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Brief Article

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Non-Anticoagulant Low Molecular Weight Heparins for Pharmaceutical Applications

Yilan Ouyang,[†,‡] Yanlei Yu,[‡] Fuming Zhang,[‡] Jianle Chen,[‡] Xiaorui Han,[‡] Ke Xia,[‡] Yiming Yao,[§] Zhenqing Zhang,[*,†] and Robert J. Linhardt[*,‡]

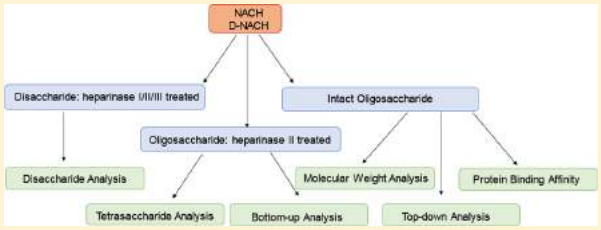
[†]Jiangsu Key Laboratory of Translational Research and Therapy for Neuro-Psycho-Diseases and College of Pharmaceutical Sciences, Soochow University, Suzhou, Jiangsu 215021, China

[‡]Center for Biotechnology and Interdisciplinary Studies, Rensselaer Polytechnic Institute, 110 8th Street, Troy, New York 12180, United States

[§]Suzhou Ronnsi Pharma Co. Ltd. Suzhou, 215125, China

Supporting Information

ABSTRACT: Heparin is a polypharmacological agent with anticoagulant activity. Periodate oxidation of the nonsulfated glucuronic acid residue results in non-anticoagulant heparin derivative (NACH) of reduced molecular weight. Similar treatment of a low molecular weight heparin, dalteparin, also removes its anticoagulant activity, affording a second heparin derivative (D-NACH). A full structural characterization of these two derivatives reveals their structural differences. SPR studies display their ability to bind to several important heparin-binding proteins, suggesting potential new therapeutic applications.



INTRODUCTION

Heparin is an *intravenous* anticoagulant drug that has been widely used since its discovery over a century ago.[1] While it is still the primary anticoagulant used in extracorporeal therapy and in surgery, low molecular weight heparins (LMWHs), prepared through its controlled chemical or enzymatic fragmentation and first introduced in the 1980s, are now routinely used *subcutaneously* for the treatment of deep vein thrombosis and other coagulation abnormalities.[2,3] Heparin's anticoagulant activity is primarily derived from its ability to bind to the serine protease inhibitor, antithrombin III (AT), and potentiate AT inhibition of blood coagulation proteases, such as thrombin.[2] While heparin is primarily thought of as an anticoagulant, it has numerous additional biological and pharmacological activities that, while known about for many years, have been infrequently exploited due to fear of hemorrhagic complications.[1,4,5] These secondary activities of heparin are the result of heparin's ability to tightly and selectively bind to and regulate a large number of heparin-binding proteins.[6,7] Heparin, a glycosaminoglycan (GAG) natural product, is extracted from then mast cell-rich tissues of food animals, such as porcine intestine, sheep intestine, and bovine intestine or lung.[8] The biological function of endogenous heparin is believed to be associated with their antiparasitic activity.[9]

The structure of heparin and the relationship of its structure to its biological and pharmacological activity have been extensively studied.[1,4,10] Heparin is a polydisperse polysaccharide with weight-average and number-average molecular weights (MW) of approximately 17.9 kDa and 13.9 kDa, respectively.[11] It is a homocopolymer of 1,4-glycosidically linked D-glucosamine (GlcN) and uronic acid (UA) residues. The structure of heparin is heterogeneous due to the differential modification of its saccharide residues during the later stages in its biosynthesis in the Golgi of mast cells.[12,13] The GlcN residues in heparin can be modified with *N*-acetyl or *N*-sulfo groups and *O*-sulfo groups at positions 3 and 6. The UA residues in heparin can be D-glucuronic acid (GlcA) or L-iduronic acid (IdoA) that can be modified with *O*-sulfo groups at position 2. The major disaccharide unit in heparin, corresponding to approximately 60−80% of its repeating units is →4) α-IdoA2S (1→4) α-GlcNS6S (1→, where S is sulfo.[1] The remaining sequences in heparin consist of less highly modified saccharide structures and include the AT pentasaccharide binding site of the structure →4) α-GlcNAc6S (1→4) β-GlcA (1→4) α-GlcNS3S6S (1→4) α-IdoAc2S (1→4) α-GlcNS6S (1→, where Ac is acetyl. Some structural variation in the AT pentasaccharide binding site is possible while retaining its anticoagulant activity.[14,15]

