Supporting Information for:

Sulfated Poly-amido-saccharides (sulPASs) Are Anticoagulants *in vitro* and *in vivo*

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Experimental Procedures

Materials and general methods

3,4,6-tri-O-benzyl-D-glucal and 3,4,6-tri-O-benzyl-D-galactal were purchased from Carbosynth, LLC (San Diego, California). All other reagents were purchased from Millipore Sigma (Burlington, MA), TCI (Portland, OR), or Chem-Impex (Wood Dale, Illinois). Solvents were purchased from Thermo Fisher Scientific (Waltham, MA). Pooled Normal Human Plasma was purchased from Innovative Research (Novi, MI). Pooled Human Plasma (Blood Derived) from Innovative Research is whole blood derived. The whole blood is collected from donors in an FDA-approved collection center and processed into plasma via cetrifugation. APTT-XL and PT reagents were purchased from Thermo Fisher Scientific (Waltham, MA). Chromogenix Coatest® Heparin kit, Bovine thrombin (FIIa), FIIa substrate (Chromogenix S-2238™), FXIa substrate (Chromogenix S-2366™) and FXIIa substrate (Chromogenix S-2302™) were purchased from Diapharma (West Chester, Ohio) . FXIIa was purchased from Biovision (Milpitas, California). FXIa was purcahsed from Haematologic Technologies, Inc. (Essex Junction, VT). Recombinant F. heparinum Heparinase I Protein, CF, Recombinant P. heparinus Heparinase II Protein, CF, Recombinant P. heparinus Heparinase III Protein, CF and Recombinant Human Active Heparanase/HPSE Protein, CF were purchased from R&D Systems, Inc. (Minneapolis, MN). Reactions were monitored by thin-layer chromatography (TLC) analysis, and stained by potassium permanganate. All ^{1}H-NMR and HSQCAD spectra were taken with compounds dissolved D_2O (D, 99%). Chemical shifts (δ) are recorded in ppm, coupling constants (J) are reported in Hz. Unless otherwise noted, all reactions were performed under a nitrogen atmosphere using anhydrous solvents and oven-dried glassware and stir bars. Lyophilization was performed using a Virtis Benchtop 4K freeze dryer Model 4BT4K2L-105 at -40 ^{o}C. ^{1}H NMR and HSQCAD spectra were recorded on a Varian INOVA 500 MHz spectrometer or Varian INOVA 400 MHz spectrometer. Infrared spectroscopy (IR) was performed on a Nicolet FT-IR with a horizontal attenuated total reflectance (ATR) adapter plate. CHNS analyses were carried out by Atlantic Microlab, Inc. (Norcross, GA). Circular dichroism (CD) studies were performed in a 1 mm path length cuvette using an Applied Photophysics CS/2 Chirascan with a standard Xenon lamp. Efforts to obtain matrix assisted laser desorption/ionization (MALDI) spectra of the sulPASs were unsuccessful in positive as well as negative modes, with the respective matrices. All animal experiments were approved by the Animal Care and Use Committee of BIDMC. All investigators adhered to NIH guidelines for the care and use of laboratory animals.

Size exclusion chromatography

THF Protected polymer molecular weights were determined by size exclusion chromatography (SEC) versus polystyrene standards (M_p = 6,570,000 g/mol, 739,500 g/mol, 128,700 g/mol, 29460 g/mol and 2880 g/mol) using THF as the eluent at a flow rate of 1.0 mL/min through two Jordi columns (Jordi Gel DVB 105 Å and Jordi Gel DVB 104 Å, 7.8 x 300 mm) at 25 °C in series with a refractive index detector. All calculations were performed using Breeze GPC software (Waters, Milford, MA). Deprotected polymer and sulfated polymer molecular weights were determined by gel permeation chromatography (GPC) versus dextran standards (M_p= 1080 g/mol, 4400 g/mol, 9890 g/mol, 21400 g/mol, 43500 g/mol, and 276,500 g/mol) using aqueous buffer (0.2 M $NaNO_3$, 0.010 M phosphate buffer, pH 7.5) as the eluent at a flow rate of 0.5 mL/min through two PL aquagel columns (OH 60 micron, 7.8 x 300 mm) at 25 °C with a refractive index detector. All calculations were performed using Cirrus GPC software (Agilent, Santa Clara, CA).

Biological assays

FXa and FIIa amidolytic activity inhibition assay in the presence of AT-III: Reagents in Chromogenix Coatest® Heparin kit and Bovine thrombin were used for FXa and FIIa amidolytic activity inhibition assay. A mixture 20 µL of AT (0.8 µM) and 20 µL of varying concentrations (0.0005- 5000 µg/mL) of analytes (glusulPAS-50-HS, glusulPAS-12-HS or Heparin) were incubated at 37 °C for 5 mins. Then, 20 µL of FXa (0.08 µM) or 20 µL of FIIa (0.08 µM) were added and mixed well and incubated at 37 °C for 1 minute. Then 40 µL of S-2222 (1 mM for FXa) or S-2238 (1 mM for FIIa) were added and incubated at 37 °C for 5 mins, after which, the reaction was quenched by the addition of 40 µL of 20% acetic acid and the absorbance at 405 nm was measured and the percent inhibition was calculated by the equation:

$$\%\ inhibition = \frac{Ao - Ai}{Ao - Ab} * 100$$

where A_i is the absorbance at 405 nm for varying concentration of analyte, A_0 is the absorbance at 405 nm in the absence of any inhibitor (positive control), A_b is the absorbance value when FXa or FIIa were completely inhibited by adding acetic acid before addition of the substrate (negative control). The assay was repeated by replacing 20 µL of AT with 20 µL of buffer to study the effect of AT-III in FXa and FIIa amidolytic activity inhibition of sulPASs.

FXIIa amidolytic activity inhibition assay: 90 µL of varying concentration of glusulPAS-50-HS (0.05- 500 µg/mL) was mixed with 60 µL of FXIIa (6.7 µM, Biovision) and was incubated at 37 °C for 5 mins. Then 40 µL of S-2302 (4 mM) was added and the mixture was incubated at 37 °C for 15 mins. The reaction was stopped by the addition of 40 µL of 20% acetic acid and the absorbance was measured at 405 nm. Heparin was used as positive control.

Evaluation of degradation of glusulPAS by heparinase: 2.38 mmoles each of Recombinant F. heparinum Heparinase I Protein, CF, Recombinant P. heparinus Heparinase II Protein, CF and Recombinant P. heparinus Heparinase III Protein, CF were dissolved in 150 µL of assay buffer (50 mM Tris, 100 mM NaCl, 2 mM $CaCl_2$, pH 7.5). 150 µL of 2 mg/mL Heparin (n=3) OR 150 µL of 2 mg/mL sulPAS (n=3) OR 150 µL of buffer control (n=3) were added to the enzyme mixture and incubated at 30 °C for 20 hours. 150 µL of buffer control and 150 µL of buffer Heparin (n=3) were also incubated at 30 °C as control. After 20 h, the solution was transferred to MilliporeSigma™ Amicon™ Ultra Centrifugal Filter Units (3kDa MWCO) and centrifuged at 10000xg for 3 minutes to remove the salts. The process was repeated after addition of 300 µL of DI H_2O and the desalted samples were lyophilized, re-dissolved in SEC buffer and retention times were evaluated.

Cytotoxicity: To determine cytotoxicity when cells were in the presence of sulPAS, glusulPAS-10-HS was used as a representative example for an in vitro cell viability assay performed using a standard MTS proliferation assay protocol (CellTiter 96® Aqueous One, Promega, Madison, WI). Cells were seeded at 25,000 cells/well in 96-well plates and incubated overnight at 37°C. The media was removed and replaced with culture media containing 2 mg/mL glusulPAS-10-HS. Cells were incubated for another 24 or 48 hours before measuring cell viability with an MTS reagent. Absorbance was recorded at 492 nm with a multi-plate reader and cell viability was calculated in relation to control cells in media. Varying concentrations of dextran, PAS and heparin were used as control for the assay.

Hemolytic assay[1]**:** Single donor human whole blood was purchased from Innovative Research (Novi, MI). 2 mL whole blood was centrifuged at 1500 rpm for 10 min at 4 °C. The plasma supernatant was removed and erythrocytes were resuspended in ice cold PBS. Cells were centrifuged at 1500 rpm for 10 min at 4 °C. This was repeated two more times to ensure the removal of any released hemoglobin. After the supernatant was removed following the last wash, the cells were resuspended in PBS to get a 20% v/v RBC solution. 100 uL each of varying concentrations (1 - 0.125 mg/mL) of the polymer and heparin prepared in PBS was incubated with 100 uL of the 20% v/v RBC solution in a 96-well plate and incubated for 24 h at 37 °C. Complete hemolysis was attained using a 2% v/v Triton-X yielding the 100% control value (positive control). After incubation, the plates were centrifuged and the supernatants were transferred to a new 96-well plate. The release of hemoglobin was determined by spectrophotometric analysis of the supernatant at 550 nm. Results were expressed as the amount of hemoglobin release induced by the conjugates as a percentage of the total.

