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Brief Article

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Enzymatic Generation of Highly Anticoagulant Bovine Intestinal Heparin

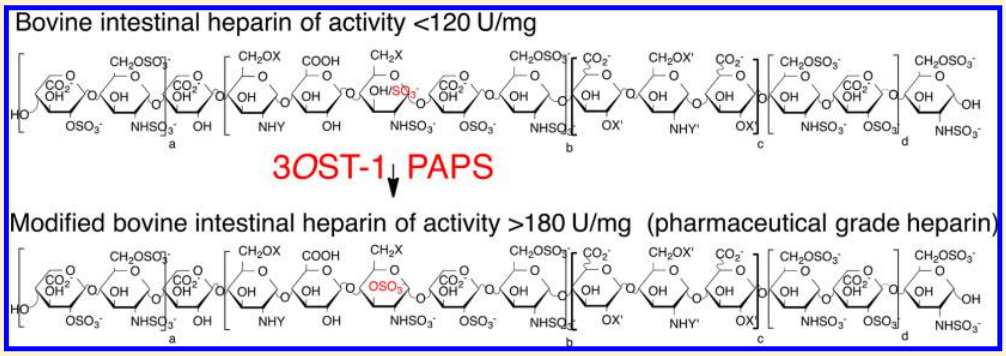
Li Fu,[†] Kevin Li,[†] Daisuke Mori,[†] Makoto Hirakane,[†] Lei Lin,[†] Navdeep Grover,[†] Payel Datta,[†] Yanlei Yu,[‡] Jing Zhao,[†] Fuming Zhang,[†,‡] Murat Yalcin,[#,▽] Shaker A. Mousa,[#] Jonathan S. Dordick,[†,‡,§,∥] and Robert J. Linhardt*[,†,‡,§,∥,⊥]

[†]Center for Biotechnology and Interdisciplinary Studies, [‡]Department of Chemical and Biological Engineering, [§]Department of Biomedical Engineering, [∥]Department of Biology, and [⊥]Department of Chemistry and Chemical Biology, Rensselaer Polytechnic Institute, Troy, New York 12180, United States

[#]The Pharmaceutical Research Institute, Albany College of Pharmacy, Rensselaer, New York 12144, United States

[▽]Department of Physiology, Veterinary Medicine Faculty, Uludag University, Gorukle 16059, Bursa, Turkey

Supporting Information



ABSTRACT: Unlike USP porcine heparin, bovine intestinal heparin (BIH) has a low anticoagulant activity. Treatment with 6-OST-1, -3, and/or 3-OST-1 afforded two remodeled heparins that met USP heparin activity and Mw specifications. We explored the pharmacodynamics and pharmacokinetics in a rabbit model. We conclude that a modest increase in the content of 3-*O*-sulfo groups in BIH increases the number of antithrombin III binding sites, making remodeled BIH behave similarly to pharmaceutical heparin.

INTRODUCTION

Heparin is a glycosaminoglycan that is obtained from animal tissues and is widely used as a clinical anticoagulant drug.[1,2] Heparin is biosynthesized in the Golgi of certain animal cells through the stepwise action of de-*N*-acetylase-*N*-sulfotransferase, C5-epimerase, 2-*O*-, 6-*O*-, and 3-*O*-sulfotransferases on the heparosan polysaccharide precursor.[3−5] Incomplete modification at each biosynthetic step leads to a large variety of chain structures. The heparin polysaccharide (Figure 1) is composed of disaccharide repeating units, including a major repeating unit that is trisulfated (TriS), →4) α-L-IdoA2S (1→4) α-D-GlcNS6S (1→, where IdoA is iduronic acid, GlcN is glucosamine, and S is sulfo. Heparin contains lesser amounts of glucuronic acid (GlcA) and *N*-acetyl glucosamine (GlcNAc) residues having varying numbers of *O*-sulfo groups.[5,6] Heparin contains a pentasaccharide sequence (i.e., →4) GlcNAc6S (1→4) GlcA (1→4) GlcNS3S6S (1→4) IdoA2S (1→4) GlcNS6S (1→) that allows it to interact with antithrombin III (AT) and inhibit blood coagulation proteases, such as factor IIa (FIIa or thrombin) and factor Xa (FXa).[1] There is some allowable structural variation in the AT-binding pentasaccharide sequences,[7,8] but all have unique, well-conserved residues, such as a central 3-*O*-sulfoglucosamine, critical for anticoagulant activity. Some unsubstituted AT precursor sites remain in heparin due to incomplete action of the 3-*O*-sulfotransferase.[9]

