De novo synthesis of a narrow size distribution low-molecular-weight heparin

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Heparin, a commonly used anticoagulant drug, is a mixture of highly sulfated polysaccharides with various molecular weights (MWs). The unique sulfation pattern dictates the anticoagulant activity of heparin. Commercial heparins are categorized into three forms according to their average MW: unfractionated heparin (UFH, MW_{avg} 14,000), low-MW heparin (LMWH, MW_{avg} 3500–6500) and the synthetic pentasaccharide (fondaparinux, MW 1508.3). UFH is isolated from porcine intestine while LMWH is derived from UFH by various methods of depolymerization, which generate a wide range of oligosaccharide chain lengths. Different degradation methods result in structurally distinct LMWH products, displaying different pharmacological and pharmacokinetic properties. In this report, we utilized a chemoenzymatic method to synthesize LMWH with the emphasis on controlling the size distribution of the oligosaccharides. A tetrasaccharide primer and a controlled enzyme-based polymerization were employed to build a narrow size oligosaccharide backbone. The oligosaccharide backbones were further modified by a series of sulfation and epimerization steps in order to obtain a full anticoagulation activity. Determination of the anticoagulation activity in vitro and ex vivo indicated that the synthetic LMWH has higher potency than enoxaparin, a commercial LMWH drug in clinical usage.

Keywords: chemoenzymatic synthesis / heparin / low-molecular-weight heparin / sulfotransferases and oligosaccharides

Introduction

Heparin, a polysaccharide-based drug, has been well known for its therapeutic anticoagulant effect for decades. Although the development of novel anticoagulation agents is increasing, heparin remains the drug of choice in several medical circumstances due to its rapid onset of action and relatively wide therapeutic window. Heparins are categorized into three forms corresponding to their average molecular weights (MWs): unfractionated heparin (UFH, MW_{avg} ~14,000), low-molecular-weight heparin (LMWH, MW_{avg} ~3500–6500) and the synthetic pentasaccharide, fondaparinux (MW 1508.3) (Linhardt and Liu 2012). These heparins differ in their pharmacokinetic profiles, providing different choices for cardiovascular treatments (Hirsh and Raschke 2004). For instance, UFH is administered by intravenous injection. It has a rapid onset of action and is metabolized by the liver at a fast clearance rate, which makes it useful in patients undergoing surgery or renal hemodialysis (Heres et al. 2001). However, UFH requires routine monitoring due to its unpredictable bioavailability. Moreover, 2–3% of patients administered UFH are prone to develop a life-threatening adverse event known as heparin-induced thrombocytopenia (HIT). Therefore, UFH usage is generally restricted to hospitalized patients. LMWH, on the other hand, has a number of advantages over UFH including predictable bioavailability by subcutaneous administration, longer half-life in plasma and lower incidences of HIT and bleeding (Hirsh and Raschke 2004). All of these advantages make LMWH useful for outpatient treatment and prophylaxis of venous thromboembolism, acute myocardial infarction and unstable angina. The advantage of synthetic pentasaccharide is a decreased risk of HIT. However, it requires renal excretion. Thus, the pentasacharide is not recommended for patients with impaired renal function. Currently, LMWH is the most commonly prescribed form of heparin (Merli and Groce 2010).

Heparin has the disaccharide-repeating unit of glucosamine (GlcN) and hexuronic acid, including glucuronic acid (GlcA) or iduronic acid (IdoA). Both GlcN and hexuronic acid residues carry sulfo groups. Heparin inhibits coagulation by forming a complex with antithrombin (AT). A unique pentasaccharide domain in heparin, -GlcNAc6S-GlcA-GlcNS3S ± 6S-IdoA2S-GlcNS6S-, plays a critical role in the binding to AT. When the pentasaccharide binds to AT, a heparin-AT complex inactivates several serine proteases such as factor Xa (FXa) and thrombin, also known as factor IIa (FIIa). The inhibition of FXa and FIIa activities prohibits the formation of a blood clot (Hirsh and Raschke 2004).

UFH is extracted from porcine intestine and bovine lung, whereas LMWH is derived from UFH by chemical and enzymatic depolymerization, resulting in oligosaccharide fragments

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(Linhardt and Gunay 1999). An investigation on LMWH prepared from a contaminated UFH revealed that the current manufacturing methods to prepare LMWH failed to eliminate oversulfated chondroitin sulfate contamination (Zhang et al. 2008). The long and poorly regulated heparin supply chain has raised concerns over the safety and reliability of animal-sourced heparin and LMWH. Recently, a demand for a synthetic form of LMWH has emerged. Although the synthetic pentasaccharide is produced using a purely chemical approach (Petitou et al. 1991), it is extremely difficult to employ this method to prepare LMWH because of the larger molecular size.

Commercially available LWMH has a broad range of oligosaccharide size distribution with component MWs ranging from 3500 to 6500 leading to complex pharmacological/pharmacokinetic effects. The polydispersity in the chain length may affect metabolic clearance since short oligosaccharides require kidney clearance while large polysaccharides are excreted by the liver (Pempe et al. 2012). In addition, different approaches to manufacture LMWH result in various product profiles across the LMWH brands with distinct biochemical and pharmacological properties. Three brands of LMWHs currently available in the United States are obtained from different methods to depolymerize UFH, resulting in different MW distributions. For example, Enoxaparin ($MW_{avg} \sim 4500$) is produced from benzylation followed by alkaline hydrolysis; Dalteparin ($MW_{avg} \sim 6000$) is derived from controlled nitrous acid depolymerization; and Tinzaparin ($MW_{avg} \sim 6500$) is prepared by controlled heparinase digestion (Merli and Groce 2010). The US FDA indicates that none of these product brands is interchangeable (Nightingale 1993). Furthermore, a structural characterization study on these three LMWHs demonstrated that the depolymerization methods potentially damage the pharmacophore of the LMWHs, reducing the anticoagulant potency (Bisio et al. 2009).

Advancement in the understanding of heparin and heparan sulfate (HS) biosynthetic pathway provides an opportunity to develop a new method to manufacture LMWH using a chemoenzymatic approach. This method employs HS biosynthetic enzymes, involving HS polymerases, an epimerase and various sulfotransferases. It has been established that HS polymerases synthesize a polysaccharide backbone consisting of -GlcA-GlcNAc- disaccharide units. *N*-Deacetylase/*N*-sulfotransferase is a bifunctional catalyst in which *N*-deacetylase removes an acetyl group from GlcNAc resulting in a free amino group (GlcNH$_2$), whereas *N*-sulfotransferase (NST) adds a sulfate group at a resultant GlcNH$_2$ thereby yielding GlcNS (Duncan et al. 2006). C$_5$-epimerase (C$_5$-epi) converts GlcA to IdoA. Various sulfotransferases cofunctioning with a sulfo donor, 3′-phospho-adenosine-5′-phosphosulfate (PAPS), add sulfo groups to their respective positions. 2-*O*-Sulfotransferase (2-OST) adds sulfo groups at the 2-OH position of both GlcA and IdoA while 6-*O*-sulfotransferase (6-OST) and 3-*O*-sulfotransferase (3-OST) add sulfo groups at the 6-OH position and 3-OH of GlcN residues, respectively (Peterson et al. 2009). An enzyme-based approach has been developed as a desirable method to produce a safer LMWH (Liu et al. 2010; Xu et al. 2011). The enzymatic method also offers an approach to rationally design a heparin-like compound with improved pharmacological effects. The enzymes regioselectively add monosaccharides and sulfo groups, eliminating the need to introduce protecting/deprotecting

steps used in chemical synthesis, thus, significantly improving the synthesis efficiency (Chappell and Liu 2012).

