Carbohydrate Polymers 295 (2022) 119825

Contents lists available at ScienceDirect

Carbohydrate Polymers

journal homepage: www.elsevier.com/locate/carbpol

# Enzymatic synthesis of low molecular weight heparins from *N*-sulfo heparosan depolymerized by heparanase or heparin lyase

Yanlei Yu [a], Li Fu [b,e], Peng He [b], Ke Xia [b], Sony Varghese [e], Jonathan Dordick [c,d,e], Hong Wang [a,*], Fuming Zhang [c,e,**], Robert J. Linhardt [b,c,d,e,***]

[a] College of Pharmaceutical Science & Collaborative Innovation Center for Yangtze River Delta Region Green Pharmaceuticals, Key Laboratory of Marine Fishery Resources Exploitment & Utilization of Zhejiang Province, Zhejiang University of Technology, 310014 Hangzhou, People's Republic of China
[b] Department of Chemistry and Chemical Biology, Center for Biotechnology and Interdisciplinary Studies, Rensselaer Polytechnic Institute, Troy, NY 12180, USA
[c] Department of Chemical and Biological Engineering, Center for Biotechnology and Interdisciplinary Studies, Rensselaer Polytechnic Institute, Troy, NY 12180, USA
[d] Department of Biology, Center for Biotechnology and Interdisciplinary Studies, Rensselaer Polytechnic Institute, Troy, NY 12180, USA
[e] Department of Biomedical Engineering, Center for Biotechnology and Interdisciplinary Studies, Rensselaer Polytechnic Institute, Troy, NY 12180, USA

ARTICLE INFO

*Keywords:*
Heparanase Bp
Heparinase III
Low molecular weight heparin
Enzymatic synthesis
Anticoagulant activity

ABSTRACT

Low-molecular-weight heparin (LMWH) is prepared from the controlled chemical or enzymatic depolymerization of animal sourced heparins. It has been widely used as an anticoagulant. Concerns about the shortcomings of animal-derived heparin and the contamination of supply chain demand biochemical approaches for synthesizing LMWH. In the present study, two LMWHs were enzymatically synthesized from low molecular weight *N*-sulfated heparosan (LMW-NSH) cleaved by recombinant hydrolase, *endo*-β-glucuronidase, (HepBp) or heparin lyase III (HepIII), followed by subsequent sulfotransferase modifications. Structural characterization shows that LMWH chains prepared using HepBp had a saturated uronic acid residue at their reducing ends, while chains of LMWH prepared using HepIII had an unsaturated uronic acid residue at their non-reducing end. Both LMWHs had anti-factor Xa and anti-factor IIa activities comparable to enoxaparin. This approach demonstrates that the hydrolase, HepBp, can be used to prepare a new type of LMWH that has no unsaturated uronic acid at its non-reducing end.

## 1. Introduction

Unfractionated heparin (UFH) and low molecular weight heparin (LMWH), both derived from porcine intestinal mucosa, are the major clinical anticoagulants approved in most countries (Mulloy et al., 2016). Heparin is a linear sulfated polysaccharide modified with an average of ~2.3 sulfo groups per disaccharide unit and has an average molecular weight (Mw) of ~18.0 kDa with a high polydispersity (Linhardt, 2003). The anticoagulant activity of heparin is primarily derived from its ability to bind antithrombin III (AT), a serine protease inhibitor that inactivates various activated coagulation serine proteases (Rezaie and Giri, 2020). LMWHs are manufactured through the controlled chemical or enzymatic depolymerization of UFH to afford a product having a Mw ranging from 3 to 7 kDa. LMWHs inhibit the coagulation cascade primarily through inactivation factor Xa since most of their chains have an insufficient number of disaccharide units to form in a ternary complex with AT and factor IIa (Gray et al., 2008). In contrast to the *intravenously* administered UFH, LMWHs are routinely clinically used *subcutaneously* for the treatment of venous thromboembolism and other coagulation abnormalities (Lee et al., 2003). Following their introduction in the 1980s, LMWHs have captured a large share of the U.S. heparin market as they show improved bioavailability, longer half-life, predictable pharmacokinetics and reduced propensity to induce thrombocytopenia (Hao et al., 2019).

The fragility of heparin supply chain, the susceptibility of the pig population to infectious agents, and the potential risk of contamination or adulteration has led the scientific community to search for alternative sources of heparin (Vilanova et al., 2019). Chemobiocatalytic synthesis

* Corresponding author.
** Correspondence to: F. Zhang, Department of Chemical and Biological Engineering, Center for Biotechnology and Interdisciplinary Studies, Rensselaer Polytechnic Institute, Troy, NY 12180, USA.
*** Correspondence to: R. J. Linhardt, Department of Biology, Department of Biomedical Engineering, Center for Biotechnology and Interdisciplinary Studies, Rensselaer Polytechnic Institute, Troy, NY 12180, USA.
*E-mail addresses:* hongw@zjut.edu.cn (H. Wang), Zhangf2@rpi.edu (F. Zhang), linhar@rpi.edu (R.J. Linhardt).

https://doi.org/10.1016/j.carbpol.2022.119825
Received 15 May 2022; Received in revised form 30 June 2022; Accepted 30 June 2022
Available online 5 July 2022

of heparin, LMWH and ultralow molecular weight heparin (ULMWH) has been proposed as an alternative approach driven by the successful expression of recombinant heparin biosynthetic enzymes including glycosyltransferases and sulfotransferases.

Heparin biosynthesis is initiated by the enzymatic synthesis of a tetrasaccharide linker (D-xylose (Xyl)-D-galactose (Gal)-Gal-glucuronic acid (GlcA)) on the serine residue of core protein in the endoplasmic reticulum (ER) (Esko and Selleck, 2002). The chains are next elongated with repeating 1-4 linked *N*-acetyl-α-D-glucosamine (GlcNAc)-GlcA disaccharide building block by exostosin glycosyltransferase (EXT) 1 and 2 to form a heparosan polysaccharide chain (Kuhn et al., 2001). Most of the *N*-acetyl groups of the GlcNAc residues are removed through the action of *N*-deacetylase/*N*-sulfotransferase (NDST) and are replaced with *N*-sulfo groups (Atienza et al., 2021). Subsequent modifications are carried out by C5-*epi*merase (C5-epi) and 2- and 6-*O*-sulfotransferases (*OST*s) (Debarnot et al., 2019; Weiss et al., 2020). The sulfation at 3-*O*-position affords an AT binding site pentasaccharide sequence, the most common one having the structure, →4) α-GlcNAc6s (1 → 4) β-GlcA (1 → 4) α-GlcNS3S6S (1 → 4) α-IdoA2S (1 → 4) α-GlcNS6S (1→ (Chang et al., 2014).

Chemobiocatalytic synthesis approach generally mimics the heparin biosynthesis, which begins with bacteria *Escherichia coli* K5 polysaccharide heparosan, and then relies on chemical and/or enzymatic treatment to afford an UFH for conversion to a low molecular weight product (Fu et al., 2016). Heparin lyase I (E.C. 4.2.2.7), II (E.C. 4.2.2.X), III (E.C. 4.2.2.8) isolated from *Flavobacterium heparinum* have been widely studied and applied since the 1970s (Dietrich et al., 1973; Lohse and Linhardt, 1992). They cleave the α-1,4 glycosidic linkage between acetylglucosamine and uronic acid residues through an eliminative mechanism resulting in unsaturated uronic acid residues at the non-reducing ends (Desai et al., 1993a, 1993b; Tripathi et al., 2012; Wu et al., 2022). Commercially available tinizaparin (Innohep®) is produced using HepI and introduces a double bond at the reducing end of LMWH chains (Maddineni et al., 2006). The *endo*-β-glucuronidase (a glycoside hydrolase) heparanase Bp (HepBp), derived from the pathogenic bacteria *Burkholderia pseudomallei*, is another enzyme capable of cleaving heparan sulfate chain first reported in 2015 (Bohlmann et al., 2015). HepBp cleaves GlcA β-1,4 linked to an GlcNAc, GlcNS or GlcNAc6S residue and is unable to cleave IdoA and GlcA residues presented in highly sulfated domains (Yu et al., 2019). HepBp has no activity on heparin making it unsuitable for directly preparing a LMWH from UFH. The products afforded by HepBp, however, preserves the chirality of the uronic acid C5 that is lost in the preparation of the LMWH tinzaparin using heparin lyase.

