Preparation of Low Molecular Weight Heparin from a Remodeled Bovine Intestinal Heparin

Sultan N. Baytas,* Sony S. Varghese, Weihua Jin, Yanlei Yu, Peng He, Marc Douaisi, Fuming Zhang, Paul Brodfuehrer, Ke Xia, Jonathan S. Dordick, and Robert J. Linhardt*

ABSTRACT: Bovine intestinal heparins are structurally distinct from porcine intestinal heparins and exhibit lower specific anticoagulant activity (units/mg). The reduced content of N-sulfo, 3-O-sulfo glucosamine, the central and critical residue in heparin’s antithrombin III binding site, is responsible for bovine intestinal heparin’s reduced activity. Previous studies demonstrate that treatment of bovine intestinal heparin with 3-O-sulfotransferase in the presence of 3′-phosphoadenosine-5′-phosphosulfate afforded remodeled bovine heparin with an enhanced activity reaching the United States Pharmacopeia’s requirements. Starting from this remodeled bovine intestinal heparin, we report the preparation of a bovine intestinal low molecular weight heparin having the same structural properties and anti-factor IIa and anti-factor Xa activities of Enoxaparin. Moreover, this bovine intestinal heparin-derived “Enoxaparin” showed comparable platelet factor-4 binding affinity, suggesting that it should exhibit similarly low levels of heparin induced thrombocytopenia, HIT.

INTRODUCTION

Heparin, a member of the sulfated glycosaminoglycan (GAG) family together with heparan sulfate (HS), chondroitin sulfate (CS), dermatan sulfate (DS), and keratan sulfate (KS), is the most negatively charged biological molecule known. Heparin and HS are comprised of 1,4-glycosidically linked β-D-glucuronic acid (GlcA) and uronic acid (UA). GlcN can be present as GlcNS, GlcNAc, GlcNAc6S, GlcNS6S, GlcNS3S6S, GlcNAcAS, and GlcNAc3S6S as a result of sulfo or acetyl substitution at the 2-, 6-, and 3-position. UA consists of α-L-iduronic acid (IdoA) or β-D-glucuronic acid (GlcA), both of which can be sulfated at their 2-position (i.e., IdoA2S and to a lesser extent GlcA2S). HS is considerably less sulfated than heparin and has a much lower anticoagulant activity. The heparin polysaccharide (Figure 1A) is composed of disaccharide repeating units, including the major repeating unit that is trisulfated (Tris), (→ 4) α-L-IdoA2S (1 → 4) α-D-GlcNS6S (1 →). The polydispersity, high negative charge, and structural variations of its chains make heparin highly heterogeneous, which allows heparin to interact with a wide range of proteins resulting in myriad biological activities. Unfractionated heparin (UFH), obtained from animal tissues, is a large polysaccharide of molecular weight (MW) ~ 20 kDa and can contain a unique pentasaccharide sequence motif, GlcNAc/NS6S → GlcA → GlcNS3S6S → IdoA2S → GlcNS6S. Heparin specifically binds to the serine protease inhibitor, antithrombin III (AT) through that pentasaccharide, resulting in inhibition of factor IIa (thrombin) and factor Xa (FXa). The sulfo group at C3 position of the glucosamine residue in the center of this pentasaccharide has a critical role in AT binding of heparin. Pentasaccharide sequences with such 3-O-sulfated glucosamine residue are randomly distributed along heparin chains, and only 30–80% of these UFH chains contain these pentasaccharide sequence motifs.

Heparin, discovered in 1916, is derived from animal tissues and has been widely used clinically as an anticoagulant since 1935. Bovine lung was the primary source of heparin until 1960, when porcine intestinal mucosa became the preferred alternative due to a simpler extraction process and a higher recovery yield. Until the mid-1990s, the U.S. Food and Drug Administration (FDA) approved the use of bovine UFH in the US. As a result of the emergence and spread of mad cow disease (bovine spongiform encephalopathy, BSE) in the UK in the late 1980s, bovine heparin products were voluntarily withdrawn from the market. Since the mid-1990s, porcine intestinal heparin is the only approved UFH in the EU and has been widely used clinically as an anticoagulant since.

Heparin in the presence of 3′-phosphoadenosine-5′-phosphosulfate afforded remodeled bovine heparin with an enhanced activity reaching the United States Pharmacopeia’s requirements. Starting from this remodeled bovine intestinal heparin, we report the preparation of a bovine intestinal low molecular weight heparin having the same structural properties and anti-factor IIa and anti-factor Xa activities of Enoxaparin. Moreover, this bovine intestinal heparin-derived “Enoxaparin” showed comparable platelet factor-4 binding affinity, suggesting that it should exhibit similarly low levels of heparin induced thrombocytopenia, HIT.
intestinal mucosa as the sole source for heparin. More than 50% of pig livestock is accounted for in China, and China exports most of the crude heparin around the world. If no other alternative anticoagulant agents emerge, due to a possible pandemic of a pig infectious disease in the near future and/or growing demand from developing countries, heparin will eventually be in shortage. In 2007–2008, a heparin contamination crisis resulted from the adulteration of crude porcine intestinal heparin with a toxic semisynthetic oversulfated chondroitin sulfate (OSCS) causing more than 100 deaths in the United States and severely disrupting the heparin supply chain. These factors have led researchers and regulatory agencies to consider alternative sources for heparins, such as the development of bioengineered heparins, synthetic heparins, or the reintroduction of bovine heparin into the pharmaceutical market.

Bovine-sourced heparins exhibit different structures and specific activities (U/mg) than porcine intestinal heparins. The MW of bovine intestinal heparin is slightly lower than that of porcine heparin; it is also more polydisperse, less highly sulfated, and more heterogeneous. Bovine intestinal heparin has also been shown to have lower content of GlcNS3S residues and a slightly higher content of GlcA residues than porcine intestinal heparin. There are also major differences in the biological activities of bovine-sourced and porcine-sourced heparins. Porcine intestinal heparin possesses significantly higher activity than bovine-sourced heparins (≈180–210 U/mg and ≈100–130 U/mg, respectively). Thus, bovine-sourced heparins need to be used in higher gravimetric doses to achieve the same antithrombotic effect as porcine heparin. Moreover, higher doses of protamine, a heparin antidote, are required to neutralize the anticoagulant effect of bovine intestinal heparin.

