SUPPORTING INFORMATION

Supporting Detailed Methods

Materials. Heparan sulfate and heparin from porcine intestinal mucosa were purchased from Celsus Laboratories, Inc. Chondroitin sulfate type A from whale cartilage, sulfur trioxide-pyridine complex, sulfur trioxide-trimethylamine complex, tributylamine, dry *N*,*N*-dimethylformamide, pyridine, methanol, dimethylsulfoxide, $NaNO_2$, $NaBH_4$, hydrogen peroxide, copper(II) acetate, acetonitrile (HPLC grade), ammonium acetate (HPLC grade), 2,2-dimethyl-2-silapentane-5-sulfonate sodium salt, tetrabutyl-ammonium chloride, and sodium trimethylsilylpropionate were purchased from Sigma-Aldrich (St. Louis, MO). OSHS, OSCS and NSOSCS were synthesized in our laboratory as described below. D_2O (99.9 atom %) and D_2O (99.96% atom %) were obtained from Cambridge Isotope laboratories (Andover, MA). The contaminated heparin sample was Baxter Heparin Sodium for Injection 5000 USP units/ mL (Lot # 107031).

Chemical *N*-deacetylation of chondroitin sulfate. Chondroitin sulfate (20 mg, 0.8 μm) was dissolved in anhydrous hydrazine (1 mL). Hydrazine sulfate (15 mg, 116 μm) was added, and the reaction was heated in a sealed tube at 105 ^oC for 15 h. The resulting product mixture was diluted with 16% NaCl (1 mL), and MeOH (9 mL) was added to precipitate the chondroitin sulfate. The precipitate was recovered by centrifugation, dissolved in H_2O for dialysis. The retentate was lyophilized and dissolved in 0.25 $NaHCO_3$ (10 mL). A solution of 0.2 M iodine in 0.4 M KI was added dropwise with continuous stirring until the solution turned yellow. Then 25% hydrazine aqueous solution was added until the mixture turned colorless. The resulting mixture was dialyzed and lyophilized to obtain the *N*-deacetylated chondroitin sulfate.

Chemical synthesis of oversulfated chondroitin sulfate, oversulfated heparan sulfate, *N*-sulfo oversulfated chondroitin sulfate. OSCS, OSHS and NSOSCS using sulfur trioxide-pyridine as the sulfonating agent. Briefly, the sodium salts of glycosaminoglycan substrates (1.0 g each of chondroitin

sulfate, heparan sulfate and *N*-deacetylated chondroitin sulfate) were each converted to their tetrabutylammonium (n-Bu_4N^+) salts by treatment with Amberlite IR-120 (H^+ form) followed by filtration and neutralization using n-Bu_4N^+ hydroxide. After freeze-drying, glycosaminoglycans in n-Bu_4N^+ salt form (~1.5 g) of each polysaccharide was dissolved in anhydrous *N*, *N'*-dimethylformamide (16 mL). Sulfur trioxide-pyridine (23.9 g, about 1 to 25 /equivalent of available hydroxyl group in glycosaminoglycan substrates) was then added and each reaction mixture was stirred overnight at 40 °C under argon atmosphere. The reaction mixtures were cooled to 0 °C and 16 mL of water was added to each to stop the reactions. Precipitation, of the resulting polysaccharide products, was accomplished by the addition of 100 mL of absolute ethanol. Centrifugation followed by filtration and freeze-drying afforded oversulfated glycosaminoglycans (2.4 g) as white powders, which were each dissolved in 10 mL of water and saturated sodium acetate in ethanol (60 mL) was added. The resulting precipitates were recovered by centrifugation, washed with ethanol, and dried under vacuum, affording corresponding oversulfated glycosaminoglycans in their sodium salt forms (~1.2 g each corresponding to ~75 %).

