UTILIZING A CHEMoenzymatic APPROACH FOR SYNTHETIC HEPARIN
TARGETING FACTOR XA AND P-SELECTIN

Kasemsiri Chandarajotí

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Approved by:
David Lawrence
Jian Liu
Michael Jarstfer
Nigel Key
Qisheng Zhang
ABSTRACT

KASEMSIRI CHANDARAJOTI: Utilizing a chemoenzymatic approach for synthetic heparin targeting Factor Xa and P-selectin
(Under the direction of Jian Liu, Ph.D.)

Heparin is a natural product composed of a mixture of highly sulfated linear polysaccharides. A unique chemical structure in the heparin chain contributes to its superb biological activity of preventing blood clots; thus, heparin is known as an anticoagulant. Heparin is widely used in various cardiovascular diseases. However, its role in inflammatory prevention and its protective effect in cancer have also been extensively explored.

Commercial heparin is isolated from the mucosal tissue of porcine intestine. Low molecular weight heparin (LMWH), a depolymerized form of heparin, is produced through either chemical or enzymatic depolymerization of heparin. The resulting LMWH fragments are not uniform in their sizes, which affect their pharmacological and pharmacokinetic properties (1) such as anticoagulation potency, metabolic clearance and reversibility to protamine neutralization.

A chemoenzymatic approach has emerged as an alternative method to produce heparin. The method takes the advantage of high regioselectivity of the biosynthetic enzymes that build the polysaccharide chain and selectively add the sulfo groups. In this study, we developed a controlled enzymatic method to produce a narrow distribution of LMWH fragments displaying less polydispersity. Our results indicated that the more uniform LMWH fractions improve the anticoagulation potency both in vitro and ex vivo. Moreover, a well-designed chemoenzymatic method offered a new generation of LMWH possessing the homogeneity and a distinct sulfation.
The homogeneous LMWH significantly improves sensitivity to protamine neutralization demonstrated \textit{in vivo}. Furthermore, the enzymatic synthesis is an effective approach to synthesize the desired size of heparin oligosaccharides that are unattainable by a chemical synthesis. The synthetic heparin was utilized to explore structural requirements for heparin binding to P-selectin, an adhesion protein involved in vascular inflammation. We demonstrated that large size heparin oligosaccharides together with \textit{N}-and \textit{6-O}-sulfo modifications are critical for P-selectin binding.
DEDICATION

To my parents: Kasem Chandarajoti and Pattarin Chandarajoti
In deepest gratitude for their love, support and optimism
ACKNOWLEDGEMENTS

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<th>Definition</th>
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<td>ΔUA</td>
<td>Δ&lt;sup&gt;4,5&lt;/sup&gt;-unsaturated uronic acid</td>
</tr>
<tr>
<td>2-OST</td>
<td>2-&lt;i&gt;O&lt;/i&gt;-sulfotransferase</td>
</tr>
<tr>
<td>3-OST</td>
<td>3-&lt;i&gt;O&lt;/i&gt;-sulfotransferase</td>
</tr>
<tr>
<td>6-OST</td>
<td>6-&lt;i&gt;O&lt;/i&gt;-sulfotransferase</td>
</tr>
<tr>
<td>AnMan</td>
<td>2,5-anhydromannitol</td>
</tr>
<tr>
<td>AT</td>
<td>Antithrombin</td>
</tr>
<tr>
<td>C5-epi</td>
<td>C&lt;sub&gt;5&lt;/sub&gt;-epimerase</td>
</tr>
<tr>
<td>DEAE</td>
<td>Diethylaminoethyl</td>
</tr>
<tr>
<td>dp</td>
<td>Degree of polymerization</td>
</tr>
<tr>
<td>EPS</td>
<td>Epimerization site</td>
</tr>
<tr>
<td>ESI-MS</td>
<td>Electrospray ionization mass spectrometry</td>
</tr>
<tr>
<td>EXT</td>
<td>Exostosin</td>
</tr>
<tr>
<td>FGF</td>
<td>Fibroblast Growth Factor</td>
</tr>
<tr>
<td>FIIa</td>
<td>Factor IIa, thrombin</td>
</tr>
<tr>
<td>FXa</td>
<td>Factor Xa</td>
</tr>
<tr>
<td>GAG</td>
<td>Glycoaminoglycan</td>
</tr>
<tr>
<td>GlcA</td>
<td>Glucuronic acid</td>
</tr>
<tr>
<td>GlcN</td>
<td>Glucosamine</td>
</tr>
<tr>
<td>GlcNAc</td>
<td>N-acetylglucosamine</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>GlcNAc6S</td>
<td>N-acetylated, 6-O-sulfated glucosamine</td>
</tr>
<tr>
<td>GlcNH₂</td>
<td>Unsubstituted glucosamine</td>
</tr>
<tr>
<td>GlcNS</td>
<td>N-sulfated-glucosamine</td>
</tr>
<tr>
<td>GlcNS6S</td>
<td>N-sulfated, 6-O-sulfated glucosamine</td>
</tr>
<tr>
<td>GlmU</td>
<td>Glucosamine-1-phosphate acetyltransferase/N-acetylglicosamine-1-phosphate uridyl transferase</td>
</tr>
<tr>
<td>GTase</td>
<td>Glycosyltransferase</td>
</tr>
<tr>
<td>HPLC</td>
<td>High performance liquid chromatography</td>
</tr>
<tr>
<td>HS</td>
<td>Heparan sulfate</td>
</tr>
<tr>
<td>IdoA</td>
<td>Iduronic acid</td>
</tr>
<tr>
<td>IdoA2S</td>
<td>2-O-sulfated-iduronic acid</td>
</tr>
<tr>
<td>IVC</td>
<td>Inferior vena cava</td>
</tr>
<tr>
<td>KfiA</td>
<td>N-acetyl glucosaminyl transferase</td>
</tr>
<tr>
<td>K5P</td>
<td>K5 polysaccharide</td>
</tr>
<tr>
<td>K5P-NS</td>
<td>N-sulfated-K5 polysaccharide</td>
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<td>K5P-NS6S</td>
<td>N-sulfated, 6-sulfated-K5 polysaccharide</td>
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<tr>
<td>K5P-NS2S6S</td>
<td>N-sulfated, 2-sulfated, 6-sulfated-K5 polysaccharide</td>
</tr>
<tr>
<td>K5P-NS3S6S</td>
<td>N-sulfated, 3-sulfated, 6-sulfated-K5 polysaccharide</td>
</tr>
<tr>
<td>LMWH</td>
<td>Low molecular weight heparin</td>
</tr>
<tr>
<td>MRRS</td>
<td>Mode of reaction recognition site</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>MWCO</td>
<td>Molecular weight cut-off</td>
</tr>
<tr>
<td>NDST</td>
<td>N-deacetylase/N-sulfotransferase</td>
</tr>
<tr>
<td>NST</td>
<td>N-sulfotransferase</td>
</tr>
<tr>
<td>OPD</td>
<td>ortho-phenylenediamine dihydrochloride</td>
</tr>
<tr>
<td>PAMN-HPLC</td>
<td>Polyamine based high performance liquid chromatography</td>
</tr>
<tr>
<td>PAPS</td>
<td>3′-phosphoadenosine 5′-phosphosulfate</td>
</tr>
<tr>
<td>PmHS2</td>
<td>Pasteurella mutocida heparosan synthase 2</td>
</tr>
<tr>
<td>pNP</td>
<td>para-nitrophenol</td>
</tr>
<tr>
<td>PSGL-1</td>
<td>P-selectin glycoprotein ligand 1</td>
</tr>
<tr>
<td>RPIP-HPLC</td>
<td>Reverse phase ion-pairing high performance liquid chromatography</td>
</tr>
<tr>
<td>RBC</td>
<td>Red blood cell</td>
</tr>
<tr>
<td>SCD</td>
<td>Sickle cell disease</td>
</tr>
<tr>
<td>SLeX</td>
<td>Sialyl lewis X</td>
</tr>
<tr>
<td>SLeX-PAA</td>
<td>Sialyl lewis X-polyacrylamide</td>
</tr>
<tr>
<td>UFH</td>
<td>Unfractionated heparin</td>
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</table>
CHAPTER I
INTRODUCTION

The discovery of heparin and its characterization

Heparin is a powerful blood anticoagulant that has been clinically used for almost a century. Although the development of novel anticoagulation agents has been highly successful, heparin remains the drug of choice in several medical circumstances (2). Heparin was unintentionally discovered by Jay McLean in collaboration with William Howell in 1916 during the search for a procoagulant (3). Heparin was named corresponding to the liver, the organ from which it was isolated. For twenty to thirty years after the discovery, the isolation techniques and the production of heparin for therapeutic applications were gradually improved. To date, the sources of animal tissue for heparin manufacturing are porcine intestine and bovine lung (4).

Heparin is a highly sulfated linear polysaccharide with a disaccharide repeating unit of glucosamine (GlcN) and uronic acids, including glucuronic acid (GlcA) or iduronic acid (IdoA). The disaccharide unit is capable of carrying sulfo groups. The GlcN residue can be sulfated at the N-, 3-O- and 6-O- positions while GlcA and IdoA only carry a sulfo group at the 2-O-postion (Figure 1). Due to the presence of an amino sugar linked to a uronic acid in a polymeric form, heparin is categorized as a member of the glycosaminoglycans (GAGs) family. Other members of the GAG family are heparan sulfate (HS), chondroitin sulfate, dermatan sulfate and keratan sulfate (5). The disaccharide building blocks contained in the GAG chains determine the types of GAGs. GAGs are widely presented on the cell surface, inside the cell and in the extracellular
matrix. Typically, one or more of the GAG chains are covalently linked to a core protein through a tetrasaccharide linkage. GAG chains tethered to a core protein are known as proteoglycans, molecules that exist throughout the animal kingdom. The diverse biological functions of GAGs mainly result from the interaction between biological proteins and GAGs due to their highly negative charge. Among the GAG members, heparin and HS share the same biosynthesis. HS and heparin participate in numerous biological processes, including embryonic development, assisting viral and bacterial infections and regulating blood coagulation. The sulfation patterns and the location of IdoA residues play critical roles in determining HS functions (6).

![Image](image.png)

**Figure 1** Disaccharide repeating unit of HS and heparin

Sulfations (R=SO₃⁻) at Carbon 6 (known as 6-O-sulfo-glucosamine) of the glucosamine is common. Sulfation at Carbon 2 of iduronic acid (known as 2-O-sulfo iduronic acid) is common. Sulfation at Carbon 3 of glucosamine (known as 3-O-sulfglucosamine) is rare. Both N-acetyl (R’ = acetyl, GlcNAc) and N-sulfo (R’=SO₃⁻, GlcNS) glucosamine is common. N-unsubstituted glucosamine (R’ = -H, GlcNH₂) is a low abundance component.
Both heparan sulfate (HS) (A) and heparin (B) consist of alternating units of glucosamine (GlcN) and hexuronic; glucuronic acid (GlcA) or iduronic acid (IdoA). HS contains a greater proportion of GlcA, whereas heparin contains more IdoA. Both molecules are sulfated (depicted by blue circles). However, heparin is more extensively sulfated than HS. HS tends to exist as proteoglycan components by tethering to a core protein. HS is expressed on the cell surface, including those of the vascular endothelium and of circulating leukocytes, which provides a general net negative charge to these surfaces (depicted by white circles). Heparin, by contrast, is co-released with histamine from degranulating mast cells, and can dissociate from its protein core to exist as free GAG chains. Picture adapted from (7).

**HS vs. Heparin**

Although HS and heparin have very similar structures, the two GAGs differ in several aspects. While HS is ubiquitously found on most cells within the body, heparin is only synthesized and stored in mast cells (Figure 2). HS is commonly made up of two domains: a highly sulfated region (NS) and lowly sulfated region (NAc). The NS domain provides binding sites for protein ligands such as antithrombin, fibroblast growth factor (FGF) and FGF receptor (5). Heparin carries more sulfo groups and contains a higher content of IdoA than HS. More than 80% of the disaccharide units composed of heparin are -IdoA2S-GlcNS6S- (8). Thus, heparin maintains approximately 2.6 sulfo groups per disaccharide while HS only maintains 0.8 (9).
Heparin in a coagulation cascade

Human blood coagulation is controlled by a process in which a series of serine proteases are consecutively activated to form a fibrin clot (Figure 3). Antithrombin (AT) is an endogenous inhibitor in a coagulation cascade. AT primarily upregulates factor Xa (FXa) and thrombin (Factor IIa), resulting in the indirect inactivation of other proteases that in turn prevent the clot formation. Heparin carries a unique pentasaccharide motif, -GlcNAc6S-GlcA-GlcNS3S±6S-IdoA2S-GlcNS6S-, within its polysaccharide chain (Figure 4). The special pentasaccharide domain induces a conformation change in AT upon binding, thereby accelerating the inactivation rate about 1000-fold. The anticoagulation effect (anti-FXa) is dependent on the presence of a specific pentasaccharide enabling binding to AT, thus potentiating its effect. The anti-IIa effect requires the specific pentasaccharide to bind to AT as well as a minimum heparin chain length of 19 saccharides to simultaneously bind to thrombin (10).

Figure 3 Role of heparin in a coagulation cascade
Heparin mainly modulates blood coagulation through inhibition of the serine-protease factor Xa and thrombin through antithrombin, a natural inhibitor in a coagulation cascade. The blood coagulation cascade consists of inactive serine proteases that need to be activated in order to activate other proteases that are linked throughout the system. The pathway is divided into two pathways: intrinsic and extrinsic pathways. The two pathways converge at factor Xa.
Commercial heparins are categorized into three forms corresponding to their average molecular weights: unfractionated heparin (UFH, $\text{MW}_{\text{avg}} \sim 14,000$), low molecular weight heparin (LMWH, $\text{MW}_{\text{avg}} \sim 3,500-6,000$), and the synthetic pentasaccharide, fondaparinux (MW 1508.3) (11). UFH is extracted from mucosal tissues of animals, currently obtained from porcine intestine and bovine lung. LMWH is derived from UFH by chemical and enzymatic depolymerization, resulting in polysaccharide fragments (4). UFH and LMWH are obtained from natural sources, but the pentasaccharide, fondaparinux, is chemically synthesized.

The three forms of heparin differ in their pharmacokinetic profiles, providing different choices for cardiovascular treatments (12) (Table 1). 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 (13). 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 its predictable bioavailability by subcutaneous administration, a longer half-life in plasma, and lower incidences of HIT and bleeding (12). All of these advantages make LMWH useful for outpatient treatment and prophylaxis of venous thromboembolism, acute myocardial
infarction and unstable angina. The advantage of the synthetic pentasaccharide is a decreased risk of HIT. However, it requires renal excretion. Thus, the pentasaccharide is not recommended for patients with impaired renal function. Currently, LMWH is the most commonly prescribed form of heparin (14).

A drawback in heparin manufacturing method

The annual worldwide consumption of crude heparin is over 200 tons, and sales revenues are in excess of $5 billion per year (15, 16). For several decades, commercial heparins have been obtained from mucous tissues, e.g., pork intestine and bovine lung. The animal-derived heparins require the extraction of polysaccharide from mucous tissues and several steps of purification to obtain the active pharmaceutical ingredient, a purified UFH. China is the worldwide leader in crude heparin production (17).

