Supporting Information

Over-sulfated chondroitin sulfate: Impact of a heparin impurity, associated with adverse clinical events, on low molecular weight heparin preparation

Zhenqing Zhang, [#] Michel Weïwer, [#] Boyangzi Li, [#] Melissa M. Kemp, [†] Tyler H. Daman, [†] Robert J. Linhardt [#,†,§]*

[#]*Departments of Chemistry and Chemical Biology,* [†]*Biology and* [§]*Chemical and Biological Engineering, Center for Biotechnology and Interdisciplinary Studies, Rensselaer Polytechnic Institute, Troy, New York, 12180*

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* Corresponding author
Phone: 518-276-3404
Fax: 518-276-3405
Email: Linhar@rpi.edu

[a] Abbreviations: GAG, glycosaminoglycan; LMWH, low molecular weight heparin; OSCS, over-sulfated chondroitin sulfate; CS, chondroitin sulfate; COSY, correlation spectroscopy; HMQC, heteronuclear multiple quantum coherence; ΔUA, 4-deoxy-α-L-threo-hex-4-enopyranosyluronic acid; AU, absorbance units; GPC, gel permeation chromatography; LC-MS, liquid chromatography-mass spectrometry; dp, degree polymerization.

Experimental protocols

Synthesis of oversulfated chondroitin sulfate (OSCS) and structural analysis by NMR.

Oversulfated chondroitin sulfate was prepared according to a modified procedure from Maruyama et al. using pyridine-sulfur trioxide complex ($Py{\cdot}SO_3$) as the sulfonating agent.[26] Briefly, chondroitin sulfate A (CS-A), sodium salt (1.0 g from bovine trachea, Celsus, Cincinnati, OH) was converted to its tetrabutylammonium (n-Bu_4N^+) salt by treatment with Amberlite IR-120 (H^+ form) followed by filtration and neutralization using tetrabutylammonium hydroxide. After freeze-drying, CS-A n-Bu_4N^+ salt, (1.5 g) was dissolved in anhydrous dimethylformamide (16 ml). $Py{\cdot}SO_3$ (23.9 g, 15 mol/equivalent of available hydroxyl group in CS-A) was then added and the reaction mixture under argon atmosphere was stirred overnight at 40 °C. The reaction mixture was cooled to 0 °C and 16 ml of water was added to stop the reaction. Precipitation of the OSCS was accomplished by the addition of 100 ml of absolute ethanol. Centrifugation followed by filtration and freeze-drying afforded OSCS PyH^+ salt (2.4 g) as a white powder. The OSCS PyH^+ was dissolved in 10 ml of water and a saturated solution of sodium acetate in ethanol (60 ml) was added. The resulting precipitate was recovered by centrifugation, washed with ethanol, and dried under vacuum, affording OSCS sodium salt (1.2 g, 76%).

NMR

NMR samples (10 mg) were dissolved in 0.5 ml D_2O (99.996%, 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. ^{1}H-, ^{13}C-NMR, COSY and HMQC

spectra were recorded on synthesized OSCS. ^{1}H-NMR was recorded on all treated OSCS samples.

PAGE

Heparin, OSCS and degraded samples were analyzed using PAGE (Mini-gel apparatus, Bio-Rad, Hercules, CA.). An equal amount of each sample (5 µl at 2 mg/ml) was combined with one volume of 50% (w/v) sucrose, and the mixture was loaded into a stacking gel of 5% (total acrylamide) and fractionated with a 22% resolving gel. Electrophoresis was performed at 200 V for 80 min. The gel was stained and fixed with Alcian Blue in 2% (v/v) acetic acid.

Preparation of LMWH by nitrous acid.

Heparin (2 mg), OSCS (2 mg), and a heparin and OSCS mixture (2 mg, 1:1 w/w) were dissolved in 80 µl of water at room temperature, 20 µl of 0.5 M sulfuric acid and then 1.4 mg sodium nitrite were then added to each (final molarities were sulfuric acid: 0.1 M, and of the sodium nitrite: 0.2 M). The reaction was accompanied by the release of gaseous nitrogen that was produced. The reactions were stopped 15 min later by adjusting the pH to 7-7.2 with 0.1 M of NaOH solution, and H_2O was added to the reaction mixtures to 0.1 ml, respectively.

Preparation of LMWH by heparinase treatment

Heparin (0.2 mg), OSCS (0.2 mg) and a heparin and OSCS mixture (0.2 mg, 1:1 w/w) were dissolved in 8 µl of water, respectively. Heparinase I (10 units, Sigma Co., St. Louis, MO) were added to these three reactions at 37 °C for 2 h. The reactions were stopped by boiling for 10 min. Denatured enzyme was removed by centrifuge at 10,000 x *g* for 10 min. H_2O (0.1 ml) was added to each of the supernatants.

