Published in final edited form as:

Carbohydr Res. 2010 January 26; 345(2): 250–256. doi:10.1016/j.carres.2009.10.024.

Preparation and characterization of ^{15}N -enriched, size-defined heparan sulfate precursor oligosaccharides

Crystal Sigulinsky^a, Ponnusamy Babu^b, Xylophone V. Victor^b, and Balagurunathan Kuberan^{a,b,c,*}

^aInterdepartmental Program in Neuroscience, University of Utah, Salt Lake City, Utah, 84112, USA

^bDepartment of Medicinal Chemistry, University of Utah, Salt Lake City, Utah, 84112, USA

^cDepartment of Bioengineering, University of Utah, Salt Lake City, Utah, 84112, USA

Abstract

We report the preparation of size-defined [^{15}N]N-acetylheparosan oligosaccharides from *Escherichia coli*-derived ^{15}N -enriched N-acetylheparosan. Optimized growth conditions of *E. coli* in minimal media containing $^{15}\text{NH}_4\text{Cl}$ yielded [^{15}N]N-acetylheparosan on a preparative scale. Depolymerization of [^{15}N]N-acetylheparosan by heparitinase I yielded resolvable, even-numbered oligosaccharides ranging from disaccharide to icosaccharide. Anion-exchange chromatography-assisted fractionation afforded size-defined [^{15}N]N-acetylheparosan oligosaccharides identifiable by ESI-TOFMS. These isotopically labeled oligosaccharides will prove to be valuable research tools for the chemoenzymatic synthesis of heparin and heparan sulfate oligosaccharides and for the study of their structural biology.

Keywords

N-Acetylheparosan; Glycosaminoglycan (GAG); Heparan Sulfate oligosaccharide; Heparin; K5 polysaccharide; Heparitinase; Isotope labeling; Mass spectrometry; Nuclear Magnetic Resonance (NMR) spectrometry

1. Introduction

Heparin and heparan sulfate (HS) are sulfated, linear polysaccharides of significant biological interest. Heparin, a primary component of the secretory granules of mast cells involved in immune response, has been exploited therapeutically for its anticoagulant activity.^{1, 2} HS localizes to both the cell surface and extracellular matrix of most animal tissues. HS has been implicated in a diverse array of physiological and pathological functions, including participation in cell–cell signaling and cell–matrix interactions that regulate numerous developmental, homeostatic and disease processes.^{3, 4} HS also participates in blood coagulation, inflammation, wound healing, lipid metabolism and host defense against pathogenic infection, among others.⁵ These biological functions of heparin and HS are

© 2009 Elsevier Ltd. All rights reserved.

*Correspondence to: Kuberan Balagurunathan Address: 30 South 2000 East, Skaggs #307, Salt Lake City, UT 84112, USA Telephone: 801-587-9474 Fax: 801-585-9119 KUBY@pharm.utah.edu.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

mediated by their specific interactions with proteins involved in these various biological processes.⁶

Heparin and HS, both members of the glycosaminoglycan (GAG) family, are highly anionic heterogeneous molecules composed of repeating disaccharide units of glucosamine and glucuronic/iduronic acid residues. During heparin and HS biosynthesis, a number of enzymes modify individual residues of the nascent growing chain.⁷ The resulting complexity of the heparin/HS chain confers specificity for high affinity interactions with various proteins, as binding is mediated by both ionic interactions and non-ionic hydrogen bonding between specific amino acids of the protein and particular domains/microstructures of heparin or HS.⁶⁻⁸ Altered biological activity of heparin/HS binding proteins has been shown to require only specific oligosaccharide domains of heparin and HS chains. However, identification of the responsible microstructure and organization of functional groups within the microstructure is largely lacking, except for antithrombin III and a few other proteins.