In this article, we report a chemoenzymatic approach to generate a narrow size distribution LMWH, namely "de novo LMWH" (dnLMWH). We demonstrate a rapid procedure to synthesize oligosaccharide backbones in a one-pot format. The use of stoichiometrically controlled starting tetrasaccharide primer and monosaccharide donors allowed us to modulate a degree of oligomerization, yielding a narrow size distribution oligosaccharide backbone. The resultant oligosaccharide backbones were characterized and identified by strong anion-exchange high-performance liquid chromatography (SAX-HPLC) and electrospray ionization mass spectrometry (ESI-MS). We utilized a series of HS sulfotransferases to install an AT-binding pentasaccharide domain within the synthetic backbones. The in vivo and ex vivo anticoagulation activity of dnLMWH was determined. Our data suggest that a carefully designed narrow size distribution LMWH has a high potency for anticoagulation activity.

Results

Controlling the degree of backbone oligomerization

We previously reported a chemoenzymatic method to synthesize oligosaccharide backbones by sequential addition of monosaccharide residues (Xu et al. 2012). Although this synthetic method produces a homogeneous oligosaccharide backbone, the method requires about 14 days for preparing a single dodecasaccharide starting from a disaccharide. Here, we developed a one-pot chemoenzymatic synthesis capable of producing the dodecasaccharides, within 2 days. In order to build the oligosaccharide backbone carrying a (-GlcNAc-GlcA-) repeating unit in a one-pot format, a glycosyltransferase capable of transferring monosaccharide donors, uridine 5′-diphosphate (UDP)-GlcNAc and UDP-GlcA, is preferable. A heparosan synthase from bacteria *Pasteurella multocida* (PmHS2), a bifunctional polymerase capable of transferring both UDP-GlcNAc and UDP-GlcA, was chosen for this synthesis (Sismey-Ragatz et al. 2007; Chavaroche et al. 2010). Moreover, PmHS2 surpasses its substrate specificity in recognizing certain unnatural UDP-GlcNs (Otto et al. 2012). Therefore, PmHS2 meets our needs for a one-pot synthesis (Sismey-Ragatz et al. 2007).

In order to control the size of the product in a one-pot reaction format, we examined four different oligosaccharide primers: disaccharide, tetrasaccharide, hexasaccharide and octasaccharide (the structures of the oligosaccharide primers are shown in Supplementary data, Table S1). Generally, the components of the one-pot synthesis consist of a starting saccharide primer, UPD-GlcNAc, UDP-GlcA and PmHS2. The starting saccharide primer can be as short as a disaccharide or it can be as long as an octasaccharide. Since the MW_{avg} of commercial LWMHs is in a range of 3500–6500 Da corresponding to 8–20 monosaccharide units, we aimed to synthesize 10–14 saccharide units for backbone oligosaccharides. We hypothesized that the size of the products can be controlled by tuning the ratio between a starting primer and the monosaccharide donors. For example, when targeting 12 saccharide units and using a tetrasaccharide as a primer, a molar ratio between the tetrasaccharide, UDP-GlcNAc and UDP-GlcA should be kept at 1:4:4. To this end, UDP-Glc[^{3}H]

NAc was used to monitor the degree of polymerization because the incorporation of the Glc[³H]NAc allowed tracing the distribution of the oligosaccharide backbones using gel filtration chromatography. When a disaccharide primer was used, we observed that the products migrated to polymers regardless of the ratio between the disaccharide primer and the UDP sugars (Figure 1A, upper). Unlike the disaccharide primer, different sizes of oligosaccharide products were obtained when a tetrasaccharide primer was employed (Figure 1A, lower). In particular, desired size oligosaccharide products were obtained when the molar ratio of tetrasaccharide:UDP-Glc[³H]NAc:UDP-GlcA was kept at 1:4:4. We found that the hexasaccharide and the octasaccharide produced the oligosaccharides in a similar pattern as observed for the tetrasaccharide primer (Supplementary data, Figure S1). SAX-HPLC was employed to analyze the size distribution of the oligosaccharide backbones (Figure 1B), demonstrating the presence of ³H-labeled oligosaccharide backbones with different sizes. Co-eluting nonradiolabeled oligosaccharides followed by ESI-MS analysis confirmed the structures of the oligosaccharide products (Supplementary data, Figure S2, Supplementary data, Table S2). The oligosaccharide distribution analysis indicated that the size of the oligosaccharide backbones was in the range of dp 8–16 (Figure 1C) where dp 10 (24.6%) and dp 12 (24.5%) are major products (Figure 1C). The tetrasaccharide primer was

therefore selected as a primer for the preparation of LMWH in the subsequent studies.

N-*Sulfation of oligosaccharide backbones*

Next, we used the one-pot format to prepare the oligosaccharides with *N*-sulfation by preparing a backbone oligosaccharide with -GlcNTFA-GlcA-, where the GlcNTFA represents *N*-trifluoroacetyl GlcN. Replacing UDP-GlcNAc with UDP-NTFA in a backbone synthetic step allowed us to prepare the backbone oligosaccharides. A molar ratio between the tetrasaccharide primer, UDP-GlcNTFA and UDP-GlcA, was selected to be 1:4:4 to prepare the products. As expected, PmHS2 is also capable of incorporating the GlcNTFA residues into the backbones yielding the (-GlcNTFA-GlcA-) repeating backbones. The (-GlcNTFA-GlcA-) backbones were identified by ESI-MS, showing desired product and partially detrifluoroacetylated products due to the fact that trifluoroacetyl group is labile under basic conditions (Figure 2, Table I). Because the (-GlcNTFA-GlcA-) repeating backbones were eventually subjected to complete alkaline detrifluoroacetylation resulting in the -GlcNH₂-GlcA-) repeating backbones, the partial detrifluoroacetylation would not affect the subsequent synthesis. The resultant (-GlcNH₂-GlcA-) backbones were further treated with NST and PAPS. ESI-MS analysis of the
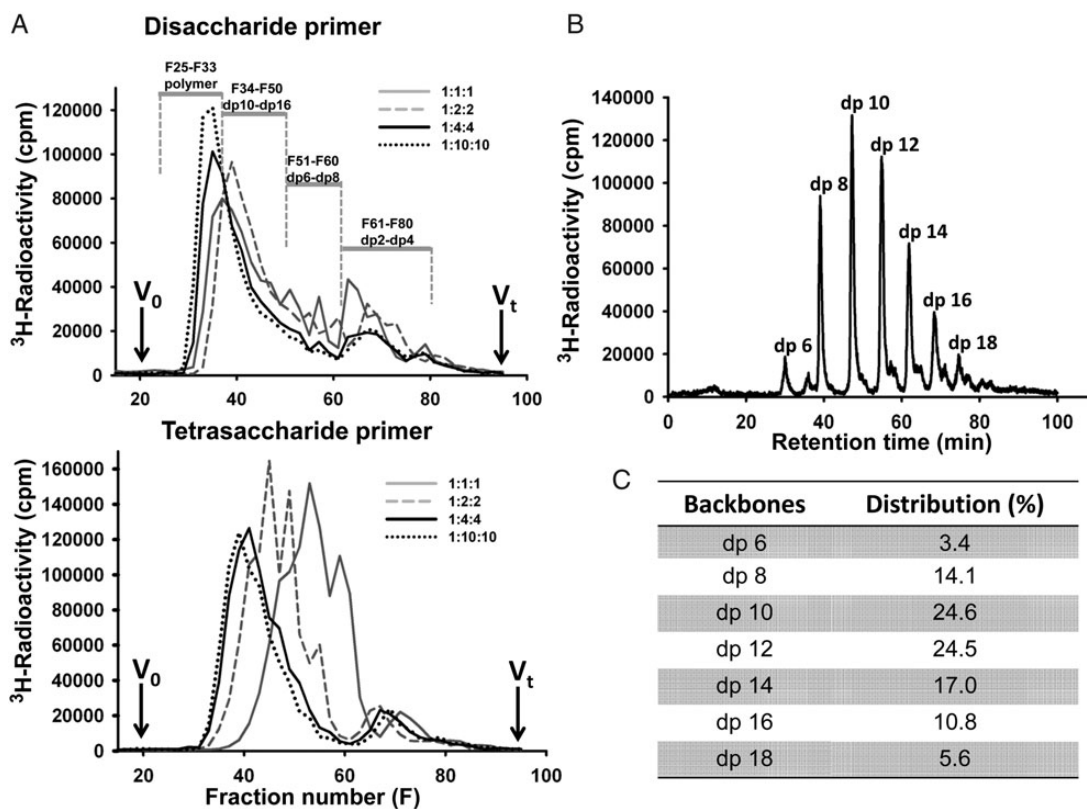

