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Preparation and anticoagulant activity of carboxybutyrylated hydroxyethyl chitosan sulfates

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Abstract

A new method of introduction carboxyl groups to chitosan sulfate by the acylation reaction between hydroxyethyl chitosan sulfates and butane dioic anhydride in homogeneous solution was used to obtain carboxybutyrylated hydroxyethyl chitosan sulfates. The structures of the derivatives were characterized by element analysis, FT-IR, ^{13}C-NMR, and gel permeation chromatography. The content and position of the carboxyl groups could be controlled favorably. Their anticoagulant activity was determined for human plasma with respect to activated partial thromboplastin time (APTT), thrombin time (TT), and prothombin time (PT). The introducing of carboxyl groups to amino groups greatly prolonged the APTT and TT. The best result occurred when the degree of substitution of the carboxyl groups was about 0.4/unit that prolonged APTT and TT with about 5 and 1.5 times compared to that of the uncarboxylated hydroxyethyl chitosan sulfates; another conclusion is that introducing of carboxyl groups into *N*,*O*-position gave better results than that just into *N*-positions. Low S% chitosan sulfate and 6-*O*-desulfated chitosan sulfate showed little anticoagulant activity but their *N*,*O*-carboxybutyrylated derivatives (0.6/unit ds) showed increased APTT or TT, while their *N*-carboxybutyrylated derivatives (0.6/unit ds) gave no improvement. Generally, the introducing of carboxyl groups could not increase PT in spite of the position introduced.

Keywords: Chitosan; Chitosan sulfate; Carboxybutyrylation; Anticoagulant activity

1. Introduction

Chitosan sulfates have obvious anticoagulant activity and the introduction of carboxyl groups could increase the activity because of better structural similarity to heparin. It was reported that carboxymethyl chitosan sulfate showed greater inhibition on the transformation of fibrinogen to fibrin than chitosan sulfate (Nishimura, Nish, & Tokura, 1986). But Hirano et al. reported that *O*-carboxylated chitosan sulfate gave poor activated partial thromboplastin time (APTT) and thrombin time (TT) delaying effect, while better inhibition on the complexation of thrombin and AT-III. Generally, C_6 sulfate groups was a pre-requisite for anticoagulant activity (Hirano et al., 1985; Nishimura et al., 1998). But Horton and Just reported a complete 6-*O*-carboxylated and *N*-sulfated chitosan derivative showed anticoagulant activity with 25.8 Iu/mg (Horton & Just, 1973). This means that the effect of carboxyl group on the anticoagulant activity of the chitosan sulfate was not so simple but regretfully no report presented a systematic study to clarify it. More than this, the previous method of introducing carboxyl into pyranose units includes carboxymethylation and oxidation reaction, which were both in heterogeneous system and could not control the reaction region and content easily.

The paper reported a profitable method to introducing carboxyl groups into the pyranose units. It was by the acylation reaction between butane dioic anhydride and hydroxyethyl chitosan sulfate in homogeneous system. The structure characterization by FT-IR, ^{13}C-NMR and gel permeation chromatography (GPC) showed that the content and position of the carboxyl groups could be easily controlled and no depolymerization was observed. Their anticoagulant activities were systematically studied by measuring the APTT, TT and prothombin time (PT) for human plasma. The APTT and TT reached the values reported by Nishimura et al. (1986). The effect of content and position of the carboxyl group on the anticoagulant activity of the chitosan sulfate was carefully studied. It

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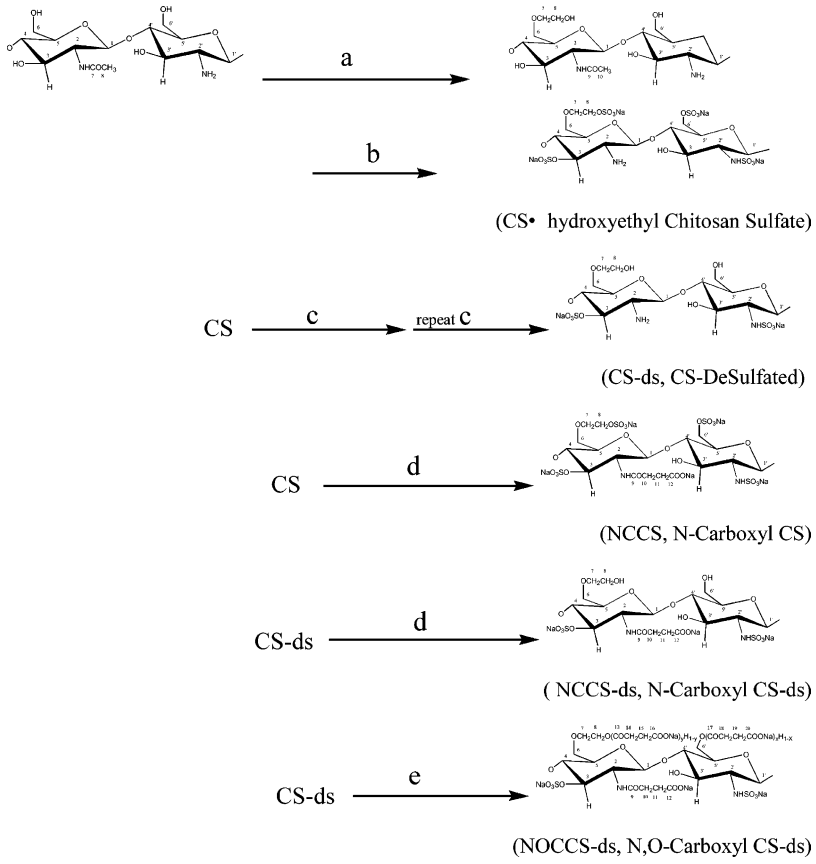

