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Preparation of sulfated-chitins under homogeneous conditions

Yuquan Zou [1], Eugene Khor *

Department of Chemistry, National University of Singapore, 3 Science Drive 3, Singapore 117543, Singapore

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ABSTRACT

Sulfated-chitins of varying degrees of sulfation were prepared by the reaction of chitin with sulfur trioxide–pyridine complex under homogeneous conditions in 5% LiCl/DMAc solvent system. Sulfation at 8 °C or room temperature was regio-selective for the C6–OH position with the degree of sulfation (D.S.) ranging from 0.53 to 1.00 depending on the reaction time. When the reaction temperature was elevated, sulfation at the C3–OH position also occurred. The extent of sulfation at the C3 position was a function of the concentration of sulfating reagent, reaction time and temperature. The structure of sulfated-chitins was established by ^{1}H, ^{13}C NMR and 2D HMQC. The degree of sulfation at the C6 position was estimated by ^{1}H NMR while that of the C3 position was by elemental analyses. The anticoagulant activity of the prepared sulfated-chitins correlated closely with D.S. The higher the D.S. yielded, the better the anticoagulant activity. In particular, a continuous sequence of 36S units was critical for obtaining high anticoagulation activity.

1. Introduction

Chitin is a biopolymer of immense interest and together with its primary derivative, chitosan, is championed for various environmental, industrial and medical applications. However, the strong hydrogen bonding that exists in chitin has led to intensive research in the chemical derivatization of chitin and chitosan, seen as a way of overcoming the processing limitations of chitin and chitosan that can also lead to derivatives offering new and interesting biological and immunological activities not obtained with the base materials (Kurita, 2001).

One derivative type that has attracted perennial interest is the sulfated-chitins and chitosans because of their similarity to heparin and therefore their potential anticoagulant activity. The sulfation of chitin was reported as far back as 1954 (Cushing, Davis, Kratovil, & MacCorquodale, 1954). Over the following several decades, effort was extended to prepare N- and/or O-sulfated-chitins and chitosans using various reaction conditions and sulfating agents. Wolfrom et al., Horton et al., Nishimura et al., Terbojevich et al., Gamzazade et al., Drozd et al. and Vongchan et al. have all reported the preparation of N-sulfated CM-chitosan, O-sulfated-chitin, 6-O-sulfated-chitosan and multi-substituted sulfated-chitosan using $HClSO_3$ (Drozd et al., 2001; Gamzazade et al., 1997; Horton & Just, 1973; Nishimura, Nishi, Tokura, Okiei, & Somorin, 1986; Terbojevich et al., 1989; Vongchan, Sajomsang, Subyen, & Kongtawelert, 2002; Wolfrom & Shen-Han, 1959). Hirano et al. and Nishimura used a SO_3–DMF complex to prepare sulfated-chitin and sulfated-6-O-(carboxymethyl)chitin, while Terbojevich et al. reported the selective 6-O-sulfation of chitosan with SO_3–pyridine complex (Hirano & Hasegawa, 1991; Hirano, Tanaka, Hasegawa, Tobetto, & Nishioka, 1985; Nishimura et al., 1986; Nishimura & Tokura, 1987). Holme et al. and Gamzazade et al. used SO_3–Me_3N complex and SO_3–pyridine complex, respectively, to prepare N-sulfated-chitosan (Holme & Perlin, 1997; Terbojevich et al., 1989). Compared to chlorosulfonic acid, the sulfur trioxide-organic solvent complexes are mild and less-destructive.

Generally, these chemical derivatization reactions were conducted under heterogeneous or semi-heterogeneous conditions due to chitin's poor solubility in common organic solvents. Consequently, the attainment of a high degree of sulfation (D.S.) was difficult, with poor selectivity for the site of sulfation (C6, C3 or N2 positions), inevitably resulting in multi-substituted derivatives unless tedious pre-protection and de-protection steps were taken (Nishimura, Kai, Shinada, Yoshida, & Tokura, 1998; Nishimura et al., 1986). The uncertainty regarding the degree of substitution at the individual positions led to structure-activity relationship ambiguities. Finally, heterogeneous reactions are known for their poor reproducibility, limiting industrial production and practical applications.

In recent years, the introduction of 5% LiCl/DMAc (w/v) as the solvent system for chitin has made it easier to use chitin as well as study its reactivity under homogeneous and mild reaction conditions. Tosylation, chlorination and bromination reactions of chitin have all been reported with this solvent system (Morita, Sugahara, Takahashi, & Ibonai, 1994; Tseng, Furuhata, & Sakamoto,

* Corresponding author. Tel.: +65 6516 2836; fax: +65 6779 1691.
E-mail address: chmkhore@nus.edu.sg (E. Khor).
[1] Present address: Center for Blood Research, University of British Columbia, 2350 Health Sciences Mall, Vancouver, BC, Canada V6T 1Z3.

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1995; Tseng, Takechi, & Furuhata, 1997; Zou & Khor, 2005). Reactions selective towards the C3 and C6 hydroxyl groups have been achieved by controlling the reaction conditions.

For chitin sulfation, Terbojevich et al. reported in 1989 on the 6-O-sulfation of chitin in this solvent system (Terbojevich et al., 1989). Apparently, there does not appear to have been any further reports of chitin sulfation under homogeneous conditions (Jayakumar, Nwe, Tokura, & Tamura, 2007). Our interests in preparing well defined and characterized chitin derivatives led us to consider and revive the study of sulfated-chitins under homogeneous conditions. In this work, we report on the sulfation of chitin in 5% LiCl/DMAc using sulfur trioxide–pyridine complex, a mild sulfating reagent to give sulfated-chitins. A systematic investigation into the effect of reaction conditions on the degree of sulfation and characterization of the resultant derivatives were conducted. The relationship between the anticoagulation activity of the obtained sulfated-chitins and the degree of sulfation is also presented.

2. Experimental

2.1. Materials

Chitin powder isolated from crab shells (Sigma, Lot. C 7170) was purified by stirring in a 5% NaOH aqueous solution for 3 days, recovered with de-ionized water, treated with 1 M HCl for 1 h, washed with de-ionized water until pH 7 and dried at 50 °C. N,N-dimethylacetamide (DMAc, Aldrich) was of HPLC grade and sulfur trioxide–pyridine complex (98%) was purchased from Aldrich (Lot. S7556). Dialysis membranes (molecular weight cut-off ~ 12,000, Lot. D9527) and deuterium chloride (Lot. 120H5953) was purchased from Sigma Aldrich Co. Deuterium oxide 99.8% (Lot. 1.13366) was purchased from Merck & Co. All other chemicals were reagent grade and used without further purification.

2.2. General methods

Elemental analyses were performed by the Microanalytical Laboratory, Department of Chemistry, National University of Singapore using a Perkin Elmer Series 2400 C, H, N, S analyzer. GPC (Gel Permeation Chromatography) was used to estimate the molecular weight profile. The set-up comprised three Polymer Lab columns in series (PL aquagel-OH), a Waters HPLC pump type 515, and a refractive index detector Waters 410. Polymer solutions were filtered through 0.45 μm millipore® filters. Pullulan (Shodex®) and 0.33 M acetic acid/0.1 M sodium acetate were used as standards and eluent, respectively.

