Enzymatic synthesis of chondroitin sulfate E to attenuate bacteria lipopolysaccharide-induced organ damage

Supplementary Information

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Experimental Procedures

Expression and purification of enzymes for CS-E synthesis - To express the chondroitin *N*-acetylgalactosamine 4-sulfate 6-*O*-sulfotransferase (GalNAc4S-6OST), the gene responsible for mouse GalNAc4S-6OST was cloned into pFastBac-Mel-HT vector[50]. Primer pair 4S6OST_F (5'-CACGATTACGCGAATTCAGAACTTCTGATTTCCTCCCCTT-3') and 4S6OST_R (5'-GGTACCGCAGGCTCTAGAGTCACGTTGTCTTCCAGGCA-3') were used to PCR amplify the GalNAc4S-6OST coding gene from cDNA clone IMAGE 4240286. The 1.4 kb PCR product was inserted into pFastBac-Mel-HT *Eco*RI/*Xba*I sites using HiFi DNA assembly master mix (NEB, MA, USA) to generate the recombinant plasmid pFastBa-Mel-HT-CS4S6OST. Sequencing was applied to make sure the correct of the DNA sequence (Eurofins Genomics, KY, USA). The sequencing correct plasmid was then transformed into *E. coli* DH10Bac cells (Invitrogen, CA, USA) for Bacmid DNA. The isolated Bacmid DNA was then transferred into insect cells SF9 (Invitrogen, CA, USA) using Cellfectin Reagent (Invitrogen, CA, USA) following the instruction manual. Infection of the recombinant virus for expression of GalNAc4S-6OST was carried out when the insect cells were at a concentration of about 2.0×10^6 cells/mL. The culture was then incubated in the shaker at 27 ℃ for another 4 to 5 days, harvested by centrifuge (4,000 rpm, 10 min). The supernatant was added 1 mM phenylmethanesulfonyl fluoride (PMSF), 0.1% Triton X-100 and 2% glycerol, adjusted pH to 7.0 by NaOH solution, then centrifuged (8,000 rpm, 30 min) to remove the precipitates. The supernatant was mixed with an equal volume of 20 mM 3-(*N*-morpholino) propanesulfonic acid (MOPS) buffer containing 0.1% Triton X-100, 2% glycerol (pH 7.0), and loaded to a heparin-Sepharose (GE Healthcare,

IL, USA) column. The target protein was eluted by a gradient of 0-100% Buffer B (20 mM MOPS, 1 M NaCl, 2% glycerol and 0.1% reduced Triton X-100, pH 7.0). Collections with chondroitin sulfotransferase activity were collected and subjected to further purification to remove chondroitinase produced by the insect cells. The collected protein from heparin column was subsequently loaded to a cobalt column. A gradient elution of 0-100% buffer B (25 mM Tris, 250 mM imidazole, 500 mM NaCl, 0.1% reduced Triton X-100, 2% glycerol, pH 7.5) was applied to elute the protein. The collected protein with chondroitin sulfotransferase activity was kept in 20% glycerol at -80 °C. Other enzymes used for chondroitin sulfates synthesis including the glycotransferase KfoC and CS 4OST were expressed and purified as previous description[24].

Measurement of chondroitinase activity - The level of contaminated viral chondroitinase in the recombinant GalNAc4S-6OST preparations was tested after nickel column purification. Briefly, 0.1 mg chondroitin (Seikagaku Corp., Japan) was incubated with 2-5 μL purified enzyme in a total of 100 μL buffer containing 50 mM MOPS (pH 7.0) at 37 ℃ overnight. The reaction mixture was diluted 10 times and then tested the absorbance at the UV 232 nm.

Preparation of co-factors for enzymatic synthesis of CSs - The co-factors used in this study including UDP-GlcA, UDP-GalNAc, and PAPS were synthesized by enzymatic approaches as described previously[24,51,52].