Scheme 1. Synthesis of chitosan derivatives. (a) $ClCH_2CH_2OH$, in NaOH/iso-propanol at 90–100 °C; (b) $ClSO_3H$/DMF, 90–100 °C; (c) (1) H^+, (2) pyridine, (3) DMF/H_2O refluxing for 24 h and then repeat the process; (d) butane dioic anhydride in CH_3COOH/CH_3OH at room temperature; (e) butane dioic anhydride in CH_3SO_3H at room temperature.

could be concluded that the introducing of carboxyl groups to amino groups greatly prolonged the APTT and TT, and introducing carboxyl groups into *N*,*O*-position gave better results than that just into *N*-positions. The preparation procedure of the chitosan derivatives is given in Scheme 1.

2. Experimental

2.1. Materials

Chitosan was bought from Yuhuan Sea Biochem. Corp., Zhejiang, China with deacetylation degree 92.85% (measured by pH titration method (Lin, Jiang, & Zhang, 1992)) and $\bar{M}_v$ 3.2×10^5 (measured by viscometer method (Wang, Bo, & Qin, 1990)); activated partial thromboplastin, prothrombin (ISI 1.22) and thrombin (standard dosage for 15, 10, and 25 times of test, respectively) were commercial reagents from Shanghai. Sun Bio. Corp. Human plasma was bought from Wuhan blood center; $ClCH_2CH_2OH$, $ClSO_3H$, methane sulfonic acid, and butane dioic anhydride, etc. were all commercial reagents at analysis grade and used without further purification; permeation film was bought from Sigma Corp. The permeable molecular weight was 3000.

2.2. Methods

FT-IR spectra were measured by a spectrum one B spectra meter (Perkin Elmer) with KBr disk; ^{13}C-NMR spectra were recorded on a Bruker ARX500 (500 MHz) spectrometer, solvent was D_2O. S% was measured in a SC-132 sulfur meter (LECO), while C, N, and H% was measured by Elemental Analyzer-MOD 1106 (Carlo Erba Strumentazione). A 320-S pH meter from Mettler Toledo was used to precede the pH titration. The average molecular weight (M_w) of samples was measured by a GPC. GPC system incorporated a TSP P100 instrument. A TSK G3000-PW column were 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 RI 150 refractive index detector. The sample concentration was ca. 0.4% (w/v). The standards used to calibrate the column were pullulan (TOSOH). All data provided by the GPC system were collected

and analyzed using the Jiangshen Workstation software package.

2.3. Preparation of chitosan derivatives

2.3.1. Hydroxyethyl chitosan sulfate (CS and LSCS) (Fang & Jiang, 1998)

The preparation of CS and LSCS was according to the reaction (a) and (b) in Scheme 1. Fifty grams of chitosan mixed with 150 ml 50 wt% NaOH solution was maintained below 0 °C for 48 h and then 400 ml mixture of 2-propanol and $ClCH_2CH_2OH$ (2:3 volume ratio) was added. The suspension was heated to 60–70 °C for 24 h, the supernatant liquid was decanted off, then the precipitate was dissolved in distilled water and dialyzed for 48 h, subsequently concentrate to give 65 g hydroxyethyl chitosan.

The C, N, and H% of the product was 47.48, 6.32, and 7.40%, respectively. The degree of substitution of the hydroxyethyl groups was 1.23/unit calculated by C%/N%.

Five grams of hydroxyethyl chitosan was added to the sulfating reagent contained 50 ml DMF and 37.5 ml $ClSO_3H$, the mixture was preserved at 80–90 °C for 4 h to give a brown solution. Two hundred milliliters of acetone was added to precipitate the solution, the precipitate was re-dissolved in distilled water and its pH was adjust to 10–11, then the solution was dialyzed for 24 h and concentrated to give hydroxyethyl chitosan sulfate (CS) 4.8 g with S wt% as 16.87%. Low sulfur content hydroxyethyl chitosan sulfate (LSCS) with S wt% as 3.87% was prepared with the same procedure but at 60–65 °C.

The S, C, N, and H% of CS was 15.25, 24.27, 3.39, and 3.10%, respectively. The calculated degree of substitution of the sulfate groups was 1.97/unit. The degree of substitution of the hydroxyethyl groups was about 1.1/unit, lower than the original hydroxyethyl chitosan. This may be due to the strong acidic environment during the sulfating reaction. The S, C, N, and H% of LSCS was 3.97, 41.32, 5.53, and 6.29%, respectively. The calculated degree of substitution of the sulfate groups was 0.31/unit.