Preparation of LMWH by periodate-oxidation.

Heparin (2 mg), OSCS (2 mg) and heparin and OSCS mixture (2 mg, 1:1 w/w) were dissolved in 17.5 µl of water, respectively. Sodium periodate (3% w/v, 50 µl) were added to these three reactions at 4 °C for 24 h in dark. The reaction mixtures were desalted using centrifuge filter (YM-3, microscale, 0.5 ml, Millipore, Bedford, MA.). The retentates were dissolved in 150 µl water and 3.2 µl of 10 M NaOH were added at room temperature for 3 h in dark. The reactions were stopped by adjusting pH to 7-7.5. H_2O was added to the supernatants to 1 ml, respectively.

Preparation of LMWH by alkaline treatment.

Benzethonium chloride (25 mg) in water (125 µl) was added to solutions of sodium heparin, (10 mg) OSCS (10 mg) and a 1:1 mixture heparin and OSCS (10 mg) in water. The precipitates (benzethonium salts) obtained were recovered by filtration washed with excess water and then dried under vacuum. The benzethonium salts, thus obtained, were dissolved in dichloromethane (100 µl) and benzyl bromide (20 µl) was added to each. The resulting solutions were stirred at room temperature for 35 h. Sodium acetate solution in methanol (90 µl of 10% w/v) was added to each reaction and the resulting precipitates were recovered by filtration, washed with methanol and dried. The resulting benzyl ester sodium salt was dissolved in 250 µl of water. This solution was heated to ~60°C and sodium hydroxide (0.9 mg) was added to each. The temperature was maintained for 1.5 h at 60°C. The reaction mixture was cooled to room temperature, and neutralized through the addition of hydrochloric acid (0.1 M) and NaCl was added to bring the salt concentration to 32% w/v. The addition of methanol to 80% v/v resulted in product precipitation. The precipitate was collected by centrifugation, freeze-dried and analyzed by

PAGE.

Preparation of LMWH by oxidation with H_2O_2.

Heparin (20 mg), OSCS (20 mg) and a 1:1 mixture of heparin and OSCS (10 mg each) were dissolved in 50 µl of deionized water and were mixed with 100 µl of Amberlite IR 120 in H^+ form, respectively. The beads were allowed to settle and the overlying solution was collected. The beads were washed with 100 µl of deionized water and again after settling the overlying liquid was collected. The combined overlying liquids had a pH of 1-1.5, which was then adjusted to pH 5 using 2 M NaOH. A solution of hydrogen peroxide (30% w/v, 25 µl) was then added and the liquid was heated in an autoclave for 15 min at 120° C. After cooling to room temperature, the pH was raised to 7.2 with 2 M NaOH. Addition of 1 ml of a saturated solution of sodium acetate in methanol allowed precipitation of the products. After centrifugation and washing with methanol, the precipitate was freeze-dried and analyzed by PAGE.

Liquid chromatography-mass spectrometric (LC-MS) analysis

LC-MS analyses were performed on Agilent 1100 LC/MSD instrument (Agilent Technologies, Inc. Wilmington, DE, U.S.A.) equipped with an ion trap, binary pump and a UV detector. The column was a 5 µm Agilent Zorbax SB-C18 (0.5 × 250 mm) from Agilent Technologies. Eluent A was water/acetonitrile (85:15), v/v and eluent B was water/ acetonitrile (35:65) v/v. Both eluents contained 12 mM tributylamine (TBA) and 38 mM NH_4OAc and their pH was adjusted to 6.5 with HOAc. A gradient of 0% B for 15 min, and 0-100% B over 85 min. was used at a flow rate of 10 µl/min. Mass spectra were obtained using an Agilent 1100 series Classic G2445D LC/MSD trap (Agilent Technologies, Inc. Wilmington, DE, U.S.A.). The

electrospray interface was set in negative ionization mode with the skimmer potential -40.0 V, capillary exit -40.0 V and a source temperature of 325 °C to obtain maximum abundance of the ions in a full scan spectra (150–1500 Da, 10 full scans/s). Nitrogen was used as a drying (5 liters/min) and nebulizing gas (20 p.s.i.). Total ion chromatograms (TIC) and mass spectra were processed using Data Analysis 2.0 (Bruker software).

Table S1. NMR chemical shifts of OSCS.