Nearly 100 metric tons of heparin is annually produced worldwide primarily from porcine intestine (PI).[10] Small amounts of bovine intestinal (BI), bovine lung, and ovine (sheep) intestinal heparins are also prepared but are not approved for clinical use in most countries. These heparins, sourced from bovine and ovine tissues, exhibit physiochemical, biological, and pharmacological differences from pharmaceutical porcine intestinal heparin.[11−17]

There has been growing concern about the stability and safety of the pharmaceutical heparin supply chain.[18] In 2007−2008 a heparin contamination crisis resulted from the adulteration of crude porcine intestinal heparin with a toxic

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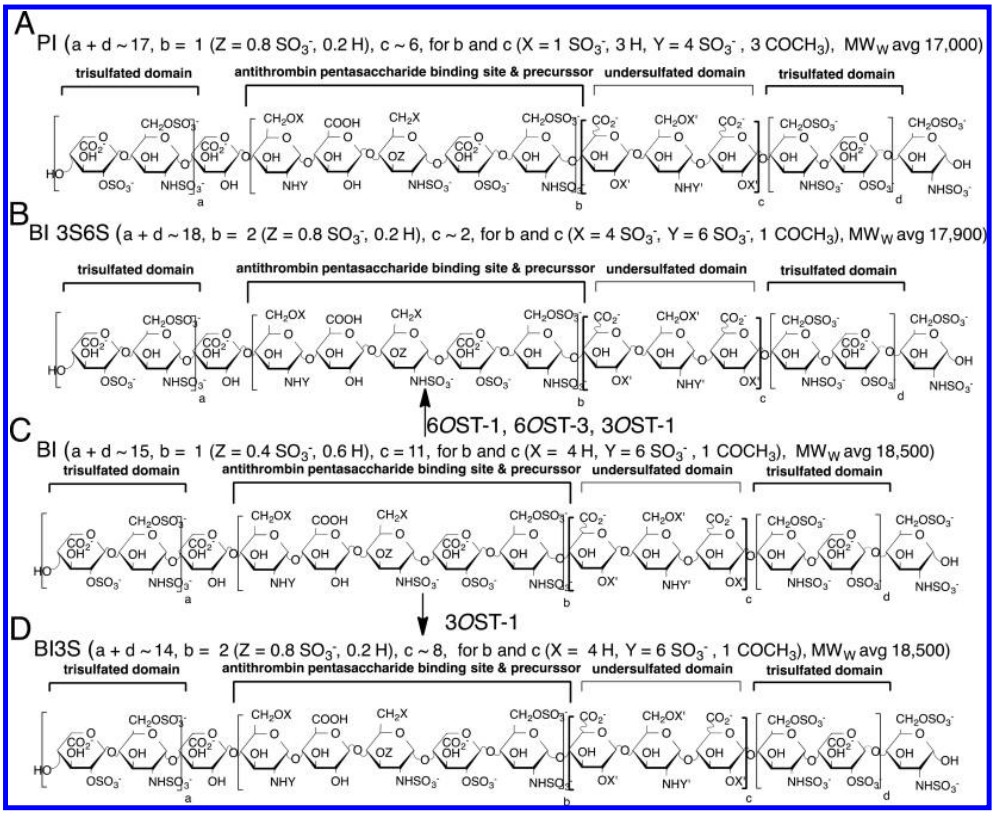

Figure 1. Structure of heparins and their remodeled structures. a + d ~ 20, b = 1, c ~ 8 for MW weight average 20 000. X or X′ = SO_3^-, H; Y or Y′ = SO_3^-, $COCH_3$, H; Z = SO_3^-, H. (A) Schematic structure of an average chain present in PI heparin. (C) Schematic structure of an average chain present in BI heparin. (B) Schematic structure of an average chain present in BI 3S6S heparin. (D) Schematic structure of an average chain present in BI 3S heparin. See Table 1 and Supporting Information Table S1 and Figure S1 for detailed MW data.

semisynthetic glycosaminoglycan, oversulfated chondroitin sulfate.[19−22] This contamination crisis was associated with multiple deaths and severely disrupted the heparin supply chain for a period of time.[20] Porcine diseases, including blue ear disease and porcine diarrhea, have also resulted in market disruptions.[18] Recently, concerns regarding illegal blending of bovine or ovine heparin products with porcine heparin have led to drug recalls.[23]