Recently, we showed that bovine intestinal heparin with low activity could be remodeled with recombinantly expressed O-sulfotransferases (OSTs) to meet USP specifications. Treatment of bovine intestinal heparin with 6-OST-1, -3, and/or 3-OST-1 increased the content of 6-O-sulfo and/or 3-O-sulfo groups and afforded two remodeled heparins that met USP heparin activity, and MW specifications. The disaccharide composition of 6-OST-1, -3, and 3-OST-1 remodeled bovine intestinal heparin was similar to that of porcine intestinal heparin. However, disaccharide composition of the bovine intestinal heparin after treatment only with 3-OST-1, was similar to untreated bovine intestinal heparin and was still dissimilar to porcine intestinal heparin. The level of heparin 3-O-sulfation correlates to heparin’s anticoagulant activity. The 3-OST-1 isoform catalyzes the 3-O-sulfation of a small number of GlcNS6X or GlcNAc6X residues, linked to GlcA/IdoA residue to afford an AT pentasaccharide binding site (Figure 1B). It has been shown that the treatment of bovine intestinal heparin with 3-OST-1 alone afforded a remodeled heparin that met the anti-Xa and anti-IIa activity requirements of USP heparin with a markedly increased level of sulfation and without enhanced platelet factor 4 (PF4) binding. Heparin and PF4 interaction is clinically significant as it may cause...
Table 1. Comparison of Anti-IIa Activity for USP Porcine Intestinal Heparin and Bovine Intestinal Heparin Starting Material and Activity of Remodeled Bovine Intestinal Heparin

<table>
<thead>
<tr>
<th>USP specs MW and activity</th>
<th>MW&lt;sub&gt;15 000–19 000&lt;/sub&gt;</th>
<th>MW&lt;sub&gt;19 000–22 44&lt;/sub&gt;/MW&lt;sub&gt;22 44–24 000&lt;/sub&gt; NLT 1.0</th>
<th>&gt;MW&lt;sub&gt;24 000&lt;/sub&gt; NMT 20%</th>
<th>anti-IIa&lt;sup&gt;*&lt;/sup&gt; NLT 180</th>
</tr>
</thead>
<tbody>
<tr>
<td>USP porcine intestinal UFH</td>
<td>meets specs</td>
<td>meets specs</td>
<td>meets specs</td>
<td>anti-IIa&lt;sup&gt;*&lt;/sup&gt;</td>
</tr>
<tr>
<td>bovine intestinal UFH</td>
<td>18 300</td>
<td>1.4</td>
<td>18.7</td>
<td>121 ± 4.04***</td>
</tr>
<tr>
<td>remodeled bovine intestinal UFH</td>
<td>18 300</td>
<td>1.4</td>
<td>18.7</td>
<td>204 ± 2.52</td>
</tr>
</tbody>
</table>

<sup>*Data are expressed as the mean of two injections. Statistical analyses were calculated via One-Way ANOVA (USP porcine intestinal UFH vs bovine intestinal UFH, ****, p < 0.0001; remodeled bovine intestinal UFH vs bovine intestinal UFH, ****, p < 0.0001; USP porcine intestinal UFH vs remodeled bovine intestinal UFH, not significant).</sup>

Table 2. Comparison of Disaccharide and Tetrasaccharide Composition of USP Porcine Intestinal Heparin, Bovine Intestinal UFH, and Remodeled Bovine Intestinal UFH<sup>a</sup>

<table>
<thead>
<tr>
<th>disaccharides and tetrasaccharides</th>
<th>USP porcine intestinal UFH</th>
<th>starting bovine intestinal UFH</th>
<th>batch reaction</th>
<th>packed bed</th>
</tr>
</thead>
<tbody>
<tr>
<td>ΔUA-GlcNAc (OS)</td>
<td>4.17</td>
<td>3.17</td>
<td>3.23</td>
<td>3.33</td>
</tr>
<tr>
<td>ΔUA-GlcNS (NS)</td>
<td>3.47</td>
<td>4.73</td>
<td>4.17</td>
<td>4.73</td>
</tr>
<tr>
<td>ΔUA-GlcNAc6S (6S)</td>
<td>3.47</td>
<td>0.93</td>
<td>0.70</td>
<td>0.50&lt;sup&gt;##&lt;/sup&gt;</td>
</tr>
<tr>
<td>ΔUA2S-GlcNAc (2S)</td>
<td>2.03</td>
<td>2.10</td>
<td>2.17</td>
<td>2.23</td>
</tr>
<tr>
<td>ΔUA2S-GlcNS6S (NS6S)</td>
<td>10.60</td>
<td>8.33</td>
<td>7.63</td>
<td>8.40</td>
</tr>
<tr>
<td>ΔUA2S-GlcNS (NS2S)</td>
<td>7.90</td>
<td>27.27</td>
<td>26.90</td>
<td>27.20</td>
</tr>
<tr>
<td>ΔUA2S-GlcNAc6S (26S6S)</td>
<td>1.43</td>
<td>0.47</td>
<td>0.40</td>
<td>0.33</td>
</tr>
<tr>
<td>ΔUA2S-GlcNS6S (Tris)</td>
<td>64.83</td>
<td>52.07</td>
<td>57.37</td>
<td>51.83</td>
</tr>
<tr>
<td>tetra-1 ΔUA-GlcNAc6S-GlcA-GlcNS3S</td>
<td>0.23</td>
<td>0.03</td>
<td>0.03</td>
<td>0.30&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>tetra-2 ΔUA-GlcNAc6S-GlcA-GlcNS3S</td>
<td>1.53</td>
<td>0.37</td>
<td>0.40</td>
<td>0.40</td>
</tr>
<tr>
<td>tetra-3 ΔUA-GlcNS6S-GlcA-GlcNS3S</td>
<td>0.20</td>
<td>0.13</td>
<td>0.17</td>
<td>0.20&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>tetra-4 ΔUA2S-GlcNAc6S-GlcA-GlcNS3S</td>
<td>0.13</td>
<td>0.10</td>
<td>0.07</td>
<td>0.07</td>
</tr>
<tr>
<td>tetra-5 ΔUA2S-GlcNS6S-GlcA-GlcNS3S</td>
<td>0.10</td>
<td>0.33</td>
<td>0.40</td>
<td>0.57&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>tetra 1–5 subtotal</td>
<td>2.20</td>
<td>1.00</td>
<td>1.07</td>
<td>1.53</td>
</tr>
</tbody>
</table>

<sup>aData are expressed as the mean of two injections. Statistical analyses were calculated via One-Way ANOVA (starting UFH vs remodeled bovine intestinal UFH. <sup>b</sup>p < 0.1. <sup>c</sup>p < 0.01.</sup>

adverse drug reaction, heparin-induced thrombocytopenia (HIT), by inducing immune responses and activating platelets.16

Low molecular weight heparin (LMWH) is obtained through the controlled depolymerization of larger UFH chains by chemical or enzymatic methods. LMWH consist of 12–22 monosaccharide units having a MWavg ∼5000 Da. Like UFH, LMWH inactivates FXa in a dose-dependent manner. LMWHs have little effect on thrombin because most LMWH do not contain sufficient saccharide units to form a ternary complex with AT and FIIa.17 In contrast, UFH has sufficient chain length (>hexadecasaccharide) to inhibit FIIa to a similar level as FXa.17 There have been efforts to prepare LMWH products from bovine-derived heparins. Structural comparisons between porcine intestinal and bovine intestinal LMWHs have shown that in bovine LMWHs, oligosaccharide components have slightly larger MWs, and in the case of the LMWH, Enoxaparin, contains considerably less 1,6-anhydro mannospyranose.18 Because the potency of bovine-sourced heparins is lower than porcine intestinal heparin, it is challenging to prepare bovine-derived LMWHs by chemical β-elimination that are biosimilar to porcine-derived LMWHs.19 In the current study, we describe the preparation of a bovine intestinal LMWH from a remodeled bovine intestinal UFH having enhanced bioactivity. This is the first time to our best knowledge, that an engineered LMWH of any type has been prepared.