2.3. NMR analyses

^{1}H and ^{13}C NMR measurements were performed on a Bruker AV 500 MHz spectrometer at 500.13 and 125.75 MHz, respectively. ^{1}H NMR spectra were recorded at 300 or 353 K depending on the experimental requirements. Spectra obtained at 353 K were allowed to stand in the magnet for 5 min to achieve thermal equilibrium prior to data acquisition. All ^{13}C NMR spectra were acquired at 300 K. Two-dimensional hetero-nuclear one bond proton carbon correlation experiments were registered in ^{1}H detected mode via multi-quantum coherence (HMQC) on a Bruker DRX 500.

2.4. Preparation of 6-O-sulfated-chitin and 3,6-O-disulfated-chitin

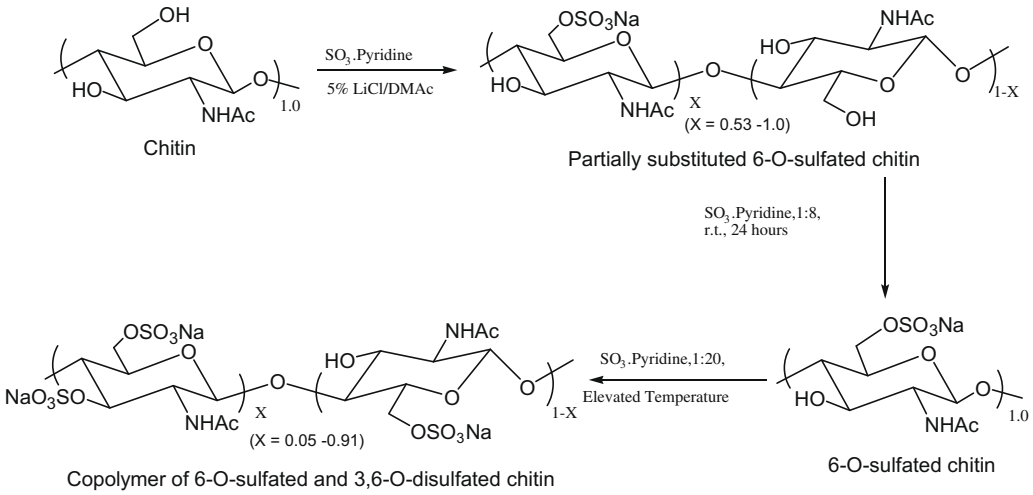
The synthesis procedure for 6-O-sulfated chitin and 3,6-O-disulfated-chitin is illustrated in Scheme 1.

2.4.1. Preparation of 6-O-sulfated-chitin in 5% LiCl/DMAc

0.4 g of purified chitin (ca. 2 mmol) was stirred in 30 ml of 5% LiCl/DMAc until dissolution was complete. 2.0 g of sulfur trioxide–pyridine complex (ca. 12 mmol) pre-dissolved in 10 ml of 5% LiCl/DMAc, was transferred into the chitin solution using a syringe. The reaction was permitted to proceed at room temperature for a pre-set reaction time. At completion, the reaction mixture was poured into 250 ml of acetone and the white fibrous precipitate filtered, collected and re-dissolved into 80 ml of water. The resultant solution was adjusted to pH ≈10 with 5% NaOH solution, loaded into the dialysis membrane assembly and dialyzed against water for 72 h. The dialyzed solution was filtered and concentrated to 30 ml by rotary evaporation. To the concentrated solution was added 150 ml of acetone, releasing a precipitate that was collected and dried at 50 °C overnight. The product was stored in a desiccator until used.

2.4.2. Preparation of 3,6-O-disulfated-chitin in 5% LiCl/DMAc

About 0.4 g of purified chitin (ca. 2 mmol) was stirred in 30 ml 5% LiCl/DMAc until dissolution was complete. 2.6 g of sulfur trioxide–pyridine complex (ca. 16 mmol) pre-dissolved in 12 ml of 5% LiCl/DMAc, was transferred into the chitin solution using a syringe. The reaction was permitted to proceed at room temperature for 24 h to give 6-O-sulfated-chitin of approximately D.S. of 1. A sec-



Scheme 1. Reaction scheme for the preparation of sulfated-chitins.

ond portion of 2.6 g of sulfur trioxide–pyridine complex pre-dissolved in 12 ml of 5% LiCl/DMAc was added. The reaction temperature was raised and allowed to continue for a pre-set period. The product was isolated following the procedure described above. After dialysis, the dialyzed solution was freeze-dried.

2.5. Anticoagulant activity study

Citrated human platelet-poor plasma (PPP) was purchased from Dade, Germany (Lot. B4244-10). The plasma powder was reconstituted by Milli-Q water before use. Innovin (B4212-50), actin FSL (B42192), thrombin reagent (B4233-25) and thromboclotin (281007) were all purchased from Dade®, Germany and used for prothrombin time (PT), activated partial thromboplastin time (APTT), fibrinogen time (FT), and thrombin time (TT) assays, respectively. The assays were conducted according to the manufacturer's instruction using an automated blood coagulation analyzer (CA-540, Sysmex Corp, Kobe, Japan). All experiments were performed in duplicate and repeated 3 times on different days. The standard deviations were less than 2% of the mean.

3. Results and discussion

3.1. The degree of acetylation (D.A.) of chitin

Ideally, chitin should be constituted of solely 2-acetamido-2-deoxy-D-glucopyranose repeat units. However, commercial chitin is very varied, typically containing a fraction (up to 30%) of 2-amino-2-deoxy-D-glucopyranose. This makes the estimation of the degree of acetylation (D.A.) of chitin an important parameter as it can have striking effects on the solubility and reactivity of chitin. Many techniques have been developed to determine the D.A. including FT-IR, UV–vis spectroscopy, titration, circular dichroism, NMR and HPLC (Brugnerotto et al., 2001; Domard, 1987; Duarte, Ferreira, Marvao, & Rocha, 2001; Ravi-Kumar, 2000; Raymond, Morin, & Marchessault, 1993; Sannan, Kurita, Ogura, & Iwakura, 1978; Tan, Khor, Tan, & Wong, 1998; Yu, Morin, Nobes, & Marchessault, 1999). Today, NMR is preferred, as it has advantages with regard to accuracy, precision, and robustness to interference by impurities. Gupta et al. reported the determination of D.A. for chitosan by liquid phase ^{1}H NMR (Lavertu et al., 2003). The D.A. was estimated conveniently by the ratio of H_{1A} (H_1 proton belonging to acetylated monomer) and H_{1D} (H_1 proton belonging to deacetylated monomer). Unfortunately, this method cannot be extended directly to chitin with a high D.A. due to its insolubility in weak acid solution. We believe that this inconvenience can be circumvented in this instance. Transforming chitin into 6-O-sulfated-chitin gives a water soluble chitin derivative and its ^{1}H NMR spectrum is readily obtained. The homogeneous reaction conditions are mild and little or no N-deacetylation is expected. Therefore, we propose that the D.A. estimated for the sulfated-chitin is a reasonable representation of the D.A. in the starting chitin material.

Fig. 1 shows the ^{1}H NMR of 6-O-sulfated-chitin with a degree of sulftion (D.S.) of 1.00. The spectrum was recorded at 353 K to remove the overlap of the H_{1A} with the H_{OD} peak.

