Chemoenzymatic Synthesis of Heparin Oligosaccharides with both Anti-factor Xa and Anti-factor IIa Activities* 5

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Background: Heparin inhibits the activity of factors Xa and IIa in the blood coagulation cascade.

Results: A series of size-defined N-sulfated oligosaccharides were synthesized to probe the size requirement for the oligosaccharides displaying anti-IIa activity.

Conclusion: Oligosaccharides that display anti-IIa activity are longer than 19 saccharide residues.

Significance: The results will direct efforts to prepare synthetic heparin with both anti-Xa and anti-IIa activities.

Heparan sulfate (HS) 2 and heparin are highly sulfated polysaccharides. Heparin is a commonly used anticoagulant drug that inhibits the activities of factors Xa and IIa (also known as thrombin) to prevent blood clot formation. Here, we report the synthesis of a series of size-defined oligosaccharides to probe the minimum size requirement for an oligosaccharide with anti-IIa activity. The synthesis was completed by a chemoenzymatic approach involving glycosyltransferases, HS sulfotransferases, and C5-epimerase. We demonstrate the ability to synthesize highly purified N-sulfo-oligosaccharides having up to 21 saccharide residues. The results from anti-Xa and anti-IIa activity measurements revealed that an oligosaccharide longer than 19 saccharide residues is necessary to display anti-IIa activity. The oligosaccharides also exhibit low binding toward platelet factor 4, raising the possibility of preparing a synthetic heparin with a reduced effect of heparin-induced thrombocytopoenia. The results from this study demonstrate the ability to synthesize large HS oligosaccharides and provide a unique tool to probe the structure and function relationships of HS that require the use of large HS fragments.

Heparan sulfate (HS) 2 is a highly sulfated polysaccharide that is widely expressed on the mammalian cell surface and in the extracellular matrix. HS is involved in regulating numerous biological processes such as blood coagulation, embryonic development, inflammatory responses, and assisting viral/bacterial infections (1). The disaccharide-building block for HS consists of GlcUA or IdoUA and glucosamine, each of which is capable of carrying sulfo groups. The position of the sulfo groups and the location of the IdoUA units dictate the functions of HS (2). The biosynthesis of HS occurs in the Golgi apparatus and involves several specialized sulfotransferases, glycosyltransferases, and an epimerase (3). HS polymerase, known as EXT-1 and EXT-2, is responsible for building the polysaccharide backbone, containing repeating units of -GlcUA-GlcNAc-. The backbone is then modified by N-deacetylase/N-sulfotransferase (which has two separate domains displaying the activity of N-deacetylase and N-sulfotransferase, respectively), C5-epimerase (which converts GlcUA to IdoUA), 2-O-sulfotransferase, 6-O-sulfotransferase, and 3-O-sulfotransferase to produce HS.

Heparin, a commonly used anticoagulant drug, is a specialized form of HS with higher levels of sulfation and IdoUA. It has remained as a top choice for treating thrombotic disorders for >50 years. Heparin achieves its anticoagulant activity by forming a 1:1 complex with antithrombin (AT). The resultant AT-heparin complex inhibits a series of proteases in the blood coagulation cascade, including factor Xa and thrombin (or factor IIa), to prevent the formation of blood clots. The heparin-exerting Xa inhibition effect requires the presence of a pentasaccharide domain with the structure of -GlcNAc6S-GlcUA-GlcNS3S±6S-IdoUA2S-GlcNS6S-, whereas the inhibition of IIa requires oligosaccharides larger than tetradecasaccharides (14-mer); the precise structures for oligosaccharides with anti-IIa activity are unknown (4, 5).

Heparin is currently isolated from porcine intestine or bovine lung through a poorly regulated supply chain. The worldwide distribution of contaminated heparin in 2007 raised concern over the safety and reliability of animal-sourced heparins (6, 7). A cost-effective method for preparing synthetic heparin and securing the safety of the heparin supply chain is highly desirable. Although heparin and HS fragments can be synthesized by a purely chemical method (8, 9), this synthesis is extremely challenging, especially for products larger than an octasaccharide. A new chemoenzymatic approach provides a promising alternative method to synthesize polysaccharides and structurally defined oligosaccharides (10–14).

