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Sulfonation of papain-treated chitosan and its mechanism for anticoagulant activity

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ABSTRACT

The novel low-molecular-weight chitosan polysulfate (MW 5120–26,200 Da) was prepared using the depolymerization of chitosan with papain (EC. 3.4.22.2). The sulfonation of depolymerized products was performed using chlorosulfonic acid in *N,N*-dimethylformamide under semi-heterogeneous conditions. The structures of the products were characterized by FTIR, ^{13}C NMR, and ^{1}H NMR (1D, 2D NMR) spectroscopy. The present study sheds light on the mechanism of anticoagulant activity of chitosan polysulfate. Anticoagulant activity was investigated by an activated partial thromboplastin assay, a thrombin time assay, a prothrombin time assay, and thrombelastography. Surface plasmon resonance also provided valuable data for understanding the relationship between the molecular binding of sulfated chitosan to two important blood clotting regulators, antithrombin III and heparin cofactor II. These results show that the principal mechanism by which this chitosan polysulfate exhibits anticoagulant activity is mediated through heparin cofactor II and is dependent on polysaccharide molecular weight.

1. Introduction

Chitosan, a deacetylated analog of chitin, is the second most abundant natural biopolymer and is a linear copolymer of β-(1→4)-linked GlcN and GlcNAc units.[1] Chitosan is a biocompatible, biodegradable, and low toxicity material with high cationic potential.[2] A variety of structural products can be prepared through modification of the three functional groups present in the parent polymer. Such modification products can have a differing degree of functionalization of these groups,[3] and can exhibit anti-viral, anti-bacterial, anti-inflammatory, and anticoagulant activities.[3–6] Among other modified chitosans, sulfonated chitosan has been well studied for its potential anticoagulant activity.[5,7] Chitosan polysulfate shows anticoagulant activity due to sulfo group substitution at C-2, C-3, and C-6 of the glucosamine residue, which are key positions in the glucosamine residue of heparin that are substituted with sulfo groups.[3] Thus, it is of interest to investigate if similarly sulfonated chitosan, possessing an identical array of negative charges, would itself be an excellent anticoagulant.

The main mechanism by which unfractionated heparin (UFH) (MW ~10,000–25,000) exerts its anticoagulant effect is by accelerating the plasma serine proteinase inhibitor, antithrombin III (ATIII),[8] inactivation of factor Xa and thrombin (Factor IIa). Heparin needs at least 18 saccharides to enhance ATIII inhibition of thrombin.[9,10] ATIII inactivation of the serine proteinase, factor Xa, requires only a heparin pentasaccharide. Thus, low-molecular-weight heparin (MW 5000–8000), containing this specific sequence, produces an anti-factor Xa effect comparable to UFH.[9,11] A second mechanism by which UFH can specifically inactivate thrombin is through its binding to a second serine protease inhibitor, heparin cofactor II (HCII). Alternative drugs for UFH are in increasing demand due to a shortage of heparin caused by its recent contamination.[12,13] Chitosan polysulfate was investigated as a possible alternative anticoagulant, and its mechanism of action was studied.

The anticoagulant activity of sulfonated polysaccharides is influenced by molecular weight, structure, and the presence and

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position of sulfo groups.[14,15] According to previous studies, chitosan polysulfate shows anticoagulant activity that is related to its molecular weight.[3,16,17] Vikhovera et al., showed that decreasing molecular weight can result in higher anti-factor Xa activity,[17] and Vongchan et al., suggested that inconsistencies in these data might be due to the other anticoagulant effects of chitosan polysulfate.[3] There are few reports available in the literature, and their inconsistent results require additional studies on the mechanism of the anticoagulant activity of sulfonated chitosan.

We hypothesized that the mechanism of the anticoagulant activity of chitosan polysulfate was more complex than that which could be explained from a simple ATIII-mediated reaction. A convenient, mild,[20] and inexpensive procedure relying on papain was used to partially depolymerize chitosan, which was then sulfonated. Three low-molecular-weight (LMW) chitosan polysulfates having molecular weights ranging from 5120 to 26,200 Da were characterized by FTIR, ^{13}C NMR, ^{1}H NMR, and 2D NMR spectral analyses. Anticoagulant activity was determined by activated thromboplastin time (APTT), thrombin time (TT), prothrombin time (PT), and thrombelastography (TEG). Surface plasmon resonance (SPR) was used to understand the interaction of LMW chitosan polysulfates with ATIII and heparin cofactor II (HCII).

2. Results and discussion

2.1. Structural analysis of LMW chitosan polysulfates

Two reduced molecular weight chitosans were produced by papain degradation from native chitosan using modified Kumar's method[18] in 5 h and 12 h, respectively. The sample was degraded for 5 h and then sulfonated using a modified Gamzazade's method,[19] fractionated by charge then size using a DOWEX® MARATHON® column and a Bio-Gel P-30 column. Then the three LMW chitosan polysulfate fractions were collected. These five samples, the two reduced molecular weight chitosans and the three LMW chitosan polysulfates, were characterized by GPC, and IR and NMR spectroscopy.