These issues have led researchers and regulatory agencies to consider alternative supplies of heparins, either the development of bioengineered heparins,[24] synthetic heparins,[25] or the reintroduction of bovine heparin into the pharmaceutical market.[18] Bovine lung heparin was successfully used in the U.S. until the 1990s when it was voluntarily withdrawn following the outbreak of bovine spongiform encephalopathy (BSE) in Europe,[18] and BI mucosa heparin has been more recently used in South America.[14,16,26]

Recently, our laboratory[11,13,15,27] and others[12,14,16,17] have been examining the physicochemical, biological, and pharmacological differences in heparins prepared from different tissues of food animals. Moreover, there have even been efforts reported to prepare low molecular weight heparin products from bovine-derived heparins.[28,29]

Some concerns have recently arisen about the reintroduction of bovine heparin, as it is clearly different from porcine intestinal heparin.[18] From a commercial standpoint, it appears that BI is a preferred source over bovine lung heparin. Since there are substantial differences between porcine intestinal heparin and BI heparin, new pharmacopeia monographs will be required. Most concerning, however, is that the substantial differences in the specific anticoagulant activities (units/mg) of BI heparin (~100−130 U/mg) and porcine intestinal heparin (~180−210 U/mg) will make these products difficult for clinicians to use and potentially interchange. For example, heparin, administered on a unit basis, is frequently neutralized with protamine sulfate (an antidote given on a weight basis), thus requiring complex calculations by surgeons if heparins with very different potencies were used. Moreover, while pharmaceutical heparin is generally considered a safe and effective drug, it does exhibit side effects in a small percentage of patients.[1] Of particular concern is heparin-induced thrombocytopenia (HIT) in which heparin binds, through primarily electrostatic interaction, to platelet factor IV (PF4), resulting in an immunologically active complex, resulting in aggregation and loss of platelets. The PF4 binding site in heparin is believed to be a dodecasaccharide or longer site rich in the highly sulfated TriS sequence.[30] Thus, there is concern that any new heparin products introduced into the market may not show enhanced PF4 binding leading to HIT, but unfortunately extensive in vivo studies are required to determine the prevalence of HIT.[31]

In the current study we extend our understanding of the structural differences between PI and BI heparins, particularly their differences in the levels of 3-*O*-sulfo and 6-*O*-sulfo content, to understand how these differences impact their anticoagulant activities. Moreover, we use recombinant 3-*O*-

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Table 1. Properties of Remodeled BI Heparins Compared to PI USP Heparin

	disaccharide composition (mol %)[a]										activity (U/mg)[b]		
heparin	0S	NS	6S	2S	NS6S	NS2S	2S6S	TriS	sulfo groups per disaccharide	Mw[c]	anti-Xa	anti-IIa	Xa/IIa
USP specs									2.5−2.7	15000−19000		>180	0.9−1.1
PI (USP) heparin	4.0	3.2	3.7	2.0	10.2	7.8	1.5	67.7	2.5	17000	200	200	1.0
BI heparin	3.3	5.9	0.8	2.5	8.2	32.2	0.2	46.8	2.3	18500	110	105	1.0
BI heparin 3S	3.9	6.3	0.0	2.5	5.7	32.1	0.0	49.5	2.3	18500	231	268	0.9
BI heparin 3S6S	3.6	5.4	0.0	2.4	6.1	15.3	0.0	67.2	2.5	17900	289	301	1.0

[a]The systematic errors of disaccharide compositional values are less than 0.1%. [b]Systematic errors of bioactivity assays are less than 5%. [c]Some of the larger chains may remain bound to the enzymes, reducing the average molecular weight of the resulting products.

and 6-*O*-sulfotransferases to prepare enzymatically modified BI heparin and compare this remodeled heparin to pharmaceutical porcine intestinal heparin.