Nuclear magnetic resonance (NMR) spectroscopy provides powerful structural and conformational information useful in identifying the precise contact points between interacting molecules. Unfortunately, NMR studies of biologically active heparin and HS have been hampered by extensive variability in length and microstructure of animal-derived and cellular-derived heparin and HS chains.^{9, 10} Recently, isotopic labeling of chemoenzymatically synthesized heparin and heparin precursor has allowed unambiguous assignment of proton peaks in NMR spectra by overcoming the extensive proton spectral overlap.¹¹ However, these analyses are restricted to the principal disaccharide unit comprising the heparin/HS chain, and rare modifications are undetectable. Preparation and use of isotope-enriched HS disaccharide units has also been reported,¹² but larger, biologically relevant structural domain information is lost. Consequently, NMR analyses on isotope-labeled oligosaccharides will provide the necessary structural information on an appropriate scale for defining heparin/HS microstructure and structure–function relationships.¹³

N-acetylheparosan, isolated from *Escherichia coli* K5 strain, has been used as a precursor to carry out chemical and enzymatic modifications and to prepare biologically active molecules, including heparin anticoagulants, over the last two decades.¹⁴⁻²⁰ This article reports for the first time the generation and characterization of ¹⁵N-labeled, size-defined oligosaccharides, up to icosaccharide, derived from the heparin/HS precursor *N*-acetylheparosan. These [¹⁵N]*N*-acetylheparosan oligosaccharides will improve microstructure determination of heparin and HS chains, facilitating future structure–function studies with heparin/HS binding proteins.

2. Results and discussion

N-acetylheparosan is the biosynthetic precursor of heparin and HS, an unmodified linear chain comprised of repeating *N*-acetylglucosamine–glucuronic acid (GlcNAc–GlcA) disaccharide units.²¹ In order to prepare ¹⁵N-enriched, size-defined oligosaccharides for the study of heparin and HS structures and their interactions with protein ligands, *N*-acetylheparosan was enriched with ¹⁵N and depolymerized using heparitinase I (Figure 1). The resulting size-defined HS precursor oligosaccharides were fractionated to homogeneity by HPLC and then subjected to ESI-TOFMS analyses to confirm structures.

2.1. Preparation of isotopically labeled *N*-acetylheparosan

E. coli strain K5 naturally synthesizes a capsular polysaccharide (K5 polysaccharide) that structurally resembles the nascent unmodified HS precursor, *N*-acetylheparosan.²¹ Therefore, [¹⁵N]*N*-acetylheparosan was prepared from *E. coli* strain K5 grown in minimal media containing ¹⁵NH₄Cl as the principal nitrogen source. Quantification of the isolated K5

polysaccharide by the carbazole assay revealed that the described optimization of *E. coli* strain K5 growth conditions afforded yields of 150 mg [^{15}N]*N*-acetylheparosan per liter of culture.

Identification of the isolated K5 product as *N*-acetylheparosan was confirmed by NMR spectroscopy and ESI-TOFMS. The ^1H NMR spectrum obtained for the isolated K5 polysaccharide product (Figure 2A) is consistent with previous reports for *N*-acetylheparosan isolated from *E. coli* strain K5, given differences in NMR experimentation.^{11, 19} The characteristic anomeric protons on GlcNAc (5.244 ppm) and GlcA (4.375 ppm) and methyl protons of the acetamido group on GlcNAc (1.975 ppm) are observed in the ^1H NMR spectrum and are consistent with the expected chemical shifts. ESI-TOFMS analysis of the disaccharides derived from exhaustive digestion of the K5 polysaccharide revealed the presence of a significant peak at m/z 379 in the total-ion chromatogram (TIC), corresponding to the predominant ^{15}N -enriched unsaturated disaccharide, $\Delta\text{UA}-[^{15}\text{N}]\text{GlcNAc}$ (Figure 3A).

^{15}N incorporation in the isolated *N*-acetylheparosan product was also assessed by NMR spectroscopy in addition to ESI-TOFMS analysis at the disaccharide level. The decoupled ^1H NMR spectrum (Figure 2A) reveals a singlet at 8.21 ppm, corresponding to the amide proton of the acetamido group of GlcNAc residues. This singlet at 8.21 ppm is largely split when recorded in the absence of ^{15}N decoupling (Figure 2B), indicating successful incorporation of ^{15}N into *N*-acetylheparosan. Detection of a minor peak at 8.21 ppm in the absence of decoupling (asterisk, Figure 2B), which corresponds to ^{14}N , reveals incomplete ^{15}N incorporation and is furthermore consistent with the 94% isotopic purity of [^{15}N]*N*-acetylheparosan calculated by ESI-TOFMS. Additional NMR analysis was conducted on isolated [^{15}N]*N*-acetylheparosan, including 2-D HSQC analysis (Figure 2C), which reveals a single correlation at ^1H , 8.21 and ^{15}N , 123.3 ppm. Disaccharide analysis of the isolated *N*-acetylheparosan using ESI-TOFMS revealed peaks at m/z 378 and m/z 379 in the TIC, corresponding to unlabeled (^{14}N) and ^{15}N -labeled $\Delta\text{UA}-\text{GlcNAc}$, respectively. Based on the relative abundance of m/z 378 and 379 peaks, the isotopic purity of the isolated [^{15}N]*N*-acetylheparosan product was assessed and determined to be 94%. Thus, the described preparation of [^{15}N]*N*-acetylheparosan from *E. coli* strain K5 grown in minimal media containing $^{15}\text{NH}_4\text{Cl}$ affords significant ^{15}N -enrichment, as compared to *E. coli* grown in LB broth (Figure 3B).