RESULTS AND DISCUSSION

Preparation of Remodeled UFH from Bovine Intestinal UFH. The starting bovine intestinal UFH was obtained by pooling a dozen bovine intestinal (bovine mucosal) heparins prepared by six different manufacturers. The MW and MW distribution and disaccharide and tetrasaccharide composition of the starting bovine intestinal UFH was determined (Tables 1 and 2). MW analysis relied on GPC-HPLC. Disaccharide analysis relied on the exhaustive treatment of this UFH with an equi-unit cocktail of heparinases (heparin lyases 1, 2, and 3) to afford primarily disaccharide products. These disaccharides were then analyzed by SAX-HPLC and their identity and concentrations determined using disaccharide standards.20 Tetrasaccharide analysis relied on the treatment of UFH starting material with heparin lyase 2, affording resistant tetrasaccharides21 that were then analyzed by SAX-HPLC and identified and quantified using tetrasaccharide standards.22 The anticoagulant activity of the starting UFH was next measured using the methods described in the current USP heparin monograph and compared to those of a USP heparin from porcine heparin (Table 1). As expected, USP heparin showed an anti-IIa activity of 200 U/mg consistent with USP requirements of >180 U/mg.23 The bovine intestinal heparin starting material showed considerably lower an anti-IIa activity of 121 U/mg (Table 1).

Both porcine intestinal and bovine intestinal heparins contain the AT binding site precursor, corresponding to the AT pentasaccharide binding site missing a 3-O-sulfo group.24 Remodeled bovine intestinal UFH was prepared from the starting material blended bovine intestinal UFH at 100 mg
The enzyme was expressed in ≥ C for pubs.acs.org/jmc Brie 58 Article (MWCO) centrifugal membrane units with After the reaction was complete, the reaction The packed bed bioreactor enables C) The anomeric signals were assigned closely resembled that of other bovine intestinal UFH and 2 groups in remodeled bovine intestinal UFH could be estimated fi repeating the desired reaction continuously. The starting and reusing the enzyme in continuous operating mode and the desired product to be obtained with high e distilled water to remove PAPS, MES bu 5 kDa MW cuto mixture was 125 filtered to remove enzyme on resin, dialyzed using 5 kDa MW cutoff (MWCO) centrifugal membrane units with distilled water to remove PAPS, MES buffer, and other small molecule impurities, and the retentate containing remodeled bovine UFH was lyophilized for further analysis. After treatment of bovine intestinal UFH with 3-OST-1 and PAPS, the resulting remodeled bovine UFH showed a greatly enhanced activity of 204 U/mg (Table 1). This remodeled bovine UFH showed a sufficiently high anticoagulant activity to meet the current USP criteria (≥180 U/mg) for pharmaceutical grade heparin. If desired, this activity could be adjusted to a value of 180 U/mg by less exhaustive treatment with 3-OST-1 and PAPS or by blending untreated bovine UFH. Disaccharide analysis of remodeled bovine UFH produced in a small-scale batch (100 mg) reaction and activity are shown in Table 2. Once we con g) to obtain remodeled bovine UFH using a packed bed bioreactor is the positive effect of the packed bed strategy to continuous enzymatic sulfation reaction. The anticoagulant activity were determined to be an anti-IIa activity of 204 IU/mg (must be (>180 IU/mg required for USP porcine heparin (Celsius, Cincinnati, OH)) and an anti-Xa activity of 195 IU/mg. The ratio of anti-Xa/anti-IIa was 0.95 (0.9—1.1 required for USP porcine heparin).

Preparation of LMWH from Remodeled Bovine Intestinal UFH. Remodeled bovine intestinal UFH was used to prepare LMWH through a chemical /-elimination method based on the Enoxaparin process (Scheme 1). Briefly, remodeled bovine intestinal UFH was added to benzethonium chloride solution to form the quaternary ammonium salt. The heparin benzethonium salt precipitated from this aqueous solution, and the precipitate was collected and dried. Dichloromethane was used to redissolve the dry remodeled bovine intestinal benzethonium salt. Benzyl chloride was added to this heparin salt, and the reaction was incubated at 35 °C for 25 h. The heparin benzyl ester was formed and recovered by filtration. Depolymerization was performed by incubating remodeled bovine intestinal UFH benzyl ester with sodium hydroxide solution. Hydrochloric acid was used to neutralize the excess sodium hydroxide after the reaction was completed. The resulting remodeled bovine intestinal LMWH products were precipitated using methanol, recovered by centrifugation, and dried.

Table 3. Adjustment of Key Processing Parameters for Preparing Remodeled Bovine Intestinal LMWHs

<table>
<thead>
<tr>
<th>bovine intestinal LMWH batch</th>
<th>reaction temp (°C)</th>
<th>time (h)</th>
<th>NaOH conc (mM)</th>
<th>conc of remodeled bovine intestinal UFH (%)</th>
<th>MW (Da)</th>
<th>anti-Xa activity (U/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>55</td>
<td>2</td>
<td>4</td>
<td>4</td>
<td>NT</td>
<td>58</td>
</tr>
<tr>
<td>2</td>
<td>50</td>
<td>2</td>
<td>4</td>
<td>5</td>
<td>NT</td>
<td>72</td>
</tr>
<tr>
<td>3</td>
<td>50</td>
<td>2</td>
<td>4.5</td>
<td>5</td>
<td>NT</td>
<td>88</td>
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<tr>
<td>4</td>
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<td>138</td>
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<td>50</td>
<td>2</td>
<td>6.6</td>
<td>6</td>
<td>5600</td>
<td>92</td>
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<tr>
<td>9</td>
<td>53</td>
<td>2</td>
<td>6.6</td>
<td>6</td>
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<td>86</td>
</tr>
<tr>
<td>10</td>
<td>50</td>
<td>2</td>
<td>5.5</td>
<td>5</td>
<td>5100</td>
<td>112</td>
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<tr>
<td>11</td>
<td>50</td>
<td>2.25</td>
<td>5.5</td>
<td>5</td>
<td>4700</td>
<td>107</td>
</tr>
<tr>
<td>12</td>
<td>50</td>
<td>2.5</td>
<td>5.5</td>
<td>5</td>
<td>3900</td>
<td>87</td>
</tr>
</tbody>
</table>

aData were the mean of two injections. NT: not tested.