	OSCS		Literature 2008 *Nat. Biotechnol.*[25]		Literature 1998 *Carbohydr. Res.* [26]
	^{1}H	^{13}C	^{1}H	^{13}C	^{1}H
N1	4.80	104.5	4.77	105.0	4.86
N2	4.04	54.4	4.06	54.0	4.10
N3	4.02	80.3	4.05	80.7	4.10
N4	4.97	77.6	4.98	78.0	5.02
N5	4.02	74.7	4.05	74.8	4.05
N6	4.25	69.1	4.28	69.3	4.30
N7 CO		177.9			
N8 Me	2.11	25.8	2.12	25.6	2.16
A1	4.88	104.76	4.87	104.5	4.97
A2	4.49	79.7	4.47	80.0	4.53
A3	4.91	79.3	4.95	79.3	4.94
A4	4.48	80.6	4.46	80.9	4.55
A5	4.13	81.8	4.12	82.0	4.20
A6		177.7			

N, *N*-acetyl galactosamine residues; A, glucuronic acid residues.

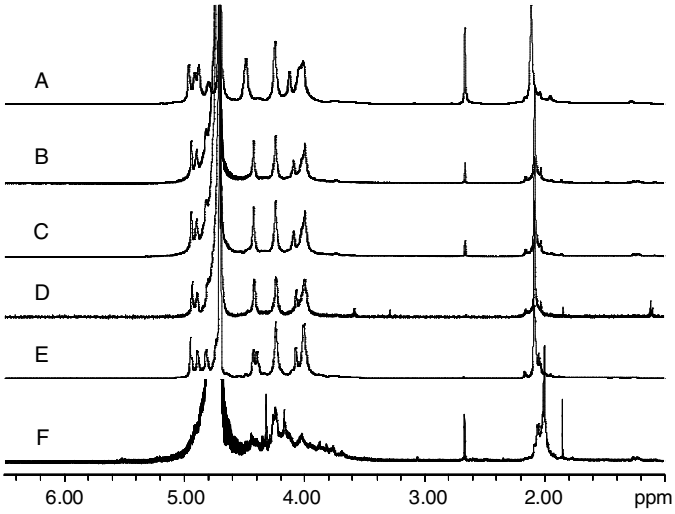

Figure S1. ^{1}H-NMR of untreated and treated OSCS. **A.** untreated OSCS; **B.** nitrous acid treated OSCS; **C.** heparin lyase I treated OSCS; **D.** periodate-oxidation treated OSCS; **E.** alkaline treated OSCS; **F.** H_2O_2 treated OSCS

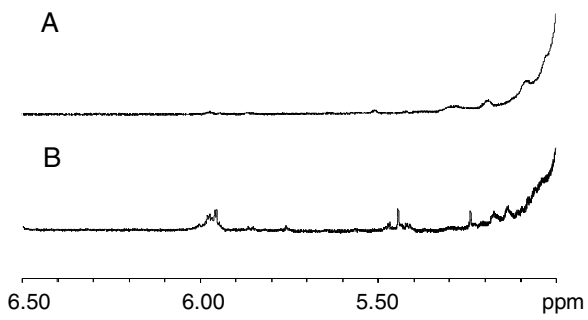

Figure S2. Partial ^{1}H-NMR of untreated OSCS and alkaline treated OSCS. **A.** Untreated OSCS; **B.** OSCS following base catalyzed β-elimination.

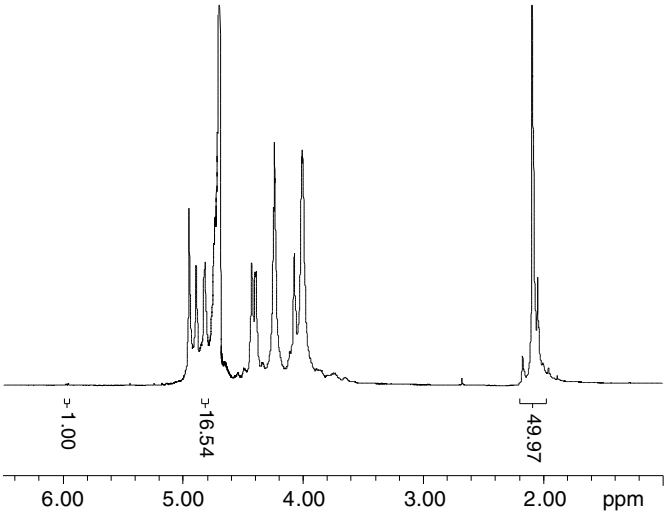

Figure S3. ^{1}H-NMR spectra of OSCS treated by base catalyzed β-elimination.