Table 1 Pharmacokinetic properties in each form of heparin

<table>
<thead>
<tr>
<th>Pharmaceutical properties</th>
<th>Unfractionated heparin (UFH)</th>
<th>Low molecular weight heparin (LMWH)</th>
<th>Synthetic pentasaccharide Fondaparinux</th>
</tr>
</thead>
<tbody>
<tr>
<td>MW&lt;sub&gt;avg&lt;/sub&gt;</td>
<td>~ 14000</td>
<td>~ 3,500-6,000</td>
<td>~ 1508.3</td>
</tr>
<tr>
<td>Indication</td>
<td>surgery, kidney dialysis</td>
<td>deep vein thrombosis (DVT), pulmonary embolism (PE)</td>
<td>Surgery, VTE, PE</td>
</tr>
<tr>
<td>Route of administration</td>
<td>intravenous</td>
<td>subcutaneous</td>
<td>subcutaneous</td>
</tr>
<tr>
<td>Half life</td>
<td>0.5-1 hour</td>
<td>5-6 hours</td>
<td>12-15 hours</td>
</tr>
<tr>
<td>Side Effect</td>
<td>bleeding, HIT (high)</td>
<td>bleeding, HIT (low)</td>
<td>bleeding</td>
</tr>
<tr>
<td>Antidote</td>
<td>protamine</td>
<td>protamine (partial)</td>
<td>no</td>
</tr>
<tr>
<td>Elimination</td>
<td>liver</td>
<td>kidney</td>
<td>kidney</td>
</tr>
<tr>
<td>Renal insufficiency</td>
<td>yes</td>
<td>dose adjustment</td>
<td>contraindication</td>
</tr>
</tbody>
</table>
Typically, the extraction process involves boiling pig intestines, collecting mucosal membranes and then drying them to obtain the crude heparin extracts. Next, the crude heparin extracts are distributed to local heparin consolidator companies, where the heparin extracts are combined for further purification and formulation as an injectable drug by foreign manufacturing companies (18). Oversulfated chondroitin sulfate (OSCS) is a synthetic material that is intentionally made to substitute the crude heparin to cut the production costs (19). During 2007-2008, OSCS caused allergic reactions and death in over a hundred dialysis patients receiving heparin (20, 21). The worldwide contamination of heparin indicated that heparin is vulnerable to contamination and adulteration due to its long and poorly regulated supply chain. The unsecured heparin supply chain has raised concerns over the safety and reliability of animal-sourced heparin and LMWH. Furthermore, an investigation on LMWH prepared from contaminated UFH revealed that the current manufacturing methods to prepare LMWH failed to eliminate OSCS contamination (22). Recently, a demand for a synthetic form of LMWH has emerged. Although the synthetic pentasaccharide is produced using a purely chemical approach, it is extremely difficult to employ this method to prepare LMWH because of its larger molecular size. Therefore, it is necessary to find alternative sources for the production of pharmaceutical heparins to meet the multiple tons of heparin demanded annually.

**Shortcomings of LMWH production**

LMWH has a more attractive pharmacokinetic profile than UFH (Table 1) and potentially has a wider range of clinical applications, such as a long-term prophylaxis of venous thromboembolism (2). LMWH is derived from UFH through chemical or enzymatic depolymerization (4). Depending on the method of depolymerization, products with different molecular weights and different pharmacological profiles are obtained. Commercially available
LWMH has a broad oligosaccharide size distribution with component molecular weights ranging from 3,500-6,000, leading to complex pharmacological/pharmacokinetic effects (1). The polydispersity in the chain length may affect metabolic clearance because short oligosaccharides require kidney clearance while large polysaccharides are excreted by the liver (23, 24). Therefore, dose adjustment is suggested prior to the administration of LMWH to kidney-impaired patients (25). The major populations to whom LMWH is prescribed are elderly or cardiovascular patients. These groups of patients tend to have kidney complications as part of their pathophysiological symptoms. Thus, those patients tend to have less benefit from LMWH, even as a first line drug (26, 27).

Furthermore, different approaches to the manufacture of LMWH result in various product profiles across the LMWH brands, with varying biochemical and pharmacological properties. The three brands of LMWHs currently available in the U.S. are obtained from different UFH depolymerization methods, resulting in different MW distributions. For example, enoxaparin (MW\text{avg} \sim 4500) is produced from benzylation followed by alkaline hydrolysis; dalteparin (MW\text{avg} \sim 6000) is derived from controlled nitrous acid depolymerization; and tinzaparin (MW\text{avg} \sim 6500) is prepared by controlled heparinase digestion (14). The U.S. FDA indicates that none of these product brands is interchangeable (28). 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 (29).

In addition to the favorable pharmacokinetic properties of LMWH over UFH, the risk of bleeding associated with LMWH (1.4%) is lower than that of UFH (2.3%) (30). However, protamine, an antidote approved for neutralizing heparins, can only partially reverse the anticoagulant activity of LMWH (31, 32). Because the interaction between protamine and
heparin is electrostatic, UFH is efficiently neutralized by protamine due to its overall highly negative charge in comparison to LMWH (33). Importantly, the polydispersity of LMWH affects the sensitivity to protamine because short chain oligosaccharides are not neutralized by protamine and remain active toward FXa (34). The shortcomings of LMWH production stemming from its manufacturing methods has provoked researchers to search for a production method that can produce a safer LMWH.

**Biosynthetic pathway of HS and heparin**

The biosynthetic pathway of HS and heparin occurs in the Golgi apparatus and involves a series of specialized enzymes (5). Generally, the pathway consists of three phases: chain initiation, chain polymerization and chain modification. The initiation phase forms a tetrasaccharide linkage that tethers to a serine residue of a core protein (Figure 5). The linkage region is initiated by the elongation of a xylose residue followed by two galactose residues and a GlcA residue, respectively (9). Next, the polymerization takes place, forming of a linear copolymer of GlcNAc and GlcA. This unmodified polysaccharide backbone undergoes chain modification by a series of sulfo modifications and epimerization.

HS biosynthetic enzymes include polymerases, epimerase and sulfotransferases (Figure 6). Two HS polymerases (encoded by exostosin gene), also known as EXT1 and EXT2, are responsible for building the non-sulfated and non-epimerized backbone polysaccharide consisting of the disaccharide repeating unit of GlcA-GlcNAc, where GlcNAc presents N-acetylated glucosamine (35). Thus, EXTs belong to the glycosyl transferase family. N-deacetylation/N-sulfotransferase (NDST) is a dual function enzyme: it removes an acetyl group from a GlcNAc residue, displaying deacetylase activity (NDase), then transfers a sulfo group to the deacetylated glucosamine residue to form an N-sulfo glucosamine (GlcNS) residue.
displaying N-sulfotransferase activity (NST). C₅-epimerase (C₅-Epi) converts a GlcA residue to an IdoA residue. Various sulfotransferases co-functioning with a sulfate donor, 3’-phosphoadenosine-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 glucosamine residues, respectively (Figure 5).

Figure 5 Biosynthetic pathway of HS and heparin
The biosynthesis of HS starts from a copolymerization of N-acetylglucosamine and glucuronic acid from a core protein by polymerase enzyme (EXT1 and EXT2). As the chains polymerize, they undergo a series of modification reactions catalyzed by at least four families of sulfotransferases and one epimerase. N-deacetylase/N-sulfotransferases (NDST) acts on a subset of N-acetylglucosamine (GlcNAc) residues to generate N-sulfated glucosamine units, some of which occur in clusters along the chain. Generally, the enzyme deacetylates N-acetylglucosamine and rapidly adds sulfate to the free amino group to form GlcNSO₃. An C₅-epimerase then acts on glucuronic acid (GlcA) residues immediately adjacent to and toward the reducing side of N-sulfated glucosamine units, followed by 2-O sulfation of the iduronic acid units generated. Next, a 6-O-sulfotransferase adds sulfate groups to the selected glucosamine residues. Finally, the sulfated sugar residues provide targets for a 3-O-sulfotransferase.
The HS biosynthetic enzymes have been extensively studied and characterized to apply their specificity toward oligosaccharides in the \textit{in vitro} synthesis of heparin. Among HS biosynthetic enzymes, NDST is a key player in the biosynthesis because the first modification step is N-sulfation, which is proven to be a critical step for subsequent modification (Figure 6). As stated earlier, NDST displays bifunctional catalytic activity. N-deacetylase is responsible for removing an acetyl group, resulting in a free amino group at GlcN. The NST domain then catalyzes the addition of a sulfo group. There are four isoforms of NDST (36). NDST-1 is found in most cells across the body, suggesting that NDST-1 is involved in HS synthesis. NDST-2, on the other hand, is found only in mast cells, implicating its role in the synthesis of heparin. NDST-3 and NDST-4 have been found only in adult brain and fetal tissues and seem to have a very narrow functionality (36-38). Currently, none of the NDST isoforms have been used in the \textit{in vitro} synthesis of heparin.

After N-sulfation, C$_5$-epimerase (C$_5$-epi) following by 2-O-sulfation take place (Figure 6). C$_5$-epi converts the D-glucuronic acid (GlcA) to L-iduronic acid (IdoA) by altering the configuration of Carbon 5 (39). The reaction proceeds through a putative cabanion intermediate in which a proton is removed from the C$_5$-position and added back to the ring, resulting in potential ring interconversion (39, 40) (Figure 8A). The presence and the location of IdoA in the HS chain dictates its biological functions (6). 2-OST catalyzes the transfer of a sulfo group to the 2-O-position of either IdoA or GlcA (Figure 6), but preferentially to the IdoA residue (41). C$_5$-epi and 2-OST each has only one isoform in the human genome.

The addition of a sulfo group at the 6-OH of GlcN is modified by 6-OSTs (Figure 6). Three isoforms of 6-OSTs have been identified (42). 6-OST recognizes and adds a sulfo groups on both GlcNAc and GlcNS to form GlcNAc6S and GlcNS6S, respectively. The substrate
specificity between the three isoforms seems to be the same (42, 43). A combination of 6-OST-1 and 6-OST-3 has been employed in the in vitro synthesis of heparin.

Another critical player in the HS backbone modification is 3-OSTs. There are 7 isoforms of 3-OSTs, including 3-OST-1, 3-OST-2, 3-OST-3A, 3-OST-3B, 3-OST-5 and 3-OST-6 (44-46). Each isoform catalyzes the addition of a sulfo group at the 3-OH of GlcN (Figure 6). However, the catalytic activity of each isoform depends on the conformation and sulfation state of the uronic acid flanking the non-reducing end of the GlcN (47). Typically, a modification at 3-OH of GlcN is rare. The abundance of 3-O-sulfation is found more on the heparin chain than HS (5). The presence of 3-O-sulfation is critical for the biological effect of HS and heparin. For example, oligosaccharides modified by 3-OST-1 are known to have anticoagulant activity. Based on their ease of production in E. coli, 3-OST-1, -3 and -5 are most commonly used in chemoenzymatic synthesis. The substrate specificity of 3-OSTs has been extensively studied. 3-OST-1 recognizes GlcA or IdoA; thus, the adjacent GlcNS is modified by 3-OST-1. 3-OST-5 can sulfate GlcNS that is flanked by IdoA2S or GlcA or IdoA. The remaining 3-OST-2, -3A, -3B, -4 and -6 recognize IdoA2S and add a sulfo group at GlcNS or GlcNH₂ (48). The knowledge of enzyme specificity aids in designing heparin structures with desired biological functions.
Figure 6 Enzymes involved in the biosynthesis of HS and heparin.

Panel A shows the elongation reactions to prepare a nonsulfated and unepimerized saccharide backbone. Both EXT1 and EXT2 enzymes are involved in the elongation reactions. Panel B shows the reactions involved in polysaccharide modifications. The sites of reactions are colored in red. Abbreviations: NDST, N-deacetylase/N-sulfotransferase; C₅-epi, C₅-epimerase; 2-OST, 2-O-sulfotransferase; 6-OST, 6-O-sulfotransferase; 3-OST, 3-O-sulfotransferase; PAPS, 3′-phosphoadenosine 5′-phosphosulfate.

**Utilizing a chemoenzymatic approach in synthetic heparin**

A number of studies on the biosynthesis of natural carbohydrates provide a platform for synthesizing complex carbohydrates that are not achievable by chemical syntheses. As a result, the synthesis has been improved significantly in regard to scale, purity and structural diversity. Advancement in the understanding of the heparin and HS biosynthetic pathway provides an opportunity to develop a new method to manufacture heparin using a chemoenzymatic approach.
This method employs HS biosynthetic enzymes, including HS polymerases, an epimerase and various sulfotransferases. The regioselectivities of the HS biosynthetic enzymes are very high, offering the opportunity to conduct the enzymatic synthesis of HS. Up to now, the chemoenzymatic method has been employed to synthesize 21 heparin oligosaccharides (10). The most representative example was to utilize this approach to synthesize an ultra-low molecular weight (ULMW) heparin construct, a heptasaccharide that has a very similar structure to the synthetic pentasaccharide, fondaparinux. Fondaparinux is currently synthesized chemically through 50 steps with the overall yield close to 0.1%. Xu and colleagues described a chemoenzymatic synthesis that shortened the synthesis to about 10 steps. The entire chemoenzymatic synthesis includes backbone elongation and saccharide modifications. After ten steps, ULMW Heparin was obtained at 45% overall yield (49).

The critical reagents for the enzymatic synthesis are the enzymes. The chemoenzymatic syntheses have evolved, using engineered enzymes and unnatural cofactors to complete the process. To date, a majority of these biosynthetic enzymes have been expressed in E. coli with some exceptions (9). EXTs and NDST enzymes cannot be expressed in E. coli. For this reason, bacterial glycosyltransferases and the N-sulfotransferase domain of NDST were used for the enzymatic synthesis of HS (50, 51).

**Strategy for controlled chemoenzymatic synthesis**

Although chemoenzymatic synthesis is an excellent way to synthesize heparin and HS oligosaccharides, understanding the substrate specificity of each HS biosynthetic enzyme used in the synthesis is critically important to conduct a successful synthesis. Several of the crystal structures of the HS biosynthetic enzymes are available, providing structural information to understand the substrate specificity of the enzymes. This information also provides the
opportunity to alter the substrate specificity to synthesize the HS oligosaccharides that cannot be achieved by the wild-type proteins(52). In addition, the order of the enzymatic modifications can significantly reduce the side-products and improve the yield. It should be noted that the sequence of the enzymatic modification order can be different depending on the structure of the target compound. So far, the procedures for the synthesis of HS oligosaccharides with different structures are still under development.

Bacterial glycosyltransferases as alternatives to EXT1 and EXT2

Glycosyltransferases (GTases) transfer a saccharide donor to a specific acceptor by utilizing a nucleotide sugar. To date, several mammalian GTases have been extensively studied, including glucosaminyltransferase and glucuronyltransferase. The difficulties in E.coli expression of these mammalian GTases have drawn interest in identifying bacterial GTase counterparts. Genetic engineering of these bacterial GTases has made large-scale syntheses of desired carbohydrate-containing products possible.