scale and PAPS through its enzymatic treatment with 3-OST-1. The enzyme was expressed in Escherichia coli as a His-tagged fusion protein and immobilized as previously described. After the reaction was complete, the reaction mixture was filtered to remove enzyme on resin, dialyzed using 5 kDa MW cutoff (MWCO) centrifugal membrane units with distilled water to remove PAPS, MES buffer, and other small molecule impurities, and the retentate containing remodeled bovine UFH was lyophilized for further analysis. After treatment of bovine intestinal UFH with 3-OST-1 and PAPS, the resulting remodeled bovine UFH showed a greatly enhanced activity of 204 U/mg (Table 1). This remodeled bovine UFH showed a sufficiently high anticoagulant activity to meet the current USP criteria (≥180 U/mg) for pharmaceutical grade heparin. If desired, this activity could be adjusted to a value of 180 U/mg by less exhaustive treatment with 3-OST-1 and PAPS or by blending untreated bovine UFH. Disaccharide analysis of remodeled bovine UFH produced in a small-scale batch (100 mg) reaction and activity are shown in Table 2. Once we confirmed required conditions in the small-scale reaction, we next performed a large-scale (1 g) to obtain remodeled bovine UFH using a packed bed strategy with recycling. The packed bed bioreactor enables the desired product to be obtained with high efficiency by reusing the enzyme in continuous operating mode and repeating the desired reaction continuously. The starting and final specifications of these heparins are shown in Tables 1 and 2. NMR analysis of the remodeled bovine intestinal UFH closely resembled that of other bovine intestinal UFH and remodeled bovine intestinal UFH, prepared and/or examined by our group. The anomeric signals were assigned and the monosaccharide composition were evaluated for remodeled bovine intestinal UFH. The number of 3-O-sulfo groups in remodeled bovine intestinal UFH could be estimated as one 3-O-sulfo group per chain based on the content of resistant tetrasaccharides. Moreover, statistical analysis has also shown that heparinase resistant tetrasaccharide composition of remodeled bovine intestinal UFH is significantly different than that of starting bovine intestinal UFH (Table 2). We believe the differences in the amount of resistant tetrasaccharides of the remodeled bovine intestinal UFH yielded in batch reaction and packed bed bioreactor is the positive effect of the packed bed strategy to continuous enzymatic sulfation reaction. The anticoagulant activity were determined to be an anti-IIa activity of 204 IU/mg (must be (>180 IU/mg required for USP porcine heparin (Celsius, Cincinnati, OH)) and an anti-Xa activity of 195 IU/mg. The ratio of anti-Xa/anti-IIa was 0.95 (0.9—1.1 required for USP porcine heparin).
USP Enoxaparin, prepared from porcine intestinal UFH, has an average MW of 4500 Da with a range of between 3800 and 5000 Da with approximately 20% (15–25%) of the Enoxaparin chains contain a 1,6-anhydro derivative at their reducing ends. Aiming to prepare bovine biosimilar of LMWH USP and to optimize depolymerization conditions, we decided to begin with the same conditions as the preparation of LMWH USP Enoxaparin from porcine intestinal UFH; an alkaline β-elimination reaction typically uses 4 mM NaOH to depolymerize heparin benzyl ester at 55 °C in 2 h.29 We first analyzed anti-Xa activity, then the MW properties, to assess the LMWH product(s) obtained. Analysis of this first LMWH product showed an anti-Xa activity below the lower limit allowed for Enoxaparin (bovine intestinal LMWH batch 1). After several trials, bovine intestinal LMWH batch 10 prepared from remodeled bovine intestinal UFH met the Enoxaparin MW and anti-Xa activity of USP specifications, but it was slightly below lower limit the allowed 1,6-anhydro content for Enoxaparin. Experiments showed that longer reaction times generally increased 1,6-anhydro levels without changing either MW properties or anticoagulant activity.27 Thus, the depolymerization reaction time needed to be longer than 2 h to obtain 1,6-anhydro derivatives within the specification range described in the USP Enoxaparin monograph (Table 3). Using longer reaction times, LMWH batch 11 and batch 12 were prepared and the MW and activity properties of these three batches, batches 10–12, of bovine intestinal LMWH were examined (Table 4). The key processing parameters for preparing LMWH batch 11 were determined as a substrate concentration of 5% by weight, alkaline concentration of 5.5 mM, reaction temperature of 50 °C, and a reaction time of 2.25 h (Table 3). Starting from same batch of remodeled benzyl bovine intestinal UFH, synthesis method for preparing LMWHs was highly reproducible. However, if the batch of bovine intestinal UFH or remodeled intermediates are changed it might be necessary to reassess the exact depolymerization conditions required to meet LMWH specifications.

MW and MW Distributions and Bioactivities of Bovine Intestinal LMWHs. According to the USP monograph, Enoxaparin has a MW of 4500 Da with a range between 3800 and 5000 Da. In addition, Enoxaparin has a MW range between 3800 to 5000 Da, and 12.0–20.0% of its components of Enoxaparin to have a MW < 2000 Da and 68.0–82.0% of its components with a MW between 2000 and 8000 Da. High performance liquid chromatography size exclusion chromatography (HP-SEC) is the most commonly used method for the measurement of such parameters. HP-SEC/multiple detector array (TDA), HP-SEC/refractive index (RI) method, and HP-SEC/multicycle laser light scattering (MALLS) technology were reported to determine MW and MW distribution of LMWHs.30,31 HPLC-GPC-refractive index (RI) method was conducted to determine the MW in this study.

The MW and activity properties of batches 10–12 of bovine intestinal LMWH were examined (Table 4). The LMWH batch 11 met all MW and MW distribution specifications for Enoxaparin. 1,6-Anhydro content of LMWH batch 10 was slightly out of the USP specifications for Enoxaparin (14.2%), and batch 12 was not meet the USP specifications a MW < 2000 Da content of 21%. The anti-Xa and anti-IIa activities of these three LMWHs batches 10–12 produced from remodeled bovine intestinal UFH were determined. Two of the batches of bovine intestinal LMWHs, batches 10 and 11, were within the USP Enoxaparin specifications for the anti-Xa activity (90–125 IU/mg), while batch 12 had slightly lower IU/mg than the required specifications. However, the anti-IIa activities of batch 10 were higher and batch 12 was lower than USP Enoxaparin limits. The anti-Xa and anti-IIa activities, and anti-Xa/anti-IIa ratio of batch 11 met the USP Enoxaparin specifications (Table 4). Thus, batch 11 was used for further structural and biological evaluation.

### Table 4. MW and Anticoagulant Activity Properties of Bovine Intestinal LMWHs Generated from Remodeled Bovine Intestinal UFH

<table>
<thead>
<tr>
<th>MW 3800–5000 Da</th>
<th>MW &lt; 2000 Da 12–20%</th>
<th>MW 2000–8000 Da 68–82%</th>
<th>MW &gt; 8000 Da NMT18%</th>
<th>1,6-anhydro 15–25%</th>
<th>anti-Xa (IU/mg) 90–125</th>
<th>anti-IIa (IU/mg) 20–35</th>
<th>anti-Xa/anti-IIa 2.5–5.5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Enoxaparin</td>
<td>4200</td>
<td>17</td>
<td>73</td>
<td>11</td>
<td>22</td>
<td>105</td>
<td>25</td>
</tr>
<tr>
<td>Bovine Intestinal LMWHs</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>batch 10</td>
<td>5100****</td>
<td>13.8**</td>
<td>72.3</td>
<td>16.2**</td>
<td>14.2****</td>
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<td>37.4****</td>
</tr>
<tr>
<td>batch 11</td>
<td>4700***</td>
<td>14.5**</td>
<td>69.5**</td>
<td>16.1**</td>
<td>17.7**</td>
<td>107</td>
<td>29.3*</td>
</tr>
<tr>
<td>batch 12</td>
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<td>21.0***</td>
<td>70.6</td>
<td>8.4</td>
<td>18.3**</td>
<td>87</td>
<td>12.5****</td>
</tr>
</tbody>
</table>

*Data are expressed as the mean of two injections. Statistical analyses were calculated via One-Way ANOVA (Enoxaparin vs Bovine intestinal LMWHs. *, p < 0.1; **, p < 0.01; ***, p < 0.001; ****, p < 0.0001.