The following two equations were used to estimate the D.A.:

$$\text{D.A.} = (H_{1A})/(H_{1A} + H_{1D}) \quad (1)$$

$$\text{D.A.} = (H_{CH3}/3)/(H_{2D} + H_{CH3}/3) \quad (2)$$

The D.A. calculated using Eqs. (1) and (2) were 98% and 97%, respectively, and in effect, the same. This indicated that the starting chitin was essentially fully acetylated and that further acetylation of the residual 2–3% of amino groups to acetyl groups was not necessary as their affect on subsequent reactions and properties would be negligible. For convenience, the D.A. of the purified chitin used in this study was approximated to 100% to simplify subsequent calculations and characterization of derivatives. This also implied that N-sulfation in this instance was not possible due to the absence of amino groups.

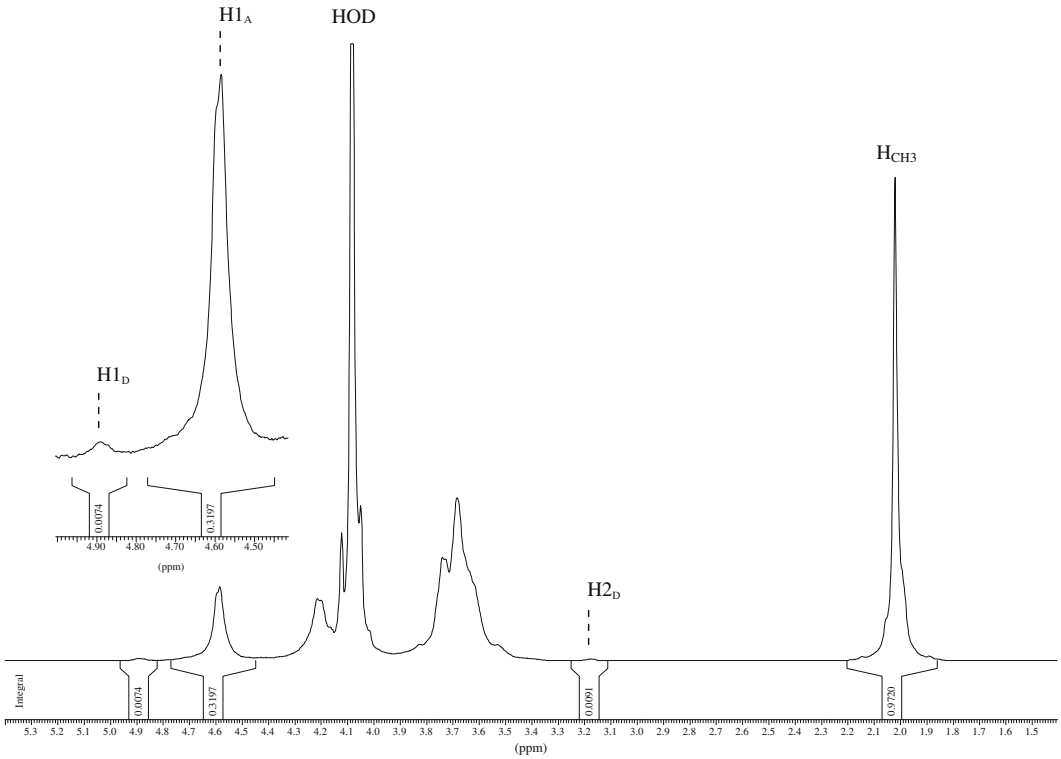


Fig. 1. ^{1}H NMR spectrum of 6-O-sulfated-chitin (D.S. ≈1.00) at 353 K in D_2O highlighting the H_1 proton region.

3.2. Sulfation at the C6 position

The feasibility of selective sulfation at the C6 position is based on the general recognition that in the N-acetyl-D-glucosamine moiety, the reactivity of the C6–OH is higher than the C3–OH (Hirano & Hasegawa, 1991). The effects of different reaction conditions, including reaction temperature, reaction time and concentration of sulfating reagent on 6-O-sulfation are summarized in Table 1. The reaction temperature was found to be critical for the regioselective sulfation. At room temperature or lower, sulfation was consistently at the C6 position. For example, at 4 °C, 12 h of reaction did not yield a water soluble product due to a very low degree of sulfation at the C6 position. At 8 °C, 36 h of reaction yielded a water soluble product with a 53% degree of sulfation. Reactions at ambient gave products with D.S. ranging from 0.53 to 1 depending on the reaction time and concentration of sulfating reagent. The selectivity of the reaction at room temperature and below is consistent with earlier reports suggesting the higher reactivity of the C6 compared to the C3 position in chitin (Terbojevich et al., 1989).

3.3. Estimation of the D.S. of 6-O-sulfated-chitin

Elemental analysis using the ratio of sulfur to nitrogen is the most commonly used method for determining the D.S. of sulfated chitins (Cushing et al., 1954; Gamzazade et al., 1997; Horton & Just, 1973; Nishimura et al., 1986; Wolfrom & Shen-Han, 1959). The sample purity requirement in elemental analysis is rather stringent, i.e. the sample must be pure, free of moisture and other contaminants that, for a biopolymer, is difficult to achieve. In addition, the D.S. calculated by elemental analysis can only reflect the overall substitution degree but not the D.S. at specific positions. Therefore, in this study, ^{1}H NMR was used to estimate the D.S. of 6-O-sulfated-chitin. ^{1}H NMR has been regarded as less effective and valuable in structural characterization of polysaccharides due to the strong overlapping of the proton signals. Here, the assignments of the peaks in the ^{1}H NMR spectra were assisted by HMQC experiments.

Fig. 2a shows the HMQC spectrum of partially substituted 6-O-substituted sulfated-chitin. The ^{1}H NMR spectra was recorded in 10% DCl/D_2O, rather than in D_2O. Under acidic conditions, all peaks shift upfield approximately 0.2 ppm, giving a well resolved signal for the H_{1A} distinct from the H_{OD} peak. The de-shielding effect of the electronegative sulfate group shifts the two protons at the sulfated C6 position downfield to 4.0 and 4.2 ppm, respectively, well