RESULTS AND DISCUSSION

Enzymatic Modification of BI Heparin. Heparin biosynthetic enzymes 6-OST-1, 6-OST-3, 3-OST-1 and PAPS regeneration enzyme AST-IV were prepared and immobilized as previously reported.[32] 6OST-1 and 6OST-3 were prepared as maltose binding protein (MBP) fusions, and 3OST-1 and arylsulfotransferase-IV (AST-IV) were prepared as His-tagged fusions. These proteins were immobilized on amylose and Ni-Sepharose resins, respectively. BI heparin was treated with a mixture of immobilized 6-OST-1, 6-OST-3, and 3-OST-1 or with only 3-OST-1. The sulfation reaction was coupled with a PAPS recycling system consisting of potassium *p*-nitrophenyl sulfate (PNPS as a sacrificial sulfo donor), 3′-phosphoadenosine 5′-phosphosulfate (PAPS), and AST-IV. After the reaction was complete, the mixtures were filtered to remove enzyme resin, dialyzed using 5K Da molecular weight cutoff (MWCO) centrifugal membrane units with distilled water to remove PNP, PAPS, MES salt, and other small molecule impurities, and the retentate containing remodeled BI heparins was lyophilized for further analysis.

Disaccharide compositional analysis of the heparin polysaccharide relies on its exhaustive treatment with a cocktail of heparinases to afford primarily disaccharide products. These disaccharides are then analyzed by strong anion exchange (SAX) high performance liquid chromatography (HPLC) and their identity and concentrations determined based on disaccharide standards.[33] The disaccharide compositions of a typical PI mucosal heparin and a BI mucosal heparin are shown in Table 1. There are major compositional differences in these two heparins. In particular, PI heparin has a higher level of fully modified TriS sequence (66% and 55% TriS in PI and BI, respectively), →4) α-L-IdoA2S (1→4) α-D-GlcNS6S (1→, the result of greatly reduced 6-*O*-sulfo group content and a higher *N*-acetyl content, resulting in enriched levels of 0S + 2S + 6S + 2S6S (8.6% and 2.9% in PI and BI, respectively). The disaccharide compositions of PI and BI heparins compare well to previously published analyses.[11,13,15,28,29]

Treatment of BI heparin with 6-OST-1, 6-OST-3, and 3-OST-1 afforded a disaccharide composition that was similar to PI heparin. In particular, the TriS content of BI 3S6S was 71%, higher than the PI heparin used in this study but comparable to many previously analyzed heparins (Table S2 in Supporting Information).[11,13,15,28,29] Finally, the disaccharide analysis of BI heparin with only 3-OST-1 was very similar to BI heparin and quite dissimilar to PI heparin. The subtle differences between BI and BI 3S heparins result from the increased levels of 3-*O*-sulfo groups resulting in a greater quantity of resistant tetrasaccharide, containing a reducing end GlcNS3S6S residue,[7,8,34] impacting the compositional makeup of the remaining disaccharides.

NMR Analysis of BI Heparin and Remodeled BI Heparin. NMR analysis[35] was performed to assess the monosaccharide composition of the starting and modified BI heparins (Figure 2 and Supporting Information Figure S2 and Table S3). Instead of using the very sophisticated quantitative 2D NMR recently developed by the Guerrini laboratory,[36] the 1D ^{1}H NMR quantification method applied indicated the types and amounts of sulfo groups. This method had been validated in our previous report to have standard deviation of 0.7%. The ^{1}H NMR of the starting BI heparin closely resembled that of more than 20 other BI heparins examined in our laboratory.[11,13,15,28,29] The anomeric signals in the partial ^{1}H NMR spectrum shown in Figure 2A could be clearly assigned and integrated (Table S3 in Supporting Information). As expected, treatment of BI heparin with 6-OST-1, 6-OST-3, and 3-OST-1 increased the content of 6-*O*-sulfo and 3-*O*-sulfo groups (62−81% and 5−19%, respectively). While the increase