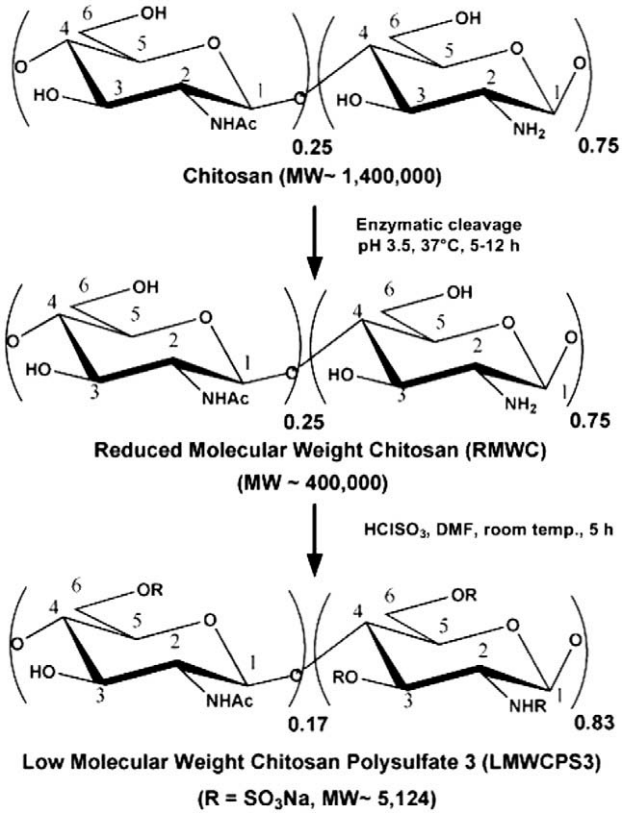
The molecular weight of native chitosan and the two reduced molecular weight chitosans were determined by gel-permeation chromatography (GPC) (Table 1). On degradation with papain for 5 h, the molecular weight was reduced by over threefold compared to that of native chitosan (Scheme 1). However, papain degradation for 12 h failed to reduce the molecular weight any further.[18,20,21] The molecular weight of the three LMW chitosan polysulfates determined by polyacrylanide gel electrophoresis (PAGE)[22] was reduced when compared to their LMW chitosan precursor. Acid hydrolysis taking place during the sulfonation process might explain this large reduction in molecular weight. Interestingly, the three LMW chitosan polysulfates had low polydispersity when compared to their precursor.

IR spectra (Fig. 1) showed the appearance of characteristic S=O and S–O bond-stretching absorptions at 1265, 1235, 1067, and 807 cm^{-1}, respectively, consistent with the sulfonation of LMW chitosan.

Table 1
Molecular weight of chitosan and sulfated chitosan[a]

Product	$\overline{M_n}$	$\overline{M_w}$	Polydispersity
Native chitosan	3.30×10^5	1.40×10^6	4.2
RMWC (5 h)	8.60×10^4	4.00×10^5	4.7
RMWC (12 h)	8.10×10^4	3.50×10^5	4.3
LMWCPS1	1.90×10^4	2.62×10^4	1.38
LMWCPS2	1.32×10^4	1.74×10^4	1.32
LMWCPS3	5.55×10^3	5.12×10^3	0.92

[a] Abbreviations include the following:
RMWC: reduced molecular weight chitosan; determination by GPC.
LMWCPS: low molecular weight chitosan; determination by PAGE.
$\overline{M_n}$: number-average molecular weight.
$\overline{M_w}$: weight-average molecular weight.



Scheme 1. Preparation of low-molecular-weight chitosan polysulfate.

1D and 2D NMR spectroscopy was next performed on LMW chitosan polysulfate (fraction 1) (Fig. 1B, C and Fig. 2A, B). Repeating residues afford relatively simple NMR spectra. The ^{1}H spectra for LMW chitosan polysaccharides (fractions 1 (Fig. 1B), 2, and 3 (not shown) were assigned based on ^{1}H–^{1}H COSY and HMQC, and the assignment is presented in Table 2. Two residues were observed in the NMR spectra, corresponding to *N*-sulfoglucosamine (GlcNS) and *N*-acetylglucosamine (GlcNAc). The chemical shifts of H-2, H-3 and H-6 of GlcNS were observed at 3.41, 4.59, and 4.26 ppm, respectively, demonstrating that positions O-3 and O-6 were fully sulfonated. Interestingly, the spectra indicated that the O-3 position of GlcNAc was unsubstituted. Interestingly, only the H'-6 of the GlcNAc residues shifted upfield, indicating that the O-3 position of GlcNAc was not sulfated. The signals corresponding to GlcNS were dominant in the NMR spectra, demonstrating that the LMW chitosan polysulfates were primarily N-sulfonated. The ^{13}C NMR showed a similar integrated ratio (Fig. 1C).

2.2. Anticoagulant activity

The preliminary purpose of this investigation was to examine the effect of molecular weight of LMW chitosan polysulfates on anticoagulant activity. The anticoagulant activities of the three LMW chitosan polysulfate fractions were next studied by a battery of in vitro assays, including aPTT, TT, and PT and TEG assays to characterize the different stages of the coagulation process (Table 3).[16] The aPTT assay measures the activity of all coagulation factors in the intrinsic pathway,[23] the TT assay screens fibrin polymerization process measuring the formation time of fibrin from fibrinogen after the addition of known amounts of thrombin to the plasma sample, and the PT assay measures the activity of the extrinsic pathway.