Chemical sulfonation of free amine groups in glycosaminoglycan substrates. The unsubstituted amino groups present in the OSHS and NSOSCS products were next *N*-sulfonated. The OSHS and NSOSCS products (10 mg) were each dissolved in H_2O (1 mL). Na_2CO_3 (36 mg) was added, and the solutions were heated to 45 ^oC. Sulfur trioxide-trimethylamine complex (3 × 12 mg) was added to each solution at 0, 2 and 4 h intervals, and the reaction was maintained at 45 ^oC for 12 h. The samples were then cooled, diluted with 16% NaCl (1 mL) and MeOH (9 mL) and precipitates were recovered by centrifugation and dissolved in H_2O for dialysis. The retentates were lyophilized to obtain fully *N*-sulfonated OSHS and NSOSCS.

1D and 2D NMR analyses. All samples were dissolved in 400 µL of D_2O (99.9 atom %) and lyophilized three-times to remove the exchangeable protons. The samples were dissolved in 400 µL of D_2O (99.96% atom %) and transferred to NMR microtubes. All NMR experiments were performed at 333 K on Bruker

Advance II 600 MHz with Topspin 2.1.6 software. One-dimensional ^{1}H spectra were recorded for 32 scans and an acquisition time of 850 msec. 2D ^{1}H-^{13}C HMQC experiments were performed with 24 scans, 1.5 sec relaxation delay, and 400 msec acquisition time.

Sample preparation. Glycosaminoglycan samples were completely degraded by controlled oxidative depolymerization using hydrogen peroxide and cupric acetate. The samples (100 μg) were dissolved in 100 μL 0.1 M sodium acetate-acetic acid solution containing 0.2 mM copper (II) acetate and adjusted to pH 7.0. Hydrogen peroxide (2 μL of 30% solution) was added with mixing and reacted at 45 °C for 3 h. Sodium bisulfite was added to terminate the reaction by removing excess unreacted hydrogen peroxide and the reaction mixture was then lyophilized.

HILIC LC-FTMS analysis. A Luna HILIC column (2.0 × 150 mm2, 200 Å, Phenomenex, Torrance, CA) was used to separate the mixture of oligosaccharides generated from oxidatively depolymerized heparin, OSHS, OSCS and NSOSCS. Mobile phase A was 5 mM ammonium acetate prepared with HPLC grade water. Mobile B was 5 mM ammonium acetate prepared in 98% HPLC grade acetonitrile with 2% of HPLC grade water. After injection of 8.0 μL mixtures (1.0 μg/μL) through an Agilent 1200 autosampler, HPLC binary pump was used to deliver the gradient from 5% A to 25% A over 35 min at a flow rate of 180 μL/min. The LC column was directly connected online to the standard ESI source of LTQ-Orbitrap XL FT MS (Thermo Fisher Scientific, San-Jose, CA). The source parameters for FTMS detection were optimized using Arixtra to minimize the insource fragmentation and sulfate loss and maximize the signal/ noise in the negative-ion mode. The optimized parameters, used to prevent in-source fragmentation, included a spray voltage of 4.2 kV, a capillary voltage of −40 V, a tube lens voltage of −50 V, a capillary temperature of 275 °C, a sheath flow rate of 30, and an auxiliary gas flow rate of 6. External calibration of mass spectra routinely produced a mass accuracy of better than 3 ppm. All FT mass spectra were acquired at a resolution 60,000 with 300−2000 Da mass range.

Analytical Quantification. Quantification analysis of OSCS (oversulfated chondroitin sulfate) in the contaminated heparin sample was performed using calibration curves constructed by separation of increasing amounts of OSCS standards (0.1, 0.5, 1.0, 5.0, 10.0, 25.0, 35.0, 50.0 μg/100 μg sample). Linearity and low limit of detection was assessed based on percentage of OSCS and peak intensity of m/z 538.96 in extract ion chromatography (EIC). All analyses were performed in triplicate. The evaluation of linearity was performed by using the correlation coefficient (r^2). The recovery test was performed using contaminated heparin samples spiked with OSCS standards. Spiked levels were 5, 10, 20 and 30 μg/100 μg of contaminated heparin, respectively. The recovery ratio percentage (REC%) and their RSD% were calculated.

Supporting Figures

Figure S1. Synthesis of oversulfated heparin sulfate, oversulfated chondroitin sulfate (OSCS), *N*-sulfo oversulfated chondroitin sulfate (NS OSCS). (Structure of polysaccharides and their persulfonated derivatives, where R is H or SO_3^- and Y is $COCH_3$, or SO_3^-.)

