pubs.acs.org/acschemicalbiology

Articles

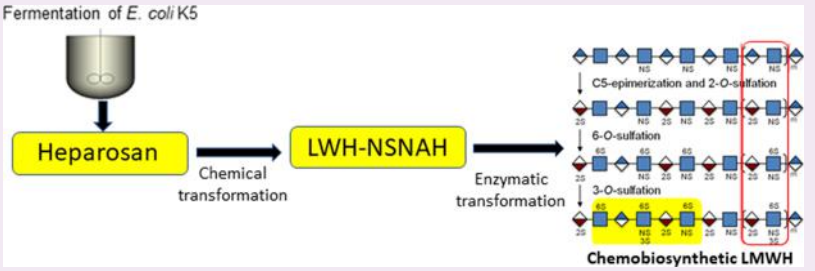
Chemobiocatalytic Synthesis of a Low-Molecular-Weight Heparin

Yanlei Yu, Li Fu, Peng He, Ke Xia, Sony Varghese, Hong Wang, Fuming Zhang,* Jonathan Dordick,* and Robert J. Linhardt*

Cite This: *ACS Chem. Biol.* 2022, 17, 637−646

Read Online

ACCESS | Metrics & More | Article Recommendations | Supporting Information



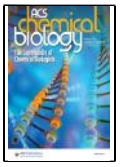
ABSTRACT: Heparin products are widely used clinical anticoagulants essential in the practice of modern medicine. Low-molecular-weight heparins (LMWHs) are currently prepared by the controlled chemical or enzymatic depolymerization of unfractionated heparins (UFHs) that are extracted from animal tissues. In many clinical applications, LMWHs have displaced UFHs and currently comprise over 60% of the heparin market. In the past, our laboratory has made extensive efforts to prepare bioengineered UFHs relying on a chemoenzymatic process to address concerns about animal-sourced UFHs. The current study describes the use of a novel chemoenzymatic process to prepare a chemobiosynthetic LMWH from a low-molecular-weight heparosan. The resulting chemobiocatalytic LMWH matches most of the United States pharmacopeial specifications for enoxaparin, a LMWH prepared through the base-catalyzed depolymerization of animal-derived UFH.

1. INTRODUCTION

Heparin is the most widely used anticoagulant drug produced from animal tissues, primarily porcine intestines.[1,2] Heparin is a linear highly sulfated polysaccharide found covalently attached to the core protein serglycin as a proteoglycan and stored in intracellular granules of mast cells.[3] It is composed of a repeating disaccharide unit comprising β-D-glucuronic acid (GlcA) or α-L-iduronic acid (IdoA) 1, 4-glycosidically linked to D-glucosamine (GlcN).[1] Unlike the synthesis of DNA and proteins, the biosynthesis of heparin is not template-driven, and thus, the resulting polysaccharides are heterogeneous in length and substitution pattern.[4] Heparin biosynthesis in certain animal cells[5] begins in the endoplasmic reticulum involving the formation of a tetrasaccharide linker [D-xylose (Xyl)-D-galactose (Gal)-Gal-GlcA] that tethers to a serine residue of its core protein. Chain polymerization next takes place through formation of a repeating disaccharide building block of *N*-acetyl-α-D-glucosamine (GlcNAc) 1,4-linked GlcA driven by two polymerases known as exostosin glycosyltransferase (EXT) 1 and EXT 2, forming heparosan, the backbone of heparin.[6,7] Subsequent modification of the backbone takes place through de-N-acetylation and *N*-sulfation, C5-epimerization, and a series 3′-phosphoadenosine 5′-phosphosulfate (PAPS)-dependent of *O*-sulfation reactions, all occurring in the Golgi compartment. These reactions are catalyzed by *N*-deacetylase/*N*-sulfotransferase to form *N*-sulfo-α-D-glucosamine (GlcNS) residues, C5-epimerase (Epi), converting GlcA residues into L-iduronic acid (IdoA), and 2-*O*-, 6-*O*-, and 3-*O*-sulfotransferases (STs) that transfer sulfo groups to the polysaccharide chain.[8−10] Pharmaceutical heparin is polydisperse and heterogeneous, having an average molecular weight of 18−20 kDa, and is currently prepared from tissues rich in heparin proteoglycan.[1]

Low-molecular-weight heparins (LMWHs) are currently produced by either controlled chemical or enzymatic depolymerization of unfractionated pharmaceutical heparins (unfractionated heparin, UFHs). LMWHs have several advantages over UFHs for therapeutic anticoagulation including high *subcutaneous* bioavailability and a more predictable pharmacokinetic profile,[11] a longer plasma half-life,[12] and lower incidences of heparin-induced thrombocytopenia (HIT).[13] Commercially available LMWHs are polydisperse, fractionated heparins with average molecular weights ranging from 3 to 8 kDa. For example, enoxaparin (~4500 Da) is produced using benzylation followed by alkaline hydrolysis,

Received: November 24, 2021
Accepted: February 4, 2022
Published: February 24, 2022



dalteparin (~6000 Da) is derived from controlled nitrous acid depolymerization, and tinzaparin (~6500 Da) is prepared by controlled heparinase digestion.[6,14] Among the three, enoxaparin (Lovenox) produced by Sanofi has the major share of the worldwide LMWH market and the most extensive clinical evidence of efficacy and safety in various applications and, hence, has the broadest range of therapeutic indications.[15] Recently, patent rights and supplementary protection certificates of originator enoxaparin have expired. The approval of generic forms of enoxaparin by the U.S. Food and Drug Administration (FDA) in 2010 has reduced the drug price, making LMWHs available to broader patient populations.[16,17] However, the quality and supply of LMWHs rely on the quality of animal-derived heparin; the supply chain is still under the threat of impurities, contamination, and adulteration. Furthermore, there is a growing concern about the shortage of porcine heparin, and in some cases, LMWHs suffer from shortcomings including impurities and an insecure supply chain, as it is derived from animal-derived heparin.[18] To this end, efforts have been directed toward developing and improving techniques and approaches toward the synthesis of UFH and LMWH.

The chemoenzymatic synthesis of UFH has become possible with successful expression of recombinant heparin biosynthetic enzymes including glycosyltransferases, C5-Epi, and 2-, 6-, and 3-OSTs.[19] A chemoenzymatic approach closely mimics the heparin biosynthetic pathway. Bioengineered UFH preparation starts with the *Escherichia coli* K5 capsular polysaccharide (CPS), heparosan, as the starting material.[20] Chemical de-*N*-acetylation and *N*-sulfonation of heparosan afford *N*-sulfoheparosan that is subsequently modified using recombinant C5-Epi and 2-, 6-, 3-OSTs.[21] This bioengineered UFH has shown chemical and biological equivalence to pharmaceutical porcine heparin.[22]

A homogeneous, monodisperse, fondaparinux-like ultra-LMWH has been chemoenzymatically synthesized from uridine-5′-diphosphate (UDP)-sugar donors and a heparosan-derived disaccharide acceptor using *N*-acetyl glucosaminyltransferase (KfiA) and heparosan synthase (pmHS2).[23,24] Furthermore, a single targeted structure of a homogeneous dodecasaccharide LMWH has also been synthesized and demonstrated to be a viable candidate to replace LMWHs in thromboprophylaxis.[25] These chemoenzymatic processes rely on the use of expensive UDP-sugar donors to iteratively synthesize homogeneous molecular species.

The new approach, described herein, relies on a less expensive chemosynthetic strategy to prepare a heterogeneous, polydisperse form of enoxaparin starting from chemically cleaved heparosan. This chemobiocatalytic LMWH is intended to serve as a comparable version of the pharmaceutical LMWH. Such chemoenzymatically synthesized LMWHs have several advantages over LMWHs prepared from animal-sourced UFH including better source material availability, better control of manufacturing processes, reduced concerns about contamination, adulteration or animal virus, or inherent impurities. This chemoenzymatic approach, modeled on that used to synthesize a chemobiosynthetic perdeuterated UFH,[22] utilizes a variety of heparin biosynthetic enzymes to synthesize a high purity chemobiosynthetic LMWH, chemically and biologically equivalent to animal-sourced LMWH.

