

Synthesis and Anticoagulant Activity of Polyureas Containing Sulfated Carbohydrates

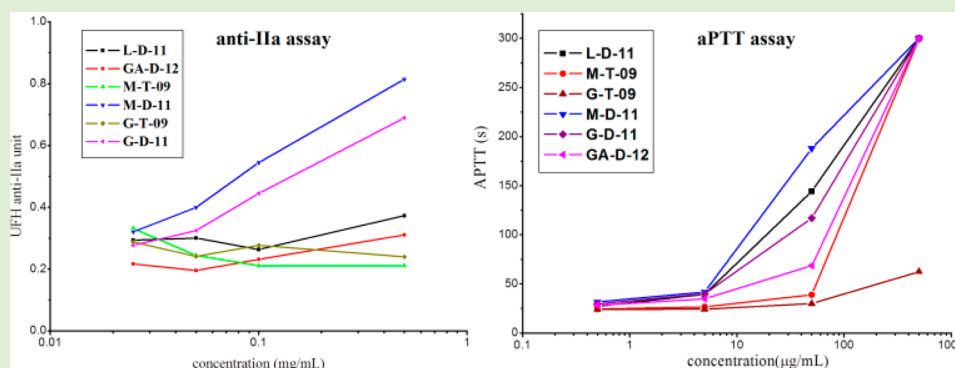
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S Supporting Information



ABSTRACT: Polyurea-based synthetic glycopolymers containing sulfated glucose, mannose, glucosamine, or lactose as pendant groups have been synthesized by step-growth polymerization of hexamethylene diisocyanate and corresponding secondary diamines. The obtained polymers were characterized by gel permeation chromatography, nuclear magnetic resonance spectroscopy, and Fourier transform infrared spectroscopy. The nonsulfated polymers showed similar results to the commercially available biomaterial polyurethane TECOFLEX in a platelet adhesion assay. The average degree of sulfation after reaction with SO_3 was calculated from elemental analysis and found to be between three and four $-\text{OSO}_3$ groups per saccharide. The blood-compatibility of the synthetic polymers was measured using activated partial thromboplastin time, prothrombin time, thrombin time, anti-IIa, and anti-Xa assays. Activated partial thromboplastin time, prothrombin time, and thrombin time results indicated that the mannose and lactose based polymers had the highest anticoagulant activities among all the sulfated polymers. The mechanism of action of the polymers appears to be mediated via an anti-IIa pathway rather than an anti-Xa pathway.

INTRODUCTION

Biomaterials have a significant positive impact on quality of life for their recipients; however, when a non-native biomaterial comes into contact with blood, a cascade reaction may be initiated and results in protein adsorption on the non-native biomaterial, cell-material surface interaction, platelet adhesion and activation, and clot formation. Therefore, a primary concern with using biomaterials is their blood compatibility. Polyurethanes have been used in blood contacting biomaterials including catheters, blood filters, and blood tubing as they typically possess excellent mechanical properties including strength, moldability, and elasticity.¹ Commercial brands of clinically used polyurethane materials include Biomer (Johnson & Johnson), Mitrathane (PolyMedica Industries, Inc.), and Pellethane and Tecoflex (The Lubrizol Corporation).^{2–5} However, typical polyurethanes exhibit poor blood compatibility due to their hydrophobicity.⁶ One strategy to improve blood compatibility of polyurethanes is to increase surface

hydrophilicity by incorporating other hydrophilic functional groups such as sulfates, carboxylates, and phosphonates. For example, propyl sulfate groups were incorporated into the hard segments of a poly(tetramethylene oxide)-based polyurethane. The sulfonated polymer was observed to promote decreased platelet deposition with a delayed thrombin time (TT), reptilase time, prothrombin time (PT), and activated partial thromboplastin time (aPTT).^{7,8} In another example, a sulfonated polyurea-urethane was prepared using a diamino disulfonic acid.⁹ Improved anticoagulant activity was observed as evidenced by a prolonged TT. Polyurethanes have been synthesized with the naturally occurring biopolymer dermatan sulfate to increase material hydrophilicity.¹⁰ Decreased protein adsorption and cell adhesion were observed, which were

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attributed to the physical and biological properties of the dermatan sulfate. Carboxylate groups can also be incorporated into polyurethane, but with lower anticoagulating capacities in platelet deposition assays compared to sulfonated polyurethanes.¹¹

Surface heparinization and heparin-mimicking polymers provide an alternative strategy to increase the hemocompatibility of biomaterials. Heparin is a linear, highly sulfated and negatively charged polysaccharide and has been widely used as an injectable anticoagulant for over 80 years.¹² The anticoagulant activity of heparin is ascribed to its unique pentasaccharide binding site, which exerts strong and specific binding with antithrombin III (ATIII) to generate a heparin–ATIII binary complex.¹³ The heparin–ATIII binary complex will further recognize and bind with thrombin or with factor Xa (FXa), both of which are important blood coagulant factors, forming an ATIII–heparin–thrombin or ATIII–heparin–FXa ternary complex.¹⁴ Although heparin is widely used as a clinical anticoagulant, it has several limitations, particularly for unfractionated heparin (UFH). Currently, pharmaceutical grade heparin with an average molecular weight of 14 kDa is extracted from mucosal tissues of slaughtered animals such as porcine (pig) intestine or bovine (cow) lung.¹⁵ Extracted heparin may possess heterogeneity in the carbohydrate structure and lead to variable dose–response relationships for different patients. The extraction from animal mucosal tissues may also introduce risks of pathogen contamination. The worldwide outbreak of bovine spongiform encephalopathy and the global distribution of contaminated heparin in 2008 highlighted these concerns.^{13,15,16} Furthermore, exposure to heparin therapy may result in adverse side effects such as heparin-induced thrombocytopenia (HIT), which is caused by the formation of an antibody against the heparin–platelet factor 4 complex.¹⁷

These drawbacks resulted in alternative forms of heparin being developed, namely low molecular weight heparin (LMWH) and ultralow molecular weight heparin (ULMWH). LMWH has an average molecular weight of 6.0 kDa and is produced from unfractionated heparin by chemical or enzymatic degradation.¹⁸ ULMWH is a synthesized drug with the trade name Arixtra and a molecular weight of 1508 Da.^{13,19,20} Compared to unfractionated heparin, LMWH and ULMWH exhibit more predictable anticoagulant doses, longer half-lives, and reduced risks of side effects.^{21,22} However, LMWH is still derived from animals with associated heterogeneity and potential pathogen contamination. Furthermore, the synthesis of ULMWH requires more than 50 steps with an overall yield of only 0.1%.²³ Although improved synthetic methods have been proposed,^{20,24} Arixtra is still the most expensive drug among the heparins. LMWH and ULMWH also exhibit limited inhibition efficiency toward thrombin due to the shorter molecular length. In summary, there remains a strong argument for safe and efficacious heparin-mimics with high anticoagulant activity and minimal side effects.

Current methods to synthesize heparin-mimicking polymers focus on using anionic sulfate groups, polymerization of sulfated saccharides, and sulfation of naturally occurring glycans. Poly(sodium-4-styrenesulfonate) (PSS) has been used to replicate the anionic sulfate domains of heparin.^{25–29} For example, poly(sodium 4-styrenesulfonate-*co*-poly(ethylene glycol) methacrylate) (pSS-*co*-pPEGMA) with a terminal dithiocarbonate group was synthesized by Maynard's group.²⁹

The dithiocarbonate was reduced to a thiol and immobilized on a gold-coated surface plasmon resonance chip to investigate electrostatic interactions of the polymer with basic fibroblast growth factor or vascular endothelial growth factor. In another example, a polymer brush containing isopropylidene protected glucose was obtained via atom transfer radical polymerization.³⁰ After deprotection and sulfation, the heparin-mimicking polymer displayed delayed clot formation and reduced complement activation. Similarly, sulfated poly-(glucosyloxyethyl methacrylate) (poly(GEMA)) has demonstrated anticoagulant activity through prolonged human blood clotting times.³¹ Direct polymerization of sulfated saccharides has also been reported. Chaikof's group^{32–39} synthesized sulfated glucose, glucosamine, and lactose monomers for polymerization via a cyanoxyl-mediated free radical polymerization. The heparin-mimicking polymers prolonged TT and aPTT.³⁵ The Hsieh–Wilson group synthesized a sulfated disaccharide monomer consisting of iduronic acid and glucosamine.^{40,41} Ring-opening metathesis polymerization of this monomer was used to prepare heparin-mimicking polymers with comparable anticoagulant activities at a concentration of 150 $\mu\text{g}/\text{mL}$ to heparin, LMWH, and ULMWH. Recently, we published a nonionic glycosaminoglycan polymer mimetic based on a methacryloyl backbone using RAFT polymerization⁴² and two heparin-mimicking polymers with glucose and mannose as pendant groups.⁴³ Naturally occurring glycans, such as dextran and chitosan, have been sulfated to produce heparin-mimicking polymers with anticoagulant effects. Baumann et al. published a detailed synthetic method for incorporation of *O*-sulfo, *N*-sulfo, *N*-acetyl, and *N*-carboxymethyl groups into chitosan derivatives.⁴⁴ Dextran-based heparin-mimicking polymers have been synthesized by ring-opening polymerization of anhydro sugar derivatives.⁴⁵ Sulfamide groups in these polymers were attributed to yield higher anticoagulant properties than *O*-sulfated groups. The Fasl group synthesized chitosan with high and low degrees of sulfation using a pyridine/ SO_3 complex or chlorosulfuric acid, respectively.⁴⁶ The highly sulfated chitosan had a comparable anticoagulant potency to heparin and dextran sulfate.