There have been a number of studies aimed at developing heparin-based therapeutics by removing heparin's prominent anticoagulant activity to obtain non-anticoagulant heparin (NACH).[16−18] Potential therapeutic applications include the treatment of cancer, atherosclerosis, and reproductive and infectious diseases. While heparin can be affinity fractionated

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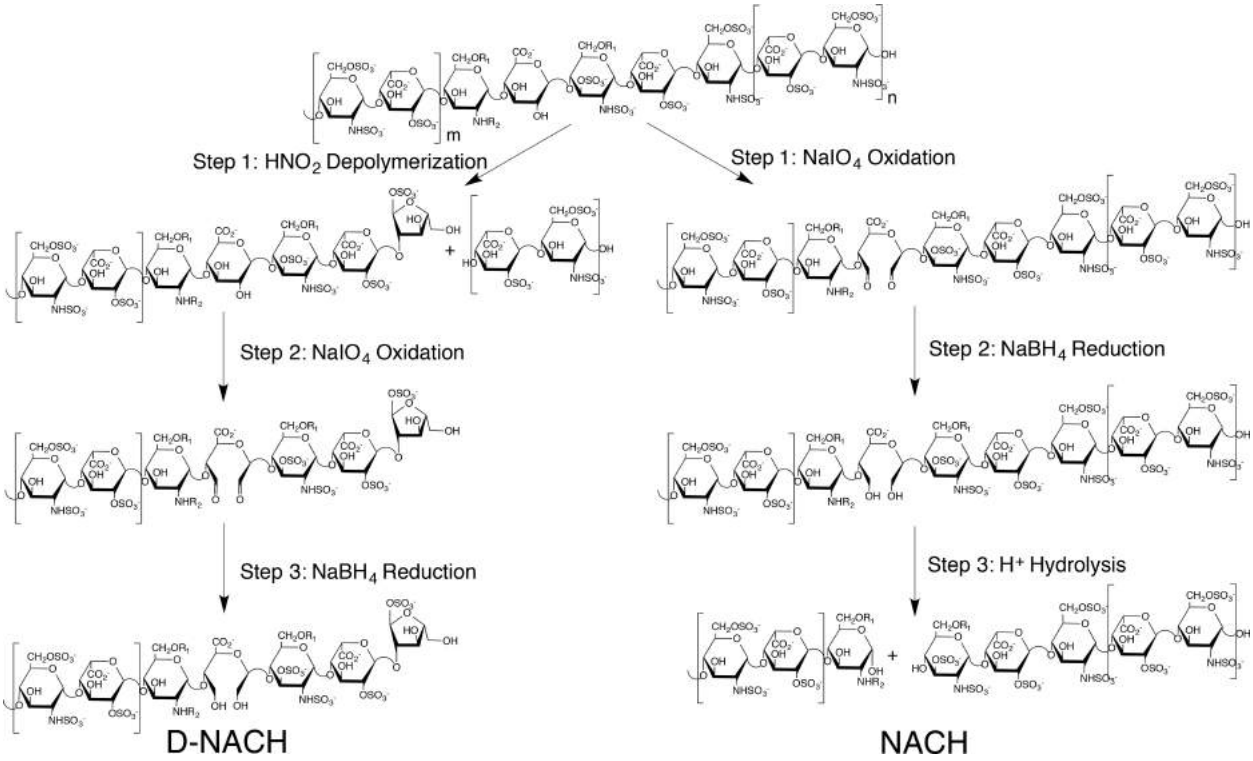

Figure 1. Structures and preparation process of non-anticoagulant low molecular weight heparins: $m + n = 16$ for MW 12 000; R_1 = R or HSO_3, R_2 = Ac or HSO_3

on a column containing resin-immobilized AT to prepare both high affinity anticoagulant heparin and low affinity NACH, this is a relatively inefficient and costly process.[19] Most NACHs are prepared through the selective chemical treatment of a heparin to remove its anticoagulant activity. The method most often applied is the periodate oxidation of heparin to selectively oxidize the vicinal hydroxyl groups present in heparin's nonsulfated UA residues, such as the GlcA residues present in heparin's AT-pentasaccharide binding site.[20−24] The current study uses sophisticated top-down and bottom-up high performance liquid chromatography−mass spectrometry (HPLC−MS) to examine the structure of NACH prepared from either porcine intestinal heparin or the low molecular weight heparin, dalteparin, and the ability of these two NACH preparations to selectively bind to heparin-binding proteins.