2.3.2. 6-O-desulfation of CS (Baumann et al., 1998) (CS-ds)

The preparation of CS-ds was according to the reaction (c) in Scheme 1. Four grams of CS was dissolved in 60 ml distilled water, then 1 mol/l HCl was added to adjust pH to 1.0–2.0 while stirring. Brown solid could be observed. Subsequently, the solution was adjusted to pH 5.6–6.0 by the addition of pyridine, then lyophilized to give ammonium salts as red powders 5.1 g. The powders were dissolved in 8 ml distilled water, subsequently 72 ml DMF was added, stirred at 90 °C for 24 h. The suspension obtained was then dissolved by the addition of appropriate distilled water and then dialyzed for 24 h, followed by lyophilized to give yellow powders. The above procedure was repeated for one more time to give 218 mg yellow powders (CS-ds) finally.

The S, C, N, and H% of CS-ds was 10.25, 31.75, 4.48, and 4.71%, respectively. The calculated degree of substitution of the sulfate groups was 1.01/unit.

2.3.3. N-carboxybutyrylated hydroxyethyl chitosan sulfate (Hirano & Matsumur, 1983)

The *N*-carboxybutyrylation reaction was according to the reaction (d) in Scheme 1. CS (4.20 g, ~10 mmol) was dissolved in 10 ml 10% acetic acid aqueous solution and 15 ml methanol was added while stirring. Then butane dioic anhydride was added at room temperature in 1 h. The mixture was stirred for 4 h, then adjusted pH to 9.0–10.0 by 1 mol/l NaOH. Subsequently, the solution was dialyzed for 24 h, and then lyophilized to give about 0.5 g yellow powders. The samples were labeled according to the usage of butane dioic anhydride added as NCCS-2 (0.2 g, ~2 mmol), NCCS-4 (0.5 g, ~4 mmol). NCCS-6 (0.8 g, ~6 mmol), NCCS-8 (1.0 g, ~8 mmol). (NCCS was the abbreviation of *N*-carboxybutyrylated CS).

LSCS (2.50 g, ~10 mmol) or CS-ds (3.20 g, ~10 mmol) were treated by the same procedure to obtain *N*-carboxybutyrylated LSCS (NCLSCS) and CS-ds (NCCS-ds), the usage of butane dioic anhydride was both 0.6 g, about ~6 mmol.

2.3.4. N,O-carboxybutyrylated hydroxyethyl chitosan sulfate (Grant, Blair, & Mackay, 1988)

The *N*,*O*-carboxybutyrylation reaction was according to the reaction (e) in Scheme 1. LSCS (2.50 g, ~10 mmol) or CS-ds (3.20 g, ~10 mmol) was dissolved in 10 ml methane sulfonic acid, then treated with butane dioic anhydride 1.0 g at 0–5 °C. The mixture were maintained below 0 °C for 24–36 h, then acetone were added. The precipitate was then dissolved in distilled water, adjusted pH to 10.0–11.0 and dialyzed for 3 days, followed by lyophilization to give yellow powders that was *N*,*O*-carboxybutyrylated LSCS (0.55 g) (NOCLSCS) and *N*,*O*-carboxybutyrylated CS-ds (0.42 g) (NOCCS-ds), respectively.

2.4. Clottability assay

The APTT, TT, and PT were measured as Lu et al. reported (Lu et al., 2000). Before test, different concentration of chitosan derivatives was added to the human plasma. The R_{APTT} (ratio of APTT at a determined sample concentration to that of the control assay), R_{TT} (ratio of TT at a determined sample concentration to that of the control assay) or R_{PT} (ratio of PT at a determined sample concentration to that of the control assay) were calculated according to the following equations

$$R_{APTT} = APTT_c/APTT_0;$$

$$R_{PT} = PT_c/PT_0;$$

$$R_{TT} = TT_c/TT_0;$$

In which $APTT_c$, PT_c, and TT_c were the APTT, TT, and PT at determined sample concentrations, respectively. While

Table 1
Elemental analysis of carboxybutyrylated hydroxyethyl chitosan sulfate

Sample	Sulfur content (%)		Carbon content (%)		Nitrogen content (%)		Hydrogen content (%)		Degree of substitution[a]
	Anal.	Calc.[b]	Anal.	Calc.[b]	Anal.	Calc.[b]	Anal.	Calc.[b]	
NCCS-2	14.45	14.42	24.35	25.10	3.20	3.20	3.17	3.24	0.18
NCCS-4	14.01	13.64	24.96	25.82	2.89	3.03	3.10	3.20	0.37
NCCS-6	12.97	12.96	25.86	26.50	2.78	2.88	3.22	3.16	0.57
NCCS-8	12.44	12.34	26.99	27.11	2.65	2.74	2.79	3.13	0.77
NCCS-ds	8.40	8.32	33.30	33.17	3.47	3.60	3.84	3.60	0.57
NOCCS-ds	8.44	8.32	32.81	33.17	3.65	3.60	4.20	3.60	0.58
NCLSCS	3.31	3.07	40.98	40.88	4.41	4.34	4.09	4.34	0.58
NOCLSCS	3.19	3.07	41.22	40.88	4.50	4.34	5.01	4.34	0.57

[a] Degree of substitution was that of carboxyl groups per pyranose unit.
[b] The calculated contents were obtained according to the mole ratio of additional butane dioic anhydride, given that the reactant was 100% reacted.