In this paper, we synthesize heparin-mimicking polyureas using step-growth polymerization with glucose, mannose, lactose, or glucosamine as pendant groups. Polyureas are functionally similar to polyurethanes and are also easily synthesized by using diamines and diisocyanates. These new synthetic heparin-mimicking polymers exhibit anticoagulant activities with respect to platelet adhesion, aPTT, PT, and TT assays. Furthermore, we have investigated the mechanism of action of these polymers using anti-FXa and anti-FIIa assays.

■ EXPERIMENTAL SECTION

Materials and Instruments. Glucose, lactose, and sodium azide were purchased from Fisher Scientific, Matherson Coleman and Bell Co., and Amresco-Inc., respectively. All other chemicals were ordered from Sigma-Aldrich and used as received without further purification. *N,N'*-bis(2-nitrobenzenesulfonyl)-ethane-1,2-diamine was synthesized according to literature procedures.⁴⁷ Pretreated standard grade, regenerated cellulose dialysis membrane with an approximate molecular weight cut off (MWCO) of 3.5 kDa was obtained from Spectrum Laboratories. The 24-well tissue culture plates were ordered from Fisher Scientific. TECOFLEX MG-8020 was kindly provided by The Lubrizol Corporation. Si wafers were purchased from Wafer World, Inc. Platelet rich plasma ($\sim 3 \times 10^5$ platelets/ μL) was purchased from the Hoxworth Blood Center at the University of

Cinninati and used the same day it was prepared from freshly drawn blood.

^1H and ^{13}C NMR measurements were performed with a Bruker Ultrashield 400 MHz (100 MHz for ^{13}C NMR) instrument. The obtained data were processed using either MestReNova or ACD/NMR Processor Academic Edition. Fourier transform infrared spectra (FT-IR) were collected on a Nicolet 6700 spectrometer and analyzed with OMNIC32 software. Mass spectrometry was performed using a Micromass Q-TOF-2 spectrometer. Galbraith Laboratories Inc. performed elemental analyses. A Ramé-hart contact angle goniometer (model 200-F1) with tilting stage was used to measure contact angles. Platelet-adhesion assays were analyzed with an EFI XL30 ESEM at 20 kV. Molecular weight averages of polymers were determined using gel permeation chromatography (GPC) with an Agilent 1100 Series HPLC equipped with a PSS SDV Lux column (5 μm) guard column and two PSS SDV Linear XL Lux Columns (5 μm) (linear range of $M_w = 100\text{--}3 \times 10^6$ g/mol), a filtered tetrahydrofuran (THF) containing 200 ppm 2,6-bis(1,1-dimethylethyl)-4-methylphenol (BHT) mobile phase at a flow rate of 1.0 mL/min at ambient temperature, miniDAWN TREOS light scattering (60 mW GaAs linearly polarized laser, 658 nm), and Optilab rEX differential refractometer (light source = 658 nm; Wyatt Technology Corporation) detectors. The polymers were dissolved in eluent THF and filtered through 0.2 μm membrane filters before injection. ASTRA software version 5.4.14 was used to determine the molecular weight averages and polydispersity.

Procedures. *Synthesis of Tetramer-Based Glucose Containing Polymer.* The synthetic route for glucose tetramer-based polymer (G-T-07 in Scheme 1) is adopted from our previously published paper.⁴³

Synthesis of Tetramer-Based Mannose Containing Polymer. Mannose tetramer containing polymer (M-T-07 in Scheme 1) is synthesized according to our previous paper.⁴³

*Synthesis of Lactose Containing Polymers. Synthesis of Compound L-D-02.*⁴⁸ Catalytic iodine (0.50 g, 0.067 equiv) was added into a suspension solution of lactose (10.00 g, 1.0 equiv) and 50 mL of acetic anhydride. This solution was stirred at room temperature for 15 min before an excess of saturated sodium thiosulfate solution was added to quench the reaction. Dichloromethane (DCM) was added to extract the solution three times. Saturated NaHCO_3 solution was added into the organic layer until no bubbles formed. The organic layer was separated, washed by brine, and concentrated to give the desired product as a white solid (19.60 g, 99%). The structure was confirmed against that reported in the literature.⁴⁹

Synthesis of Compound L-D-03.^{50,51} Benzyl amine (6.32 g, 2.0 equiv) was added to a solution of L-D-02 (20.00 g, 1.0 equiv) in 200 mL of THF. This solution was stirred at room temperature for 20 h before the solvent was removed. DCM was added to dissolve the residue, and this solution was washed with 1.0 M HCl three times, followed by saturated NaHCO_3 , brine, and was dried over anhydrous sodium sulfate. After concentration, the crude product was purified by flash column chromatography (hexanes:EtOAc = 1:1 to 1:2), which gave the desired product as white solid (18.00 g, 96%). This structure was confirmed with the reported literature.^{50,51}

Synthesis of Compound L-D-04.^{50,51} L-D-03 (8.97 g, 1.0 equiv) was placed in a flame-dried round-bottom flask. After it was evacuated and refilled with nitrogen three times, 50 mL of anhydrous DCM was injected, followed by trichloroacetonitrile (10.17 g, 5.0 equiv). This solution was cooled using an ice bath for 5 min before catalytic 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) (0.21 g, 0.1 equiv) was added. The solution was warmed to room temperature slowly within 3 h. Solvent was removed, and the crude product was purified by flash column chromatography (hexanes:EtOAc = 3:2), which gave product as a light yellow solid 9.80 g (89%). This structure was confirmed with the reported literature.^{50,51}

*Synthesis of Compound L-D-05.*⁵¹ L-D-04 (23.78 g, 1.0 equiv) was placed in a flame-dried round-bottom flask. After it was evacuated and refilled with nitrogen three times, 150 mL of anhydrous DCM was injected, followed by 2-bromethanol (11.42 g, 3.0 equiv). This solution was cooled with dry ice/acetone for 30 min before boron trifluoride diethyl etherate (3.84 mL, about 48% BF_3 basis, 1.0 equiv) was added.

Then, it was stirred and warmed to room temperature overnight. Excess triethylamine was added to quench the reaction, and DCM was added to dilute the solution. Then the solution was washed with 1.0 M HCl solution three times, saturated NaHCO_3 , and brine and then dried over anhydrous sodium sulfate and concentrated. The crude product was purified by flash column chromatography (hexanes:EtOAc = 3:2), which gave light yellow product 14.68 g (65%). This structure was confirmed with the reported literature.⁵¹

*Synthesis of Compound L-D-06.*⁵¹ L-D-05 (12.50 g, 1.0 equiv) and NaI (4.28 g, 1.7 equiv) were dissolved in 150 mL of dry acetone. This solution was refluxed for 3 h before the solvent was removed. The crude product was purified by flash column chromatography (hexanes:EtOAc = 1:1), which gave a light yellow product 12.90 g (97%). This structure was confirmed with the reported literature.⁵¹

Synthesis of Compound L-D-07. L-D-06 (12.90 g, 3.0 equiv), *N,N'*-bis(2-nitrobenzenesulfonyl)-ethane-1,2-diamine (2.34 g, 1.0 equiv), and potassium carbonate (3.01 g, 4.0 equiv) were placed in a flame-dried flask. After it was evacuated and refilled with nitrogen three times, anhydrous DMF (50 mL) was injected under nitrogen protection. This solution was stirred at 50 $^\circ\text{C}$ for 24 h before dimethylformamide (DMF) was removed. DCM was added to dissolve the residue, and the solution was filtered through Celite and concentrated. The crude product was purified by flash column chromatography (hexanes:EtOAc = 1:1 to EtOAc), which gave a light yellow product 9.45 g (99%).

Synthesis of Compound L-D-08. L-D-07 (0.36 g, 1.0 equiv) and potassium carbonate (0.14 g, 5.0 equiv) in 4 mL of anhydrous DMF were degassed with nitrogen before thiophenol (PhSH) (0.090 g, 4.0 equiv) was added. This solution was stirred at room temperature overnight. DMF was removed, and DCM was added to dissolve the residue. After filtration through Celite and concentration, the crude product was purified by flash column chromatography (DCM to DCM:MeOH:Et₃N = 400:100:5), which gave 0.25 g (88%) of a white solid foam.