In the current study we applied HepBp to the controlled depolymerization of *N*-sulfo, *N*-acetyl heparosan (NSH) to obtain LMW-NSH, followed by the enzymatic synthesis, to obtain a LMWH without unsaturated uronic acid residues. We compared this new LMWH with a LMWH similarly prepared using HepIII and chemically depolymerized enoxaparin both containing unsaturated uronic acid residues at the non-reducing ends of their chains. Our enzymatic synthesis strategy, which mimics the heparin biosynthesis, appear to afford a LMWH biologically equivalent to animal-sourced enoxaparin. Moreover, unlike all the current LMWH products that contain process artifacts (i.e., unsaturated uronic acid residues, 1,6-anhydro mannose residues, 2,5-anhydromannitol residues, etc.), this new LMWH is process artifact-free.

## 2. Experimental section

### 2.1. Materials

Recombinant *F. heparinum* heparin lyase I, II, III (EC Nots. 4.2.2.7, 4.2.2.X, and 4.2.2.8, respectively) were expressed in *E. coli* and purified in our laboratory or purchased (R&D Systems, Minneapolis, MN, USA). Recombinant heparanase Bp from pathogenic bacteria *B. pseudomallei* was expressed in *E. coli* and purified in our lab as described (Yu et al., 2019). 2-*O*-sulfotransferase, 6-*O*-sulfotransferase-3, 3-*O*-sulfotransferase-1 and C5-epimerase were prepared in our lab as previously described (Fu et al., 2017; Zhang et al., 2014; Zhang et al., 2015). Biophen heparin anti-Xa (2 stages) and anti-IIa (2 stages) kits were purchased from Aniara (West Chester, OH, USA). Enoxaparin LMWH standard was obtained from the United States Phaemacopeia (USP, Rockville, MD). Human AT and platelet factor 4 (PF4) were purchased from Hyphen BioMed (Neuville-sur-Oise, France). Unsaturated heparin disaccharide standards were purchased from Iduron (Manchester, UK).

### 2.2. Treatment of LMW-NSH with HepBp or HepIII

*E. coli* K5 heparosan Mw ~49 kDa was treated with hydrochloric acid to remove 3-deoxy-D-*manno*-oct-2-ulosonic acid primer (Yan et al., 2020) and then treated with sodium hydroxide followed by trimethylamine-sulfur trioxide complex to afford an NSH (with 15 % of the GlcN residues being GlcNS and 85 % being GlcNAc) with a Mw of ~10 kDa (Wang et al., 2011). HepBp was used to cleave the NSH to obtain LMW-NSH, targeted at a Mw of approximately 4.0 kDa. Enzymatic reactions were carried out as follows: NSH was dissolved in 50 mM ammonium acetate buffer (pH 4.5) and digested for 1 to 60 min at 37 °C with different amount of HepBp for optimization. The samples were taken out for several time points and terminated by boiling for 5 min. The reaction time, substrate concentration and enzyme amount were optimized to obtain the targeted Mw of LMW-NSH profiled by GPC analysis.

NSH was partially digested with HepIII to obtain LMW-NSH of Mw of 4.0 kDa. The reaction was incubated for 1 to 60 min at 37 °C in digestion buffer (50 mM ammonium acetate containing 2 mM calcium chloride, pH 7.2) for optimization and deactivated by boiling for 5 min. The reaction time, substrate concentration and enzyme amount were optimized.

### 2.3. Enzymatic synthesis of LMW-N-sulfo, 2-O-sulfo heparosan using 2-OST and C5-epi

LMW-NSH intermediate (50 mg) acquired from HepBp or HepIII cleavage were treated with C5-epi and 2-*OST* to afford low molecular weight *N*-sulfo, 2-*O*-sulfo heparosan (LMW-NS2SH). Immobilized C5-epi and 2-*OST* enzymes were mixed in a tube in a 50 % slurry concentrated at 1 mg/mL. The intermediate and 3′-phosphoadenosine 5′-phosphosulfate (PAPS) were added into the slurry at final concentration of 1 mg/mL and 5 mM, respectively. The reaction was incubated in 50 mM 2-(*N*-morpholino) ethane sulfonic acid buffer (MES, pH 7.2) with 0.05 % $NaN_3$ and 125 mM NaCl for 120 h at 37 °C. Disaccharide compositional analysis was used to monitor and confirm the sulfation reaction. The mixture was filtered to remove resin and dialyzed using 1 kDa molecular weight cut-off (MWCO) membrane to remove salt and other small molecular impurities after the reaction was completed.

### 2.4. Enzymatic synthesis of LMW-N-sulfo, 2-O-sulfo, 6-O-sulfo heparosan by 6-OST

The resulting LMW-NS2SH intermediate was subjected to 6-*OST* modification to obtain LMW-*N-sulfo*, *2-O-sulfo*, *6-O-sulfo heparosan* (TriS). The intermediate and PAPS were added into immobilized 6-*OST* resin to obtain final concentration of 3 mg/mL and 15 mM, respectively. The reaction was incubated in 50 mM MES buffer (pH 7.2) with 0.05 % $NaN_3$ and 125 mM NaCl for 24 h at 37 °C. Disaccharide compositional analysis was used to monitor and confirm the reaction. Once the reaction was complete, remove the resin and dialyze using 1 kDa MWCO membrane.