Measurement of the sulfotransferase activity of GalNAc4S-6OST - Chondroitin sulfotransferase activity of GalNAc4S-6OST was determined by incubating 2 to 5 μL purified proteins with 5 μg chondroitin sulfate A (Seikagaku, Japan) and 1-5 × 10^5 cpm of [^{35}S]PAPS (5 μM) in 100 μL buffer containing 50 mM MOPS (pH 7.0). The reaction was

incubated at 37 ℃ for 1 h and quenched by adding 400 μL UPAS buffer (50 mM NaOAc, 150 mM NaCl, 4 M urea, 1 mM EDTA and 0.1% Triton X-100, pH5.5). Then the samples were loaded to 200 μL DEAE-Sepharose columns and washed by 4 mL UPAS buffer, 4 mL washing buffer (50 mM NaOAc, 250 mM NaCl and 0.1% Triton X-100, pH5.5). The [^{35}S]-chondroitin was eluted by 1 mL elution buffer (50 mM NaOAc, 1 M NaCl and 0.1% Triton X-100, pH5.5) and then subjected to liquid scintillation counting.

Synthesis of chondroitin backbone oligosaccharides (CS-0S) - The synthesis of CS-E oligosaccharides was started from monosaccharide, GlcA-pNP. Briefly, KfoC was used for elongation of GlcA-pNP to appropriate sized backbones as previously reported[24]. It alternately added GalNAc and GlcA residues to the start material using UDP-GalNAc and UDP-GlcA as the co-factors. The CS backbones were then subjected to sulfo-modification by *N*-acetylgalactosamine 4-*O*-sulfotransferase (CS-4OST) to generate CS-A oligosaccharides. CS-A oligosaccharides were subsequently used as the substrates of CS 4S-6OST that transfers the sulfo group from PAPS to the C-6 position of GalNAc4S residues to give CS-E oligosaccharides.

Step **a** was the elongation involving the addition of GalNAc residue to the CS backbones. 2.5 mmol GlcA-pNP was dissolved in 1 L buffer containing 25 mM Tris-HCl (pH 7.5), 20 mM $MnCl_2$, 3 mmol UDP-GalNAc and 250 mg KfoC, incubated at 37°C overnight or until the reaction completely.

Step **b** was to add a GlcA residue to the CS backbones. Disaccharide GalNAc-GlcA-pNP (2.5 mmol) was dissolved in a buffer containing 50 mM Tris-HCl (pH 7.5), 20 mM $MnCl_2$, 3.7 mmol UDP-GlcA and 500 mg KfoC. The total reaction volume was 1 L and incubated at 37°C overnight.

Step **a** and step **b** were alternately used for the elongation of CS backbone to the desired size. All the reactions involving step **a** and step **b** were analyzed by HPLC using Polyamine II column (250 × 4.6 mm, S-5 μm, 12 nm, YMC). Gradient elution was applied using KH_2PO_4 buffer (1 M) for the elution as described previously[24]. Products were purified by C18 (Biotage) columns eluted by gradient of MeOH (0 - 70% in 120 minutes). Absorption at 310 nm was used to detect the chondroitin compounds.

4-O-sulfation of CS-0S oligosaccharides using CS 4OST -Step **c** was the addition of sulfo-group to the C-4 position of GalNAc residues using CS 4OST, which transfers sulfo-group to the C-4 position of GalNAc residues. To generate CS-A 7-mer, chondroitin sulfate backbone **CS-0S 7-mer** 0.1 mmol (152 mg) was incubated with 0.48 mmol PAPS, 10 mL purified CS-4OST in 200 mL buffer containing 50 mM MES (2-(*N*-morpholino)ethanesulfonic acid, pH 6.5, Sigma), 20 mM $CaCl_2$ and 2 mM DTT. After incubation at 37 ℃ overnight, the product (**CS-A 7-mer**) was purified by Q-Sepharose column and then subjected to dialysis using 1 kD membrane (Spectrum Laboratories, CA, USA). The production of **CS-A 13-mer** was originally from **CS-0S 13-mer**. Briefly, about 0.035 mmol (90 mg) **CS-0S 13-mer** and 0.3 mmol PAPS were incubated in 200 mL reaction buffer containing 50 mM MES (pH 6.5), 20 mM $CaCl_2$, 2 mM DTT and 5 mL CS-4OST. After incubated overnight at 37°C, the product (**CS-A 13-mer**) was purified, dialyzed and subjected to the reaction for **CS-E 13-mer**. Using the similar procedures, 0.317 mmol **CS-0S 19-mer** (1180 mg) was incubated with 4.5 mmol PAPS and 60 mL purified CS-4OST in a 1 L of the reaction mixture containing 50 mM MES (pH 6.5), 20 mM $CaCl_2$ and 2 mM DTT to generate **CS-A 19-mer**. The reaction was incubated at 37°C overnight. After purification and desalting, **CS-A 19-mer** was used as the substrate for

CS-E 19-mer.