2. EXPERIMENTAL SECTION

2.1. Materials. *Escherichia coli* K5 heparosan CPS was prepared through fermentation as previously described.[26] The 2-, 6-, 3-OST and C5-Epi enzymes were prepared in our laboratory as previously described.[10,27−29] Enoxaparin LMWH standard and enoxaparin sodium molecular weight calibrant A (1400, 2250, 4550, and 9250 Da) and B (1800, 3350, and 6650 Da) were purchased from the United States Pharmacopeia (USP, Rockville, MD). Human antithrombin III (AT) and platelet factor 4 (PF4) were purchased from Hyphen BioMed (Neuville-sur-Oise, France). Recombinant *Flavobacterium heparinum* heparin lyase I, II, and 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 as previously described.[30] Unsaturated heparin disaccharide standards were purchased from Iduron (Manchester, UK). Biophen heparin anti-Xa (2 stages) and anti-IIa (2 stages) kits were purchased from Aniara (West Chester, OH, USA).

2.2. Removal of the Glycolipid Terminus of K5 Heparosan. The heparosan CPS prepared from *E. coli* K5 was treated with hydrochloric acid to remove glycolipid acceptor 3-deoxy-D-manno-oct-2-ulosonic acid (Kdo). Heparosan was dissolved in hydrochloric acid solution and adjusted pH to 1 and then incubated at 90 °C for 1 h. The solution pH was re-adjusted to 7 by sodium hydroxide, and the solution was desalted by dialysis. The results were determined by nuclear magnetic resonance (NMR) analysis.

2.3. Preparation of Low-Molecular-Weight N-Sulfo, N-Acetyl Heparosan by Chemical Cleavage. De-N-acetylation and depolymerization of Kdo-free heparosan were undertaken by a controlled alkaline reaction. The sample (20 g/L) was dissolved in 50 mL of 2 N NaOH and incubated for 48 h at 65 °C in a shake flask, cooled to room temperature, and pH-adjusted to 7.0 with HCl. Controlled re-acetylation was undertaken by adding methanol (3.5 mL), anhydrous sodium carbonate (130 mM/L), and acetic anhydride (53 μM/L each for four times with 20 min intervals). The amount of acetic anhydride was optimized to reach 10−15% of the *N*-acetyl group as determined by NMR. The re-*N*-sulfation was next undertaken by adding an equal portion of anhydrous sodium carbonate (130 mM/L) and trimethylamine sulfur trioxide (76 mM/L) and mixed for 48 h at 47 °C. The sulfation level was monitored by measuring unsubstituted amines using an *o*-phthaldialdehyde (OPA) assay.[31] The sulfate and the acetyl group ratio were determined by NMR. The low-molecular-weight *N*-sulfo, *N*-acetyl heparosan (LMW-NSNAH) was precipitated with 85% methanol at 4 °C overnight. The remaining salt was removed by washing four times with 85% methanol and centrifuged at 1800 × *g*.

2.4. Preparation of a Chemobiosynthetic LMWH by Enzymatic Modifications. LMW-NSH sample (50 mg) was treated with C5-Epi and 2-OST to afford low-molecular-weight *N*-sulfo, *N*-acetyl, 2-sulfo heparosan (LMW-NSNA2SH). The detailed reaction conditions are as follows: substrate concentration of 1 mg/mL, PAPS concentration of 5 mM, and each immobilized enzyme[27] (C5-Epi/2-OST) at 1 mg/mL in a 50% slurry. The reaction was incubated in 50 mM 2-(*N*-morpholino) ethane sulfonic acid buffer (pH 7.2) with 0.05% NaN_3 and 125 mM NaCl for 120 h at 37 °C. After exhaustive treatment, the reaction was complete and the mixture was filtered to remove enzyme resin and dialyzed using a 1 kDa molecular weight cutoff membrane tube against distilled water to remove salt and other small molecular impurities. Disaccharide compositional analysis was used to monitor and confirm the sulfation reaction.[32] The controlled 6-OST and 3-OST reactions were next undertaken using immobilized enzymes to yield chemobiocatalytic LMWH. The reaction conditions were similar to that used in the C5-Epi/2-OST reaction. Disaccharide composition analysis and anti-Xa activity assay were used to monitor the reaction status.

2.5. Molecular Weight Determination by Gel Permeation Chromatography. Molecular weights were determined by gel permeation chromatography (GPC)−high performance liquid chromatography (HPLC) using enoxaparin sodium molecular weight calibrants.[33] 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,

https://doi.org/10.1021/acschembio.1c00928
ACS Chem. Biol. 2022, 17, 637−646

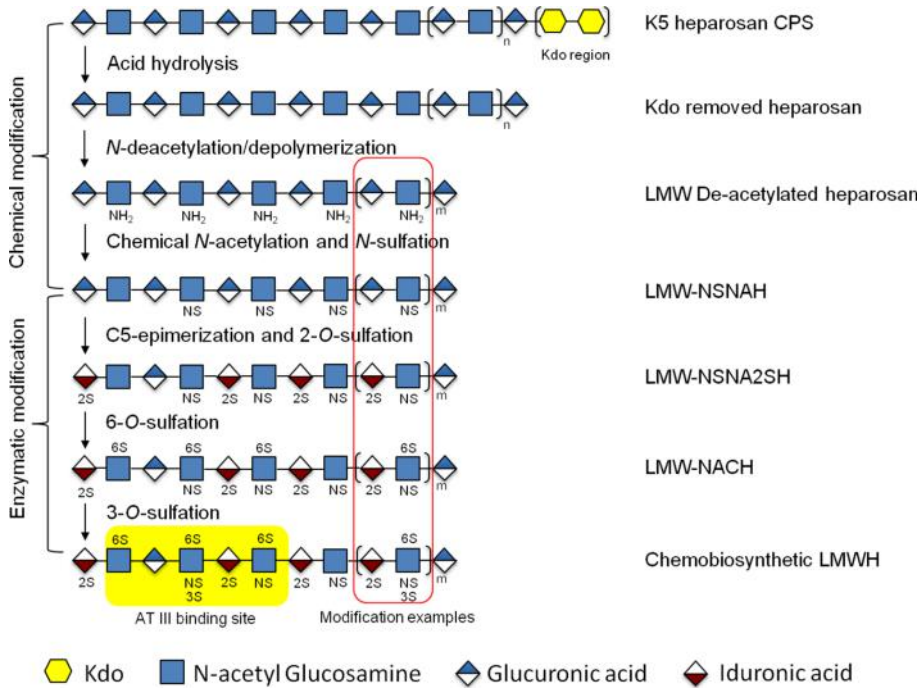


Figure 1. Scheme of chemoenzymatic synthesis of chemobiocatalytic LMWH.

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 the concentration at 5 mg/mL.

2.6. Anticoagulant Activity. The anticoagulant activities of the products were determined using Biophen heparin anti-Xa (2 stages) and Anti-IIa (2 stages) kits following the protocols provided by the manufacturer.[34] Briefly, AT (anti-Xa reagent 1 (r1)), factor Xa (r2), and factor Xa specific chromogenic substrate (r3) were used for anti-Xa activity, and AT (anti-IIa reagent 1 (R1)), human thrombin (R2), and factor IIa specific chromogenic substrate (R3) were used for anti-II activity. Each reagent was reconstituted with 1 mL of distilled water and shaken until fully dissolved. After a 1/5 dilution in the appropriate buffer (Tris-EDTA-NaCl-PEG, pH 8.4) for r1/R1 and r2/R2, distilled water was used for r3/R3 to restore the reagents immediately before use. Reference standard and dilute samples were prepared to the appropriate concentrations. Samples (40 μL) were added into a 96-well plate and incubated for 5 min at 37 °C, and 40 μL of r1/R1 was added and mixed well and incubated for 2 min, 40 μL of r2/R2 was next added and incubated for 2 min, and 40 μL of r3/R3 was added last and incubated for another 2 min. The reactions were stopped by adding 80 μL of 50 mM acetic acid. The absorbance was then determined at 405 nm. The anti-Xa and anti-IIa activities were calculated using a standard curve of different concentrations of enoxaparin standards.