### 2.5. Enzymatic synthesis of LMWH by treatment with 3-OST

LMW-TriS intermediate was next treated with 3-*OST* to afford an

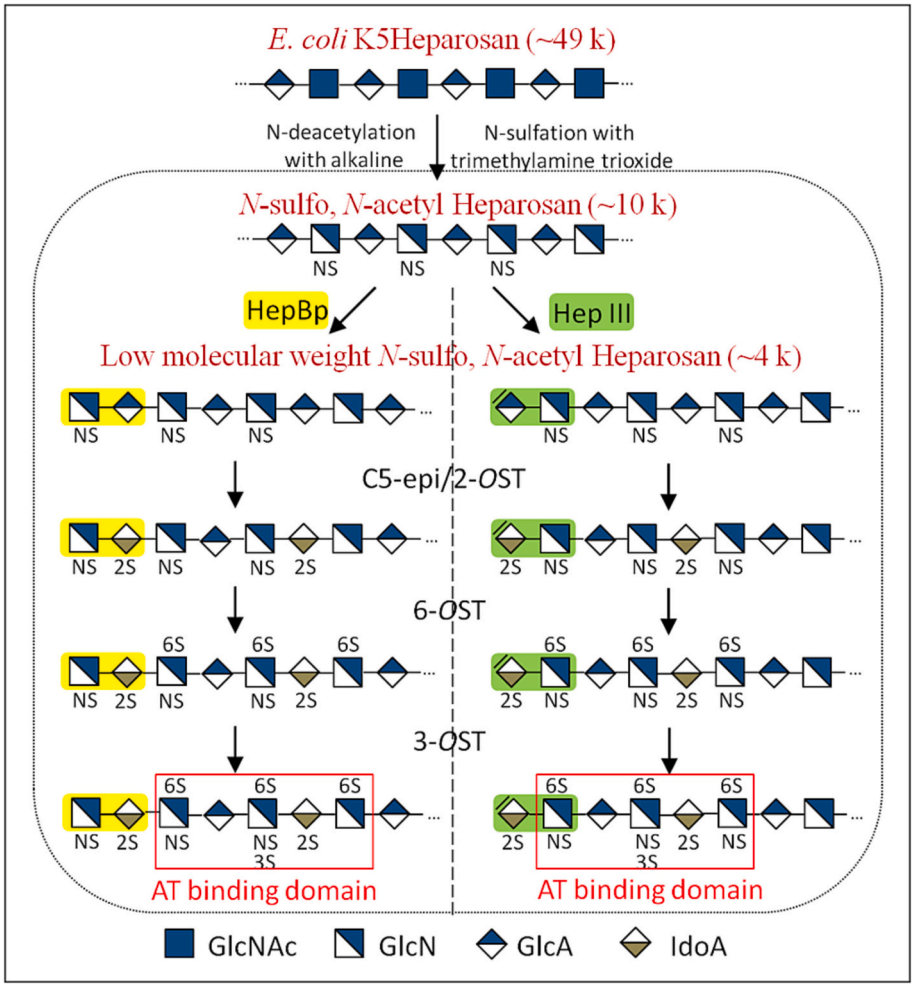


**Fig. 1.** Scheme for enzymatic biosynthesis of LMWH sourced using HepBp and HepIII cleavage.

enzymatic biocatalytic LMWH. The intermediate and PAPS were added into immobilized 3-*OST* resin to obtain final concentration of 10 mg/mL and 7.5 mM, respectively. The reaction was incubated in 50 mM MES buffer (pH 7.2) with 0.05 % $NaN_3$ and 125 mM NaCl for 120 h at 37 °C. Anti-Xa activity test was used to monitor the reaction.

### 2.6. Molecular weight distribution determined by gel permeation chromatography

The molecular weight distribution was determined by high performance liquid chromatography (HPLC)-gel permeation chromatography (GPC) using enoxaparin calibrants as standards. HPLC system is consisting of Shimadzu LC-10Ai pump, a Shimadzu CBM-20A controller and a Shimadzu RID-10A refractive index detector (Shimadzu, Kyoto, Japan). A guard column BioSuite 7.5 × 75 mm was used to protect two series connected analytical columns: Waters BioSuite™ 125, 5 μm HR SEC 7.8 × 300 mm column (Waters Corporation, Milford, MA). The mobile phase was 0.5 M lithium nitrate, and the flow rate was set at 0.6 mL/min. The sample injection volume was 20 μL with concentration at 5 mg/mL.

### 2.7. Disaccharide and tetrasaccharide composition analysis

Disaccharide and tetrasaccharide composition were determined by strong anion exchange (SAX)-HPLC with ultraviolet detector performed on Shimadzu LC-2030 (Shimadzu, Kyoto, Japan). Each intermediate and final product (100 μg) were exhaustive digested using a mixture of heparin lyase I, II and III (10 mU each) in digestion buffer at 37 °C for 2 h. The reaction was terminated by boiling for 10 min and the denatured enzymes were removed by centrifugation at 10,000 ×*g* for 10 min. The supernatant concentrated at 1 μg/μL was used for HPLC analysis. A Spherisorb SAX chromatography column (4.0 × 250 mm, 5.0 μm, Waters) was used. Mobile phase A: 1.8 mM monobasic sodium phosphate, pH 3.0, mobile phase B: 1.8 mM monobasic sodium phosphate with 2 M sodium perchlorate, pH 3.0. A gradient elution was used in which mobile phase B was increased from 5 % to 50 % in 30 min, held for 5 min then changed to 5 % and held for another 15 min.

### 2.8. Anticoagulant activity

The anticoagulant activities of 3-*OST* modified intermediates and final product were determined using BIOPHEN Heparin Anti-Xa (2 stages) and Anti-IIa (2 stages) kits following the protocols provided by the manufacturer. Briefly, reagent AT (r1), factor Xa (r2) and factor Xa specific chromogenic substrate (r3) for anti-Xa, AT (R1), human thrombin (R2) and factor IIa specific chromogenic substrate (R3) for anti-IIa were reconstituted with 1 mL distilled water and shaken until fully dissolved. The reagents were restored by a 1/5 dilution in the appropriate buffer (Tris-EDTA-NaCl-PEG, pH 8.4) for r1/R1 and r2/R2, distilled water for r3/R3 immediately before use. Samples and standards (40 μL) were added into a 96-well plate and incubated for 5 min at 37 °C, 40 μL r1/R1 was added and mixed well and incubated for 2 min, 40 μL r2/R2 was next added and incubated for 2 min, 40 μL r3/R3 was added last and incubated for another 2 min. The reactions were stopped by

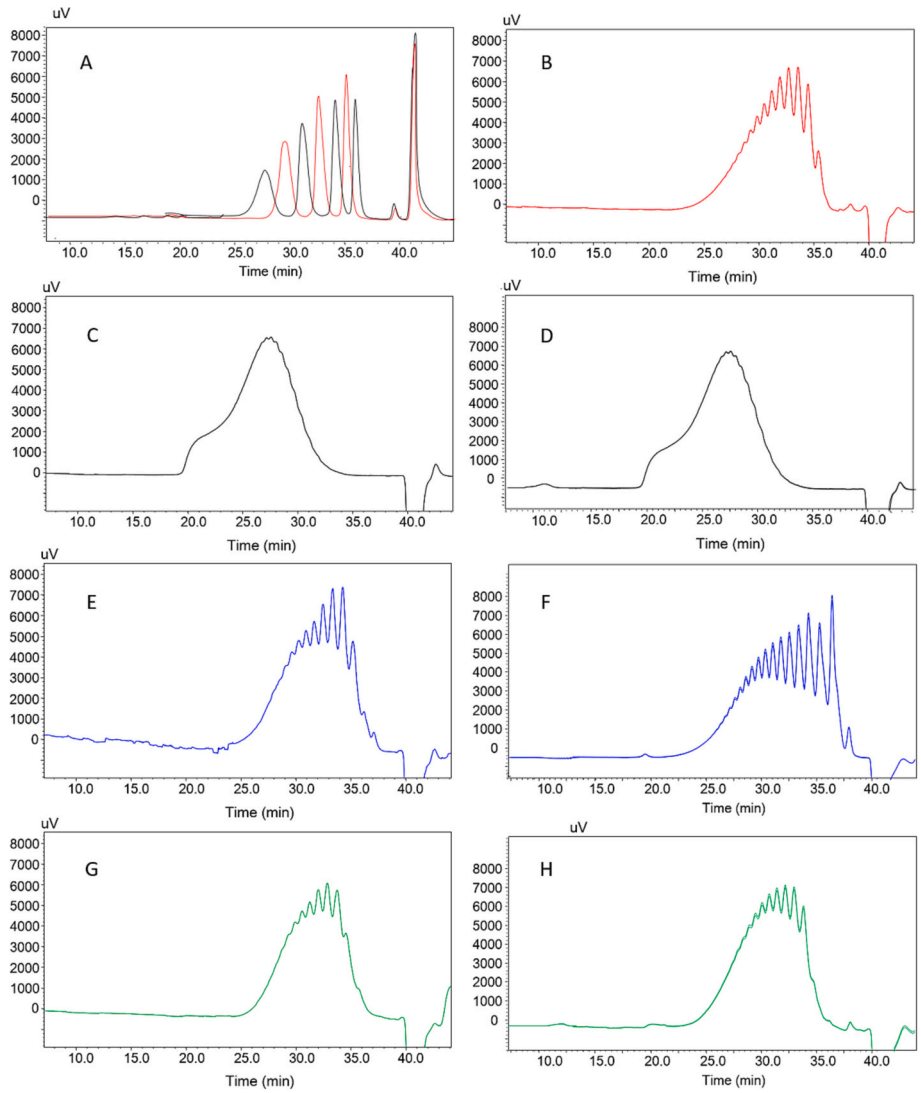


**Fig. 2.** Molecular weight distribution analyzed by GPC. A. enoxaparin sodium molecular weight calibrant A, black, peaks from left to right were 9250, 4550, 2250, 1400 Da, enoxaparin calibrant B red, peaks from left to right were 6650, 3350, 1800 Da; B. enoxaparin sodium, C & D. NSH raw material, E. LMW-NSH cleaved by HepBp, F. LMW-MSH cleaved by HepIII, G. LMWH (HepBp), H. LMWH (HepIII).