Step **d** was the sulfation of the C-6 of GalNAc4S by CS-4S6OST using the products of step **c** as the substrates. The aforementioned **CS-A 7-mer** was subsequently incubated with 0.48 mmol PAPS and 12.5 mL CS-4S6OST in 200 mL reaction buffer containing 50 mM MES (pH 6.5), 20 mM $CaCl_2$ and 2 mM Re-GSH. The reaction was incubated at 37°C overnight. Product (142 mg) was purified by Q-Sepharose column (GE Health). Similarly, CS-E 13-mer was produced by incubating of the reaction mixture containing 0.03 mmol CS-A 13-mer, 0.3 mmol PAPS, 5 mL CS-4OST, 50 mM MES (pH 6.5) and 20 mM $CaCl_2$ at 37°C overnight, 86 mg product was purified by Q- Sepharose column. For CS-E 19-mer production, 0.09 mmol (400 mg) CS-A 19-mer was taken out from total 1300 mg product to be used as the substrate. CS-A 19-mer was incubated with 1.4 mmol PAPS and 40 mL CS-4S6OST in 1 L reaction buffer (50 mM MES, 20 mM $CaCl_2$) at 37°C for overnight. Total 393 mg product was harvest after purification.

HPLC analysis-Analysis of the products of step **c** and step **d** were carried out via a DNA-NPR column (75 × 4.6 mm, 2.5 μm, Tosoh). Gradient elution using 0-1 M NaCl in 20 mM NaOAc in 30 min was employed at flow rate 0.4 mL/min. UV absorption at 310 nm was used for the detection of synthetic CS oligosaccharides. For the purification of the sulfated CS products, Q-Sepharose fast flow column (from GE Healthcare Life Science) was eluted with a linear gradient 0-100% 2 M NaCl in 20 mM NaOAc-HOAc (pH 5.0) in 3 h at a flow rate of 1 mL/min. The sizes of Q-Sepharose columns were chosen by the binding affinity of the products and reaction scale.

*Determination of the efficiency using CS sulfotransferases to prepare **CS-E 19-mer*** – This experiment was carried with an attempt to synthesize **CS-E 19-mer** using route with using GalNAc4S 6-OST. 10 μg of CS-0S 19-mer or CS-C 19-mer was incubated with 5 μL purified

CS4OST in 100 μL reaction buffer containing 1-5 ×10^5 cpm of [^{35}S]PAPS (5 μM) and 50 mM MOPS (pH 7.0). The reaction was incubated at 37 ℃ for 1 hour and quenched by adding 400 μL UPAS buffer. Then the samples were loaded into 200 μL DEAE sepharose columns and washed by 1 mL UPAS buffer for 4 times, 1 mL washing buffer for 4 times and finally eluted by 1 mL elution buffer containing 1 M NaCl, 50 mM NaOAc and 0.1% triton X-100. The radioactivity of the product that with [^{35}S] labeled sulfo groups was counted by liquid scintillation. Similar as CS4OST, CS6OST (5 μL) was incubated with CS-0S 19-mer (10 μg) and CS-A 19-mer (10 μg) separately with [^{35}S]PAPS (5 μM) in 100 μL buffer containing 50 mM MOPS (pH 7.0) at 37 ℃ for 1 hour. 5 μL purified CS4S6OST was incubated with 10 μg of **CS-0S 19-mer**, CS-A 19-mer and CS-C 19-mer separately in 100 μL buffer. The steps including quench the reaction, load to the DEAE column and measure the radioactivity counts are the same as CS4OST.

Biotinylation of CS oligosaccharides – Conversion of pNP-tagged CS oligosaccharides to biotinylated counterparts was carried out similar as previously reported[53]. In brief, 23 mg of **CS-E 7-mer**, 10 mg of **CS-E 13-mer**,13 mg of **CS-E 19-mer** or 10 mg of **CS-0S 19-mer** was dissolved in 20 mM NaOAc, pH 5.0 in a total volume of 4 mL respectively with 0.5 mg Pd/C. Reaction mixture was vacuumed and refilled with H_2 three times, then incubated at room temperature for 4 h. Filter via a 0.22 μm membrane (Millipore) was applied to remove charcoal. After adjusting pH to 8.5 using 1 M Na_2HPO_4, succinimidyl 6-azidohexanoate (20-50 molar equivalents of starting materials) was added to the filtered solution, and the reaction was incubated at 60 °C overnight. The azido tagged products were purified by DEAE-HPLC column, dialyzed in water for removing salts. Azido-tagged oligosaccharides (4-8 mg) were dissolved in N_2 bubbled PBS buffer with 8 mM $CuSO_4$, 40 mM Tris(3-hydroxypropyltriazolylmethyl)amine (THPTA) (Sigma), 24 mM sodium ascorbate and 5-15 mM biotin-PEG_4-alkyne (Sigma). The reaction mixture was incubated at 37 °C overnight. Biotinylated products were purified by DEAE -HPLC column. All the

reactions were monitored using HPLC and MS.