$APTT_0$, PT_0, and TT_0 were that of the control assays in which the concentration of the sample was 0 μg/ml. All the data were the mean ($d = 4$).

3. Results and discussion

3.1. Structural characterization

3.1.1. Elemental analysis of the carboxybutyrylated hydroxyethyl chitosan sulfate

Table 1 gives the elemental analysis of the carboxybutyrylated hydroxyethyl chitosan sulfate. The contents of analysis were closely matching the ones calculated. S and N% decreased, while C% increased as the usage of butane dioic anhydride increased like the calculated contents shown. This means that the degree of substitution of the carboxybutyryl groups was related with the usage. The calculation result showed that the anhydride was nearly 100% reacted as showed. For this, the content of the carboxyl groups could be favorably controlled by the mole ratio of additional butane dioic anhydride in the reaction.

3.1.2. FT-IR spectra of the carboxybutyrylated hydroxyethyl chitosan sulfate

The FT-IR spectra could easily give the information about degree and position of substitution. Fig. 1 shows the FT-IR spectra of CS and its *N*-carboxybutyrylated derivatives. All spectra have absorption bands related to sulfate groups (1250, 800 cm^{-1}). Compared to the spectra of CS, that of the *N*-carboxybutyrylated derivatives appear obvious symmetric and asymmetric stretching vibrations (~1410, 1560 cm^{-1}) (Peniche-Covas et al., 1999) of the carbonyl groups and the strength of the bands increased as the usage of butane dioic anhydride increased and no stretching vibration of the carbonyl groups of the ester could be observed. This means that *N*-carboxybutyrylation reaction had a favorite selectivity and a series of chitosan derivatives with different content of *N*-carboxyl groups had been successfully prepared.

The FT-IR spectra of LSCS, NCLSCS and NOCLSCS are shown in Fig. 2. It could be seen that two new absorption bands due to the symmetric (1410 cm^{-1}) and asymmetric (1560 cm^{-1}) stretching vibration of carbonyl groups (Peniche-Covas et al., 1999) appeared after *N*-carboxybutyrylation and one more absorption band (1721 cm^{-1}) (Zong et al., 2000) due to the stretching vibration of carbonyl groups of esters appeared after *N*,*O*-carboxybutyrylation. (No absorption bands due to sulfate groups (1250, 800 cm^{-1}) (Horton & Just,1973; Nishimura et al., 1998) were observed because of the low sulfur contents.)

Fig. 3 presents the FT-IR spectra of CS-ds, NCCS-ds, and NOCCS-ds. The ratio of absorption strength of the sulfate group to that of C–O–C (1068 cm^{-1}) (Anguelles--Monal & Peniche-Covas, 1988) decreased when compared to CS. This indicates that some sulfate groups had been removed from the pyranose units. After *N*- or *N*,*O*-carboxybutyrylation, the stretching vibration of carbonyl groups could be distinctly observed like NCLSCS and NOCLSCS. It means that differently from that in CH_3-$COOH/CH_3OH$ environment, the acylation reactions in methane sulfonic acid had no position selective, the carboxyl groups could be introduced in not only amino groups but also in hydroxyl groups. This could also be confirmed by the ^{13}C-NMR spectra.

3.1.3. ^{13}C-NMR spectra of the carboxybutyrylated hydroxyethyl chitosan sulfate

The signal of the carbon atom of chitosan derivatives (CS, NCCS-4, NCCS-ds, NOCCS-ds) is very complicated, but much useful information could still be found from their ^{13}C-NMR spectra (Fig. 4). The spectrum of CS shows very weak chemical shift of C–O (179 ppm).

After *N*-carboxybutyrylation, the spectrum of NCCS-4 shows two new peaks, 185 and 184 ppm, that could be assigned to the carbonyl groups of amide (C_9) (Kim, Kim, & Lee, 1994) and CO_2^- (Lu et al., 2000) groups (C_{12}),