Supplementary figures

Figure S1. ^{1}H NMR of gluPAS-12(D_2O).

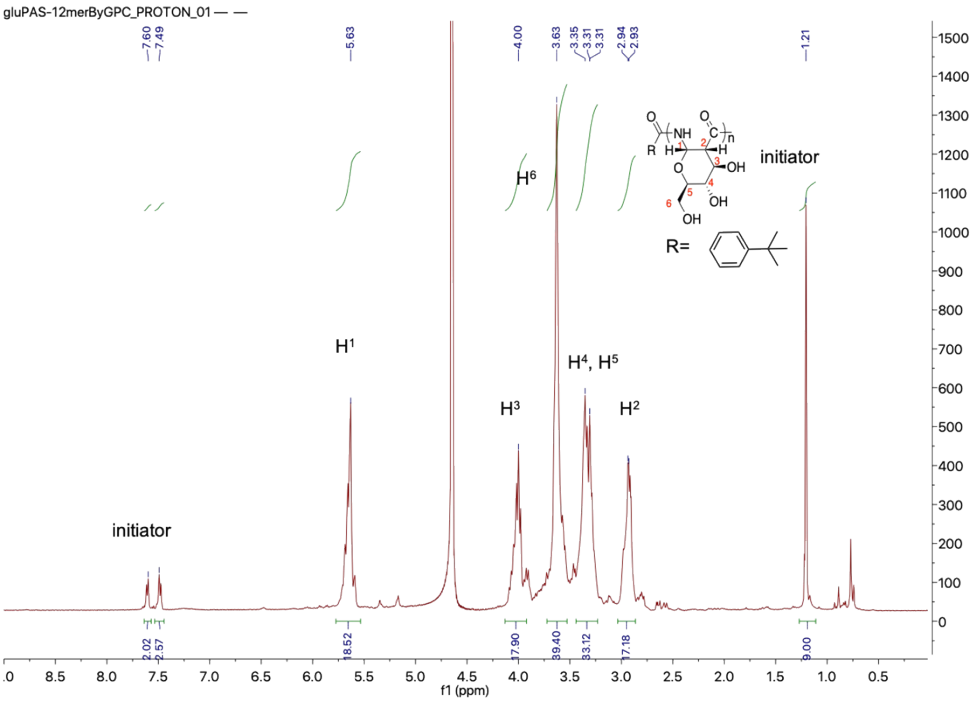


Figure S2. ^{1}H NMR of gluPAS-25(D_2O).

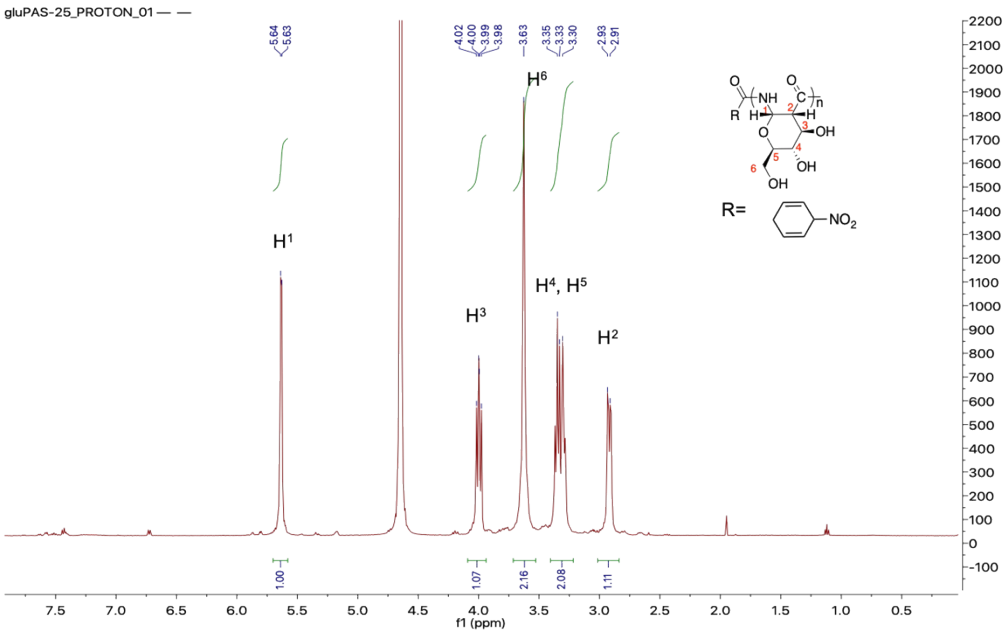


Figure S3. ^{1}H NMR of gluPAS-50 (D_2O).

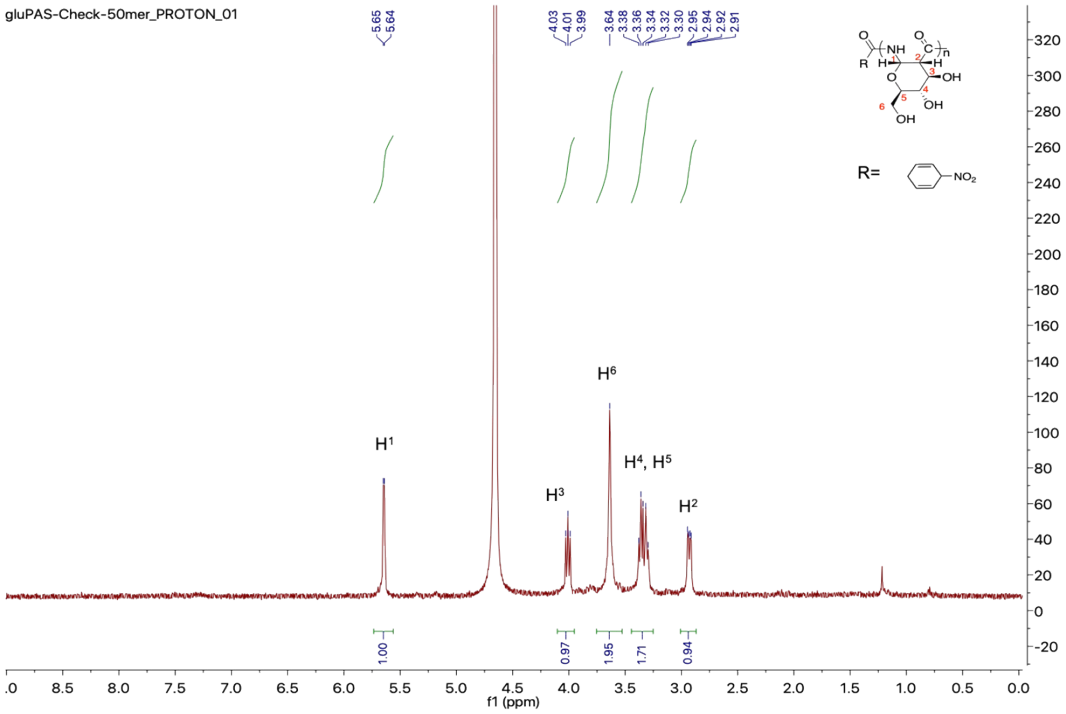


Figure S4. ^{1}H NMR of galPAS-12 (D_2O).