2.2. Preparation of size-defined [^{15}N]*N*-acetylheparosan oligosaccharides

Enzymatic digestion of *N*-acetylheparosan is routinely performed to generate oligosaccharides and disaccharides for fine structural analyses. Heparitinase I cleaves *N*-acetylglucosaminyl α -(1 $\rightarrow$ 4)-glucuronic acid (GlcNAc- α -(1 $\rightarrow$ 4)-GlcA) linkages, removing one glucuronic acid- β -(1 $\rightarrow$ 4)-*N*-acetylglucosamine (GlcA- β -(1 $\rightarrow$ 4)-GlcNAc) unit in an elimination reaction.²² Under appropriate conditions with heparitinase I, *N*-acetylheparosan can be partially digested, yielding a series of even-numbered oligosaccharides (Figure 1).^{23, 24}

Partial digestion of [^{15}N]*N*-acetylheparosan under the described conditions yielded the desired size-defined, isotope-labeled oligosaccharides. Anion-exchange chromatography of the partially digested [^{15}N]*N*-acetylheparosan revealed a series of highly resolved even-numbered oligosaccharides, ranging in size from 2- to 22-mers (Figure 4). Tetracosaccharide (dp24) and hexacosaccharide (dp26) were also detected, albeit in low abundance (Figure 4). Fractionation by preparative-scale anion-exchange chromatography permitted purification of the oligosaccharides of various sizes to homogeneity. Fractions containing oligosaccharides of various sizes were concentrated, desalted and further characterized by ESI-TOFMS.

2.3. ESI-TOFMS analysis

Negative-ion ESI-TOFMS spectra were obtained for each size-defined [^{15}N]*N*-acetylheparosan oligosaccharide to confirm its structure (Figures 5 and 6). The mass spectra (Figures 5 and 6) show the molecular ions for the most abundant charge state of each resolved structure ranging in size from DP 2- to DP 20-mers. Although each oligosaccharide produces several multiply charged molecular ions under ESI conditions, the ESI-TOFMS analysis allowed identification of the predominant charge state of each oligosaccharide. Comparison of ^{15}N -labeled and unlabeled (^{14}N) DP 10- and DP 20 heparosan oligosaccharides clearly revealed the expected shift in mass values that result from differences in the mass-to-charge ratios of the oligosaccharides due to ^{15}N incorporation (Figure 6). The calculated and observed m/z values for all size-defined ^{15}N -enriched heparosan oligosaccharides are summarized in Table 1.

3. Concluding remarks

Structure–function relationships for heparin and HS will require detailed mapping of heparin/HS microstructure at the oligosaccharide level. Such efforts have begun, as unlabeled *N*-acetylheparosan oligosaccharides have been isolated and subjected to NMR analysis. While use of size-uniform oligosaccharides reveals additional fine structure information in the NMR spectrum, unambiguous assignment of proton peaks requires heteronuclear NMR analysis. Due to low natural abundance of nuclei with a net spin, isotope-enrichment is required for enhanced sensitivity. Thus, the present study describes the generation and characterization of such size-defined ^{15}N -enriched oligosaccharides for use in microstructure mapping and future binding affinity studies with heparin/HS binding proteins. The optimized methods described here allow for the preparative scale production of [^{15}N]*N*-acetylheparosan oligosaccharides necessary for structural, biological and bioanalytical studies.