In this work, we attempted to synthesize oligosaccharides with anti-IIa activity using the chemoenzymatic approach. A...
series of size-defined N-sulfo-oligosaccharides were synthesized using bacterial glycosyltransferase, an unnatural UDP-sugar, and N-sulfotransferase with high efficiency and purity. We demonstrate for the first time the preparation of oligosaccharides with up to 21 saccharide residues. We converted these N-sulfo-oligosaccharides to O-sulfated oligosaccharides to measure their anti-Xa and anti-IIa activities. Our data suggest that the minimum size for the oligosaccharides to present anti-IIa activity is 19 residues long and that a size of 21 saccharide residues is required to display full anti-IIa activity. The results will assist our long-term efforts to prepare synthetic heparin residues is required to display full anti-IIa activity. The results will assist our long-term efforts to prepare synthetic heparin.

**EXPERIMENTAL PROCEDURES**

*Expression of HS Biosynthetic Enzymes—*A total of eight enzymes were used for the synthesis, including N-sulfotransferase, C5-epimerase, 2-O-sulfotransferase (2-OST), 6-OST-1, 6-OST-3, 3-OST-1, KfiA, and Pasteurella multocida heparosan synthase 2 (pmHS2). All enzymes were expressed in Escherichia coli and purified by appropriate affinity chromatography as described previously (13).

*Preparation of Enzyme Cofactors—*A sulfo donor, 3′-phosphoadenosine 5′-phosphosulfate (PAPS), was prepared using adenosine phosphokinase and ATP-sulfurylase (13). The preparation of UDP-N-trifluoroacetylglucosamine (GlcNTFA) was started from glucosamine (Sigma), which was first converted to GlcNTFA by reacting with S-ethyl trifluorothioboacetate (Sigma) following the protocol described previously (13). The resultant GlcNTFA was converted to GlcNTFA 1-phosphate using N-acetylhexosamine 1-kinase (15). The plasmid expressing KfiA (30 mg), pmHS2, UDP-GlcNTFA, and UDP-GlcUA as described (13). The resultant UDP-GlcNTFA synthesis was completed by transforming GlcNTFA 1-phosphate using N-acetylhexosamine 1-kinase (15). The plasmid expressing C5-epimerase was a generous gift from Prof. Peng Wang (Georgia State University), and the expression of the enzyme was carried out in E. coli as reported (15). The UDP-GlcNTFA synthesis was then ready for the elongation reaction using KfiA as described below.

*Preparation of Oligosaccharide Backbone—*A disaccharide (GlcUA-anhydromannitol (AnMan)) was prepared from heparosan as described (13). The disaccharide was then elongated to N-trifluoroacetylated (N-TFA) oligosaccharides by repetitive exposure to KfiA, pmHS2, UDP-GlcNTFA, and UDP-GlcUA as shown in Fig. 1. Briefly, the disaccharide (20 mg), KfiA (30 μg/ml), and UDP-GlcNTFA (500 μM) were mixed in 120 ml of 50 mM Tris buffer. The reaction was incubated overnight at room temperature. The completion of the reaction was monitored by the disappearance of UDP-GlcNTFA using a silica-based polyamine HPLC column (Waters). Once the reaction was completed, pmHS2 (30 μg/ml) and UDP-GlcUA (750 μM) were added, and the reaction was incubated for another 24 h at room temperature. The resultant product was a tetrasaccharide, which was purified using a Bio-Gel P-2 column (0.75 × 200 cm) that was equilibrated with 0.1 M ammonium bicarbonate at a flow rate of 6 ml/h. The fractions were then subjected to electrospray ionization (ESI) MS analysis. The fractions containing the tetrasaccharide were pooled. The procedures for synthesizing the N-TFA pentadecasaccharide (15-mer), N-TFA heptadecasaccharide (17-mer), N-TFA nonadecasaccharide (19-mer), and N-TFA hentacosaccharide (21-mer) were essentially the same, completed by repeating the above cycle with the designated times.

When the backbone was elongated beyond octasaccharide, a special method was employed to deplete UDP-GlcNTFA and UDP-GlcNAc prior to further elongation by pmHS2. If unreacted UDP-GlcNTFA or UDP-GlcNAc was present in the GlcUA elongation step involving pmHS2, an uncontrolled elongation occurred, resulting in a mixture. It was therefore especially important to avoid the formation of mixtures when the synthesis reached octasaccharide and beyond because the P-2 column cannot separate oligosaccharides larger than octasaccharides. To this end, the disaccharide GlcUA-AnMan was added to the reaction to exhaust the residual UDP-GlcNTFA and GlcNAc at the reaction, and the resultant trisaccharide was removed using the P-2 column.