Fig. 1. Optimization of a primer and a molar ratio in a one-pot reaction. A disaccharide (**A**, upper) and a tetrasaccharide (A, lower) were used as primers in a one-pot synthesis. The oligosaccharide products were obtained by gel filtration chromatography. The ratios between the primers, UDP-[³H]GlcNAc and UDP-GlcA are shown (1:1:1 = grey-solid, 1:2:2 = grey-dash, 1:4:4 = black-solid, 1:10:10 = black-dot). V_0, void volume and V_t, total volume. The ³H-oligosaccharide products obtained from a reaction when the tetrasaccharide was used at 1:4:4 were pooled. A size distribution of the ³H-labeled products was analyzed by strong anion-exchange HPLC. The nonradiolabeled oligosaccharides were co-eluted and identified by ESI-MS analysis; dp, degree of polymerization (**B**). The distribution of the oligosaccharide chain length was estimated as a percentage (**C**).
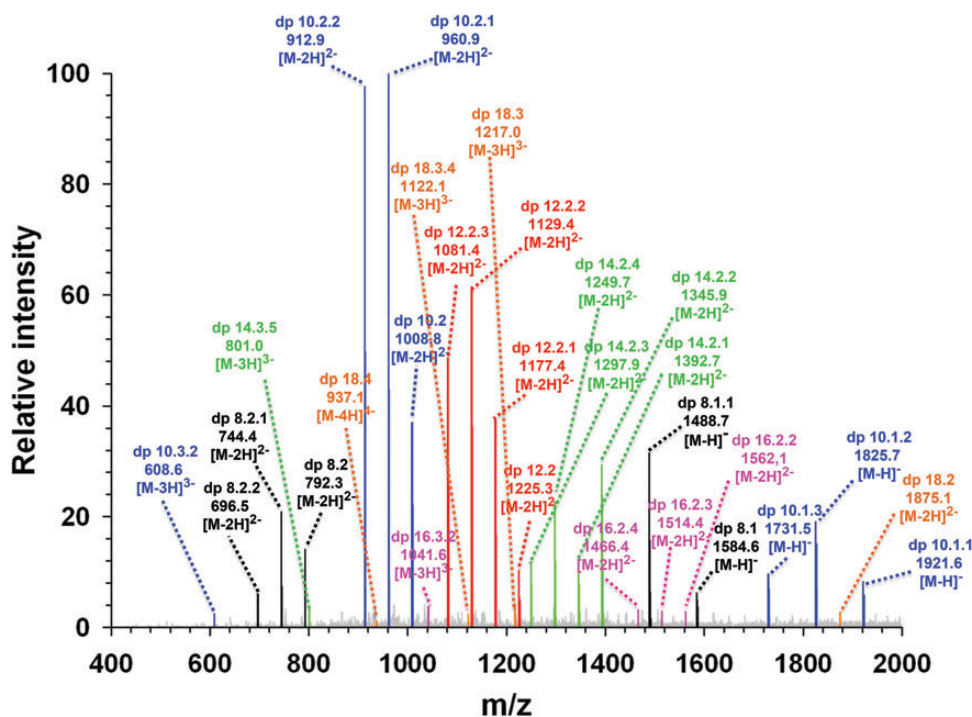
Fig. 2. MS analysis of the NTFA backbones. ESI (negative mode)-mass spectrum analysis of oligosaccharide backbones when a ratio between a tetrasaccharide primer, UDP-GlcN-TFA and UDP-GlcA was kept at 1:4:4. Line corresponds to the oligosaccharide chain length (dp8 = octasaccharide, dp10 = decasaccharide, dp12 = dodecasaccharide, dp14 = tetradecasaccharide, dp16 = hexadecasaccharide, dp18 = octadecasaccharide). Subnumbered molecular species indicated multiply charged and detrifluoroacetylated species, respectively. For example, dp 12.2.3 (MW 1081.4) is a double charge dodecasaccharide with the loss of three fluoroacetyl groups (see Table I).

Table I. ESI-MS analysis of (-GlcA-GlcNTFA-) backbones

Name	Abbreviated structure	M_r	
		Calculated	Measured
Octasaccharide (dp 8)	(GlcA-GlcNTFA)$_2$-GlcA-GlcNAc-GlcA-AnMan	1586.2	1586.5 ± 0.6
Decasaccharide (dp 10)	(GlcA-GlcNTFA)$_3$-GlcA-GlcNAc-GlcA-AnMan	2019.5	2019.3 ± 1.3
Dodecasaccharide (dp 12)	(GlcA-GlcNTFA)$_4$-GlcA-GlcNAc-GlcA-AnMan	2452.7	2453.2 ± 0.8
Tetradecasaccharide (dp 14)	(GlcA-GlcNTFA)$_5$-GlcA-GlcNAc-GlcA-AnMan	2886.0	2885.1 ± 1.2
Hexadecasaccharide (dp 16)	(GlcA-GlcNTFA)$_6$-GlcA-GlcNAc-GlcA-AnMan	3319.3	3318.9 ± 0.5
Octadecasaccharide (dp 18)	(GlcA-GlcNTFA)$_7$-GlcA-GlcNAc-GlcA-AnMan	3752.6	3752.2 ± 1.1

backbones resolved from Bio-Gel P-2 after *N*-sulfation step indicated that the (-GlcNS-GlcA-) repeating backbones were produced (Supplementary data, Figure S3, Supplementary data, Table S3).