Due to limitations expressing EXTs in the bacterial system, the search for bacterial GTases that can polymerize the oligosaccharide backbone has emerged. The bacteria producing capsular polysaccharides were investigated for the possibility of applying their GTases in the enzymatic synthesis. The E. coli K5 strain and Pasteurella multocida (P. multocida) are capsular bacteria whose polysaccharide chains have a similar disaccharide repeating unit to that found in the unmodified chain of HS and heparin. Currently, two bacterial GTases are employed in heparin synthesis. The N-acetyl glucosaminyl transferase from E. coli K5 (KfiA) (51) is used to transfer the GlcN residue, while Pasteurella multocida heparosan synthase 2 (PmHS2) (50) is used to transfer the GlcA residue (Figure 6A).
Typically, GTase is known for its one enzyme-one-linkage property. However, PmHS2 has two independent catalytic sites for UDP-GlcA and UDP-GlcNAc (50). Therefore, PmHs2 can simultaneously elongate the sugar residues to form polysaccharide backbones. However, PmHS2 catalyzes the transfer of GlcA at a higher rate than that of GlcNAc (53, 54). The discovery of these two heparosan synthases has made the synthesis of unmodified heparin oligosaccharides possible.

Unnatural UDP-sugar analogs

The high cost of saccharide substrates and the inavailability of their derivatives due to their difficult syntheses hinder large-scale production in heparin synthesis. The development of unnatural nucleotide analogs that can be recognized by bacterial glycosyltransferases is beneficial for large-scale carbohydrate production. Masuko and co-workers developed a library of the unnatural UDP-hexosamine analogs by utilizing a chemoenzymatic synthesis (55). The synthesis employed N-acetylglucosamine-1-phosphate uridyltransferase (GlmU) to catalyze a reaction between UTP and GlcNAc, resulting in UDP-GlcNAc analogs. These analogs were tested for their incorporation into a polysaccharide by PmHSs. This study demonstrated the feasibility of using unnatural nucleotide sugars to synthesize polysaccharides. The most useful substrate produced by GlmU that is currently being used to synthesize HS/heparin is UDP-N-acetyltrifluoroglucosamine (UDP-N-TFA) (10, 23, 34, 49, 56).

N-sulfation

The formation of N-sulfo glucosamine (GlcNS) is an initial step in the biosynthesis of HS (Figure 6B), suggesting its important role in directing the subsequent modifications to form a highly sulfated product. In cells, N-deacetylase/N-sulfotransferase (NDST) is used to convert a GlcNAc residue to a GlcNS residue (Figure 6B). However, NDST is not suitable for
chemoenzymatic synthesis for two reasons. First, NDST is a large protein with more than 800 amino acid residues, and it is difficult to express in E. coli in high levels. Second, the conversion of GlcNAc to GlcNS by NDST has a special pattern, depending on the isoform (57). The use of NDST does not have the freedom to specifically position the GlcNS residue in an oligosaccharide product.

Currently, the introduction of an N-sulfo group is accomplished by using KfiA coupled with an unnatural UDP-monosaccharide, UDP-GlcNFTA (Figure 7). KfiA recognizes UDP- N-acetyl glucosaminyl transferase GlcN-TFA (56) as a substrate and transfers the GlcN-TFA to the acceptor. In addition, the GlcN-TFA residue at the nonreducing end of the acceptor substrate is also an excellent substrate for pmHS2 to transfer a GlcA residue. Using this process, one can synthesize an oligosaccharide with a -GlcA-GlcN-TFA- disaccharide repeating unit. The conversion of NTFA to N-SO₃H is readily accomplished by a chemical detrifluoroacylation followed by N-sulfation using N-sulfotransferase. It should be noted that NST is the C-terminal of NDST, consisting of only the N-sulfotransferase domain. The size of NST is only 260 amino acid residues, and it is expressed in E. coli at a very high level (58). This method is highly effective to build oligosaccharides with a designed distribution of GlcNAc/GlcNS at sizes of up to a nonadecasaccharide (19 saccharide residues long) (10).
An unnatural UDP-monosaccharide, UDP-GlcN-TFA, is the synthesis. The GlcN-TFA is incorporated into the oligosaccharide acceptor by KfiA. PmHS2 enzyme further elongates the acceptor by transferring another GlcA residue. The resultant oligosaccharide is exposed to a mild basic condition to remove the trifluoroacetyl group. The resultant unsubstituted glucosamine residues are sulfated by N-sulfotransferase (NST).

C5-epimerization

The C5-epimerization step converts a GlcA to an IdoA residue under the catalysis of C5-epimerase (C5-epi). In this reaction, C5-epi deprotonates at the C5 position of the GlcA residue to form a putative carbanion intermediate (Figure 8A). C5-epi is a unique enzyme that serves as a two-way catalyst; C5-epi converts a GlcA residue to an IdoA residue and also converts an IdoA residue to a GlcA residue. The N-sulfation at the nonreducing end of the GlcA residue, also known as the epimerization site, is required for the action of C5-epi (Figure 8B). The two-way catalytic property of C5-epi has complicated the process of installing IdoA using a chemoenzymatic method. Indeed, the attempt to synthesize oligosaccharides containing multiple IdoA residues resulted in a complex mixture (10, 23, 59). So far, introducing several IdoA residues in a structurally defined oligosaccharide by the enzymatic approach remains a challenge.
Figure 8 Substrate specificity of C\textsubscript{5}-epi
Panel A shows the reaction catalyzed by C\textsubscript{5}-epi. C\textsubscript{5}-epi removes the proton from C\textsubscript{5} of the GlcA residue to form a putative carbanion intermediate. Conversely, C\textsubscript{5}-epi can catalyze the reverse reaction, namely to convert an IdoA residue to a GlcA residue. Panel B shows the reaction mode of C\textsubscript{5}-epi in response to different octasaccharides. The designated epimerization site (EPS) is at the residue 0. The residue -1 must be a GlcNS residue to serve as an EPS. If residue -1 is a GlcNAc residue, the octasaccharide is not reactive to C\textsubscript{5}-epi (see reaction e). The N-substitution status at the mode of reaction recognition site (MRRS) determines the mode of reaction of C\textsubscript{5}-epi. If the MRRS site (residue -3) is a GlcNS or GlcNH\textsubscript{2} residue or unoccupied, C\textsubscript{5}-epi displays a reversible reaction mode (see reaction a, c and b). If a GlcNAc residue is at the MRRS site, C\textsubscript{5}-epi displays an irreversible reaction mode (see reaction d). The grey shaded box indicates the pentasaccharide domain recognized by C\textsubscript{5}-epi. Both Panel A and B are adapted from a previously published paper by Sheng and colleagues (60).
A recent study of C₅-epi reveals a very interesting aspect of its substrate specificity. In this study (60), Sheng and colleagues discovered that C₅-epi can display both reversible and irreversible catalytic modes by recognizing the structure of a pentasaccharide domain, designated as the C₅-epi recognition pentasaccharide (Figure 8B). This pentasaccharide domain consists of an epimerization site (EPS) and a mode of reaction recognition site (MRRS). The MRRS is located three residues away from the non-reducing end side of the EPS (Figure 8B). The N-substitution status of the MRRS residue dictates the mode of action of C₅-epi. A GlcNAc residue directs C₅-epi to display an irreversible reaction mode, while a GlcNS or GlcNH₂ residue (or the MRRS site being unoccupied) drives C₅-epi to display a reversible reaction mode. The presence of GlcNS at the -1 residue is essential for rendering the susceptibility to C₅-epi modification. The results from the substrate specificity study of C₅-epi could lead to a new strategy for synthesizing oligosaccharides with multiple IdoA residues.

**Enzymatic synthesis of a sulfate donor**

In addition to the oligosaccharide synthesis, sulfate modifications crucially dictate the biological activity of HS and heparin. The sulfation steps by HS sulfotransferases require a sulfate donor known as PAPS (3’-phosphoadenosine-5’-phosphosulfate). PAPS is expensive for even the milligram-scale synthesis in an academic lab. The availability of PAPS determines the cost-effectiveness when performing a large-scale synthesis. Although a PAPS regeneration system has been employed to prepare heparin, a combination of this system with HS sulfotransferases yields unexpected byproducts. Zhou and co-workers developed an enzymatic approach (61) to synthesize PAPS in a one-pot format using ATP sulfurylase (from *Kluyveromyces lactis*), APS kinase (from *Penicillium chrysogenum*) and pyrophosphatase (from *E.coli*). Bacterial expression of all three enzymes yielding crude extracts is efficient enough to
convert ATP and sulfate to PAPS in 6 hours. A synthetic scheme shown in Figure 9 describes a method for PAPS synthesis.

**Figure 9 The steps involved in PAPS synthesis**

Sodium sulfate and adenosine 5’-triphosphate (ATP) are first converted to adenosine 5’-phosphosulfate (APS), and then the second phospho group is added to produce PAPS. PPI represents pyrophosphate, and Pi represents phosphate. An enzymatic scheme is adapted from a previously published paper by Zhou and colleagues (61).

**Product purification**

The current method to separate heparin oligosaccharides and enzymes are achieved by using a BioGel P-2 column, which is eluted with 0.1 M ammonium bicarbonate. The sample is detected by mass spectrometry directly from the eluted fractions. Further purification also includes the use of a strong anion exchange column, *i.e.*, Q-Sepharose. The anion exchange column is able to further purify the sample if it contains incomplete sulfated by-products. One technical issue of using Q-Sepharose is monitoring the sample during the purification. To overcome this problem, a UV tag can be introduced to the HS oligosaccharide products. To this end, *para*-nitrophenyl glucuronide (GlcA-pNP) is used as a starting material to conduct the synthesis (62). The strong absorbance from the pNP tag provides easy detection during column purification, increasing the purity of the product to greater than 95%.
STATEMENT OF PROBLEM

Heparin is the most widely used anticoagulant in a broad range of clinical applications including surgery, kidney dialysis and various cardiovascular diseases due to its powerful anticoagulation activity. The biological functions of heparin not only limit to its anticoagulation effect, several studies showed that heparin is a potential candidate in inflammatory and cancer diseases.

In contrast to the synthetic pentasaccharide, UFH and LMWH are currently obtained from animal sources. The worldwide contamination of heparin suggested that heparin is vulnerable to contamination and adulteration. Among the three forms heparins, LMWH is the most commonly used in clinics owing to the predictable anticoagulation activity. However, the current manufacturing methods produce polydisperse LMWH fragments that affect the pharmacological and pharmacokinetic properties such as anticoagulation potency, metabolic clearance and the sensitivity to the neutralization by an antidote. Although the synthetic pentasaccharide has been successfully produced by chemical synthesis, a complex structure of LMWH has limited the total synthesis suggesting that other production sources are required. With the availability of HS biosynthetic enzymes and the high regioselective functions of those enzymes, an enzymatic approach is a promising method to synthesize LMWH with desired properties.

The main goal of this dissertation was to utilize a chemoenzymatic method to produce heparin oligosaccharides, particularly LMWH. The synthetic heparins obtained from the enzymatic synthesis not only offer a potential candidate for a safer heparin but they also provide insight into the structural requirements for heparin-protein interaction. In this dissertation, we hypothesized that a controlled chemoenzymatic method is feasible to synthesize a narrow range
of oligosaccharide lengths, resulting in the enhanced potency in anticoagulation activity. In addition, we proposed that the improvement in homogeneity and a specific sulfo modification of a new generation LMWH increase neutralization sensitivity to an antidote, protamine. Furthermore, we proposed that the interaction between heparin oligosaccharides and P-selectin, an adhesion receptor involved in the initiation phase of an inflammatory event, requires a minimum length of the oligosaccharides as well as a sulfation pattern.
CHAPTER II
MATERIALS AND METHODS

Preparation of synthetic enzymes

A total of nine enzymes were used for the synthesis, including N-Acetyl-D-glucosaminyl transferase (KfiA), Pasteurella multocida (PmHS2), N-sulfotransferase (NST), C₅-epimerase (C₅-epi), 2-OST, 6-OST-1, 6-OST-3, 3-OST-1 and 3-OST-5. All enzymes were expressed in E. coli and purified by appropriate affinity chromatography as described previously (56).

Preparation of K5 polysaccharide

K5 polysaccharide (K5P) was derived from E. coli K5 (63). E. coli K5 (Bi 8337-41) (50 μL stock) was grown in 100 mL LB media as a stock culture for 8 h (37 ºC). The culture was expanded from the stock culture by adding 13 mL in 1 litre (L) of LB media and incubated for 16-18 h (37 ºC). For larger scale preparation, 6 L of LB media were used to repeat the same procedure. The cells were pelleted (7000 x g, 30 min), and the supernatant containing K5P was filtered and adjusted to pH 5. The solution at pH 5 was diluted 1:1 with buffer A (20 mM sodium acetate, 50 mM NaCl, pH 4.0), and loaded onto a column containing 100 mL DEAE per 12 L of the diluted supernatant (flow rate 10mL/min). After loading, the column was washed with 1 L buffer A and eluted with 400 mL of buffer B (20 mM sodium acetate, 1 M NaCl, pH 4.0) or until eluent was clear. The collected K5P eluent was ethanol precipitated overnight at -20°C by mixing the eluent with an equal volume of 100% reagent ethanol. The precipitate was
centrifuged at 7000 rpm for 30 minutes and resuspended in water (H$_2$O). A solution of saturated ammonium sulfate was added to the give a 1:1 mixture. The mixture was cooled on ice for 15 min, then centrifuged (3500 x g, 30 min). The supernatant was removed and the gel-like precipitate was saved. The gel precipitate was resuspended in 10 mL water and dialyzed overnight in water with MWCO 12,000-14,000 membrane (Spectrapor), leaving 2 times the amount of headspace for expansion. The resultant K5P was then lyophilized. The average MW of heparosan isolated from *E. coli* K5 strain is about 30 kDa (63).

**Preparation of monosaccharide donors**

UDP-GlcNAc and UDP-GlcA were purchased from Sigma-Aldrich while UDP-N-trifluoroacetylglucosamine (GlcN-TFA) was synthesized by a chemoenzymatic approach as previously described (56). In brief, GlcN-TFA was generated from a reaction between GlcNH$_2$-1-phosphate (Sigma-Aldrich) and S-ethyl trifluorothioacetate (Sigma-Aldrich). The resultant GlcN-TFA was converted to GlcN-TFA 1-phosphate using *N*-acetylhexosamine 1-kinase. The expression of *N*-acetylhexosamine 1-kinase was carried out in *E. coli* as reported (64). A coupling of UTP and GlcN-TFA 1-phosphate resulting in UDP-GlcN-TFA was completed by glucosamine-1-phosphate acetyltransferase/*N*-acetylglucosamine-1-phosphate uridyltransferase (GlmU). UDP-[N-[$^3$H]acetyl]GlcNAc was synthesized by an enzymatic approach as previously described (51).

**Preparation of a sulfate donor, PAPS**

A sulfate donor, 3’-phosphoadenosine 5’-phosphosulfate (PAPS), was prepared from ATP and sodium sulfate using adenosine phosphokinase and ATP-sulfurylase (61). The purity of PAPS was more than 95% as measured by PAMN-HPLC and its structure was confirmed by
ESI-MS as described in a previous publication (61).

