	16.51	1.11	1.00	0.93
6	92.96	113.04	122.24	15.67	15.74	16.21	0.94	0.99	0.95
7	95.45	139.37	150.65	15.15	17.37	17.83	0.96	0.99	1.02
8	65.07	74.58	90.86	13.87	13.22	13.11	0.92	1.02	1.05
9	70.43	86.40	99.40	11.72	11.78	11.25	1.00	0.99	1.03
10	60.78	88.64	96.92	12.14	11.27	9.60	0.95	0.98	0.94
Control assay		86.62			14.46			1.00	

Each value represents the mean of four parallel assays that the discrepancies among their clotting times were less than 1 s, the PT in seconds was 15.58 s.

negative charge density and the arrangement of chitosan sulfate.

1. Experimental

1.1. Materials

Chitosan was bought from Yuhuan Sea Biochem. Corp. (Zhejiang, China) with degree of acetylation 0.15 (measured by pH titration method[28]) and M_v 3.2×10^5 (measured by a viscometric method[29]). Activated partial thromboplastin, prothrombin (ISI 1.22) and thrombin were commercial reagents from Shanghai. Sun Biochemical Corp. Normal human platelet-poor plasma was bought from Wuhan Blood Center, Wuhan, China. Chlorosulfonic acid ($ClSO_3H$), *n*-caproic anhydride, and *n*-propanoic anhydride were all commercial reagents of analytical grade and used without further purification. 2,3-Epoxypropyl trimethylammonium chloride was prepared in our laboratory. Permeation film (molecular weight cutoff, 3000) was bought from Sigma Chemical Co. The standards used to calibrate the column in GPC were pullulan from TOSOH, Japan.

1.2. General methods

FTIR spectra were measured by a spectrum one B Spectra Meter (Perkin–Elmer) with a KBr disk. ^{13}C and ^{1}H NMR spectra were recorded on a Mercury Vx-300 (Varian, 300 MHz) spectrometer in D_2O. Sulfur (S%) was measured with an SC-132 Sulfur Meter (LECO), and C, H, and N was measured by Elemental Analyzer-MOD 1106 (Carlo Erba Strumentazione). A 320-S pH meter (Metler) was used to follow the pH titration. The M_v of chitosan sulfate and its derivatives was measured by GPC, which incorporated a TSP P100 instrument, a TSK G3000-PW column, a RI 150 refractive index detector with 0.1 M aq NaCl eluent flowing at 1.0 mL/min at 30 °C. The sample concentration was approx 0.4% (w/v). The Mark–Houwink equations to calibrated M_v were $[\eta] = 1.75 \times 10^{-5}\ M_v^{0.98}$ (0.1 M NaCl, 25 °C) for samples **1**–**10**,[4] and $[\eta] = 0.0185 M_v^{0.67}$ for pullulan standards.[23]

1.3. Chitosan sulfate (1)[30]

Chitosan (15 g) was added to a sulfating reagent consisting of formamide (150 mL) and $ClSO_3H$ (100 mL). The mixture was stirred at 80–90 °C for 4 h, and C_3H_6O (500 mL) was added to precipitate the product. The precipitate was redissolved in distilled water, and the pH was adjusted to pH 10–11. The solution was dialyzed for 24 h and concentrated to give chitosan sulfate (12.3 g) with S wt% as 10.90%, which is a degree of sulfation of 0.8 per glucosamine unit calculated from the elemental analysis of S/C shown in Table 1.

1.4. *N*-Acylated chitosan sulfates (2–7)[31]

Chitosan sulfate (**1**, 2.34 g, ~0.01 mol glucosamine unit) was dissolved in 10% aq HOAc (10 mL), and MeOH (15 mL) was added while stirring. Caproic anhydride (for **2**–**4**) or propanoic anhydride (for **5**–**7**) was added at room temperature in 1 h with different molar ratios to glucosamine units (see Table 1). The mixture was stirred for 4 h, and the pH was adjusted to pH 9.0–10.0 by addition of 1 M NaOH. Subsequently the solution was dialyzed for 24 h, and then lyophilized to yield acylated chitosan sulfates (0.9–1.2 g) as yellow powders.

1.5. Quaternized chitosan sulfates (8–10)[27]

Chitosan sulfate (**1**, 2.34 g, ~0.01 mol glucosamine unit) was dissolved in distilled water (20 mL), and 2,3-epoxypropyltrimethylammonium chloride was added with different molar ratios to glucosamine units (see Table 1). The mixture was stirred at 40 °C for 24 h, then dialyzed for 72 h, and finally lyophilized to yield quaternized chitosan sulfates (1.2–1.5 g) as yellow powers.

1.6. In vitro coagulation assay

The anticoagulant activities of **1**–**10** were determined by activated APTT, TT, and PT,[7] measured according to the methods provided by the biological reagents provider, Sun Biochemical Corp. Before assay, chitosan sulfate or its derivatives were added to the platelet-poor human plasma in different concentrations. The APTT, TT or PT at various sample concentrations were measured by comparing to the results of control assays in which neither chitosan sulfate nor its derivatives were added. In this paper all the data were the mean of four parallel assays with discrepancies among them less than 1 s.

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