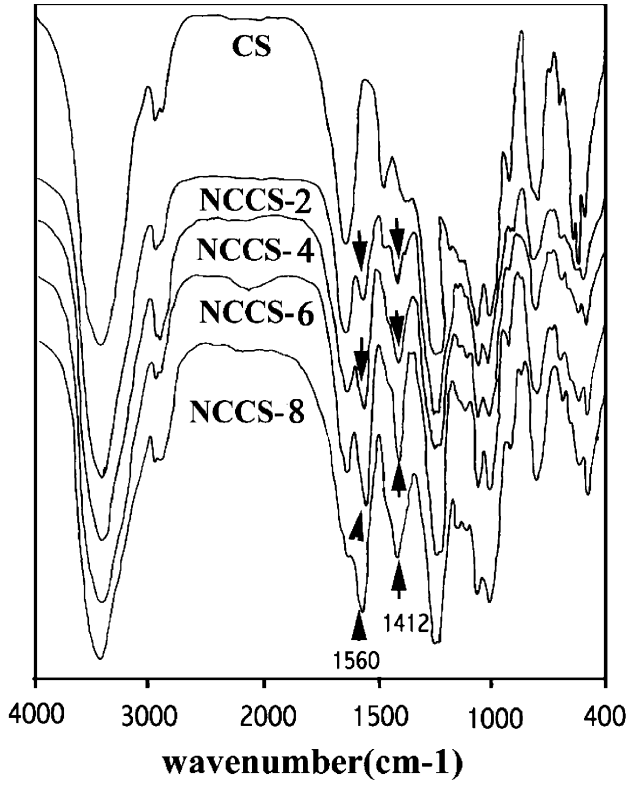

Fig. 1. FT-IR spectra of NCCS-2, NCCS-4, NCCS-6, and NCCS-8 compared to CS.

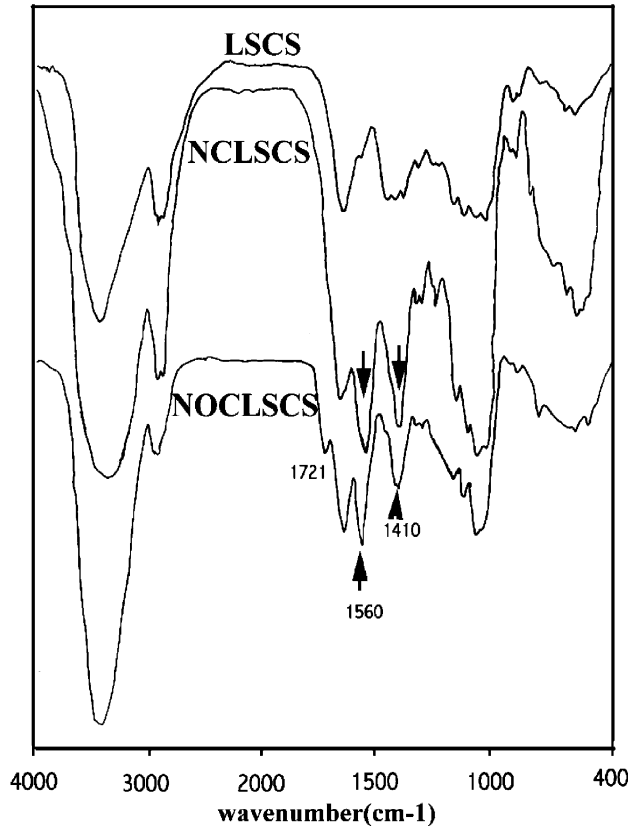

Fig. 2. FT-IR spectra of LSCS, NCLSCS, and NOCLSCS.

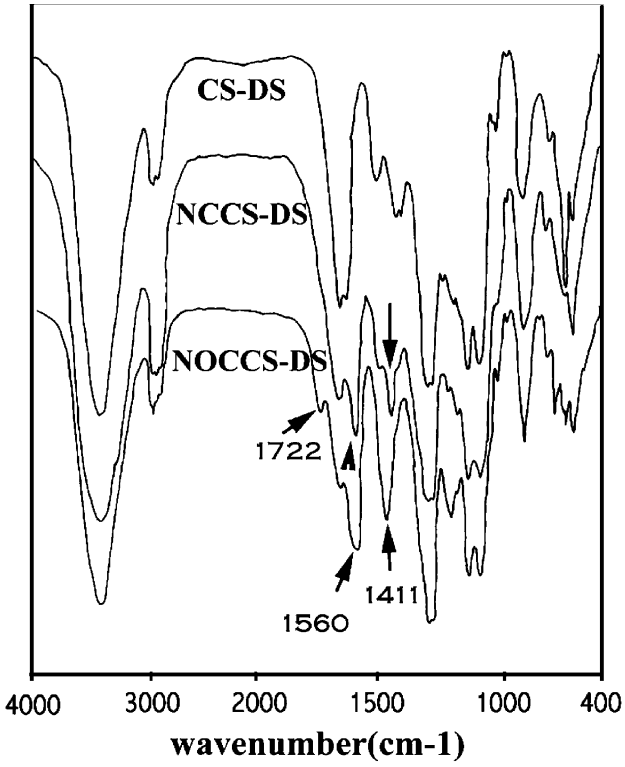

Fig. 3. FT-IR spectra of CS-ds, NCCS-ds, and NOCCS-ds.