■ RESULTS AND DISCUSSION

NACH is most commonly prepared from heparin by treatment with sodium periodate to cleave the C2−C3 vicinal diol moiety in the unsulfated GlcA and IdoA residues present in this polysaccharide. The resulting ring-opened dialdehyde residue is then reduced with sodium borohydride and cleaved with acid to afford a NACH having of decreased molecular weight.[20,21,24] This literature approach was repeated using unfractionated porcine intestinal heparin affording NACH as well as the LMWH prepared through nitrous acid depolymerization of porcine intestinal heparin, dalteparin, as a starting material affording a dalteparin (D)-NACH (Figure 1). The average MWs of NACH and D-NACH, measured by gel permeation chromatography (GPC), were 5300 and 3870 Da, respectively (see Figure S1). As anticipated, D-NACH exhibited a lower MW than NACH. Their purities are all over 95% as determined by compositional analysis. The anti-Xa potency of NACH and D-NACH was measured as 1 and 6 U/mg, respectively, with BIOPHEN heparin anti-Xa (2 stages) kit. Theses values are much lower than those of heparin and enoxaparin (the most commonly prescribed LMWH), 196 and 94 U/mg, respectively.[25,26] One-dimensional ^{1}H NMR spectra of NACH and D-NACH were similar (see Figure S2), although the spectrum for D-NACH showed peaks associated with the characteristic anhydromannitol[24,27−30] afforded in the oxidative cleavage by nitrous acid used in the preparation of dalteparin.

While limited characterization of NACH using GPC and NMR had been previously reported,[20−24] recent advances by our laboratory and others now make more detailed structural EIC of disaccharides analyses including, disaccharide compositional analysis, bottom-up analysis, and top-down analysis of heparin products possible. USP heparin, prepared from porcine intestinal heparin, D-NACH, and NACH were each exhaustively treated with an equiunit mixture of heparin lyase I, II, and III and then subjected to disaccharide analysis using HPLC−MS.[31] The extracted ion chromatograms of each product mixture together with the structures of the resulting disaccharide products are provided in Figure 2. As expected, USP heparin showed disaccharide compositions that contained all eight of the disaccharide common to heparin (Figure 2 B). The content of trisulfated disaccharide (8) is about 64% (Table S1). In contrast, D-NACH and NACH primarily (>95%) comprised trisulfated disaccharide (8) and very small amounts of disaccharide NS6S (5) and NS2S (6) (Figure 2C,D). This is consistent with the destruction of all the

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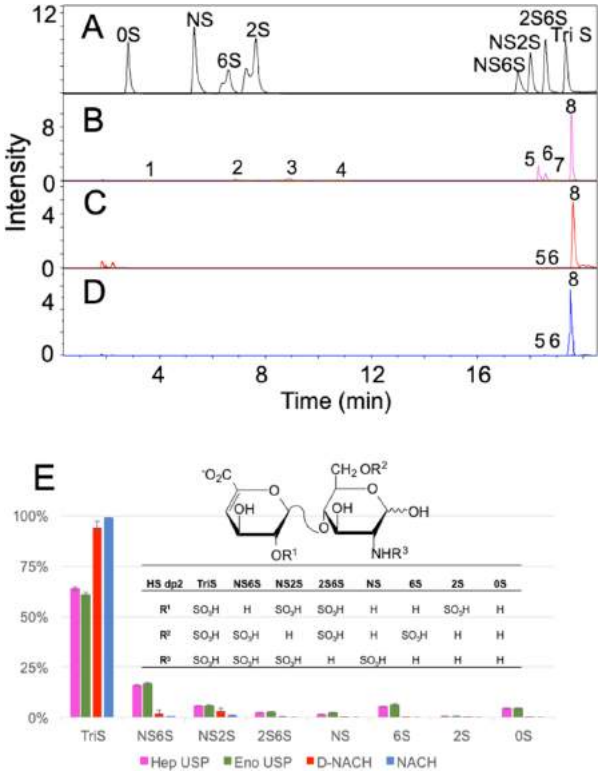

Figure 2. Disaccharide analysis of D-NACH and NACH using RPIP-MS. Structure of 8 major unsaturated disaccharides afforded on exhaustive heparin lyase depolymerization of heparin products (R^1 and R^2 can be H or SO_3^- and R^3). Disaccharides are TriS, NS6S, NS2S, NS, 6S, 2S, 0S. (A) Extracted-ion chromatogram (EIC) of 8 major unsaturated disaccharide standards. (B) EIC of disaccharides from porcine intestinal heparin (heparin USP). (C) EIC of disaccharides from D-NACH and (D) EIC of disaccharides from NACH. (E) Percentage of individual dp2 of heparin USP, enoxaparin USP, D-NACH, and NACH. Extracted-ion chromatograms in parts A–C are at intensity of 10^5, and part D is at intensity of 10^6.

unsulfated IdoA and GlcA residues by the periodate treatment used in the preparation of D-NACH and NACH (Figure 1).