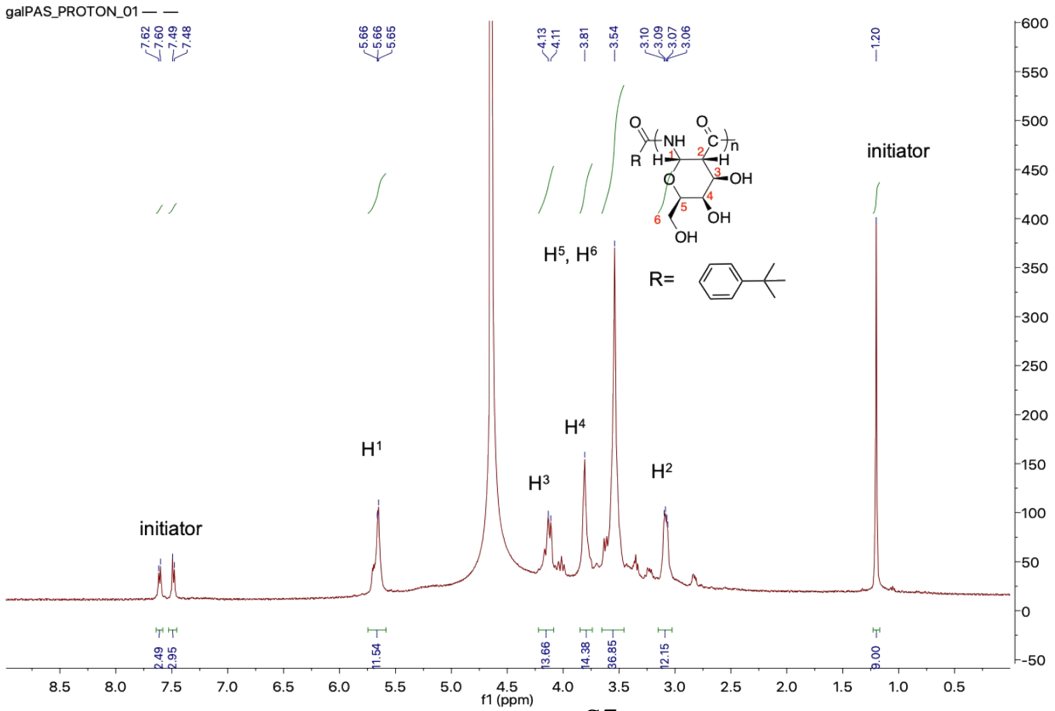


Figure S5. ^{1}H NMR of glusulPAS-12mer-LS (D_2O).

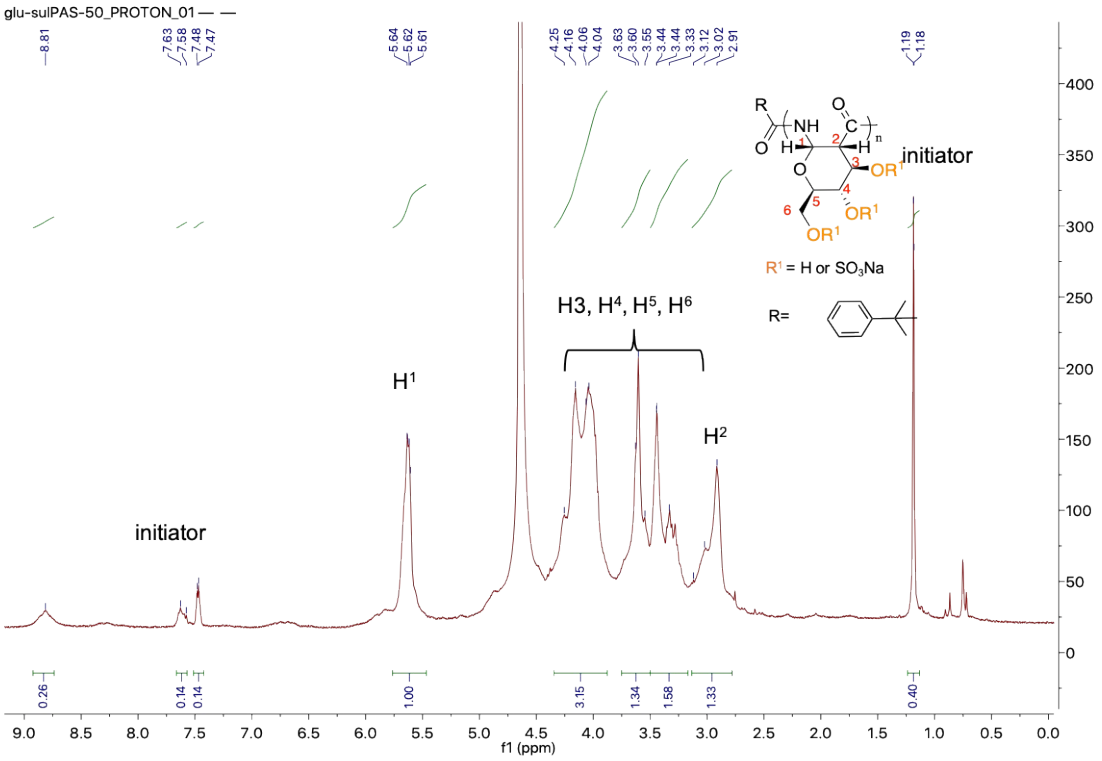


Figure S6. ^{1}H NMR of glusulPAS-12mer-HS (D_2O).

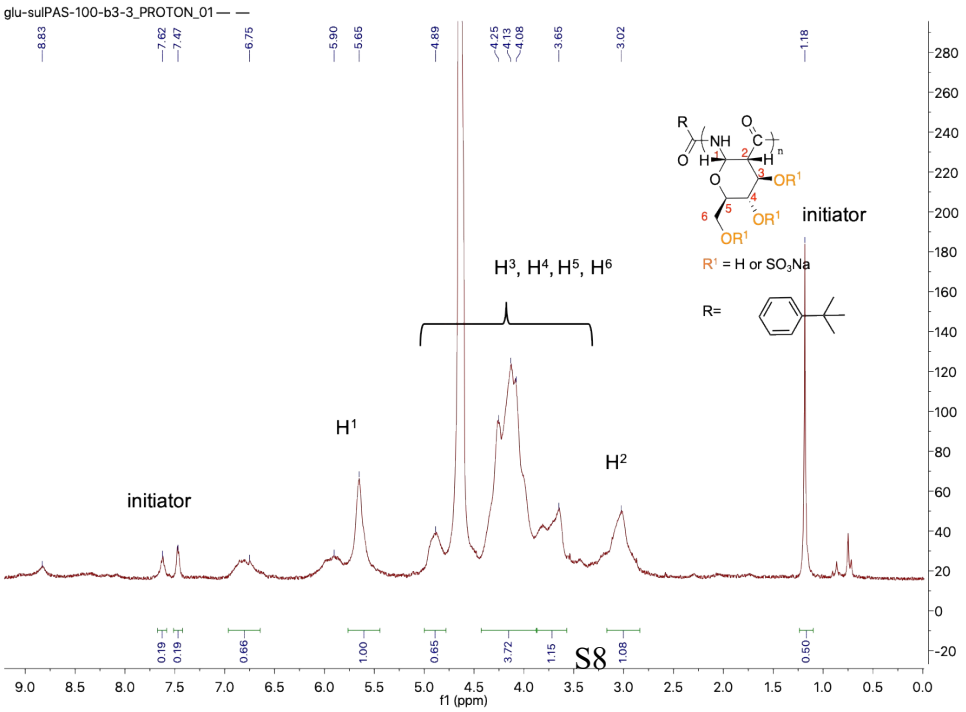


Figure S7. ^{1}H NMR of glusulPAS-25mer-HS (D_2O).

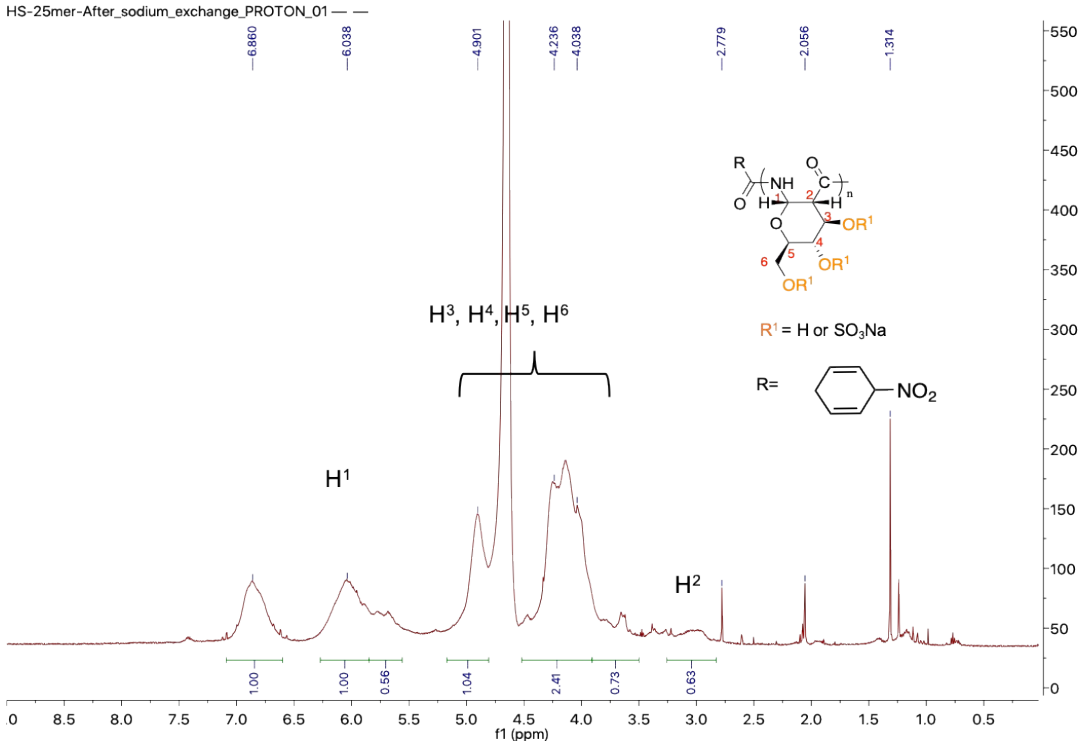


Figure S8. ^{1}H NMR of glusulPAS-50mer-HS (D_2O).