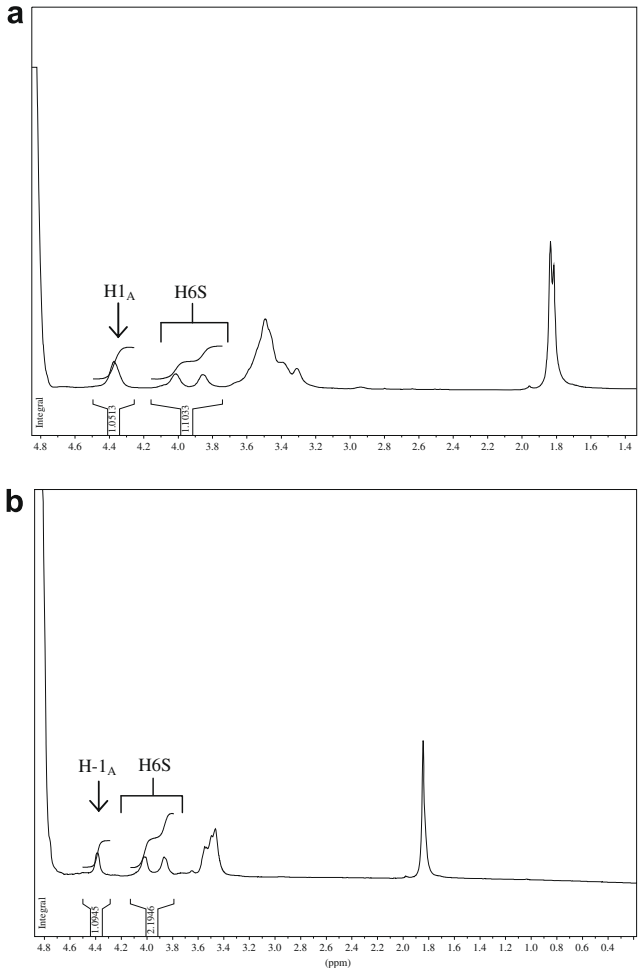


Fig. 2. ^{1}H NMR of 6-O-sulfated-chitin in 10% DCl/D_2O at 300 K. (a) D.S. = 0.53 (SC6-53); (b) D.S. = 1.00 (SC6-100).

Table 1
Conditions for the sulfation of chitin and the corresponding degree of sulfation (D.S.).

Product[a]	Step 1			Step 2[b]			D.S.[c]		
	SO_3.pyr:pyranose	Temp (°C)[b]	Time (h)	SO_3.pyr:pyranose	Temp (°C)[b]	Time (h)	C6		C3
							NMR	E.A.	E.A.
[d]SC6-00	6	4	12	←	N.R.	→	N.R.		N/A
SC6-53	6	8	36	←	N.R.	→	0.53	0.49	N/A
SC6-74	6	r.t.	12	←	N.R.	→	0.74	0.70	N/A
SC6-81	6	r.t.	24	←	N.R.	→	0.81	0.80	N/A
SC6-100	8	r.t.	24	←	N.R.	→	1.00	0.94	N/A
SC36-05	8	r.t.	24	8	45	12	1.00		0.05
SC36-14	8	r.t.	24	8	55	12	1.00		0.14
SC36-34	8	r.t.	24	8	65	12	1.00		0.34
SC36-48	8	r.t.	24	8	75	24	1.00		0.48
SC36-52	8	r.t.	24	20	90	1	1.00		0.52
SC36-65	8	r.t.	24	20	75	6	1.00		0.65
SC36-74	8	r.t.	24	20	65	12	1.00		0.74
SC36-84	8	r.t.	24	20	70	12	1.00		0.84
SC36-91	8	r.t.	24	20	75	12	1.00		0.91

N.R., No reaction.
[a] SC6 and SC36 represent 6-O-sulfated-chitin and 3,6-O-disulfated-chitin, respectively.
[b] All the SC36 series products were sulfated at room temperature for 24 h with 8-fold of SO_3–pyridine complex before a higher temperature was applied.
[c] Both ^{1}H NMR and elemental analyses were applied to estimate the D.S. at C6 position, whereas only elemental analyses were used to estimate the D.S. at the C3 position.
[d] The product was not water soluble.

resolved from the other proton peaks in the sugar ring. The protons of the un-substituted C6 were at 3.5 and 3.7 ppm, respectively.

The D.S. was estimated using the integration of the proton peaks of H_{6S} and H_1 ($H_1 \approx H_{1A}$): where D.S. = H_{6S}/H_{1A}.

The methyl group was not chosen as the reference peak because of the potential for its integration factor to include contributions from trace residual acetone.

Fig. 3a and b present the ^{1}H NMR spectra of 6-O-sulfated-chitin with D.S. of 0.53 and 1.00, respectively. Comparing the data of the D.S. obtained by ^{1}H NMR and elemental analyses summarized in Table 1, we note that the D.S. for SC6-53, 74, 81 and 100 determined by ^{1}H NMR were 8.2%, 5.7%, 1.3%, 6.4%, respectively, higher than that obtained by elemental analyses. The difference of the two methods could be attributed to the presence of moisture. Although all samples were dried at 110 °C overnight, moisture uptake during material transfer and weighing of samples in elemental analyses were inevitable due to the hygroscopic nature of sulfated-chitins. The D.S. obtained by elemental analyses would therefore be expected to be lower than that obtained by ^{1}H NMR.

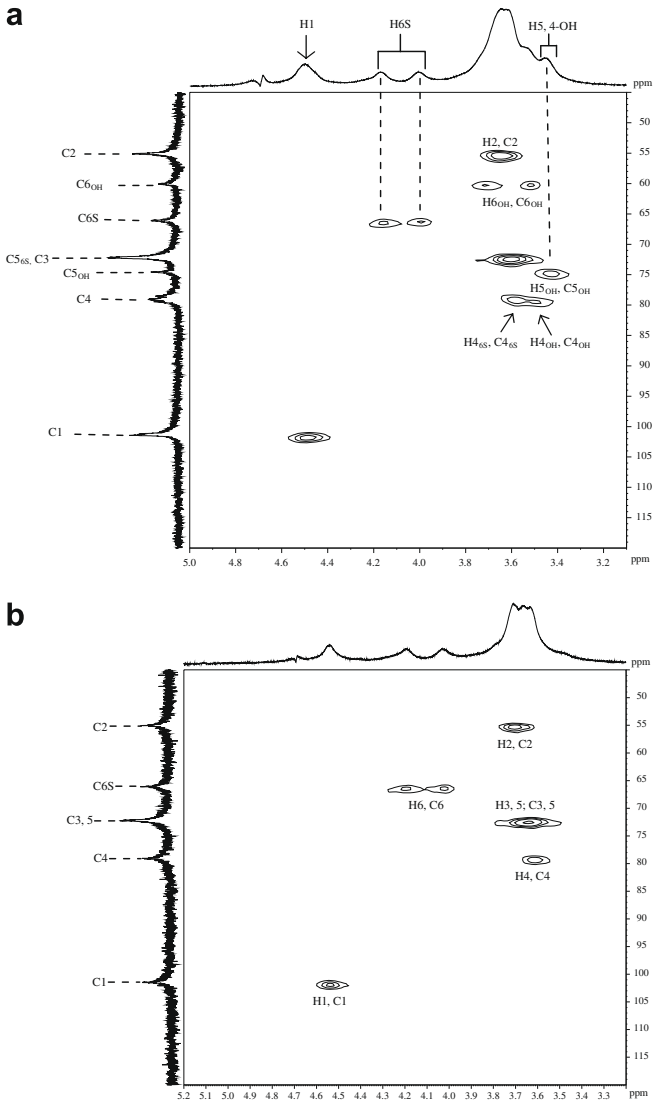


Fig. 3. HMQC of partially substituted 6-O-sulfated-chitin. (a) D.S. = 0.53; (b) D.S. ≈ 1.00.

3.4. Sulfation at the C3 position

The sulfation of chitin at the C3 position was accomplished by a two-step reaction. First, chitin was converted to fully substituted (D.S. = 1.00) 6-O-sulfated-chitin at ambient followed by further sulfation at the C3 position at elevated temperatures. The purpose of utilizing the two-step reaction instead of direct sulfation at high temperatures was to avoid the possible structural heterogeneity resulting from incomplete sulfation at the C6 position.