*N-Detrifluoroacetylation—*Oligosaccharide backbones (1–2 mg) were resuspended in solution (20 ml) containing 2:2:1 (v/v/v) CH3OH, H2O, and (C2H5)3N. The reaction was incubated overnight at 50 °C. The samples were dried and reconstituted in H2O to recover de-N-TFA oligosaccharides.

*N-Sulfation of Oligosaccharides—*N-Sulfation of oligosaccharides was carried out by incubating the oligosaccharide substrates with N-sulfotransferase and PAPS. The reaction mixture typically contained 1–2 mg of de-N-TFA oligosaccharide, 500 μM PAPS, 50 mM MES (pH 7.0), and 1 mg of N-sulfotransferase in a total volume of 15 ml. The reaction mixture was incubated overnight at 37 °C.

*Sulfation and Epimerization Modifications of Oligosaccharide Backbones—*The conversion of N-sulfo-oligosaccharides to the final products involved three steps, including C5-epimerization/2-O-sulfation, 6-O-sulfation, and 3-O-sulfation. N-Sulfo-oligosaccharides (1–2 mg) were incubated in a reaction mixture containing 50 mM MES (pH 7.0), 0.03 mg/ml C5-epimerase, and 2 mM CaCl2 in a total volume of 40 ml. After incubation for 30 min at 37 °C, 2-OST (0.03 mg/ml) and 200 μM PAPS were added, and the reaction was incubated overnight at 37 °C. The products were purified using a DEAE column as described previously (14). For 6-O-sulfation, the substrate was incubated overnight at 37 °C in a reaction mixture containing 50 mM MES (pH 7.0) and 500 μM PAPS in the presence of 6-OST-1 (0.03 mg/ml) and 6-OST-3 (0.03 mg/ml) in a total volume of 20 ml. The products were purified using a DEAE column. For 3-O-sulfation, the substrate was incubated overnight at 37 °C in a reaction mixture containing 3-OST-1 (0.03 mg/ml), 10 mM MnCl2, 5 mM MgCl2, and 500 μM PAPS in a total volume of 20 ml.

**MS Analysis of Oligosaccharides—**Analyses were performed with a Thermo Scientific LCQ-Deca system. The non-sulfated oligosaccharide (1 μl) eluted from Bio-Gel P-2 column was directly diluted in 200 μl of 9:1 MeOH/H2O. A syringe pump (Harvard Apparatus) was used to introduce the sample via direct infusion (35 μl/min). Experiments were carried out in negative ionization mode with the electrospray source set to 5 kV and 275 °C. The sulfated oligosaccharide (1 μl) was diluted...
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in a different working solution containing 200 μl of 70% acetonitrile and 10 mM imidazole. Experiments for sulfated oligosaccharides were carried out in negative ionization mode with the electrospray source set to 2 kV and 200 °C. The automatic gain control was set to 1 × 10^7 for full-scan MS. The MS data were acquired and processed using Xcalibur 1.3.

Inhibition of Factor Xa and Ila Activities—Assays were based on a previously published method (16, 17). Briefly, factor Xa (Enzyme Research Laboratories, South Bend, IN) and thrombin (Sigma) were diluted at 80 and 100 nM, respectively, with PBS containing 1 mg/ml BSA. Human AT (Cutter Biological) was diluted with PBS containing 1 mg/ml BSA to give a stock solution at a concentration of 0.4 μM. The chromogenic substrates S-2765 (for factor Xa assay) and S-2238 (for factor IIa assay) were prepared at 1.3 and 1.5 mM in water. The synthesized oligosaccharides or heparin was dissolved in PBS at various concentrations (1–30 nM). The reaction mixture, which consisted of 70 μl of AT stock solution and 15 μl of sample solution, was incubated at room temperature for 2 min. Factor Xa or thrombin (10 μl) was added. After incubation at room temperature for 4 min, 30 μl of S-2765 or S-2238 was added. The initial reaction rates as a function of concentration were used to calculate the IC50 values. The absorbance of the reaction mixture was measured at 405 nm continuously for 10 min. The absorbance values were plotted against the reaction time. The initial reaction rates as a function of concentration were used to calculate the IC50 values.