2.7. Disaccharide and Tetrasaccharide Composition Analysis. Disaccharide and tetrasaccharide compositions were determined by strong anion exchange (SAX)-HPLC with an ultraviolet detector, which was performed on a Shimadzu LC-2030 system (Shimadzu, Kyoto, Japan). Samples (100 μg) were exhaustively digested using a mixture of heparin lyase I, II, and III (10 mU each) in digestion buffer (50 mM ammonium acetate containing 2 mM calcium chloride, pH 7.0) at 37 °C for 2 h. The reaction was terminated by boiling for 10 min, and the denatured enzymes were removed by centrifugation at 10000 ×g for 10 min. The supernatant concentrated at 1 μg/μL was analyzed by an HPLC system coupled with a Shimadzu LC-20 AD pump, CBM-20A controller, SIL-20AHT auto-sampler, and SPD-20AV UV detector. A Spherisorb SAX chromatography column (4.0 × 250 mm, 5.0 μm, Waters) was equilibrated with mobile phase A (1.8 mM monobasic sodium phosphate, pH = 3), followed by gradient elution using mobile phase B (1.8 mM monobasic sodium phosphate with 2 M sodium perchlorate, pH = 3). Disaccharide analysis used a gradient of mobile phase B that increased from 5 to 50% in 30 min, held for 5 min, then changed to 5%, and held for 15 min. Tetrasaccharide analysis used a gradient of 15−32.5% mobile phase B from 0 to 40 min, 42.5% mobile phase B at 50 min, 50% at 54 min and maintained for 1 min at a flow rate of 0.45 mL/min.

2.8. NMR Spectroscopy Analysis. The 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. The sample was dissolved in 0.4 mL of 99.96% D_2O and lyophilized and then repeated twice. $^{1}H/^{13}C$ 1D NMR was carried out at 298 k.

2.9. 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-coated chip based on the manufacturer's protocol. Competition studies between surface heparin and LMWH binding to proteins were performed using SPR through measurement of IC_{50}. AT (250 nM) or PF4 (125 nM) mixed with different concentrations of LMWHs in HBS-EP buffer (0.01 M 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), 0.15 M NaCl, 3 mM ethylenediaminetetraacetic acid (EDTA), and 0.005% surfactant P20, pH 7.4) were 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 a fully regenerated surface after each run. For each set of competition experiments, a control experiment was performed to ensure that the surface was completely regenerated, and the results obtained between runs were comparable.

3. RESULTS AND DISCUSSION

The general method for the chemoenzymatic synthesis of chemobiocatalytic LMWH is shown in Figure 1.

3.1. Preparation of Kdo-Free Heparosan. Heparosan is an acidic CPS of an engineered strain of *E. coli* K5 with fructosyl transferase removed[26] and serves as the critical precursor in heparin biosynthesis and as an intermediate in the chemoenzymatic synthesis of chemobiosynthetic heparins.[35]

https://doi.org/10.1021/acschembio.1c00928
ACS Chem. Biol. 2022, 17, 637−646

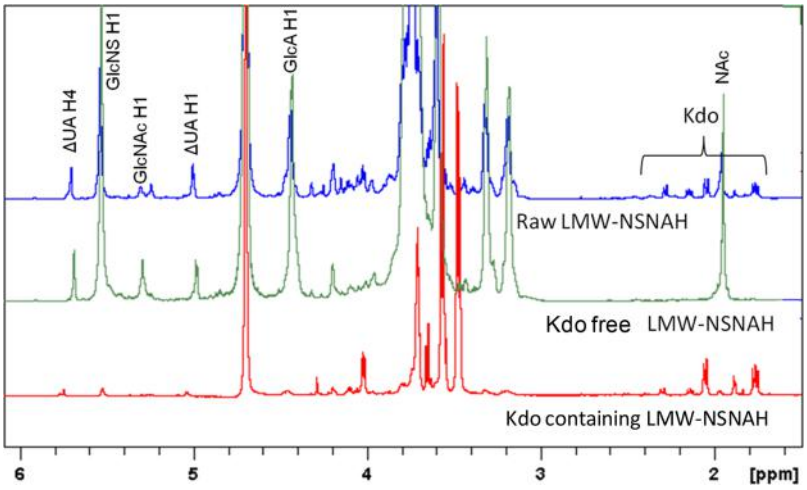


Figure 2. ^{1}H NMR analysis of Kdo removal of low-molecular-weight NSH.

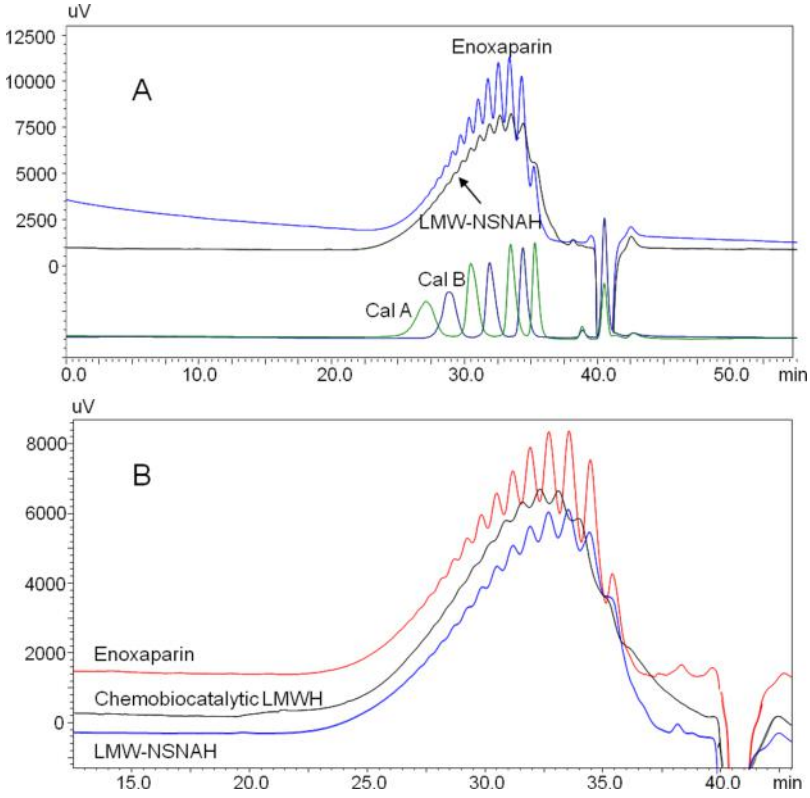


Figure 3. Molecular weight analysis of chemobiosynthetic low-molecular-weight heparin by GPC. (A) LMW-NSNAH; (B) chemobiocatalytic LMWH.

Heparosan is a linear chain of repeating structure →4)-*β*-GlcA) (1 → 4)-*α*-GlcNAc (1→. A major difference between heparosan intermediate in animals and the heparosan in CPS in bacteria is the acceptors on which they are biosynthesized. In animals, the heparosan is assembled on an acceptor corresponding to the tetrasaccharide linkage region (Xyl-Gal-Gal-GlcA) attached to serine residue of the core protein serglycin. However, the biosynthesis of heparosan CPS in *E. coli* K5 initiates on a glycolipid acceptor, which is composed of multiple linked Kdo residues.[20,36−38] The glycolipid terminus needs to be removed before chemoenzymatic synthesis of chemobiosynthetic LMWH because it is not found in porcine-derived LMWH products. Acid hydrolysis can degrade the Kdo chains. Harsh conditions will remove the Kdo but also can hydrolyze *N*-acetyl groups and reduce heparosan molecular weight. Using ^{1}H NMR and GPC analyses, we determined that treating CPS with HCl at pH = 1 for 1 h at 90 °C releases Kdo without modifying heparosan chains. The Kdo-free heparosan upon acid hydrolysis was then removed using a 1 kDa molecular weight cutoff membrane. The Kdo signals observed at 1.5−2.5 ppm in ^{1}H NMR were missing in the retentate, indicating that the Kdo had been successfully removed (Figure 2).