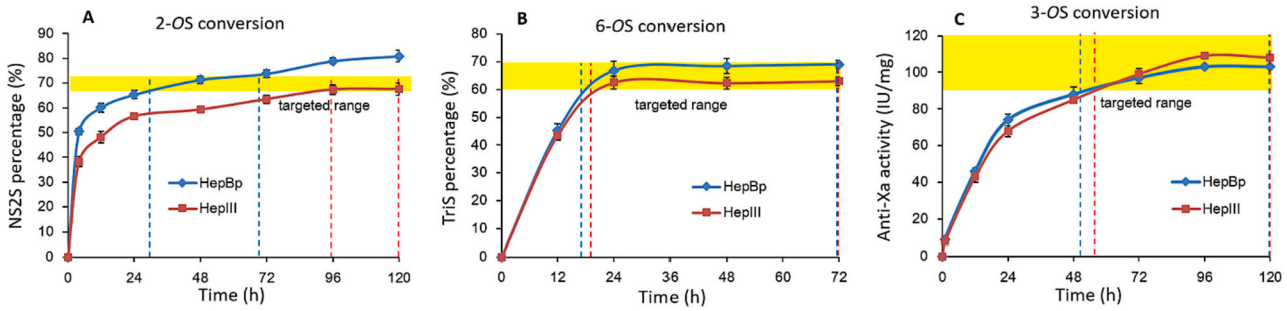


**Fig. 3.** Sulfotransferase modifications of enzymatic biosynthesis LMWHs. A. NS2S conversion by 2-*O*-sulfotransferase and C5-epimerase reaction; B. TriS conversion by 6-*O*-sulfotransferase reaction; C. Anti-Xa activity by 3-*O*-sulfotransferase reaction.

adding 80 μL of 50 mM acetic acid. The absorbance was then determined at 405 nm.

### 2.9. Nuclear magnetic resonance spectroscopy analysis

The nuclear magnetic resonance (NMR) spectra were obtained on a Bruker 800 MHz (18.8 T) standard-bore NMR spectrometer equipped with a $^{1}H/^{2}H/^{13}C/^{15}N$ cryoprobe with z-axis gradients. Sample was dissolved in 0.4 mL of 99.96 % $^{2}H_2O$ and lyophilized, and then repeated twice. A $^{1}H/^{13}C$ NMR was carried out at 298 k.

**Table 1**
Summary of anticoagulant activity and $IC_{50}$ values of LMWHs.

| | Mw (Da) | Anticoagulant activity | | | $IC_{50}$ (μg/mL) | |
|---|---|---|---|---|---|---|
| | | Anti-Xa (IU/mg) | Anti-IIa (IU/mg) | ratio | AT III | PF4 |
| USP criteria | 3800–5000 | NMT 125, NLT 90 | NMT 35, NLT 20 | 3.3–5.3 | | |
| Enoxaparin | 4170 ± 25 | 110.0 ± 1.0 | 24.7 ± 1.5 | 4.4 ± 0.3 | 11.0 ± 0.2 | 2.7 ± 0.1 |
| HepBp | 4250 ± 40 | 102.3 ± 0.6 | 23.3 ± 1.5 | 4.5 ± 0.2 | 9.5 ± 0.1 | 2.5 ± 0.3 |
| HepIII | 4480 ± 75 | 109.3 ± 1.5 | 27.3 ± 1.5 | 4.0 ± 0.2 | 12.0 ± 0.3 | 2.2 ± 0.1 |

### 2.10. Surface plasmon resonance analysis

Surface plasmon resonance (SPR) measurements were performed on a BIAcore 3000 instrument (GE, Uppsala, Sweden) operated using BIAcore 3000 control and BIAevaluation software (version 4.0.1). Biotinylated heparin prepared by conjugating the reducing end of heparin to amine-$PEG_3$-Biotin (Pierce, Rockford, IL) was immobilized to a streptavidin (SA)-coated chip based on the manufacturer's protocol. Competition studies between surface heparin and enzymatic biocatalytic LMWH binding to proteins was performed using SPR through measurement of $IC_{50}$. AT (250 nM) or PF4 (125 nM) mixed with different concentration of chemobiocatalytic LMWH in HBS-EP buffer was injected over the chip at a flow rate of 30 μL/min. Dissociation and regeneration were performed using sequential injection with 10 mM glycine-HCl (pH 2.5) and 2 M NaCl to obtain fully regenerated surface after each run.

## 3. Results and discussion

The heparin supply chain depends on a vulnerable animal population putting the world supply at risk, furthermore the disease epidemics in swine population aggravates these concerns. Commercially available LMWHs are also at risk as they are currently all derived from porcine heparin. Modern chemobiocatalytic or biological synthesis capabilities offer an alternative source of heparin (Thacker et al., 2022). Recently, our laboratory developed a chemobiocatalytic approach of LMWH synthesis sourced from heparosan (Yu et al., 2022). However, a depolymerization process using basic reagents or heparin lyase can produce large amounts of a process artifact at the non-reducing end, unsaturated uronic acid residues. Moreover, the impact of nonreducing end, unsaturated uronic acid residues on epimerase and sulfotransferase activities and on platelet factor 4 binding has not been studied. The recent discovery of HepBp offers an alternative approach for the cleavage of large polysaccharide chains to a chain size appropriate for a LMWH. HepBp is an *endo*-β-glucuronidase that hydrolyzes the 1,4-linkage between GlcA and GlcNAc (or GlcNS) residues. HepBp has a substrate preference for polysaccharides with a low level of sulfation (Bohlmann et al., 2015; Yu et al., 2019). Compared to our previous chemobiocatalytic approach, enzymatic synthesized LMWH sourced from HepBp cleaved LMW-NSH provide a new LMWH without a process artifact at the non-reducing end of the resulting chains. The proposed approach for the enzymatic biocatalysis synthesis of LMWH relying on HepBp or HepIII is shown in Fig. 1. Starting from NSH, HepBp or HepIII was used for the controlled cleavage of chains to reach the targeted Mw range, ~4 kDa, after which enzymatic modification was used to synthesize LMWH.

### 3.1. Depolymerization of NSH using HepBp or HepIII

We used controlled HepBp or HepIII enzymatic depolymerization to obtain the LMW-NSH of Mw targeted from 3.8 to 4.5 kDa. The amount of

**Table 2**
Disaccharide and tetrasaccharide compositional analysis.

| | USP heparin (%) | Enoxaparin (%) | Hep III (%) | HepBp (%) |
|---|---|---|---|---|
| ΔU-GlcNAc (0S) | 4.2 ± 0.26 | 3.1 ± 0.10 | 2.7 ± 0.26 | 3.8 ± 0.35 |
| ΔU-GlcNS (NS) | 3.3 ± 0.31 | 3.0 ± 0.15 | 6.0 ± 0.36 | 2.5 ± 0.17 |
| ΔU-GlcNAc, 6S (6S) | 3.5 ± 0.45 | 3.6 ± 0.15 | 3.0 ± 0.31 | 3.2 ± 0.21 |
| ΔU2S-GlcNAc (2S) | 2.0 ± 0.15 | 1.9 ± 0.26 | 0.0 | 0.0 |
| ΔU-GlcNS, 6S (NS6S) | 10.6 ± 0.67 | 10.3 ± 0.50 | 16.8 ± 0.83 | 13.2 ± 0.57 |
| ΔU2S-GlcNS (NS2S) | 7.7 ± 0.35 | 7.0 ± 0.20 | 8.5 ± 0.20 | 4.6 ± 0.06 |
| ΔU2S-GlcNAc, 6S (2S6S) | 1.4 ± 0.40 | 1.8 ± 0.40 | 0.8 ± 0.15 | 0.1 ± 0.03 |
| Tetra 1 6S-NS3S | 0.1 ± 0.03 | 0.4 ± 0.27 | 0.1 ± 0.04 | 0.3 ± 0.10 |
| ΔU2S-GlcNS, 6S (TriS) | 65.6 ± 1.20 | 66.3 ± 1.03 | 60.1 ± 0.60 | 71.2 ± 0.91 |
| Tetra 2 6S-NS3S6S | 1.4 ± 0.12 | 1.9 ± 0.35 | 1.3 ± 0.31 | 0.0 |
| Tetra 3 NS6S-NS3S6S | 0.1 ± 0.04 | 0.4 ± 0.10 | 0.4 ± 0.15 | 0.3 ± 0.10 |
| Tetra 4 2S6S-NS3S6S | 0.1 ± 0.04 | 0.3 ± 0.06 | 0.1 ± 0.08 | 0.3 ± 0.06 |
| Tetra 5 TriS-NS3S6S | 0.1 ± 0.06 | 0.1 ± 0.04 | 0.2 ± 0.12 | 0.6 ± 0.15 |
| Tetra 1–5 subtotal | 1.8 ± 0.28 | 3.1 ± 0.82 | 2.2 ± 0.69 | 1.5 ± 0.41 |
| Total (dp2 & 4) | 100.0 | 100.0 | 100.0 | 100.0 |