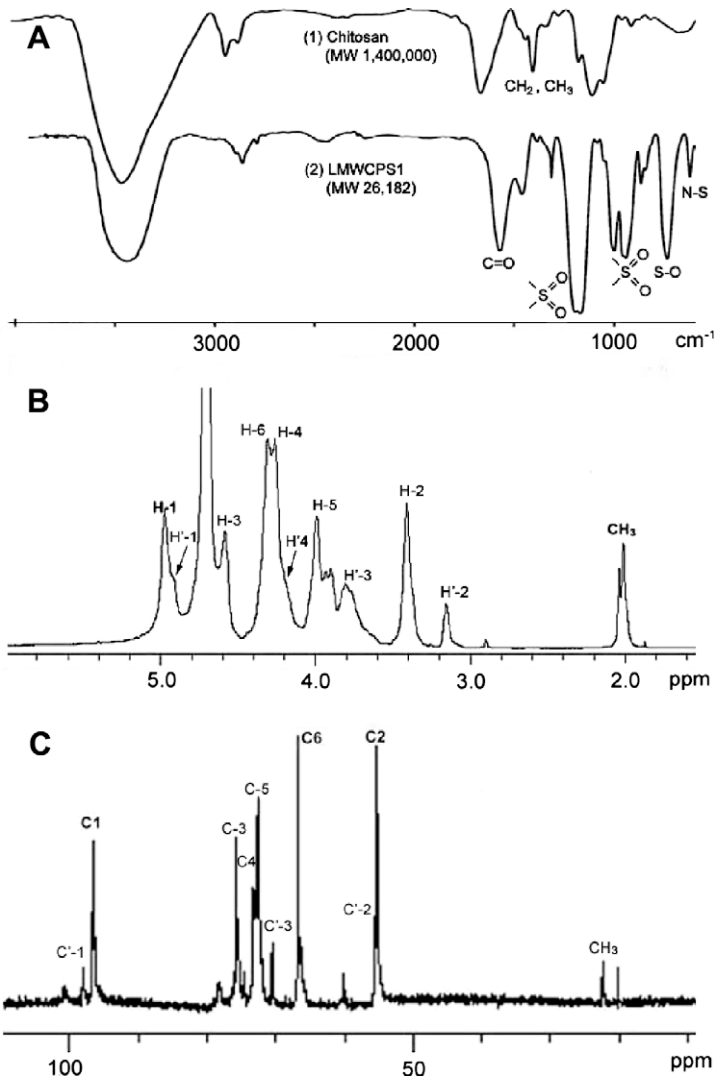


Figure 1. Characterization of LMWCPS1 (A) IR spectra of (1) chitosan; (2) LMWCPS1 (B) ^{1}H NMR spectrum. (C) ^{13}C NMR spectrum.

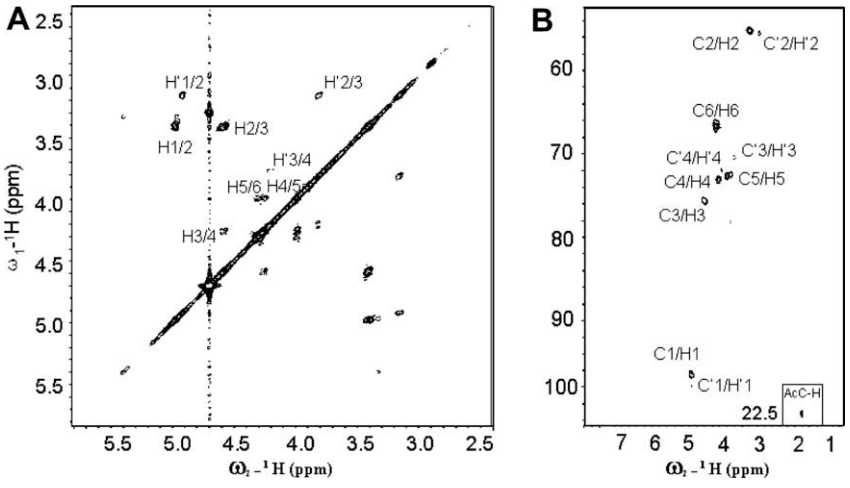


Figure 2. 2D NMR spectrum of LMWCPS1 (A) ^{1}H–^{1}H COSY of LMWCPS1. Two series of assignments, H is the residue of *N*-sulfoglucosamine (GS); H′ is the residue of *N*-acetylglucosamine (GA); (B) HMQC of LMWCPS1. Two series of assignments, C/H is residue of *N*-sulfoglucosamine (GS); C′/H′ is residue of *N*-acetylglucosamine (GA).

The PT study showed that none of the LMW chitosan polysulfate fractions had an effect on PT (Table 3). Heparin and heparinoids typically interfere with the last step of the intrinsic coagulation pathway and show no effect on the extrinsic pathway. The lowest

Table 2
The ^{1}H and ^{13}C chemical shifts of the LMWCPS

	^{1}H NMR			^{13}C NMR	
	GS	GA		GS	GA
H1	4.98	4.91	C1	96.5	97.8
H2	3.41	3.15	C2	55.2	55.6
H3	4.59	3.81	C3	75.6	70.5
H4	4.29	4.20	C4	73.1	72.1
H5	3.98	3.98	C5	72.7	72.7
H6	4.26	4.26	C6	66.6	66.6
	83%	17%			

GS: *N*-sulfoglucosamine; GA: *N*-acetylglucosamine; NMR solvent: D_2O; NMR reference frequency: 600 MHz.

molecular weight fraction of LMW chitosan polysulfate showed the greatest increase in anticoagulant activity as assessed by aPTT (Table 3).[3,16,17,24] In contrast, the higher molecular weight fractions of LMW chitosan polysulfate showed the greatest anticoagulant activity as assessed using the TT assay. A previous study showed that chitosan polysulfate with size range of 8–10 kDa had a higher anti-Xa:anti-IIa ratio than chitosan polysulfate in the size range of 15–30 kDa.