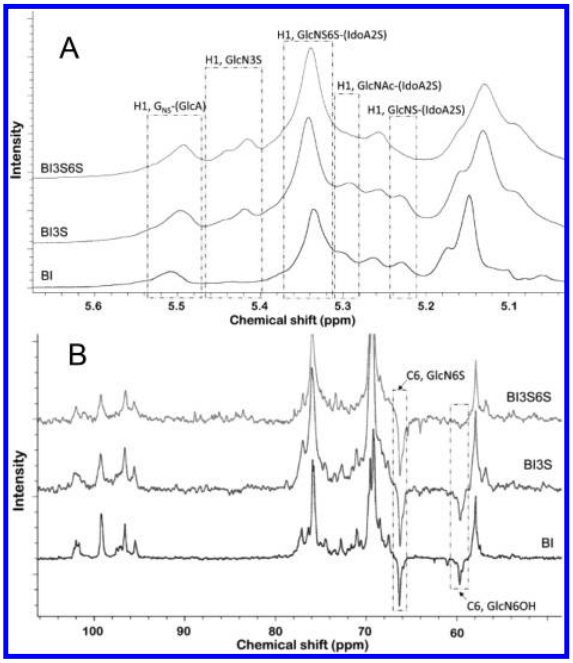

Figure 2. NMR analysis of BI heparin and modified BI heparins: (A) 1D ^{1}H NMR spectra of bovine heparins BI, BI 3S, BI 3S6S; (B) 1D DEPT-135 ^{13}C NMR spectra of bovine heparins BI, BI 3S, BI 3S6S.

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in 3-*O*-sulfo groups in both enzyme treated BI heparins could be clearly demonstrated, it is much harder to demonstrate the increase in 6-*O*-sulfo groups because of the structural heterogeneity of these polysaccharides. To that end, 1D distortionless enhancement by polarization transfer (DEPT)-135 ^{13}C NMR spectra were obtained to examine definitively the change in 6-*O*-sulfo group content of the BI heparin treated with 6-OST-1, 6-OST-3, and 3-OST-1 (Figure 2B and in Supporting Information Table S3). DEPT spectroscopy results in a negative signal for the primary C6 carbon allowing the clear assignment of the C6 of GlcNS6S and GlcNS resonances. These experiments confirm that only the sample treated with 6-OST-1 and 6-OST-3 showed increased GlcNS6S content and with decreased GlcNS content (Figure 2B).

Tetrasaccharide Mapping Analysis. Tetrasaccharide mapping analysis was next undertaken by treating each heparin sample with heparinase II followed by analysis of the resulting resistant tetrasaccharides[34] with reverse phase ion pairing (RPIP) HPLC and mass spectral (MS) detection (Figure 3).[7,8]

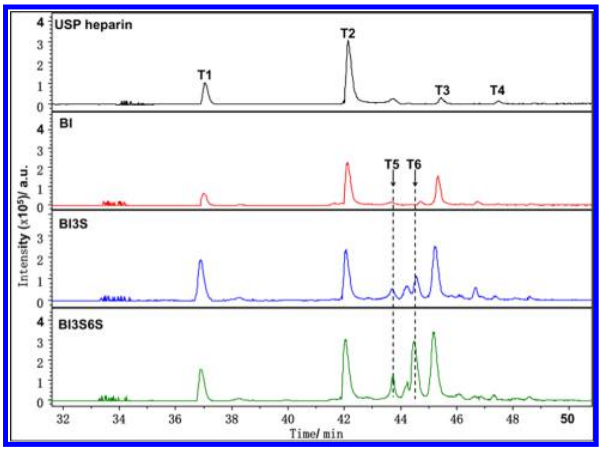

Figure 3. Extracted ion chromatogram of tetrasaccharide mapping on BI heparins and PI heparin. T1 (*m*/*z* 477) is ΔUA-GlcNAc6S-GlcA-GlcNS3S. T2 (*m*/*z* 517) is ΔUA-GlcNAc6S-GlcA-GlcNS3S6S. T3 (*m*/*z* 536) is ΔUA-GlcNS6S-GlcA-GlcNS3S6S. T4 (*m*/*z* 557) is ΔUA-GlcNAc2S6S-GlcA-GlcNS3S6S. T5 (*m*/*z* 536) and T6 (*m*/*z* 536) have unknown structures.

Standard tetrasaccharides carrying a GlcNS3S6S residue at their reducing end have been prepared through heparinase II treatment of heparin, purified and characterized.[7,8] Analysis of PI heparin affords the major resistant tetrasaccharides (Figure 3 T1, T2, T3, and T4) expected, while BI heparin shows lower amounts of these and minor amounts of unidentified resistant tetrasaccharides (T5 and T6). Treatment of BI heparin with either 6-OST-1, 6-OST-3 and 3-OST-1 or 3-OST-1 alone enhances the content of tetrasaccharides (Figure 3) but to different degrees. The presence of 6-OST-1 and 6-OST-3 increases the sulfation level of the resulting resistant tetrasaccharides as evidenced from the higher intensity of the peaks eluting later (having greater net charge) and their molecular mass (Table 1).