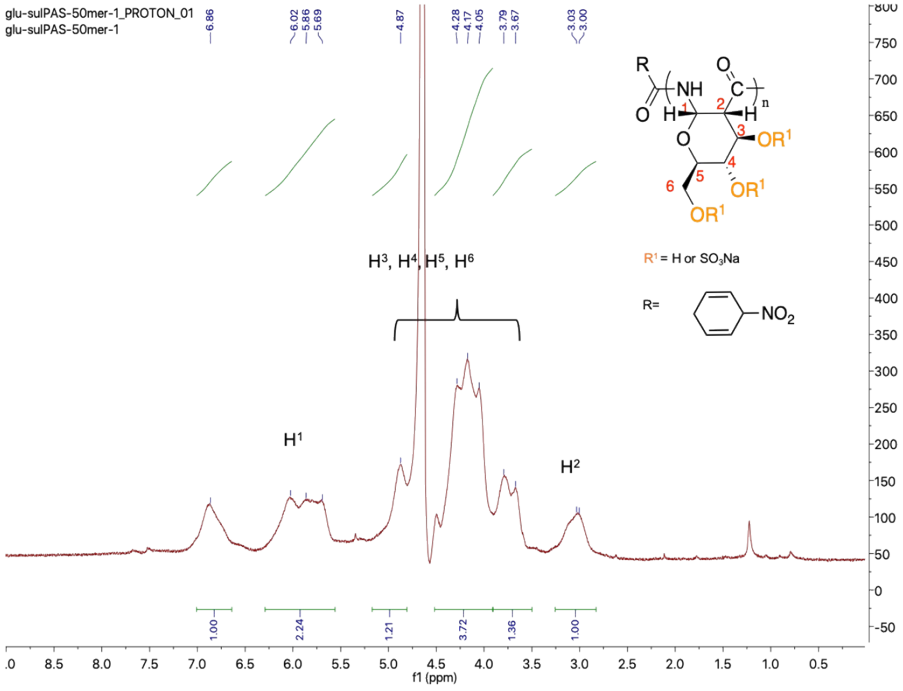


Figure S9. ^{1}H NMR of galsulPAS-12mer-HS (D_2O).

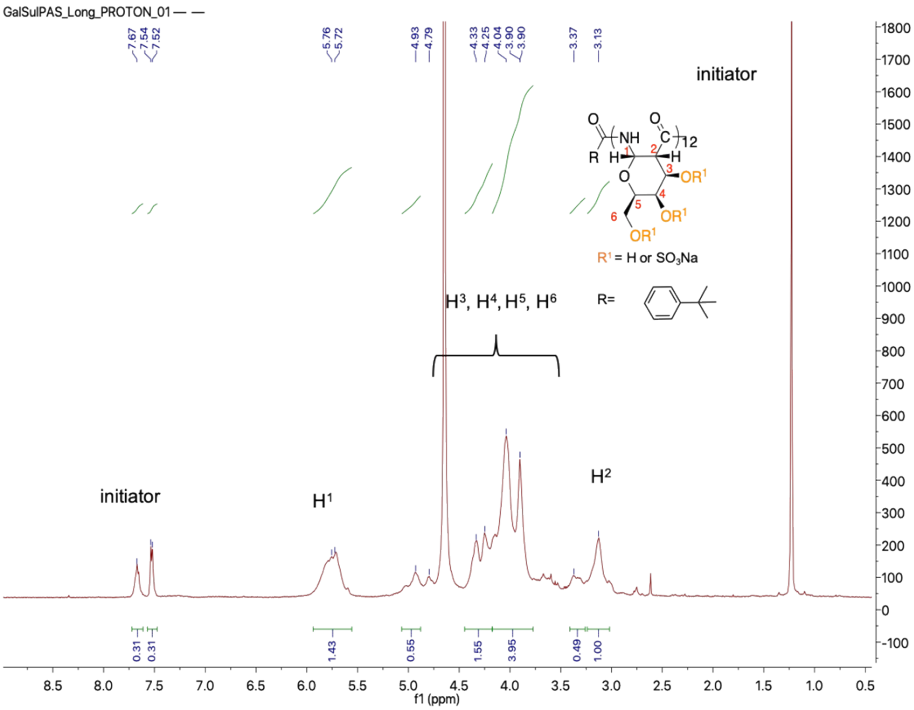


Figure S10. HSQCAD of glusulPAS-12mer-LS (D_2O).

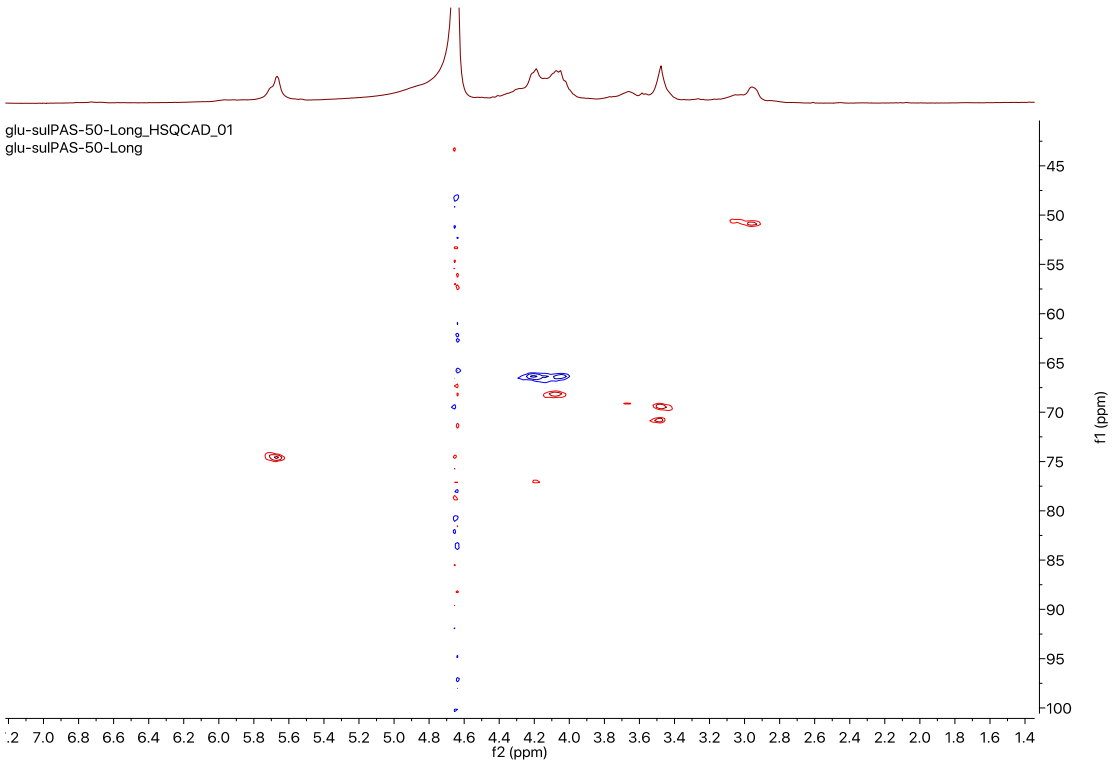


Figure S11. HSQCAD of glusulPAS-12mer-HS (D_2O)