MS analysis - The low-resolution analyses were performed at a Thermo LCQ-Deca. CS oligosaccharides were directly diluted in 500 μL water. A syringe pump (Harvard Apparatus) was used to introduce the sample by direct infusion (50 μL/min). Experiments were carried out in negative ionization mode. The electrospray source was set to 3 KV and 150 °C. The MS data were acquired and processed using Xcalibur 1.3.

High-resolution ESI-MS analysis was conducted on Thermo LTQ XL Orbitrap under the following conditions. A Luna hydrophilic liquid interaction chromatography (HILIC) column (2.0 × 50 mm, 200 Å, Phenomenex) was used to separate the oligosaccharide mixture. Mobile phase A was 5 mM ammonium acetate prepared with HPLC-grade water. Mobile phase B was 5 mM ammonium acetate prepared in 98% HPLC-grade acetonitrile with 2% of HPLC-grade water. After injection of 8.0 μL oligosaccharide mixture (1.0 μg/μL) through an Agilent 1200 autosampler, an HPLC binary pump was used to deliver the gradient from 10% A to 35% A over 10 min at a flow rate of 150 μL/min. The LC column was directly connected online to the standard electrospray ionization source of LTQ-Orbitrap XL Fourier transform (FT) mass spectrometer (MS) (Thermo Fisher Scientific). The optimized parameters 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 of 60,000 with 300–2,000 Da mass range.

NMR analysis - NMR experiments were performed on Bruker Avance 700 MHz and 850 MHz spectrometer with Topsin 3.2 software. Samples (5 to 20 mg) were each dissolved in 0.5 mL D_2O (99.996%, Sigma-Aldrich) and lyophilized two times to remove the exchangeable protons. The samples were re-dissolved in 0.5 mL D_2O and transferred to NMR microtubes (O.D. 5 mm, Norrell). 1D ^{1}H-NMR experiments "zg" pulse sequence were performed with 64 scans and an acquisition time of 3.8 sec. 1D ^{13}C-NMR experiments "zgdc30" pulse sequence was performed with 10,000 scans and an acquisition time of 1.0 sec. 2D ^{1}H-^{13}C HSQC experiments "hsqcgpph" pulse sequencing was performed with 48 scans, 512 increments, 1.5 s relaxation delay, and 120 milliseconds acquisition time. 2D spectra were recorded with GARP carbon decoupling. 48 dummy scans were used prior to the start of acquisition. 2048 total points were collected in f2. ^{13}C transmitter offset was set at 90.0 ppm.

Preparation of histone H3 and histone mixture biochip -Histone H3/ histone mixture was immobilized on research grade CM5 chips according to standard amine coupling protocol (GE healthcare, Uppsala, Sweden) to obtain kinetic data for H3/ histone mixture and CS-E oligosaccharide interactions. Briefly, carboxymethyl groups on the CM5 chip surface were first activated using an injection pulse of 35 µL (flow rate, 5 µL/min) of an equimolar mix of *N*-ethyl-*N*-(dimethyaminopropyl) carbodiimide and *N*-hydroxysuccinimide (final concentration 0.05 M, mixed immediately prior to injection). Following activation, H3/ histone mixture was diluted to 200 µg/mL in 100 mM sodium acetate (pH 4.5) buffer and injected over the activated biosensor surface. Excess unreacted sites on the sensor surface were deactivated with a 35-µL injection of 1 M ethanolamine. A reference flow cell was prepared using an injection pulse of 35 µL (flow rate, 5 µL/min) of an equimolar

mix of *N*-ethyl-*N*-(dimethyaminopropyl) carbodiimide and *N*-hydroxysuccinimide followed with a 35-µL injection of 1 M ethanolamine.