Next, we examined the size dependency of TEG clotting inhibition of LMW chitosan polysulfates using normal human blood. In standard TEG tracings, MA measures clot strength, which is dependent on both fibrinogen level and platelet function. Liquid whole blood transmits little or no torque in TEG, producing no amplitude on the TEG tracing, even in blood samples with high viscosity. The MA of the TEG tracing increases with higher levels of fibrinogen and greater platelet number in the blood sample.[25] The data presented in Table 3, obtained with the whole blood TEG assay with LMW chitosan polysulfate fractions, show that higher molecular weight fraction effectively inhibits clot strength, the *R* and *k* values increased and large α values were obtained. In contrast, the lower molecular weight fractions were much less effective. Each LMW chitosan polysulfate fraction showed a dose-dependent inhibition (data not shown). The lower molecular weight of chitosan polysulfate affords more prolonged the plasma clotting time based on aPTT, but the whole blood TEG assay showed the same results as plasma TT test. This suggests that LMW chitosan polysulfate has the ability to inhibit a platelet/fibrin clot. Although chitosan polysulfate reportedly inhibits the coagulation cascade by forming a complex with antithrombin III,[3] the results of our TT and TEG experiments suggest other activities that potentially affect thrombin.

2.3. Characterization of binding of ATIII and HCII to LMW chitosan polysulfate

The anticoagulant activities of glycosaminoglycans can be mediated by both ATIII and HCII, which are the members in the 'serpin' family.[8] When binding with negatively charged glycosaminoglycans, ATIII and HCII inhibit proteases involved in coagulation.[26] ATIII particularly inhibits thrombin (Factor IIa) and factor Xa, whereas HCII only inhibits thrombin (Factor IIa). Our plasma and whole blood assays suggest that the mechanism by which LMW chitosan polysulfate exhibits anticoagulant activity involves more than simply ATIII. SPR experiments were undertaken to investigate the effect of the molecular weight of LMW chitosan polysulfate on the key inhibitors of coagulant cascade, ATIII and HCII. Biotinylated LMW chitosan polysulfates were immobilized onto streptavidin (SA) sensor chips through the strong, non-covalent biotin–streptavidin interaction. Various concentrations of proteins were then flowed over these chips. Sensorgrams of ATIII binding to LMW chitosan polysulfates showed a slow climb in RU signal during the association phase, corresponding to a modest on-rate (k_{on}), and a gradual signal decrease during the dissociation phase corresponding to slow off-rate (k_{off}), resulting in weak binding between ATIII and the LMW chitosan polysulfates, corresponding to K_d values of 8–58 μM (Fig. 3A–C). However, the LMW chitosan polysulfate–HCII interaction exhibited a faster on-rate with comparable off-rates, giving a stronger binding corresponding to K_d values of 1–2 μM for the two larger LMW chitosan polysulfates. Interestingly, the smallest LMW chitosan polysulfate bound very weakly to HCII with a $K_d > 100$ μM. The differences in the HCII binding affinity of LMW chitosan polysulfate fractions 1, 2, and 3 are reflected in the TT and TEG values observed for these fractions (Fig. 3D–F). Interactions with both ATIII and HCII were concentration dependent. The binding kinetics fit well to a 1:1 Langmuir binding model.[27] The results, including kinetic analysis k_{on}, k_{off}, and the binding affinity (K_d), are presented in Table 4.

Chitosan polysulfate does not exhibit consistent ATIII-mediated inhibition of anticoagulant activity.[3,17] Our present study sheds light on the mechanism of chitosan polysulfate and its anticoagulant activities. The binding exhibited by the three LMW chitosan polysulfate fractions shows SPR curves consistent with monophasic binding to ATIII and gives kinetics that fit a 1:1 Langmuir binding model. In contrast, heparin shows biphasic binding, consistent with an induced conformational change in the binding of heparin to ATIII.[27] The conformation change in ATIII binding with heparin is reportedly important in ATIII-mediated inhibition of many serine proteases.[28] Although LMW chitosan polysulfate showed structural similarities to heparin, especially the presence of a 3-*O*-sulfo glucosamine residue, which is critical for heparin's ATIII-mediated anticoagulant activity,[29,30] heparin is more heterogeneous in chemical structure than chitosan polysulfate and also more specific in its interaction with ATIII. Moreover, a unique pentasaccharide sequence in the heparin chain is crucial for the high-affinity binding to ATIII.[11,30]

In summary, the current study suggests that the main mechanism of anticoagulant activity of chitosan polysulfate is mediated by HCII and is molecular-weight dependent, which is in agreement with previous reports.[3] Furthermore, chitosan polysulfate appears to show direct inhibition of thrombin activity, and while binding

Table 3
PT, aPTT, TT, and TEG for LMWCPS

Products	PT[a,b] (INR)	aPTT [a,c]	TT[a,c]	TEG [d]			
				R (min)	*k* (min)	α (angle)	MA (mm)
PPP	0.92	24.8	12.1	8.0	2.2	38.8	64.0
LMWCPS1	0.88	40.3	19.7	39.8	21.8	10.6	27.4
LMWCPS2	0.98	39.4	16.1	22.6	10.4	20.5	38.6
LMWCPS3	0.86	51.7	12.6	14.8	3.3	48.0	59.1

[a] Each value represents the mean of duplicate of three independent determinations and used normal human platelet poor plasma.
[a,b] Products used in PT assay were 0.5 μg/reaction.
[a,b,c] Products used in aPTT and TT assays were 1 μg/reaction; PPP, platelet-poor plasma.
[d] Measurement in singlicate using whole blood at 10 μg/reaction.