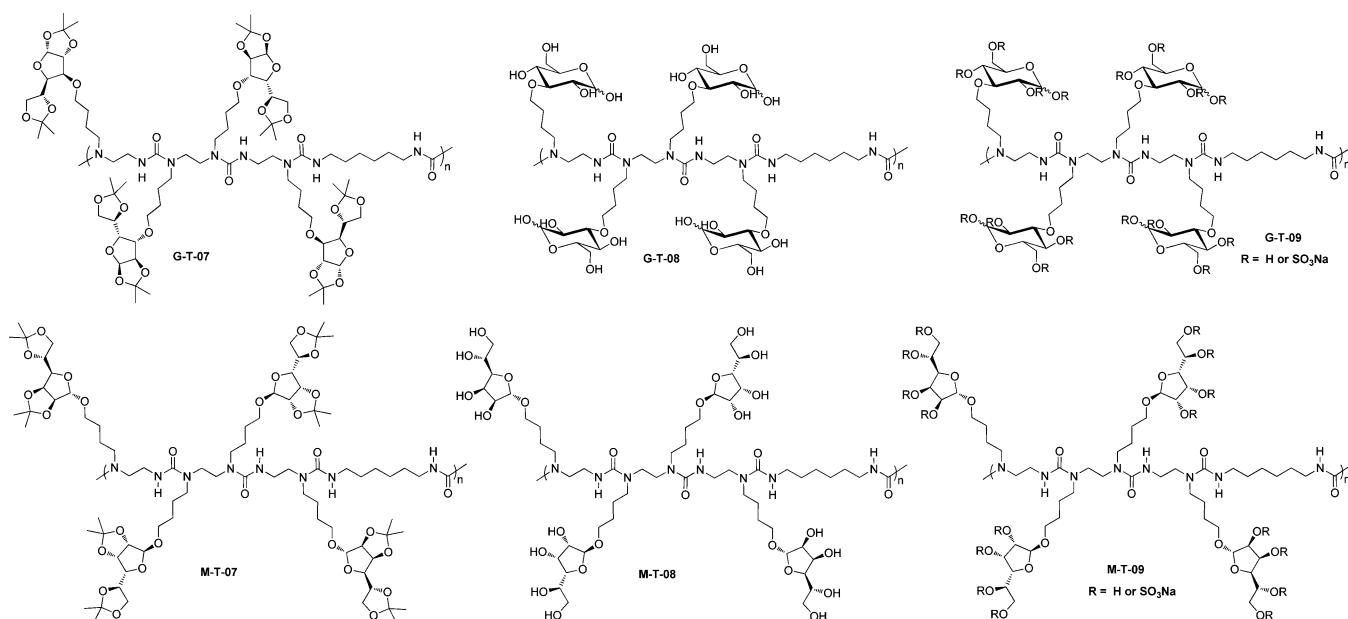
Synthesis of Polymer L-D-09. L-D-08 (2.678 g, 1.0 equiv) was added into a flame-dried flask. After it was evacuated and refilled with nitrogen three times, 13 mL of anhydrous DMF was injected followed by hexamethylene diisocyanate (0.341 g, 1.05 equiv). This solution was stirred at 60 $^\circ\text{C}$ for 40 h before the DMF was removed. The residue was dissolved in a minimum amount of DCM and precipitated in cold ether three times. The solvent was removed under vacuum to yield 2.850 g of product as a white powder in 93% yield.

Synthesis of Polymer L-D-10. In a flame-dried flask, polymer L-D-09 (2.85 g) was dissolved in 35 mL of anhydrous methanol, followed by the addition of a catalytic amount of MeONa/MeOH solution (25% by weight). This solution was stirred at room temperature for 3 h during which time a precipitate formed. The solution was poured into a beaker. MeOH was used to wash the precipitate three times and combined with the original solution. The precipitate was dried over high vacuum to yield 1.20 g of product (66%). The remaining solution was added to Dowex 50WX8 hydrogen form resin, and the pH was adjusted to approximately 6.0. After filtration to remove the Dowex 50WX8 resin, the solvent was removed to yield 0.60 g (33%) of white product. The two products were then combined (1.20 g + 0.60 g and overall 99% yield).

Synthesis of Polymer L-D-11. Polymer L-D-10 (0.98 g, 1.0 equiv) and $\text{SO}_3/\text{pyridine}$ complex (5.20 g, 33.3 equiv) were added into a flame-dried flask. After it was evacuated and refilled with nitrogen three times, 30 mL of anhydrous pyridine was injected. Then, this solution was heated at 90 $^\circ\text{C}$ for 16 h. After the solvent was removed, saturated NaHCO_3 solution was added to adjust the pH to 8.0, and the polymer was isolated through dialysis and lyophilization to give a white powder. Yield = 2.20 g, 92%.

Synthesis of Glucosamine Containing Polymers. To prepare the glucosamine-containing monomer, the amino group of glucosamine was first protected using an azide group, followed by a similar synthetic strategy as used for L-D-08. After polymerization with hexamethylene diisocyanate, Pd/C hydrogenation was used to reduce the azide group to an amine, followed by deprotection of the acetate protecting groups. The polymer was sulfated to obtain GA-D-12 (1.20 g, 83% yield). The

Scheme 1. Structures of Glucose and Mannose Tetramer Polymers, G-T-07 and M-T-07; Deprotected Polymers, G-T-08 and M-T-08; and Sulfated Polymers, G-T-09 and M-T-09. The Numbers Correspond to Products in Ref 43



detailed synthetic route, as well as the full spectra, is presented in the Supporting Information.

Synthesis of Glucose and Mannose Based Polymers Containing Dimer-Based Diamine Monomers. The synthetic routes for glucose and mannose dimer-based diamines, G-D-08 and M-D-08, are the same as those used for the lactose dimer. After polymerization, deprotection, and sulfation, the sulfated polymers G-D-11 (1.50 g, 93% yield) and M-D-11 (1.50 g, 94%) were obtained. The details of the synthetic routes and full spectra are presented in the Supporting Information.

Contact Angle Measurements. Surface hydrophilicity/hydrophobicity of the synthesized polymers was determined by measuring the contact angles with a Ramé-hart contact angle goniometer (Model 200-F1). DCM or methanol solutions of the synthesized polymers were solvent-cast onto a polished Si wafer and dried overnight to produce solvent-cast thin films. Ten μL of distilled water was placed onto the polymer surface using a micropipette. Contact angles were calculated with a tangent method using the software supplied with the goniometer. Each sample was measured at least three times with different drops of water, and the averaged data are reported. The advancing and receding contact angles were recorded by tilting the base to 35° .

Platelet Adhesion Assay. The synthesized polymers (10 mg/mL in MeOH or H_2O) were spin-coated onto a polished Si wafer at 3000 rpm and dried in vacuum for 1 h to obtain the polymer-coated Si wafers, which were then cut and placed into the wells of a 24-well tissue culture plate. CaCl_2 (1.0 mol/L) was added to platelet rich plasma to a final concentration of 2.0 mmol/L. This solution (1.0 mL) was immediately added into the 24-well tissue culture plates and incubated at 37°C for 1 h with the coated wafers. After the platelet rich plasma was pipetted out, the polymer-coated Si wafers were rinsed with Tris-buffered saline solution (TBS, 1.0 mL) three times, followed by incubation in 2% glutaraldehyde/TBS solution (1.0 mL) for 30 min to fix the adhered platelets. The wafers were removed from the plates and rinsed with TBS solution (1.0 mL) two times, followed by rinses with a series of ethanol solutions (50%, 70%, 90%, and 100%). Finally, the adhered platelets were analyzed by scanning electron microscopy (SEM). For each wafer, the average number of platelets was counted by using nine images of $100 \times 100 \mu\text{m}^2/\text{field}$ using ImageJ software.

Activated Partial Thromboplastin Time (aPTT). Activated partial thromboplastin times (aPTTs) were initiated in mouse platelet poor plasma (PPP) via use of Sta-PTT reagent on a STA-Hemostasis Analyzer (Diagnostica Stago). Sulfated polymers were dissolved in

HEPES buffered saline (pH 7.3) and spiked into plasma prior to the initiation of the aPTT as 1/10 volume of the sample. Control specimens were spiked with HEPES buffered saline only. The final concentrations of the final sulfated polymers are as indicated in the text.

Prothrombin Time (PT) Assay. Thromboplastin (Biopol) was reconstituted with 5 mL of water and warmed alongside wild-type mouse plasma that contained the polymer under study (prepared as for the aPTT assay) to 37°C . The thromboplastin and plasma were mixed in a 1:1 ratio and agitated until a clot was observed.