3.2. Optimization of de-N-Acetylation and N-Sulfation of Low-Molecular-Weight Heparosan. Heparosan CPS had an average molecular weight of 49 kDa, much larger

https://doi.org/10.1021/acschembio.1c00928
ACS Chem. Biol. 2022, 17, 637−646

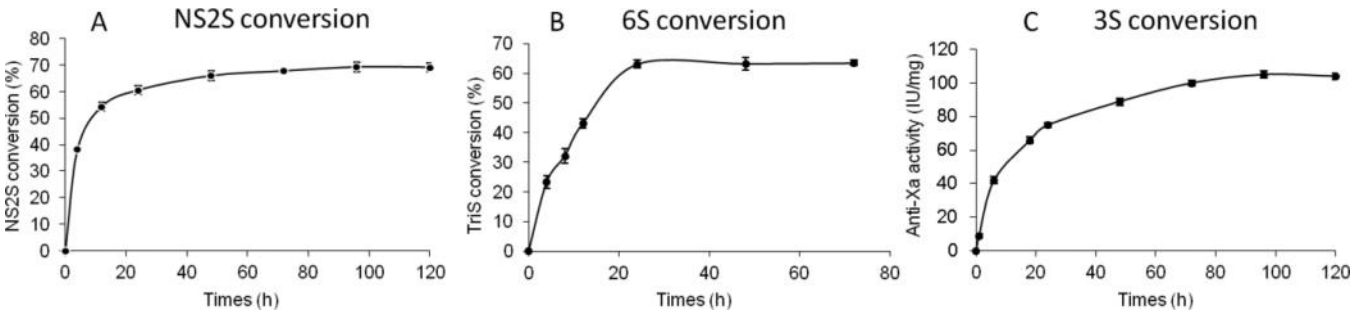


Figure 4. Enzymatic synthesis of chemobiosynthetic LMWH. (A) NS2S conversion by 2-*O*-sulfotransferase and C5-epimerase reaction; (B) TriS conversion by 6-*O*-sulfotransferase reaction; and (C) anti-Xa activity by 3-*O*-sulfotransferase reaction.

than the molecular weight of commercial UFH and LMWH. The controlled depolymerization of CPS heparosan is necessary prior to its conversion to chemobiosynthetic LMWH. The chemical de-N-acetylation of heparosan by base hydrolysis results in partial (or complete) removal of *N*-acetyl groups of the GlcNAc residues and polysaccharide chain depolymerization through β-elimination. Our goal is to obtain low-molecular-weight heparosan having an average molecular weight ranging from 3.8 to 4.5 kDa accompanied by ~85 to 90% de-N-acetylation. We initially focused on the reaction temperature (55, 60, 65, and 70 °C) and time (24, 48, 72, and 96 h). After a 48 h de-N-acetylation reaction time at 65 °C, the average molecular weight of the product decreased to 3.9 kDa as determined by GPC. Control of the *N*-sulfo/*N*-acetyl ratio in the *N*-sulfation step was next examined. Due to the much harsher conditions in the de-N-acetylation step, no acetyl group (100% de-N-acetylation) was found based on NMR analysis. We re-N-acetylated by adding an optimized amount of acetic anhydride after base treatment and prior to *N*-sulfation. As a result of these optimization experiments, we obtained 146 mg *N*-sulfo, *N*-acetyl low-molecular-weight heparosan from 1 g heparosan starting material, with a molecular weight of 4200 Da (Figure 3A). ^{1}H NMR analysis of the 5.31 ppm peak, corresponding to the GlcNS residue, and the 5.55 ppm peak, corresponding to the GlcNS residue, afforded an *N*-acetyl/*N*-sulfo ratio ranging from 10 to 15%, affording a product matching the USP criteria for enoxaparin.

3.3. Enzymatic Modifications of Low-Molecular-Weight N-Sulfo, N-Acetyl Heparosan. A strategy like that used in the chemoenzymatic synthesis of heparin was undertaken to investigate the synthesis of chemobiocatalytic LMWH. We were uncertain whether these enzymatic modifications could meet the USP enoxaparin specifications because the chain length of substrates are much smaller than those of UFH. This enzymatic process can be divided into three steps: (1) conversion of *N*-sulfo, *N*-acetyl heparosan (NSNAH) into *N*-sulfo, *N*-acetyl, 2-*O*-sulfo IdoA-containing heparosan (NSNA2SH) using C5-Epi and 2-*O*ST; (2) conversion of NSNA2SH into non-anticoagulant heparin (NACH) using 6-*O*ST-1 and 6-*O*ST-3; and (3) conversion of NACH into chemobiosynthetic anticoagulant heparin using 3-*O*ST. The sulfo donor is PAPS, resulting in the formation of 3′-phosphoadenosine-5′-phosphate. The first step catalyzed by C5-Epi converts GlcA residues into IdoA residues in a reversible reaction that is locked in place with 2-*O*-sulfation to afford IdoA2S residues, which is critical and most difficult.[21,39] The C5-Epi/2-*O*ST and 6-*O*ST reactions were monitored by disaccharide compositional analysis, and 3-*O*ST reaction was monitored by anti-Xa activity assay. Disaccharide compositional analysis is critical for determining the sulfation status, with a targeted range NS2S of 68−74% based on commercial enoxparins. The conversion of NSNAH to NSNA2SH was determined at 4, 12, 24, 48, 72, 96, and 120 h time points (Figure 4A). The synthesis of a chemobiocatalytic LMWH was much slower than the synthesis of a chemobiosynthetic UFH due to reduced activity of these enzymes on shorter chain substrates. The maximum conversion percentage reached was 69.3% of NS2S at the 96 h time point, which met the required specifications. Next, the conversion of NS2S to NS2S6S was completed in 24 h, as shown in Figure 4B. UFHs have chains of sufficient length to bind both AT and thrombin to afford a ternary complex, inactivating thrombin and thus preventing clot formation. In contrast, LMWHs are composed of smaller chains than UFH, and most of these are only of sufficient length for binding AT, inactivating factor Xa.[40] Thus, in the synthesis of LMWH, we monitored the reaction through anti-Xa activity. The potency of anti-factor Xa of enoxaparin is no less than (NLT) 90 IU/mg and no more than (NMT) 125 IU/mg on the dried basis. This activity could be reached after 120 h of treatment with 3-*O*ST (Figure 4C), and there was no increased anticoagulant activity on further enzymatic reaction.

3.4. Molecular Weight Analysis by GPC. GPC was used to determine the molecular weight of chemobiocatalytic LMWH using USP enoxaparin sodium molecular weight calibrants. The USP criterion of weight average molecular weight for enoxaparin sodium is 4500 Da, the range being between 3800 and 5000 Da. Because sulfation increases the molecular weight of the final product, we set the target molecular weight of low-molecular-weight for the NSNAH intermediate of 3800−4500 Da. As expected, starting at 4200 Da for low-molecular-weight of NSNAH, the molecular weight of our final LMWH product had increased to 4350 Da (Figure 3B).

3.5. Anticoagulant Activity. The anticoagulant activity of the NACH intermediate and final LMWH product was measured using the methods described in the current USP enoxaparin monograph. The target anticoagulant activity of enoxaparin sodium has a potency of NLT 90 and NMT 125 anti-factor Xa International Units (IU)/mg and NLT 20.0 and NMT 35.0 anti-factor IIa IU/mg, calculated on a dry basis. The ratio of anti-Xa to anti-IIa activity is between 3.3 and 5.3. We periodically removed 20 μL of reaction solution at various time points and analyzed anti-Xa activity and concentration analysis by HPLC-GPC. The anti-Xa activity (Figure 4C) increased in the first 48 h and slowed down in the following reaction until reaching the maximum activity at 105 IU/mg. The final chemobiocatalytic LMWH product has an anti-Xa of

https://doi.org/10.1021/acschembio.1c00928
ACS Chem. Biol. 2022, 17, 637−646

Table 1. Summary of Anticoagulant Activity and IC_{50} Values of LMWHs from Triplicated Preparations

	anticoagulant activities			IC_{50}	
	anti-Xa (IU/mg)	anti-IIa (IU/mg)	anti-Xa/IIa ratio	AT (μg/mg)	PF4 (μg/mg)
Enoxaparin	110 ± 4.4	28 ± 2.6	3.9 ± 0.22	11.0 ± 0.53	2.7 ± 0.26
Chemobiocatalytic LMWH	105 ± 2.6	24 ± 1.0	4.4 ± 0.13	12.0 ± 0.29	2.8 ± 0.35
USP criteria	90−125	20.0−35.0	3.3−5.3		

105 and 24 IU/mg of anti-IIa activity with an anti-Xa/IIa ratio of 4.4, consistent with USP requirements (Table 1).