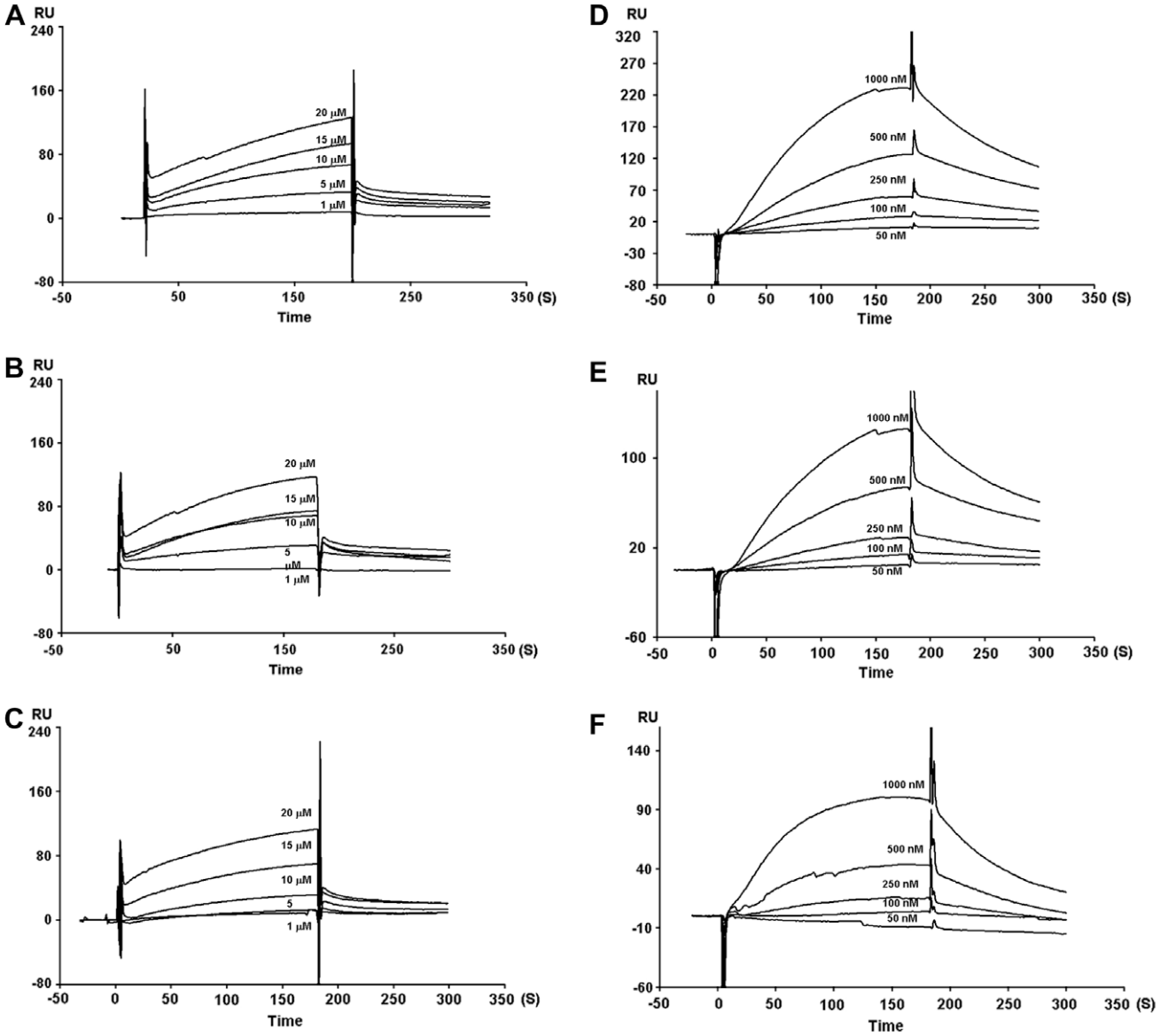


Figure 3. Sensorgrams showing the interaction of ATIII (20, 15, 10, 5, 1 μM) with (A) LMWCPS1; (B) LMWCPS2; (C) LMWCPS3 and the interaction of HCII (1000, 500, 250, 100, 50 nM) with (D) LMWCPS1 ; (E) LMWCPS2; (F) LMWCPS3.

Table 4
Kinetic parameters for antithrombin III (ATIII) and heparin cofactor II (HCII) with LMWCPSs using surface plasmon resonance (SPR) sensorgraphy

Protein	Products	k_{on} (1/*MS*)	k_{off} (1/*S*)	K_D (M)
ATIII	LMWCPS1	217	1.69×10^{-3}	7.82×10^{-6}
	LMWCPS2	382	2.22×10^{-3}	5.8×10^{-5}
	LMWCPS3	204	2.63×10^{-3}	1.29×10^{-5}
HCII	LMWCPS1	4420	7.25×10^{-3}	1.64×10^{-6}
	LMWCPS2	4070	7.37×10^{-3}	$1.81 \times \times 10^{-6}$
	LMWCPS3	157	0.0172	1.10×10^{-4}

to ATIII, fails to produce a conformational change critical for the action of this serpin on Factor Xa and thrombin. It should be pointed out that no in vivo evaluation of these LMW chitosan polysaccharides has been performed. The activity of these heparinoids resembles that of oversulfated chondroitin sulfates that recently caused deaths in patients receiving contaminated heparin.[12,13] Thus, caution should be exercised in evaluating the in vivo activity of these agents.