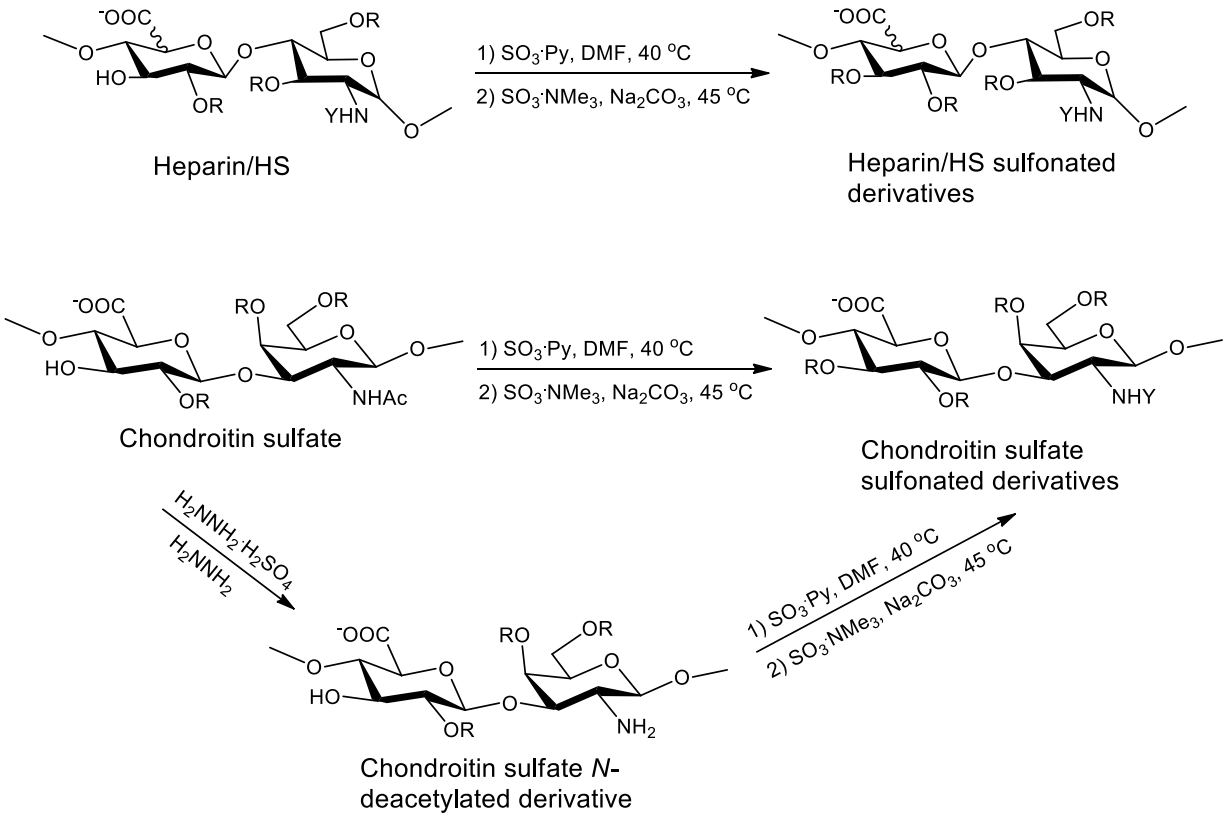