Measurement of interaction between CS-E oligo and H3/ histone mixture using BIAcore– CS-E oligo samples were diluted in HBS-EP buffer (0.01 M HEPES, 0.15 M NaCl, 3 mM EDTA, 0.005% surfactant P20, pH 7.4). Different dilutions of CS-E oligo samples were injected at a flow rate of 30 µL/min. At the end of the sample injection, HBS-EP buffer was flowed over the sensor surface to facilitate dissociation. After a 3 min dissociation time, the sensor surface was regenerated by injecting 30 µL of 2 M NaCl to get fully regenerated surface. The response was monitored as a function of time (sensorgram) at 25 °C.

*Removal of endotoxin from **CS-E 19-mer*** - Endotoxin was removed from **CS-E 19-mer** before the animal studies. Briefly, a 50 mL centrifugal filter unit (Amicon Ultra-15, Ultracel-100k; Merck Millipore) was employed to remove the endotoxin in **CS-E 19-mer** solution by centrifugation at 4000 rpm for 30 min. The filtered solution was collected. The level of endotoxin was confirmed by the Limulus Amebocyte Lysate (LAL) kit (Associated of Cape Cod Inc.) according to the instruction.

Animals - C57BL/6 male mice (8-12 weeks-old) were purchased from Jackson Laboratories (Bar Harbor, ME, USA) and allowed to acclimate to our animal facility for 2 weeks prior to use. All studies were followed the protocols that have been approved by the Institutional Animal Care and Use Committee of University of North Carolina at Chapel Hill (Chapel Hill, NC) and complied with National Institutes of Health guidelines. Mice were randomized to treatment groups blindly.

Histone cytotoxicity assay –EA.hy 926 cells (5×10^5 cells/well) were seeded in 12-well plates using DMEM medium (Gibco) with 10% FBS and 1% Penicillin-Streptomycin to confluence, and followed by 18 h incubation in serum-free DMEM medium. Cells were next incubated for 1 h in serum-free DMEM containing 30 μg/mL histone H3 (Roche, Germany) with varying concentrations of chondroitin sulfate oligosaccharides (0-50 μg/mL). Then, PBS was used to wash cells for two times and 0.05% trypsin –EDTA was used to detach cells. The cells were collected by centrifugation (500 g for 5 min) and resuspended in 1 mL PBS buffer containing 10 μg/mL propidium iodide (Sigma). After 10 min incubation in the dark, cells were subjected to flow cytometry on Becton Dickinson LSRFortessa to test cell viability.

Mouse model of histones induced endotoxemia – According to the previous report[11], calf thymus histones (Sigma) was injected retro-orbitally into the anesthetized mice at 75 mg/kg with/without **CS-E 19-mer** pre-injection (75 mg/kg). The mice were monitored for 1 h after histones injection for the survival experiment. Histones (65 mg/kg) were also injected to the mice with or without **CS-E 19-mer** (75 mg/kg) to study characteristics of sepsis. After 3 h monitoring, mice were euthanized by **cervical dislocation under** methoxyfluane inhalation. Tissues of lung, liver and kidney were collected for histological analysis.

Mouse model of LPS induced endotoxemia – LPS (*Escherichia coli* serotype O111:B4, Sigma, St Louis, MO) was dissolved in sterile saline and injected *intraperitoneally* into mice at 6 mg/kg. **CS-E 19-mer** diluted by sterile saline was subcutaneously injected to the mice anesthetized by inhalation of methoxyflurane (2-3%). The injection of **CS-E 19-mer** with 20 mg/kg was carried out at 0 h, 6 h, 12 h and every 12 h thereafter. At the same

time, equivalent volumes sterile saline was injected to the control group. The survival study was monitored to 72 h after LPS injection.

Measurement of the biomarkers for liver/kidney damage - For measurement of plasma ALT, AST, BUN and creatinine levels, blood samples were collected from inferior vena cava (IVC) into sodium citrate (final concentration about 0.5%) 24 h after LPS injection. Plasma was prepared by centrifugation of blood samples at 4000 rpm for 15 minutes at 4 °C, and it was stored at -80 °C until analysis. ALT and AST were measured using the ALT Infinity reagent and AST Infinity reagent (Thermo Fisher, MD, USA) following the manufacturer's instructions. BUN and creatinine were measured using the Urea Nitrogen (BUN) Colorimetric Detection Kit from Invitrogen by Thermo Fisher Scientific (MD, USA) and Mouse Creatinine kit from Crystal Chem (IL, USA) according to their respective instructions.