The disaccharide composition of three batches of LMWHs and their parent remodeled bovine intestinal UFH are similar (Figure 2 and Supporting Information, Table S1). The 1H NMR spectra for Enoxaparin and batch 11 are essentially the same, suggesting that the samples are similar. Indeed, the monosaccharide compositions determined by NMR analysis (Supporting Information, Table S3), are comparable.

The heparin lyase 2-resistant tetrasaccharides T1, ΔUA-GlcNAc6S-GlcA-GlcNS3S; T2, ΔUA-GlcNAc6S-GlcA-GlcNS3S6S; T3, ΔUA-GlcNS6S-GlcA-GlcNS3S6S; T4, ΔUA2S-GlcNAc6S-GlcA-GlcNS3S6S; T5, ΔUA2S-GlcNS6S-GlcA-GlcNS3S6S were identified. Their molar ratio provides
Thus, the reduction step allows the was used to determine Competitive SPR binding assayBecause competition SPR provides an indirect Article Measurement of LMWH Batch 11 Inhibiting AT

The resonances of \( \Delta U 4 \) (\( \delta 5.90, 108.83 \) ppm) was assignable. The signal peaks at \( 5.48, 100.62 \) ppm; \( 5.43, 99.92 \) ppm; \( 5.33, 99.50 \) ppm; \( 5.13, 102.26 \) ppm; \( 5.07, 102.55 \) ppm; and \( 4.51, 104.84 \) ppm corresponded to the anomeric hydrogen; carbon signals of GlcNS6S-(GlcA), GlcNS3S6S, GlcNS6X-(Ido2S)/GlcNAc6X-(GlcA), IdoA2S, IdoA, and GlcA, respectively. The common Enoxaparin peaks of GlcNS6X (H2, 3.19 ppm), GlcNAc6X (CH3, 1.95 ppm), and GlcNS (H6, 3.7S ppm) were observed.

\section*{Monosaccharide Composition} NMR analysis provides information on composition at the monosaccharide level. Quantitative analysis using 1D NMR \(^3\) was used to determine the content of IdoA, IdoA2S, and GlcNAc6X of bovine intestinal LMWH batch 11 and porcine heparin-derived Enoxaparin (Figure 5). The GlcNS3S peak was overlapped with H1 of \( \Delta U 2 S \) in range from 5.44 to 5.42 ppm, H2 GlcNS peak was overlapped with H2 of 1,6-an.Man in the range from 3.23 to 3.08 ppm, and GlcA peak was overlapped with H2 signals of GlcNS3S from 3.41 to 3.25 ppm. Thus, the content of these monosaccharides could not be determined quantitatively. However, GlcNAc6X, IdoA2S, and IdoA contents were determined. GlcNAc6X peak was integrated from 1.97 to 1.91 ppm. The IdoA2S peak was integrated from 5.17 to 5.09 ppm, the IdoA peak was integrated from 5.09 to 5.06 ppm. The peaks and the temperature (\( 25^\circ \)C) selected provide minimum spectral overlap and ensure the most accurate integration values. Bovine intestinal LMWH batch 11 showed higher IdoA2S, but lower GlcNac and IdoA levels than porcine heparin-derived Enoxaparin.

\section*{IC\textsubscript{50} Measurement of LMWH Batch 11 Inhibiting AT and PF4 Binding to Surface Heparin by SPR Solution Competition Assay} Competitive SPR binding assay correlate well with anti-Xa activity as well as AT binding site content.\(^3\) Because competition SPR provides an indirect measurement of binding, competitive binding experiments between AT (or PF4) and surface heparin and soluble LMWH batch 11 as well as remodeled bovine intestinal UFH and Enoxaparin were performed to determine IC\textsubscript{50} (Figure 6).

AT (or PF4) were mixed with different concentrations of heparin in HBS-EP buffer were injected over heparin chip. Once the active binding sites on AT (or PF4) are occupied by LMWH in the solution, the binding of AT (or PF4) to the

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure3.png}
\caption{Disaccharide and tetrasaccharide composition analysis. From bottom to top: red, USP heparin; blue, USP Enoxaparin; pink, remodeled bovine intestinal UFH; black, bovine intestinal LMWH. See Supporting Information, Table S1 and S2, for details.}
\end{figure}

\section*{NMR Analysis of Bovine Intestinal LMWH} The \(^1\)H NMR spectrum of bovine intestinal LMWH batch 11 is relatively complex, as it represents a pattern of overlapping signals from the many protons in a different environment.\(^1\)H NMR of the porcine heparin-derived Enoxaparin and bovine LMWH batch 11, and heteronuclear multiple quantum coherence (HMQC) spectra of bovine intestinal LMWH batch 11 were used to perform assignments (Figure 4).\(^33,34\) The resonances of \( \Delta U 4 \) (\( \delta 5.90, 108.83 \) ppm) was assignable. The signal peaks at \( 5.48, 100.62 \) ppm; \( 5.43, 99.92 \) ppm; \( 5.33, 99.50 \) ppm; \( 5.13, 102.26 \) ppm; \( 5.07, 102.55 \) ppm; and \( 4.51, 104.84 \) ppm corresponded to the anomeric hydrogen; carbon signals of GlcNS6S-(GlcA), GlcNS3S6S, GlcNS6X-(Ido2S)/GlcNAc6X-(GlcA), IdoA2S, IdoA, and GlcA, respectively. The common Enoxaparin peaks of GlcNS6X (H2, 3.19 ppm), GlcNAc6X (CH3, 1.95 ppm), and GlcNS (H6, 3.7S ppm) were observed.

\section*{Figure 2} Disaccharide compositional analysis of remodeled bovine intestinal UFH and bovine intestinal LMWHs. See Supporting Information, Table S1, for details.

The points present at the higher levels in Enoxaparin with the exception of T5, which was at the similar levels in both samples (Figure 3).

1,6-Anhydro hexosamine reducing ends are generated due to the alkaline \( \beta \)-eliminate cleavage. The 1,6-anhydro content is determined in order to compare it with the FDA-approved Enoxaparin. About 20% of the Enoxaparin chains contain a 1,6-anhydro derivative on the reducing end, within the allowed range of 15–25%. To determine 1,6-anhydro contents, the LMWHs were digested to basic building blocks using the cocktail heparinases. Disaccharides with undervatized reducing ends were reduced to alditols by sodium borohydride, whereas the 1,6-anhydro derivatives remained unchanged. The reduction of the oligosaccharides decreases their retention time, whereas the retention time of the 1,6-anhydro derivatives remains unchanged.\(^3\) Thus, the reduction step allows the separation of 1,6-anhydro derivatives on the Spherisorb SAX column, which were poorly resolved before reduction reaction. One of the batches of LMWH, prepared from remodeled bovine intestinal UFH, batch 10 was slightly out of the USP Enoxaparin specifications for 1,6-anhydro content (Table 4). However, using a longer depolymerization time, batch 11 and batch 12 met 1,6-anhydro specifications for USP Enoxaparin.

\section*{Tables S1 and S2} for details.
surface-immobilized heparin results in a reduction in signal. The IC_{50} values (concentration of competing analyte resulting in a 50% decrease in response units (RU)) can be calculated from the plots (AT (or PF4) binding signal (normalized) against the concentration of LMWH in solution.