In contrast to 6-O-sulfation, 3-O-sulfation could only be accomplished at elevated temperatures. A perusal of Table 1 revealed that although the reaction time, reaction temperature and concentration of sulfating reagent all affected sulfation at the C3 position, the reaction temperature was the most critical parameter. Sulfation at the C3 position was not obtained at room temperature or lower. Only when the reaction temperature was above 45 °C was sulfation at the C3 position observed. The other two parameters, the concentration of sulfating reagent and reaction time, were found to be more important for controlling the D.S. at the C3 position. For the same reaction temperature, a longer reaction time and higher concentration of sulfating reagent yielded higher degrees of substitution. An 8-fold amount (with respect to chitin) of sulfating reagent gave a derivative with a D.S. of 0.48 after 24 h reaction at 75 °C. With a 20-fold amount of sulfating reagent at the same temperature and reaction time, a D.S of 0.91 was obtained. Therefore, adjusting these three parameters, a D.S. ranging from 0.05 to 0.91 at the C3 position was achieved.

Another event observed during sulfation at the C3 position was the occurrence of gelling as the reaction progressed. Gel formation is a consequence of increasing hydrophilic character as the number of sulfate groups attached to the chitin backbone increases, leading to poor solubility in the organic reaction solvent.

The ^{1}H NMR of 3,6-O-disulfated-chitin was found to be unsuitable for the estimation of the D.S. because of severe peak overlapping. Therefore, only elemental analyses were used to estimate the D.S. of 3,6-O-disulfated-chitins with the calculation based on the assumption that the C6 position was fully sulfated.

3.5. Structural elucidation of 6-O-sulfated-chitin by 2D HMQC NMR

Structural characterization of sulfated-chitins and sulfated-chitosans by ^{1}H and ^{13}C NMR is of importance in understanding their structure-activity relationships (Drozd et al., 2001; Hirano et al., 1985; Nishimura & Tokura, 1987; Terbojevich et al., 1989). While one-dimensional NMR does provide some aspects in structural elucidation of the sulfated-chitins, severe overlapping of peaks in both the ^{1}H and ^{13}C NMR spectra limit their usefulness. This is exacerbated by the variation of D.S. among the samples, congesting the spectra further.

In this study, 2D HMQC NMR was used to facilitate the structural analyses of sulfated-chitins. In the HMQC spectra of 6-O-sulfated-chitin with a D.S. of 0.53 and 1.00 (Fig. 2a and b), the peaks for the substituted H_{6S} and un-substituted H_{6OH} were found at 4.0, 4.2 ppm and 3.5, 3.7 ppm, respectively, correlating well with the peaks for the C_{6S} at 66 ppm and C_{6OH} at 60 ppm.

Compared to Fig. 2a, Fig. 2b shows a relatively simple HMQC spectrum. The disappearance of the un-substituted C_6 and C_5 peaks at 60 and 72 ppm supports the complete conversion of chitin to 6-O-sulfated-chitin. The chemical shifts for the C_1–C_4 carbon peaks were identical to those in Fig. 2a.

The downfield shifts of both ^{1}H and ^{13}C signals were attributed to the electronegative effect of the sulfate group. In addition, the peak at 74 ppm was assigned to the C_5 of the un-reacted pyranose unit as it correlated with the high field proton at 3.4 ppm. The substituted C_5 peak was found to shift upfield to 72 ppm, combining with the C_3 carbon. The cross peak relating the H_4 and C_4 were

split into two, suggesting the existence of two types of H_4 and C_4. Although the substituted and un-substituted C_4 peaks were not well resolved in the ^{13}C NMR spectrum due to the small differences in chemical shifts, the obvious broadening and partial splitting of the peak in Fig. 2 suggested the existence of both types of C_4. Carbon peak signals for C_3, C_2 and C_1 were not obviously affected by 6-O-sulfation. Combining these data, we conclude that the effect of 6-O-sulfation on atoms in the sugar ring diminished as the bond distance increased.

3.6. Structural elucidation of 3,6-O-disulfated-chitin by 2D HMQC NMR

In contrast to 6-O-sulfation, sulfation at the C3 position imposed a more striking effect on the chemical environment of the sugar ring, reflected by a much more complicated spectrum for 3,6-O-disulfated-chitin. Fig. 4a and b presents the HMQC spectra for 3,6-O-disulfated-chitins having D.S. of 0.52 and 0.91 at the C3 position, respectively.

In Fig. 4a, the carbon peak at 79 ppm showed correlation with two types of protons at 3.6 and 4.4 ppm, respectively. The proton at 3.6 ppm was assigned to the H_4 of 6-O-sulfated-chitin (6_S) units, while the proton at 4.4 ppm was assigned to the H_3 of the 3,6-O-disulfated-chitin (36_S) units. Therefore, the carbon peak at 79 ppm contained two types of carbons, the C_4 of 6_S units and the C_3 of 36_S units. The new emerging carbon peak at 75 ppm was assigned as the C_4 of 36_S units. This upfield shift is consistent with the upfield shift of the C_5 peak influenced by sulfation at the C6 position (Fig. 2). The carbon peak at 73 ppm comprised three types of carbons, C_3 and C_5 of 6_S units and C_5 of 36_S units. The C_2 at 55 ppm showed correlation with two types of protons at 3.7 and 3.9 ppm and assigned to H_2 at 6_S and 36_S units, respectively. The C_1 peak was found to split into two peaks due to its sensitivity to the change in chemical environment. The carbon peak at 99 ppm was assigned to the C_1 of 36_S units.

In Fig. 4b, the minor peak at 101 ppm in the ^{13}C NMR spectrum and 3.5–3.6 ppm in the ^{1}H NMR spectrum suggested the presence of trace amounts of un-substituted 6-O-sulfated sugar units. The carbon peaks of C_1–C_6 were found at 99, 55, 79, 76, 73 and 66 ppm, respectively. Again, as was found for 6-O-sulfated-chitin, in contrast with the sample with low D.S. (Fig. 4a), the carbon peaks of highly substituted 3,6-O-disulfated-chitin were comparatively narrow and the peak splits were nominal, indicating a more uniform and homogeneous structure.