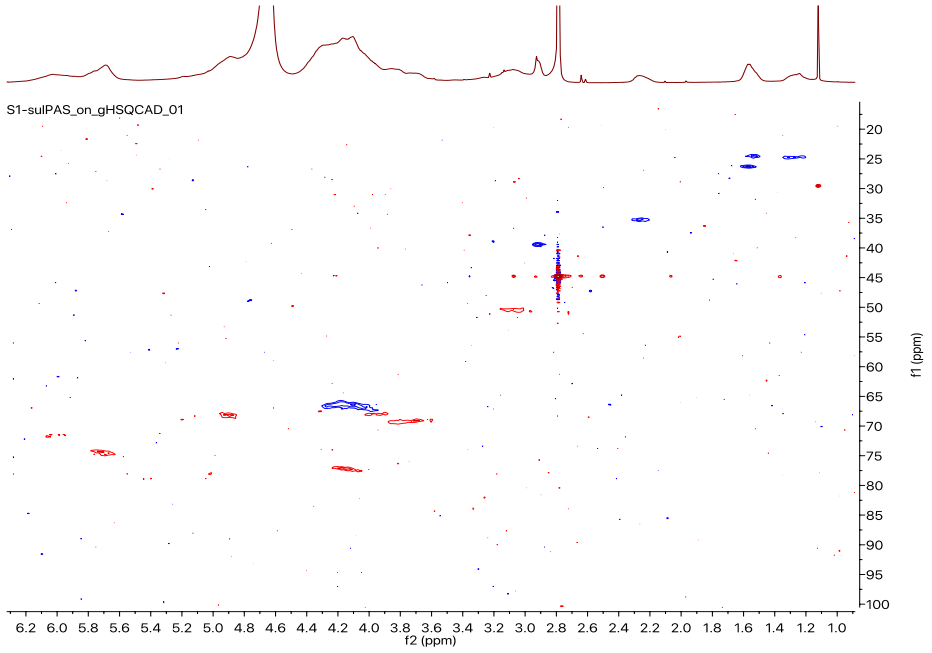


Figure S12. HSQCAD of glusulPAS-25mer-HS (D_2O)

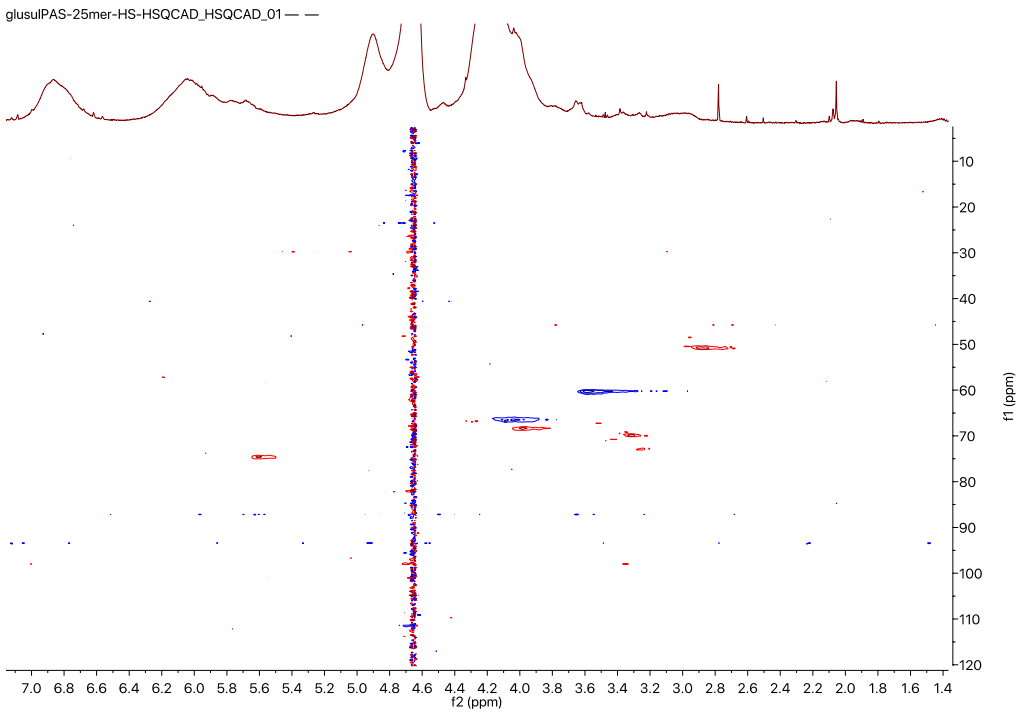


Figure S13. HSQCAD of glusulPAS-50mer-HS (D_2O)

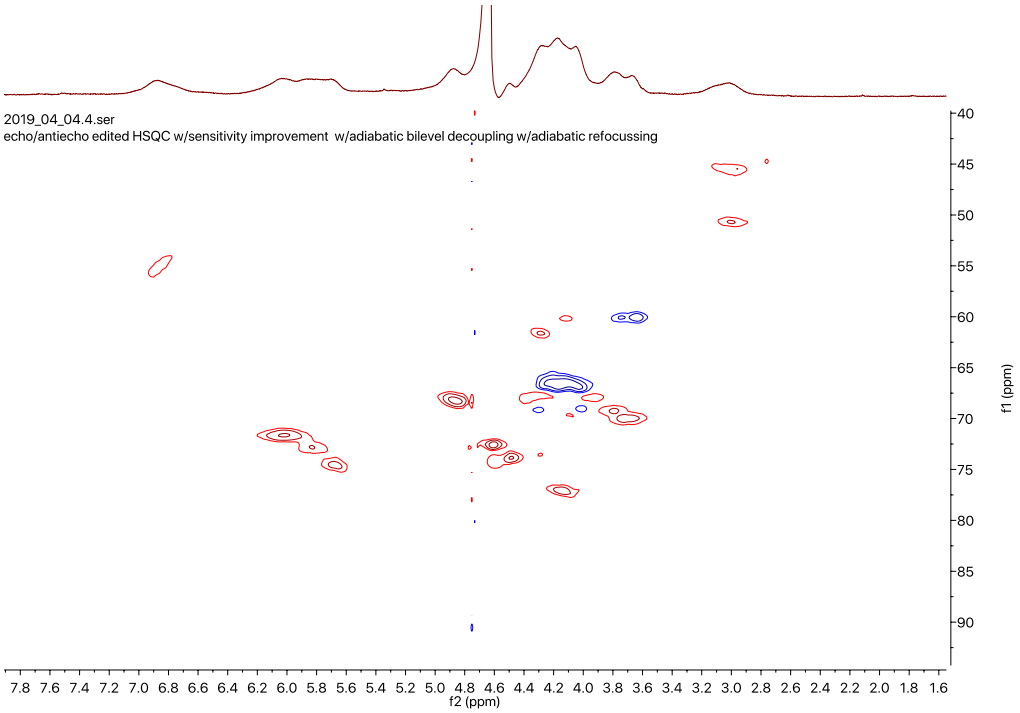


Figure S14. HSQCAD of galsulPAS-12-HS (D_2O)

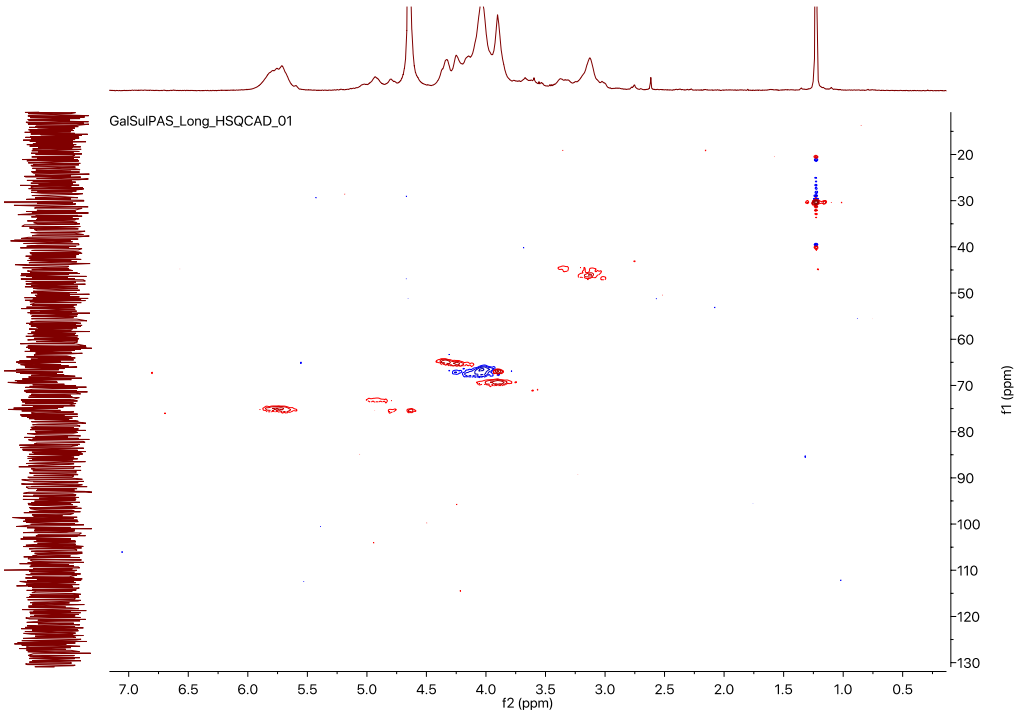


Figure S15. IR spectra gluPAS, glusulPAS-12-LS, glusulPAS-12-HS, glusulPAS-25-HS, glusulPAS-50-HS