Vascular permeability – Mice were injected retro-orbitally with 1% (wt/vol) Evans blue dye in PBS buffer (50 μL each mouse) at 23.5 h post-LPS to evaluate the leakage of endothelial barrier. After 30 min, mice were anesthetized and perfused with cold PBS after ICV blood draw. Organs including lung, liver and kidney were collected. Dye was extracted from the tissues for 2 days in formamide (4 mL/g of tissues) at 37 °C. Extracted dye concentration was determined by measuring the absorbance at 620 nm using 740 nm as the reference. To avoid the bias cause by injection, the concentration of Evans Blue in plasma was used for correction.

$$\frac{(\mathrm{A}\ 620 - \mathrm{A}\ 740)\text{in tissues}}{(\mathrm{A}\ 620 - \mathrm{A}\ 740)\text{in plasma}} = \text{Leakage of Evans Blue}$$

Immunoblot - Biotinylated CS oligomers (**CS-E 19-mer**, **CS-E 13-mer**, **CS-E 7-mer**, and **CS-0S 19-mer**, 50 μg each sample) were mixed with 50 μg histone H3 (Roche, Germany) in PBS buffer and incubated at 37 °C for 1 hour to screen the binding ability to histone proteins of CS oligosaccharides. Then, Pierce high capacity streptavidin agarose (Thermo Fisher) was used to isolated biotinylated CS oligosaccharide bound complexes. After washing by PBS for 5 times, samples were eluted with LDS buffer. Eluted samples were separated using NuPAGE 4-12% Bis-tris protein gels and detected via Western blotting using anti-histone H3 antibody (Abcam) and goat anti-rabbit HRP (Abcam). Biotinylated **CS-E 19-mer** (10 μg) was mixed with 50 μL mouse plasma and incubated at the conditions described above to confirm the binding of **CS-E 19-mer** with histones *ex vivo*. Pierce high capacity streptavidin agarose was used to isolate the binding complexes, and Western blotting was applied for the detection of histone H3 in the eluent.

Western blotting was carried out similar as previous reported[53]. Circulating histone H3 was detected by Western blotting. Briefly, 10 μL mice plasma was used for each sample to load to the SDS-PAGE gene for separation. After transferring the protein from the gel to the membrane, 1000 times dilution of anti-histone H3 antibody (Abcam) was used as the primary antibody and subsequently 10000 times dilution of goat anti-rabbit HRP (Abcam) was used as the secondary antibody. SuperSignal West Pico PLUS Chemiluminescent Substrate (Thermo Scientific, IL) was used for detection of the bands.

Histology – Mice organs including lung, liver and kidney tissues were fixed in 10% neutral buffered formalin for 24 h at room temperature, paraffin-embedded, and sectioned. Embedding, sectioning and hematoxylin-eosin (H&E) staining were performed at the Animal Histopathology and Laboratory Medicine Core Facility at UNC Chapel Hill. Images

were captured using an HD camera attached to a bright field microscope (Leica DM 1000 LED, Leica Microsystems Inc., IL, USA)

Statistical Analysis - All data are presented as mean ± SEM. Statistics analysis was performed by unpaired Student *t* test. The survival curves were analyzed by log-rank test and differences between multiple groups was analyzed by one-way ANOVA followed by Tukey's multiple comparison's test using GraphPad Prism software (version 7.03; GraphPad Software, Inc.) Statistically significant was considered at P value <.05.

*Determination of the binding of LPS to **CS-E 19-mer*** - **CS-E 19-mer** (0.039 mM) was incubated with various doses of LPS (0 – 0.04mM) in 1 mL PBS buffer for 30 minutes at room temperature. Then the mixtures were transferred to the 100 kDa centrifugal filter (Millipore) and then centrifuged for 10 minutes at 4,000 rpm. The concentration of **CS-E 19-mer** in the run-through liquid was tested by UV at maximum 310 nM, and the value was compared to that of the solution prior to the centrifugation to obtain percentage of the decrease. LPS was measured by the Limulus Amebocyte Lysate (LAL) kit (Associated of Cape Cod Inc.) following the manual from the manufacturer. LPS did not penetrate 100 KDa centrifugal filter under this condition.