Next, a bottom-up analysis was used to analyze D-NACH and NACH relying on treatment of these products with a single enzyme, heparin lyase II, followed by HPLC–MS analysis of all disaccharide and oligosaccharide products formed (Figure 3). This analysis shows that D-NACH and NACH afforded the same major peak [1,0,0,1,0,0,3] ([ΔUA; open-UA; UA; HexN; anhydromannitol; Ac; SO_3]), on heparin lyase II treatment, corresponding to the TriS disaccharides. A relatively complex mixture of products was afforded on heparin lyase treatment of D-NACH, due to the presence of the anhydromannitol residue resulting from the nitrous acid treatment used to prepare the dalteparin starting material (Table 1).[33,34] As expected, saturated uronic acid residues, arising from the original nonreducing residue in each chain, were more prevalent (6% compared to 1%) in the lower molecular weight D-NACH than in the NACH derived products.

The top-down approach directly analyzes the intact chains in a LMW heparin. D-NACH and NACH contained oligosaccharide chains from degree of polymerization (dp) 2 (disaccharide) to 14 (tetradecasaccharide) and dp2 through

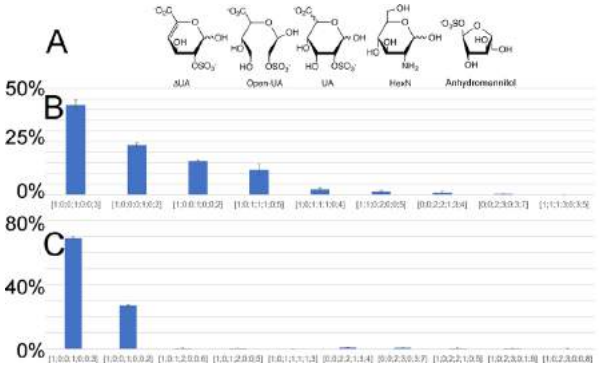

Figure 3. Bottom-up analysis for oligosaccharides of nonanticoagulant low molecular weight heparin digested by heparin lyase II. (A) Structures of saccharide units shown in the brackets on the *x*-axis of parts B and C [ΔUA; open-UA; UA; HexN; anhydromannitol; Ac; SO3]. (B) Relative percentage of oligosaccharides obtained from D-NACH on treatment with heparin lyase II. (C) Relative percentage of oligosaccharides obtained from NACH on treatment with heparin lyase II.

Table 1. Assignment and Quantitative Analysis of D-NACH and NACH Digested by Heparin Lyase II[a]

peak no.	assignment	D-NACH	NACH
1	unsaturated dp2-1S1Ac	ND	0.2%
2	saturated dp2-2S	0.3%	ND
3	unsaturated dp2-2S	0.9%	ND
4	unsaturated dp2-3S	0.2%	ND
5	unsaturated dp2-2S	4.7%	6.0%
6	unsaturated dp2-2S1Ac	ND	0.1%
7	unsaturated dp2-2S_anhydromannitol	7.4%	ND
8	unsaturated dp4-3S1Ac	0.5%	ND
9	saturated dp2-3S	4.6%	ND
10	saturated dp2-3S	ND	0.5%
11	saturated dp2-3S	ND	0.2%
12	unsaturated dp2-3S	42.7%	91.7%
13	unsaturated dp2-3S	4.3%	ND
14	unsaturated dp4-4S1Ac	1.0%	ND
15	unsaturated dp4-4S_anhydromannitol	2.8%	ND
16	unsaturated dp4-4S	ND	0.1%
17	unsaturated dp4-4S_anhydromannitol	2.2%	ND
18	unsaturated dp4-5S1Ac	1.0%	ND
19	saturated dp4-5S_anhydromannitol	1.0%	ND
20	unsaturated dp4-5S_anhydromannitol	2.7%	ND
21	unsaturated dp4-5S_anhydromannitol	23.6%	ND
22	unsaturated dp4-5S	ND	0.4%
23	unsaturated dp4-6S	ND	0.1%
24	unsaturated dp4-6S	ND	0.3%