The AT-binding affinity of heparin is dependent on the presence of an AT-binding site within a UFH or LMWH chain. PF4-binding affinity is dependent on the MW of the heparin product being tested. The calculated IC_{50} value for bovine intestinal LMWH batch 11 for AT was 2.16 μM, which is lower than the IC_{50} of Enoxaparin (~10 μM). The calculated IC_{50} value for bovine intestinal LMWH batch 11 for PF4 was 0.16 μM, which is also lower than the IC_{50} of Enoxaparin (0.7 μM). The AT-binding affinity of a LMWH is generally lower than a UFH because of the loss of a number of AT-binding sites during chemical or enzymatic depolymerization. This was confirmed by the lower IC_{50} value (13.5 nM) for remodeled bovine intestinal UFH for AT than LMWH batch 11 (IC_{50} = 2.16 μM). Our results (Figure 6B,F) also demonstrate that the binding affinity of remodeled bovine intestinal UFH (IC_{50} = 20 nM) to PF4 was stronger than for bovine intestinal LMWH batch 11 (IC_{50} = 0.16 μM), suggesting a lower potential of HIT for bovine intestinal LMWH than for UFH.

### CONCLUSION

Heparin products, used in the U.S. and Europe, are currently produced from porcine intestinal tissues. Recently, problems in the heparin supply chain have suggested a need for alternative sources of heparin. Bovine heparins were previously used as anticoagulant drugs. Unfortunately, bovine heparin has lower
The MW of porcine intestinal UFH generally ranges from 12,000 to 25,000 Da, while the average MW of LMWHs prepared by depolymerization reactions are generally less than 8000 Da. Enoxaparin sodium, the most widely used LMWH, is produced by the esterification of porcine intestinal UFH followed by depolymerization through alkaline β-elimination. The structure of either one or both termini, of the newly generated oligosaccharide chains, is usually modified. The reducing end can be a glucosamine residue or a 1,6-anhydro derivative and the nonreducing end can be a uronic acid residue or a ΔUA2S (or a ΔUA) residue. Because drug efficacy and safety are related to drug structure, complex drugs, such as LMWHs, must have well characterized structures. Both the U.S. FDA and European Medicines Agency (EMA) require comprehensive structural characterization with sophisticated analytical techniques using multiple assays to evaluate the bioequivalence or biosimilarity between generic and innovator LMWH. In this project, by changing reaction conditions such as reaction time and temperature and UFH substrate and sodium hydroxide concentration, independent batches of LMWH were prepared from remodeled bovine intestinal heparin using a chemical β-eliminative process. Key chemical reaction parameters were controlled to prepare a bovine...
"Enoxaparin" with MW properties meeting the requirements for USP Exonaparin. The structural and biological properties between bovine intestinal heparin-derived Exonaparin were then examined using several state-of-the-art analytical techniques.

**Experimental Section**

**Enzyme Expression and Immobilization.** Briefly, 3-OST-1 and arylsulfotransferase IV (AST IV) were expressed as fusion proteins in Escherichia coli grown in LB medium (MP Biomedicals) at 37 °C using rotary air shaker (New Brunswick Scientific Innova 44R). Recovered cell pellets were stored at −80 °C for further immobilization. His-tagged fusion proteins (3-OST-1 and AST IV) were immobilized on amyllose and Ni sepharose resins (New England Biolabs), respectively. Briefly, cell paste (10 g) was suspended in 40 mL of ice-cold lysis buffer A (25 mM Tris-HCl, pH 7.4, containing 500 mM NaCl). The cell suspension was sonicated on ice at power of 4.5 for 1 min (30 strokes, 1 s on and 1 s off), using the Q700 sonicator (Qsonica, Newtown, CT). The step was repeated four times. The insoluble fraction was centrifuged at 18,000 g for 4 °C for 30 min. The supernatants were collected and immobilized with amyllose and Ni-sepharose resins for corresponding protein at 4 °C. Resins were washed with ice-cold lysis buffer A for purification. The purified immobilized enzyme was kept at 4 °C for reactions. The activity of the immobilized enzyme was assayed by following the color of p-nitrophenol generated when p-nitrophenyl sulfate was used as a sacrificial sulfon donor in the presence of AST IV. The enzyme activity used in the remodeling reactions was sufficient to ensure complete reaction in a 24 h period.11

**Remodeled Bovine Intestinal UFH Preparation from Bovine Intestinal Heparin.** Bovine intestinal UFH from 12 different manufacturers were obtained from the USP and were blended in equal amounts to obtain starting material bovine intestinal UFH. Remodeled bovine intestinal UFH was prepared by treatment of starting material bovine intestinal UFH with CNBr-immobilized 3-OST-1, either in a batch reaction, where the catalyst resin was kept in suspension with agitation, or in a packed bed reaction, where the low activity substrate was passed through a column of catalyst resin. The reaction conditions were as follows: the reaction mix (mobile phase) consisted of 40 g/L low activity heparin substrate, 7.5 mM 3′-phosphoadenosine-5′-phosphosulfate (PAPS) in 50 mM MES, 125 mM NaCl, pH 7 (MFS/NaCl); the reaction mix was added to the 3-OST-1 catalyst, equilibrated in MES/NaCl, at the concentration of 1 mg of immobilized enzyme per mL of reaction mix. The enzymatic reactions were carried at 37 °C under agitation until the desired anticoagulant activity was reached; samples were tested for anti-IIa activity at regular intervals. For the packed bed reaction, the reaction mix was passed through a 5 mL column, placed in a 37 °C chamber, at an appropriate flow rate, and the product was recirculated through the column until the targeted anti-IIa activity was reached.

**MW and Concentration Determinations.** MWs were measured with gel permeation chromatography (GPC), in accordance with the USP 40 Heparin monograph method.17 A guard column BioSuite SEC Guard Column, 5 μm, 6 mm × 40 mm diameter, was used in series with two analytical columns: BioSuite SEC columns, 125 Å, 5 μm, 7.8 mm × 300 mm. These columns were connected to an HPLC system consisting of Shimadzu LC-20AD pump, a Shimadzu CBM-20A controller, Shimadzu SIL-20AHT autosampler, Shimadzu CTO-20AC column oven, and a Shimadzu RID20A refractive index detector (RID) (Shimadzu, Kyoto, Japan). Columns and RID detector were maintained at 30 °C. The mobile phase was 0.5 M lithium nitrate, and the flow rate was 0.6 mL/min. A sample injection volume was 20 μL. The GPC chromatograms were recorded with the LC Solution version 5.73 software (Shimadzu, Kyoto, Japan), and analyzed with its “GPC Postrun” function. For MW determination, USP Exonaparin MW Calibrant A and USP Exonaparin MW Calibrant B dissolved in lithium nitrate were used as calibrants, and USP Exonaparin Identification standard (USP, MD, US) was used to confirm system suitability. Also, USP Exonaparin molecular weight identification RS, prepared as several concentrations, were injected to get a standard curve to calculate the concentration of reaction samples using area under the curve (AUC). All data passed System Suitability requirements as stated in the USP monograph. For calculation, a third-order polynomial equation was used. The definitions of MW parameters are given below. The weight-average MW is calculated with \( \sum NM_i \), where \( N_i \) is the number of molecules of molecular weight \( M_i \). The weight-average MW for the sample solution should be 3800 and 5000 Da. The acceptance criteria for MW 2000 is between 12.0% and 20.0%, MW 2000–8000 is between 68.0% and 82.0%, and MW 8000 is NMT 18.0%.