**Saccharide primer preparations**

A disaccharide precursor, 2,5-anhydromannitol (GlcA-AnMan) was obtained from a chemical depolymerization of heparosan K5 polysaccharide as previously described (62). 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 MnCl₂ 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 x 200 mm Bio-Gel P-2 (Bio-Rad, Hercules, CA) that was equilibrated with 0.1 M ammonium bicarbonate (NH₄HCO₃) at a flow rate of 4 mL/hour. The fractions were directly subjected to ESI-MS analysis and the fractions containing trisaccharide were pooled. The trisaccharide dissolved in NH₄HCO₃ 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 ug/mL) in a buffer containing 25 mM MnCl₂ 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 ahexasaccharide (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[^3H]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 MnCl₂. 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-[3H]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 1M NaCl. The ^3H-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.

**Synthesis of -(GlcA-GlcN-TFA)- 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-GlcN-TFA (1.4 mM) and UDP-GlcA (1.4 mM) were incubated at 1:4:4 molar ratio with PmHS2 (30 μg/mL) at room temperature overnight. The reaction was purified using Bio-Gel P-2. Then, the resultant -(GlcA-GlcN-TFA)- 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.

**Strong anion exchange (SAX)-HPLC analysis**

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

**N-sulfation of oligosaccharide backbones**

N-sulfation was initiated by detrifluoroacetylation of the GlcN-TFA residue (de-NTFA). The de-NTFA step required overnight incubation of a reagent containing 2:2:1 (v/v/v) H_2O,
CH\textsubscript{3}OH and (C\textsubscript{2}H\textsubscript{5})\textsubscript{3}N at 50°C in 50 mL. The reaction mixture was dried and reconstituted in water to obtain the resultant -(GlcNH\textsubscript{2}-GlcA)- backbones. The addition of sulfo groups at the GlcNH\textsubscript{2} residue was carried out by incubation of -(GlcNH\textsubscript{2}-GlcA)- backbones with NST (10 μg/mL) and PAPS (500 μM) at 37°C overnight in a buffer containing 50 mM MES (pH 7.4) in a total volume of 40 mL.

**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\textsubscript{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\textsubscript{5}-epi (20 μg/ml) and 2 mM CaCl\textsubscript{2} in a total volume of 40 ml. After incubation for 30 minutes 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 (10). 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\textsubscript{2}, 5mM MgCl\textsubscript{2} and 50 mM MES in a total volume of 30 ml.
Deacetylation of K5 polysaccharide with NaOH

Twenty milligrams of heparosan was lyophilized. This sample was resuspended in 4 mL of 2M NaOH and rotated at 68°C for 3 h. This reaction was neutralized with 2M HCl and dialyzed against water. The deacetylated K5P was lyophilized.

Chemical N-sulfation of deacetylated K5 polysaccharide

Deacetylated K5P (2 mg) was dissolved in 1 mL of water. 0.1 M NaOH was added dropwise until the pH reached between 9.5-10. The number of free amino groups were calculated and multiplied by 15 in order to get the amount of sulfur trioxide pyridine that needed to be added. About 1/5th of the amount of sulfur trioxide pyridine was added to the mixture. After 30 minutes, the sample was re-titrated with 0.1 M NaOH to pH 9.5. Then another 1/5th of the sulfur trioxide pyridine was added. The procedure was repeated until all of the sulfur trioxide pyridine was added. When the pH was stable at 9.5 for about an hour, the sample was dialyzed for 16 hours in a 0.1 N ammonium bicarbonate buffer.

Enzymatic modification of N-sulfated K5 polysaccharide

Epimerization and 2-O-sulfation: N-sulfated K5P (2 mg) was incubated in a reaction mixture containing 50 mM MES (pH 7.4) C5-epi (20 μg/ml) and 2 mM CaCl₂ in a total volume of 12 ml. After incubation for 30 minutes at 37 °C, 2-OST (20 μg/ml) and 500 μM PAPS were added, and the reaction was incubated overnight at 37 °C.

6-O-sulfation: Either N-sulfated K5P or N-, 2-O-sulfated K5P 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 12 ml.

3-O-sulfation: N-, 2-, 6-O-sulfated K5P was incubated with 3-OST-1 (10 μg/ml) and 3-
OST-5 (10 μg/ml) and 500 μM PAPS at 37 °C overnight in a reaction mixture containing, 10 mM MnCl₂, 5mM MgCl₂ and 50 mM MES in a total volume of 12 ml.

The sulfated products in each sulfation step were purified using a DEAE column as described previously (10). The resultant sulfated products were dialyzed against water prior to the next sulfation step.

**Syntheses of trideca- (13 mer) and heptadeca (17 mer)- saccharides**

First, a hepatasaccharide (7 mer) was synthesized. The conversion of a starting material GlcA-pNP to 7 mer involves seven steps, including six elongation steps, one detrifluoroacetylation/N-sulfation step. Elongation of GlcA-pNP to GlcA-GlcN-TFA-GlcA-GlcN-TFA-GlcA-GlcN-TFA-GlcA-pNP was completed in 6 steps using two bacterial glycosyl transferases, KfiA and PmHS2. To introduce a GlcN-TFA residue, GlcA-pNP (20 mg, from Sigma-Aldrich) (mM) was incubated with KfiA (20 μg/ml) in 45 ml buffer containing Tris (25 mM, pH 7.5), MnCl₂ (15 mM) and UDP-GlcN-TFA (1.5 mM) at room temperature overnight. To introduce a GlcA residue, disaccharide substrate, GlcN-TFA-GlcA-pNP (1.2 mM), was incubated with PmHS₂ (20 μg/ml) in a buffer containing Tris (25 mM, pH 7.5), MnCl₂ (15 mM) and UDP-GlcA (1.5 mM) at room temperature overnight. The product after each elongation step was purified using a C₁₈ column (0.75 × 20 cm; Biotage), which was eluted with a linear gradient of 0–100% acetonitrile in H₂O and 0.1% TFA in 60 min at a flow rate of 4 ml/min. The eluent was monitored by the absorbance at 310 nm, and the identity of the product was confirmed by ESI-MS. The addition of GlcN-TFA and GlcA residues was repeated two more time to form a dp7 backbone for the subsequent N-detrifluoroacetylation/N-sulfation.

The 7 mer backbone was further subjected to detrifluoroacetylation, followed by N-sulfation with N-sulfotransferase (NST). The 7 mer backbone was dried and resuspended at 10
mg/ml in 0.1 M LiOH. The reaction mixture was incubated on ice for 2 h. The degree of completion of the detrifluoroacetylation reaction was monitored by PAMN-HPLC analysis and ESI-MS. Upon the completion of detrifluoroacetylation, the pH of the reaction mixture decreased to 7.0. The detrifluoroacetylated-7 mer was incubated with 50 mM MES (pH 7.0), 10 µg/ml NST and 1 mM PAPS at 37°C overnight, where the amount of PAPS was about 1.5-times molar amount of NH2 groups in the heptasaccharide. N-sulfated product was purified by Q-Sepharose (GE Health Care), and the purified product with the structure of GlcA-GlcNS-GlcA-GlcNS-GlcA-GlcA-pNP was dialyzed.

To GlcA-GlcNS-GlcA-GlcNS-GlcA-GlcNS-GlcA-pNP heptasaccharide, GlcN-TFA and GlcA were continuously introduced using KfiA and PmHS2 following the procedure described above. The reaction was monitored by PAMN-HPLC to observe a shift in retention time of the peak with the absorbance at 310 nm. The reaction mixture was then purified by Q-Sepharose column to obtain tridecasaccharide (13 mer). The detrifluoroacetylation, followed by N-sulfation with NST and PAPS was repeated until N-sulfo-tridecasaccharide (13 mer-NS) was obtained. 13 mer-NS was then subjected to 6-O-sulfation by 6-OST-1 and 6-OST3. 13 mer-NS 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 130 ml. Once dp13NS6S was obtained, it was used to alternately add 4 residues of GlcN-TFA and GlcA to obtain heptadecasaccharide (17 mer). The enzymatic cycle for N- and 6-O-modifications were repeated until 17 mer-NS6S was obtained. The 17 mer-NS6S was then subjected to the 3-O-sulfation by incubating with 3-OST-1 (10 µg/ml) and 3-OST-5 (10 µg/ml) and 500 µM PAPS at 37 °C overnight in a reaction mixture containing, 10 mM MnCl2, 5mM MgCl2 and 50 mM MES in a total volume of 130 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 5kV 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:H₂O. The MS data were acquired and processed using XCalibur 13 software.

**Disaccharide analysis**

By heparin Lyase: The oligosaccharide backbones or the modified K5P carrying ³⁵S-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 x 200 mm Bio-Gel P-2 gel.

By nitrous acid degradation: The oligosaccharide backbones or the modified K5P carrying radiolabeled 3-O-sulfo groups were deacetylated by hydrazine at 95°C for 3 h 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 (65).

The resultant ³⁵S-labeled disaccharides were resolved using a C₁₈ reverse-phase column (0.46x25 cm; Vydac) under reverse-phase ion-pairing HPLC conditions (66). The identities of the disaccharides were determined by co-elution with the appropriate ³⁵S-labeled disaccharide standards.

**Determination of AT-binding activity**

³⁵S-labeled oligosaccharide is required for AT-binding activity assay (66). Approximately
$1 \times 10^4$ cpm of each oligosaccharide was incubated with 5 μg of human antithrombin (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 hours. The beads were then washed 3 times with 1 ml of binding buffer, and the amount of $^{35}$S-labeled radioactivity was determined by scintillation counting.

**Sialyl Lewis X ELISA inhibition assay**

ELISA inhibition assay was done by the method previously reported (67). 96-well ELISA plate was coated with 200 ng of polyacrylamide-SLe$^X$ (SLe$^X$-PAA; Glycotech, Rockville, MD) by overnight incubation at 4°C in 100 μl of 50 mM sodium carbonate/bicarbonate buffer, pH 9.5. Plates were then blocked with 200 μl/well of assay buffer: 20 mM Hepes (Sigma), 125 mM NaCl, 2 mM CaCl$_2$, 2 mM MgCl$_2$, 1% protease-free BSA (Sigma), pH 7.45 for a minimum of 3 h at 4°C. During the blocking step, the selectin chimeras (R&D systems) were pre-incubated separately at 4°C with the secondary antibody, peroxidase-conjugated goat anti-human IgG (Jackson Immuno Research Laboratories, Inc., West Grove, PA) in assay buffer for 1 h. Final selectin-Rg concentration was 20 nM, and the optimal secondary antibody dilution was determined to be 1:1,000 for the particular serum used. A series of the modified K5P or heparin oligosaccharides were serially diluted in assay buffer at twice the final required concentration. The selectin-Rg/secondary antibody stock was aliquoted into tubes containing an equivalent volume of inhibitor solution; buffer alone for the positive control, or buffer with 10 mM Na$_2$EDTA, pH 7.5, for the negative control. These tubes were preincubated at 4°C for 30 min, and added to ELISA plates, in duplicates, at final well volume of 100 μl. After 4 h of plate
incubation at 4°C, plate was washed three times with 200 µl/well of assay buffer at 4 C, followed by development with 150 µl/well of o-phenylenedi-amine dihydrochloride (OPD) solution at RT: 0.002 mg OPD/ml in 50 mM sodium citrate, 50 mM disodium phosphate buffer, pH 5.2 containing 1 µl/ml 30% H₂O₂. Using a timer, each well was sequentially quenched with 40 µl of 4 M H₂SO₄ after 10 minutes of peroxidase reaction. The reaction was determined and recorded absorbance at 492 nm. Before curve fitting, the data were changed into percentages for comparative purposes, using the formula: [(average of duplicates)−(negative control)]/[(positive control)-(negative control)]×100.

**In vitro anti-FXa activity**

An assay was carried out based on a published method (68). In brief, human AT (Cutter Biological) was diluted with phosphate buffer saline (PBS) containing 1 mg/mL bovine serum albumin (BSA) to give a stock solution at a concentration of 0.4 µM. Human Factor Xa (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 factor Xa, S-2765 (Diapharma, West Chester, Ohio) was dissolved in water at a concentration of 1 mM. HS from bovine kidney, heparin, enoxaparin (local pharmacy), dnLMWH and all the modified K5P 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 minutes. Then 10 µL of FXa was added. After incubation at room temperature for 4 minutes, 30 µL of S-2765 was added. The absorbance at 405 nm was continuously measured for 5 minutes. The absorbance values were plotted against the reaction time. The initial reaction rates as a function of concentration were used to calculate IC₅₀ 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 gram were purchased from Jackson Laboratory (Bar Harbor, ME). Under anesthesia by isoflurane (2 percent in oxygen), PBS, enoxaparin (1.5 mg/kg), dnLMWH (1.5 mg/kg) and compounds 1-5 were subcutaneously administered to the mice. After 30 minutes, 1, 2, 4 and 8 hours, mice were anesthetized and a midline laparotomy was performed. Blood (300-500 μL) from inferior vena cava (IVC) 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 g at 4°C for 15 minutes 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 room temperature. After 4 minutes, S-2765 (30 μL) was then added. FXa activity was calculated as a percent when PBS mice were defined as 100 %.

**Neutralization of synthetic LMWHs by protamine in mice**

The study was performed on 8-week-old male C57BL/6J mice (Jackson Laboratories, Bar Harbor, ME) (*n* = 4 per group). The mouse experiments were approved by the University of North Carolina Animal Care and Use Committees and complied with National Institutes of Health guidelines. Under isoflurane anesthesia, mice were subcutaneously administered with PBS, UFH (3 mg/kg), enoxaparin (3 mg/kg) or compound 5 (0.6 mg/kg) 30 min before a protamine administration. Protamine (15 mg/kg) or PBS was administered intravenously via retro-orbital plexus injection, and 5 min later, blood samples were drawn from the IVC into
syringes preloaded with 3.2% solution of sodium citrate (final volume ratio 9:1). To obtain mouse plasma, blood samples were centrifuged at 4,000g for 15 min at 4 °C. Mouse plasma was then used to determine anti-FXa activity. *Ex vivo* analysis of anti-FXa activity was done similarly to the *in vitro* study described above. Briefly, plasma (10 μl) from different groups of mice was incubated with 80 nM human factor Xa (10 μl) at room temperature for 4 min and S-2765 (1 mg/ml, 30 μl) was then added. The anti-FXa activity in the mouse plasma from the PBS injected mice was defined as 100%. Statistical analysis for multiple comparisons was performed by two-way analysis of variance (ANOVA) with Bonferroni’s post hoc test (GraphPad Prism Software).

**Mouse model of tail bleeding**

Under isoflurane anesthesia, 8-week-old male C57BL/6J mice (*n* = 8 per group) were administered with either PBS or compound 5 (0.6 mg/kg) subcutaneously, and 30 min later PBS or protamine (15 mg/kg) was administered via retro-orbital intravenous injection. After 5 min the distal part of the tail was transected at the constant diameter (1.5 mm), approximately 3–4 mm from the end, resulting in both arterial and venous bleeding. The tail was immediately placed in a 15-ml Falcon tube containing 13 ml of pre-warmed PBS (37 °C), and blood loss was observed for 30 min.