respectively, and at 30–40 ppm the chemical shift of saturated carbon atoms (C_{10}, C_{11}) could be observed. The desulfation reaction would selectively remove the sulfate groups on $C_{6'}$ (Baumann et al., 1998). The C_8 sulfate groups will also be removed because of low steric hindrance to DMF. The chemical shifts of sulfated $C_{6'}$ and C_8 are at 69 ppm (Gamzade et al., 1997). The disappearance of 69 ppm peaks on the spectrum of NOCCS-ds and NCCS-ds indicates the success of the desulfation reactions. New peaks at 183 and 178 ppm assigned to the carbonyl carbon of amide groups (C_9) and CO_2^-(C_{12}) and at 30–40 ppm assigned to saturated carbon atoms (C_{10}, C_{11}) appeared at the spectrum of NCCS-ds similar to that of NCCS-4. These indicate that the *N*-carboxybutyryl groups had been introduced. The spectrum of NOCCS-ds gives a new peak at 148 ppm compared to that of NCCS-ds; more than this, four peaks at 40–30 ppm were shown while that of NCCS-ds showed only three. It might be concluded that carboxyl groups had been introduced not only at amino, but also at hydroxyl groups in the acylation reaction with methane sulfonic acid.

3.1.4. Molecular weight of the derivatives

The anticoagulant activity of the chitosan sulfate was greatly influenced by molecular weight of the samples. Generally, the suitable molecular weight was 2.6×10^4 (Nishimura et al., 1986). Table 2 presents the GPC elution volume of the samples. Because the Mark–Houwink equation of pullulan was not fit to chitosan sulfate, the M_w

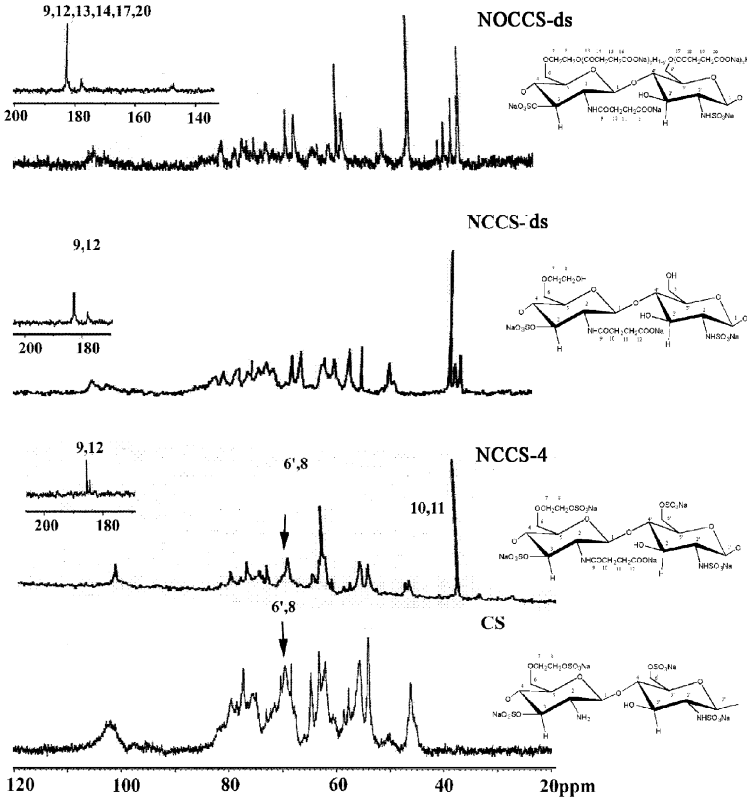

Fig. 4. ^{13}C-NMR spectra of CS, NCCS-4, NCCS-ds, and NOCCS-ds.

of the chitosan samples were not given. It could be seen that in the acylation procedure no depolymerization occurred. NCCS-2, NCCS-4, NCCS-6 and NCCS-8 had similar elution volume to CS; NCLSCS and NOCLSCS had similar elution volume to LSCS; NCCS-ds and NOCCS-ds had similar elution volume to CS-ds. The anticoagulant activity comparison of CS, LSCS, and CS-ds with their acylated derivatives would not be affected by molecular weight.

3.2. Anticoagulant activity

3.2.1. APTT assay

The APTT of CS in the experiments was 360 s at 40 μg/ml, near to that of *N*,*O*-sulfated chitosan. Hirano had reported (Hirano et al., 1985), 270 s at 15 μg/ml. The APTT of the samples increased with the content of the carboxyl group. At 40 μg/ml, CS showed the R_{APTT} as 2.25, NCCS-2 and NCCS-4 showed as 2.78 and 11.65, respectively, by up to more than four times increase. But the R_{APTT} of NCCS-6, NCCS-8 decreased when compared to that of CS (Fig. 5(a)). This may be due to the low sulfur content after a great many of carboxyl groups introduced.