Placing AT-binding site inside the oligosaccharide backbones by epimerization and O-sulfation modification

Heparin displays its anticoagulant activity by binding to AT through the unique pentasaccharide region (Hirsh and Raschke 2004). An important step in heparin synthesis is to properly install the AT-binding site within the oligosaccharide backbones. Structural analysis of heparin revealed that the AT-binding site is randomly distributed along the polysaccharide chain (Oscarsson et al. 1989) providing flexibility for placing the AT-binding region. We found that by placing a GlcNAc residue at the

nonreducing end of *N*-sulfo oligosaccharide backbones, the pentasaccharide motif could be readily produced by the latter epimerization and sequential sulfo modifications (Sheng et al. 2012). An installation of the GlcNAc residue directs the irreversible catalytic mode of C$_5$-epi such that GlcA at residue D is irreversibly converted to IdoA (Figure 3). The GlcNAc elongation was catalyzed by an *N*-acetyl glucosaminyl transferase of *Escherichia coli* K5 strain (KfiA) as described elsewhere (Chen et al. 2006). We confirmed the completion of GlcNAc elongation by ESI-MS analysis (Supplementary data, Figure S4 and Table S4). Once the GlcNAc elongation was achieved, we used recombinant C$_5$-epi for epimerization as well as a series of HS sulfotransferases for *O*-sulfo addition. The presence of the GlcA at residue B controls a specific addition of a sulfo group at residue C but not residue E by 3-OST-1 (Moon et al. 2012). The *O*-sulfated products were purified by diethylaminoethyl (DEAE) anion-exchange
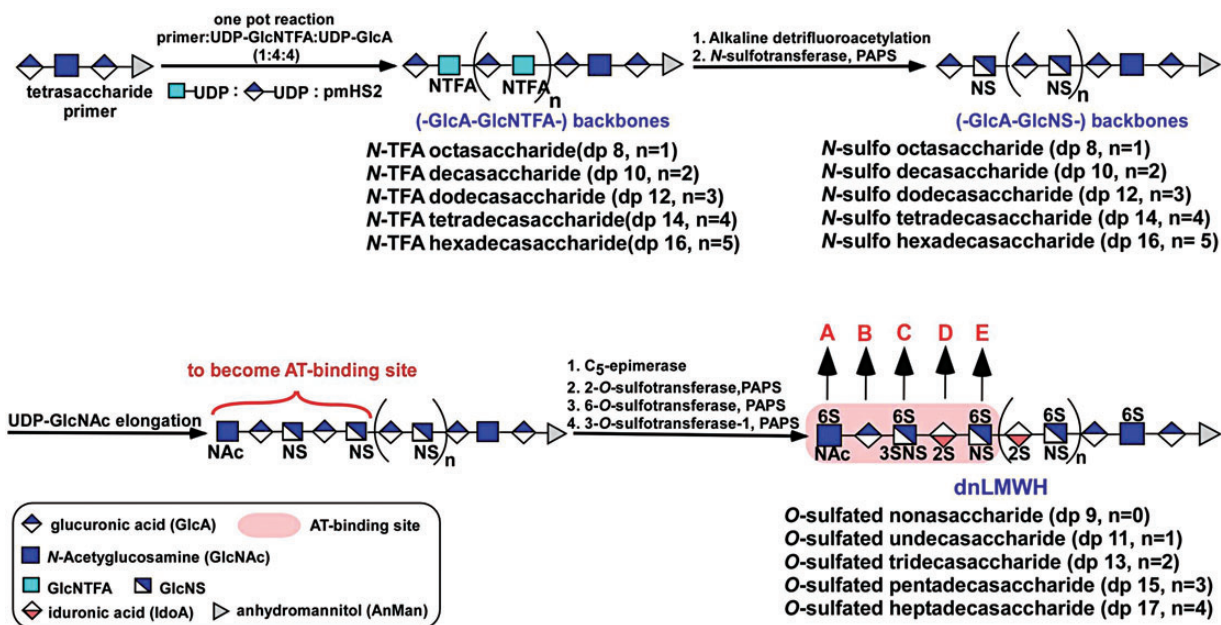
Fig. 3. Schematic synthesis of dnLMWH. A scheme illustrates the synthetic steps starting from an incubation of a tetrasaccharide primer with UDP-GlcN-TFA and UDP-GlcA at a 1:4:4 molar ratio (upper panel). The (-GlcNTFA-GlcA-) repeating backbones were subjected to detrifluoroacetylation and N-sulfation. The GlcNAc elongation aided in an installation of the AT-binding site. Sequential modification by epimerization and 2-*O*-, 6-*O*- and 3-*O*-sulfation were required to achieve dnLMWH (lower panel).

chromatography. We ensured the completion of each *O*-sulfation by performing a small-scale reaction using PAP^{35}S to monitor the reaction.

It is important to demonstrate that one-pot synthesis can be performed in a reproducible way to produce narrow size distribution of the oligosaccharide backbones. We performed three individual synthetic batches. ESI-MS analyses confirmed that the (-GlcNTFA-GlcA-) and the (-GlcNH$_2$-GlcA-) backbones are similar across the three batches. The (-GlcNH$_2$-GlcA-) backbones were treated with NST and PAP^{35}S yielding (-GlcN^{35}S-GlcA-) backbones. The backbones were purified and subjected to DEAE-HPLC analysis. The result shows that all three batches have similar oligosaccharide distributions (Figure 4A). We also tested the oligosaccharide distribution by conducting a [^{3}H] GlcNAc elongation of the (-GlcNS-GlcA-) backbones. The ^{3}H-labeled backbones showed similar results (Figure 4B).

Structural characterization of dnLMWH
After the modifications with C$_5$-epi and *O*-sulfotransferases, ESI-MS analysis for dnLMWH products was difficult due to the structural complexity resulting from incomplete *O*-sulfation and epimerization. Instead, disaccharide analysis was used to characterize the structures of the product. To this end, two approaches for cleaving dnLMWH into disaccharide units were employed: an enzymatic digestion and a chemical degradation. Heparin lyases recognize saccharide units carrying sulfo groups at the 2-*O*- and 6-*O*-positions. However, oligosaccharides carrying 3-*O*-sulfo groups are resistant to heparin lyases (Shriver et al. 2000), thus requiring a nitrous acid degradation (Shively and Conrad 1976). ^{35}S-Label was also introduced to facilitate the disaccharide analysis. To analyze the ^{35}S-labeled-2-*O*- and 6-*O*-oligosaccharides, we treated the compound with three

heparin lyases, I, II and III. We observed that the majority of GlcNS residues carried 6-*O*-sulfo groups (Supplementary data, Figure S5A). Furthermore, the content of the IdoA2S found in the corresponding IdoA2S-GlcNS6S unit suggested that the product has similar structure to heparin (Supplementary data, Figure S5B). A critical modification that drives the binding of a pentasaccharide to AT is a 3-*O*-sulfo modification at GlcNS ± 6S within the pentasaccharide (Duncan et al. 2004). We determined AT-binding activity of dnLMWH since it is an initial step prior to FXa inhibition. Moreover, AT-binding activity also suggested the presence of 3-*O*-sulfo modification on dnLMWH (Figure 5A). The knowledge of substrate specificity suggests that 3-OST-1 inserts a sulfo group at GlcNS6S that is adjacent to the GlcA at the reducing end (-GlcA-GlcNS3S* ± 6S-) (Liu et al. 1996; Moon et al. 2012). A disaccharide, GlcA-AnMan3S6S, indicated the presence of a 3-*O*-sulfo group on dnLMWH (Figure 5B); however, the level of 3-O-sulfated disaccharide is higher than expected. Although dnLMWH was designed for a placement of a 3-*O*-sulfo group only at the AT-binding site, it was expected that a mixture of GlcA-GlcNS6S residue outside the AT-binding region, resulted from incomplete C$_5$-epimerization and 2-*O*-sulfation, might be subjected to a 3-*O*-sulfo modification. Further, the results are largely consistent with our previous publication (Xu et al. 2012). In this study, a fully sulfated 15-mer compound that was prepared using similar approaches contained 26% 3-*O*-sulfated disaccharide (GlcA-AnMan3S6S).