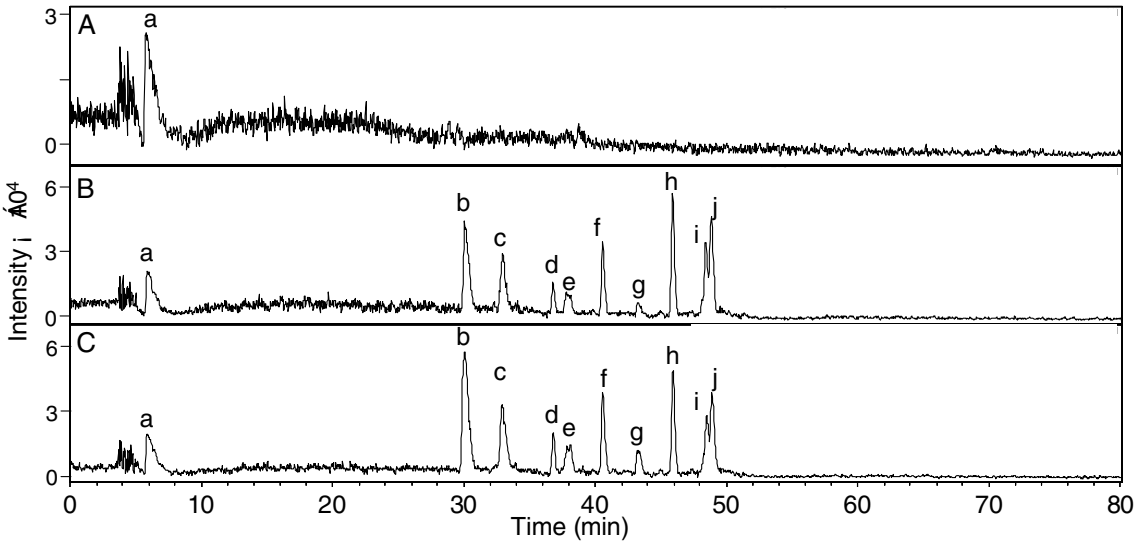

Figure S4. LC-MS analysis for H_2O_2 degraded OSCS. A, products formed at pH 2; B, products formed at pH 5; C, products formed at pH 7.

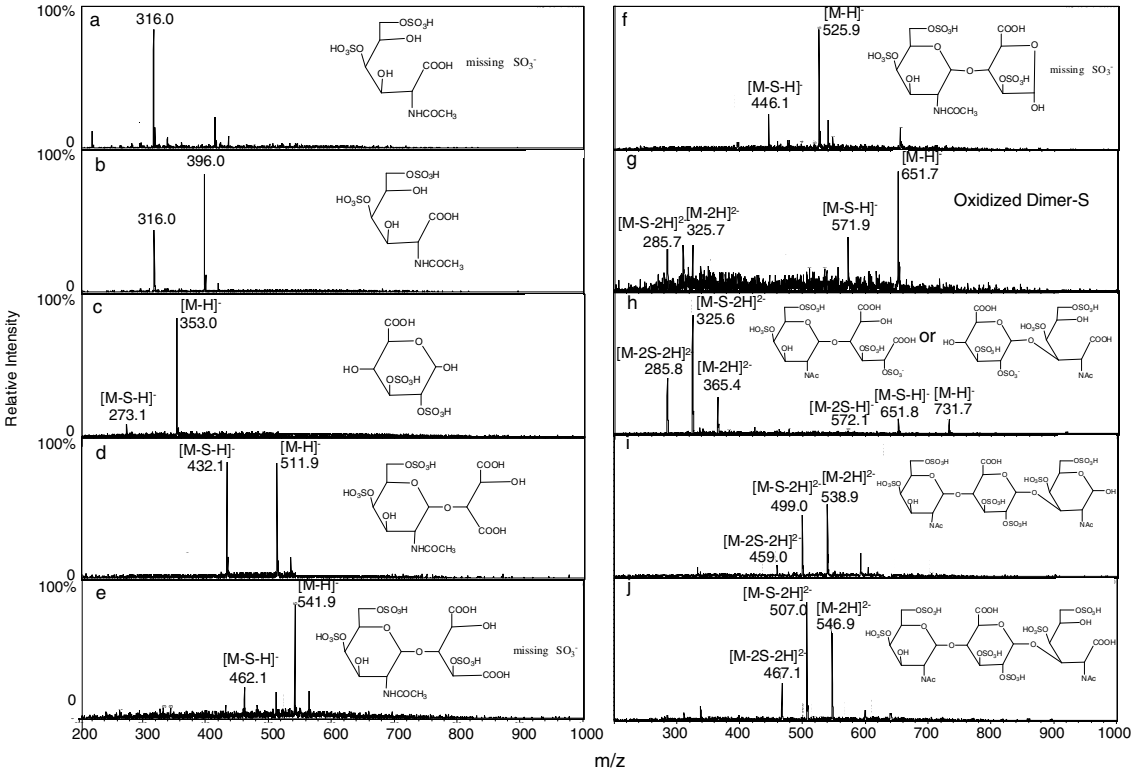

Figure S5. Mass spectra of all peaks observed in Figure S3.