3.6. Disaccharide and Tetrasaccharide Analysis. Disaccharide compositional analysis of the chemobiocatalytic LMWH and its intermediates relies on exhaustive treatment with heparin lyases I, II, and III to afford eight different disaccharide products based on sulfation levels and positions (Figure S1). These disaccharides are then analyzed by SAX-HPLC to monitor the intermediate biosynthesis and final product (Figure S2). The disaccharide compositions of heparin, enoxaparin controls, and final products are shown in Table 2. Treatment of heparosan with chemical *N*-sulfation, 2-*O*ST/C5-Epi, 6-*O*ST, and 3-*O*ST afforded a disaccharide composition that was similar to enoxaparin (Figure 5). The TriS content was 62.6% of chemobiocatalytic LMWH compared to enoxaparin at 66.3%. The NS6S of chemobiocatalytic LMWH was 17.3%, higher than enoxaparin of 10.3%, while NS2S of chemobiocatalytic LMWH was 3.5%, lower than 7.0% of enoxaparin. This suggests that the 2-*O*ST conversion was lower than 6-*O*ST. It is important to note that 3-*O*-sulfated glucosamine residues are resistant to heparin lyases cleavage. Thus, in addition to anticoagulant activity analysis, tetrasaccharide analysis was undertaken next by treating with heparinase I, II, and III, followed by analysis of the resulting resistant tetrasaccharides with SAX-HPLC. Five 3-*O*-sulfo-containing tetrasaccharides, (1) ΔUA-GlcNAc6S-GlcUA-GlcNS3S (where ΔUA is deoxy-α-L-*threo*-hex-4-enopyranosyluronic acid); (2) ΔUA-GlcNAc6S-GlcUA-GlcNS3S6S; (3) ΔUA-GlcNS6S-GlcUA-GlcNS3S; (4) ΔUA2S-GlcNAc6S-GlcUA-GlcNS3S6S; and (5) ΔUA2S-GlcNS6S-GlcUA-GlcNS3S6S, have been fully characterized, and their structures are presented in Figure S3.[41] The results revealed that chemobiocatalytic LMWH has a similar 3-*O*ST-containing tetrasaccharide distribution compared to enoxaparin (Table 2). Our chemobiocatalytic LMWH is highly close to enoxaparin in disaccharide and tetrasaccharide composition analysis.

Table 2. Disaccharide and Tetrasaccharide Composition Analysis of LMWHs from Triplicated Preparations

	heparin control (%)	enoxaparin (%)	chemobiocatalytic LMWH (%)
0S	4.2 ± 0.15	3.1 ± 0.28	3.9 ± 0.21
NS	3.3 ± 0.10	3.0 ± 0.35	3.8 ± 0.21
6S	3.3 ± 0.12	3.6 ± 0.07	4.4 ± 0.14
2S	2.0 ± 0.21	1.9 ± 0.07	0.1 ± 0.08
NS6S	10.6 ± 0.23	10.3 ± 0.49	17.3 ± 0.49
NS2S	7.7 ± 0.21	7.0 ± 0.14	3.5 ± 0.0
2S6S	1.5 ± 0.23	1.8 ± 0.28	0.9 ± 0.39
Tetra 1	0.3 ± 0.06	0.4 ± 0.08	0.3 ± 0.08
TriS	65.1 ± 0.81	66.3 ± 0.07	62.6 ± 0.01
Tetra 2	1.7 ± 0.21	1.9 ± 0.17	1.8 ± 0.14
Tetra 3	0.1 ± 0.05	0.4 ± 0.09	0.5 ± 0.09
Tetra 4	0.1 ± 0.06	0.3 ± 0.02	0.6 ± 0.07
Tetra 5	0.1 ± 0.03	0.1 ± 0.04	0.1 ± 0.04
Tetra 1−5 subtotal	2.3 ± 0.31	3.1 ± 0.40	3.3 ± 0.40
Total (di & tetra)	100.0	100.0	100.0

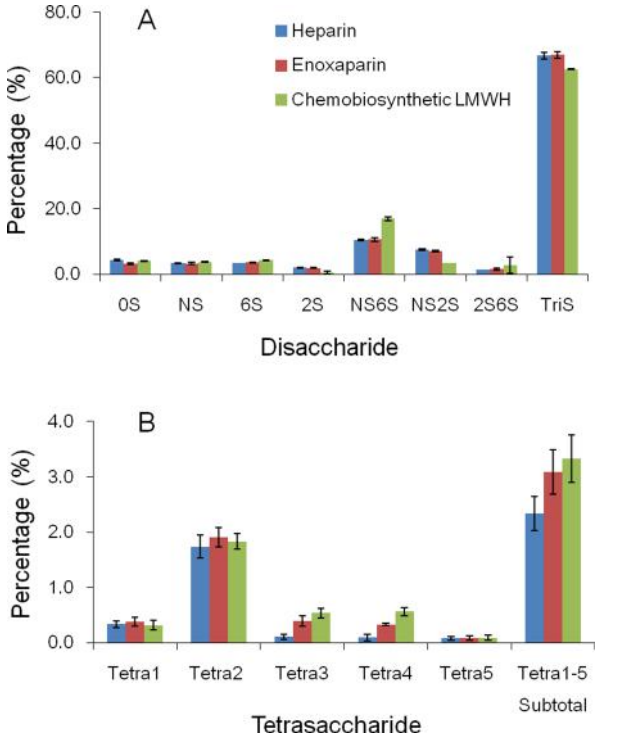


Figure 5. Disaccharide and tetrasaccharide composition analysis of chemobiosynthetic LWMH. (A) Disaccharide composition; (B) five 3-O-sulfo-containing tetrasaccharide compositions.

3.7. NMR Analysis. One-dimensional ^{1}H and ^{13}C NMR spectra were performed to characterize the structure of chemobiocatalytic LMWH and enoxaparin. The enoxaparin ^{1}H peaks can be all assigned.[42] The spectra of the two LMWHs looked quite similar but have some differences (Figures 6 and S4). The GlcNS3S peak overlapped H1 of ΔUA2S from 5.44 to 5.42 ppm. The IdoA2S peak was assigned from 5.17 to 5.09 ppm. The H4 ΔUA intensity at 5.90 ppm of chemobiocatalytic LMWH was lower compared to enoxaparin. The signal peaks at 5.48, 5.43, 5.33, 5.13, 5.07, and 4.51 ppm corresponded to the anomeric hydrogen. Our chemobiocatalytic LMWH has two more peaks from 5.09 to 5.00 ppm, which could be IdoA2S or impurities. In comparison to enoxaparin or generic enoxaparins, our chemobiocatalytic synthetic LMWH has only very small amounts of 1,6-anhydromannose. In order to meet compendial requirements for enoxaparin, the appropriate amount of 1,6-anhydromannose containing chains would need to be introduced.