Binding of Oligosaccharides to Platelet Factor 4—A filter-trapping assay was used to compare the binding of the different oligosaccharides to platelet factor 4 (PF4) (18). The 35S-labeled oligosaccharides (~6000 cpm) were incubated with 0–15 μg/ml PF4 in 50 mM Tris and 250 mM NaCl (pH 7.3) for 30 min at 37 °C to allow complex formation. The mixture was then spotted onto a nitrocellulose membrane, which binds to proteins nonspecifically, allowing the capture of complexes of PF4 and 35S-labeled oligosaccharide. The membrane wells were washed with buffer and excised, and the bound radioactivity was quantified using a scintillation counter.

Neutralizing Effect of PF4 on Anti-Xa Activity of Heparin and Oligosaccharide—HS oligosaccharides were incubated in a 96-well plate with 70 μl of BSA (1 mg/ml in PBS), 10 μl of AT (0.2 mg/ml in PBS), and 0–16 μl of PF4 (0.45 mg/ml) for 2 min at room temperature. Ten microliters of Xa was then added, and after 4 min, 30 μl of chromogenic substrate S-2765 (1 mg/ml) was added to initiate the color change reaction. Sequential absorbance readings at 405 nm were started immediately using a Bio-Tek ELx808 plate reader. The rate of increased absorbance relative to the rate of a control sample was used to define Xa activity. Quantities of oligosaccharides showing ~8% Xa activity in the absence of PF4 were used.

HPLC Analysis—Both DEAE-HPLC and polyamine-based anion exchange HPLC were used to determine the purity of the oligosaccharides. The elution conditions for the HPLC analysis have been described elsewhere (13).

Disaccharide Analysis of 35S-Labeled Oligosaccharides—The 35S-labeled compound was deacetylated and degraded with nitrous acid at pH 4.5 and then at pH 1.5, followed by reduction with sodium borohydride as described by Shively and Conrad (19). The resultant 35S-labeled disaccharides were resolved using a C18 reverse-phase column (0.46 × 25 cm; Vydac) under reverse-phase ion-pairing HPLC conditions. The identities of the disaccharides were determined by co-elution with the appropriate 35S-labeled disaccharide standards.

RESULTS

Chemoenzymatic Synthesis of N-Sulfo-oligosaccharide Backbones—Although a pentasaccharide is sufficiently large to exhibit anti-Xa activity (4), a much longer heparin is required to carry anti-IIa activity (5). The architecture of the IIa inhibitor is believed to contain a domain that binds to AT, a domain that binds to IIa, and a linker domain consisting of low sulfated (or non-sulfated) -GlcUA-GlcNAc- repeating units (Fig. 1) (20). Our first step toward the synthesis of HS oligosaccharides carrying anti-IIa activity involved synthesizing the oligosaccharide backbone with specific N-sulfo distribution. This unique N-sulfation pattern permitted the subsequent C5-epimerization and O-sulfations to yield products with three segments: an AT-binding domain, a linker domain, and a thrombin-binding domain (as shown in Fig. 1). The synthesis was initiated from a disaccharide, which was elongated to the desired size using two bacterial glycosyltransferases, N-acetylgalcosamine transferase from E. coli strain K5 (KfiA) and pmHS2 (14). During the synthesis, GlcNTFA residues were strategically introduced into the backbone, where the AT-binding and IIa-binding domains are located, as the N-TFA group is readily converted to an N-sulfo group by a chemoenzymatic approach. The linker region contains GlcNAc residues.

A total of four N-TFA oligosaccharides were synthesized from a decasaccharide designated as the “AT-binding site and linker backbone” (Fig. 1). The structural difference in the four N-TFA oligosaccharides was that each oligosaccharide contained distinct numbers of the -GlcUA-GlcNTFA- repeating unit in the IIa-binding domain. It should be noted that all four compounds contain very similar structures in the AT-binding and linker domains. The size of the -GlcUA-GlcNTFA- repeating unit was controlled by the cycles of modifications using KfiA and pmHS2. For example, the preparation of the N-TFA pentadecasaccharide (15-mer) involved five enzymatic steps (three KfiA modification steps and two pmHS2 modification steps), whereas the synthesis of the N-TFA hensicosasaccharide (21-mer) required 11 enzymatic steps (six KfiA modification steps and five pmHS2 modification steps). The N-TFA oligosaccharides were synthesized in milligram scales.