There are numerous applications for these size-defined [^{15}N]*N*-acetylheparosan oligosaccharides. Currently, there is no means of accurately quantifying oligosaccharides during mass spectrometric analyses due to functional domain effects on ion intensity.

Use of isotopically labeled disaccharides as internal standards has greatly improved quantification of heparin/HS disaccharide composition.¹² In a similar manner, one can anticipate the indispensable utility of these ^{15}N -enriched HS precursor oligosaccharides. Additionally, the size-defined [^{15}N]*N*-acetylheparosan oligosaccharides will prove valuable as substrates for use in studying the mechanism(s) of heparin and HS biosynthesis. Furthermore, defined chemical and enzymatic modifications of [^{15}N]*N*-acetylheparosan oligosaccharides will allow identification of the critical functional groups conferring specific affinity for various heparin and HS binding proteins, and therefore, the microstructure necessary for biological activity.

4. Experimental

4.1. Materials

^{15}N -enriched *N*-acetylheparosan was produced from *E. coli* strain K5 [O10:K5 (L):H4] (ATCC). Detailed conditions for the bacterial culture, isolation and purification of [^{15}N]*N*-acetylheparosan are described below. Enzymes used in this study included: Solid pronase from *Streptomyces griseus* (Sigma–Aldrich), Mung Bean Nuclease (Sigma–Aldrich), and Heparitinase I from *Flavobacterium heparinum* was cloned and expressed as described before.²² All other chemicals, including stable isotopes and mass spectrometry grade solvents, were obtained from Sigma–Aldrich. Water was purified using a Milli-Q filtration apparatus (Millipore Co., Bedford, MA, USA).

4.2. Preparation of [^{15}N]N-acetylheparosan

4.2.1 Production of ^{15}N -enriched N-acetylheparosan and *E. coli* growth

conditions—[^{15}N]N-acetylheparosan was prepared from cultures of *E. coli* strain K5 grown in minimal media containing $^{15}\text{NH}_4\text{Cl}$. Optimized growth of *E. coli* strain K5 in minimal media was accomplished using bacterial starter cultures grown in LB broth and supplementation of minimal media with glycerol. Specifically, bacterial starter cultures were grown in either 10 mL of LB broth for 8 h or 50 mL of LB broth for 20 h and incubated at 37 °C with shaking (250 rpm). Starter culture (10 mL) was used to inoculate 0.5 L of minimal media containing 2 g/L $^{15}\text{NH}_4\text{Cl}$ as the principle nitrogen source. Optimal growth was achieved by including 40 mL of pre-autoclaved 50% (v/v) glycerol solution per liter of minimal media (1% v/v glycerol, working concentration). Incubation was carried out at 37 °C in a 2-L Erlenmeyer flask with shaking (250 rpm) for 24 h, at which point growth began to plateau and the cells and medium were autoclaved.

4.2.2 Isolation & purification of ^{15}N -enriched N-acetylheparosan— ^{15}N -enriched N-acetylheparosan was isolated from *E. coli* strain K5 cultures according to the procedure reported by Fritz and colleagues with minor modifications.²⁵ Specifically, following adjustment of the autoclaved medium to 0.1 M NaOH and incubation for 15 min at room temperature, the medium was adjusted to pH 6.5–7.0 with HOAc. The medium was further adjusted to 1 mM CaCl_2 and subjected to protease treatment for 24 h while being incubated at 37 °C with minimal shaking (50 rpm). For optimal results, the protease (solid pronase from *Streptomyces griseus*, 1.0 mg/mL) was added twice in 12-h intervals. Insoluble material was removed by centrifugation (8000 rpm, 30 min), and the supernatant was diluted to 3 times the original volume with deionized water. Diluted supernatant was applied to a previously equilibrated DEAE-Sephacel column. Column equilibration was accomplished by 3 washes (3 column volumes/wash) with equilibration buffer (0.1 M NaCl in 20 mM NaOAc, pH 6) while performing vacuum filtration. Following washing with 15–20 column volumes of equilibration buffer, the loaded heparosan product was eluted with 6 column-volumes of 0.5 M NaCl in 20 mM NaOAc (pH 6). The eluate was adjusted to 1 M NaCl, and 4 volumes of pure EtOH were added to precipitate the polysaccharide. After 24 h at 4 °C, the resulting [^{15}N] N-acetylheparosan precipitate was recovered by centrifugation (8000 rpm, 30 min, 4 °C) and removal of the supernatant, then it was allowed to air-dry overnight. Purification of the isolated ^{15}N -enriched K5 polysaccharide precipitate was accomplished by sequential nuclease and dialysis treatments. Briefly, the dried precipitate was resuspended in water and subjected to nuclease treatment for 24 h using Mung Bean Nuclease (95 units/0.1 L solution, twice with a 12-h interval) and incubation at 37 °C with minimal rotation (50 rpm). This solution was transferred to Spectra/Por 7 dialysis tubing (1000 MW cutoff, 29 mm diameter, Spectrum Laboratories, Inc., Rancho Dominguez, USA) to remove residual salts. The final [^{15}N]N-acetylheparosan product was recovered following 96 h of dialysis by freeze-drying the sample for 48 h.