Determination of in vitro and ex vivo anti-FXa activity
Heparin achieves its anticoagulant activity by binding to AT, and the complex of heparin and AT inhibits the activities of FXa and FIIa. Although heparin has both anti-FXa and anti-FIIa activities
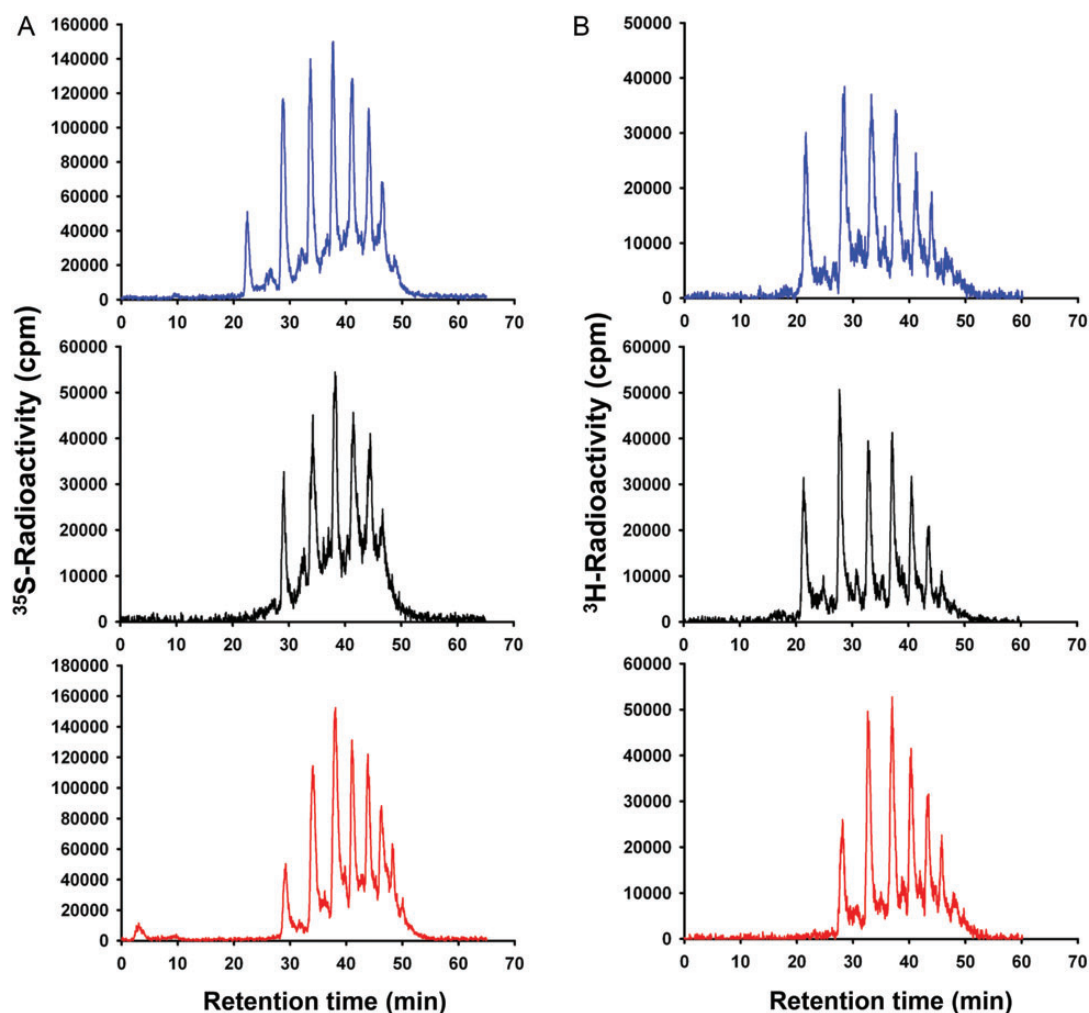
Fig. 4. Reproducibility of the synthesis of *N*-sulfated backbones using one-pot synthesis format. Reproducibility of oligosaccharide size distribution by the one-pot method was assessed by three individual batch syntheses (1 = upper; 2 = middle; 3 = lower panels). The similarity of the distribution profiles between each batch indicates the reproducibility of the method. (**A**) ^{35}S-radioactivity. In the first test, the (-GlcNTFA-GlcA-) backbones from each batch were subjected to *N*-sulfation by using PAP[35]S. (**B**) ^{3}H-radioactivity. In the second test, the (-GlcNS-GlcA-) backbones from each batch were subjected to Glc[^{3}H]NAc elongation. The resultant [35]S- and ^{3}H-labeled oligosaccharides were resolved by anion-exchange (DEAE)-HPLC (A and B, respectively). PAPS, 3′-phosphoadenosine-5′-phosphosulfate, a sulfo donor.

(Petitou et al. 1999), LMWH displays higher activity toward FXa than toward FIIa. Previously, we demonstrated that only those oligosaccharides >19 saccharide residues have anti-FIIa activity (Xu et al. 2012). Since 10 and 12 saccharide units are the major products found in dnLMWH, dnLMWH should exhibit only anti-FXa activity. Thus, we determined the anti-Xa activity using a chromogenic substrate. Enoxaparin was used to compare the activity since its MW_{avg} is similar to dnLMWH. Enoxaparin or dnLWMH was incubated with human AT allowing the AT-dnLMWH complex to inhibit FXa. Indeed, dnLMWH (IC_{50} 40 ng/mL) showed superior anti-FXa activity in comparison with enoxaparin (IC_{50} 96 ng/mL) (Figure 6A). Next, we confirmed that dnLMWH possesses a high potency of anti-FXa activity and anticipated pharmacokinetic profiles when administered to mice. Enoxaparin and dnLWMH were given subcutaneously to the mice at 1.5 mg/kg while phosphate buffer saline (PBS)-treated mice were used as a control group. As demonstrated in Figure 6B, dnLMWH exhibits significantly higher

anti-FXa activity compared with enoxaparin up to 2 h after injection, with a maximum effect observed 30 min after injection. Both the in vitro and ex vivo experiments indicate that dnLMWH displays stronger anticoagulant activity than a commercially available LMWH.