3.8. SPR Analysis. The anticoagulant activity of heparin is primarily mediated through its binding and regulation of AT.

https://doi.org/10.1021/acschembio.1c00928
ACS Chem. Biol. 2022, 17, 637−646

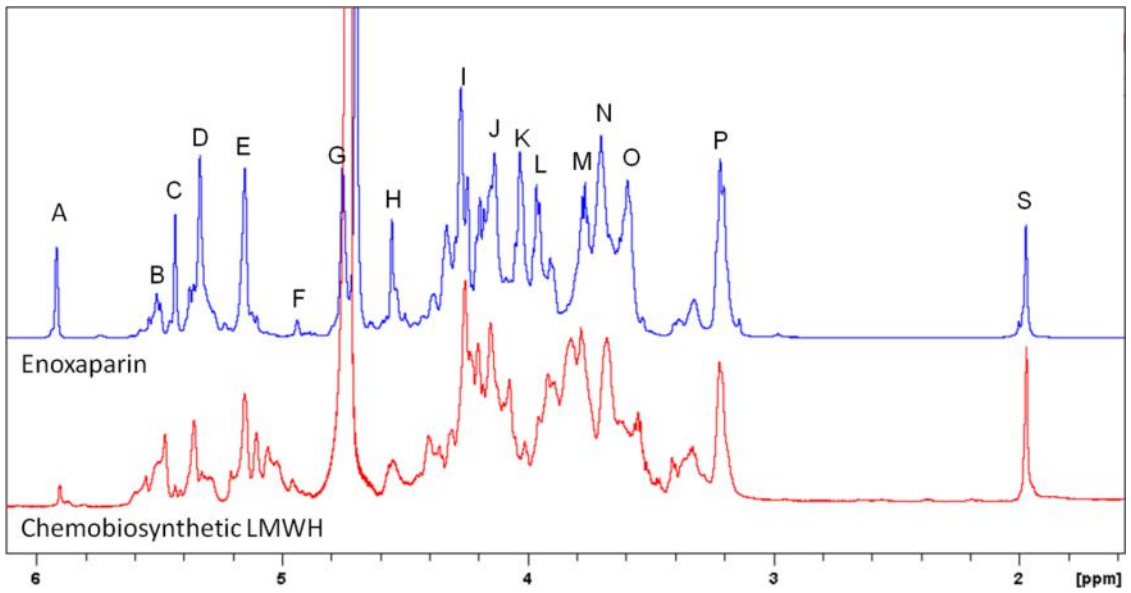


Figure 6. ^{1}H NMR of enoxaparin and chemobiosynthetic LMWH. (A) H4 ΔU2S; (B) H1 A_{NS6S}^{-}(G); (C) H1 ΔU2S; (D) H1 A_{NS6X}^{-}(I_{2S}) and A_{NAc6X}^{-}(G); (E) H1 I_{2S}; (F) I (A_{NS6S}); (G) H5 I_{2S}; (H) H1 G; (I) H2 I_{2S}; (J) H6 A_{NS6S}; (K) H3 I_{2S}; (L) H4 I_{2S}; (M) H6 A_{NS}; (N) H4 A_{NS6S}; (O) H3 A_{NS6X}; (P) H2 A_{NS6X}; and (Q) CH_3 A_{NAc}.

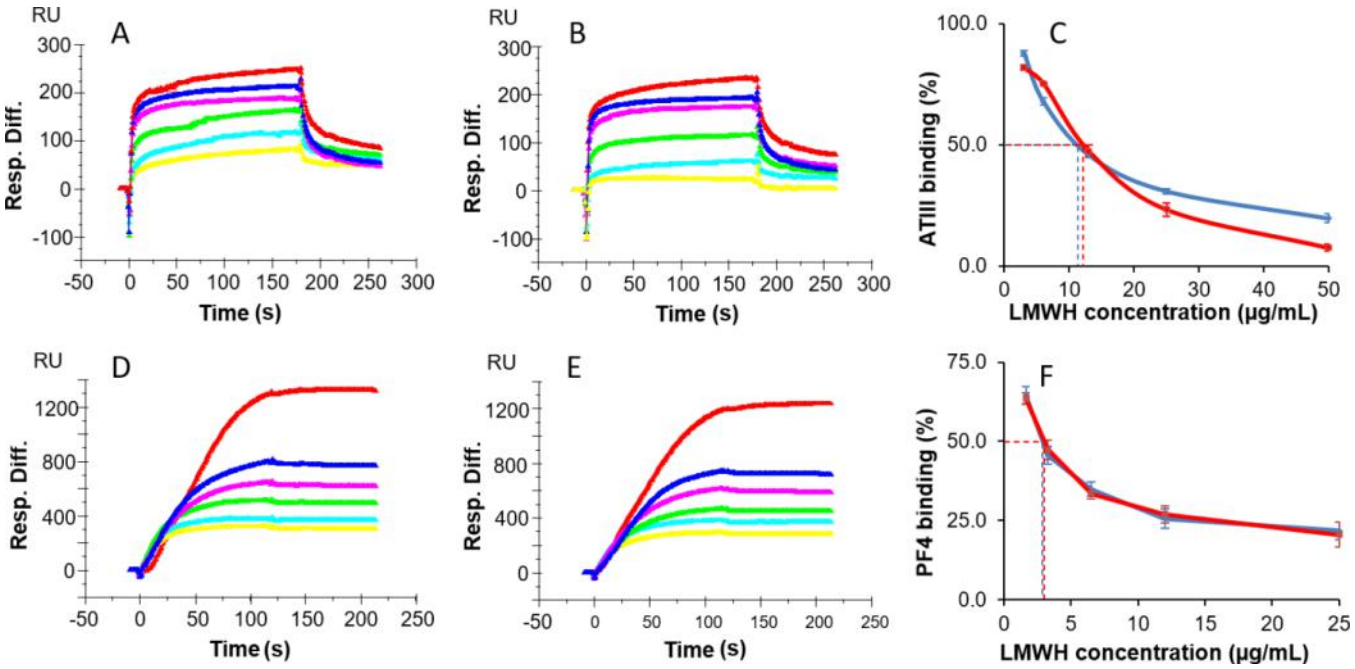


Figure 7. (A,B) SPR sensorgrams of AT binding to heparin surface competing with different LMWH samples. (A) Enoxaprain and (B) chemobiocatalytic LMWH. The concentration of AT was 250 nM. LMWH concentrations in solution (from top to bottom) were 0, 3.13, 6.25, 12.5, 25, and 50 μg/mL, respectively. (C) IC_{50} calculation of LMWHs using AT inhibition data; blue: enoxaparin; red: chemobiocatalytic LMWH. (D,E) SPR sensorgrams of PF4 binding to the heparin surface competing with different LMWH samples. (D) Enoxaprain and (E) chemobiocatalytic LMWH. The concentration of PF4 was 125 nM. LMWH concentrations in solution (from top to bottom) were 0, 1.57, 3.13, 6.25, 12.5, and 25 μg/mL. (F) IC_{50} calculation of LMWHs using PF4 inhibition data; blue: enoxaparin; red: chemobiocatalytic LMWH.

Accordingly, the interaction between heparin and AT is a crucial step for the anticoagulation process.[43,44] Competition SPR was used to measure the competitive AT binding of analyte LMWHs in the solution phase vs USP heparin immobilized on the chip surface. The IC_{50} values resulting in a 50% decrease in response units can be calculated from the plots over a range of LMWH solution concentrations (up to 50 μg/mL). The results of IC_{50} values for enoxaparin and chemobiocatalytic LMWH were 11.0 and 12.0 μg/mL, respectively (Figure 7A−C). Hence, the AT binding activity of chemobiocatalytic LMWH was slightly lower than enoxaparin, but it is in the acceptance range.[34] Of particular concern is HIT caused by interaction of heparin and platelet factor IV (PF4), resulting in an adverse immunological disorder. The analysis of HIT potential for chemobiocatalytic LMWH is important prior to its clinical application.[45] A rapid method developed in our laboratory was used to evaluate the PF4 binding and calculate the IC_{50} value through solution

https://doi.org/10.1021/acschembio.1c00928
ACS Chem. Biol. 2022, 17, 637−646

competition SPR.[46] The measured IC_{50} for chemobiosynthetic LMWH was 2.8 μg/mL compared to enoxaparin of 2.7 μg/mL (Figure 7D−F). These results were comparable to the reported different LMWHs ranged from 2.4 to 2.9 μg/mL. The binding affinity of LMWH to PF4 is much smaller than UFH, resulting in a lower potential of HIT for LMWH.

4. CONCLUSIONS

Enoxaparin sodium, the most world widely used LMWH, is obtained by alkaline depolymerization of heparin benzyl ester exclusively from porcine intestinal mucosa. However, the variability of animal-sourced heparin, the limited availability and poor control of source materials, and their inherent impurities suggest a need for new approaches for LMWH production. In this project, we developed a new chemo-biocatalytic LMWH without the requirement of a depolymerization step from porcine-sourced UFH. We used K5 heparosan from an engineered strain of *E. coli* as the backbone precursor. An alkali depolymerization method was optimized to obtain an appropriate chain length backbone; furthermore, C5-Epi, 2-*O*-, 6-*O*-, and 3-*O*-sulfotransferases were modified on the backbone. An optimized and controlled modification step at each biosynthetic step leads to a chemobiosynthetic LMWH that has biosimilarity activity compared to enoxaparin.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acschembio.1c00928.