4.3. Quantification of ^{15}N -enriched K5 polysaccharide product

Purified [^{15}N]N-acetylheparosan product was subjected to the carbazole assay for uronic acids for quantification using glucuronolactone-derived standard curves. All glucuronolactone standards and [^{15}N]N-acetylheparosan samples were performed in triplicate.

4.4. Preparation of N-acetylheparosan disaccharides

^{15}N -labeled or unlabeled N-acetylheparosan (30–40 μg) was suspended in a buffer containing 3 mM $\text{Ca}(\text{OAc})_2$, 40 mM NH_4OAc (pH 7.0) and 0.1 mg/mL BSA. Exhaustive digestion of the polysaccharide into disaccharides was accomplished using 10 mU of heparitinase I enzyme and incubation at 37 °C for 24 h. The digestion was terminated by boiling for 2 min.

4.5. Preparation of size-defined [^{15}N]N-acetylheparosan oligosaccharides

^{15}N -enriched K5 polysaccharide (500 μg) was resuspended in a buffer containing 3 mM Ca (OAc) $_2$, 40 mM NH $_4$ OAc (pH 7.0) and 0.1 mg/mL BSA. Partial digestion of the polysaccharide into size-defined oligosaccharides was accomplished using 10 mU of heparitinase I enzyme and incubation at 37 °C for 15 min. The digestion was terminated by boiling for 2 min.

4.6. Anion-exchange HPLC separation of oligosaccharides

[^{15}N]N-acetylheparosan oligosaccharides of varying degree of polymerization were purified by fractionation using anion-exchange HPLC. The oligosaccharides were bound to a Phenosphere SAX column (250 $\times$ 4.6 mm, Phenomenex, Inc., Torrance, USA) in the presence of double-distilled water (pH 3.5) and eluted from the column with a linear gradient of 2 M NaCl (pH 3.5) from 0 to 150 min at a flow rate of 1 mL/min. Fractions (~1 mL) containing purified, size-defined [^{15}N]N-acetylheparosan oligosaccharides were pooled together and desalted by dialysis.

4.7. ESI-TOFMS

Mass spectra were acquired in the negative-ion mode on a LCT Premier XE electrospray ionization time-of-flight (ESI-TOF) mass spectrometer (Waters Corporation, Milford, MA). The instrument was calibrated using a solution of NaI (2 ng/ μL , Sigma–Aldrich) in 1:1 H $_2$ O–2-PrOH through the reference nozzle in the negative-ion mode. Nitrogen was used as a desolvation and nebulizing gas. Conditions for ESI-TOFMS were as follows: cone gas flow 50 L/h, nozzle temperature 140 °C, nitrogen was used as a drying gas at a flow rate of 400 L/h, spray tip potential 2.8 kV, nozzle potential 50 V. For each oligosaccharide analysis, negative-ion spectra were generated by scanning the range of m/z 100–2500. Scan resolution was measured as full width at half maximum (fwhm), which was at least 10,000 ppm for all spectra allowing isotopically-resolved signals. Spectra were analyzed using MassLynx V4.1 software (Waters Corporation, Milford, MA).

4.8. ^1H NMR spectroscopy

One-dimensional 500-MHz ^1H and two-dimensional HSQC spectra were recorded on a Varian Inova 500 NMR spectrometer (Varian, Palo Alto, USA). One-dimensional ^1H spectra were acquired with and without waltz decoupling of ^{15}N during acquisition. Samples of [^{15}N]N-acetylheparosan (5 mg) were repeatedly dissolved in D $_2$ O and freeze dried, then reconstituted in D $_2$ O to a concentration of 2.5 mg/mL prior to NMR analysis. Sample temperature was set to 25 °C. The ^1H signal is referenced to the DSS methyl at 0 ppm and 25 °C. The ^{15}N signal is referenced indirectly (Markley et al., 1998).