[a]ND: not detected.

dp16 (hexadecasaccharide), respectively (Figure 4). D-NACH and NACH contained oligosaccharides of even and odd chain lengths, although the even chains were most abundant. Of the [0;1;2;3;0;0;8]/[0;0;3;2;1;0;8] and [0;1;2;3;0;1;7] chain structures identified in D-NACH and NACH, respectively, both samples showed process signatures associated with periodate treatment, ring open sugars, but only D-NACH showed ring contracted anhydromannitol sugars associated with nitrous acid treatment. Smaller oligosaccharides contain less ring open sugar as milder acid hydrolysis cut more opened sugar ring out.

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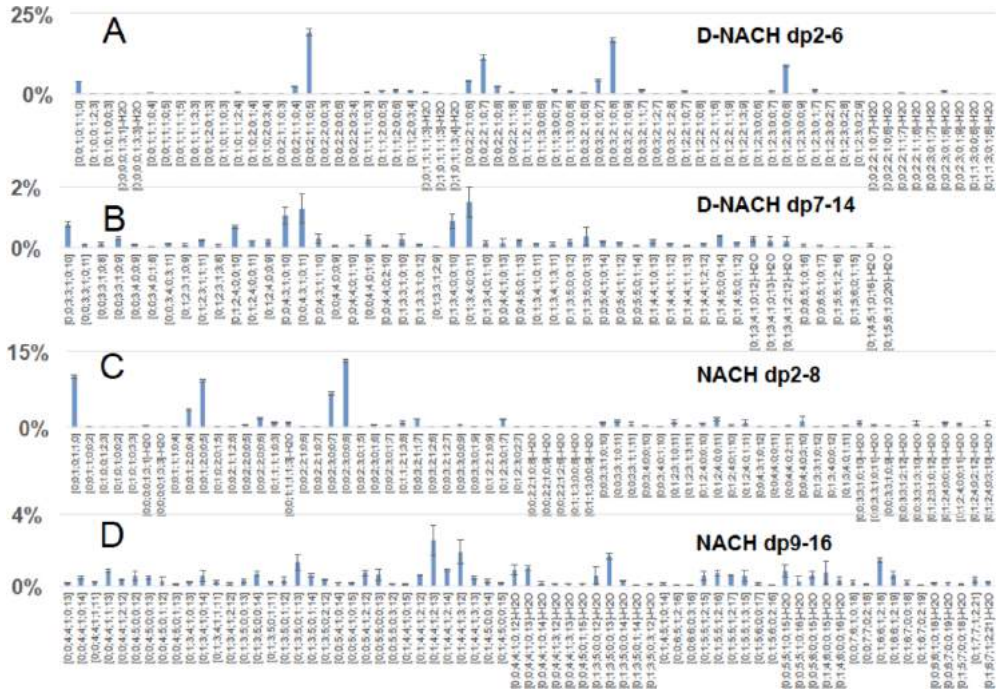

Figure 4. Top-down analysis for intact oligosaccharides of non-anticoagulant low molecular weight heparins using HILIC−MS. (A−D) Relatively quantitative of D-NACH dp2−6, D-NACH dp7−14, NACH dp2−8, NACH dp9−16, respectively. Oligosaccharide compositions are given as [ΔUA; ppen-UA; UA; HexN; anhydromannitol; Ac; SO_3].

The binding of D-NACH and NACH to a selection of biologically and therapeutically relevant heparin-binding proteins, including AT, FGF2, HGF, and TGFβ1, was next compared to anticoagulant unfractionated USP porcine intestinal heparin and anticoagulant LMWH, enoxaparin using competition SPR (Figure 5). First, we examined the ability of D-NACH and NACH to compete with surface-immobilized heparin for binding to AT. While USP heparin and enoxaparin (positive controls) effectively competed with surface-immobilized heparin for AT binding, D-NACH and NACH failed to compete. This result confirmed that periodate treatment completely destroyed the AT pentasaccharide binding sites in these samples. D-NACH showed the tightest binding to HGF and FGF2, while NACH showed the tightest binding to TGFβ1.[32] The binding of D-NACH and NACH to these three heparin-binding proteins was stronger than the binding of either of the anticoagulant heparins, USP heparin and enoxaparin. This is consistent with the higher content of TriS in D-NACH and NACH and the known preference of HGF, FGF2, and TGFβ1 for the more highly sulfated domain in heparin. It was possible to extract quantitative binding constants from the competition SPR data (Table 2, Figure S4). It is particularly noteworthy that D-NACH bound to HGF with picomolar affinity.