Discussion

LMWH has been used in cardiovascular disorders for almost 30 years. To date, LMWH is clinically favored over UFH due to its superior pharmacokinetic properties. However, among commercial brands, LMWHs differ in polysaccharide size distribution and the active pentasaccharide content (Bisio et al. 2009). The polydispersity of LMWH affects the duration of action, drug potency and clinical safety (Fareed et al. 1998). Therefore, controlling LMWH size distribution is essential for more predictable pharmacological responses. While the current research on
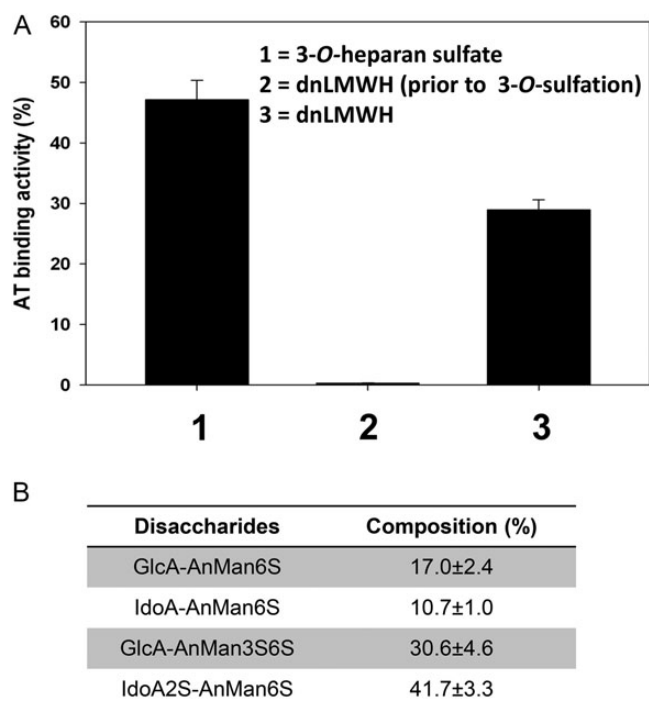
Fig. 5. Investigation of 3-*O*-sulfation on dnLMWH. The AT-binding assay requires a radiolabeled material for a measurement of percent-binding activity to AT (**A**). The ^{35}S-labeled at 3-*O*-postion of HS was used as a positive control (1). ^{35}S-labeled only at 2-*O*- and 6-*O*- positions of dnLMWH was used as a negative control (2). ^{35}S-labeled at 3-*O*-position of dnLMWH was shown in (3). The data presented represent the average of three independent determinations ± SD. Oligosaccharides obtained after *N*-sulfation and GlcNAc elongation steps were ^{35}S-labeled at 2-*O*-sulfo and 6-*O*-sulfo and 3-*O*-sulfo positions. A presence of GlcA-AnManNS3S6S was analyzed by nitrous acid degradation. The resultant disaccharides were analyzed by reverse-phase ion-pairing HPLC. The distribution of the disaccharide units was estimated as a percentage. The percentages of disaccharide are presented as mean ± SD (**B**).

improving LMWH production methods is moving forward, most of the approaches remain focused on obtaining oligosaccharide fragments from UFH derived from animals. Thus, the development of a novel approach that avoids the use of animal sources and obtains a high-potency LMWH is beneficial for clinical applications.

Although a homogenous oligosaccharide product is the ultimate goal of LMWH production, to date, a chemical approach has reached its limit due to the complex structure of LMWH. In this paper, we demonstrated a quick chemoenzymatic method to produce a narrow size distribution oligosaccharide backbone, providing a suitable platform to design an LMWH with high anticoagulant activity. We demonstrated that the degree of polymerization could be controlled by selecting an appropriate starting primer and by tuning the molar ratio of the primer and the UDP sugars. The controlled one-pot oligosaccharide synthesis using a bifunctional polymerase is a feasible approach to produce restricted size distribution oligosaccharides. Heparosan synthases from *P. multocida* were characterized into two isoforms: PmHS1 and PmHS2. Although PmHS1 was found to promote a formation of a monodisperse polysaccharide, the polysaccharide products are in a high-MW range (Sismey-Ragatz et al. 2007; Chavaroche et al. 2011). PmHS2, on the other hand, produces a short-length oligosaccharide that is the targeted size for LMWH production. In addition, PmHS1 lacks the capability to incorporate an unnatural sugar, e.g., UDP-GlcN-TFA (Sismey-Ragatz et al. 2007). Therefore, PmHS2 served as a suitable glycosyltransferase in this synthetic fashion. In a one-pot synthetic method, the presence of a starting primer potentially bypasses the initiation phase of an elongation process by PmHS2, such that the UDP-sugar donors are successively transferred to the acceptor primer. A tetrasaccharide primer contributing to oligosaccharides instead of polysaccharides indicated that a longer saccharide unit is at least required to occupy an acceptor site on PmHS2. We found that this hypothesis is consistent with several reports (Jing and DeAngelis 2004; Sismey-Ragatz et al. 2007; Otto et al.
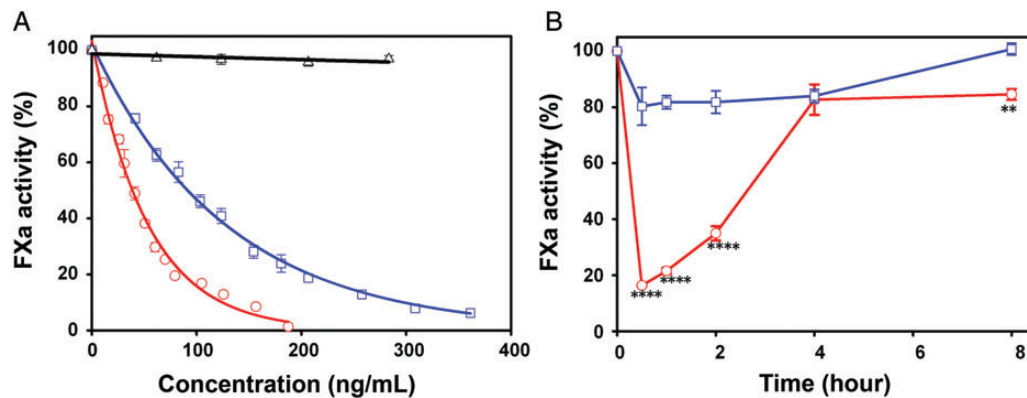

Fig. 6. Determination of anti-FXa activity of dnLMWH. (**A**) In vitro anti-FXa activity determined by the rate of an increase in absorbance of a chromogenic substrate of human FXa at the wavelength of 405 nm (an average from three independent experiments). The IC$_{50}$ was determined relatively to FXa activity when the activity without drug is defined as 100%. Compounds are shown: HS (triangle), enoxaparin (square) and dnLMWH (circle). (**B**) Ex vivo anti-FXa activity of the plasma collected from the mice injected subcutaneously with dnLMWH (red) or enoxaparin (blue) (**$P < 0.05$ and ****$P < 0.0001$; $n = 3$–5 mice for each time point and treatment).

2012). A previous study showed that PmHS2 is likely to catalyze a new chain initiation preferably using UDP-GlcNAc as an acceptor resulting in the generation of endogenous sugar backbones, e.g., GlcA-GlcNAc-UDP (Chavaroche et al. 2011). However, we were unable to observe the endogenous products generated. We found that only even numbered-oligosaccharide products were present. This observation is expected given the fact that PmHS2 catalyzes the transfer of UDP-GlcA at a higher rate than UDP-GlcNAc (Chavaroche et al. 2011; Otto et al. 2012).

Apart from offering an expedited method to produce oligosaccharide backbones, a one-pot synthesis is an easy approach for installation of an AT-binding site. After a backbone synthetic step, the anticoagulation activity can be achieved by five additional modification steps. The method is clearly reproducible in providing products with consistency in size distribution of the oligosaccharide backbones. The anti-Xa activity, demonstrated in vitro and ex vivo, indicated that dnLMWH has a greater anti-Xa potency than Enoxaparin. The minimal heterogeneity as well as the increased proportion of active pentasaccharide permitted a high potency with respect to anti-Xa activity. It is known that 3-O-sulfated decasaccharide or larger are metabolized by the liver (Pempe et al. 2012). dnLMWH might be beneficial in patients in whom synthetic pentasaccharides are contraindicated due to renal insufficiency.