3. Materials and methods

3.1. Materials

Chitosan, obtained from marine crab shell and that had a degree of deacetylation (DA) of $\geqslant 0.75$ and average molecular weight of 1.4×10^6, was purchased from Sigma–Aldrich Co. Chemical, St. Louis, MO, USA. *N*,*N*-Dimethylformamide (DMF) was distilled under reduced pressure over calcium hydride (CaH_2).

All commercial reagents were of analytical grade and were used without further purification. The following reagents were used for anticoagulant activity determination: Control Plasma N (Dade Behring), Thromborel® S (Dade Behring), Test thrombin reagent (Dade Behring), Dade® Actin® FS Activated PTT Reagent (Dade Behring). Heparin sodium (5000 IU/mL) was purchased from LEO Pharmaceutical Products Bullerup, Denmark. Papain (2X crystallized, lyophilized powder from *Carica papaya* latex, EC. 3.4.22.2) was purchased from Sigma Chemical Co., St. Louis, MO, USA. ATIII was purchased from HYPHEN Biomed, Neuville-sur-Oise, France. HCII was

purchased from Haematologic Technologies, Essex Junction, VT, USA. Amine-PEO3-Biotin was from Pierce, Rockford, IL, USA. The sensor SA chips were from BIAcore (Biacore AB, Uppsala, Sweden).

3.2. Preparation of low-molecular-weight chitosan

Reduced molecular weight chitosan was prepared via a modification of the method of Kumar et al.[18] Chitosan was dissolved in 1% HOAc with pH 3.5, and was treated with papain in the ratio of 100:1 (w/w) before incubating for different periods at optimal conditions. The reaction was stopped by heat denaturing and the pH of the reaction mixture was adjusted to 7.0 using 10% w/v NaOH. The precipitate obtained was washed with distilled water followed by absolute methanol.

3.3. Preparation of low-molecular-weight chitosan polysulfate

Sulfonation of LMW chitosan was performed according to the method of Gamzazade et al., with several modifications.[19] $HClSO_3$ (4.5 mL) was added dropwise with stirring to 30 mL of *N*,*N*-dimethylformamide (DMF) dropwise with stirring which had been previously cooled to 0–4 °C. The reaction mixture was then stirred without cooling until the solution reached room temperature. One gram of solvent including RMWC was added to the sulfonating reagent, and the reaction mixture was stirred overnight at room temperature. The polymer was neutralized with 20% NaOH and precipitated in cold MeOH. The precipitate obtained was dissolved and dialyzed using a 3500 molecular weight cut-off membrane against distilled water for 48 h and then freeze-dried.

3.4. Separation of low-molecular-weight chitosan polysulfate

The crude LMW chitosan polysulfate was applied to a DOWEX® MARATHON® [Sigma] column and equilibrated with distilled water. The column was eluted with 15–20% NaCl at a flow rate of 20 mL/h. Fractions of 2 mL were collected and assayed by spectrophotometry at 210 nm and using a dye-binding method relying on 1,9-dimethylene blue.[11] Each fraction of LMW chitosan polysulfate diluted with NaCl solution from the DOWEX® MARATHON® column was applied to a Bio-Gel P30 column (Bio-Rad) and equilibrated with distilled water. The flow rate of the column was 20 mL/h. Fractions of 2 mL were collected and assayed by the dye-binding method and by spectrophotometry.

3.5. Gel-permeation chromatography (GPC)

Molecular weights of chitosan were measured by GPC on a PL-110 column (Polymer Laboratories) using NaOAc buffer (0.5 M HOAc containing 0.2 M NaOAc, pH 4.5) as the mobile phase with detection by a refractive index (RI) detector. Pullulans (MW 5800–788,000) were used to calibrate the GPC column.

3.6. Polyacrylamide gel electrophoresis (PAGE)

The average molecular weight of LMW chitosan polysulfates obtained after gel-permeation chromatography was estimated by PAGE. Aliquots containing 5–15 μg of LMWCPS obtained by Bio-Gel P30 chromatography were dissolved in the buffer solution pH 8.3, which consist of 100 mM Tris base, 100 mM boric acid, 2 mM EDTA and 2 M sucrose and 0.2% bromphenol blue, which used as the tracking dye. Heparin oligosaccharides were used as molecular weight markers.[22] The solutions were subjected to electrophoresis on 1.5-mm-thick 15% polyacrylamide gel (16 × 20 cm) in 100 mM Tris base, 100 mM boric acid, 2 mM EDTA pH 8.3. The gel was stained with 0.3% alcian blue in 3% HOAc.

3.7. Fourier-transform infrared spectroscopy (FTIR)

The LMW chitosan polysulfate fractions were dried overnight at 60 °C under reduced pressure. The dried sample was mechanically well blended with 100 mg of KBr and pressed on a vacuum-connected disk instrument at 10 tons/inch^2 for 5 min. The thickness of the KBr disc was 0.5 mm. The IR spectrum was recorded using air as the reference.