Figure S1: Eight disaccharides structures. Figure S2: Disaccharide and tetrasaccharide spectrum analysis by SAX-HPLC. Figure S3: Five 3-O-sulfated containing tetrasaccharide structures. Figure S4: 13C NMR analysis of enoxaparin and bioengineered LMWH (PDF)

■ AUTHOR INFORMATION

Corresponding Authors

Fuming Zhang − *Department of Chemical and Biological Engineering and Center for Biotechnology and Interdisciplinary Studies, Rensselaer Polytechnic Institute, Troy, New York 12180, United States;* orcid.org/0000-0003-2803-3704; Email: zhangf2@rpi.edu

Jonathan Dordick − *Department of Chemical and Biological Engineering, Department of Biology and Department of Biomedical Engineering, and Center for Biotechnology and Interdisciplinary Studies, Rensselaer Polytechnic Institute, Troy, New York 12180, United States;* orcid.org/0000-0001-7802-3702; Email: dordick@rpi.edu

Robert J. Linhardt − *Department of Chemistry and Chemical Biology, Department of Chemical and Biological Engineering, Department of Biology and Department of Biomedical Engineering, and Center for Biotechnology and Interdisciplinary Studies, Rensselaer Polytechnic Institute, Troy, New York 12180, United States;* orcid.org/0000-0003-2219-5833; Email: linhar@rpi.edu

Authors

Yanlei Yu − *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*

Li Fu − *Department of Chemistry and Chemical Biology and Center for Biotechnology and Interdisciplinary Studies, Rensselaer Polytechnic Institute, Troy, New York 12180, United States*

Peng He − *Department of Chemistry and Chemical Biology and Center for Biotechnology and Interdisciplinary Studies, Rensselaer Polytechnic Institute, Troy, New York 12180, United States*

Ke Xia − *Department of Chemistry and Chemical Biology and Center for Biotechnology and Interdisciplinary Studies, Rensselaer Polytechnic Institute, Troy, New York 12180, United States*

Sony Varghese − *Center for Biotechnology and Interdisciplinary Studies, Rensselaer Polytechnic Institute, Troy, New York 12180, United States*

Hong Wang − *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;* orcid.org/0000-0003-0058-060X

Complete contact information is available at: https://pubs.acs.org/10.1021/acschembio.1c00928

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

This work was partially supported by (1) GlycoMIP a National Science Foundation Materials Innovation Platform funded through Cooperative Agreement DMR-1933525; (2) Key Research and Development Program of Zhejiang (2021C03084); and (3) High-Level Talent Special Support Plan of Zhejiang Province (2019R52009).

■ REFERENCES

(1) Linhardt, R. J. 2003 Claude S. Hudson Award Address in Carbohydrate Chemistry. Heparin: Structure and Activity. *J. Med. Chem.* **2003**, *46*, 2551−2564.

(2) Mulloy, B.; Hogwood, J.; Gray, E.; Lever, R.; Page, C. P. Pharmacology of heparin and related drugs. *Pharmacol. Rev.* **2016**, *68*, 76−141.

(3) Humphries, D. E.; Wong, G. W.; Friend, D. S.; Gurish, M. F.; Qiu, W. T.; Huang, C.; Sharpe, A. H.; Stevens, R. L. Heparin is essential for the storage of specific granule proteases in mast cells. *Nature* **1999**, *400*, 769−772.

(4) Guerrini, M.; Rudd, T. R.; Mauri, L.; Macchi, E.; Fareed, J.; Yates, E. A.; Naggi, A.; Torri, G. Differentiation of generic enoxaparins marketed in the United States by employing NMR and multivariate analysis. *Anal. Chem.* **2015**, *87*, 8275−8283.

(5) Esko, J. D.; Selleck, S. B. Order out of chaos: assembly of ligand binding sites in heparan sulfate. *Annu. Rev. Biochem.* **2002**, *71*, 435−471.

(6) Chandarajoti, K.; Liu, J.; Pawlinski, R. The design and synthesis of new synthetic low-molecular-weight heparins. *J. Thromb. Haemost.* **2016**, *14*, 1135−1145.

(7) Lind, T.; Tufaro, F.; McCormick, C.; Lindahl, U.; Lidholt, K. The putative tumor suppressors EXT1 and EXT2 are glycosyltransferases required for the biosynthesis of heparan sulfate. *J. Biol. Chem.* **1998**, *273*, 26265−26268.