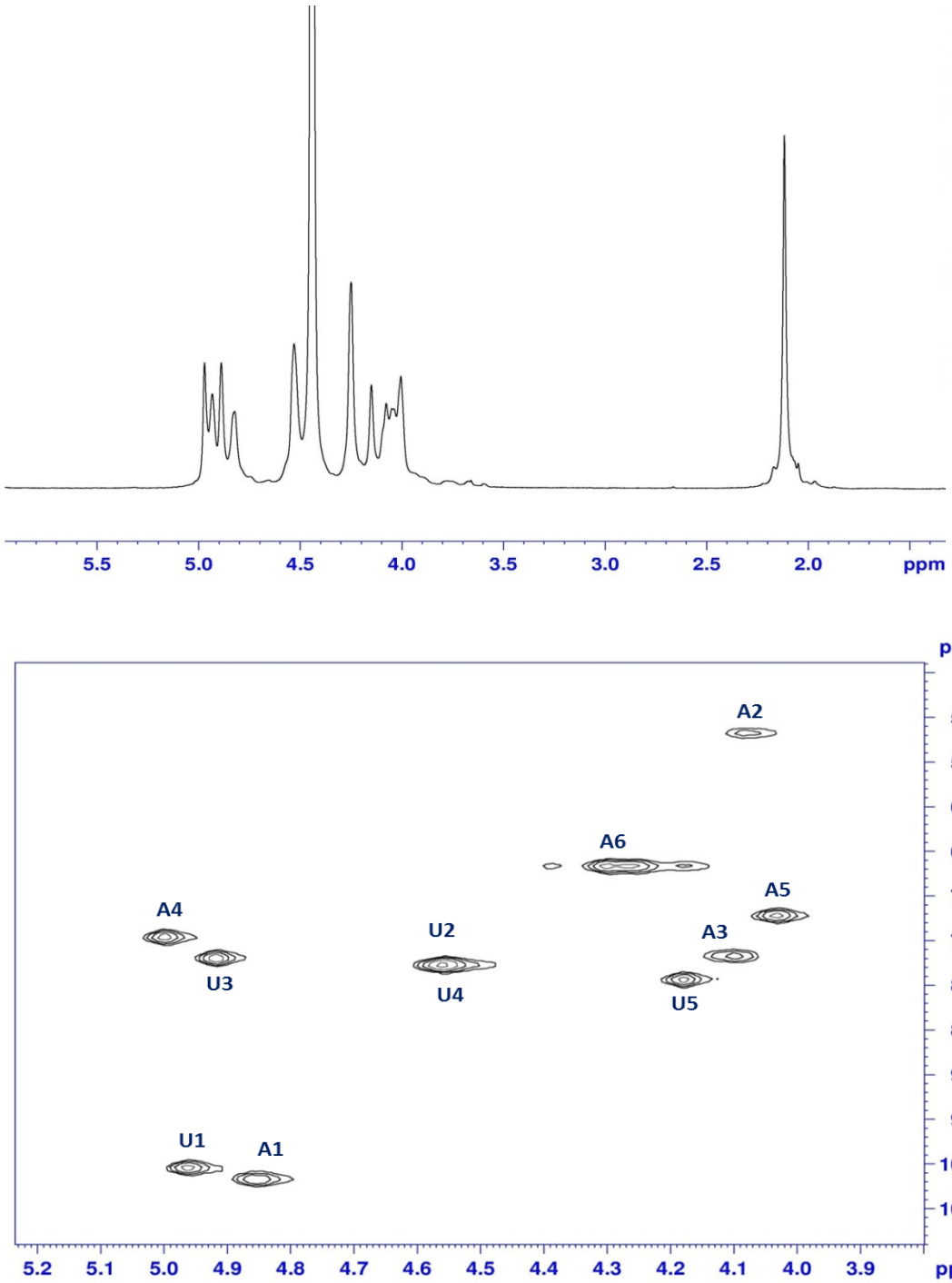