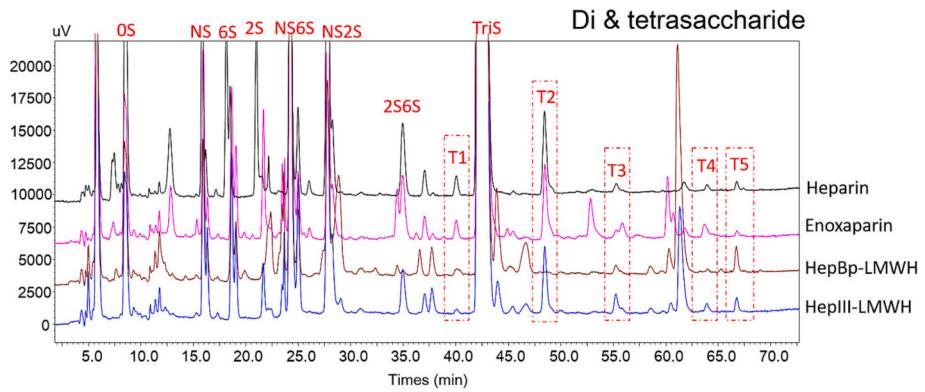


**Fig. 4.** Disaccharide and tetrasaccharide compositional analysis of enzymatically synthesized LMWHs.

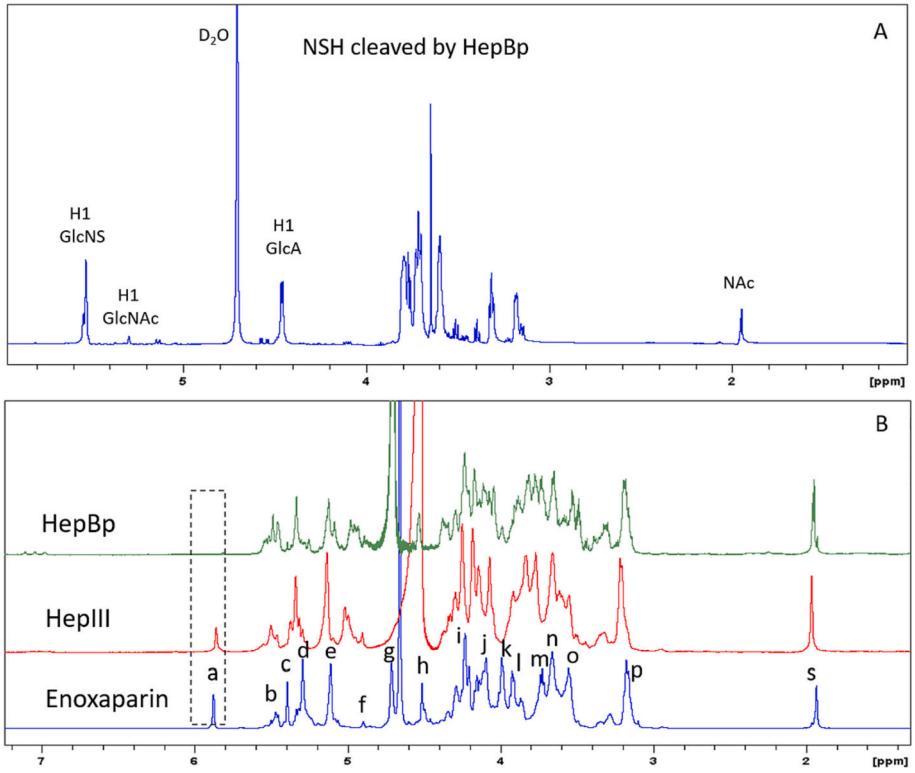


**Fig. 5.** A. $^{1}$H NMR of HepBp cleaved LMW-NSH, B. $^{1}$H NMR of LMWH (HepBP), LMWH (HepIII) and enoxaparin. Enoxaparin assignment: a. H4 ΔU2S, b. H1 GlcNS6S-(GlcA), c. H1ΔU2S, d. H1 GlcNS6X-(IdoA2S) and GlcNAc6X-GlcA, e. H1 IdoA2S. f. H1 IdoA-(GlcNS6S), g. H5 IdoA2S, h. H1 GlcA, i. H2 IdoA2S, j. H6 GlcNS6S, k. H3 IdoA2S, l. H4 IdoA2S, m. H6 GlcNS, n. H4 GlcNS6S, o. H3 GlcNS6X, p. H2 GlcNS6X, s. $CH_3$ GlcNAc.

substrate, amount of enzyme and reaction time were optimized, and the results were determined using GPC analysis. The original Mw of NSH was 10.0 ± 0.2 kDa determined by GPC (Fig. 2C & D). The optimal enzymatic digestion conditions for HepBp were: 100 μg NSH substrate was treated with 2 μL HepBp (0.7 mg/mL) in 100 μL 50 mM ammonium acetate buffer (pH 4.5) for 15 min at 37 °C. The optimal enzymatic digestion conditions for HepIII were: 100 μg NSH substrate was treated with 5 μL HepIII (10 U/mg) in 50 mM ammonium acetate buffer (pH 7.0) for 5 min at 37 °C. The LMW-NSH obtained using HepBp had a Mw of 3950 ± 30 Da (Fig. 2E) and for HepIII was 4250 ± 50 Da (Fig. 2F), Using the same GPC analysis, enoxaparin standard (Fig. 2B) had a Mw of 4150 ± 50 Da based on three independent experiments. The chromatogram shown in green corresponds to the final enzymatic synthesized LMWH products and had a Mw of 4250 ± 40 Da (Fig. 2G) produced using HepBp and 4500 ± 50 Da (Fig. 2H) produced using HepIII. These results show the expected increase in Mw following sulfotransferase modification.

### 3.2. Enzymatic synthesis of LMWHs

The enzymatic modification relied on four steps: (1) conversion of GlcA into IdoA by C5-*epi*; (2) sulfation at C2 position of IdoA or GlcA by 2-*O*ST; (3) sulfation at C6 position of the GlcN residue by 6-*O*ST; (4) sulfation at C3 position of GlcN residue by 3-*O*ST (Wang et al., 2020). The conversion of GlcA residue to IdoA is catalyzed by C5-epi, a reversible reaction, which can then be locked in place with 2-*O*-sulfation to afford IdoA2S (and minor amounts of GlcA2S) residue (Zhang et al., 2008). Therefore, C5-epi and 2-*O*ST catalysts are used at the same time and the resulting product is monitored by disaccharide compositional analysis. Both LMW-NSH processes could reach 2-*O*-sulfation levels of 68 %–74 %, however, the presence of unsaturated uronic acid on the non-reducing end negatively impacted the activity of 2-*O*ST (Fig. 3A). Next, the conversion of LMW-NS2SH to LMW-TriS relied on 6-*O*ST catalysis and both processes reached ~65 % TriS content within 24 h (Fig. 3B). The 3-*O*ST reaction was monitored by anti-Xa activity assay (Babin et al., 2017). The anti-Xa activity of enoxaparin should be no less than (NLT) 90 IU/mg and no more than (NMT) 125 IU/mg. The HepBp and HepIII LMWH were 102.3 ± 0.6 IU/mg and 109.3 ± 1.5 IU/mg, respectively (Fig. 3C).