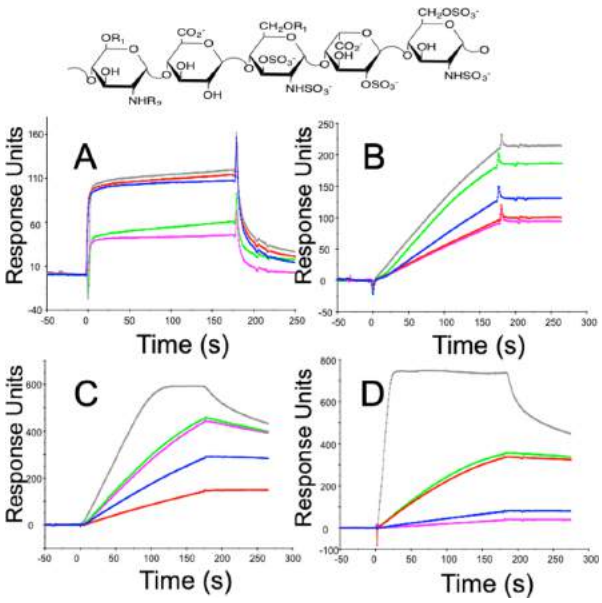

Figure 5. Competitive binding of soluble heparin samples with surface immobilized porcine intestinal heparin for protein analytes determined using surface plasmon resonance. The anti-thrombin binding site is shown at the top where R_1 is H or sulfo and R_2 is acetyl or sulfo. Panels A−D correspond to SPR solution competition experiments for AT, HGF, FGF2, TGFβ: negative control (gray), positive control USP heparin (magenta), enoxaparin (green), D-NACH (red), and NACH (blue) lines.

■ CONCLUSION

The current study shows the preparation of two non-anticoagulant derivatives, D-NACH and NACH from two different commercial heparin products. Both of these non-anticoagulant derivatives were devoid of anticoagulant activity and neither bound to AT. D-NACH and NACH were both enriched in highly sulfated heparin sequences with TriS disaccharide corresponding to >95% of their structure. Top-down and bottom-up analysis confirmed the structural differences between these two non-anticoagulant derivatives,

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Table 2. Summary of Kinetic of D-NACH and FGF2, HGF, TGFβ1

interaction	k_a (1/ms)	K_d (1/s)	K_D (M)
FGF2/D-NACH	4.0×10^7 ($\pm 9.4 \times 10^5$)	0.11 ($\pm 2.9 \times 10^{-3}$)	2.7×10^{-9}
TGF1/D-NACH	2.9×10^4 ($\pm 1.0 \times 10^3$)	0.039 ($\pm 6.8 \times 10^{-4}$)	1.4×10^{-6}
HGF/D-NACH	1.5×10^6 ($\pm 4.2 \times 10^4$)	1.5×10^{-4} ($\pm 1.7 \times 10^{-5}$)	1.0×10^{-10}

with D-NACH being considerably more structurally complex than NACH. Both D-NACH and NACH bound with high affinity to heparin-binding proteins preferring highly sulfated heparin domains and D-NACH bound with extremely high affinity to HGF.

EXPERIMENTAL SECTION

Non-Anticoagulant Low Molecular Weight Heparins Preparation. NACH was prepared from heparin by periodate oxidation as described in our previous work.[20] Briefly, 1 g of heparin sodium (>95% purity) aqueous solution (8.75 mL, pH 5.0) was added to freshly prepared 140 mM $NaIO_4$ solution (25 mL, pH 5.0) in a single portion with stirring. The oxidation was carried out at 4 °C for 24 h in the dark before the solution was desalted using 3 kDa cutoff spin membrane. $NaBH_4$ (50 mg) was added to the reaction to reduce the generated aldehydes. The pH of the reaction solution was adjusted to 4.0 with stirring for additional 15 min to remove the opened sugar ring (hydrolysis step). Diluted NaOH solution was used to adjust the pH to 7.0. The NACH was precipitated by adding ethanol to 70−80% (v/v). After desalting, 0.45g of NACH was obtained. D-NACH was kindly provided by Ronnsi Pharma. LMWH (>95% purity) was prepared from heparin sodium by nitrous acid depolymerization. This LMWH was oxidized by $NaIO_4$ and reduced by $NaBH_4$ following exactly the same reaction procedures used to prepare NACH but without hydrolysis. The preparation processes are shown in Figure 1. Peak areas are proportional to concentration in GPC. GPC and disaccharide analyses confirmed the >95% purity of D-NACH, and NACH using USP enoxaparin was used as a standard.