LSCS showed poor anticoagulant activity because of it low sulfur content (Fig. 5(b)). CS-ds showed coagulant activity because of the removal of C_6 sulfate groups (Nishimura et al., 1998). Their *N*-carboxybutyrylated derivatives NCLSCS and NCCS-ds presented little anticoagulant or coagulant activity, too. But their *N*,*O*-carboxybutyrylated derivatives, NOCLSCS and NOCCS-ds give considerable anticoagulant activity. The R_{APPT} of NOCCS-ds and NOCLSCS increased while the concentration of the samples increased. For NOCLSCS, the R_{APTT} at 125 μg/ml was 1.27; at 250 μg/ml, 1.36 and at 375 μg/ml, 1.45 (Fig. 5(b)). For NOCCS-ds, the R_{APTT} increased from 1.3 at 10 μg/ml to 2.7 at 40 μg/ml. Compared to this, R_{APTT} of NCCS-ds decreased from 0.82 at 10 μg/ml to 0.78 at 40 μg/ml

Table 2
M_w of the samples characterized by GPC elution volume

M_w of pullulan (× 10^4)	Elution volume (min)	Samples	Elution volume (min)	Samples	Elution volume (min)	Samples	Elution volume (min)
4.73	6.02	CS	7.76	LSCS	6.30	CS-ds	7.84
2.28	6.53	NCCS-2	7.75	NCLSCS	6.27	NCCS-ds	7.67
1.8	6.92	NCCS-4	7.77	NOCLSCS	6.23	NOCCS-ds	7.60
0.59	7.26	NCCS-6	7.76				
		NCCS-8	7.77				

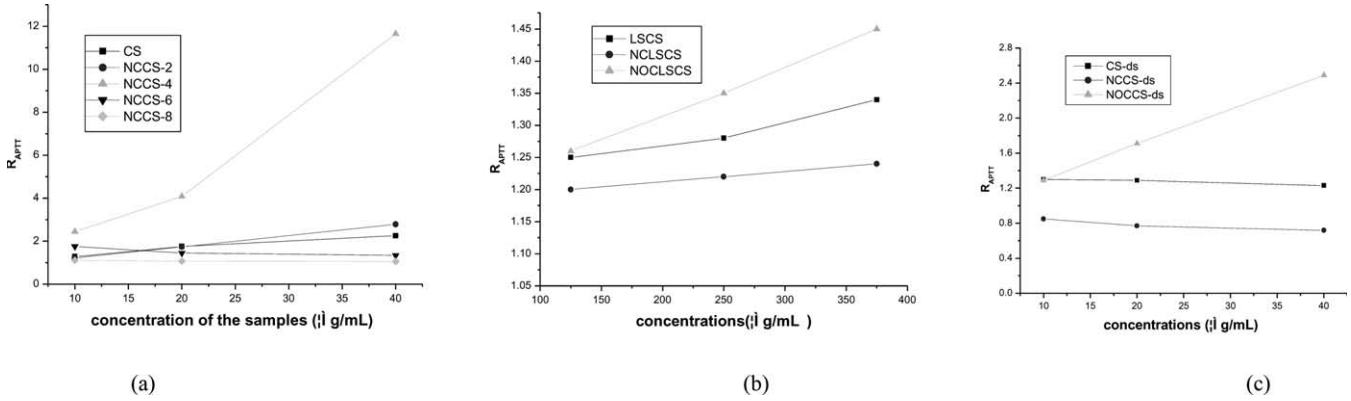

Fig. 5. Ratio of APTT (R_{APTT}) of the samples at different concentrations to that of the control assay.

(Fig. 5(c)). NOCCS-ds give obvious anticoagulant activity.

3.2.2. PT assay

Generally, the influence of the samples on the PT was not so obvious as R_{APTT}. The R_{PT} showed little change if the concentration of the samples was less than 100 μg/ml. So we determined the concentration for PT assay as 100–400 μg/ml (Fig. 6).

Differently from R_{APTT}, the R_{PT} decreased while the carboxyl group was introduced (Fig. 6(a)). R_{PT} of CS at 125, 250, and 375 μg/ml were 2.28, 4.64, and 11.71, respectively, and that of NCCS-4, which showed highest R_{APTT} among *N*-carboxybutyrylated derivatives, were 1.81, 2.10, and 7.04. The change of R_{PT} according to carboxyl group's content presents irregularity. NCLSCS and NCCS-ds did not show anticoagulant, but coagulant activity like LSCS or CS-ds. Even NOCLSCS and NOCCS-ds had no anticoagulant activity but coagulant activity (Fig. 6(b) and (c)). The R_{PT} of NOCLSCS, NOCCS-ds at 125, 250, and 375 μg/ml were 0.82, 0.70, 0.61, and 0.87, 0.82, 0.76, respectively, even lower than that of NCCS-ds and NCLSCS. These means that the introducing of carboxyl groups could not enhance the antiprothrombin activity of the chitosan derivatives but lower it.

3.2.3. TT assay

The TT of the CS in the experimental was 20 s at 10 μg/ml. Hirano reported *N*,*O*-sulfated chitosan as 20 s at 10 μg/ml, too. The introducing of carboxyl groups greatly increased the antithrombin activity of the samples. The R_{TT} of NCCS-4 at 40 μg/ml was 2.18, compared to that of CS, 1.47; increased by about 50%. Similarly to APTT assay, too many carboxyl groups introduced lowered the sulfur content and decreased the antithrombin activity of the samples, such as NCCS-5 and NCCS-8.