### 3.3. Anticoagulant activity and molecular weight distribution of enzymatic synthesis LMWHs

The synthesized LMWHs were next subjected to anticoagulant activity analysis and Mw determination. The LMWH synthesized from HepBp cleavage had an anti-Xa activity of 102.3 ± 0.6 IU/mg and anti-IIa activity of 23.3 ± 1.5 IU/mg with an anti Xa/IIa ratio of 4.5 ± 0.2. The LMWH synthesized from HepIII cleavage has an anti-Xa activity of 109.3 ± 1.5 IU/mg and anti-IIa activity of 27.3 ± 1.5 IU/mg with anti Xa/IIa ratio of 4.0 ± 0.2. Both processes afforded a LMWH that met the USP potency criteria for enoxaparin, NLT 90 and NMT 125 IU/mg for anti-Xa activity, NLT 20.0 and NMT 35.0 IU/mg for anti-IIa activity, the ratio of anti-Xa to anti-IIa is between 3.3 and 5.3 (Table 1). The Mw of final LMWH products sourced from HepBp and HepIII cleavage was 4250 Da and 4480 Da, respectively (Table 1), meet the USP criteria for enoxaparin ranged from 3800 to 5000 Da (Arnold et al., 2017). Furthermore, the peak shapes were quite similar compared to those of enoxaparin (Fig. S1). Starting from LMW-NSH of 3950 Da and 4250 Da, sulfation modifications slightly increased the Mw of final products through the addition of sulfate groups.

### 3.4. Disaccharide and tetrasaccharide analysis

Disaccharide compositional analysis of the final enzymatic synthesized LMWH products and their intermediates was used to monitor the catalytic reactions and determine their similarity compared to

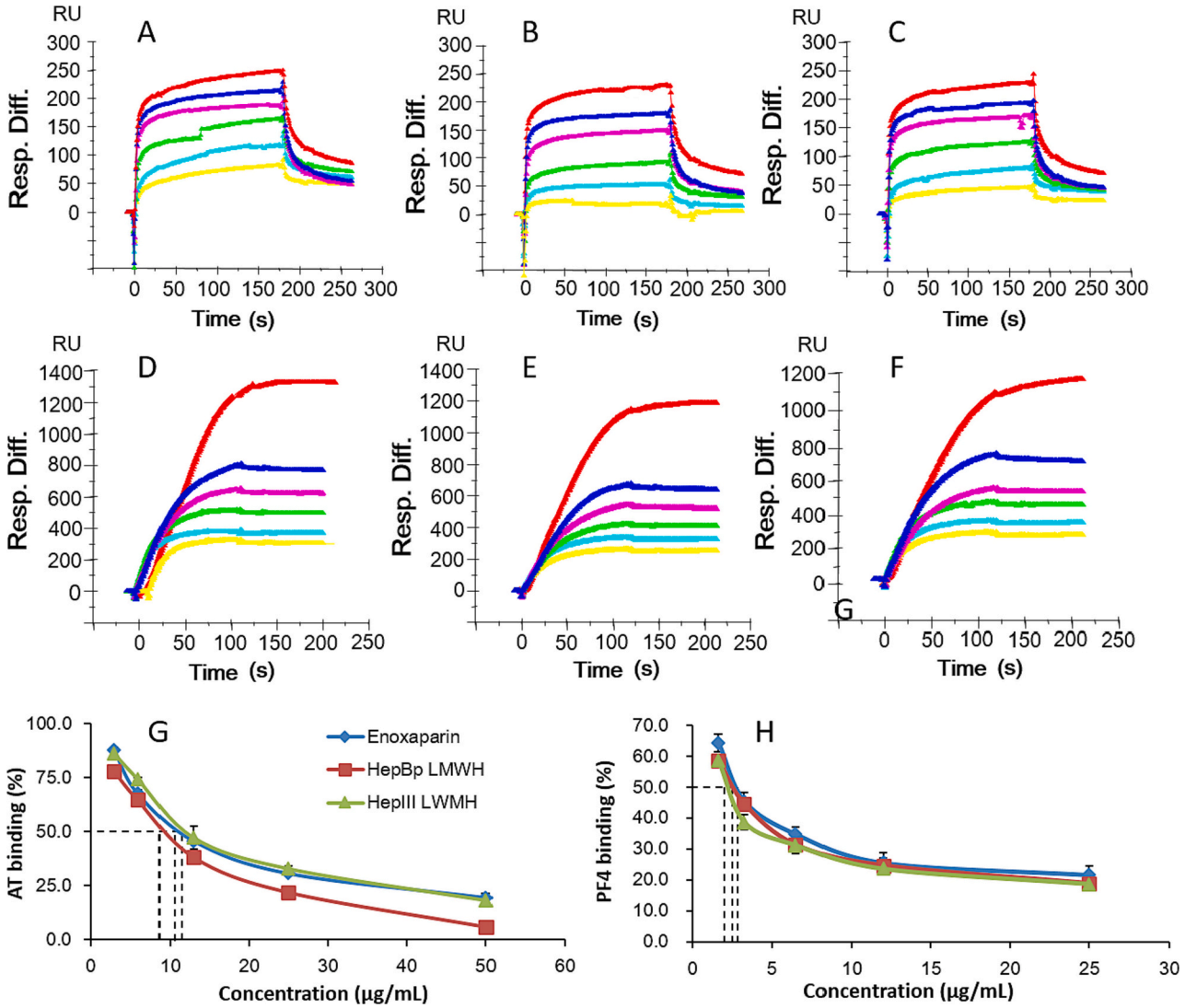


**Fig. 6.** SPR sensorgrams of AT (A–C) and PF4 (D–F) binding to heparin surface competing with different LMWH samples. A. Enoxaparin and AT, B. HepBp-LMWH and AT, C. HepIII-LMWH and AT, D. Enoxaparin and PF4, E. HepBp-LMWH and PF4, F. HepIII-LMWH and PF4, G. $IC_{50}$ of AT binding, H. $IC_{50}$ of PF4 binding.

enoxaparin. Tetrasaccharide profiling is necessary in addition to disaccharide analysis since 3-*O*-sulfated glucosamine residues related to anticoagulant activity are resistant to heparin lyase cleavage in our current digestion conditions (Beecher et al., 2016; Karlsson et al., 2021). Five 3-*O*-sulfo containing tetrasaccharides: (1) ΔUA-GlcNAc6S-GlcUA-GlcNS3S; (2) ΔUA-GlcNAc6S-GlcUA-GlcNS3S6S; (3) ΔUA-GlcNS6S-GlcUA-GlcNS3S; (4) ΔUA2S-GlcNAc6S-GlcUA-GlcNS3S6S; (5) ΔUA2S-GlcNS6S-GlcUA-GlcNS3S6S were determined (Chen et al., 2017). Eight disaccharides and five 3-*O*-sulfo containing tetrasaccharides were eluted in 70 min using SAX-HPLC analysis (Fig. 4).

The results showed that enzymatic modifications of LMW-NSH cleaved by HepBp or HepIII afforded an enzymatic synthesized LMWHs that have disaccharide composition similar to enoxaparin. The disaccharide with NS2S6S content of LMWH sourced from HepBp or HepIII cleavage were 71.2 ± 0.91 % and 60.1 ± 0.60 %, respectively (Table 2). Although the NS2S6S contents of both processes were within the targeted range, it was difficult for HepIII-LMWH process to reach 60 %. This suggests that the presence of unsaturated uronic decreases 2-*OS* modification, resulting in high TriS content in HepBp and low TriS content in HepIII. The total amount of tetrasaccharide 1 to 5 of HepBp and Hep III was 1.5 ± 0.41 % and 2.2 ± 0.69 %, respectively, compared to 1.8 ± 0.28 % of UFH and 3.1 ± 0.82 % of enoxaparin. Tetrasaccharide 2 (ΔUA-GlcNAc6S-GlcUA-GlcNS3S6S) was not detected in HepBp-LMWH, and it had a higher content of tetrasaccharide 5 (ΔUA2S-GlcNS6S-GlcUA-GlcNS3S6S).