Binding Analysis. Heparin anticoagulant activity results from the binding of heparin to the AT site on its blood factor targets. There are a number of approaches for determining AT binding site content. These include activity assays, AT-based affinity fractionation of heparin chains, fluorescence binding assays, and competitive SPR. The competitive binding assay was recently developed by our laboratory[37] and was found to be a very specific method correlating well with anti-Xa activity and AT binding site content. Thus, we compared the protein binding of the modified BI heparins to BI and PI heparins based on a competitive binding study using surface plasmon resonance (SPR) (Figure 4). Since the resistant tetrasaccharide

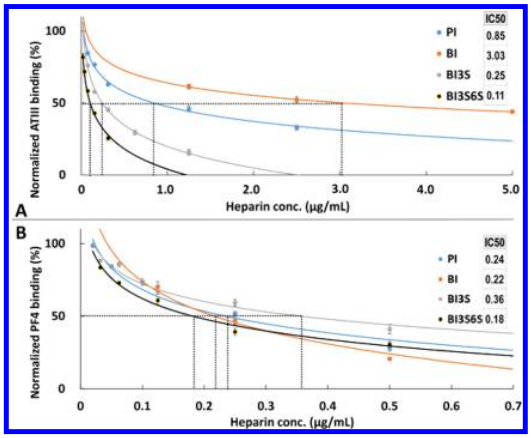

Figure 4. Surface plasmon resonance IC_{50} measurement of bovine heparins using surface competition SPR: (A) IC_{50} for AT binding of heparins; (B) IC_{50} for PF4 binding of heparins.

content increased on treatment with 6-OST-1, 6-OST-3 and 3-OST-1, or 3-OST-1 alone, we expected that the AT-binding activity would be similarly enhanced.[34] Unmodified BI heparin required over 3-fold higher concentration to compete with USP heparin than did PI heparin (Figure 4A). In contrast, BI heparin treated with 3-OST-1 alone or 6-OST-1, 6-OST-3 and 3-OST-1 showed 3-fold and 5-fold lower concentrations than PI heparin to displace AT from surface-bound USP heparin. These results clearly demonstrate that competent AT binding sites had been generated in BI heparin with both treatments, consistent with the results obtained in the tetrasaccharide mapping experiment.

SPR competition studies performed with PF4 (Figure 4B) showed that PI and BI heparins had comparable binding in terms of IC_{50}, but the shapes of the binding curves were dissimilar, with BI heparin showing much more PF4 binding at high concentrations than PI heparin. This is particularly concerning since much higher concentrations of BI heparin are required compared to PI heparin for the anticoagulant activity effect. The 6-OST-1, 6-OST-3 and 3-OST-1 treated BI heparin had significantly higher affinity for PF4 consistent with its higher level of sulfation and its particularly high TriS content (74% compared to 66% for PI and 50% for BI heparin, respectively) (Table 1). In contrast, the 3-OST-1 treated BI heparin showed considerably weaker binding to PF4. This is consistent with the fact that 3-OST-1 treatment cannot increase TriS content (TriS has no 3-*O*-sulfo group and can only be enhanced by treatment with 6-OST-1 and/or 6-OST-3, which can convert →4) α-L-IdoA2S (1→4) α-D-GlcNS (1→ into → 4) α-L-IdoA2S (1→4) α-D-GlcNS6S (1→ (TriS)). It is noteworthy that while 3-OST-1 treatment slightly alters the relative TriS content based on disaccharide analysis (55% for BI heparin compared to 54% for BI 3S (Table 1), this increase is an artifact of the increased level of resistant tetrasaccharides (Figure 3), corresponding to AT-binding sites (Figure 4A).

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Anticoagulant Activity. The anticoagulant activity of the remodeled BI heparins was next measured using the methods described in the current USP heparin monograph and compared to those of PI and BI heparins (Table 1). As expected, PI heparin showed an anti-FXa activity of 200 U/mg, an anti-FIIa activity of 200 U/mg with an anti-FXa/anti-FIIa ratio of 1.0, consistent with USP requirements of >180 U/mg with an anti-FXa/anti-FIIa ratio of 0.9−1.1.[38] BI heparin showed considerably lower anti-FXa activity of 110 U/mg, an anti-FIIa activity of 105 U/mg with an anti-FXa/anti-FIIa ratio of 1.0. BI 3S, and BI3S6S heparins both showed activities greatly enhanced over PI heparin. Although remodeled BI heparin shows relatively higher anticoagulant activity, it meets all current USP criteria for pharmaceutical grade heparin. Two possible approaches to reduce activity closer to 180 U/mg are less exhaustive treatment of BI heparin with 3-OST-1 or blending untreated BI heparin with 3-OST-1-treated BI heparin.