The N-TFA oligosaccharides were next converted to N-sulfo-oligosaccharides in two steps: alkaline detrifluoroacetylation and treatment with N-sulfotransferase. To this end, four N-sulfated oligosaccharides, ranging from pentadeca- to hensicosasaccharides (1-4), were obtained. The purity of the N-sulfation products was analyzed by high resolution DEAE-HPLC analysis, and their molecular weights were determined by ESI-MS. As shown in Fig. 2A, the 35S-labeled N-sulfo-hensicosasaccharide (4) was eluted in a predominant single symmetric peak, suggesting that the compound is substantially pure. The results of the ESI-MS analysis revealed the molecular weight of Compound 4 to be 4263.2 ± 1.0, very close to the calculated value of 4261.5, suggesting that Compound 4 is a...
Henicosasaccharide carrying eight sulfo groups (Fig. 2 B and Table 1). We completed the purity analysis using DEAE-HPLC and molecular weight determination using ESI analysis (supplemental Figs. S1–S3) for Compounds 1–3. All of these N-sulfo-oligosaccharides had the anticipated molecular weights (Table 1) and displayed high purity. Our data suggest that we obtained the expected products.

Preparation of Oligosaccharides Carrying IdoUA Residues and O-Sulfations—Incubation of N-sulfo-oligosaccharides with C5-epimerase, 2-OST, 6-OST, and 3-OST yielded oligosaccharides carrying IdoUA residues and O-sulfations (5–8).

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Summary of disaccharide analysis of the 15–17-mers without 3-O-sulfation degraded by heparin lyases

Oligosaccharides were 35S-labeled at both the 2-O-sulfur and 6-O-sulfur positions. The oligosaccharides were then degraded with a mixture of heparin lyases I–III. The disaccharides were resolved by reverse-phase ion-pairing HPLC. ∆UA, ∆5'-unsaturated uronic acid.

| Compound | Name | Abbreviated structure | M_r Calculated | M_r Measured |
|----------|------|-----------------------|----------------|--------------|
| 1        | N-Sulfo-pentadecasaccharide (15-mer) | GlcNS-(GlcUA-GlcNS)₃-GlcUA-GlcNaC₆S-GlcUA-GlcNS-GlcUA-AnMan | 3009.5 | 3010.5 ± 1.1 |
| 2        | N-Sulfo-heptadecasaccharide (17-mer) | GlcNS-(GlcUA-GlcNS)₄-GlcUA-GlcNaC₆S-GlcUA-GlcNS-GlcUA-AnMan | 3426.8 | 3428.1 ± 1.0 |
| 3        | N-Sulfo-nonadecasaccharide (19-mer) | GlcNS-GlcUA-GlcNS₃-GlcUA-GlcNaC₆S-GlcUA-GlcNS-GlcUA-AnMan | 3844.2 | 3846.2 ± 1.2 |

| TABLE 3 | Summary of disaccharide analysis of Compounds 5–8 degraded by nitrous acid |
|----------|------------------------|------------------|------------------|------------------|------------------|
|          | 15-mer without 3-O-sulfation | 17-mer without 3-O-sulfation | 19-mer without 3-O-sulfation | 21-mer without 3-O-sulfation |
| ∆UA-GlcNaC₆S | 30.9% | 30.0% | 24.3% | 26.6% |
| ∆UA-GlcN₆S | 31.0% | 27.0% | 17.4% | 35.6% |
| ∆UA2S-GlcNS | <1% | <1% | 1.5% | 2.1% |
| ∆UA2S-GlcN₆S | 38.0% | 43.0% | 57.6% | 35.6% |
| O-Sulfo group/disaccharide* | 1.4 (1.2)* | 1.5 (1.4) | 1.5 (1.6) | 1.5 (1.4) |

* The number of O-sulfos per disaccharide was determined based on the specific 35S radioactivity of [35S]PAPS.
* The numbers given in parentheses are the values based on disaccharide analysis.

The presence of monosaccharide in the nonreducing ends of Compounds 5–8 was also demonstrated. Heparin lyase-degraded Compounds 5–8 were analyzed using Bio-Gel P-2. The elution profiles of the degraded oligosaccharides from the Bio-Gel P-2 column show the presence of 35S-labeled monosaccharide, suggesting that these oligosaccharides have a GlcNS6S residue at the nonreducing end (supplemental Fig. S5).