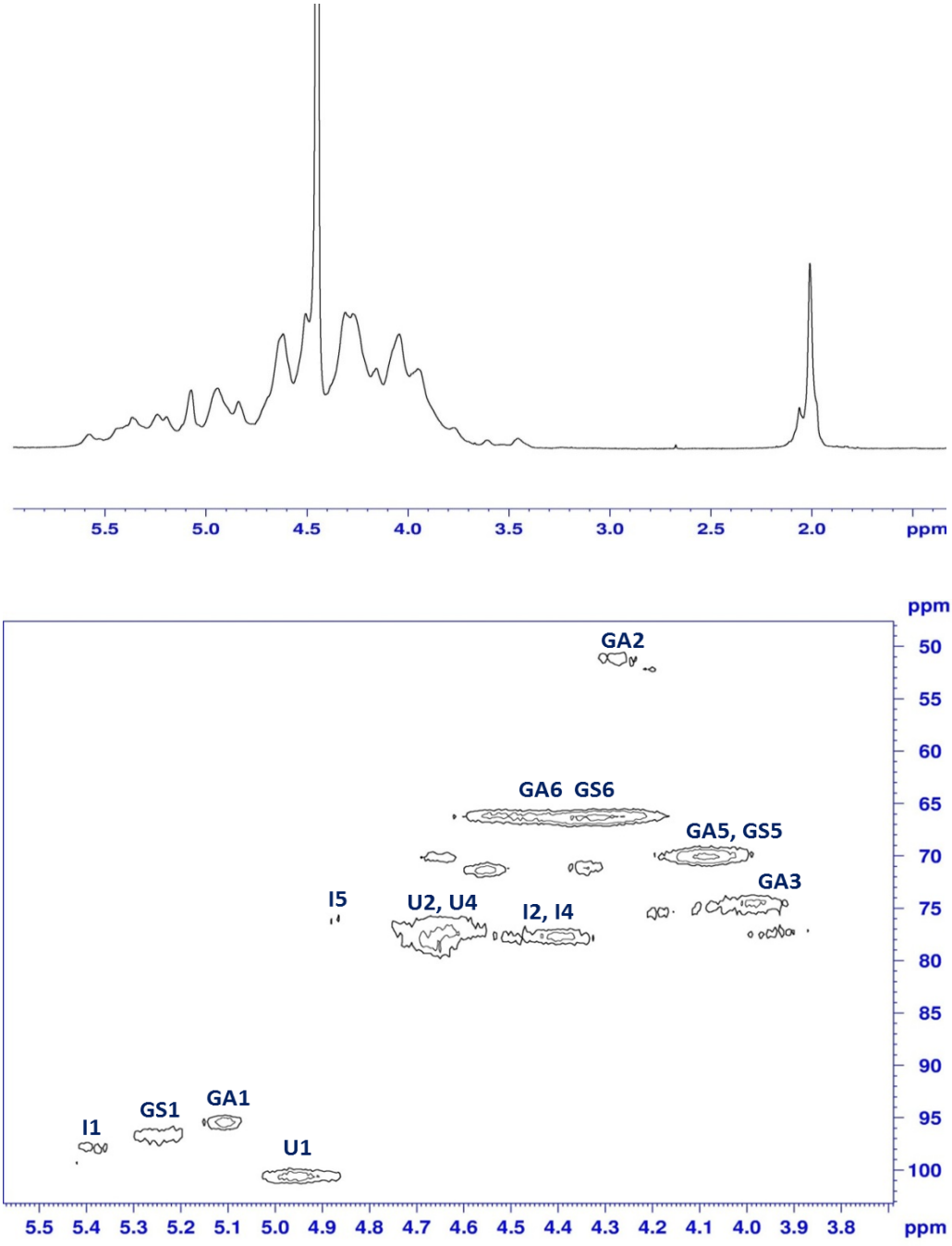