### 3.5. NMR analysis

NMR analyses showed that our two different enzymatic cleaved biocatalytic LMWHs have similar structures. Using published assignments (Guerrini et al., 2015; Jiang et al., 2022) NMR analysis was used to monitor the intermediates in each step. The $^1$H NMR of LMW-NSH cleaved by HepBp is shown in Fig. 5A. LMW-NSH is composed of GlcNAc/GlcNS-GlcA disaccharide repeating units, we can find H1 signal of GlcNS, GlcNAc and GlcA at 5.44, 5.25 and 4.51 ppm, respectively. The intermediate LMW-TriS after C5-*epi*, 2-*OST* and 6-*OST*, and before 3-*OST* modification is shown in Fig. S2. The $^1$H NMR analysis of final LMWH products was compared to enoxaparin shown in Fig. 5B. The proton signal (a) at ~5.8 ppm is assigned to H4 of ΔU2S, which indicated the presence of an unsaturated uronic acid residue at the non-reducing end. The absence of a signal at 5.8 ppm of HepBp-cleaved LMWH preparation demonstrates that there were no unsaturated uronic acid residues. IdoA2S corresponding to the signal at (e) is missing. The anomeric proton signals at b, c, d, e, f, and h can be assigned to H1 of GlcNS6S (-GlcA), ΔU2S, GlcNS6X(-IdoA2S)/GlcNAc6X(-GlcA), IdoA2S, IdoA (-GlcNS6S), and GlcA in enoxaparin, respectively. The signals detected

at the similar positions in the spectra of HepBp-LMWH and HepIII-LMWH preparations could be assigned in the same way, although minor differences were detected in the chemical shift and the peak shape of signals. It is noteworthy, that the signals between e and f (5.05 ppm) in HepBp-LMWH and HepIII-LMWH are not present in enoxaparin and require further identification.

### 3.6. Surface plasmon resonance (SPR) analysis

In addition to anticoagulant activity, the determination of interaction between heparin and AT is a crucial step for anticoagulation process. The SPR measurement is mainly based on a competitive effect between USP heparin biotinylated and immobilized on the SA chip and a LMWH sample that is pre-mixed with AT. The results were calculated as $IC_{50}$ values which were a 50 % decrease in response units (RU) from the plots over a range of LMWH concentrations. We compared the anticoagulant activities of LWMHs sourced from HepBp and HepIII cleavage to enoxaparin (Fig. 6A–C). The resulting $IC_{50}$ values for enxoaparin, HepBp, HepIII chemobiocatalytic LMWH were $11.0 \pm 0.2$, $9.5 \pm 0.1$ and $12.0 \pm 0.3$ μg/mL, respectively (Fig. 6G and Table 2). They were in the agreement with the range and consistent with the colorimetric analysis. The heparin-induced thrombocytopenia (HIT) potential caused by platelet factor IV (PF4) binding can result in an adverse immunological disorder needs particular concern for new LMWHs (Arepally, 2017; Warkentin et al., 1995). The measured $IC_{50}$ for chemobiosynthetic LMWH and PF4 binding is shown in Fig. 6D–F. The results show that the $IC_{50}$ were $2.5 \pm 0.3$ μg/mL and $2.2 \pm 0.1$ μg/mL for HepBp and Hep III LMWH compared to $2.7 \pm 0.1$ μg/mL of enoxaparin control (Fig. 6H and Table 2). As expected, the binding affinity of enzymatically synthesized LMWHs is at the level observed for enoxaparin. The saturated non-reducing end LMWH cleaved by HepBp did not appear to impact the PF4 binding.

## 4. Conclusion

Commercially available LMWHs are currently prepared by controlled chemical or enzymatic depolymerization of unfractionated heparins extracted from porcine intestine mucosal. Due to the growing concerns about shortages of animal-derived heparin and potential impurities and contamination of supply chains, our laboratory prepared chemobiocatalytic LMWHs. In the current study, we used two enzymes, HepBp and HepIII to prepare new LMWHs. The results showed that these two novel LMWHs were successful enzymatically synthesized and their structure and activity showed they were biosimilar to enoxaparin. Furthermore, the absence of unsaturated uronic acid residues at the non-reducing end cleaved by HepBp removes a process artifact associated with enoxaparin.

## CRediT authorship contribution statement

**Yanlei Yu:** Conceptualization, Investigation, Writing – original draft, Writing – review & editing, Funding acquisition. **Li Fu:** Conceptualization, Investigation. **Peng He:** Investigation. **Ke Xia:** Investigation, Supervision. **Sony Varghese:** Investigation. **Jonathan Dordick:** Resources, Supervision, Project administration, Funding acquisition. **Hong Wang:** Resources, Writing – review & editing, Supervision, Project administration, Funding acquisition. **Fuming Zhang:** Conceptualization, Resources, Supervision, Project administration, Funding acquisition. **Robert J. Linhardt:** Conceptualization, Resources, Writing – original draft, Writing – review & editing, Supervision, Project administration, Funding acquisition.

## Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

## Data availability

Data will be made available on request.

## Acknowledgements

This work was supported by GlycoMIP a National Science Foundation Materials Innovation Platform funded through Cooperative Agreement DMR-1933525; Key Research and Development Program of Zhejiang (2021C03084); and High-Level Talent Special Support Plan of Zhejiang Province (2019R52009).

## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.carbpol.2022.119825.