To validate the method, the positive and negative control experiments involved the use of antithrombin (AT) two HS hexasaccharides: GlcNS6S-GlcA-GlcNS3S6S-IdoA2S-GlcNS6S-pNP (hexasaccharide 1) and GlcNS6S-GlcA-GlcNS6S-GlcA-GlcNS6S-GlcA-pNP (hexasaccharide 2). Hexasaccharide 1 binds to antithrombin, but hexasaccharide 2 does not. Briefly, different concentrations of hexasaccharide 1 (0 - 0.103 mM) was incubated with 0.108 mM AT, or hexasaccharide 2 (0 - 0.07 mM) was incubated with 0.039 mM AT in 1 mL PBS buffer for 30 minutes at room temperature.

Each binding mixture was centrifuged in a 30 kDa centrifugal filter (Millipore) at 4,000 rpm for 30 minutes. The concentrations of hexasaccharides in the run-through liquid were measured by UV absorbance at 310 nm, and the values were compared to those of the solutions prior to the centrifugation to obtain percentage decrease.

*Determination of the effect of **CS-E 19-mer** on the expression TNF-α from monocyte cells* - Human monocyte leukemia THP-1 cells (kindly provided by Dr. Nigel Mackman) were cultured in RPMI 1640 (Gibco) with 10% fetal bovine serum (VWR), 50 μM 2-mercaptoethanal (Sigma), and 1% penicillin –streptomycin cocktail (Sigma). Various doses of **CS-E 19-mer** (0.5 – 10 μM) or 50 μM TAK-242 (Sigma) were preincubated with THP-1 cells (about 1 $\times 10^6$ cells/mL) at 37 ℃ for 15 minutes prior to simulated by LPS (10 μg/mL). After incubation with LPS for 5 hours at 37 ℃, THP-1 cells were harvest by centrifuge for 15 minutes at 4000rpm and the supernatant were subjected to measure the TNFα levels using an ELISA kit (R&D system).

*Heparin-induced thrombocytopenia (HIT) ELISA Assay***-**The enzyme-linked immunosorbant assay (ELISA) for detection of HIT antibodies was performed under three conditions: PF4 alone, PF4 in complex with **CS-E 19-mer** (1:1 molar ratio) and PF4-polyanion complexes (PF4-polyvinylsulfonate). Plates were first blocked using 3% bovine serum albumin in phosphate buffered saline pH 7.4 followed by addition of 1:50 dilution of patient samples incubated for 30 min at 37°C. Following four washes, goat anti-Human IgG (Fc) was added for 30 min at 37°C. The plates were washed again and PNPP was added and incubated in the dark for 45 min at room temperature. Absorbance was read at 405nm and expressed as optical density (O.D).

Determination of the in vitro anti-FXa and anti-FIIa activity **-** Anti-FXa and Anti-FIIa assays were both performed according to a previous report[54]. Unfractionated heparin (UFH) (US Pharmacopeia) and fondaparinux (Auromedics Pharma LLC, NJ) were used as the control. For the anti-FXa activity assays, reaction mixture, which consisted of 60 μL of antithrombin (35 μg/mL, Cutter Biologics) and 10 μL of the solution containing different concentration of heparin or oligosaccharides, was incubated at room temperature for 5 min. Factor Xa (100 μL, Enzyme Research Laboratories) was diluted to 50 U/mL by PBS and then added to the reaction mixture. After incubation at room temperature for 4 min, 30 μL of chromogenic S-2765 substrate (Diapharma, diluted to 1 mg/ml in water) was added. The absorbance of the reaction mixture was measured at 405 nm continuously for 5 min. The absorbance values were plotted against the reaction time to measure the reaction rate. The initial reaction rates as a function of concentration were used to calculate the IC_{50} values. For the anti-FIIa activity assays, the reaction mixture, which consisted of 60 μL of antithrombin (35 μg/mL, Cutter Biologics) and 10 μL of the solution containing the sample at various concentrations, was incubated at room temperature for 2 min. 100 μL thrombin (Sigma-Aldrich, diluted to 10 U/mL in PBS with 1 mg/mL bovine serum albumin) was then added. After incubating at room temperature for 4 min, 30 μL of chromogenic substrate S-2238 (Diapharma, diluted to 1 mg/mL by PBS) was added. The absorbance of the reaction mixture was measured at 405 nm continuously for 5 min. The absorbance values were plotted against the reaction time. The initial reaction rates as a function of concentration were used to calculate the IC_{50} values.

Safety Statement

No unexpected or unusually high safety hazards were encountered.