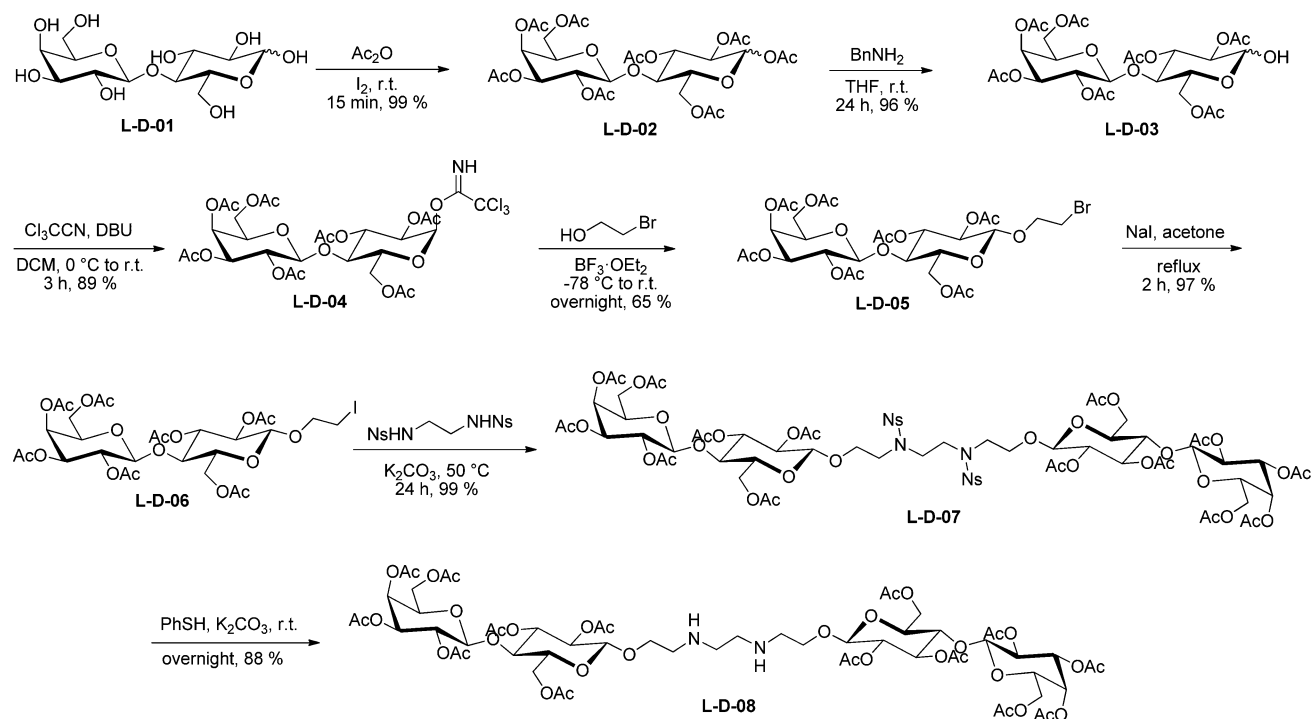
Thrombin Time (TT) assay. A solution of 20 units/mL of thrombin and 400 mM CaCl_2 in HEPES buffered saline was prepared and kept on ice until use. Separately, wild-type mouse plasma containing the polymer under study (prepared as for the aPTT assay) was warmed to 37°C . The thrombin solution and wild-type mouse plasma were then mixed in a 1:10 ratio and agitated until a clot was observed.

Anti-Xa and Anti-IIa Assay. Anti-Xa and anti-IIa assays were performed in human PPP. For the anti-Xa assay, a Coatest Heparin assay kit (Chromogenix) was obtained and performed via the manufacturer's recommendations adapted for a 96-well plate. Again, 1/10 volume sulfated polymers were spiked into the plasma prior to determination of the anti-Xa activity. For the anti-IIa assay, S-2238 (Chromogenix), human thrombin, and human antithrombin (Enzyme Research Laboratories) were utilized. The anti-IIa assay was adapted for a 96-well plate from the Chromogenix protocol for S-2238. The sulfated polymers were spiked into the plasma prior to initiation of the assay. To determine the ATIII dependence of the sulfated polymers, the anti-IIa assay was repeated with ATIII depleted human plasma (Affinity Biologicals) and omitted addition of exogenous ATIII. For all assays, pharmaceutical grade heparin (APP Pharmaceuticals) was used for the standard curves.

RESULTS AND DISCUSSION

Polymer Synthesis. We designed polymers containing glucose, mannose, lactose, and glucosamine sugars. Our aim was to compare polymers with different molecular structures with respect to their performance in in vitro blood-compatibility assays. We chose to use glucose and mannose since they are monosaccharides with different stereochemistry. Lactose was selected to compare monosaccharide and disaccharide pendant groups.^{35,37} Finally, the glucosamine

Scheme 2. Synthetic Route for the Lactose Dimer-Based Diamine Monomer



unit provides an amino group for *N*-sulfonation, which has been reported to enhance hemocompatibility over *O*-sulfation.^{52–55} In our previous publication,⁴³ we synthesized the glucose and mannose based polymers shown in Scheme 1. Isopropylidene groups were selected to protect glucose and mannose because of their resistance to the basic reaction conditions used in the glucose and mannose tetramer-based monomer synthesis and their easy deprotection in acidic solutions.

The number-average molecular weight (M_n) and polydispersity index (M_w/M_n) for G-T-07 and M-T-07 were 41.9 kDa and 1.14 and 37.8 kDa and 2.55, respectively.⁴³ The isopropylidene protecting groups were deprotected with an excess of an 80% $\text{CF}_3\text{CO}_2\text{H}/\text{H}_2\text{O}$ solution to generate G-T-08 and M-T-08, which were then sulfated using a $\text{SO}_3/\text{pyridine}$ complex in pyridine solution to give the targeted heparin-mimicking polymers, G-T-09 and M-T-09. Elemental analyses of the sulfated polymers indicated that the polymers possessed an average of 3.5 sulfates per saccharide unit in both G-T-09 and M-T-09.

In the current work, when isopropylidene protecting groups were used to protect lactose, the products were unstable.^{56,57} We therefore switched to acetyl groups to protect the sugar hydroxyls. Furthermore, we prepared the simplified dimer-based monomers rather than the tetramer-based versions shown in Scheme 1. The rationale for the simplification was based on making the synthesis, purification, and characterization easier with a minimal loss of information in the final polymers. The synthetic route for the lactose monomer is as follows: lactose was acetylated with acetic anhydride and catalytic iodine, followed by selective anomeric deprotection using benzyl amine to give L-D-03. The trichloroacetimidate product (L-D-04) was obtained by reacting L-D-03 with trichloroacetonitrile and a catalytic amount of DBU. Glycosylation of L-D-04 with 2-bromoethanol and $\text{BF}_3 \cdot \text{Et}_2\text{O}$ resulted in L-D-05, which was converted to the iodide (L-D-06)

derivative under reflux with NaI in acetone. Excess L-D-06 was reacted with *N,N'*-bis(2-nitrobenzenesulfonyl)-ethane-1,2-diamine at 50 °C to ensure the complete consumption of the diamine. The obtained product, L-D-07, was deprotected using thiophenol to produce diamine L-D-08, which has been fully characterized by NMR and FT-IR spectroscopy and mass spectrometry. The complete synthesis is shown in Scheme 2.

Polymer L-D-09 was prepared by reacting L-D-08 with hexamethylene diisocyanate in anhydrous DMF at 60 °C for 40 h, as shown in Scheme 3. Polymer L-D-09 was purified by precipitation into cold ethyl ether, and gel permeation chromatography (GPC) analysis showed the M_n , M_w , and PDI to be 27.0 kDa, 47.3 kDa, and 1.75, respectively (Table 1).

The deprotection of the acetyl groups from L-D-09 was performed in anhydrous methanol solution with a catalytic amount of NaOMe, which yielded the deprotected polymer L-D-10. The successful deprotection was confirmed using ^1H NMR spectroscopy by the disappearance of the acetyl CH_3 group's signals at 1.96–2.15 ppm. ^{13}C and DEPT-135 NMR spectroscopy were performed to further corroborate the deprotection of all the acetyl groups using the unique primary (CH_3) and acetyl quaternary ($\text{C}=\text{O}$) carbon peaks. As expected, none of these peaks were observed in either spectrum, which indicates the complete removal of the acetyl groups. Furthermore, the existing peaks at 102.39, 102.86, and 159.71 ppm in the ^{13}C NMR spectrum indicate the disaccharide units and the carbonyl groups from the urea functional groups were still intact, and no side reactions occurred. The disappearance of the strong absorption peak at 1740 cm^{-1} for the protected ester group and the new strong absorption peak at 3311 cm^{-1} due to the free hydroxyl groups were also observed in the FT-IR spectrum (Figure 1).