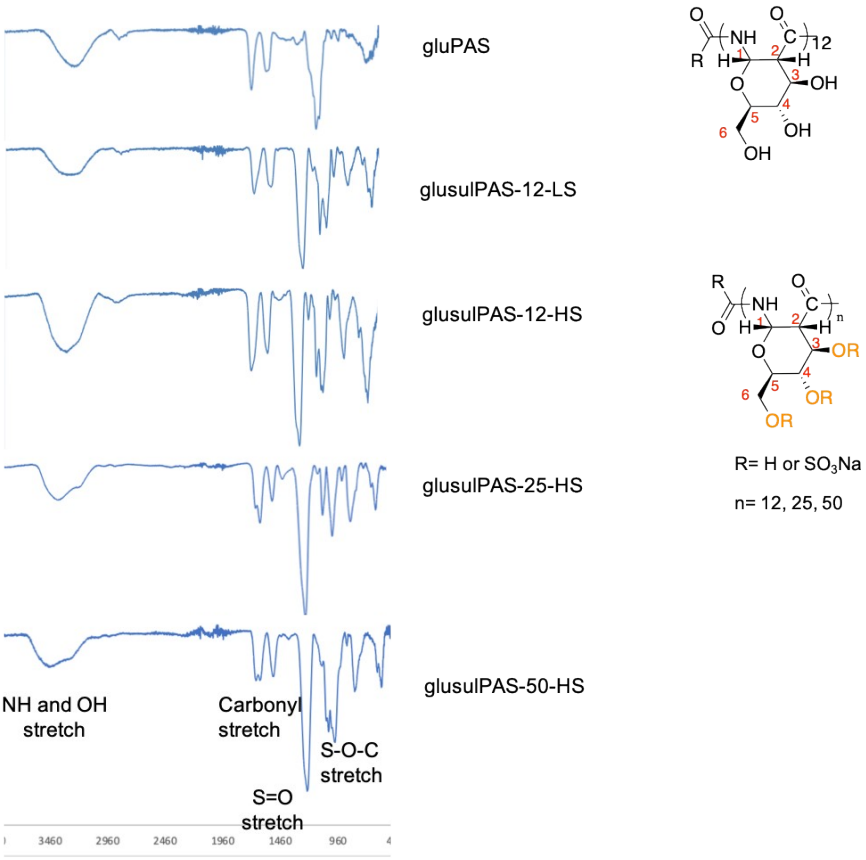


Figure S16. IR spectra of galPAS, galPAS-12-HS

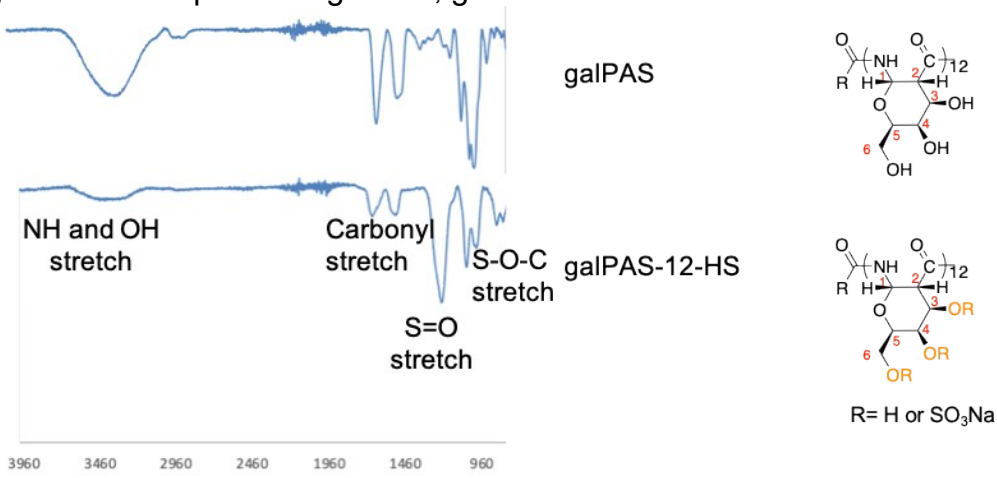
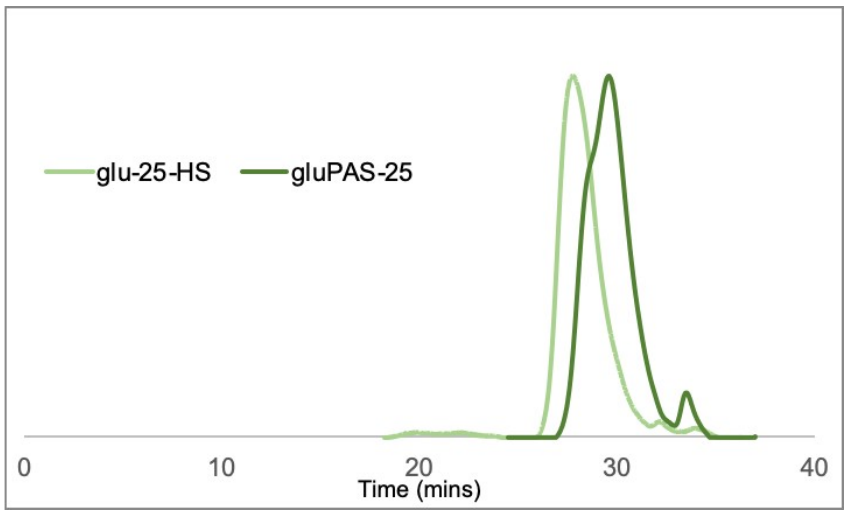


Figure S17. Example GPC traces of glusulPAS-HS-25 and gluPAS-25



glusulPAS-25-HS move to lower retention time after sulfation, indicating increase in molecular weight.

Figure S18. Circular Dichroism spectra of gluPAS-12, glu-12-LS and glu-12-HS

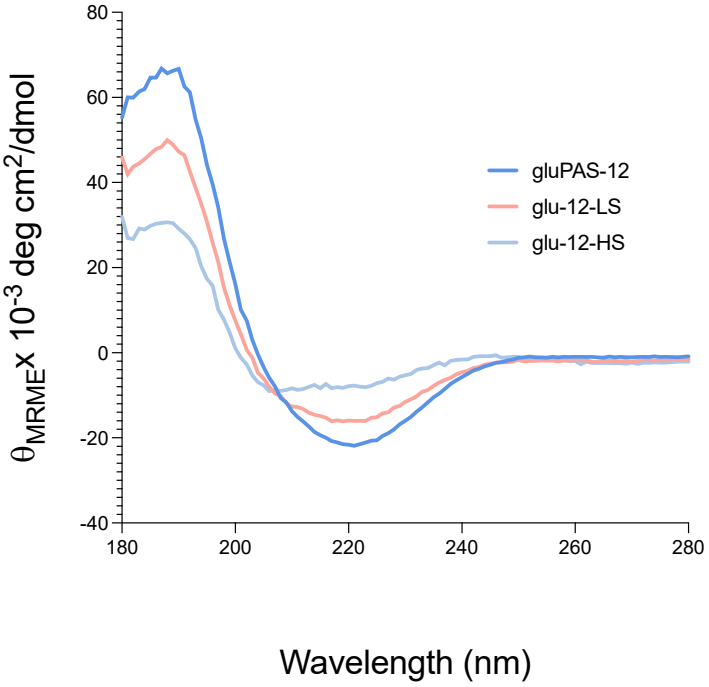


Figure S19. Coagulation cascade[3]