3.8. Nuclear magnetic resonance (NMR)

NMR samples (10 mg) were dissolved in 0.5 mL of D_2O (99.96%, Sigma) and freeze-dried three times to remove the exchangeable protons. The samples were re-dissolved in 0.5 mL D_2O. Spectra were recorded at 300 K on Bruker Avance II 600 MHz spectrometers equipped with cryogenically cooled HCN-probes with *z*-axis gradients. ^1H, ^{13}C NMR, COSY and HMQC spectra were recorded on LMWCS.

3.9. Anticoagulant assays

The anticoagulant activities of LMW chitosan polysaccharides were determined in vitro by aPTT, TT, and PT assays to characterize different stages of the coagulation cascade, according to a method provided by Sysmex Corporation, Kobe, Japan. The reactions were performed using citrated human platelet-poor plasma prepared from healthy adults by a standard protocol. Briefly, the Vacuette (Greiner & Sohne, Kremsmunster, Austria) contains 3.2% buffer trisodium citrate and 3.15 mL of blood to each tube. The citrate/blood ratio was 1:9, before any assay. Various concentrations of LMW chitosan polysulfate and standard therapeutic heparin (Heparin sodium 5000 IU/mL, LEO Pharmaceutical Products, Ballerup, Denmark) were added to platelet-poor plasma at various final concentrations. The aPTT, TT, or PT values at various sample concentrations were measured in parallel with a saline control. The specific anticoagulant activities of LMW chitosan polysulfate were evaluated by linear fit to a standard curve. All assays were performed in duplicate and repeated at least three times on different days.

3.10. Thrombelastography

Blood samples from healthy donors were obtained using the same Vacuette system. Citrated whole blood was spiked with heparin or LMWCPS to final concentrations of 0–0.2 μg/mL or 0–100 μg/mL. TEG was performed on Thromboelastograph® (TEG®) Hemostasis Analyzer TEG-5000 series (Hemoscope, Skokie, IL) according to the manufacturer's instructions and as reported by Mousa et al.[31] All disposable supplies were purchased from Hemoscope. Briefly, 340 μL of citrated blood was mixed with 20 μL of $CaCl_2$ solution in a plain plastic cup. The reaction started immediately after mixing. A second set of TEG was performed in plain plastic cup in which LMW chitosan polysulfate was included; 330 μL of blood was mixed with 10 μL of LMW chitosan polysulfate, followed by the addition of 20 μL of $CaCl_2$. TEG assay results were quantified according to the time a detectable clot was formed designated as *R* for reaction time. When there were no values given owing to blood samples that would not form a clot, the test was terminated at 60 min or beyond. With these values, the effects of LMW chitosan polysulfate on TEG clotting were determined.

3.11. Biotinylation of heparin, and LMW chitosan polysulfate for SPR studies

Heparin and LMW chitosan polysulfate were immobilized to the streptavidin (SA) sensor chip surface by noncovalent capture of

biotin to streptavidin. The biotinylated LMW chitosan polysulfate was prepared by reaction of Amine-PEO3-Biotin with a free carboxyl group. Heparin, LMW chitosan polysulfate (2 mg), and Amine-PEO3-Biotin (2 mg) were dissolved in 200 μL of H_2O and 10 mg of $NaCNBH_3$ was added. The reaction mixture was heated at 70 °C for 24 h, after which time an additional 10 mg of $NaCNBH_3$ was added, and the reaction mixture was heated at 70 °C for another 24 h. After cooling, the mixtures were desalted using centrifuge filter (YM-3, Millipore, Bedford, MA). Biotinylated samples were collected, freeze-dried, and used for SA chip.

3.12. Affinity and kinetic study of the interaction of protein with LMW chitosan polysulfate

Different concentrations of protein (ATIII in HBS-EP running buffer, HCII in PBS) were injected at a flow rate of 30 μL/min over a chip with LMW chitosan polysulfate immobilized flow cells at 25 °C. A kinetic injection mode was used, leading the protein to flow for 3 min and to be dissociated for the next 3 min. The surface was generated by injection of 30 μL of 2 M NaCl, followed by 30 μL of NaOH (50 mM) containing 1 M NaCl. The response was monitored as a function of time (sensorgram).