**Disaccharide and Tetrasccharide Compositional Analysis.** For disaccharide and tetrasccharide compositional analysis of low molecular weight heparin, the samples (50 μg) were completely digested using heparinase I, 2, and (3 μg each) mixture in 50 mM ammonium acetate, 2 mM CaCl₂, in a 96-well plate (MicroAmp Fast Optical 96-well reaction plate). The samples were digested using Applied Biosystems Veriti (96 well fast thermal cycler) at 35 °C for 2 h, followed by 95 °C for 15 min. Once completed, temperature of the sample was maintained at 4 °C.

The analyses were performed using the HPLC system LC-2030 (Shimadzu, Kyoto, Japan) (Note: this system has controller, pumps, degasser, UV detector, column oven, and autosampler), and the chromatograms were recorded using Labsolutions software lite version 5.82 (Shimadzu). The data analyses were carried out using Postrun function of the Labsolutions software. The column (Spherisorb strong-anion exchange (SAX) chromatography column, 4.0 mm × 250 mm, 5 μm (Waters Corporation)) was equilibrated with 1.8 mM monobasic sodium phosphate (eluent A, pH 3.0) and followed with ingredient elution after injection using 1.8 mM monobasic sodium phosphate with 1 M sodium perchlorate (eluent B, pH 3.0). The column temperature was maintained at 40 °C. The flow was low-pressure gradient with a flow rate of 0.45 mL/min. The gradient for eluent B is as follows: 0.01 min—10%, 10 min—30%, 50 min—65%, 60 min—85%, 65 min—100%, 68 min—10%, and 90 min—stop. The chromatograms were measured using a UV detector: D2 lamp, + polarity, cell temperature of 40 °C, and wavelengths 232 and 280 nm. The injection volume was 20 μL. Disaccharide standards were purchased from Iduron (Manchester, UK). Digested USP heparin and Exonaparin were used to identify tetrasccharide peaks by comparison.

**1,6-Anhydro Analysis.** Analysis of the 1,6-anhydro components of LMW heparin samples was performed as previously described. Enoxaparin and LMWH samples were digested in sodium/calcium acetate pH 7.0 solution prepared by dissolving 10 mg of bovine serum albumin and 32 mg of calcium acetate in 60 mL of water. Glacial acetic acid (580 mL) was added, and the pH adjusted to 7.0 with 2 M sodium hydroxide. The solution was transferred to a 100 mL volumetric flask and diluted with water to volume. A mixture was prepared of 400 μg of each sample in 40 μL of water, 49 μL of water, 70 μL of sodium/calcium acetate pH 7.0 solution, and 31 μL of the heparinases 1, 2, 3 solutions. This solution was mixed gently by inversion and allowed to stand for at least 48 h in a 25 °C water bath. A reduction mixture was prepared by dissolving 12 mg of sodium borohydride in 400 μL of water. A 10 μL aliquot of freshly prepared sodium borohydride solution was added to 60 μL each of the depolymerized sample. The mixture was allowed to stand at room temperature for 4 h. The resulting solution was analyzed by HPLC and the (w/w) percentage of the three main 1,6-anhydro derivatives were obtained after depolymerization of Exonaparin sodium using the following formula: \( \% \text{1,6-anhydro, (w/w)} = \left( \frac{100 \times MW \times A_i}{\sum (MW \times A_i)} \right) \times \left( \frac{\text{Area of } 1,6-\text{anhydro peak } i, \text{ respectively}}{\text{MW} \times \text{Area } X} \right) \times \left( \frac{\text{Area } \Delta IS 1,6-\text{anhydro}}{\text{Area IS 1,6-}} \right) \) in which MW and \( A_i \) are the MW and the area of the 1,6-anhydro peak \( i, \) respectively; and MW, and \( A_i \) are the MW and the areas, respectively, of either the peak \( X \) or the zone \( X \) specified by its retention time.

The molar percentage of components containing a 1,6-anhydro structure at the reducing end of their chain in the Exonaparin sodium test sample were calculated according to the following formula: \( \% \text{1,6-anhydro} = 100 \times \left( \frac{\text{Area } \sum (MW \times A_i)}{\text{Area } \Delta IS 1,6-} \right) \).
and Area $\Delta$H (1.6-anhydro + Area $\Delta$S-1.5 anhydro), in which MW is the weight-average molecular mass, MW, and Area, are the MW and the area, respectively, of either the peak X or the range X specified by its retention time. The peaks were identified by comparison with peaks from Enoxaparin standard.

The analyses were performed using the HPLC system LC-2030 (Shimadzu, Kyoto, Japan) with controller, pumps, degasser, UV detector, column oven, and autosampler. Chromatograms were recorded using LabSolutions software lite version 5.82 (Shimadzu). The data analyses were carried out using Procrun function of the LabSolutions software. The column (Spherisorb SAX chromatography column, 4.0 mm x 250 mm, 5 $\mu$m (Waters Corporation)) was equilibrated with 1.8 mM monobasic sodium phosphate (eluent A, pH 3.0) and followed with ingredient elution after injection using 1.8 mM monobasic sodium phosphate with 1 M sodium perchlorate (eluent B, pH 3.0). The column temperature was maintained at 50 °C. The flow was low-pressure gradient with a flow rate of 0.45 mL/min. The gradient for eluent B is as follows: 001 min—3%; 20 min—35%; 50 min—100%; 61 min—3%; 79 min—Stop 3%. The chromatograms were measured using a UV detector: D2 lamp with + polarity and cell temperature of 50 °C, at wavelengths 232 and 280 nm. The injection volume was 10 $\mu$L.

The system suitability as stated in USP monograph 32 was first confirmed. Depolymerization: The ratio of the peak area of 1,6-anhydro-$\Delta$S-1.5 (TriS) to that of 1,6-anhydro-$\Delta$H (TriS) in the depolymerized standard solution 2 should be no more than 1.15. Column performance: The resolution between reduced $\Delta$A (286S) and 1,6-anhydro-$\Delta$S (TriS) should be no less than 1.5. Reduction: The ratio of the peak area $\Delta$S to that of reduced $\Delta$H in the depolymerized, and reduced standard solution 3 should be no more than 0.2%.

NMR Spectroscopy. Samples were dissolved in 500 $\mu$L of D$_2$O (99.9%) and lyophilized three times to substitute the exchangeable protons with deuterium, and then transferred to NMR microtubes after dissolving in 500 $\mu$L of D$_2$O. $^1$H, $^3$H, $^13$C HSQC NMR spectra were recorded on a Bruker 600 spectrometer (Madison, WI, USA) with topspin 3.2 software at 298.15 K. The spectra were processed using the MestReNova 14.1.1 (Mestrelab Research, Santiago de Compostela, Spain). The quantification methods had been validated in our earlier reports. The estimated standard deviation was approximatively 0.7%.