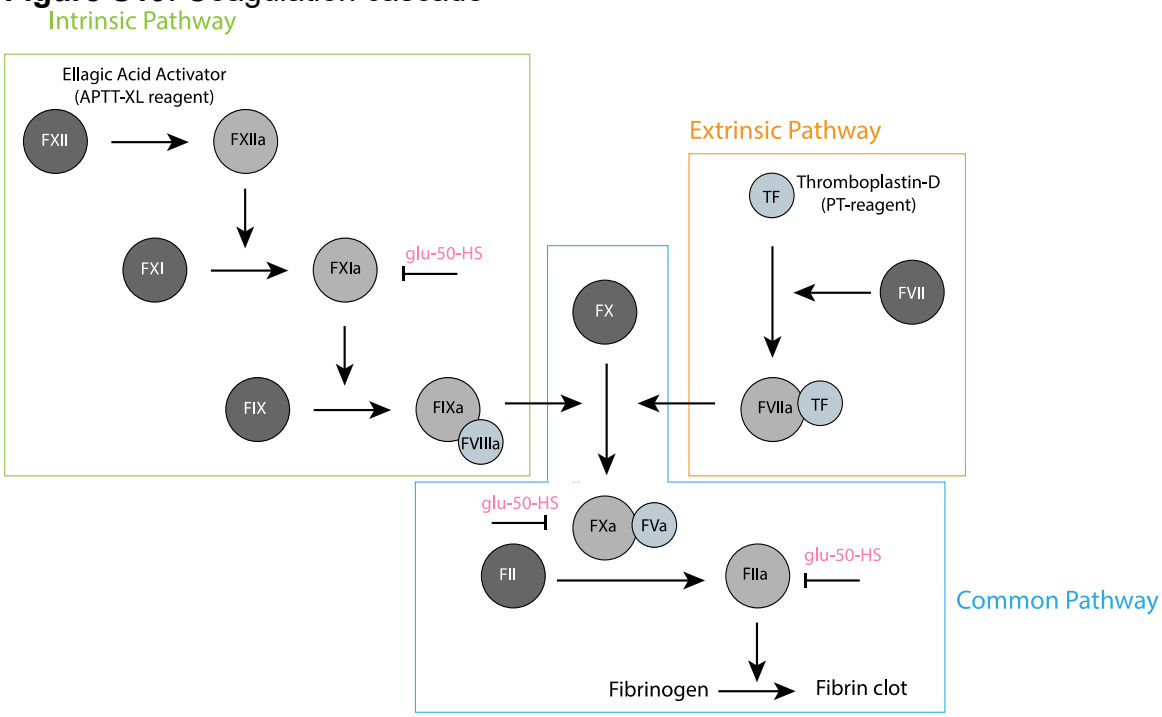


Figure S20. Results of a) APTT and b) PT assays with concentration unit IU/mL

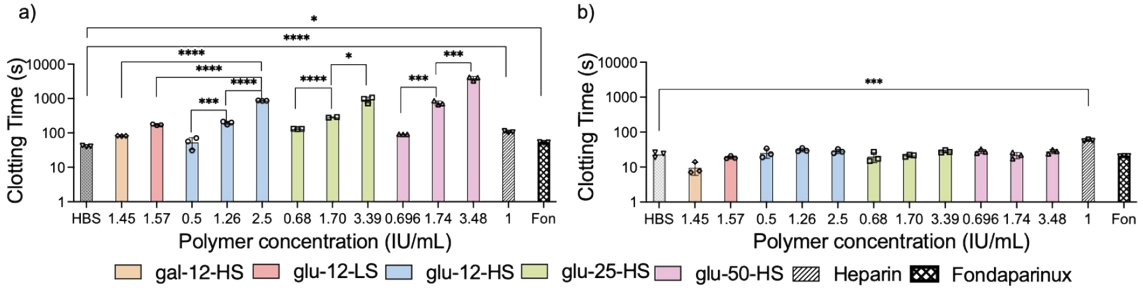


Figure S21. APTT and PT of PASs

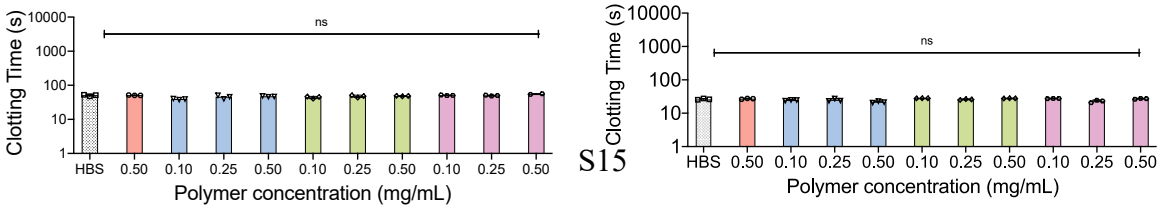


Figure S22. Calibration curve of APTT using Heparin

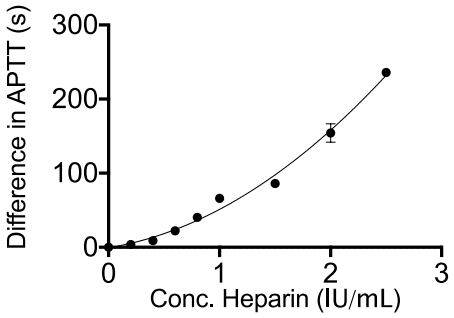


Figure S23. FXa assay- Heparin and Fondaparinux

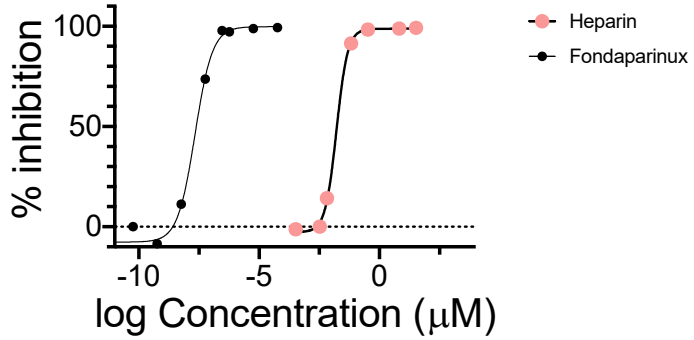


Figure S24. FIIa assay- Heparin and glusulPAS-50-HS

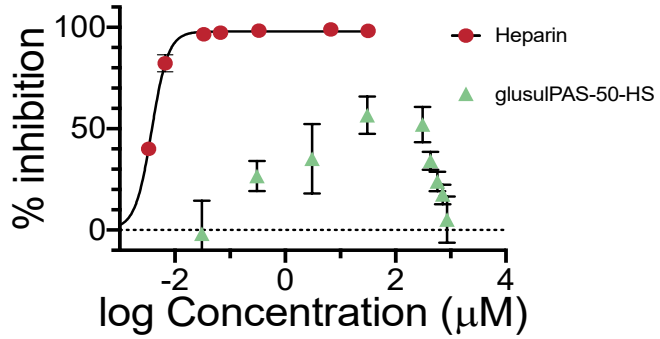


Figure S25. FXa inhibition assay with and without AT-III

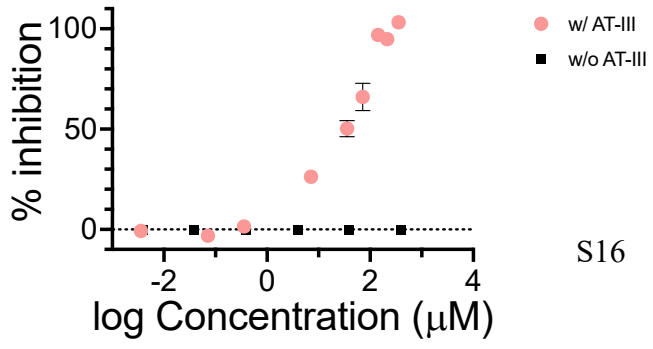


Figure S26. Thrombin inhibition assay with and without AT-III

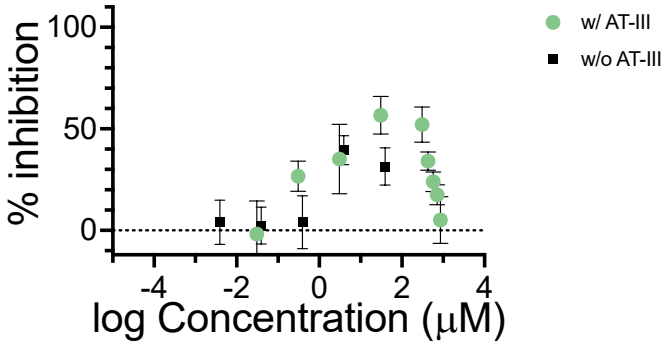


Figure S27. FXIa assay- fondaparinux

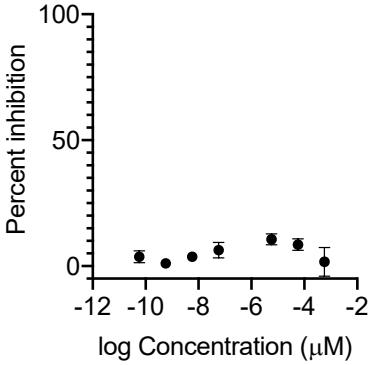


Figure S28. FXIIa assay- glusulPAS-50-HS

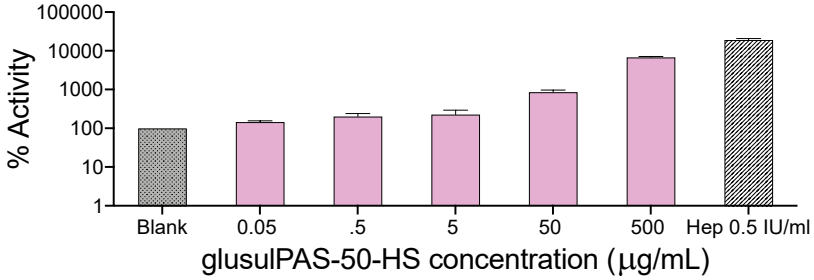
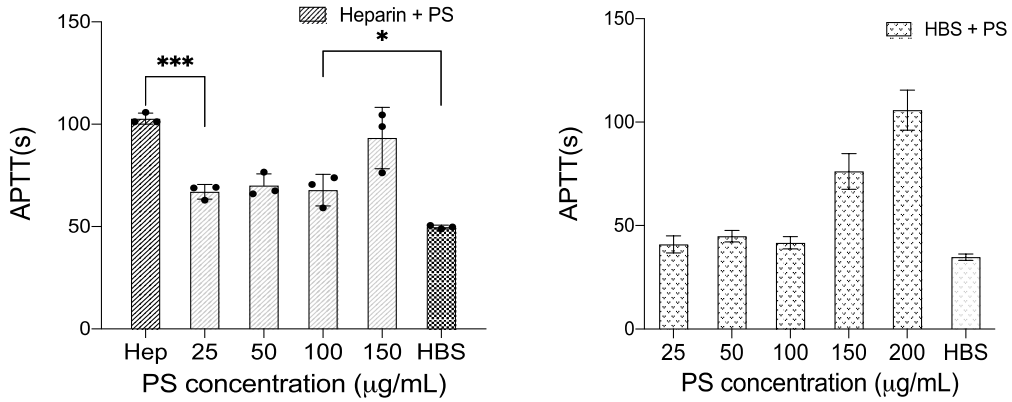


Figure S29. Neutralization of heparin by protamine sulfate



Protamine sulfate lowered clotting time of 1 IU/mL heparin with addition of 25 μg/mL PS. PS increased clotting time above concentration of 100 μg/mL. This is consistent with the weak anticoagulant property of PS. [2]

Figure S30. Cytotoxicity Assay

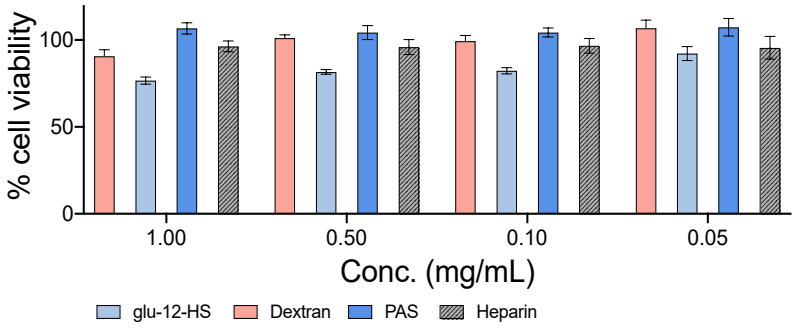
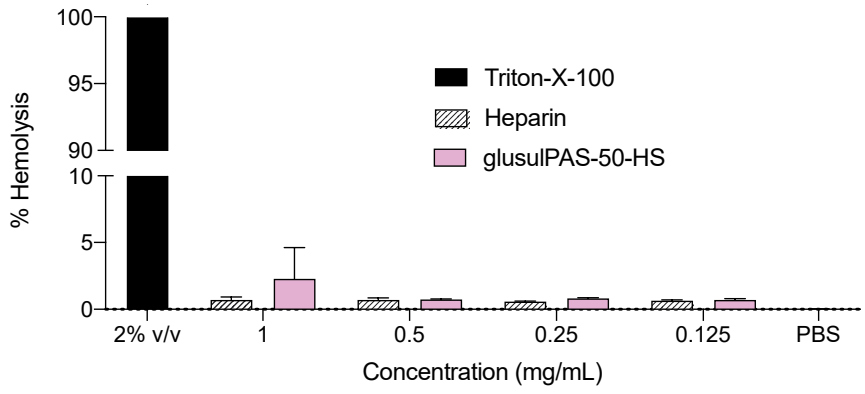


Figure S31. Hemolysis assay



Supplementary tables

Table S1. CHNS analysis of sulPAS

Polymer	%C	%S	DS
glusulPAS-12-LS	25.73	9.99	1.01
glusulPAS-12-HS	19.16	14.33	1.96
glusulPAS-25-HS	23.47	11.48	1.28
glusulPAS-50-HS	21.79	11.92	1.43
galsulPAS-12-HS	21.71	13.00	1.57

Degree of sulfation from CHNS was calculated using the equation,

$DS = 2.625 * S\% \,/C\%$

Table S2. Calculation of activity from calibration curve

Polymer	Concentration (mg/mL)	Average increase in clotting time compared to HBS (s)	Activity from calibration curve (IU)	Activity (IU/mg)	Average Activity (IU/mg)
glusulPAS-12-LS	0.1	9.45 ± 2.24	0.343068588	3.430685883	3.15±0.5
glusulPAS-12-LS	0.25	49.48± 3.37	0.895981432	3.583925728	
glusulPAS-12-LS	0.5	131.64 ± 5.83	1.222670918	2.445341837	
glusulPAS-12-HS	0.1	22.61 ± 6.10	0.634464361	6.344643615	5.03±1.1