Materials and methods

Preparation of PmHs2 and other HS biosynthetic enzymes

PmHS2 was expressed as an N-terminal fusion to His_6 using PET-15b vector (Novagen) (Sismey-Ragatz et al. 2007). The expression of PmHS2 was carried out in BL21 star (DE3) cells (Invitrogen) coexpressing bacteria chaperone proteins, GroEL and GroES. A total of seven enzymes were used for the synthesis, including N-acetyl-D-glucosaminyl transferase (KfiA), NST, C_5-epi, 2-O-sulfotransferase (2-OST), 6-OST-1/3 and 3-OST-1. All enzymes were expressed in E. coli and purified by appropriate affinity chromatography as described previously (Liu et al. 2010).

Preparation of monosaccharide donors and a sulfate donor

UDP-GlcNAc and UDP-GlcA were purchased from Sigma–Aldrich while UDP-N-trifluoroacetylglucosamine (GlcNTFA) was synthesized by a chemoenzymatic approach as previously described (Liu et al. 2010). In brief, GlcNTFA was generated from a reaction between $GlcNH_2$-1-phosphate (Sigma–Aldrich) and S-ethyl trifluorothioacetate (Sigma–Aldrich). The resultant GlcNTFA was converted to GlcNTFA 1-phosphate using N-acetylhexosamine 1-kinase. The plasmid expressing N-acetylhexosamine 1-kinase was a gift from Prof. Peng Wang, and the expression of the enzyme was carried out in E. coli as reported (Zhao et al. 2010). A coupling of UTP and GlcNTFA 1-phosphate resulting in UDP-GlcNTFA was completed by GlcN-1-phosphate acetyltransferase/N-acetylglucosamine-1-phosphate uridyltransferase (GlmU). UDP-[N-[^{3}H]acetyl]GlcNAc was synthesized by an enzymatic approach as previously described (Chen et al. 2006). A sulfo donor, PAPS, was prepared starting from Na_2SO_4 and ATP (Sigma–Aldrich) using ATP sulfurylase and adenosine phosphokinase (Zhou et al. 2011).

Saccharide primer preparations

A disaccharide precursor, 2,5-anhydromannitol (GlcA-AnMan) was obtained from a chemical depolymerization of heparosan K5 polysaccharide as previously described (O'Leary et al. 2013). A tetrasaccharide primer was initiated from a reaction mixture of GlcA-AnMan (5 mg), UDP-GlcNAc (0.5 mM) and KfiA (20 μg/mL) in a 30 mL buffer containing 25 mM $MnCl_2$ in 50 mM Tris–HCl (pH7.5) at 37°C overnight to obtain a trisaccharide (GlcNAc-GlcA-AnMan). The resultant trisaccharide was purified using 0.75 × 200 mm Bio-Gel P-2 (Bio-Rad, Hercules, CA) that was equilibrated with 0.1 M ammonium bicarbonate (NH_4HCO_3) at a flow rate of 4 mL/h. The fractions were directly subjected to ESI-MS analysis and the fractions containing trisaccharide were pooled. The trisaccharide dissolved in NH_4HCO_3 was then dried (Centrivap, LABCONCO, Kansas City, MO). To elongate a trisaccharide to a tetrasaccharide, the trisaccharide was reconstituted in water and incubated with UDP-GlcA (0.5 mM) PmHS2 (20 μg/mL) in a buffer containing 25 mM $MnCl_2$ in 50 mM Tris–HCl (pH 7.5) at 37°C overnight. The resultant tetrasaccharide (GlcA-GlcNAc-GlcA-AnMan) was purified and pooled as described above. Other oligosaccharide primers were synthesized by sequential elongation of UDP sugars to the tetrasaccharide to obtain a hexasaccharide (GlcA-GlcNAc-GlcA-GlcNAc-GlcA-AnMan) and an octasaccharide (GlcA-GlcNAc-GlcA-GlcNAc-GlcA-GlcNAc-GlcA-AnMan).

Optimization of a one-pot enzymatic reaction using radiolabeled UDP-Glc[^{3}H]NAc

All PmHS2 catalyzed reactions were performed at room temperature (RT) overnight in a 200 μL buffer containing 50 mM Tris, pH 7.5 containing 25 mM $MnCl_2$. An individual reaction mixture consisted of various molar ratios (1:1:1, 1:2:2, 1:4:4 and 1:10:10) between saccharide primers, UDP-GlcNAC and UDP-GlcA. UDP-[^{3}H]GlcNAc (6.7×10^4 cpm/μL) was mixed with UDP-GlcNAc prior adding to the reaction. The oligosaccharide products were purified by 0.75 × 200 cm Bio-Gel P-10 column (Bio-Rad, Hercules, CA) which was equilibrated with a buffer containing 20 mM Tris–HCl buffer (pH 7.5) in 1 M NaCl. The ^{3}H-labeled-oligosaccharide fractions were identified (Packard Tri-Carb 2500 TR) and pooled. Then, it was dialyzed against H_2O and subjected to SAX-HPLC analysis as described below.

SAX-HPLC analysis

The distribution of oligosaccharide backbones was demonstrated using an SAX-HPLC (5 μM × 80 Å × 250 mm × 4.6 mm, Phenomenex, Torrance, CA). The dialyzed ^{3}H-radiolabeled oligosaccharide backbones were eluted with a linear gradient of 0–100% 0.5 M NaCl (pH 3) at a flow rate of 0.5 mL/min in 150 min.

Synthesis of (-GlcA-GlcNTFA-) oligosaccharide backbones

The oligosaccharide backbone synthesis was carried out corresponding to the optimal condition described above. Briefly, a mixture of the tetrasaccharide primer (0.35 mM), UDP-GlcNTFA (1.4 mM) and UDP-GlcA (1.4 mM) were incubated at 1:4:4 molar ratio with PmHS2 (30 μg/mL) at RT overnight.

The reaction was purified using Bio-Gel P-2. Then, the resultant (-GlcA-GlcNTFA-) repeating backbones were subjected to ESI-MS analysis and pooled. The backbones dissolved in NH_4HCO_3 were dried prior to the N-sulfation step.

N-Sulfation of oligosaccharide backbones

N-Sulfation was initiated by detrifluoroacetylation of the GlcN-TFA residue (de-N-TFA). The de-N-TFA step required overnight incubation of a reagent containing 2:2:1 (v/v/v) H_2O, CH_3OH and $(C_2H_5)_3N$ at 50°C in 50 mL. The reaction mixture was dried and reconstituted in water to obtain the resultant -(GlcNH$_2$-GlcA)- backbones. The addition of sulfo groups at the $GlcNH_2$ residue was carried out by incubation of -(GlcNH$_2$-GlcA)- backbones with NST (10 µg/mL) and PAPS (500 µM) at 37°C overnight in a buffer containing 50 mM 2-(N-morpholino ethane sulfuric acid) (MES) (pH 7.4) in a total volume of 40 mL.