In Vitro Anticoagulant Activity Measurement. The anti-Xa and anti-IIa activities of UFH samples and LMWH samples were determined using BIOPHEN Heparin anti-Xa (two stage) and anti-IIa (two stage) kits (Aniara, West Chester, Ohio). Human AT 40 $\mu$g/mL in 80 $\mu$L of R1 buffer (Tris 0.05 M, NaCl 0.175 M, EDTA 0.0075 mM, at pH 8.40 containing polyethylene glycol at 0.1%, and sodium azide as preservative) was used for anti-Xa assay. Human AT 10 $\mu$g/mL in 80 $\mu$L of R2 buffer (Tris 0.05 M, NaCl 0.175 M, EDTA 0.0075 mM, at pH 8.40, containing bovine serum albumin at 0.2%, and sodium azide as preservative) mixed with different masses of heparin (range from 0.5, 5, 10, 15, and 20 ng) were incubated for 2 min at 37 °C. Then, purified bovine factor Xa (320 ng in 40 $\mu$L of R1 buffer) or purified human thrombin (960 $\mu$L in 40 $\mu$L of R2 buffer) preincubated at 37 °C were added and incubated for 2 min. After the addition of chromogenic substrate specific for factor Xa (CS01(65), 1.2 mM, 40 $\mu$L) or the chromogenic substrate specific for thrombin (CS-01(38), 1.25 mM, 40 $\mu$L). The reaction mixture was incubated at 37 °C for 2 min for anti-Xa assay and 1 min for anti-IIa assay and then stopped with acetic acid (20 mg/mL, 80 $\mu$L). The absorbance was measured at 405 nm. Anti-Xa and anti-IIa activities were calculated using a standard curve of different concentrations of heparin and Enoxaparin standards.

Surface Plasmon Resonance (SPR) Analysis of AT/PF4 Binding to LMWH. Materials. USP sodium heparin (from porcine intestinal mucosa) was from Celsus (Cincinnati, OH). AT and PF4 were from Hyphen BioMed (Neuville-sur-Oise, France). Biotinylated streptavidin Chip was from GE Healthcare (Uppsala, Sweden).

Preparation of Heparin Biochip. The preparation of biotinylated heparin was followed our previous protocol. 33 In brief, in 200 $\mu$L of water, 2 mg of heparin and 2 mg of amine-PEG-biotin (Thermo Scientific, Waltham, MA) were mixed with 10 mg of NaCNBH$_3$. The initial reaction was carried at 70 °C for 24 h, and then a further 10 mg of NaCNBH$_3$ was added to continue running the reaction for another 24 h. After completion of the reaction, the mixture was desalted with a spin column (3000 MW cutoff) and was freeze-dried for chip preparation. The biotinylated heparin was immobilized to a SA chip based on the manufacturer’s protocol. In brief, 20 $\mu$L of solution of the heparin–biotin conjugate (0.1 mg/mL) in HBS-EP running buffer was injected over flow cell 2 of the SA chip at a flow rate of 10 $\mu$L/min. The successful immobilization of heparin was confirmed by the observation of a ~200 RU increase in the sensor chip. The control flow cell 1 was prepared by 1 min injection with a saturated solution of biotin.

**SPR Solution Competition Study of LMWH.** Solution competition study between surface heparin and soluble different LMWH or UFH (as control) to measure $IC_{50}$ was performed using SPR. 33 In brief, AT (250 nM) or PF4 (125 nM) samples mixed with different concentrations of LMWH in buffer (0.1 M HEPES, 1.5 M NaCl, 30 mM EDTA, 0.5% v/v surfactant P20) were injected over a heparin chip at a flow rate of 40 $\mu$L/min, respectively. After a 2 min dissociation time, the sensor surface was regenerated by sequence injecting with 40 $\mu$L of 2 M NaCl (for AT) or 10 mM glycine-HCl pH 2.5 buffer (for PF4) to get fully regenerated surface. The response was monitored as a function of time (sensorgram) at 25 °C. For each set of competition experiments on SPR, a control experiment (only protein without LMWH or UFH) was performed to make sure the surface is completely regenerated and that the results obtained between runs are comparable. Once the active binding sites on AT (or PF4) molecules are occupied by LMWH or UFH in the solution, the binding of AT (or PF4) to the surface-immobilized heparin should decrease, resulting in a reduction signal. The $IC_{50}$ values (concentration of competing analyte resulting in a 50% decrease in RU) can be calculated from the plots AT (or PF4) binding signal (normalized) as a function of LMWH or UFH concentration in solution.

**Statistical Analysis.** Significance in experiments with two groups was determined via One-Way ANOVA using GraphPad Prism 9.0.0 software.

### ASSOCIATED CONTENT

#### Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/10.1021/acs.jmedchem.0c02019.

Additional figures stacked $^1$H NMR spectrum of Enoxaparin and remodeled bovine LMWH batch 11, and disaccharide and tetrasaccharide compositions and 1,6-anhydro analysis of prepared bovine intestinal LMWH (PDF)

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Notes
The authors declare no competing financial interest.

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ABBREVIATIONS USED
1,6-an.A, 2-amino-1,6-anhydro-2-deoxy-β-n-glucopyranose; 1,6-an.M, 2-amino-1,6-anhydro-2-deoxy-β-L-mannopyranose; AST IV, arylsulfotransferase IV; AT, antithrombin III; BSE, bovine spongiform encephalopathy; CS, chondroitin sulfate; DS, dermatan sulfate; ΔUA, 4-deoxy-α-L-threo-3-hex-4-nopyranosyluronic acid; EMA, European Medicines Agency; FDA, Food and Drug Administration; FXa, factor Xa; GAG, glycosaminoglycan; GLcA, glucuronic acid; GLcN, glucosamine; GPC, gel permeation chromatography; HIT, hepatic induced thrombocytopenia; HMQC, heteronuclear multiple quantum coherence; HPLC, high performance liquid chromatography; HP-SEC, high performance liquid chromatography size exclusion chromatography; HS, heparan sulfate; IdoA, iduronic acid; KS, keratan sulfate; LMWH, low molecular weight heparin; MES, 2-(4-morpholino)ethanesulfonic acid; MW, molecular weight; MWCÖ, molecular weight cutoff; NAC, N-acetyl; NS, ΔUA-GlcNS; NS6S, ΔUA-GlcNS6S; NS2S, ΔUA2S-GlcNS; OST, O-sulfotransferase; OSCS, oversulfated chondroitin sulfate; PAPS, 3’-phosphoadenosine-5’-phosphosulfate; PF4, platelet factor 4; RU, resonance unit; US, ΔUA-GlcNaC; 6S, ΔUA-GlcNaC6S; 2S, ΔUA2S-GlcNaC; 256S (ΔIA), ΔUA2S-GlcNaC6S; SAX, strong anion exchange; SPR, surface plasmon resonance; T1, ΔUA-GlcNaC6S-GLcA-GlcNS3S; T2, ΔUA-GlcNaC6S-GLcA-GlcNS3S6S; T3, ΔUA-GlcNaC6S6S-GLcA-GlcNS3S6S; T4, ΔUA2S-GlcNaC6S-GLcA-GlcNS3S6S; T5, ΔUA2S-GlcNaC6S-GLcA-GlcNS3S6S6S; TriS (Δ)S, ΔUA2S-GlcNaS6S; UFH, unfractionated heparin; USP, United States Pharmacopeia

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