Pharmacodynamics and Pharmacokinetics. Finally, in vivo pharmacodynamics/pharmacokinetics analysis (PD/PK)[39] was undertaken in the rabbit model (n = 3) with intravenous administration of BI 3S heparin at a dose of 6.4 mg/kg (Figure 5A). The blood was sampled prior to dosage and up to 2 h after

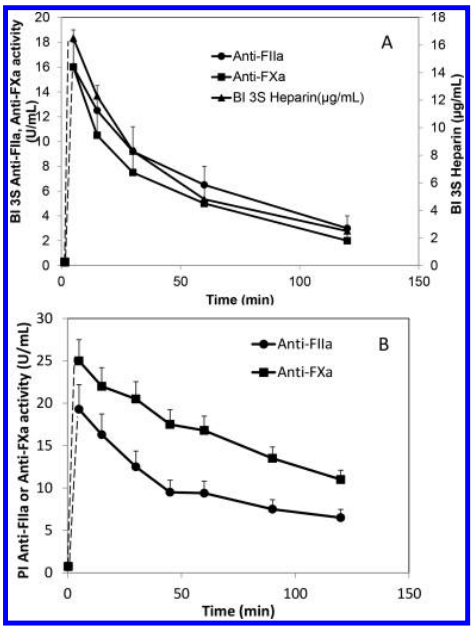

Figure 5. Pharmacodynamics/pharmacokinetics (PK/PD) of heparins in the rabbit: (A) BI 3S heparin (in triplicate, ±SD); (B) PI heparin (n = 7 USP PI heparins, each in triplicate, ±SD).

administration. PD analysis showed BI 3S heparin had a $t_{1/2}$ of 28 and 44 min based on anti-FXa activity and anti-FIIa activity, respectively. The area under the curve (AUC) for these activities was 665 and 826 U·min/mL, respectively. PK was next determined using disaccharide analysis relying on multiple reaction monitoring (MRM) as previously described.[39] The concentration of heparin in the plasma was calculated and afforded a $t_{1/2}$ of 30 min and an AUC of 714 μg·min/mL. The PD data compared favorably with historic data from our laboratory in rabbits (by the same route and same dose) that examined eight different contemporary USP (PI) heparins (Figure 5B). PI heparin had a $t_{1/2}$ of 105 and 45 min, AUC of 1925 and 1164 U·min/mL, respectively, based on anti-FXa activity and anti-FIIa activity.

CONCLUSION

The current study provides proof-of-concept experiments that bovine heparin of low activity can be remodeled to meet USP specifications. The results of this study show that it is possible to increase the activity of BI heparin to that of PI heparin through its treatment with 6-OST-1, 6-OST-3 and 3-OST-1. Surprisingly, treatment of BI heparin with 3-OST-1 alone also afforded a remodeled heparin that met the ant-FXa and anti-FIIa activity requirements of PI heparin. While treatment with 6-OST-1, 6-OST-3 and 3-OST-1 provided BI 3S6S heparin that was closer in composition to PI heparin and had the highest anticoagulant activity, this approach had two limitations. First, the resulting heparin had a very high level of sulfation and was particularly rich in the fully modified TriS sequence. This sequence, known to be responsible for PF4 binding, showed enhanced binding to PF4 and thus might increase the incidence of the HIT side effect associated with the intravenous use of heparin. Second, the use of three enzymes each requiring PAPS cofactor would significantly increase the cost of remodeling BI heparin to meet the USP activity specifications. Thus, we undertook a more selective remodeling strategy that relied only on 3-OST-1 and PAPS to enhance only the AT-binding activity without enhancing the PF4 binding and also decreasing the cost and complexity of the remodeling process. The result was a remodeled BI 3S heparin that met USP activity specifications without enhanced PF4 binding. Preliminary PD/PK studies showed that this remodeled BI heparin behaved similarly to PI (USP) heparins. Cautionary notes are required for this study. The authors are aware that heparin, particularly crude unprocessed raw heparin, is under continued risk from illegal blending, adulteration, and processing. Remodeling of BI heparin for human use (alone or blended with other heparins) is not acceptable without appropriate regulatory oversight and approval. Additional in vivo evaluation of remodeled BI heparin is required prior to human use. Remodeled BI heparin is structurally different from PI heparin. Ultimately, the avoidance of animal-sourced heparins might be the only way to prevent future contamination and secure the heparin supply.