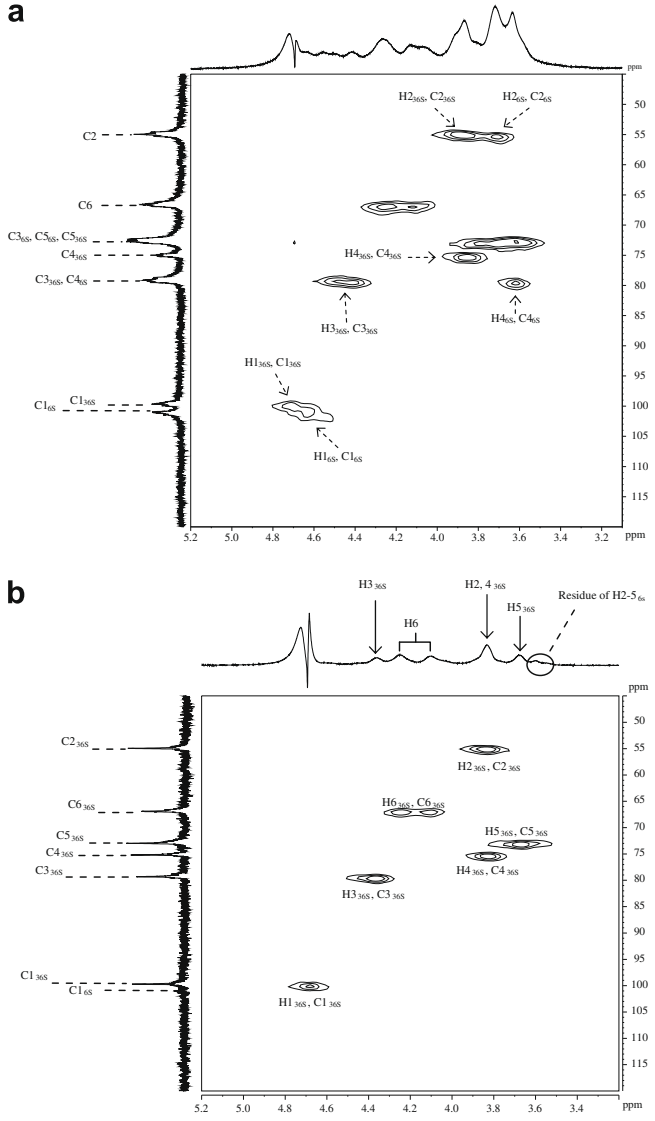


Fig. 4. HMQC spectrum of 3, 6-O-disulfated-chitin. (a) D.S. at C3 = 0.52; (b) D.S. at C3 = 0.91.

3.7. Structural elucidation of the H1 and CH_3 region of ^{1}H NMR

The majority of the proton signals in sulfated-chitins were aggregated in a narrow range, making them of little value to structural elucidation. However, the H_1 proton was well resolved from the other protons and is known to be sensitive to structural variations that could provide further details to the effects of sulfation on the structure of sulfated-chitins.

Fig. 5a presents the ^{1}H NMR spectra obtained at 353 K for the H1 region of sulfated-chitins with D.S. from 1 to 1.84. A higher temperature (353 K) was applied to resolve the H1 signal from that of the H_{OD} signal. The H1 of SC6-100 was at 5.28 ppm, typical of the H1 resonance of 6-O-sulfated-chitins with D.S from 0.53 to 1.0. Sulfation at the C6 position did not cause any splitting of the H1 signal. This may be due to the long distance between the H_1 and H_6, diminishing the effects of 6-O-sulfation on H_1.

A weak shoulder peak was observed in the 5.35–5.43 ppm region for SC36-5, assigned as the H1 resonance in the 36_S units. In contrast to 6-O-sulfation that had minimal effect on the position of the H1 in the NMR spectrum, 3-O-sulfation caused noticeable downfield shifts of the H1 even at very low D.S. The complicated multiple peaks in this region suggested a random distribution of the 36_S units in the polymer chain at the initial stage of 3-O-sulfation. Varum et al. reported that the C1 peak was not only sensitive to the structural change of the sugar ring, but the sequence of the neighboring sugar units (Varum, Anthonsen, Grasdalen, & Smidsrod, 1991). A similar relationship could also be applicable to the H1 peak here. When the D.S. at C3 was low, the 6S–36S sequence dominates the distribution sequence for 36_S units. As the percentage of 36S units in the polymer chain increases, the 36S–36S gradually becomes the dominating sequence. When the D.S. at C3 was above 0.50 (SC36-65, 84), the $H1_{36S}$ with two different sequences became well resolved at 5.37 and 5.43 ppm, respectively. Similarly, an increasing percentage of the 36S units also changed the distribution sequence of the $H1_{6S}$ from 6S–6S to 6S–36S. When the D.S. at C3 was above 0.50, the 6S–36S sequence became dominant and the intensity of the signal of the $H1_{6S-6S}$ at 5.28 diminished, whereas the signal at 5.22 representing the $H1_{6S-36S}$ became predominant.

Besides the H1 region, the CH_3 of the amide group was also investigated to study the effect of sulfation on structural changes. In contrast to the H_1, sulfation at the C6 position caused noticeable

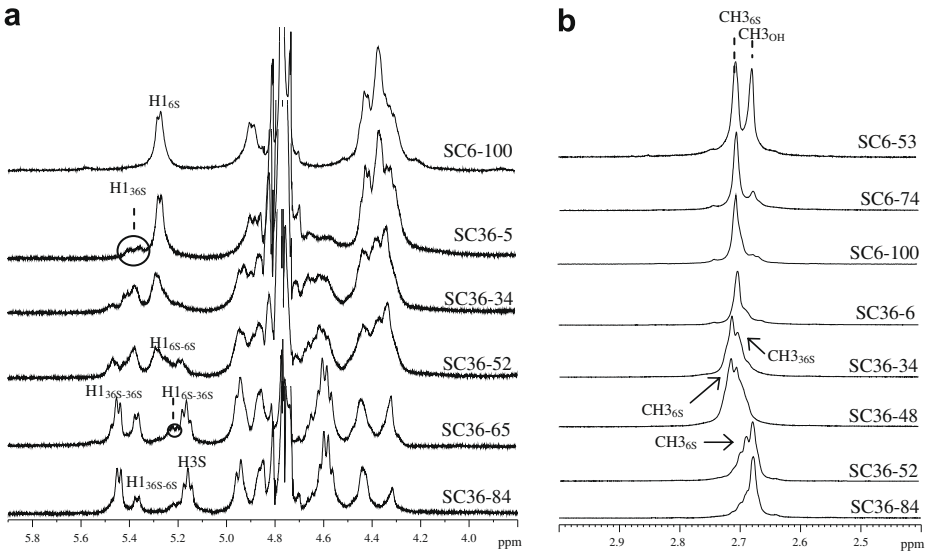


Fig. 5. ^{1}H NMR spectra at 353 K of sulfated-chitins with different D.S. values. (a) H1 region; (b) CH_3 region.

shifts of the H_{CH3}. In Fig. 5b, the resonance of the H_{CH3} in the 6S units ($CH3_{6S}$) and un-substituted pyranose units ($CH3_{OH}$) were observed at 2.71 and 2.68 ppm, respectively, for 6-O-sulfated-chitin. The intensities of the two types of peaks varied with the D.S. but the chemical shifts remained essentially, unchanged. The introduction of the 3-O-sulfate group not only changed the intensities of the $CH3_{6S}$ and $CH3_{36S}$ (resonance of H_{CH3} in the 36S units), but caused the chemical shifts to vary with the D.S. The resonances of the $CH3_{6S}$ and $CH3_{36S}$ peaks were at 2.713 and 2.705 ppm when the D.S. was below 0.50, whereas they were observed at 2.688 and 2.680 ppm, respectively, when the D.S. was above 0.50. The obvious upfield shift suggests that the chemical environment of sulfated-chitin changed sharply when the D.S. at C3 was around 0.50. This finding is consistent with what was observed in the H_1 region (Fig. 5a).

3.8. The effect of reaction conditions on structural integrity

In this study, gel permeation chromatography (GPC) was applied to investigate the effect of reaction conditions on the molecular weight of sulfated-chitins. The weight average molecular weight (M_w) of sulfated-chitins is summarized in Table 2. Due to the mildness of the sulfating reagent sulfur trioxide–pyridine complex, its effect on the molecular weight was not considered.