## References

Arepally, G. M. (2017). Heparin-induced thrombocytopenia. *Blood, 129*, 2864–2872.
Arnold, K. M., Capuzzi, S. J., Xu, Y., Muratov, E. N., Carrick, K., Szajek, A. Y., et al. (2017). Modernization of enoxaparin molecular weight determination using homogeneous standards. *Pharmaceuticals, 10*, 66.
Atienza, J., Tkachyova, I., Tropak, M., Fan, X., & Schulze, A. (2021). Fluorometric coupled enzyme assay for N-sulfotransferase activity of N-deacetylase/N-sulfotransferase (NDST). *Glycobiology, 31*, 1093–1101.
Babin, J. L., Traylor, K. L., & Witt, D. M. (2017). Laboratory monitoring of low-molecular-weight heparin and fondaparinux. *Seminars in Thrombosis and Hemostasis, 43*, 261–269.
Beecher, C. N., Manighalam, M. S., Nwachuku, A. F., & Larive, C. K. (2016). Screening enoxaparin tetrasaccharide SEC fractions for 3-O-sulfo-N-sulfoglucosamine residues using [(1)H, (15)N] HSQC NMR. *Analytical and Bioanalytical Chemistry, 408*, 1545–1555.
Bohlmann, L., Tredwell, G. D., Yu, X., Chang, C., Haselhorst, T., Moritz, W., et al. (2015). Functional and structural characterization of a heparanase. *Nature Chemical Biology, 11*, 955–957.
Chang, C., Lico, L. S., Huang, T., Lin, S., Chang, C., Arco, S. D., et al. (2014). Synthesis of the heparin-based anticoagulant drug fondaparinux. *Angewandte Chemie International EditionIn English, 53*, 9876–9879.
Chen, Y., Lin, L., Agyekun, I., Zhang, X., St. Ange, K., Yu, Y., et al. (2017). Structural analysis of heparin-derived 3-O-sulfated tetrasaccharides: antithrombin binding site vatiants. *Journal of Pharmaceutical Science, 106*, 973–981.
Debarnot, C., Monneau, Y. R., Roig-Zamboni, V., Delauzun, V., Le Narvor, C., et al. (2019). Substrate binding mode and catalytic mechanism of human heparan sulfate d-glucuronyl C5 epimerase. *Proceedings of the National Academy of Sciences of the United States of America, 116*, 6760–6765.
Desai, U. R., Wang, H. M., & Linhardt, R. J. (1993a). Specificity studies on the heparin lyases from Flavobacterium heparinum. *Biochemistry, 32*, 8140–8145.
Desai, U. R., Wang, H. M., & Linhardt, R. J. (1993b). Substrate specificity of the heparin lyases from Flavobacterium heparinum. *Archives of Biochemistry and Biophysics, 306*, 461–468.
Dietrich, C. P., Silva, M. E., & Michelacci, Y. M. (1973). Sequential degradation of heparin in Flavobacterium heparinum. Purification and properties of five enzymes involved in heparin degradation. *Journal of Biological Chemistry, 248*, 6408–6415.
Esko, J. D., & Selleck, S. B. (2002). Order out of chaos: Assembly of ligand binding sites in heparan sulfate. *Annual Review of Biochemistry, 71*, 435–471.
Fu, L., Li, K., Mori, D., Hirakane, M., Lin, L., Grover, N., et al. (2017). Enzymatic generation of highly anticoagulant bovine intestinal heparin. *Journal of Medicinal Chemistry, 60*, 8673–8679.
Fu, L., Suflita, M., & Linhardt, R. J. (2016). Bioengineered heparins and heparan sulfates. *Advanced Drug Delivery Reviews, 97*, 237–249.
Gray, E., Mulloy, B., & Barrowcliffe, T. W. (2008). Heparin and low-molecular-weight heparin. *Thrombosis and Haemostasis, 99*, 807–818.
Guerrini, M., Rudd, T. R., Mauri, L., Macchi, E., Fareed, J., Yates, E. A., et al. (2015). Differentiation of generic enoxaparins marketed in the United States by employing NMR and multivariate analysis. *Analytical Chemistry, 87*, 8275–8283.
Hao, C., Sun, M., Wang, H., Zhang, L., & Wang, W. (2019). Low molecular weight heparins and their clinical applications. *Progress in Molecular Biology and Translational Science, 163*, 21–39.
Jiang, H., Li, X., Ma, M., Shi, X., & Wu, X. (2022). Quality control and product differentiation of LMWHs marketed in China using 1H NMR spectroscopy and chemometric tools. *Journal of Pharmaceutical and Biomedical Analysis, 209*, Article 114472.
Karlsson, R., Chopra, P., Joshi, A., Yang, Z., Vakhrushev, S. Y., Clausen, T. M., et al. (2021). Dissecting structure-function of 3-O-sulfated heparin and engineered heparan sulfates. *ScienceAdvances, 7*, Article eabl6026.
Kuhn, J., Gotting, C., Schnolzer, M., Kempf, T., Brinkmann, T., & Kleesiek, K. (2001). First isolation of human UDP-D-xylose: Proteoglycan core protein beta-D-xylosyl-

transferase secreted from cultured JAR choriocarcinoma cells. *Journal of Biological Chemistry, 276*, 4940–4947.
Lee, A. Y. Y., Levine, M. N., Baker, R. I., Bowden, C., Kakkar, A. K., Prins, M., et al. (2003). Low-molecular-weight heparin versus a coumarin for the prevention of recurrent venous thromboembolism in patients with cancer. *New England Journal of Medicine, 349*, 146–153.
Linhardt, R. J. (2003). Heparin: Structure and activity. *Journal of Medicinal Chemistry, 46*, 2551–2554.
Lohse, D. L., & Linhardt, R. J. (1992). Purification and characterization of heparin lyases from Flavobacterium heparinum. *Journal of Biological Chemistry, 267*, 24347–24355.
Maddineni, J., Walenga, J. M., Jeske, W. P., Hoppensteadt, D. A., Fareed, J., Wahi, R., et al. (2006). Product individuality of commercially available low-molecular-weight heparins and their generic versions: Therapeutic implications. *Clinical and Applied Thrombosis Hemostasis, 12*, 267–276.
Mulloy, B., Hogwood, J., Gray, E., Lever, R., & Page, C. P. (2016). Pharmacology of heparin and related drugs. *Pharmacological Review, 68*, 76–141.
Rezaie, A. R., & Giri, H. (2020). Anticoagulant and signaling functions of antithrombin. *Journal of Thrombosis and Haemostasis, 18*, 3142–3153.
Thacker, B. E., Thorne, K. J., Cartwright, C., Park, J., Glass, K., Chea, A., et al. (2022). Multiplex genome editing of mammalian cells for producing recombinant heparin. *Metabolic Engineering, 70*, 155–165.
Tripathi, C. K., Banga, J., & Mishra, V. (2012). Microbial heparin/heparan sulphate lyases: Potential and applications. *Applied Microbiology and Biotechnology, 94*, 307–321.
Vilanova, E., Tovar, A. M. F., & Mourão, P. A. S. (2019). Imminent risk of a global shortage of heparin caused by the African swine fever afflicting the Chinese pig herd. *Journal of Thrombosis and Haemostasis, 17*, 254–256.
Wang, Z., Li, J., Cheong, S., Bhaskar, U., Akihiro, O., Zhang, F., Dordick, J. S., & Linhardt, R. J. (2011). Response surface optimization of the heparosan N-deacetylation in producing bioengineered heparin. *Journal of Biotechnology, 156*, 188–196.
Wang, T., Liu, L., & Voglmeir, J. (2020). Chemoenzymatic synthesis of ultralow and low-molecular weight heparins. *BBA-Proteins and Proteomics, 1868*, Article 140301.
Warkentin, T. E., Levine, M. N., Hirsh, J., Horsewood, P., Roberts, R. S., Gent, M., et al. (1995). Heparin-induced thrombocytopenia in patients treated with low-molecular-weight heparin or unfractionated heparin. *New England Journal of Medicine, 332*, 1330–1335.
Weiss, R. J., Spahn, P. N., Toledo, A. G., Chiang, A. W. T., Kellman, B. P., Li, J., et al. (2020). ZNF263 is a transcriptional regulator of heparin and heparan sulfate biosynthesis. *Proceedings of the National Academy of Sciences of the United States of America, 117*, 9311–9317.
Wu, J., Chopra, P., Boons, G., & Zaia, J. (2022). Influence of saccharide modifications on heparin lyase III substrate specificities. *Glycobiology, 32*, 208–217.
Yan, L., Fu, L., Xia, K., Chen, S., Zhang, F., Dordick, J. S., & Linhardt, R. J. (2020). A revised structure for the glycolipid terminus of E. coli K5 heparosan capsular polysaccharide. *Biomolecules, 10*, 1516.
Yu, Y., Fu, L., He, P., Xia, K., Varghese, S., Wang, H., et al. (2022). Chemobiocatalytic synthesis of a low-molecular-weight heparin. *ACS Chemical Biology, 17*, 637–646.
Yu, Y., Williams, A., Zhang, X., Fu, L., Xia, K., Xu, Y., et al. (2019). Specificity and action pattern of heparanase Bp, a β-glucuronidase from Burkholderia pseudomallei. *Glycobiology, 29*, 572–581.
Zhang, J., Suflita, M., Li, G., Zhong, W., Li, L., Dordick, J. S., et al. (2015). High cell density cultivation of recombinant Escherichia coli strains expressing 2-O-sulfotransferase and C5-epimerase for the production of bioengineered heparin. *Applied Biochemistry and Biotechnology, 175*, 2986–2995.
Zhang, J., Suflita, M., Fiaschetti, C. M., Li, G., Li, L., Zhang, F., et al. (2014). High cell density cultivation of a recombinant escherichia strain expressing a 6-O-sulfotransferase for the production of bioengineered heparin. *Journal of Applied Microbiology, 118*, 92–98.
Zhang, Z., McCallum, S. A., Xie, J., Nieto, L., Corzana, F., Jime'nez-Barbero, J., et al. (2008). Solution structures of chemoenzymatically synthesized heparin and its precursors. *Journal of the American Chemical Society, 130*, 12998–13007.