EXPERIMENTAL SECTION

Enzymatic Sulfation of Bovine Heparins. Two BI heparin samples (20 mg each) were treated in parallel either with both 6-OST-1, -3 and 3-OST-1 or with only 3-OST-1. The sulfation reaction was coupled with a PAPS recycling system that consisted of potassium 4-nitrophenyl sulfate (PNPS), PAPS, and AST-IV. The detailed reaction conditions are as follows: substrate concentration of 1 mg/mL, each enzyme concentration of 0.5 mg/mL for 50% slurry, PNPS and PAPS concentrations of 10 mM and 250 μM, respectively. All reactions were incubated at 37 °C for 40 h in 50 mM 2-(*N*-morpholino)ethanesulfonic acid (MES) buffer (pH 7.2). After the reaction was complete, the mixtures were filtered to remove enzyme resin, dialyzed using 5K Da molecular weight cutoff (MWCO) centrifugal membrane units with distilled water to remove PNP, PAPS, MES salt, and other small molecule impurities, and the retentates were lyophilized for further analysis. NMR and disaccharide compositional analysis confirmed the purity of each polysaccharide product to be ≥95%.

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■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jmedchem.7b01269.

Heparin sources, enzyme expression and immobilization, disaccharide and tetrasaccharide analysis, NMR spectroscopy, molecular weight and concentration determinations, surface plasmon resonance binding analysis, in vitro anticoagulant activity measurement, purity and quality analysis of modified bovine intestinal heparins, animal studies and determination of PD, PK studies, supplementary tables, and supplementary figures (PDF)

■ AUTHOR INFORMATION

Corresponding Author

*Phone: 1-518-276-3404. E-mail: linhar@rpi.edu.

ORCID

Fuming Zhang: 0000-0003-2803-3704

Robert J. Linhardt: 0000-0003-2219-5833

Author Contributions

L.F. carried out synthesis, purification, and NMR characterization of modified bovine heparins. P.D. prepared enzymes. K.L. and N.G. purified and immobilized enzymes. D.M. did disaccharide and Mw assays for the modified bovine heparins. M.H. prepared PAPS. Y.Y. and M.H. did anticoagulant assays. L.L. did tetrasaccharide analysis. F.Z. and J.Z. did SPR analysis. M.Y. and S.A.M. did animal studies, and J.S.D. helped plan the studies and write the manuscript. R.J.L. designed and supervised the project and wrote the manuscript.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

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■ ABBREVIATIONS USED

USP, United States Pharmacopeia; 6-OST-1 and -3, 6-*O*-sulfotransferase isoform-1 and -3; 3-OST, 3-*O*-sulfotransferase isoform-1; IdoA, iduronic acid; GlcN, glucosamine; S, sulfo; GlcA, glucuronic acid; GlcNAc, *N*-acetyl glucosamine; TriS, trisulfated; AT, antithrombin III; FIIa, factor IIa or thrombin; FXa, factor Xa; BSE, bovine spongiform encephalopathy; HIT, heparin-induced thrombocytopenia; PF4, platelet factor IV; API, active pharmaceutical ingredient; MBP, maltose binding protein; PNPS, *p*-nitrophenyl sulfate; PAPS, 3′-phosphoadenosine 5′-phosphosulfate; MWCO, molecular weight cutoff; AST-IV, arylsulfotransferase-IV; SAX, strong anion exchange; HPLC, high performance liquid chromatography; PI, porcine intestinal; BI, bovine intestinal; 1D, one-dimensional; DEPT, distortionless enhancement by polarization transfer; RPIP, reverse phase ion pairing; MS, mass spectral; SPR, surface plasmon resonance; PD, pharmacodynamics; PK, pharmacokinetics; AUC, area under the curve; MRM, multiple reaction monitoring; MES, 2-(*N*-morpholino)ethanesulfonic acid; GPC, gel permeation chromatography; M_W, weight-average molecular weight; EDTA, ethylenediaminetetraacetic acid

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