A $\text{SO}_3/\text{pyridine}$ complex in pyridine was used to convert the sugar hydroxyl groups to sulfate groups.³⁰ The sulfated heparin-mimicking polymer L-D-11 was purified by dialyzing in water and isolated by lyophilization. FT-IR spectroscopy was used to

Scheme 3. Synthetic Route for Lactose Containing Polymer, L-D-09; Deprotected Polymer, L-D-10; and Sulfated Heparin-Mimicking Polymer, L-D-11

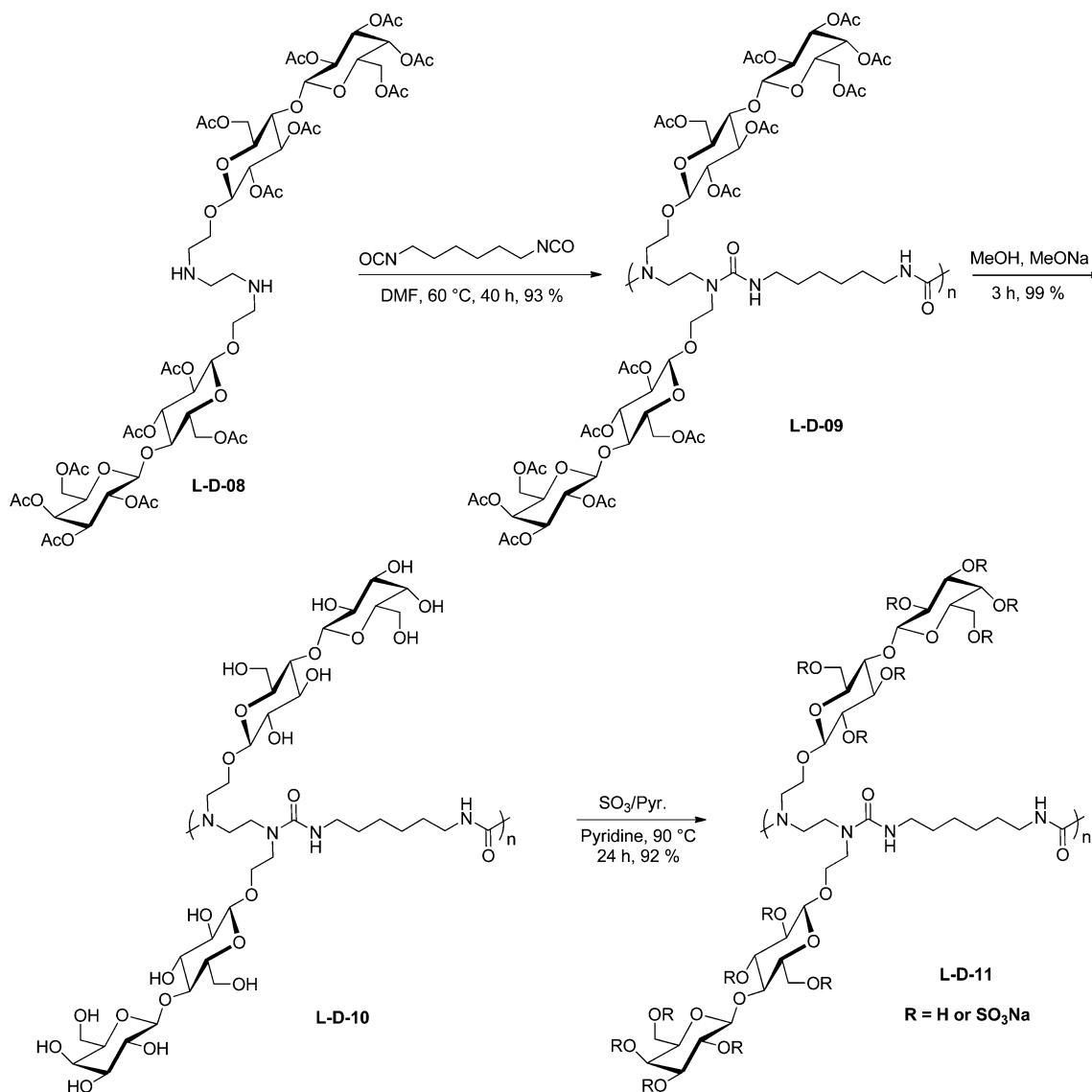


Table 1. GPC Data for the Polymers.^a (Note the Nomenclature G, M, GA, L, T, and D Stand for Glucose, Mannose, Glucosamine, Lactose, Tetramer, and Dimer, Respectively.)

polymer	M_n (kDa) ^b	M_w (kDa) ^c	M_w/M_n
L-D-09	27.0	47.3	1.75
GA-D-09	15.7	26.8	1.71
G-D-09	26.7	44.9	1.68
M-D-09	22.7	32.0	1.41

^aData were collected using THF as the mobile phase at a flow rate of 1.0 mL/min with refractive index and light scattering detectors. ^b M_n = number-average molecular weight. ^c M_w = weight-average molecular weight.

confirm the successful sulfation reaction, and signals from the O=S=O group and the C–O–S bonds were observed at 1225, 1004, and 930 cm⁻¹ (Figure 1). The average degree of sulfation was calculated to be 6.88 sulfates per disaccharide (Table 2) using elemental analysis.

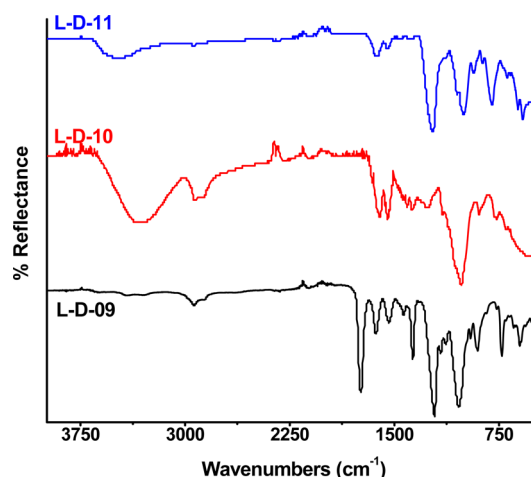


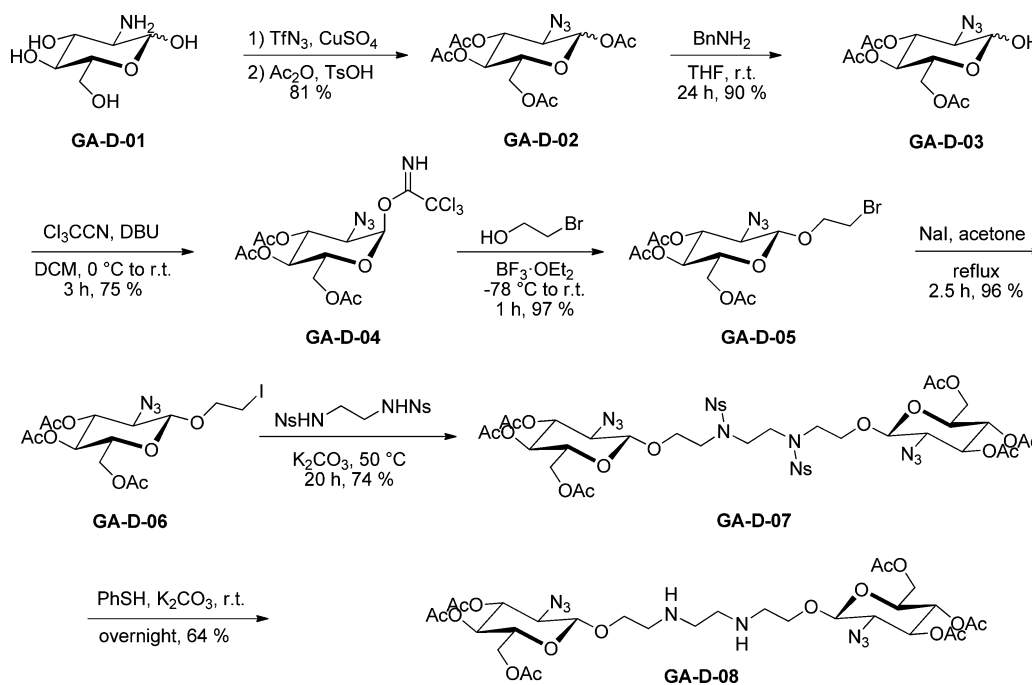
Figure 1. FT-IR spectra of polymers L-D-09, L-D-10, and L-D-11.

The glucosamine-containing monomer was prepared using a similar strategy to that of the lactose monomer (Scheme 4).

Table 2. Elemental Analysis Data of the Sulfated Polymers. (Note the Nomenclature G, M, GA, L, T, and D Stand for Glucose, Mannose, Glucosamine, Lactose, Tetramer, and Dimer, Respectively.)

polymer	carbon	hydrogen	nitrogen	sodium	sulfur	DS ^a
L-D-11	18.08%	3.44%	2.36%	10.27%	15.42%	6.88 ^b
GA-D-12	25.33%	4.50%	6.30%	7.57%	14.09%	3.68
G-D-11	20.28%	3.42%	3.87%	10.81%	16.45%	3.95
M-D-11	19.57%	3.55%	3.63%	10.81%	16.45%	3.99

^aDS = average degree of sulfation per saccharide. ^bPer disaccharide.