glusulPAS-12-HS	0.25	152.53 ± 12.59	1.271849674	5.087398697	
glusulPAS-12-HS	0.5	821.26 ± 11.90	1.833944629	3.667889258	
glusulPAS-25-HS	0.1	90.04 ± 2.59	1.095853921	10.9585392	6.79±3.0
glusulPAS-25-HS	0.25	241.55 ± 7.85	1.425343896	5.70137558	
glusulPAS-25-HS	0.5	883.74 ± 149.35	1.858427839	3.71685568	
glusulPAS-50-HS	0.1	51.11 ± 1.58	0.90675907	9.06759074	6.96±1.8
glusulPAS-50-HS	0.25	706.17 ± 82.28	1.78353563	7.1341425	
glusulPAS-50-HS	0.5	3803.41 ± 412.30	2.34617518	4.69235036	
galsulPAS-12-HS	0.1	13.67 ± 5.54	0.46653621	3.73228966	2.91±0.7
galsulPAS-12-HS	0.25	30.43 ± 3.49	0.73367846	2.93471382	
galsulPAS-12-HS	0.5	72.91 ± 4.40	1.02537848	2.05075697	

Table S3. Heparanase Assay

Polymer	Average Max RT w/o Heparanase (Mins)	Average Max RT w/ Heparanase (Mins)
Heparin	25.54 ± 0.30	27.04 ± 0.42
glusulPAS-25-HS	27.86 ± 0.38	27.92 ± 0.38

Heparin peak shifts to a later retention time after treatment with heparanase (unpaired t test, P = 0.071) indicating depolymerization. Retention time of sulPAS did not vary significantly with or without heparanase (unpaired t test, P = 0.8556). All incubations were repeated 3 times and SEC characterization was carried out for each trial.

Table S4. Heparinase Assay

Polymer	Average Max RT w/o Heparinase (mins)	Average Max RT w/ Heparinase (mins)
Heparin	25.39 ± 0.03	27.00 ± 0.22
glusulPAS-50-HS	26.97*	26.98 ± 0.01

* n =1

Heparin peak shifts to a later retention time after treatment with heparanase (unpaired t test, P = 0.0002) indicating depolymerization. Retention time of sulPAS did not vary significantly with or without heparanase (unpaired t test, P = 0.4226). All incubations except glusulPAS-50-HS were repeated 3 times and SEC characterization was carried out for each trial.

References

1 H.-T. Chen, M. F. Neerman, A. R. Parrish and E. E. Simanek, *J. Am. Chem. Soc.*, 2004, **126**, 10044–10048.

2 D. Bolliger, F. Szlam, M. Azran, K. Koyama, J. H. Levy, R. J. Molinaro and K. A.

Tanaka, *Anesth. Analg.*, 2010, **111**, 601–608.

3 V. Kumar, A. Abbas, J. Aster, & S. Robbins 2018. Robbins basic pathology (Tenth edition). Elsevier.