Figure S2. ^{1}H and 2D ^{1}H-^{13}C HMQC NMR data of oversulfated chondroitin sulfate (D_2O, 333 K)



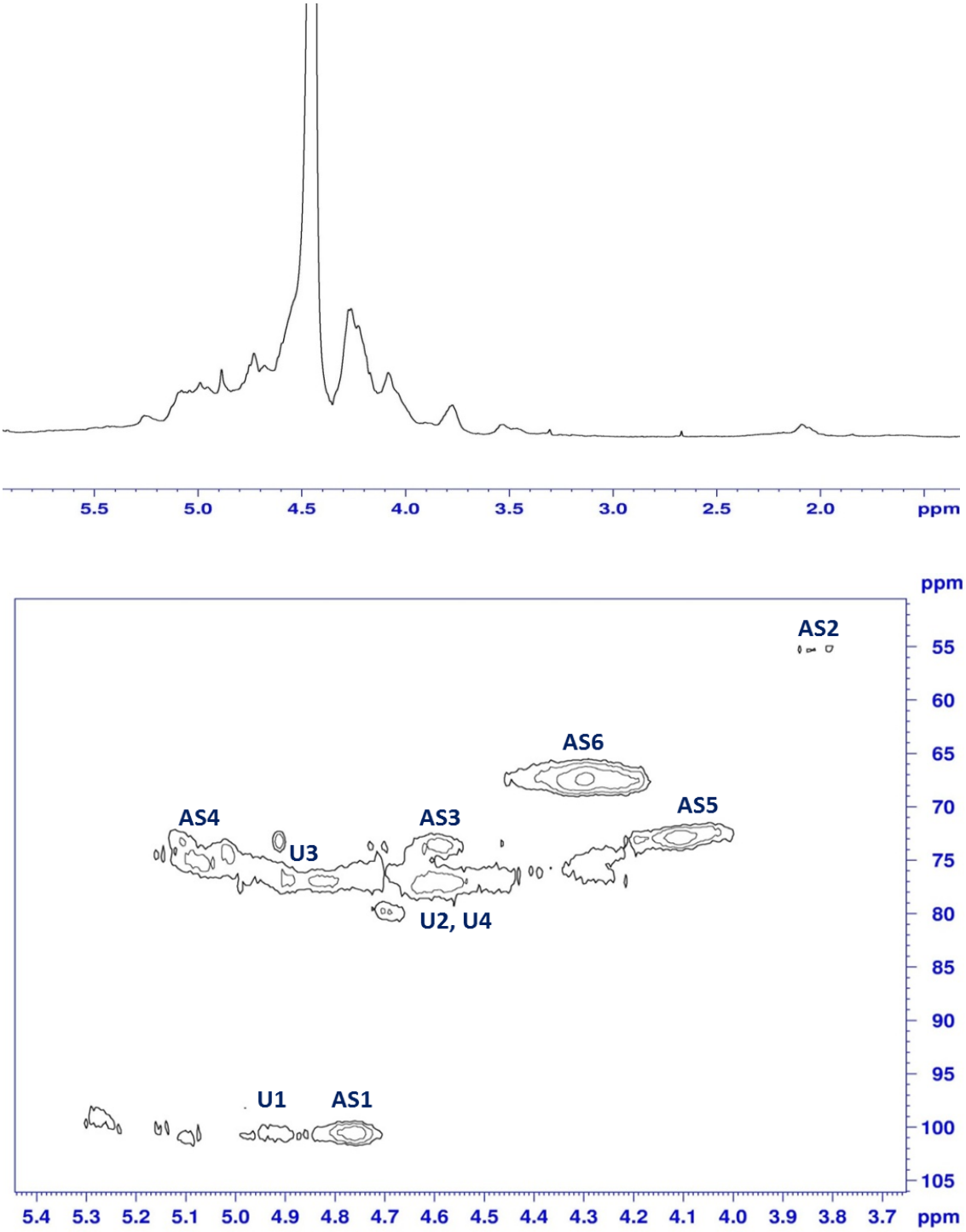
Signals for 4,6-O-sulfo-N-acetylgalactosamine (A) and of 2,3-O-sulfo-glucuronic acid (U) are labeled in the 2D ^{1}H-^{13}C HMQC NMR spectroscopy.