Scheme 4. Synthetic Route for the Glucosamine Dimer-Based Diamine Monomer

The azido group was selected to protect the amino group because of its resistance to both acidic and basic reaction conditions and its easy reduction to an amine by Pd/C hydrogenation. The structure of the glucosamine dimer, GA-D-08, was confirmed by NMR and FT-IR spectroscopy and mass spectrometry. The unique peaks at 2.85 and 2.89–2.97 ppm in the ¹H NMR spectrum are assigned to the protons from the four CH₂ groups connected to N atoms, and the peak at 2110 cm⁻¹ in the FT-IR spectrum is assigned to the azide absorption. Mass spectrometry also confirmed the successful synthesis of GA-D-08, where a molecular ion peak was observed at *m/z* = 775.3101 M+H⁺ (calculated: 775.3110). The synthesized GA-D-08 monomer was polymerized with hexamethylene diisocyanate via a step-growth polymerization (Scheme 5).

The *M_n*, *M_w*, and PDI values for polymer GA-D-09 are 15.7 kDa, 26.8 kDa, and 1.71, respectively (Table 1). The reduction of azido to amino groups was performed using Pd/C hydrogenation at 60 psi for 7 days. The ¹³C NMR and DEPT-135 spectra confirmed the complete reduction. The azido absorption peak at 2111 cm⁻¹ was also absent in the FT-IR spectrum, while the strong NH₂ absorption peak at 3288 cm⁻¹ further corroborated the complete reduction of azido groups to amino groups.

The deprotection of the acetyl groups was carried out in anhydrous methanol with a catalytic amount of NaOMe to generate polymer GA-D-11. The removal of the acetyl groups was confirmed using NMR and FT-IR spectroscopy similarly to

the lactose-containing polymer. Polymer GA-D-11 was sulfated in the same way as the lactose polymer, which gave the heparin-mimicking polymer GA-D-12. The successful sulfation reaction was confirmed by FT-IR and ¹³C NMR spectroscopy. A comparison of the FT-IR spectra for polymers GA-D-09, GA-D-10, GA-D-11, and GA-D-12 is presented in Figure 2. The average degree of sulfation was calculated from elemental analysis as 3.68 SO₃ groups per saccharide.

Polymers prepared from dimer-based glucose and mannose diamine monomers with acetyl protecting groups were also synthesized. The glucose-containing polymer is designated as G-D-09, and the mannose-containing polymer is designated as M-D-09. The molecular structures are provided in Scheme 6. This allowed us to probe structure/property relationships compared to the polymers prepared with the tetra-functionalized monomers.⁴³ Specifically, the influence of the position on the ring where the sugar is attached to the polymer and also the effect of a pyranose ring versus a furanose ring for the mannose-containing polymers were examined. The experimental details are similar to those used for lactose and are provided in the Supporting Information. The *M_n*, *M_w*, and PDI values were 26.7 kDa, 44.9 kDa, and 1.68 for polymer G-D-09 and 22.7 kDa, 32.0 kDa, and 1.41 for M-D-09, respectively. After deprotection and sulfation, polymers G-D-11 and M-D-11 were obtained. The average degree of sulfation was calculated to be 3.95 and 3.99 per saccharide, respectively.

Scheme 5. Synthetic Route for Glucosamine Dimer Polymer, GA-D-09; Deprotected Polymer, GA-D-11; and Sulfated Polymer, GA-D-12

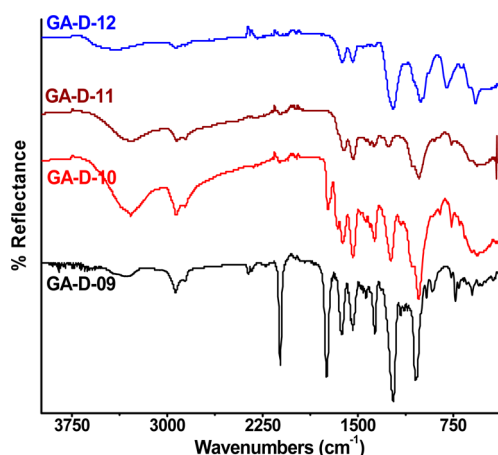
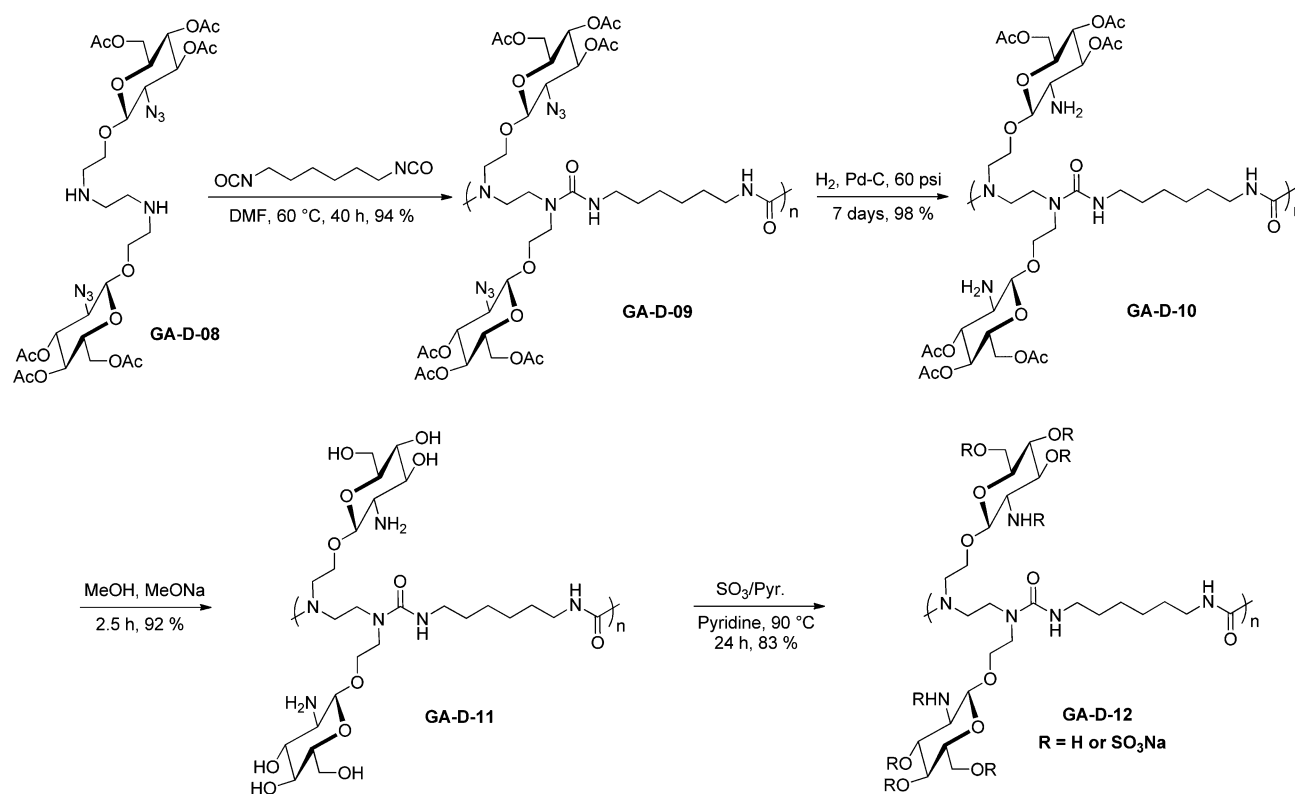


Figure 2. FT-IR spectra of polymers GA-D-09, GA-D-10, GA-D-11, and GA-D-12. See Scheme 5 for the structures of each polymer.

Contact Angle Measurements. The blood compatibility of biomaterials can be greatly affected by the surface hydrophilicity of materials.⁵⁸ Contact angle measurement is an accepted method to characterize a surface with respect to its hydrophilicity or hydrophobicity. Solvent-cast thin films of the synthesized polymers were used to measure contact angles at different locations on the films, and the static, receding, and advancing contact angles were recorded (Table 3). TECOFLEX is a commercial polyurethane that possesses carbamate functional groups. We used TECOFLEX as a comparison with our synthesized polymers because of its wide application in blood-contacting biomaterials, including cardiovascular devices.^{59–62} For glucose and mannose polymers from the tetramer-based monomers, the static contact angles were 73°

and 71° for G-T-07 and M-T-07, respectively. This indicated that the films were hydrophobic as expected from the hydrophobic isopropylidene protecting groups. The deprotected glucose and mannose polymers, G-T-08 and M-T-08, were measured to be hydrophilic with static contact angle values of 38° and 32°, respectively. Compared to the isopropylidene-protected polymers, the contact angles of acetyl-protected polymers had much lower values. The static contact angles of L-D-09, GA-D-09, G-D-09, and M-D-09 were 43°, 39°, 42°, and 47°, respectively. These values indicated that the acetyl-protected polymers were hydrophilic due to the polar ester groups in the polymer structures. After deprotection, the polymers, L-D-10, GA-D-11, G-D-10, and M-D-10, were observed to be hydrophilic with similar contact angles as those of G-T-08 and M-T-08. Solvent-casting thin films of TECOFLEX afforded a static contact angle of 61°, which indicates that it is more hydrophobic than our polymers. No contact angles could be measured for the sulfated polymers because they are completely wetting surfaces, and the solvent-cast thin films dissolved upon contact with water. Therefore, all of the sulfated polymers are highly hydrophilic, which implies that they are candidates for blood compatible materials.