(8) Rong, J.; Habuchi, H.; Kimata, K.; Lindahl, U.; Kusche-Gullberg, M. Substrate specificity of the heparan sulfate hexuronic acid 2-O-sulfotransferase. *Biochemistry* **2001**, *40*, 5548−5555.
(9) Habuchi, H.; Tanaka, M.; Habuchi, O.; Yoshida, K.; Suzuki, H.; Ban, K.; Kimata, K. The occurrence of three isoforms of heparan sulfate 6-O-sulfotransferase having different specificities for hexuronic acid adjacent to the targeted N-sulfoglucosamine. *J. Biol. Chem.* **2000**, *275*, 2859−2868.
(10) Fu, L.; Li, K.; Mori, D.; Hirakane, M.; Lin, L.; Grover, N.; Datta, P.; Yu, Y.; Zhao, J.; Zhang, F.; Yalcin, M.; Mousa, S. A.; Dordick, J. S.; Linhardt, R. J. Enzymatic generation of highly anticoagulant bovine intestinal heparin. *J. Med. Chem.* **2017**, *60*, 8673−8679.
(11) Hirsh, J.; Bauer, K. A.; Donati, M. B.; Gould, M.; Samama, M. M.; Weitz, J. I. Parenteral anticoagulants: American college of chest physicians evidence-based clinical practice guidelines (8th Edition). *Chest* **2008**, *133*, 141S−159S.
(12) Bradbrook, I. D.; Magnani, H. N.; Moelker, H. C.; Morrison, P. J.; Robinson, J.; Rogers, H. J.; Spector, R. G.; Van Dinther, T.; Wijnand, H. ORG 10172: a low molecular weight heparinoid anticoagulant with a long half-life in man. *Br. J. Clin. Pharmacol.* **1987**, *23*, 667−675.
(13) Junqueira, D. R.; Zorzela, L. M.; Perini, E. Unfractionated heparin versus low molecular weight heparins for avoiding heparin-induced thrombocytopenia in postoperative patients. *Cochrane Database Syst. Rev.* **2017**, *4*, No. cd007557.
(14) Merli, G. J.; Groce, J. B. Pharmacological and clinical differences between low-molecular-weight heparins: implications for prescribing practice and therapeutic interchange. *Pharm. Ther.* **2010**, *35*, 95−105.
(15) Sabatine, M. S.; Morrow, D. A.; Dalby, A.; Pfisterer, M.; Duris, T.; Lopez-Sendon, J.; Murphy, S. A.; Gao, R.; Antman, E. M.; Braunwald, E.; ExTRACT-TIMI 25 Investigators. Efficacy and safety of enoxaparin versus unfractionated heparin in patients with ST-segment elevation myocardial infarction also treated with clopidogrel. *J. Am. Coll. Cardiol.* **2007**, *49*, 2256−2263.
(16) Voelker, R. FDA approves generic enoxaparin. *J. Am. Med. Assoc.* **2010**, *304*, 844−844.
(17) Thompson, C. A. FDA approves first generic enoxaparin product. *Am. J. Health Syst. Pharm.* **2010**, *67*, 1486−1488.
(18) Kishimoto, T. K.; Viswanathan, K.; Ganguly, T.; Elankumaran, S.; Smith, S.; Pelzer, K.; Lansing, J. C.; Sriranganathan, N.; Zhao, G.; Galcheva-Gargova, Z.; Al-Hakim, A.; Bailey, G. S.; Fraser, B.; Roy, S.; Rogers-Cotrone, T.; Buhse, L.; Whary, M.; Fox, J.; Nasr, M.; Dal Pan, G. J.; Shriver, Z.; Langer, R. S.; Venkataraman, G.; Austen, K. F.; Woodcock, J.; Sasisekharan, R. Contaminated heparin associated with adverse clinical events and activation of the contact system. *N. Engl. J. Med.* **2008**, *358*, 2457−2467.
(19) Zhang, X.; Lin, L.; Huang, H.; Linhardt, R. J. Chemoenzymatic synthesis of glycosaminoglycans. *Acc. Chem. Res.* **2020**, *53*, 335−346.
(20) Cress, B. F.; Englaender, J. A.; He, W.; Kasper, D.; Linhardt, R. J.; Koffas, M. A. G. Masquerading microbial pathogens: capsular polysaccharides mimic host-tissue molecules. *FEMS Microbiol. Rev.* **2014**, *38*, 660−697.
(21) Zhang, Z.; McCallum, S. A.; Xie, J.; Nieto, L.; Corzana, F.; Jiménez-Barbero, J.; Chen, M.; Liu, J.; Linhardt, R. J. Solution structures of chemoenzymatically synthesized heparin and its precursors. *J. Am. Chem. Soc.* **2008**, *130*, 12998−13007.
(22) Cress, B. F.; Bhaskar, U.; Vaidyanathan, D.; Williams, A.; Cai, C.; Liu, X.; Fu, L.; M-Chari, V.; Zhang, F.; Mousa, S. A.; Dordick, J. S.; Koffas, M. A. G.; Linhardt, R. J. Heavy heparin: A stable isotope-enriched, chemoenzymatically-synthesized, poly-component drug. *Angew. Chem. Int. Ed.* **2019**, *58*, 5962−5966.
(23) Xu, Y.; Masuko, S.; Takieddin, M.; Xu, H.; Liu, R.; Jing, J.; Mousa, S. A.; Linhardt, R. J.; Liu, J. Chemoenzymatic synthesis of homogeneous ultralow molecular weight heparins. *Science* **2011**, *334*, 498−501.
(24) Sismey-Ragatz, A. E.; Green, D. E.; Otto, N. J.; Rejzek, M.; Field, R. A.; DeAngelis, P. L. Chemoenzymatic synthesis with distinct *Pasteurella* heparosan synthases: monodisperse polymers and unnatural structures. *J. Biol. Chem.* **2007**, *282*, 28321−28327.
(25) Xu, Y.; Chandarajoti, K.; Zhang, X.; Pagadala, V.; Dou, W.; Hoppensteadt, D. M.; Sparkenbaugh, E. M.; Cooley, B.; Daily, S.; Key, N. S.; Severynse-Stevens, D.; Fareed, J.; Linhardt, R. J.; Pawlinski, R.; Liu, J. Synthetic oligosaccharides can replace animal-sourced low molecular weight heparins. *Sci. Transl. Med.* **2017**, *9*, No. eaan5954.
(26) Wang, Z.; Dordick, J. S.; Linhardt, R. J. *Escherichia coli* K5 heparosan fermentation and improvement by genetic engineering. *Bioeng. Bugs.* **2011**, *2*, 63−67.
(27) Zhang, J.; Suflita, M.; Li, G.; Zhong, W.; Li, L.; Dordick, J. S.; Linhardt, R. J.; Zhang, F. High cell density cultivation of recombinant Escherichia coli strains expressing 2-O-sulfotransferase and C5-epimerase for the production of bioengineered heparin. *Appl. Biochem. Biotechnol.* **2015**, *175*, 2986−2995.
(28) Zhang, J.; Suflita, M.; Fiaschetti, C. M.; Li, G.; Li, L.; Zhang, F.; Dordick, J. S.; Linhardt, R. J. High cell density cultivation of a recombinant Escherichia coli strain expressing a 6-O-sulfotransferase for the production of bioengineered heparin. *J. Appl. Microbiol.* **2015**, *118*, 92−98.
(29) Vaidyanathan, D.; Paskaleva, E.; Vargason, T.; Ke, X.; McCallum, S.; Linhardt, R. J.; Dordick, J. S. Elucidating the Unusual Reaction Kinetics of D-Glucuronyl C5-Epimerase. *Glycobiology* **2020**, *30*, 847−858.
(30) Linhardt, R. J.; Turnbull, J. E.; Wang, H. M.; Loganathan, D.; Gallagher, J. T. Examination of the substrate specificity of heparin and heparan sulfate lyases. *Biochemistry* **1990**, *29*, 2611−2617.
(31) Go, K.; Horikawa, Y.; Garcia, R.; Villarreal, F. J. Fluorescent method for detection of cleaved collagens using *O*-phthaldialdehyde (OPA). *J. Biochem. Biophys. Methods* **2008**, *70*, 878−882.
(32) Skidmore, M. A.; Guimond, S. E.; Dumax-Vorzet, A. F.; Yates, E. A.; Turnbull, J. E. Disaccharide compositional analysis of heparan sulfate and heparin polysaccharides using UV or high-sensitivity fluorescence (BODIPY) detection. *Nat. Protoc.* **2010**, *5*, 1983−1992.
(33) The United States Pharmacopeial Convention. Revision Bulletin. Enoxaparin Sodium. 2011.
(34) Yu, Y.; Zhang, F.; Renois-Predelus, G.; Amster, I. J.; Linhardt, R. J. Filter-entrapment enrichment pull-down assay for glycosaminoglycan structural characterization and protein interaction. *Carbohydr. Polymers* **2020**, *245*, No. 116623.
(35) Wang, Z.; Ly, M.; Zhang, F.; Zhong, W.; Suen, A.; Hickey, A. M.; Dordick, J. S.; Linhardt, R. J. *E. coli* K5 fermentation and the preparation of heparosan, a bioengineered heparin precursor. *Biotechnol. Bioeng.* **2010**, *107*, 964−973.
(36) Willis, L. M.; Stupak, J.; Richards, M. R.; Lowary, T. L.; Li, J.; Whitfield, C. Conserved glycolipid termini in capsular polysaccharides synthesized by ATP-binding cassette transporter-dependent pathways in Gram-negative pathogens. *Proc. Natl. Acad. Sci. U. S. A.* **2013**, *110*, 7868.
(37) Yan, L.; Fu, L.; Xia, K.; Chen, S.; Zhang, F.; Dordick, J. S.; Linhardt, R. J. A revised structure for the glycolipid terminus of Escherichia coli K5 heparosan capsular polysaccharide. *Biomolecules* **2020**, *10*, 1516.
(38) Doyle, L.; Ovchinnikova, O. G.; Myler, K.; Mallette, E.; Huang, B. S.; Lowary, T. L.; Kimber, M. S.; Whitfield, C. Biosynthesis of a conserved glycolipid anchor for Gram-negative bacterial capsules. *Nat. Chem. Biol.* **2019**, *15*, 632−640.
(39) Chen, J.; Avci, F. Y.; Muñoz, E. M.; McDowell, L. M.; Chen, M.; Pedersen, L. C.; Zhang, L.; Linhardt, R. J.; Liu, J. Enzymatically redesigning of biologically active heparan sulfate. *J. Biol. Chem.* **2005**, *280*, 42817−42825.
(40) Fu, L.; Zhang, F.; Li, G.; Onishi, A.; Bhaskar, U.; Sun, P.; Linhardt, R. J. Structural and activity of a new low-molecular-weight heparin produced by enzymatic ultrafiltration. *J. Pharm. Sci.* **2014**, *103*, 1375−1383.
(41) Chen, Y.; Lin, L.; Agyekum, I.; Zhang, X.; St. Ange, K.; Yu, Y.; Zhang, F.; Liu, J.; Amster, I. J.; Linhardt, R. J. Structural analysis of

https://doi.org/10.1021/acschembio.1c00928
ACS Chem. Biol. 2022, 17, 637−646

heparin-derived 3-O-sulfated tetrasaccharides: antithrombin binding site variants. *J. Pharm. Sci.* **2017**, *106*, 973−981.
(42) Baytas, S. N.; Varghese, S. S.; Jin, W.; Yu, Y.; He, P.; Douaisi, M.; Zhang, F.; Brodfuehrer, P.; Xia, K.; Dordick, J. S.; Linhardt, R. J. Preparation of low molecular weight heparin from a remodeled bovine intestinal heparin. *J. Med. Chem.* **2021**, *64*, 2242−2253.
(43) Gray, E.; Hogwood, J.; Mulloy, B. The anticoagulant and antithrombotic mechanisms of heparin. In *Heparin-a Century of Progress*; Springer: Berlin Heidelberg, 2012, pp. 43−61.
(44) Zhao, J.; Liu, X.; Malhotra, A.; Li, Q.; Zhang, F.; Linhardt, R. J. Novel method for measurement of heparin anticoagulant activity using SPR. *Anal. Biochem.* **2017**, *526*, 39−42.
(45) Warkentin, T. E.; Levine, M. N.; Hirsh, J.; Horsewood, P.; Roberts, R. S.; Gent, M.; Kelton, J. G. Heparin-induced thrombocytopenia in patients treated with low-molecular-weight heparin or unfractionated heparin. *N. Engl. J. Med.* **1995**, *332*, 1330−1336.
(46) Zhang, F.; Datta, P.; Dordick, J. S.; Linhardt, R. J. Evaluating heparin products for heparin-induced thrombocytopenia using surface plasmon resonance. *J. Pharm. Sci.* **2020**, *109*, 975−980.

https://doi.org/10.1021/acschembio.1c00928
ACS Chem. Biol. 2022, 17, 637−646