**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 2-group comparison of continuous data, 2-tailed Student’s t test was used. A p-value ≤ 0.05 was regarded as significant.
CHAPTER III
CHEMOENZYMATIC SYNTHESIS OF A NARROW SIZE DISTRIBUTION LOW MOLECULAR WEIGHT HEPARIN

Introduction

Heparin, a commonly used anticoagulant drug, is a mixture of highly sulfated polysaccharides with various molecular weights. The unique sulfation pattern dictates the anticoagulant activity of heparin. Commercial heparins are categorized into three forms according to their average molecular weight (MW): unfractionated heparin (UFH, MW_{avg} 14,000), low molecular weight heparin (LMWH, MW_{avg} 3,500-6,500) 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 (1).

In this chapter, we utilized a chemoenzymatic method to synthesize LMWH, namely “de novo LMWH” (dnLMWH) with an emphasis on controlling the size distribution of the oligosaccharides. We demonstrate a rapid procedure to synthesize oligosaccharide backbones in a one-pot format. The use of a stoichiometrically controlled starting tetrasaccharide primer and monosaccharide donors allowed us to modulate the degree of oligomerization, yielding a narrow size distribution of oligosaccharide backbones. A tetrasaccharide primer and a controlled
enzyme-based polymerization were employed to build a narrow size oligosaccharide backbone. The resultant oligosaccharide backbones were characterized and identified by strong anion exchange (SAX)-HPLC and ESI-MS. The oligosaccharide backbones were further modified by a series of HS sulfotransferases to install an AT-binding pentasaccharide domain within the synthetic backbones. 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 use. Our data suggest that a carefully designed narrow size distribution LMWH has highly potent anticoagulation activity.

**Controlling the degree of backbone oligomerization**

We previously reported a chemoenzymatic method to synthesize oligosaccharide backbones by the sequential addition of monosaccharide residues (10). Although this synthetic method produces a homogeneous oligosaccharide backbone, the method requires about fourteen days to prepare a single dodecasaccharide when starting from a disaccharide. Here, we developed a one-pot chemoenzymatic synthesis capable of producing the dodecasaccharides within two 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 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 (50, 53). Moreover, PmHS2 surpasses its substrate specificity in recognizing certain unnatural UDP-GlcNs (54). Therefore, PmHS2 met our needs for a one-pot synthesis (50).

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 (Table 2). 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 3,500-6,000 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[3H]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 size exclusion chromatography (gel filtration). 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 10A). Unlike the disaccharide primer, different sizes of oligosaccharide products were obtained when a tetrasaccharide primer was employed (Figure 10B). 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 (Figure 10C) and the octasaccharide (Figure 10D) produced the oligosaccharides in a similar pattern as observed for the tetrasaccharide primer. Strong anion exchange HPLC (SAX-HPLC) was employed to analyze the size distribution of the oligosaccharide backbones (Figure 12A), demonstrating the presence of $^{3}$H-labeled oligosaccharide backbones with different sizes. Co-eluting non-radiolabeled oligosaccharides followed by electrospray ionization mass spectrometry (ESI-MS) analysis confirmed the structures of the oligosaccharide products (Figure 11, Table 3). The
oligosaccharide distribution analysis indicated that the size of the oligosaccharide backbones was in the range of dp 8 to dp 16 (Fig. 12B) where dp 10 (24.6%) and dp 12 (24.5%) are major products (Fig. 12B). The tetrasaccharide primer was therefore selected as a primer for the preparation of LMWH in the subsequent studies.

Figure 10 Optimization of a primer and a molar ratio in a one-pot reaction
A disaccharide (Panel A), a tetrasaccharide (Panel B), a hexasaccharide (Panel C) and an octasaccharide (Panel D) 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 in different colors (1:1:1 = grey, 1:2:2 = black, 1:4:4 = magenta, 1:10:10 = green). V₀ = void volume and Vₜ = total volume.
Figure 11 ESI-MS analysis of -(GlcA-GlcNAc)- 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. Different color corresponds to the oligosaccharide chain length (brown = hexasaccharide, black = octasaccharide, blue = decasaccharide, red = dodecasaccharide, green = tetradecasaccharide, pink = hexadecasaccharide, orange = octadecasaccharide). Subnumbered molecular species indicated multiply charged species. For example, dp 12.2 (M.W. 1117.3) is a double charge dodecasaccharide with the loss of two protons. See table 3. dp = degree of polymerization.

Figure 12 Analysis of oligosaccharide distribution by SAX-HPLC

The $^3$H-oligosaccharide products obtained from a reaction when the tetrasaccharide was used at 1:4:4 were pooled. A size distribution of the $^3$H-labeled products were analyzed by strong anion exchange-HPLC. The non-radiolabeled oligosaccharides were co-eluted and identified by ESI-MS analysis. dp = degree of polymerization (Panel A). The distribution of the oligosaccharide chain length was estimated as a percentage (Panel B).
Table 2 ESI-MS analysis of oligosaccharide primers

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<th>Primers</th>
<th>Structure</th>
<th>Calculated</th>
<th>Measured</th>
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<tr>
<td>disaccharide</td>
<td>AnMan</td>
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<td>tetrasaccharide</td>
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<td>GlcA-GlcNAc-GlcA-GlcNAc-GlcA-GlcNAc-GlcA-AnMan</td>
<td>1478.2</td>
<td>1476.6±0.7</td>
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</tbody>
</table>

Table 3 ESI-MS analysis of -(GlcA-GlcNAc)- backbones

<table>
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<th>Name</th>
<th>Abbreviated structure</th>
<th>Calculated</th>
<th>Measured</th>
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<tbody>
<tr>
<td>Hexasaccharide (dp 6)</td>
<td>(GlcA-GlcNS)₁-GlcA-GlcNAc-GlcA-AnMan</td>
<td>1098.9</td>
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<td>Octasaccharide (dp 8)</td>
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<td>Decasaccharide (dp 10)</td>
<td>(GlcA-GlcNS)₃-GlcA-GlcNAc-GlcA-AnMan</td>
<td>1857.6</td>
<td>1857.6±1.6</td>
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<td>Dodecasaccharide (dp 12)</td>
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<td>2236.9</td>
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<td>Tetrasaccharide (dp 14)</td>
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<td>Hexadecasaccharide (dp 16)</td>
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<td>Octadecasaccharide (dp 18)</td>
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<td>3374.8</td>
<td>3375.9±1.2</td>
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</table>
**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 -GlcN-TFA–GlcA-, where the GlcN-TFA represents N-trifluoroacetyl glucosamine. Replacing UDP-GlcNAc with UDP-N-TFA in a backbone synthetic step allowed us to prepare the backbone oligosaccharides. A molar ratio between the tetrasaccharide primer, UDP-GlcN-TFA and UDP-GlcA, was selected to be 1:4:4 to prepare the products. As expected, PmHS2 is also capable of incorporating the GlcN-TFA residues into the backbones yielding the -(GlcN-TFA-GlcA)- repeating backbones. The -(GlcN-TFA-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 13, Table 4). Because the -(GlcN-TFA-GlcA)- repeating backbones were eventually subjected to complete alkaline detrifluoroacetylation resulting in the -(GlcNH$_2$-GlcA)- repeating backbones, the partial detrifluoroacetylation would not affect the subsequent synthesis. The resultant (GlcNH$_2$-GlcA-) backbones were further treated with NST and a sulfate donor, 3’-phosphoadenosine 5’-phosphosulfate (PAPS). ESI-MS analysis of the backbones resolved from Bio-Gel P-2 after N-sulfation step indicated that the -(GlcNS-GlcA)- repeating backbones were produced (Figure 14, Table 5).
Figure 13 ESI-MS analysis of the N-TFA 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. Different color corresponds to the oligosaccharide chain length (black = octasaccharide, blue = decasaccharide, red = dodecasaccharide, green = tetradecasaccharide, pink = hexadecasaccharide, orange = octadecasaccharide). Subnumbered molecular species indicated multiply charged and detrifluoroacetylated species, respectively. For example, dp 12.2.3 (M.W. 1081.4) is a double charge dodecasaccharide with the loss of three fluoroacetyl groups (see table 4).
### Table 4 ESI-MS analysis of -(GlcA-GlcN-TFA)- backbones

<table>
<thead>
<tr>
<th>Name</th>
<th>Abbreviated structure</th>
<th>Calculated</th>
<th>Measured</th>
</tr>
</thead>
<tbody>
<tr>
<td>Octasaccharide (dp 8)</td>
<td>(GlcA-GlcN-TFA)_2-GlcA-GlcNAc-GlcA-AnMan</td>
<td>1586.2</td>
<td>1586.5±0.6</td>
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<tr>
<td>Decasaccharide (dp 10)</td>
<td>(GlcA-GlcN-TFA)_3-GlcA-GlcNAc-GlcA-AnMan</td>
<td>2019.5</td>
<td>2019.3±1.3</td>
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<tr>
<td>Dodecasaccharide (dp 12)</td>
<td>(GlcA-GlcN-TFA)_4-GlcA-GlcNAc-GlcA-AnMan</td>
<td>2452.7</td>
<td>2453.2±0.8</td>
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<tr>
<td>Tetrasaccharide (dp 14)</td>
<td>(GlcA-GlcN-TFA)_5-GlcA-GlcNAc-GlcA-AnMan</td>
<td>2886.0</td>
<td>2885.1±1.2</td>
</tr>
<tr>
<td>Hexadecasaccharide (dp 16)</td>
<td>(GlcA-GlcN-TFA)_6-GlcA-GlcNAc-GlcA-AnMan</td>
<td>3319.3</td>
<td>3318.9±0.5</td>
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<tr>
<td>Octadecasaccharide (dp 18)</td>
<td>(GlcA-GlcN-TFA)_7-GlcA-GlcNAc-GlcA-AnMan</td>
<td>3752.6</td>
<td>3752.2±1.1</td>
</tr>
</tbody>
</table>

### Table 5 ESI-MS analysis of -(GlcA-GlcNS)- backbones

<table>
<thead>
<tr>
<th>Name</th>
<th>Abbreviated structure</th>
<th>Calculated</th>
<th>Measured</th>
</tr>
</thead>
<tbody>
<tr>
<td>Octasaccharide (dp 8)</td>
<td>(GlcA-GlcNS)_2-GlcA-GlcNAc-GlcA-AnMan</td>
<td>1554.3</td>
<td>1554.5±0.4</td>
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<td>Decasaccharide (dp 10)</td>
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<td>1972±1.0</td>
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<td>Dodecasaccharide (dp 12)</td>
<td>(GlcA-GlcNS)_4-GlcA-GlcNAc-GlcA-AnMan</td>
<td>2389.0</td>
<td>2389.5±1.1</td>
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<tr>
<td>Tetrasaccharide (dp 14)</td>
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<td>2807.1±1.1</td>
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<td>Hexadecasaccharide (dp 16)</td>
<td>(GlcA-GlcNS)_6-GlcA-GlcNAc-GlcA-AnMan</td>
<td>3223.7</td>
<td>3223.2±1.0</td>
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<td>Octadecasaccharide (dp 18)</td>
<td>(GlcA-GlcNS)_7-GlcA-GlcNAc-GlcA-AnMan</td>
<td>3641.0</td>
<td>3641.0±1.2</td>
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</tbody>
</table>
Figure 14 ESI-MS analysis of -(GlcA-GlcNS)- 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. Different color corresponds to the oligosaccharide chain length (black = octasaccharide, blue = decasaccharide, red = dodecasaccharide, green = tetradecasaccharide, pink = hexadecasaccharide, orange = octadecasaccharide). Subnumbered molecular species indicated multiply charged species. For example, dp 10.2.2 (M.W. 904.7) is a double charge decasaccharide with the loss of two sulfo groups and two protons. See table 5.
Figure 15 ESI-MS analysis of GlcNAc elongation at the nonreducing end of -(GlcA-GlcNS)- 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. Different color corresponds to the oligosaccharide chain length (black = octasaccharide, blue = decasaccharide, red = dodecasaccharide, green = tetradecasaccharide, pink = hexadecasaccharide, orange = octadecasaccharide). Subnumbered molecular species indicated multiply charged species. For example, dp 13.2.1 (M.W. 1214.7) is a double charge tridecasaccharide with the loss of two sulfo groups and two protons. See table 6.
<table>
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<th>Name</th>
<th>Abbreviated structure</th>
<th>Calculated</th>
<th>Measured</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nonasaccharide (dp 9)</td>
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<td>1757.5</td>
<td>1757.5±1.4</td>
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<tr>
<td>Undecasaccharide (dp 11)</td>
<td>GlcNAc-(GlcA-NS)3-GlcA-GlcNAc-GlcA-AnMan</td>
<td>2174.8</td>
<td>2174.8±1.6</td>
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<tr>
<td>Tridecasaccharide (dp 13)</td>
<td>GlcNAc-(GlcA-NS)4-GlcA-GlcNAc-GlcA-AnMan</td>
<td>2592.2</td>
<td>2592.6±0.7</td>
</tr>
<tr>
<td>Pentadecasaccharide (dp 15)</td>
<td>GlcNAc-(GlcA-NS)5-GlcA-GlcNAc-GlcA-AnMan</td>
<td>3009.5</td>
<td>3010±1.0</td>
</tr>
<tr>
<td>Heptadecasaccharide (dp 17)</td>
<td>GlcNAc-(GlcA-NS)6-GlcA-GlcNAc-GlcA-AnMan</td>
<td>3426.8</td>
<td>3427.4±1.3</td>
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<tr>
<td>Nonadecasaccharide (dp 19)</td>
<td>GlcNAc-(GlcA-NS)7-GlcA-GlcNAc-GlcA-AnMan</td>
<td>3844.2</td>
<td>3844.5±0.8</td>
</tr>
</tbody>
</table>
Placing antithrombin 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 (12). 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 (69) providing flexibility for placing the AT binding region. We found that by placing a GlcNAc residue at the non-reducing end of N-sulfo oligosaccharide backbones, the pentasaccharide motif could be readily produced by the latter epimerization and sequential sulfo modifications (60). An installation of the GlcNAc residue directs the irreversible catalytic mode of C5-epimerase such that GlcA at residue D is irreversibly converted to IdoA (Figure 16). The GlcNAc elongation was catalyzed by a N-Acetyl glucosaminyl transferase of *E. coli* K5 strain (KfiA) as described elsewhere (51). We confirmed the completion of GlcNAc elongation by ESI-MS analysis (Figure 15, Table 6). Once the GlcNAc elongation was achieved, we used recombinant C5-epimerase 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 3OST-1 (59). The O-sulfated products were purified by DEAE anion-exchange chromatography. We ensured the completion of each O-sulfation by performing a small-scale reaction using PAP^{35}S to monitor the reaction.
Figure 16 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 -(GlcN-TFA-GlcA)- repeating backbones were subjected to detrifluoroacetylation and N-sulfation. The GlcNAc elongation aided in an installation of the antithrombin binding site. Sequential modification by epimerization and 2-O-, 6-O- and 3-O-sulfation were required to achieve dnLMWH (lower panel).