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References

1. Robert, G. A. F. *Chitin Chemistry*; McMillan: Houndmills and London, 1992.
2. Kweon, D. K.; Song, S. B.; Park, Y. Y. *Biomaterials* **2003**, *24*, 1595–1601.
3. Vongchan, P.; Sajomsang, W.; Subyen, D.; Kongtawelert, P. *Carbohydr. Res.* **2002**, *337*, 1239–1242.
4. Nishimura, S. I.; Kai, H.; Shinada, K.; Yoshida, T.; Tokura, S.; Kurita, K.; Nakashima, H.; Yamamoto, N.; Uryu, T. *Carbohydr. Res.* **1998**, *306*, 427–433.
5. Jayakumar, R.; Nwe, N.; Tokura, S.; Tamura, H. *Int. J. Biol. Macromol.* **2007**, *40*, 175–181.
6. Jordan, J. L.; Henderson, S.; Elson, C. M.; Zhou, J.; Kydonieus, A.; Downie, J.; Lee, T. D. *Urology* **2007**, *70*, 1014–1018.
7. Franz, G.; Pauper, D.; Alban, S. *Proc. Phytochem. Soc. Eur.* **2000**, *44*, 47–58.
8. Bjork, I.; Olson, S. T.; Shore, J. D. In *Chemical and Biological Properties, Clinical Applications*; Lane, D. A., Lindahl, U., Eds.; Edward Arnold: London, 1989; pp 229–255.
9. Lane, D. A.; Denton, J.; Flynn, A. M.; Thunberg, L.; Lindahl, U. *Biochem. J.* **1984**, *218*, 725–732.
10. Laurent, T. C.; Tengblad, A.; Thunberg, L.; Hook, M.; Lindahl, U. *Biochem. J.* **1978**, *175*, 691–701.
11. Choay, J.; Petitou, M.; Lormeau, J. C.; Sinay, P.; Casu, B.; Gatti, G. *Biochem. Biophys. Res. Commun.* **1983**, *116*, 492–499.
12. Kishimoto, T. K.; Viswanathan, K.; Ganguly, T.; Elankumaran, S.; Smith, S.; Pelzer, K.; Lansing, J. C.; Sriranganathan, N.; Zhao, G.; Galcheva-Gargova, Z.; Al-Hakim, A.; Bailey, G. S.; Fraser, B.; Roy, S.; Rogers-Cotrone, T.; Buhse, L.; Whary, M.; Fox, J.; Nasr, M.; Dal Pan, G. J.; Shriver, Z.; Langer, R. S.; Venkataraman, G.; Austen, K. F.; Woodcock, J.; Sasisekharan, R. *N. Eng. J. Med.* **2008**, *358*, 2457–2467.
13. Guerrini, M.; Beccati, D.; Shriver, Z.; Naggi, A.; Viswanathan, K.; Bisio, A.; Capila, I.; Lansing, J. C.; Guglieri, S.; Fraser, B.; Al-Hakim, A.; Gunay, N. S.; Zhang, Z.; Robinson, L.; Buhse, L.; Nasr, M.; Woodcock, J.; Langer, R.; Venkataraman, G.; Linhardt, R. J.; Casu, B.; Torri, G.; Sasisekharan, R. *Nat. Biotechnol.* **2008**, *26*, 669–675.
14. Maruyama, T.; Toida, T.; Imanari, T.; Yu, G.; Linhardt, R. J. *Carbohydr. Res.* **1998**, *306*, 35–43.
15. Nardella, A.; Chaubet, F.; Boisson-Vidal, C.; Blondin, C.; Durand, P.; Jozefonvicz, J. *Carbohydr. Res.* **1996**, *289*, 201–208.
16. Huang, R.; Du, Y.; Yang, J.; Fan, L. *Carbohydr. Res.* **2003**, *338*, 483–489.
17. Vikhoreva, G.; Bannikova, G.; Stolbushkina, P.; Panov, A.; Drozd, N.; Makarov, V.; Varlamov, V.; Gal'braikh, L. *Carbohydr. Polym.* **2005**, *62*, 327–332.
18. Vishu Kumar, A. B.; Varadaraj, M. C.; Lalitha, R. G.; Tharanathan, R. N. *Biochim. Biophys. Acta* **2004**, *1670*, 137–146.
19. Gamzazade, A.; Skyyar, A.; Nasibov, S.; Suskov, A.; Knirel Yu, A. *Carbohydr. Polym.* **1997**, *34*, 113–116.
20. Kumar, A. B.; Gowda, L. R.; Tharanathan, R. N. *Eur. J. Biochem.* **2004**, *271*, 713–723.
21. Kumar, B. A.; Varadaraj, M. C.; Tharanathan, R. N. *Biomacromolecules* **2007**, *8*, 566–572.
22. Edens, R. E.; al-Hakim, A.; Weiler, J. M.; Rethwisch, D. G.; Fareed, J.; Linhardt, R. J. *J. Pharm. Sci.* **1992**, *81*, 823–827.
23. Matsubara, K.; Matsuura, Y.; Bacic, A.; Liao, M.; Hori, K.; Miyazawa, K. *Int. J. Biol. Macromol.* **2001**, *28*, 395–399.
24. Hirano, S.; Tanaka, Y.; Hasegawa, M.; Tobetto, K.; Nishioka, A. *Carbohydr. Res.* **1985**, *137*, 205–215.
25. Iqbal, O.; Mousa, S. A.. In Mousa, S. A., Ed.; Anticoagulants, Antiplatelets and Thrombolytics; Humana: Totowa, New Jersy, 2004; pp 9–20.
26. Tollefsen, D. M. *Adv. Exp. Med. Biol.* **1992**, *313*, 167–176.
27. Yu, H.; Munoz, E. M.; Edens, R. E.; Linhardt, R. J. *Biochim. Biophys. Acta, Gen. Sub.* **2005**, *1726*, 168–176.
28. Olson, S. T.; Bjork, I.. In *Advances in Experimental Medicine and Biology*; Lane, D. A., Ed.; Plenum: New York, 1992; Vol. 313, pp 155–165.
29. Olson, S. T.; Srinivasan, K. R.; Bjork, I.; Shore, J. D. *J. Biol. Chem.* **1981**, *256*, 11073–11079.
30. Lindahl, U.; Backstrom, G.; Thunberg, L.; Leder, I. G. *Proc. Natl. Acad. Sci. U.S.A.* **1980**, *77*, 6551–6555.
31. Mousa, S. A.; Khurana, S.; Forsythe, M. S. *Arterioscler. Thromb. Vasc. Biol.* **2000**, *20*, 1162–1167.