Platelet Adhesion Assay. Platelet adhesion assays are a widely used method to evaluate the blood-compatibility of blood contacting materials. The number of adherent platelets quantifies the activation of platelets to the materials, and a higher number of adherent platelets is inferred as a less blood-compatible material.^{10,63–65} The nonsulfated polymers and TECOFLEX were spin-coated on Si wafers and incubated in platelet rich plasma for 1 h. After the adhered platelets were fixed using 2% glutaraldehyde/TBS solution, the wafers were examined by SEM. TECOFLEX was used as a comparison

Scheme 6. Chemical Structures of Glucose and Mannose Containing Polymers, G-D-09 and M-D-09; Deprotected Polymers, G-D-10 and M-D-10; and Heparin-Mimicking Sulfated Polymers, G-D-11 and M-D-11

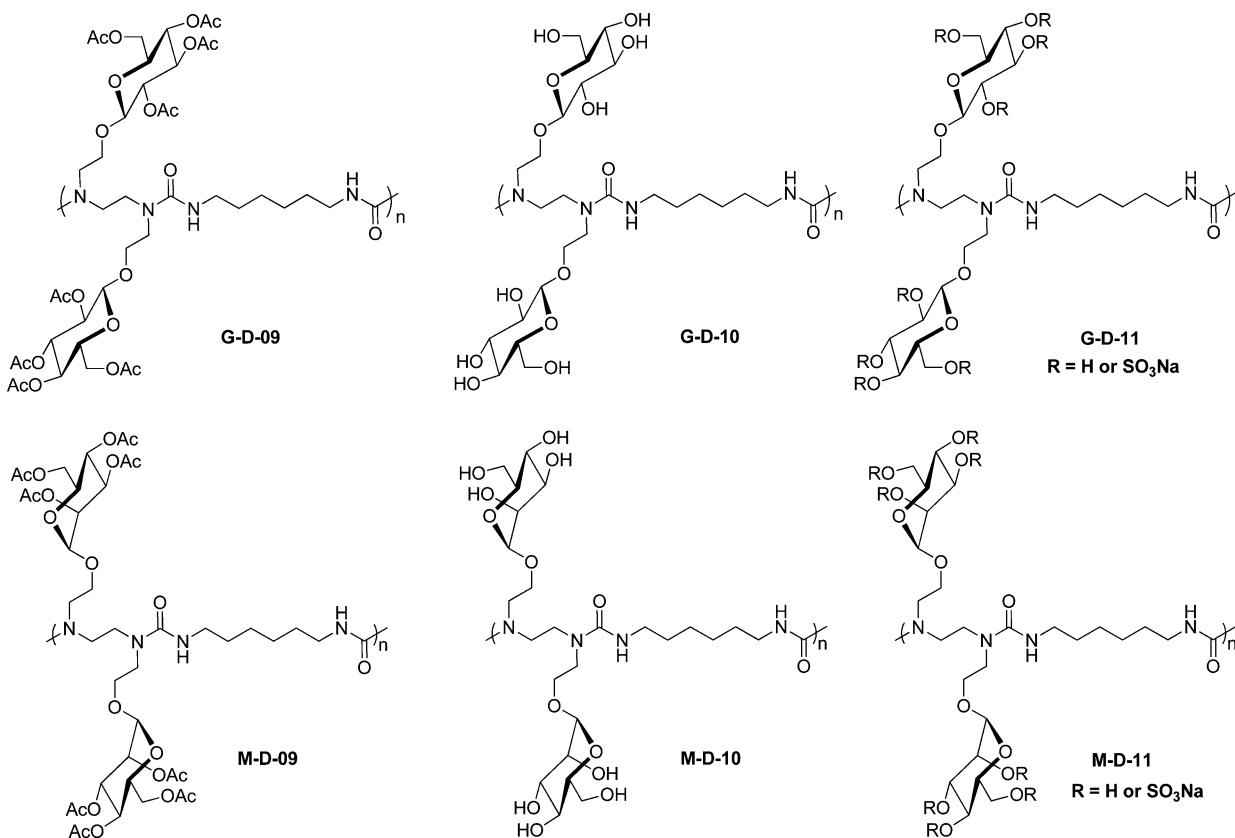


Table 3. Contact Angle Measurements of the Synthesized Polymers and TECOFLEX. (Note the Nomenclature G, M, GA, L, T, and D Stand for Glucose, Mannose, Glucosamine, Lactose, Tetramer, and Dimer, Respectively.)

polymer	θ (deg) (static)	θ (deg) (advancing)	θ (deg) (receding)
G-T-07	73	81	61
G-T-08	38	44	24
M-T-07	71	80	67
M-T-08	32	38	22
L-D-09	43	49	31
L-D-10	30	45	20
GA-D-09	39	46	29
GA-D-11	30	42	20
G-D-09	42	47	31
G-D-10	28	39	21
M-D-09	47	54	40
M-D-10	33	45	26
TECOFLEX	61	68	53

since it is a widely used biomaterial.^{59–62} The number of adherent platelets for TECOFLEX was similar to that of M-T-08. Although hydrophilicity plays an important role in blood-compatibility,^{63,66–69} other factors also play important roles in many biomaterials (e.g., charged ionomers).⁶⁴ This effect is seen in the difference between our nonsulfated polymers and TECOFLEX. TECOFLEX has a much higher static contact angle than do the nonsulfated polymers, but it exhibited comparable platelet adhesion results. We anticipate that in similar assays the sulfated polymers would demonstrate improved performance over the nonsulfated polymers because

of factors including increased osmotic pressure at the interface and electrostatic repulsion of factors such as fibrin that can promote platelet adherence. However, results from spin-cast films of the sulfated polymers would be challenging to interpret because of the stability issues described for contact angle measurements.

Anticoagulant Assay. Sulfated sugars have been proven in literature to exhibit anticoagulant activities by prolonging blood clotting time using assays such as aPTT, TT, blood recalcification time, PT, etc.^{30,31,34–37,40} The aPTT assay is widely used to measure the hemostatic efficiency of both the intrinsic and common pathways, which include factors II, V, VIII, IX, X, XI, XII, and fibrinogen.⁷⁰ It is a standard clinical assay for monitoring unfractionated therapeutic levels. A prolonged clotting time indicates an increase in anticoagulant activity, while a shorter clotting time demonstrates a faster conversion of fibrinogen into insoluble fibrin and the formation of thrombus. In this case, “prolonged” refers to an extended time for clot formation versus “normal”. A “normal” aPTT would be 28–30 s in our assays. The anticoagulant activities of our synthesized polymers were investigated by measuring the aPTT in murine plasma spiked with the sulfated polymers. The nonsulfated, hydroxylated polymers exhibited no anticoagulant activities as measured by this assay, with the exception of a mild prolongation with deprotected glucosamine. This shows the importance of characterizing these novel biomaterials in assays beyond platelet adhesion assays. The average aPTT values are reported in Table S2 of the Supporting Information and are compared graphically in Figure 3 for the sulfated polymers. The aPTT values are all in seconds and are absolute times rather than an extended time past normal. The sulfated polymers

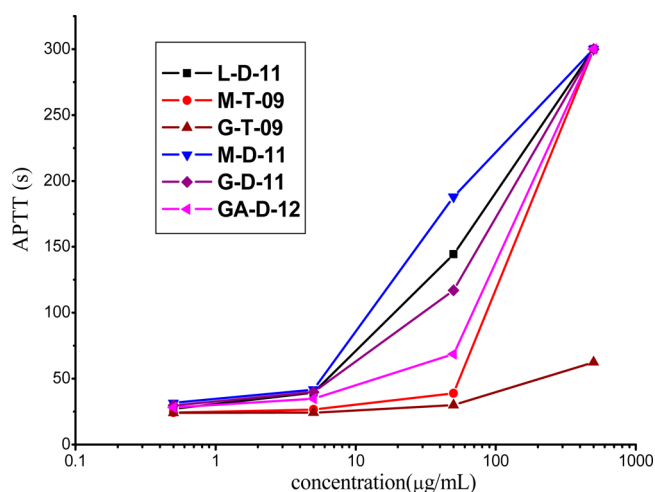


Figure 3. aPTT of sulfated heparin-mimicking polymers at various concentrations. The nomenclature used in the key is defined in the text.

induced a significantly prolonged aPTT. The mild prolongation seen with deprotected glucosamine was significant compared to plasma both with and without other deprotected polymers ($p < 0.05$). At a concentration of 500 $\mu\text{g/mL}$, the mean aPTT was 41.6 s, as opposed to >300 s with sulfated glucosamine. The range of aPTTs at 50 $\mu\text{g/mL}$ of L-D-11, G-D-11, and M-D-11 include values in and above the typical clinical therapeutic range for unfractionated heparin.