Figure S3. ^{1}H and 2D ^{1}H-^{13}C HMQC NMR data of oversulfated heparan sulfate (D_2O, 333 K)



Signals for 3,6-O-sulfo-N-acetylglucosamine (GA), 3,6-O-sulfo-N-sulfoglucosamine (GS) and of 2,3-O-sulfo-glucuronic acid (U), 2,3-O-sulfo-iduronic acid (I) are labeled in the 2D ^{1}H-^{13}C HMQC NMR spectroscopy.

Figure S4. ^{1}H and 2D ^{1}H-^{13}C HMQC NMR data of *N*-sulfo oversulfated chondroitin sulfate (D_2O, 333 K)



Signals for 4,6-O-sulfo-N-sulfogalactosamine (AS) and of 2,3-O-sulfo-glucuronic acid (U) are labeled in the 2D ^{1}H-^{13}C HMQC NMR spectroscopy.

Figure S5. The total ion chromatograms (TICs are from HILIC LC-MS*)* of ROS radical depolymerizated OSCS in different concentrations of H_2O_2. Peaks assigned : 1 (1,1,1,4); 2 (1,2,2,6); 3 (1,1,1,4)-A; 4 (1,2,2,6)-A; 5 (2,2,2,8); 6 (2,3,3,10); 7 (2,2,2,8)-A; 8 (2,3,3,10)-A. The oligosaccharide compositions are given as (HexA, HexN, Ac, SO_3), and during the depolymerization, the reducing end had been oxidated to acid, "A" represents the oxidated oligomers.

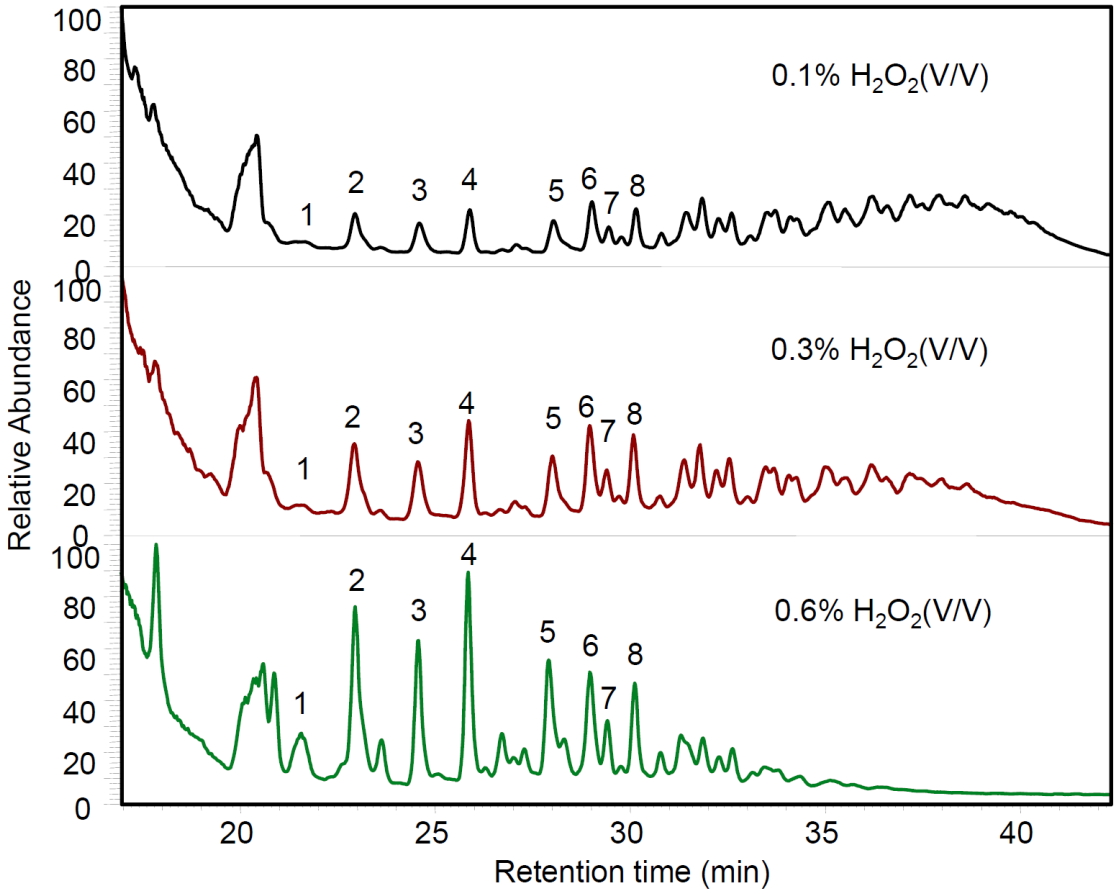


Figure S6. Extracted the ion m/z 538.9639 (±5ppm) peaks in different content of OSCS in the heparin samples.