Chitosan derivative with low sulfur content, such as LSCS enhanced the thrombin activity. But after *N*- or *N*,*O*-carboxybutyrylated, such as NCLSCS and NOCLSCS, the derivatives showed a little antithrombin activity. Their R_{TT} increased as the concentrations increasing. The R_{TT} of NCLSCS and NOCLSCS at 10, 20, and 40 μg/ml were 0.94, 0.95, 0.95, and 1.02, 1.05, 1.08 (Fig. 7(b)).

Chitosan derivatives that lack C_6 sulfate groups (CS-ds) had antithrombin activity but much lower than that of CS, its R_{TT} at 10, 20, and 40 μg/ml were 0.82, 0.88, and 0.92. The activity was not improved by the introducing of carboxyl groups on both amino and hydroxyl groups. The R_{TT}s of NCCS-ds and NOCCS-ds were 0.83, 0.88, 0.94, and 0.87, 0.89, 0.97, respectively (Fig. 7(c)). This means that both *N*- and *N*,*O*-carboxybutyrylation could not enhance the antithrombin activity.

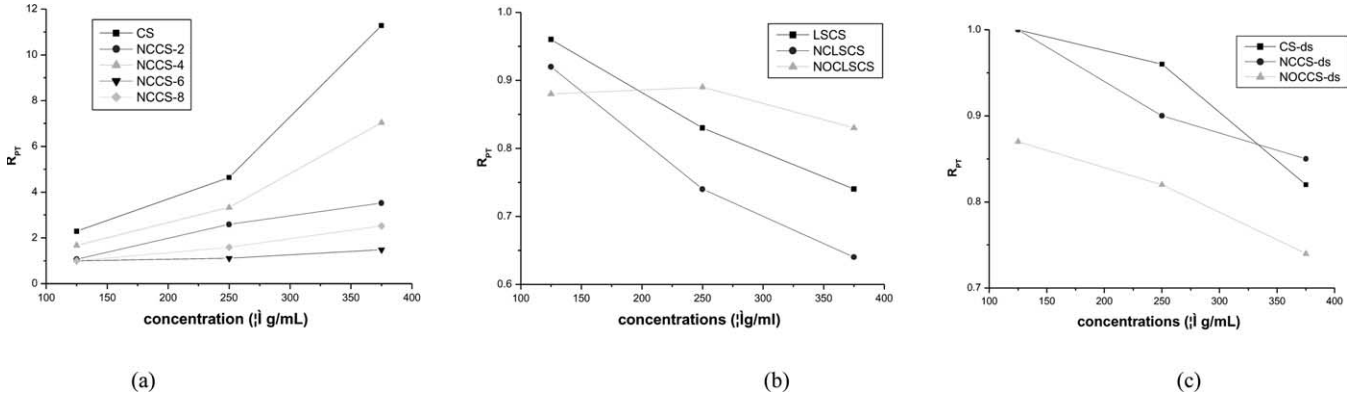

Fig. 6. Ratio of PT (R_{PT}) of the samples at different concentrations to that of the control assay.

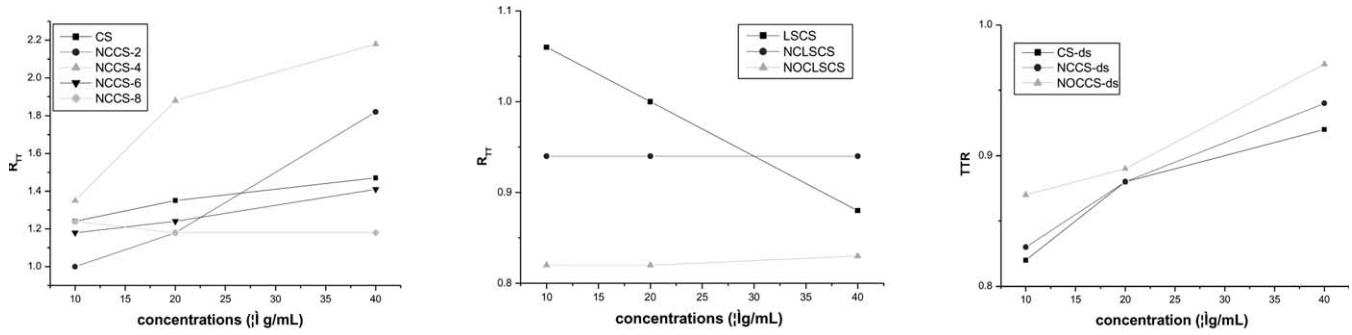

Fig. 7. Ratio of TT (R_{TT}) of the samples at different concentrations to that of the control assay.

4. Conclusions

A new method for introducing carboxyl groups was successfully carried out. The content and position of the carboxyl groups could be controlled favorably. The influence of carboxyl groups on the anticoagulant activity was studied with respect to APTT, TT, and PT. It was shown that, the introducing of carboxyl groups to amino groups increased the APTT and TT. The position of the carboxyl groups also influenced the anticoagulant activity of the chitosan derivatives, introducing carboxyl groups into amino and hydroxyl groups gave better results than that just into amino groups. Generally, the introducing of carboxyl groups could not increase PT, but decrease it in spite of the position introduced.

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