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 -(GlcN-TFA-GlcA)- and the -(GlcNH2-GlcA)- backbones are similar across the three batches. The -(GlcNH2-GlcA)-backbones were treated with NST and PAP35S yielding -(GlcN35S-GlcA)- backbones. The backbones were purified and subjected to DEAE-HPLC analysis. The result shows that all three batches have similar oligosaccharide distributions (Figure 17A). We also tested the oligosaccharide distribution by conducting a [3H]GlcNAc elongation of the -(GlcNS-GlcA)-backbones. The 3H-labeled backbones showed similar results (Figure 17B).
Reproducibility of oligosaccharide size distribution by the one-pot method was assessed by three individual batch syntheses (1= blue, 2= black, 3= red). The similarity of the distribution profiles between each batch indicates the reproducibility of the method. Panel A shows $^{35}$S-radioactivity. In the first test, the -(GlcN-TFA-GlcA)- backbones from each batch were subjected to N-sulfation by using PAP$^{35}$S. Panel B shows $^3$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 (Panel A and Panel B, respectively). PAPS = 3’-phosphoadenosine-5’-phosphosulfate, a sulfate donor.

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 (70), thus requiring a nitrous acid degradation (65). \(^{35}\)S-labeled 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 (Figure 18A). Furthermore, the content of the IdoA2S found in the corresponding IdoA2S-GlcNS6S unit suggested that the product has similar structure to heparin (Figure 18B).

![Figure 18 Disaccharide analysis of the 2-, 6-\(O\)-sulfated products](image)

Oligosaccharides obtained after N-sulfation and GlcNAc elongation steps were \(^{35}\)S-labeled at 2-\(O\)-sulfo and 6-\(O\)-sulfo positions. The oligosaccharides were then treated with heparin lyases I, II and III. The resultant disaccharides were resolved by reverse phase ion-pairing (RPIP) HPLC. \(\Delta UA = \Delta^{1,5}\)–unsaturated uronic acid, (Panel A). A presence of the IdoA2S-GlcNS6S repeating disaccharides were further confirmed by exposing the \(^{35}\)S-labeled 2, 6-\(O\)-sulfated products to nitrous acid at both pH 4.5 and 1.5 (Panel B). red = components of \(\Delta UA\)-GlcNS6S, blue = components of \(\Delta UA2S\)-GlcNS6S.
A critical modification that drives the binding of a pentasaccharide to AT is a 3-O-sulfo modification at GlcNS±6S within the pentasaccharide (71). 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 19A). 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)- (44, 59). 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 a reversible epimerization, might be subjected to a 3-O-sulfo modification. A disaccharide, GlcA-AnMan3S6S, indicated the presence of a 3-O-sulfo group on dnLMWH (Figure 19B).

Figure 19 Investigation of 3-O-sulfation on dnLMWH
The AT-binding assay requires a radiolabeled- material for a measurement of percent binding activity to AT (Panel A). The 35S-labeled at 3-O-position of heparan sulfate was used as a positive control (1). 35S-labeled only at 2-O- and 6-O- positions of dnLMWH was used as a negative control (2). 35S-labeled at 3-O-position of dnLMWH was shown in (3). The data presented represent the average of three independent determinations ± S.D. Oligosaccharides obtained after N-sulfation and GlcNAc elongation steps were 35S-labeled at 2-O-sulfo and 6-O-sulfo and 3-O-sulfo positions. A presence of GlcA-AnMan3S6S 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±s.d (Panel B).

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 (72), LMWH displays higher activity toward FXa than toward FIIa. Previously, we demonstrated that only those oligosaccharides larger than 19 saccharide residues have anti-FIIa activity (10). 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 factor Xa. Indeed, dnLMWH (IC_{50} 40 ng/mL) showed superior anti-FXa activity in comparison to enoxaparin (IC_{50} 96 ng/mL) (Figure 20A). 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 PBS treated mice were used as a control group. As demonstrated in Figure 20B, dnLMWH exhibits significantly higher anti-FXa activity compared to Enoxaparin up to 2 hours after injection, with a maximum effect observed 30 minutes after injection. Both the in vitro and ex vivo experiments indicate that dnLMWH displays stronger anticoagulant activity than a commercially available LMWH.
Panel A shows 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 in different color: Heparan sulfate (black), enoxaparin (blue), dnLMWH (red). Panel B shows ex vivo anti-FXa activity of the plasma collected from the mice injected subcutaneously with dnLMWH (red) or enoxaparin (blue). (** indicates p<0.05 and **** indicates p <0.0001; n=3-5 mice for each time point and treatment).

**Conclusion**

LMWH has been used in cardiovascular disorders for almost thirty 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 (29). The polydispersity of LMWH affects the duration of action, drug potency and clinical safety (73). Therefore, controlling LMWH size distribution is essential for more predictable pharmacological responses. While the current research on 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 a 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 molecular weight range (50, 74). 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 (50). 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 (50, 54, 75) A previous study showed that PmHS 2 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 (74). 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 (50, 53).

    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 (23). dnLMWH might be beneficial in patients in whom synthetic pentasaccharides are contraindicated due to renal insufficiency.
CHAPTER IV
HOMOGENEOUS LMWH WITH DISTINCT SULFATE MODIFICATION EXHIBITS INCREASED SENSITIVITY TO PROTAMINE NEUTRALIZATION

Introduction

Heparin has long been the mainstay in the treatment of a diverse array of cardiovascular diseases; for example, UFH is necessary for patients undergoing hemodialysis as well as knee and hip surgery. LMWH is commonly prescribed for the treatment and prophylaxis of venous thromboembolism and acute coronary syndrome (2). Moreover, LMWH is usually prescribed for cancer patients because thromboembolism is associated with tumor progression (24). Many of the patients who are diagnosed with venous thromboembolism and cancer are elderly and have reduced kidney function (76). Safety concerns regarding the use of LMWH in kidney impaired-patients have arisen, but there is a lack of evidence regarding its safety.

Due to its specific interaction with AT through its pentasaccharide motif, heparin increases the action of AT about 1000-fold in the inhibition of clot formation. Both FXa and thrombin are downstream targets of the heparin-AT complex. The powerful action of heparin in the coagulation cascade can cause an unwanted bleeding side effect, like other anticoagulants. The risk of bleeding depends on several factors such as the heparin dose, duration of treatment, indication of treatment and concomitant use of thrombolytic drugs (77). UFH has a higher risk of bleeding than LMWH (30). The risk of this hemorrhagic event results in the routine monitoring
of patients receiving UFH who are likely to be hospitalized for close observation for the bleeding side effect.

Protamine is known to reverse the bleeding side effect of UFH. Protamine is a highly basic protein molecule predominantly composed of arginine residues. It was firstly described in the nucleus of the spermatozoa of fish to act as a DNA stabilizer (2). Currently, protamine products are obtained from the sperm ducts of salmon and through recombinant technology (77). The polycationic structure of protamine interacts with highly negative charged heparin. The U.S. FDA approved protamine for use as an antidote for UFH in cases of bleeding complications (2).

Introduced in 1990s, LMWH, a depolymerized form of UFH, has better bioavailability than UFH; therefore, LMWH exhibits more predictable anticoagulant activity (2). LMWH is widely used in many clinical settings. The benefit of its bioavailability makes it safe for outpatients who need long-term treatment and prophylaxis. In addition, LMWH has lower bleeding risk in comparison to UFH (30).

Protamine efficiently reverses the anticoagulant activity of UFH. However, it only partially neutralizes the bleeding side effect of LMWH (77). The incomplete reversal of LMWH by protamine is thought to be due to a reduced sulfate charge in LMWH fragments (33). Furthermore, LMWH is a mixture of oligosaccharide fragments whose size distribution varies from 4 to 18 sugar residues. The broad size distribution of these fragments results in the differences in their affinities toward protamine. Larger oligosaccharides can be efficiently neutralized by protamine while smaller fragments that are unable to bind protamine still display anticoagulant activity (34). Developing a LMWH that is homogenous in size will certainly improve the neutralizing capacity by protamine. In this chapter, we demonstrated that homogeneous LMWH with a distinct sulfate modification has a high affinity to protamine.
Distinct sulfation demonstrates substantial sensitivity to protamine neutralization

Recently, a series of homogeneous heparin oligosaccharides (compounds 1-5) with potent anti-FXa activity were synthesized (34) (Figure 21). These oligosaccharides were designed to probe the binding to the stabilin receptor as well as the sensitivity toward protamine neutralization. As the compounds were synthesized in different sizes, ranging from hexasaccharide to dodecasaccharide, they were capable of carrying different numbers of IdoA2S residues. For example, compound 1 can only carry one IdoA2S residue whereas compound 4 can carry four IdoA2S residues. The size of compound 5 is as same as compound 4, but compound 5 carries an extra sulfo group (Figure 21).

To probe the affinity toward protamine, the sensitivity to protamine neutralization of compounds 1-5 were determined in vitro. The study was straightforward such that the compounds were incubated with protamine prior to the determination of FXa activity. The anti-FXa activities of compounds were compared to that of UFH as well as a widely used LMWH, enoxaparin. The results showed that the FXa activity of compound 5 is more reversible than enoxaparin and showed the same protamine reversibility as UFH while compound 4 was partially reversed by protamine. In contrast, compounds 1-3 had fairly low sensitivity to protamine (Figure 22).
Figure 21 Chemical structures of compounds 1-5
We further determined protamine neutralization in mice. Because compound 5 showed high sensitivity to protamine, we selected compound 5 to further confirm that compound 5 can be neutralized by protamine in vivo. In this study, C57BL6/J mice were used. There were four groups of mice; the groups received PBS, UFH (3 mg/kg), enoxaparin (3 mg/kg) or compound 5 (0.6 mg/kg), subcutaneously. In each group of treatment, mice were either given protamine (15 mg/kg) or PBS. The dose of each compound used in this experiment was calculated based on the results obtained from in vitro study (34). In our previous study, we observed that the maximum effect of UFH and LMWH administered subcutaneously was at 30 minutes. Therefore, we intravenously administered protamine to the mice 30 minutes after heparin injection. Five minutes after protamine injection, the blood was collected and mouse plasma was used to
determine FXa activity. We expected that in the absence of protamine, FXa is inhibited by heparin. Therefore, the FXa activity should be suppressed. However, when protamine is given, FXa is released from heparin. Thus, FXa is reversed back to normal. Indeed, compound 5 showed similar result to UFH such that in the presence of protamine, FXa activity was reversed to levels similar to the PBS-treated group. As expected, enoxaparin was only partially neutralized by protamine (Figure 23).

![Figure 23 Ex vivo protamine neutralization](image)

**Figure 23 Ex vivo protamine neutralization**

*Ex vivo* reversibility of anti-FXa activity by protamine. The inhibition of FXa activity by the test compounds was significantly affected in the presence of protamine (*P* < 0.05 and ****P* < 0.0001). The study was performed on 8-week-old male C57BL/6J male mice (*n* = 4 per group). Under isoflurane anesthesia, mice were subcutaneously administered with PBS, UFH (3 mg/kg), enoxaparin (3 mg/kg) or compound 5 (0.6 mg/kg) 30 min before a protamine administration. Protamine (15 mg/kg) or PBS was administered intravenously via retro-orbital plexus injection, and 5 min later, blood samples were drawn from the inferior vena cava. Blood samples were centrifuged at 4,000g for 15 min at 4 °C to obtain mouse plasma. Mouse plasma was then used to determine anti-FXa activity. *Ex vivo* analysis of anti-FXa activity was done similarly to the *in vitro* study described earlier. Statistical analysis for multiple comparisons was performed by two-way analysis of variance (ANOVA) with Bonferroni’s *post hoc* test. Data presented is the average of three to five determinations ± s.d.

To further demonstrate the ability of protamine to stop bleeding in mice that were given with compound 5, we used a mouse-tail-clip bleeding model. The experimental steps were carried out as described in the *ex vivo* study. Five minutes after protamine injection, we cut the distal part of the mouse’s tail. The tail was placed in a saline solution and observed for blood loss. We recorded the time that bleeding occurred during 30 minutes. The primary bleeding time
was defined as the time to the first cessation of bleeding (Figure 24A). Subsequently, time for each reinitiated bleeding was also recorded, and used to calculate total bleeding time (Figure 24B). Indeed, protamine shortened the prolongation of both primary bleeding time and total bleeding time induced by compound 5. The blood collected in PBS was used to calculate total blood volume loss. Indeed, protamine reversed the FXa activity of compound 5; the volume of blood loss was similar to a control group (Figure 24C). The results from tail bleeding model confirmed the sensitivity of compound 5 to protamine neutralization in vivo.
A. Mouse model of tail bleeding

Effect of compound 5 and protamine on tail-bleeding time after tail transection. Under isoflurane anesthesia, 8-week-old male C57BL/6J mice (n = 8 per group) were administered with PBS or compound 5 (0.6 mg/kg) subcutaneously, and 30 min later PBS or protamine (15 mg/kg) was administered via retro-orbital intravenous injection. After 5 min the distal part of the tail was transected. The tail was immediately placed in the pre-warmed PBS (37 °C), and blood loss was observed for 30 min. The primary bleeding time was defined as the time to the first cessation of bleeding. Subsequently, time for each reinitiated bleeding was also recorded, and used to calculate total bleeding time. One mouse in the treatment group of compound 5/protamine received an inaccurate protamine injection and was excluded from the study. The blood collected in PBS was used to calculate total blood volume loss with a standard curve generated from the known amounts of blood. Statistical analysis between each group was performed by one-way ANOVA followed by Bonferroni’s multiple-comparison test.
Conclusion

It has been established that the sulfation pattern as well as the size of oligosaccharide fragments are key factors contributing to the interaction between heparin and heparin-binding proteins. In order to design a new generation of the synthetic LMWH that is safe for patients with renal insufficiency, the pharmacokinetic and pharmacologic properties of LMWH were taken into consideration. In addition to the potent anticoagulation property, hepatic clearance and a high sensitivity to protamine neutralization are also critical aspects for developing a new generation of LMWH.

The size and the sulfation pattern of LMWH are factors that dictate whether LMWH is metabolized by the liver or excreted by the kidney (23). As demonstrated previously in a cell based-assay, oligosaccharides larger than a decasaccharide with 3-O-sulfo-modification showed increased binding to stabilin, a scavenger receptor that mediates heparin clearance in the liver (23). Compound 1-5 were also tested for their affinities to stabilin-2 both in vitro and in vivo; the results showed that the larger oligosaccharides, compound 3, 4 and 5, were significantly internalized by stabilin-2 and retained in the liver, in contrast to the smaller oligosaccharides, compound 1 and 2 (34).

The excellent sensitivity toward protamine neutralization shown by compound 5 suggests that both sulfation pattern and size contribute to its sensitivity to neutralization. Enoxaparin is a mixture of oligosaccharides with a broad size distribution fragments, having different affinities toward protamine. Our data suggested that protamine only neutralized fragments that are larger than decasaccharides. Smaller chains, pentasaccharide to decasaccharide, still display anticoagulant activity even though they are not protamine reversible. Therefore, only partial neutralization of FXa activity for commercial LMWH can be achieved with protamine. Compound 5 has a uniformly high affinity to protamine because it is a structurally homogeneous
compound. In addition, our results demonstrate that an extra 3-\(O\)-sulfate group significantly increases the sensitivity for protamine neutralization, suggesting that the sulfation pattern has a critical role in protamine binding.