Determinations of Anti-Xa and Anti-IIa Activities of Oligosaccharides—All anticoagulant HS structures exhibiting anti-Xa and anti-IIa activities bind to AT. Indeed, an analysis using affinity co-electrophoresis demonstrated that Compounds 5–8 bound to AT effectively (supplemental Fig. S6). We next determined the anti-Xa and anti-IIa activities. Both anti-Xa and anti-IIa activities were measured using specific chromogenic substrates (17). As expected, all four O-sulfated oligosaccharides displayed anti-Xa activity with a similar potency to heparin as well as the previously synthesized ultra low molecular weight (ULMW) heparin 1 (14) (Fig. 3A and Table 4). The results suggest that anti-Xa activity is independent of the size of the oligosaccharides. This conclusion is consistent with previous findings, namely that the minimum size of the HS oligosaccharide with anti-Xa activity is a pentasaccharide (5). In stark contrast, the anti-IIa activity measurement revealed a dependence on the size of the oligosaccharide. The oligosaccharides shorter than a heptadecasaccharide did not exhibit detectable anti-IIa activity (Table 4). The nonadecasaccharide (7, 19-mer) began to show weak anti-IIa activity (the
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Inhibition of anti-Xa and anti-IIa by O-sulfated oligosaccharides

| Compound       | IC_{50} (nM) | Ratio of anti-Xa to anti-IIa |
|----------------|--------------|-----------------------------|
| ULMW heparin 1*| 2.8          | 1:1                         |
| 9              | 1.2          | ND                          |
| 10             | 1.5          | ND                          |
| 11             | 0.8          | 4.2                         |
| 12             | 1.0          | 1.7                         |
| Heparin        | 0.7          | 0.7                         |

* ULMW heparin 1 is a heptasaccharide using a chemoenzymatic approach with a structure of GlcNAc6S-GlcUA-GlcNS3S6S-IdoUA2S-GlcNS6S-GlcUA-AnMan (14). IC_{50} represents the half-maximum inhibitory concentration. ND, not detectable; NA, not applicable.

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anti-Xa/anti-IIa ratio was determined to be 1:5). The heparicosasaccharide (8, 21-mer) showed stronger anti-IIa activity, with an anti-Xa/anti-IIa ratio of 1:2 (Fig. 3B and Table 4). The anti-Xa/anti-IIa ratio for heparin was 1:1 (Table 4). Our data suggest that the oligosaccharides should be longer than 21 saccharide units to fully exhibit both anti-Xa and anti-IIa activities comparable with those observed for heparin. In a previous report, a synthetic heparin mimetic with a size larger than 16 saccharide units was the minimum length for displaying anti-IIa activity (20). Our data demonstrated a slightly larger oligosaccharide for anti-IIa activity than reported previously for heparin. This result is not completely unexpected given the fact that two saccharide units at the reducing end of Compound 8 are unlikely to participate in binding to AT or IIa. Furthermore, a cluster of highly sulfated glucose residues was used to mimic the IIa-binding domain in the synthetic heparin mimetic due to the complexity of the chemical synthesis process. In fact, highly sulfated glucose residues are not present in heparin or HS isolated from natural sources (3).

Determination of the Binding of Oligosaccharides to PF4—PF4 is a positively charged chemokine. PF4 binds to heparin avidly, and the resultant complex induces the production of anti-PF4/heparin antibody, leading to a life-threatening thrombotic disorder known as heparin-induced thrombocytopenia (23). Thus, reducing the binding between heparin and PF4 could potentially decrease the risk of heparin-induced thrombocytopenia associated with the use of heparin. To this end, we tested the binding of the oligosaccharides and PF4. Although all compounds bound to PF4, the oligosaccharides bound to PF4 to a lower extent compared with full-length HS (Fig. 4A). It should be noted that ULMW heparin 1 did not bind to PF4, consistent with the previous finding indicating that the minimum size of HS oligosaccharides that bind to PF4 is an octasaccharide (24, 25). Furthermore, the use of fondaparinux, a synthetic ULMW heparin, effectively eliminates the risk of heparin-induced thrombocytopenia (26, 27).