The clotting times (aPTT) increased as the concentration of all the sulfated polymers exhibited increased. Except for G-T-09, the clotting times exceeded 300 s when the concentration reached 500 $\mu\text{g/mL}$. These results are comparable with related literature.^{35,40} For example, a lactose/acrylamide copolymer reported by Chaikof used concentrations of 79.9 and 184.0 $\mu\text{g/mL}$ to attain aPTTs of 100 and 200 s, respectively.³⁵ Similarly, a recently published heparin-mimicking polymer by Hsieh–Wilson group reported an aPTT of 119.4 ± 0.5 s for 150 $\mu\text{g/mL}$ of polymer.⁴⁰ The hierarchy of the anticoagulant activities of our polymers can be seen from Figure 3 in the order of mannose (dimer) > lactose > glucose (dimer) > glucosamine > mannose (tetramer) > glucose (tetramer). The sulfated glucose and mannose tetramer polymers, G-T-09 and M-T-09, showed the lowest anticoagulant activity, which might be attributed to the free anomeric group of G-T-09 and the furanose ring of M-T-09. The glucosamine containing polymer, GA-D-12, afforded a lower anticoagulant activity than did M-D-11, L-D-11, or G-D-11, which while initially surprising, is consistent with some previously reported results.³⁵

To further show the blood-compatibility of our polymers and verify the results of the aPTT assay, we performed a PT assay. The results are collected in Table 4, where the data are the PT values in seconds. For comparison purposes, a HEPES buffer solution was used as a negative control with a recorded PT value of 14.5 s, and a 0.7 IU/mL heparin solution was used as a positive control, with a recorded PT value of >60 s.

Similar to aPTT, the nonsulfated, hydroxylated polymers exhibited no anticoagulant activities in the PT assay at all concentrations. Unlike the aPTT assay, this assay did not prove to be discriminative between the dimer-based polymers beyond a binary yes or no result with respect to blood compatibility based on the control measurement. Despite this fact, the assay does show the blood compatibility of the dimer-based

Table 4. PT Assay Times in Seconds with Various Concentrations of Polymers Prepared in This Work. The Data Is an Average of Duplicate Measurements and Reported to the Nearest 0.5 Seconds

polymer	polymer concentration ($\mu\text{g/mL}$)			
	0.5	5.0	50	500
G-T-09	15.0	14.0	14.0	13.5
M-T-09	14.0	13.5	13.5	15.5
G-D-11	14.0	14.0	>60	>60
M-D-11	14.0	14.0	>60	>60
L-D-11	14.5	13.0	>60	>60
GA-D-12	14.0	14.0	14.0	>60

polymers, and in particular, those containing lactose and mannose sugars. Similarly, we performed a TT assay, and the results are shown in Table 5. For comparison purposes, a

Table 5. TT Assay Times in Seconds with Various Concentrations of Polymers Prepared in This Work. The Data Is an Average of Duplicate Measurements and Reported to the Nearest 0.5 Seconds

polymer	polymer concentration ($\mu\text{g/mL}$)			
	0.5	5.0	50	500
G-T-09	23.0	23.5	>75	>75
M-T-09	23.0	20.5	>75	>75
G-D-11	24.0	27.5	>75	>75
M-D-11	22.0	>75	>75	>75
L-D-11	25.0	>75	>75	>75
GA-D-12	23.0	24.5	>75	>75

HEPES buffer solution with wild-type mouse plasma was used as a negative control with a recorded TT value of 21 s, and heparin solutions with concentrations of 0.1–0.7 units/mL were used as positive controls, and all gave recorded TT values of >75 s.

As was the case for the PT assay, the TT assay did not prove to be discriminative between the polymers beyond a binary yes or no result with respect to blood compatibility based on the control measurement. However, the polymers containing lactose and mannose based diamines proved to show the highest blood compatibility as measured by the TT assay.

Anti-Fxa and Anti-FIIa Activity. The primary mechanism of action for anticoagulation using heparin is the facilitation of binding the serine protease inhibitor (SERPIN) ATIII with two of the common pathway hemostatic serine proteases: factors Xa and thrombin (IIa). To directly determine the effect of sulfated polymers on the activity of these two enzymes, chromogenic anti-Xa and anti-IIa assays were used. These assays measure the chromogenic activity of exogenous Xa or IIa when added to plasma that contains heparin. The assays use exogenous ATIII to minimize sample-to-sample variation, which allows the heparin level to be the primary variable in the assay. The anti-Xa assay is the most common version of this assay used in clinical practice. Plasma aliquots were spiked with sulfated polymers to final concentrations from 125–500 $\mu\text{g/mL}$. Only polymers L-D-11 and M-D-11 exhibited any anti-Xa activity in this assay. At 500 $\mu\text{g/mL}$, both polymers had activity consistent with 0.1 units/mL of heparin (data not shown), which is less activity than needed for therapeutic treatment with UFH; however, it is unclear at this stage if this activity would be

sufficient for using these polymers as blood-contacting biomaterials.

A chromogenic anti-IIa assay was then used to determine if the observed prolongation of the aPTT was due primarily to anti-IIa activity rather than anti-Xa activity. Again, plasma was spiked with sulfated polymers prior to the determination of anti-IIa activity. With this assay, the highest anti-IIa activity was observed at 500 $\mu\text{g}/\text{mL}$ of G-D-11 and M-D-11, with heparin activity of 0.8 units/mL and 0.7 units/mL, respectively. Polymer L-D-11 also exhibited therapeutic-range anti-Xa activity, at 0.4 units/mL (Figure 4). To test if the sulfated

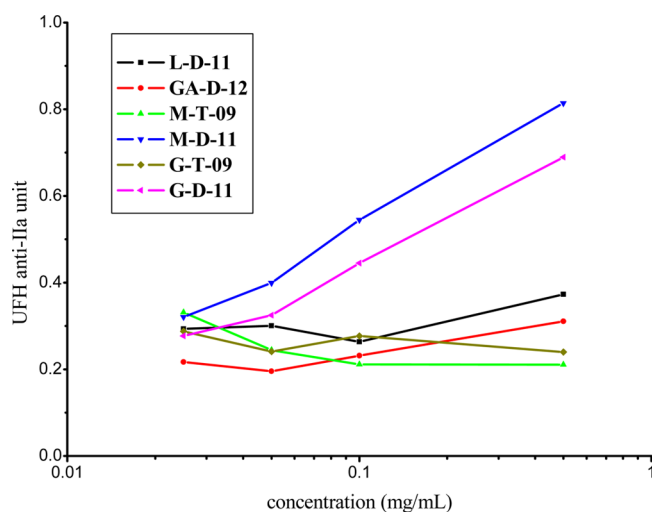


Figure 4. Activity of the sulfated heparin-mimicking polymers at various concentrations in the anti-IIa assay.

polymers inhibit thrombin activity via an ATIII-dependent mechanism, we repeated the anti-IIa assay in ATIII deficient plasma and removed exogenous ATIII from the reaction mixture. This assay was run with the three polymers with anti-IIa activity that fell into the therapeutic range. Heparin, as expected, had no effect on thrombin activity in the absence of ATIII; however, the sulfated polymers exhibited a dose-dependent inhibition of thrombin activity in the absence of ATIII. The relative activity of these three polymers in this assay was M-D-11 > L-D-11 > G-D-11; hence, the sulfated polymers may inhibit IIa activity by both ATIII-dependent and ATIII-independent mechanisms.

CONCLUSIONS

Step-growth polymerization was used to synthesize six *N*-alkyl urea containing polymers with glucose, mannose, glucosamine, or lactose as pendant groups. The obtained polymers were deprotected and sulfated to obtain highly hydrophilic heparin-mimicking glycopolymers. The blood compatibility of the sulfated polymers has been demonstrated by prolonged aPTT, PT, and TT. Polymers M-D-11 and L-D-11 exhibited the highest anticoagulant activity as determined by these assays. None of the polymers expressed any significant anti-Xa activity; however, three polymers, M-D-11, L-D-11, and G-D-11, demonstrated therapeutic level anti-IIa activity at a concentration of 500 $\mu\text{g}/\text{mL}$. The mechanism of thrombin inhibition remains unclear at this stage and may be via both ATIII-dependent and ATIII-independent routes.

ASSOCIATED CONTENT

Supporting Information

The syntheses and characterizations of glucose, mannose, lactose, and glucosamine polymers as well as the platelet adhesion, aPTT assay, anti-IIa assay, and anti-Xa assay results. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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Author Contributions

The manuscript was written through contributions of all authors. Y.H. performed all of the synthesis work and characterization of the monomers and polymers. M.A.S. and E.S.M. performed and interpreted the aPPT, PT, TT, anti-Xa, and anti-IIa assays. T.L.K. assisted with the design and execution of the platelet adhesion assay. All authors have given approval to the final version of the manuscript.

Notes

The authors declare no competing financial interest.

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