Taken together, the clearance study and our study suggested that compound 5 serves as a good candidate for a new generation of LMWH. Although new oral anticoagulants, direct FXa and direct thrombin inhibitors have been recently approved by US FDA, the antidotes for these oral drugs are under development and await approval by a regulatory agency. Compound 5 is a promising LMWH that can be neutralized by protamine, an FDA-approved antidote for UFH.
CHAPTER V
PROBING HEPARIN-LIKE OLIGOSACCHARIDES FOR P-SELECTIN BINDING

Introduction

Although heparin has been commonly used for treatment of thromboembolic disorders for more than 70 years, the potential uses of heparin toward inflammatory diseases have long been extensively studied (7). Heparin is known to interact with a diverse array of proteins, including biological proteins that play roles in immunological responses (7). A growing body of literature reported that UFH, LMWH and some size defined-heparin oligosaccharides inhibit selectins (7, 67, 79-81), the adhesion receptors whose main function is to promote adhesion between leukocytes and platelets to endothelial cells.

Selectins are transmembrane glycoproteins expressed on certain cell types. The selectin family comprises L-(leukocyte), P-(platelet) and E-(endothelial) selectins. The fundamental functions of selectins are to bind their carbohydrate-containing ligand and mediate the rolling of leukocytes on activated endothelial cells (82). P-selectin glycoprotein ligand 1 (PSGL-1) is a glycoprotein that carries tyrosine sulfate as well as a sugar moiety, sialyl Lewis X (SLex) at its N-terminus (83). These two modifications on the glycoprotein dictate its role in binding to selectins.

Inflammation is a multi-step event, which takes place upon injury or infection. At the site of inflammation, leukocytes are recruited to the injured tissues in order to invade into the cells and release the proteolytic mediators to protect tissue injury. Selectins participate in the
initial inflammatory process by mediating the adhesion and the rolling of leukocytes to the cell surface (84). At the site of inflammation, P-selectin is found expressed on activated endothelial cells. Leukocytes that express PSGL-1 can interact with the P-selectin. Therefore, P-selectin promotes the adhesion and the rolling of leukocytes. The immobilized leukocytes can further interact with integrin to invade the cells.

Sickle cell disease (SCD) is a hematologic disorder caused by a point mutation of beta globulin chain leading to an abnormal shape of hemoglobin, resulting in sickled red blood cells (RBC) that are less flexible, adhere to the endothelium and are prone to hemolysis (85). Sickling of RBC causes vaso-occlusion, which is responsible for most of the severe complications of SCD (86). The adhesion of sickle RBC to the vascular endothelium is important to the generation of vascular occlusion that leads to vascular inflammation (87). Vascular inflammation, in turn, contributes to coagulation (85). Several studies suggested the role of P-selectin in the pathophysiology of SCD (88-90). For example, expression levels of P-selectin were found to be elevated in patients with SCD (88). In addition, the disruption of PSGL-1 can reduce inflammation associated with P-selectin in sickle cell mice (87).

Heparin is a component that is synthesized and stored in mast cells. In an inflammatory event, heparin is released from mast cells with other mediators such as histamine and proteases. Heparin is known to inhibit the binding between PSGL-1 and P-selectin (67, 79-81, 91-92). Whether heparin has a protective effect against excessive inflammation remains to be investigated. However, heparin has been shown to reduce inflammation through the inhibition of selectins (92). Moreover, the parental effect of heparin is its anticoagulation effect. Taken together, these two biological properties of heparin would be of benefit for SCD patients. Although the function of heparin in the coagulation cascade has been known for a long time,
information on the structural requirements for heparin binding to selectin are limited. For more than a decade, the defined structures of heparin-like oligosaccharides have been awaited to investigate their binding to selectins due to difficulties in obtaining homogeneous compounds to probe the binding activity. With a promising approach of an enzymatic method, in this study, different size of heparin oligosaccharides were enzymatically synthesized. We utilized these oligosaccharides to probe the structural requirements for P-selectin binding.

**Investigation of short oligosaccharides for P-selectin binding**

Previous studies indicated that heparin interferes with L- and P- but not E-selectins binding to their ligands (67, 80-81). P-and E-selectins are expressed at reasonably high density on the lumina plasma membrane of vascular endothelial cells at the site of inflammation (92). Therefore, we narrowed our study and focused only on P-selectin. Furthermore, the studies suggested that certain sulfation patterns as well as lengths of oligosaccharides are required for P-selectin binding. In this study, we aimed to probe the size and the sulfation pattern of heparin oligosaccharides that are sufficient for P-selectin binding. Because selectins are carbohydrate recognition receptors that bind to sialylated and fucosylated glycoprotein ligands, we utilized a well-developed competition ELISA assay in which sialyl Lewis X polyacrylamide (SLex-PAA) was immobilized on a 96-well plate. In this study, oligosaccharides with defined length and sulfation were assayed for their ability to inhibit binding between the immobilized SLex and P-selectin chimera. Recently, we successfully synthesized oligosaccharides ranging from octa- to dodeca-saccharides (8 mer, 10 mer and 12 mer) (34). These oligosaccharides are size-defined and differ in their number of the IdoA2S-GlcNS6S repeating units (Figure 25 upper, Table 7). We tested the inhibitory effects of these compounds on P-selectin and found that all of them failed to bind P-selectin (Figure 25 lower). This result showed that oligosaccharides with the
lengths up to 12 sugar residues were too short to inhibit P-selectin. Furthermore, we observed that a high density of sulfo groups on these compounds did not enhance the binding activity similarly to a result observed from unmodified K5 polysaccharide (K5P). K5P is a capsular polysaccharide obtained from the *E. coli* K5 strain whose structure consists of a disaccharide repeating unit of GlcA-GlcNAc similar to the unmodified backbone of heparin. Our results are largely consistent with the previous studies (67, 79-80, 91).

Figure 25 Probing of short oligosaccharides (8 mer-12 mer) for P-selectin binding
Upper panel shows the structures of short size oligosaccharides.
Lower panel shows inhibition of P-selectin-Ig binding to immobilized PAA-SLe³. Inhibition curves were generated using heparin (black), K5P (blue), 8 mer (orange), 10 mer (light green), 12 mer (purple), K5P (blue). Inclusion of EDTA blocked binding by more than 90%. Each point represents the average of duplicate determinations, and the data are representative of 3-5 separate experiments.
### Table 7 Summary of compounds used to probe P-selectin binding

<table>
<thead>
<tr>
<th>Oligosaccharides</th>
<th>M.W. (Da)</th>
<th>Structure</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Short oligosaccharides</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>8 mer</td>
<td>2368.9</td>
<td>GlcNS6S-GlcA-GlcNS3S6S-(IdoA2S-GlcNS6S)$_1$-GlcA-pNP</td>
</tr>
<tr>
<td>10 mer</td>
<td>2946.4</td>
<td>GlcNS6S-GlcA-GlcNS3S6S-(IdoA2S-GlcNS6S)$_2$-GlcA-pNP</td>
</tr>
<tr>
<td>12 mer</td>
<td>3523.7</td>
<td>GlcNAc6S-GlcA-GlcNS3S6S-(IdoA2S-GlcNS6S)$_3$-GlcA-pNP</td>
</tr>
<tr>
<td><strong>Medium-length oligosaccharides</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>13 mer-NS6S</td>
<td>3297.1</td>
<td>GlcA-GlcNS6S-GlcA-GlcNS6S-(GlcA-GlcNS6S)$_4$-GlcA-pNP</td>
</tr>
<tr>
<td><strong>Polysaccharides</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Heparin</td>
<td>~14,000</td>
<td>-(IdoA2S-GlcNS6S)$_n$</td>
</tr>
<tr>
<td>K5P</td>
<td>~49,000</td>
<td>-(GlcA-GlcNAc)$_n$</td>
</tr>
</tbody>
</table>

**P-selectin binding requires N-sulfation and 6-O-sulfation**

A number of studies investigating heparin binding to P-selectin reported consistent findings on the significant presence of a sulfo group at the 6-O-position of GlcN residues. However, whether removing sulfo groups at N-, 2-O- and 3-O-positions from heparin affects the binding of heparin to P-selectin is less conclusive (81, 93). A chemoenzymatic synthesis of heparin was first employed in the synthesis of structurally heterogeneous polysaccharides. In this method, a bacterial capsular polysaccharide isolated from *E. coli* K5 strain is subjected to a series of enzymatic modifications, including N-sulfation, epimerization, 2-O-, 6-O- and 3-O-sulfations. The products have desired biological activities as long as the appropriate sulfation is introduced, including anticoagulant activity (94-95).
K5P has the same -(GlcA-GlcNAc)ₙ structure as the precursor polysaccharide generated in heparin biosynthesis (Figure 26). Due to the similarity of the repeating units in K5P and the unmodified backbone of heparin, we utilized K5P to add defined sulfo groups in order to identify the sulfation patterns that are required for P-selectin binding. The result obtained from using the modified K5P is useful for the later design of heparin oligosaccharides targeting P-selectin. In heparin biosynthesis, N-sulfation is critical for later modifications. A bifunctional enzyme, N-deacetylase /N-sulfotrasferase (NDST), is responsible for removing acetyl groups and adding sulfo groups to the GlcNAc residues. However, the conversion of GlcNAc to GlcNS residue by NDST has a special pattern, depending on the isoform (57). Therefore, we implemented an alkaline treatment to remove acetyl groups from K5P and further treated the resultant free amino-K5P (K5P-NH₂) with N-sulfotransferase (NST). However, K5P-NH₂ was largely insoluble in water. Therefore, prior to enzymatic sulfation by NST, a chemical N-sulfonation was introduced to improve the solubility of the modified K5P. Chemical N-sulfation partially added sulfo groups. We further completed the N-sulfation using NST and a sulfate donor, PAPS. The resulting NS modified-K5P (K5P-NS) was tested for P-selectin binding activity. As expected, we found that N-sulfation modification alone was not sufficient enough for the polysaccharide to interact with P-selectin (Figure 27A).
Figure 26 Disaccharide repeating units of the polysaccharides used in the study

K5P = K5 polysaccharide derived from *E. coli* K5. The unmodified K5P has similar structure to the unmodified heparin, thus K5P can be used to probe the sulfation patterns required for P-selectin binding. K5P-NS = K5 polysaccharide carries a sulfo group at N-position of GlcN, K5P-NS6S = K5 polysaccharide carries sulfo groups at N- and 6-O-positions of GlcN. K5P-NS2S6S = K5 polysaccharide carries sulfo groups at N- and 6-O-positions of GlcN and at 2-O-position of IdoA. K5P-NS6S = K5 polysaccharide carries sulfo groups at N-, 3- and 6-O-positions of GlcN.

It is important to note that heparin comprises 70-80% disaccharide IdoA2S-GlcNS6S repeating units. Next, we intended to determine whether the presence of IdoA2S is critical for P-selectin binding. We attempted to compare P-selectin binding activity between the repeating structures of -GlcA-GlcNS6S- and -IdoA2S-GlcNS6S- (Figure 26). To this end, K5P-NS was subjected to two separate reactions. The first reaction was to modify K5P-NS with C5-epimerase and 2-OST prior to 6-O-sulfation. In the second reaction, K5P-NS was directly treated with 6-OSTs. The presence of 2- and 6-O-sulfo groups on K5P was investigated by disaccharide analysis. The result showed that more than 80% of K5P carry the desired sulfo groups (Figure 27B). The resulting N-,2-O-, 6-O-K5P (K5P-NS2S6S) and N-,6-O-K5P (K5P-NS6S) showed similar binding activity to P-selectin, suggesting that the K5PNS6S can bind P-selectin regardless of the presence of the IdoA2S (Figure 27A, Table 7). Our results are largely consistent with several previous studies (81, 93, 96).
Panel A shows inhibition of P-selectin-Ig binding to immobilized PAA-SLe\(^x\). Inhibition curves were generated using heparin (black), K5P-NS (dark blue), K5P-NS6S (light blue), K5P-NS2S6S (light purple). Inclusion of EDTA blocked binding by more than 90%. Each point represents the average of duplicate determinations, and the data are representative of 3-5 separate experiments.

Panel B shows disaccharide analysis of the modified K5P. The K5P-NS6S was \(^{35}\text{S}\)-labeled at N- and 6-O-sulfo positions (Panel B, upper). K5P-NS2S6S was \(^{35}\text{S}\)-labeled at N-, 2-O and 6-O-sulfo positions (Panel B, lower). The polysaccharides were then treated with heparin lyases I, II and III. The resultant disaccharides were resolved by reverse phase ion-pairing (RPIP) HPLC. \(\Delta \text{UA} = \Delta^{\text{i, j}}\)-unsaturated uronic acid.

**Investigation of medium-length oligosaccharides for P-selectin binding.**

Based on the results obtained in the previous sections, we learned that N- and 6-O-sulfations are critical for oligosaccharide and P-selectin interactions. Moreover, the length of the oligosaccharide is also important for P-selectin binding. Our goal was to synthesize a homogeneous compound with a defined structure that can inhibit P-selectin at a higher potency than that of heparin. Since the target size of the oligosaccharide was unknown, we proposed to
synthesize oligosaccharides between 13 and 17 sugar residues due to the fact that the oligosaccharides need to be at least longer than 12 sugar residue for P-selectin binding. The oligosaccharides were designed to present a repeating structure of –(GlcA-GlcNS6S)- (Table 7). We utilized a commercially available 1-O-(para-nitrophenyl)-glucuronide (GlcA-pNP) as a starting monosaccharide. We elongated the monomer by alternately adding UDP-N-trifluoroacetyl (UDP-N-TFA) and UDP-GlcA to obtain an oligosaccharide backbone of the desired size. Using unnatural UDP-GlcN-TFA aids in the sulfo group insertion at the N-position of GlcN. To synthesize tridecasaccharide (13 mer), the GlcA-pNP was first elongated to a heptasaccharide (7 mer); the trifluoroacetyl groups on 7 mer were then removed by LiOH treatment, and the sulfo groups were added by NST coupling with PAPS resulting in an N-sulfo-heptasaccharide (7 mer-NS). 7 mer-NS was elongated to 13 mer, which was later subjected to N-sulfation. Once an N-sulfo-tridecasaccharide (13 mer-NS) was obtained, the compound was treated with 6-OSTs yielding N-, 6-O-sulfo-13 mer (13 mer-NS6S). The homogeneous 13 mer-NS6S was tested for P-selectin binding. The purity of 13 mer-NS6S is shown in Figure 28. We observed that 13 mer-NS6S still lacked P-selectin binding activity (Figure 30A upper). We then elongated 13 mer-NS6S to a heptasaccharide (17 mer). Once 17 mer was obtained, the N-sulfation and 6-O-sulfation were repeated, resulting in the N-,6-O-sulfo-heptasaccharide (17 mer-NS6S). The purity of 17 mer-NS6S is shown in Figure 29. We found that 17 mer-NS6S began to show some binding activity (Figure 30A, upper). Nevertheless, the binding activity of 17 mer-NS6S (55 μg/mL) was less than that of K5P-NS6S (0.4 μg/mL) and heparin (1.5 μg/mL) (Table 8).
Figure 28 Structural characterization and purity of 13 mer-NS6S.