Disaccharide Analysis with Online Reversed Phase Ion Pair (RPIP) LC−MS. Disaccharides of NACHs were prepared by exhaustive digestion using a mixture of heparin lyases I, II, III[35] (each lyase 0.1 IU/1.0 mg sample) in digestion buffer (50 mM ammonium acetate containing 2 mM calcium chloride adjusted to pH 7.0) at 37 °C for overnight. Enzymatic digestion was terminated by boiling samples for 10 min and removing the denatured enzymes with centrifugation at 13 400g for 10 min. Supernatants were freeze-dried and redissolved to 1 μg/μL for LC−MS analysis. Online RPIP LC tandem Iontrap MS on an Agilent 1200 LC-Iontrap was used to analyze disaccharides from NACHs. LC was performed on an Agilent 1200 LC system at 45 °C using an Agilent Poroshell 120 EC-C18 (2.7 μm, 2.1 mm × 100 mm) column. Mobile phase A (MPA) was 38 mM ammonium acetate and 12 mM tributylammonium acetate in 15% acetonitrile aqueous solution (pH = 6.5), and the mobile phase B (MPB) was 38 mM ammonium acetate and 12 mM tributylammonium acetate in 65% acetonitrile aqueous solution (pH = 6.5). The mobile phase passed through the column at a flow rate of 100 μL/min and gradient from 2% to 40% MPB in 25 min, then rose to 60% MPB in following 0.2 min, and a 5 min flow of 60% MPB was applied to elute all compounds. The MS parameters in negative mode were as follows: scan range 300−700 m/z, nebulizer 40 psi, dry gas 8 L/min, dry temperature 350 C, capillary 1 nA.[31]

Tetrasaccharide Analysis with Online RPIP LC−MS. Tetrasaccharides from NACHs were obtained by digestion only using heparin lyase II (0.1 IU/1.0 mg sample) in digestion buffer at 37 °C for 2 h.[36] LC−MS parameters and condition were the same as for the disaccharide analysis described above, except flow rate was 120 μL/min and mobile phase gradient was from 2% to 30% MPB in 40 min, then rose to 60% MPB in following 15 min.

Bottom-Up Analysis for Oligosaccharides Digested by Heparin Lyase II. Oligosaccharides of NACHs were obtained same as described above in tetrasaccharide analysis. HILIC LC tandem ESI-LTQ-Orbitrap-FTMS (Thermo Fisher Scientific) was used to analyze digested NACHs. A Luna HILIC column (2.0 mm × 50 mm, 200 Å, Phenomenex, Torrance, CA) was used to separate. Mobile phase A (MPA) was 5 mM ammonium acetate prepared with HPLC grade water. MPB was 5 mM ammonium acetate prepared in 98% HPLC grade acetonitrile with 2% of HPLC grade water. The gradient was used from 5% A to 70% A in 7 min, then reset to 5% A at a flow rate of 250 μL/min after 8.0 μL digested NACHs (2.0 μg/μL) injected. The source parameters for FTMS detection were optimized to minimize the in-source fragmentation and sulfate loss and maximize the signal/noise in the negative-ion mode.[37] The source parameters included 4.2 kV spray voltage, −40 V capillary voltage, −50 V tube lens voltage, 275 °C capillary temperature, 30 L/min sheath flow rate, and 6 L/min auxiliary gas flow. Better than 3 ppm of mass accuracy was obtained routinely by external calibration of mass spectra. All FT mass spectra were acquired at a resolution 60 000 with 400−2000 Da mass range.

Top-Down Analysis for Intact Oligosaccharides of NACHs. LC−MS conditions and parameters are the same with bottom-up analysis described above except Luna HILIC column (2.0 × 150 mm^2, 200 Å, Phenomenex, Torrance, CA) was used and gradient from 10% A to 35% A over 40 min at a flow rate of 150 μL/min.[38]