Table 2 shows that the M_w of the samples obtained at ambient or lower temperatures (SC6-53, SC6-74, 81 and 100) were similar, indicating that reactions at ambient or lower caused little chain scission even at prolonged reaction times. The average M_w (240,800 Da) of these 4 samples could be roughly regarded as the M_w of the original chitin.

The M_w of SC36-5, SC36-14 and SC36-34 obtained at 45, 55 and 65 °C, respectively, after 12 h of reaction decreased moderately by 1.7%, 3.8% and 7.0%, respectively. There was also little difference in the M_w of reactions conducted between 65 and 70 °C. Reactions at 75 °C resulted in a sharp decrease of M_w. SC36-65, SC36-91 and SC36-48 obtained from 6, 12 and 24 h reactions, respectively, at 75 °C had M_w decreases of 7.8%, 16.8% and 31.1%. This result shows that elevated temperature results in depolymerization of chitin during sulfation, the extent a function of the reaction time. In particular, chain scission was accelerated when the reaction temperature was above 75 °C. The mechanism of depolymerization could be thermal degradation or chain scission by weak acidity caused by the sulfating reagent.

It is worth noting that elevated temperature reactions also resulted in lower yields of sulfated-chitins. SC36-48 and SC36-52 obtained at 75 and 90 °C, respectively, gave yields of 69% and 77%, noticeably lower than those of samples obtained at lower temperatures. The decrease of yield could be attributed to the increasing

Table 2
The molecular weight of sulfated-chitins.

Product	Reaction conditions		$M_w \times 10^4$ (Da)	$M_n \times 10^4$ (Da)	Polydispersity	Yield (%)
	Temp (°C)	Time (h)				
SC6-53	8	36	24.11	9.84	2.45	92
SC6-74	r.t.	12	24.07	10.01	2.39	90
SC6-81	r.t.	24	24.20	9.89	2.46	95
SC6-100	r.t.	24	23.94	10.34	2.32	89
SC36-5	45	12	23.68	10.12	2.34	93
SC36-14	55	12	23.16	9.98	2.32	89
SC36-34	65	12	22.39	9.26	2.42	88
SC36-48	75	24	16.60	8.10	2.35	69
SC36-52	90	1	22.79	9.70	2.35	77
SC36-65	75	6	22.20	9.30	2.39	90
SC36-74	65	12	22.24	8.90	2.49	87
SC36-84	70	12	22.32	9.41	2.37	88
SC36-91	75	12	19.99	8.33	2.40	85

portion of low molecular weight sulfated-chitin that was eliminated during dialysis. Finally, it is noted that the polydispersity (M_w/M_n) for all the samples were around 2.50, typical of biopolymers.

3.9. Anticoagulation activity of sulfated-chitins

There have been many efforts extended to relate the relationship between the structure and activity of sulfated-chitins and sulfated-chitosans. In general, the presence of the sulfate group is essential for the anticoagulation activity, while the position of the sulfate group is critical for the anticoagulation activity. N-sulfated-chitosan and 3-O-sulfated-chitin showed no anticoagulant activity, while 3,6-O-sulfated-chitin and fully sulfated-chitosan were found to have a 2-fold and 45% anticoagulation activity, respectively, over that of heparin (Nishimura et al., 1998, 1986; Warner & Coleman, 1958). In contrast to the structure-activity relationship studies, the relationship between the degree of sulfation (D.S.) and anticoagulant activity is seldom mentioned as the preparation of sulfated-chitins with defined D.S. are not straightforward. We discuss here, the effects of D.S. on the anticoagulation activity of sulfated-chitins.

3.9.1. Activated partial thromboplastin time (APTT) and thrombin time (TT)

APTT and TT are the two most frequently used assays for monitoring heparin or heparinoid anticoagulation. In this study, heparin (Sigma, 181 IU/mg) was used as the standard to evaluate the anticoagulation activity of sulfated-chitins. To quantitatively evaluate the anticoagulation activity of sulfated-chitin, standard curves of heparin-APTT and TT were first obtained.

The dependence of APTT and TT to heparin concentration is plotted as Fig. 6. At low heparin concentrations (0–0.3 IU/ml), the relationship of APTT to heparin concentration shows good linearity, consistent with results reported by Hirano et al. (1985). However, as the concentration of heparin increases, the relationship deviates from linearity and became unsuitable for linear approximation as R decreases (0.9320 and 0.9781) obtained by exponential approximation.

The functions were expressed as:

$$T_{APTT} = 17.72e^{3.23C} + 16.11 \quad (R^2 = 0.9992)$$

$$T_{TT} = 3.34e^{5.91C} + 12.25 \quad (R^2 = 0.9971)$$

where T_{APTT} and T_{TT} represent the time for clot formation for APTT and TT assays, respectively, and C is the concentration of the heparin solutions. The anticoagulation activity of sulfated-chitin were first screened by APTT and TT assays and subsequently converted to heparin activity based on the two functions derived from the standard curves. The relationship of anticoagulation activity of sulfated-chitin with D.S is illustrated in Fig. 7.

Fig. 7 shows that anticoagulation activity of sulfated-chitins was closely related to the D.S. Generally, when the D.S. increased, there was a corresponding higher observed anticoagulation activity. This is consistent with the relationship of D.S. and anticoagulation activity for several other polysaccharides (Alban, Schauerte, & Franz, 2002; Hattori et al., 1998; Nardella et al., 1996). The anticoagulation activity of SC6-53 (D.S. = 0.53) with respect to APTT and TT was 6.67 and 16.9 IU/mg, respectively. However, the anticoagulation activity of SC36-91 (D.S. = 1.91) with respect to APTT and TT was 173.1 and 129.1 IU/mg, respectively, which was equivalent to 95.6% and 71.3% of heparin activity, respectively. The anticoagulation activity with respect to APTT was lower than that reported by Hirano et al. (331–379 IU/mg), while the anticoagulation activity with respect to TT was higher than their report (70–87 IU/ml) Hirano et al., 1985. The difference of the results could be attributed to the different instrumentation and reagents used and the different data processing method.

A more thorough evaluation revealed that sulfation at the C6 and C3 positions affected the anticoagulation activity differently. All 6-O-sulfated chitin derivatives showed very low anticoagulation activity. For example, the fully C6 sulfated sample SC6-100 (D.S. = 1.0) had heparin activity only equivalent to 8.8% and 12.4% with respect to APTT and TT. Therefore, the increase of D.S. at C6 did not contribute to noticeable increases of anticoagulation activity.

However, the further C3 sulfation of 6-O-sulfated-chitin greatly increased the anticoagulation activity of sulfated-chitins. The anticoagulation activity of SC36-91 was almost 10-fold and 6-fold of that of SC6-100, respectively, in APTT and TT. This indicates that the presence of 3,6-O-sulfate groups was critical for obtaining high anticoagulation activity. Moreover, Fig. 7 shows that the anticoagulation activity of sulfated-chitins had a sharp increase at the D.S. range of 1.34–1.65. The reason for the sharp change of anticoagulation activity could be attributed to the dramatic structural change at this D.S. range (Fig. 5).