MS analysis of oligosaccharide backbones

All MS analyses were carried out in a negative electrospray ionization mode using a Thermo Scientific LCQ-Deca system. The electrospray source was set to 5 kV and 275°C and a syringe pump (Harvard Apparatus) was used to inject the samples via direct infusion (35 µL/min). The oligosaccharide backbones (5 µL) purified from a Bio-Gel P-2 column in each synthetic step were directly diluted in 200 µL of 8:2 MeOH:H_2O. The MS data were acquired and processed using Xcalibur 13 software.

Placement of AT-binding site at the nonreducing end of the (-GlcA-GlcNS-) backbones

To create the AT-binding site within the oligosaccharide backbones, GlcNAc elongation, C_5-epimerization, 2-O-, 6-O- and 3-O-sulfation modifications were required. The GlcNAc elongation was completed by KfiA as described under "Saccharide Primer Preparations". The GlcNAc-(GlcA-GlcNS)- backbones were incubated in a reaction mixture containing 50 mM MES (pH 7.4) C_5-epi (20 µg/mL) and 2 mM $CaCl_2$ in a total volume of 40 mL. After incubation for 30 min at 37°C, 2-OST (20 µg/mL) and 500 µM PAPS were added, and the reaction was incubated overnight at 37°C. The products were purified using a DEAE column as described previously (Xu et al. 2012). The resultant 2-O-sulfated products were dialyzed against water prior to the next sulfation step. 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 (20 µg/mL) and 6-OST-3 (20 µg/mL) in a total volume of 40 mL. For 3-O-sulfation, the substrate was incubated with 3-OST-1 (10 µg/mL) and 500 µM PAPS at 37°C overnight in a reaction mixture containing, 10 mM $MnCl_2$, 5 mM $MgCl_2$ and 50 mM MES in a total volume of 30 mL.

Disaccharide analysis of dnLMWH

The oligosaccharide backbones carrying ^{35}S-sulfo groups at 2-O- and 6-O-positions were exposed to heparin lyase I, II and III (0.1 mg/mL each) in 200 µL of 50 mM sodium phosphate (pH 7) at 37°C. The reaction was terminated by boiling at 100°C for 5 min and was desalted by 0.75 × 200 mm Bio-Gel P-2 gel. The 3-O-sulfated backbones were 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 (Shively and Conrad 1976). The resultant ^{35}S-labeled disaccharides were resolved using a C_{18} reverse-phase column (0.46 × 25 cm; Vydac) under reverse-phase ion-pairing HPLC conditions (Duncan et al. 2006). The identities of the disaccharides were determined by co-elution with the appropriate ^{35}S-labeled disaccharide standards.

Determination of AT-binding activity

Approximately 1×10^4 cpm of each oligosaccharide were incubated with 5 µg of human AT in 50 µL of binding buffer containing 10 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1 mM Mn^{2+}, 1 mM Mg^{2+}, 1 mM Ca^{2+}, 10 µM dextran sulfate, 0.0004% Triton X-100 and 0.02% sodium azide for 30 min at RT, respectively. Concanavalin A-Sepharose gel (Sigma, 200 µL of 1:1 slurry) was then added, and the reaction was shaken at RT for 2 h. The beads were then washed three times with 1 mL of binding buffer, and the amount of ^{35}S-labeled radioactivity was determined by scintillation counting.

In vitro anti-FXa activity

An assay was carried out based on a published method (Chen et al. 2007). In brief, human AT (Cutter Biological) was diluted with PBS containing 1 mg/mL bovine serum albumin (BSA) to give a stock solution at a concentration of 0.4 µM. Human FXa (Enzyme Research Laboratories, South Bend, IN) was diluted with PBS containing 1 mg/mL bovine serum albumin at a concentration of 80 nM. A chromogenic substrate for FXa, S-2765 (Diapharma, West Chester, OH) was dissolved in water at a concentration of 1 mM. HS from bovine kidney, enoxaparin (local pharmacy) and dnLMWH were dissolved in PBS at various concentrations (5–100 ng/µL). The assay was initiated by incubation of the drugs with 80 µL human AT stock solution at RT for 2 min. Then 10 µL of FXa was added. After incubation at RT for 4 min, 30 µL of S-2765 was added. The absorbance at 405 nm was continuously measured for 5 min. The absorbance values were plotted against the reaction time. The initial reaction rates as a function of concentration were used to calculate IC_{50} values. The activity without drug was defined as 100%.

Ex vivo anti-FXa activity

The Institutional Animal Care and Use Committee at UNC approved all animal procedures. Female C57BL/6J mice weighing 20–25 g were purchased from Jackson Laboratory (Bar Harbor, ME). Under anesthesia by isoflurane (2% in oxygen), PBS, enoxaparin (1.5 mg/kg) and dnLMWH (1.5 mg/kg) were subcutaneously administered to the mice. After 30 min, 1, 2, 4 and 8 h, mice were anesthetized and a midline laparotomy was performed. Blood (300–500 µL) from inferior vena cava was collected into syringes containing 3.8% sodium citrate solution at the final blood to sodium citrate volume 9:1. The blood samples were centrifuged at $4000 \times g$ at 4°C for 15 min to obtain mouse plasma. The plasma was frozen at −80°C until the analyses were performed. The plasma containing the drug at each time point was used to determine FXa activity. The FXa activity assay was carried out similar to the in vitro experiment. Briefly, the assay mixture consisted of PBS (80 µL), mouse plasma (10 µL) and 80 nM human FXa (10 µL) was incubated

at RT. After 4 min, S-2765 (30 µL) was then added. FXa activity was calculated as a percent when PBS mice were defined as 100%.

Statistical analysis

All statistical analyses were performed using Sigma Plot Software (version 11; Systat Software, Inc., San Jose, CA). Data are represented as mean ± SEM. For two-group comparison of continuous data, two-tailed Student's *t*-test was used. A *P*-value of ≤0.05 was regarded as significant.

Supplementary data

Supplementary data for this article is available online at http://glycob.oxfordjournals.org/.

Funding

Acknowledgement

Conflict of Interest

None declared.

Abbreviations

AT, antithrombin; C$_5$-epi, C$_5$-epimerase; DEAE, diethylaminoethyl; dp, degree of polymerization; FXa, factor Xa; FIIa, factor IIa; GlcA, glucuronic acid; GlcN, glucosamine; GlcNAc, *N*-acetylglucosamine; GlcNS6S, *N*,6-*O*-sulfo glucosamine; GlcNS3S6S, *N*,3,6-*O*-sulfo glucosamine; Glc-NTFA, *N*-trifluoroacetylglucosamine; HIT, heparin-induced thrombocytopenia; HPLC, high-performance liquid chromatography; HS, heparan sulfate; IdoA, iduronic acid; KfiA, *N*-acetyl glucosaminyl transferase of *E. coli* K5 strain; LMWH, low-molecular-weight heparin; MES, 2-(*N*-morpholino ethane sulfuric acid); NST, *N*-sulfotransferase; PAPS, 3′-phosphoadenosine-5′-phosphosulfate; PBS, phosphate buffer saline; PmHS2, *Pasteurella multocida* heparosan synthase 2; RT, room temperature; SAX, strong anion exchange; UDP, uridine 5′-diphosphate; UFH, unfractionated heparin; 2-OST, 2-*O*-sulfotransferase; 3-OST-1, 3-*O*-sulfotransferase isoform 1; 6-OST-1/3, 6-*O*-sulfotransferase isoform 1/isoform 3.

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