Data Processing. Charge deconvolution was autoprocessed by DeconTools software. NACHs structural assignment was done by automatic processing using GlycReSoft 2.0 software, which established NACHs' structural features, which involved 2,5-anhydromannitol affording the oxidative cleavage by nitrous acid, glycol-split uronic acid obtained by sodium periodate oxidation used in the preparation of D-NACH (see Figure 1), and odd saccharides affording the preparation of NACH.[20,34,39−41] (http://code.google.com/p/glycresoft/downloads/list). For automatic processing, GlycReSoft 2.0 parameters were set as follows: Minimum Number of Scans, 1; Molecular Weight Lower Boundary, 150 Da; Molecular Weight Upper Boundary, 6000 Da; Mass Shift, ammonium; Match Error (E_M), 5.0 ppm; Grouping Error (E_G), 80 ppm; Adduct Tolerance (E_A), 5.0 ppm. In order to simplify database, [ΔUA; open-UA; UA; HexN; anhydromannitol; Ac; SO_3] presents the number of unsaturated uronic acid, glycol-split uronic acid, saturated uronic acid, glucosamine, anhydromannitol, acetyl, and sulfate groups, respectively.

Surface Plasmon Resonance (SPR) Competition Mode. Binding between NACHs and proteins was measured by SPR utilizing a competitive inhibition strategy with a Biacore 3000 (GE, Uppsala, Sweden). It is a rapid and real-time method to evaluate the binding affinity between analytes and proteins.[42] Unfractionated heparin standard or D-NACH was biotinylated and immobilized on three channels of the SA chip. The intensity should have no less than 200 resonance unit (RU) increase to make sure it was immobilized successfully. One flow cell containing immobilized biotin served as a control. Each analyte prepared at 20 μg/mL was premixed with AT solution (323 nM/L) in running buffer (HBS-EP). The running buffer was eluted at a flow rate of 30 μL/min at 25 °C. The response was monitored in real time. Before every analysis, 30 μL of 2 M NaCl was used to regenerate the chip surface and 30 μL of running buffer (HBS-EP) was used to equilibrate the chip.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jmedchem.8b01551.

Materials, measurement of molecular weight of D-NACH and NACH, interaction analyses, refractive index profile (Figure S1); one-dimensional ^{1}H NMR of D-NACH and NACH (Figure S2); profile comparison of

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non-anticoagulant low molecular weight heparins treated by heparinase II (Figure S3); total ion chromatograms (TICs) of D-NACH and NACH (Figures S4 and S5); SPR sensorgrams of proteins with D-NACH interaction (Figure S6); disaccharide percentage of D-NACH and NACH digested by heparinases I/II/III mixture using RPIP-MS (Table S1); summary of kinetics between heparin, enoxaparin and FGF2, HGF, TGFβ1 (Table S2) (PDF)

AUTHOR INFORMATION

Corresponding Authors

*Z.Z.: e-mail, z_zhang@suda.edu.cn; fax, +86-512-65882593.
*R.J.L.: e-mail, linhar@rpi.edu; phone, +1-518-276-3405; fax, +1-518-276-3405.

ORCID

Fuming Zhang: 0000-0003-2803-3704
Robert J. Linhardt: 0000-0003-2219-5833

Author Contributions

Y.O. carried out preparation of NACH, disaccharide analysis, tetrasaccharide analysis, and bottom-up, top-down, and SPR analysis. Y.Yu measured molecular weight of NACHs by GPC and anti-Xa activity. F.Z. did SPR data process. J.C. did 1D ^{1}H NMR analysis of NACHs. X.H. helped typesetting manuscript. K.X. helped with top-down analysis. Y.Yao prepared the D-NACH sample. R.J.L. and Z.Z. designed and supervised the project and wrote the manuscript.

Notes

The authors declare the following competing financial interest(s): Yiming Yao is an employee of Suzhou Ronnsi Pharma, a heparin manufacturer.

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ABBREVIATIONS USED

AM.ol, 2, 5-anhydro-D-mannitol; AT, antithrombin III; NACH, non-anticoagulant heparin; D-NACH, dalteparin-similar non-anticoagulant heparin; GAG, glycosaminoglycan; UA, uronic acid; GlcN, glucosamine; GlcA, glucuronic acid; IdoA, iduronic acid; HPLC−MS, high performance liquid chromatography−mass spectrometry; GPC, gel permeation chromatography; NMR, nuclear magnetic resonance; Ac, acetyl group; SO_3, sulfate group; FGF2, fibroblast growth factor 2; HGF, hepatocyte growth factor; TGF β1, transforming growth factor β1; SPR, surface plasmon resonance; EDTA, ethylenediaminetetraacetic acid; RPIP, reverse phase ion pairing; MPA, mobile phase A; MPB, mobile phase B; HILIC, hydrophilic interaction chromatography; ESI, electrospray ionization; LTQ, linear trap mass spectrometer; FTMS, Fourier transform mass spectrometry; RU, resonance unit

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