In Fig. 5a, the intensity of proton (H1A) representing the 36S–36S sequence increased dramatically at the range 1.34–1.65. Therefore, we speculate that instead of single 3,6-O-sulfate groups (abbreviated as 36S), continuous 36S–36S sequences were critical in high anticoagulation activity for sulfated-chitins. When the D.S. at C3 was low (0.05–0.34), the 36S was the minor group randomly distrib-

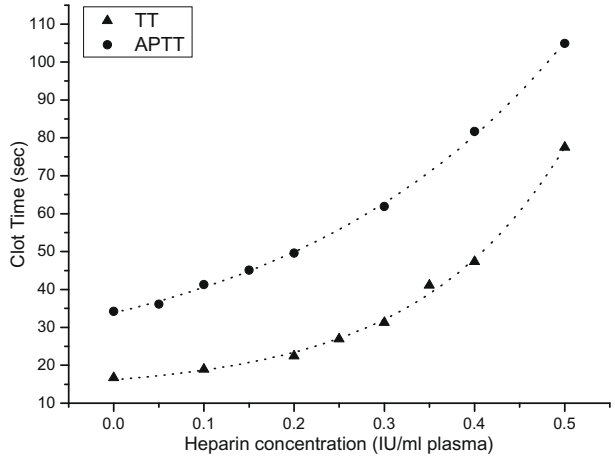


Fig. 6. The dependence of APTT and TT on heparin concentration.

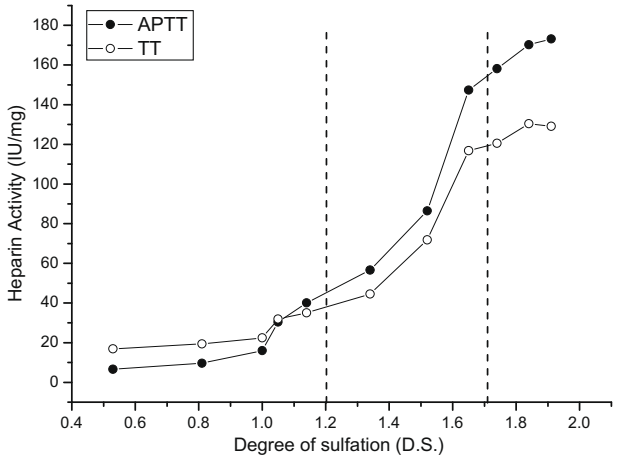


Fig. 7. The relationship of anticoagulation activity of sulfated-chitins with D.S.

uted on the polymer backbone (SC36-5, 34, Fig. 5a). The 36S–6S was therefore the primary sequence on the polymer backbone, while the 36S–36S was the secondary sequence. Due to the low content of 36S group in this D.S. range, the 36S–36S sequences were nominal and only a negligible increase in anticoagulation activity was obtained. When the D.S. at C3 increased, the ratio of 36S–36S sequence on the polymer backbone gradually increased. In particular, when the D.S. at the C3 position was around 0.5, the 36S–36S sequence dramatically accelerates because 36S is now the dominant group. Finally, at the high range of D.S. at the C3 position (0.74–0.91), the anticoagulation activity of sulfated-chitin again slowly increased due to the slow increase of 36S–36S sequences. Combining this result and the report by Nishimura et al. that 3-O-sulfate-chitin almost had no anticoagulation activity, we conclude that single 6-O-sulfated or 3-O-sulfated-chitin does not yield the potent anticoagulant (Nishimura et al., 1998). Only when there were many 36S–36S sequences, did sulfated-chitin show a high anticoagulation activity. However, it remains unclear the number of 36S units in this sequence. It is noteworthy that this finding is consistent with previous report that a specific penta-saccharide sequence in heparin is critical for its anticoagulation activity due to its strong affinity for binding antithrombin III (Weitz, 1997; Simmonneau et al., 1997).

3.9.2. Fibrinogen time (FT) and prothrombin time (PT)

The measure of the fibrinogen concentration could be considered as a toxicity evaluation of the sulfated-chitin while the PT test was conducted to determine whether the anticoagulant activity followed the extrinsic pathway. Fig. 8 shows that all sulfated-chitins did not cause noticeable shortening of fibrinogen time. The maximum difference of fibrinogen time between sulfated-chitins and the control (blank plasma) was less than 2%. Therefore, sulfated-chitin can be considered to have little effect on fibrinogen concentration in plasma. The prolonged APTT and TT for sulfated-chitins were therefore attributed to their heparinoid behavior. Similarly, the PT was not sensitive to sulfated-chitin. The maximum PT difference between sulfated-chitin and control blank sample was less than 3%. This could be explained by the fact that sulfated-chitin, as a heparinoid, mainly affects the intrinsic pathway and therefore has little effect on the assays reflecting extrinsic pathway.

4. Conclusion

This paper presents carefully prepared and characterized sulfated-chitins whose anticoagulant reactivity can be specifically related to the structure of the material. 6-O and 3,6-O-sulfated-chitins with D.S. ranging from 0.53 to 1.91 were prepared under mild and homogeneous conditions, in a controllable manner in 5% LiCl/DMAc solvent system. Sulfation at room temperature yielded only mono substituted 6-O-sulfated-chitins whereas elevated temperatures gave 3,6-O-disulfated-chitins. Sulfation at the two positions resulted in different effects on the structural features of sulfated-chitins, the C3 position being more subject to structural variation than the 6-O position. At low D.S., both the 6-O and 3,6-O-sulfated-chitins showed structural heterogeneity that eased as the D.S. increased becoming, more homogeneous and uniform. The H1 and CH3 NMR signals of the amide group were useful for evaluating the progress of sulfation qualitatively as well as estimating the distribution of sulfated groups on the polymer chain. There was a sharp change of chemical environment at the C3 position when the D.S. was around 0.50. This work presents the first reports using HMQC NMR spectra for supporting and assigning the partially substituted sulfated-chitin derivatives. Anticoagulant activity of the prepared sulfated-chitins correlated closely with D.S. The higher the D.S. yielded, the better the anticoagulant activity. In particular, the continuous sequence of 36S units was critical for obtaining high anticoagulation activity. The highest anticoagulation activity of 3,6-O-sulfated-chitin (D.S. = 1.91) with respect to APTT and TT was equivalent to 95.6% and 71.3% of heparin activity, respectively.

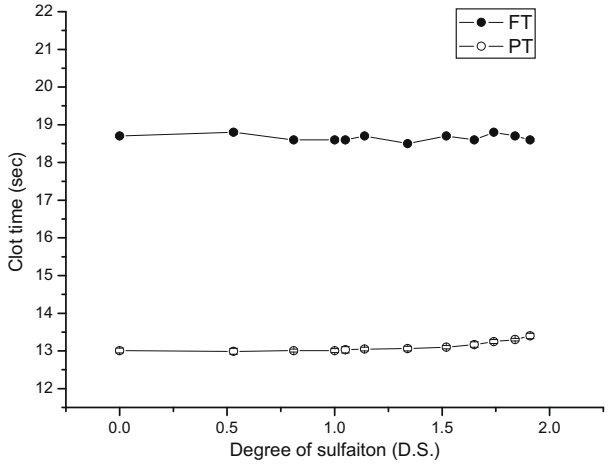


Fig. 8. The relationship of FT and PT of sulfated-chitins with degree of sulfation (D.S.).

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