Next, we compared the binding affinity between O-sulfated oligosaccharides and full-length heparin using a PF4 neutralization assay (24). In this experiment, heparin and O-sulfated oligosaccharides were incubated with AT and Xa. Under these conditions, the activity of Xa is low because of the presence of AT-saccharide complexes. Upon the addition of PF4 to the reaction mixture, PF4 displaced AT by interacting with the saccharides. Consequently, AT lost the ability to inhibit the activity of Xa. As shown in Fig. 4B, PF4 effectively neutralized the inhibition effect of heparin at an ED_{50} of ~1.2 μg/ml. In contrast, Compound 8 displayed a much higher ED_{50} (3.4 μg/ml), suggesting that the synthesized O-sulfo-heparicosasaccharide has reduced binding affinity for PF4. As expected, PF4 was unable to neutralize the anti-Xa activity of ULMW heparin 1 because ULMW heparin 1 does not bind to PF4 (Fig. 4B).

DISCUSSION

In this study, we have reported the synthesis of large HS oligosaccharides that display anti-IIa activity. Although HS inhibits the activities of Xa and IIa through interaction with AT, the HS domain that exhibits anti-IIa activity is ~3–4 times longer than the domain that exhibits anti-Xa activity. To date, the synthesis of genuine HS oligosaccharides with both anti-Xa and anti-IIa activities had not been accomplished for the fol-
Chromogenic substrate was added to the mixture, and the rate of increase in the absorbance at 405 nm was used to determine Xa activity. AT inhibited the activity of Xa by binding to HS; PF4 competed with AT for binding to HS and therefore neutralizes the anti-Xa activity of AT.

Determination of the binding of PF4 to O-sulfated oligosaccharides. A, binding between the 35S-labeled oligosaccharides and PF4 using a filter-trapping assay. 35S-Labeled oligosaccharides were incubated with increasing amounts of PF4 and spotted onto a nitrocellulose membrane. Complexes of PF4 and 35S-labeled oligosaccharide were captured by the membrane, excised, and quantified using a scintillation counter. 3-O-Sulfated [35S]HS was used as a positive control, and it was prepared by incubating HS from bovine kidney and purified 3-OST-1 enzyme in the presence of [35S]PAPS. B, neutralizing effects of PF4 on the anti-Xa activity of Compound 8, heparin, and ULWM heparin 1. The HS compounds were incubated with AT and varying amounts of PF4 before the addition of Xa. AT inhibited the activity of Xa by binding to HS; PF4 competed with AT for binding to HS and therefore neutralizes the anti-Xa activity of AT. Chromogenic substrate was added to the mixture, and the rate of increase in the absorbance at 405 nm was used to determine Xa activity.

The chemical synthesis of heparin-like oligosaccharides with anti-IIa activity has been completed by Petitou et al. (20). In this impressive piece of work, a series of oligosaccharides up to an icosasaccharide (20-mer) were synthesized. However, none were authentic HS oligosaccharides, but rather heparin mimetics. The IIa-binding domain in these compounds consists of methylated glucose sulfate residues, an unnatural saccharide, to reduce the complexity of the synthesis. These compounds, especially a hexadecasaccharide (16-mer), showed very promising anticoagulant activity in vitro and in vivo, with the possibility to become a new generation of synthetic heparin (28). The hexadecasaccharide was also used in crystal structural studies of the ternary complex of AT-thrombin (factor IIa)-heparin to understand the mechanism of action of the heparin-mediated deactivation of IIa (29, 30). Unfortunately, the hexadecasaccharide reportedly failed in a recent clinical trial due to excessive bleeding effects (31).

Unlike chemical synthesis, the chemoenzymatic approach produces authentic heparin molecules and involves a much shorter synthetic route. Although we were unable to synthesize structurally homogeneous O-sulfated oligosaccharides in this study, the results clearly show that this approach can be used to prepare N-sulfo-oligosaccharides up to 21 saccharide residues. The availability of these N-sulfo-oligosaccharides permitted us to probe the size requirement for oligosaccharides with anti-IIa activity. We believe that the structural heterogeneity stemmed from the modification steps using C5-epimerase and 2-O-sulfation because we have demonstrated the synthesis of a structurally pure dodecasaccharide carrying both N-sulfation and 6-O-sulfation in a previously published report (13). Further investigation into the control of the placement of IdoUA2S residues is needed to synthesize large heparin oligosaccharides carrying IdoUA2S residues.

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