The top panel shows the structure of 13 mer-NS6S. Panel A shows the purity of 13 mer-NS6S after Q-sepharose purification resolved by high resolution DEAE-HPLC. Panel B shows the ESI-MS spectra of 13 mer-NS6S.
Figure 29 Structural characterization and purity of 17 mer-NS6S
The top panel shows a structure of 17 mer-NS6S. Panel A shows the purity of 17 mer-NS6S after Q-sepharose purification resolved by high resolution DEAE-HPLC. Panel B shows the ESI-MS spectra of 17 mer-NS6S.

*N, 3-O-,6-O-heparosan demonstrated both anti-P-selectin and anti-FXa activity.*

It has been well established that 3-O-sulfo modification at GlcNS±6S is critical for oligosaccharide binding to AT (6, 69), thus inhibiting FXa. To this end, we used 3-OST-1 and 3-OST-5 to add sulfo groups on 17 mer-NS6S to obtain a repeating structure of -(GlcA-GlcNS3S6S)-. Using two isoforms of 3-OSTs aids in driving the addition of 3-O-sulfo groups to completion. After the reaction, the purity of the 3-O-sulfated product (17 mer-NS3S6S) was approximately 80%. We presumed the presence of 3-O-sulfo groups on 17 mer-NS3S6S enhances P-selectin binding activity due to the increase in charge density. However, the addition of 3-O-sulfo groups only increased 4-fold binding activity of 17 mer-NS3S6S in comparison to 17 mer-NS6S. In addition, the binding activity of 17 mer-NS3S6S (14 μg/mL) was still
approximately 10-fold less than heparin (1.5 μg/mL) (Table 8). From the results, we speculated that P-selectin might require larger sized oligosaccharides for binding. Therefore, we decided not to continue the elongation on 17 mer but to modify K5P-NS6S with 3-OSTs to determine whether the inhibition effect would increase. The presence of the repeating -(GlcA-GlcNS3S6S)- on K5P is shown in Figure 30B. Interestingly, the resultant 3-O-sulfo-K5PNS6S (K5P-NS3S6S) exhibited about 30-fold better binding than heparin (Figure 30A lower, Table 8). Next, we determined the anti-FXa activity of 17 mer-NS3S6S and K5P-NS3S6S. Surprisingly, K5P-NS3S6S was about 2.5-fold more potent in FXa inhibition than 17 mer-NS3S6S and heparin (Figure 30C). A summary of the IC_{50} values of the compounds is provided in Table 8.

<table>
<thead>
<tr>
<th>Compounds</th>
<th>IC_{50} P-selectin (μg/mL)</th>
<th>FXa (ng/uL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heparin</td>
<td>1.5</td>
<td>17</td>
</tr>
<tr>
<td>17 mer-NS6S</td>
<td>55</td>
<td>N.D.</td>
</tr>
<tr>
<td>17 mer-NS3S6S</td>
<td>14</td>
<td>18</td>
</tr>
<tr>
<td>K5P-NS6S</td>
<td>0.4</td>
<td>N.D.</td>
</tr>
<tr>
<td>K5P-NS2S6S</td>
<td>0.4</td>
<td>N.D.</td>
</tr>
<tr>
<td>K5P-NS3S6S</td>
<td>0.05</td>
<td>7</td>
</tr>
</tbody>
</table>

N.D. = not detected
Figure 30 Inhibition of P-selectin and FXa when 3-O-sulfo groups were introduced

Panel A shows inhibition of P-selectin-Ig binding to immobilized PAA-SLe\(^\text{c}\). Inhibition curves were generated using heparin (black), K5P-NS6S (light blue), 13 mer-NS6S (red), 17 mer-NS6S (dark red), 17 mer-NS3S6S (magenta), K5P-NS3S6S (blue). Inclusion of EDTA blocked binding by more than 90%. Each point represents the average of duplicate determinations, and the data are representative of 3-5 separate experiments.

Panel B shows disaccharide analysis of the modified K5P-NS3S6S. A presence of GlcA-AnManNS3S6S was analyzed by nitrous acid degradation. K5P was radiolabeled at N-, 3-O-, 6-O-positions. The \(^3\)S-labeled 3-O-sulfated product was deacetylated by hydrazine and exposed to nitrous acid at both pH 4.5 and 1.5. The resultant disaccharides were analyzed by reverse-phase ion-pairing HPLC.

Panel C shows 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 in different color: Heparin (black), K5P-NS3S6S (blue), 17 mer-NS3S6S (magenta), K5P-NS6S (light blue).

Conclusion

For the past decades, many efforts have been made to understand the pathophysiology of SCD. Vaso-occlusive crisis is a major cause of morbidity and mortality in SCD patients. The vaso-occlusion is a result of adhesive interactions between circulating sickle red blood cells, leukocytes and endothelial cells. Evidence suggested that P-selectin presenting on endothelial
cells plays a key role in leukocyte recruitment as well as the adhesion of sickle red blood cells to endothelium. Currently, the available treatment for SCD patients is considered to be supportive therapy, as mechanism-based drugs remain to be investigated. Various approaches to interfering with P-selectin-mediated adhesion are under study or development. Several classes of P-selectin inhibitors have been developed, for example, an antibody for P-selectin (82), P-selectin aptamer (88), and a recombinant truncated form of PSGL-1-immunoglobulin fusion protein. Interestingly, an orally absorbable semisynthetic sulfated polysaccharide showed 10 times the potency of heparin in P-selectin inhibition and is now in a phase II clinical trial (89).

Heparin is known to have an inhibitory effect on P-selectin; thus, heparin has a potential use for treatment of diseases whose pathophysiology associated is with P-selectin. However, the potent anticoagulation effect of heparin has raised concerns about bleeding complications in treatment of diseases in which anticoagulation is not a primary goal. However, SCD patients might benefit from the anticoagulant property of heparin. Recently, Sparkenbaugh and colleagues demonstrated that FXa contributes to vascular inflammation in a mouse model of SCD (97). They demonstrated that rivaroxaban, a direct FXa inhibitor, significantly reduced the inflammation markers in sickle cell mice through the inhibition of FXa. Their results suggest that in addition to blocking P-selectin, inhibition of FXa would have a synergistic effect in inflammatory prevention in SCD. Based on this finding, heparin oligosaccharides that inhibit both P-selectin and FXa would be an alternative treatment for SCD patients.

Our primary goal was to synthesize a homogeneous oligosaccharide targeting both P-selectin and FXa. The homogeneity can enhance the potency for both anticoagulation and anti-inflammation activities in comparison to heparin, which is a heterogeneous compound. Moreover, a defined sulfation pattern can define the structural requirements for P-selectin
binding, which is still unknown. Several studies showed that a smaller size pentasaccharide, fondaparinux, does not bind P-selectin, while a portion of high-molecular-weight oligosaccharides in tinzaparin, a LMWH composed primarily larger fragments, has greater affinity to P-selectin (67, 80). This was consistent with the finding that the anticoagulant property of heparin is related to the selectin-binding property. Our results are consistent with the others in that smaller oligosaccharides have no binding activity to P-selectin, as observed from 8 mer, 10 mer, 12 mer and 13 mer. Furthermore, the result from modified K5P emphasized a critical role of N- and 6-O-sulfation (81). Unexpectedly, the size of 17 mer-NS6S could not improve the binding activity to P-selectin. Our result indicated that the size of oligosaccharide required for P-selectin binding needs to be longer than 17 sugar residues. Otherwise, a specific interaction contributing from sulfation pattern is required for binding.

Challenges in the synthesis of multiple IdoA2S residues as well as structural characterization of larger oligosaccharides have limited our study. Since the medium-size oligosaccharides used in this study lack repeating IdoA2S residues, IdoA2S participation in binding to P-selectin remains to be addressed. Furthermore, whether 3-O-sulfation is essential to a specific interaction with P-selectin needs to be further explored. Both K5P-NS3S6S and 17 mer-NS3S6S greatly enhanced the binding activity compared to their non-3-O-sulfated parents. However, 17 mer-NS3S6S, which is more homogeneous than K5P-NS3S6S, showed only a small improvement in binding activity (Table 8), suggesting that the enhanced binding activity was likely a result of the increased intensity of charge interaction. Furthermore, the results from modified K5P indicated that 3-O-sulfation is more important for the interaction than 2-O-sulfation, as observed from the binding activity between K5P-NS2S6S and K5P-NS3S6S.
We showed that 17 mer-NS3S6S has comparable anti-FXa activity to heparin whereas K5P-NS3S6S is more potent than heparin in FXa inhibition (Figure 30C). We previously showed that the homogeneity of heparin oligosaccharides greatly enhanced its ability to inhibit FXa (34). Although K5P-NS3S6S is heterogeneous in its size and structure, the strong inhibition of FXa potentially came from the presence of GlcNS3S6S that is critical for AT binding.

In summary, we compared the binding activity to P-selectin in three groups of heparin: short, medium-length and large oligosaccharides. Our results determined that short length oligosaccharides have no binding activity to P-selectin. Our results also suggest that a larger size oligosaccharide plays role in the inhibition of P-selectin. In addition, we showed that N- and 6-O-sulfation are critical for oligosaccharide binding to P-selectin. Although the semi-synthetic K5P-NS3S6S is a mixture, its high potency in P-selectin and FXa inhibition suggest that K5P-NS3S6S can serve as a candidate to further investigating its role in sickle cell mice. Although a specific structure required for binding with P-selectin remains to be explored, our results provide a foundation for a basic structure of a heparin oligosaccharide that contributes to P-selectin binding.
A chemoenzymatic approach has served as a useful alternative to synthesize complex carbohydrate-containing molecules that surpass the limitations of organic syntheses. An enzyme-based approach has been developed as a method to produce a safer LMWH (10, 34, 49, 56). The enzymatic method also offers a means to rationally design heparin-like compounds with improved pharmacological effects. The use of biosynthetic enzymes not only provides a high regio- and stereo-selectivity for the formation of glycosidic bonds, but also avoids the protection and deprotection steps required for chemical synthesis, thus significantly improving the synthesis efficiency. The discovery of bacterial counterparts and advances in bioengineering technologies led to a breakthrough in complex carbohydrate synthesis. The method offers a new strategy to prepare synthetic heparin with a goal of replacing the heparin that is isolated from animal tissues to secure the safety of heparin supply chain (98).

Among the three forms of heparin, the most favorable form is LMWH owing to its improved pharmacokinetic properties, reduced risk of bleeding side effects and minimized HIT. Commercial LMWHs are produced by the depolymerization of the larger size heparin, resulting in heparin fragments that are not uniform in their sizes. The polydispersity of LMWH products affect metabolic clearance such that smaller oligosaccharides are excreted by kidney while larger oligosaccharides are cleared by the liver, thus limiting its use in kidney impaired patients. In addition, LMWH is partially neutralized by protamine. Protamine can only neutralize LMWH
fragments that are larger than a decasaccharide, leaving the smaller oligosaccharides exposed to AT. Taken together, the new generation of a synthetic LMWH that will meet the clinical requirements should display high purity, liver clearance and a high sensitivity to protamine.

The first step in preparing synthetic heparin is to build a sugar backbone. Typically, a production of an oligosaccharide backbone, for example, a decasaccharide, requires about 10-12 days. Using a controlled enzymatic method by varying the ratio between a sugar donor and a sugar acceptor combined with the use of a bifunctional glycosyltransferase, the production time decreases to 2-3 days. We demonstrated that the controlled one-pot method is capable of producing desired oligosaccharides with enhanced potency demonstrated in vivo. However, the one-pot method is not ideal for creating a homogeneous compound because the method can only minimize polydispersity. With the knowledge of the enzyme specificity, we were able to synthesize a homogeneous compound carrying high potency in the inhibition of FXa. In addition, the synthetic compound indeed shows the excellent sensitivity to protamine as observed in a tail bleeding model in mice. This synthetic LMWH is also taken up by a scavenger receptor in the mouse liver. The results suggest that this new generation of LMWH will have benefits in patients with renal insufficiency.

Heparin not only binds to AT; its highly negative charge can interact with a diverse array of biological proteins, including P-selectin. P-selectin is identified to play role in both inflammation and cancer. Heparin oligosaccharides carry both anti-FXa and anti-P-selectin activities. The molecule would be beneficial to SCD patients who suffer from both vascular inflammation and coagulation. Our results suggested that N- and 6-O-sulfo modifications on heparin chain are critical for P-selectin binding.
In summary, a chemoenzymatic method offers a promising platform to prepare HS and heparin for desired clinical applications and for further studying the functions of biological proteins.
APPENDIX
CURRICULUM VITAE
KASEMSIRI CHANDARAJOTI

2701 Homestead Road, Chapel Hill, NC 27516 • (919) 537-2038 • chandara@email.unc.edu

Education
Ph.D. candidate, Pharmaceutical Sciences 2009-present
Division of Chemical Biology and Medicinal Chemistry
Eshelman School of Pharmacy
University of North Carolina at Chapel Hill

Bachelor of Pharmacy (B. Pharm.) with highest honor 2002-2007
Faculty of Pharmaceutical Sciences (major: Pharmaceutical chemistry)
Prince of Songkla University, Songkhla, Thailand

Research Experience
Doctoral Candidate, Dr. Jian Liu laboratory, UNC-Chapel Hill 2010-present
Developing a chemoenzymatic method to synthesize heparin oligosaccharides targeting Factor Xa and P-selectin.

Research Assistant, Dr. Jasadeee Kaewsrichan, Prince of Songkla University, Songkhla, Thailand. Investigating the role of FGF2 and BMP2 in marrow stromal cells.

Publications


**Fellowship**
The Royal Thai Government Award Pre-doctoral Fellowship 2009-2014

**Professional Experience**

**Laboratory Mentor**
Monitored a summer project for North Carolina School of Science and Math student. Project: Endotoxin removal of heparin oligosaccharides produced from a chemoenzymatic method.

**Laboratory Mentor**
Monitored a summer project for biophysical society student. Project: Optimization of large scale K5 polysaccharide preparation.

**Graduate teaching assistant, Medicinal Chemistry II and Biochemistry I**
Chemical Biology and Medicinal Chemistry, Eshelman School of Pharmacy, UNC-Chapel Hill. Proctored and graded tests and homework, updated Blackboard, held weekly office hours, facilitated and graded presentations.

**Graduate teaching assistant, Medicinal Chemistry I**
Chemical Biology and Medicinal Chemistry, Eshelman School of Pharmacy, UNC-Chapel Hill. Graded tests and updated Blackboard.

**Lecturer, Pharmaceutical Analysis (B. Pharm.) and Drug Analysis (Pharm D. )**
Department of Pharmaceutical Chemistry, Faculty of Pharmaceutical Sciences, Prince of Songkla University, Songkhla, Thailand.

**Poster Presentations**

**Chandarajoti K, Xu Y, and Liu, J.** Developing a chemoenzymatic method for low molecular weight heparin. Abstract for poster presentation, Graduate Student Recruitment Weekend, Division of Chemical Biology and Medicinal Chemistry, Eshelman School of Pharmacy, Chapel Hill, NC, 2011.

REFERENCES