Acknowledgments

This work was supported by a National Institutes of Health grant (GM075168) and an American Heart Association national scientist development award to BK. We acknowledge the Interdepartmental Program in Neuroscience for support of CS. We thank Professor Ireland for providing access to mass spectrometry. We acknowledge funding from the NIH, grant RR06262, for NMR instrumentation awarded to the University of Utah Health Sciences NMR Center and Dr. Jack Skalicky for technical assistance with the NMR experiments.

References

1. Damus PS, Hicks M, Rosenberg RD. Nature 1973;246:355–7. [PubMed: 4586320]
2. Rosenberg RD. Heparin action. Circulation 1974;49:603–5. [PubMed: 4817700]
3. Bernfield M, Gotte M, Park PW, Reizes O, Fitzgerald ML, Lincecum J, Zako M. Annu. Rev. Biochem 1999;68:729–77. [PubMed: 10872465]
4. Bishop JR, Schuksz M, Esko JD. Nature 2007;446:1030–7. [PubMed: 17460664]

5. Rosenberg RD, Shworak NW, Liu J, Schwartz JJ, Zhang L. *J. Clin. Invest* 1997;99:2062–70. [PubMed: 9151776]
6. Capila I, Linhardt RJ. *Angew. Chem. Int. Ed. Engl* 2002;41:391–412. [PubMed: 12491369]
7. Salmivirta M, Lidholt K, Lindahl U. *FASEB J* 1996;10:1270–9. [PubMed: 8836040]
8. Lindahl U. *Thromb. Haemost* 2007;98:109–15. [PubMed: 17598000]
9. Hricovini M, Guerrini M, Bisio A, Torri G, Naggi A, Casu B. *Semin. Thromb. Hemost* 2002;28:325–34. [PubMed: 12244479]
10. Cros S, Petitou M, Sizun P, Perez S, Imberty A. *Bioorg. Med. Chem* 1997;5:1301–9. [PubMed: 9377090]
11. Zhang Z, McCallum SA, Xie J, Nieto L, Corzana F, Jiménez-Barbero J, Chen M, Liu J, Linhardt RJ. *J. Am. Chem. Soc* 2008;130:12998–13007. [PubMed: 18767845]
12. Zhang Z, Xie J, Liu H, Liu J, Linhardt RJ. *Anal. Chem* 2009;81:4349–55. [PubMed: 19402671]
13. Mobli M, Nilsson M, Almond A. *Glycoconjugate J* 2008;25:401–14.
14. Leali D, Belleri M, Urbinati C, Coltrini D, Oreste P, Zoppetti G, Ribatti D, Rusnati M, Presta M. *J. Biol. Chem* 2001;276:37900–8. [PubMed: 11473122]
15. Kuberan B, Beeler DL, Lawrence R, Lech M, Rosenberg RD. *J. Am. Chem. Soc* 2003;125:12424–5. [PubMed: 14531682]
16. Kuberan B, Lech MZ, Beeler DL, Wu ZL, Rosenberg RD. *Nat. Biotechnol* 2003;21:1343–6. [PubMed: 14528313]
17. Kusche M, Hannesson HH, Lindahl U. *Biochem. J* 1991;275(Pt 1):151–8. [PubMed: 1902083]
18. Lindahl U, Li JP, Kusche-Gullberg M, Salmivirta M, Alaranta S, Veromaa T, Emeis J, Roberts I, Taylor C, Oreste P, Zoppetti G, Naggi A, Torri G, Casu B. *J. Med. Chem* 2005;48:349–52. [PubMed: 15658847]
19. Casu B, Grazioli G, Razi N, Guerrini M, Naggi A, Torri G, Oreste P, Tursi F, Zoppetti G, Lindahl U. *Carbohydr. Res* 1994;263:271–84. [PubMed: 7805054]
20. Chen J, Jones CL, Liu J. *Chem. Biol* 2007;14:986–93. [PubMed: 17884631]
21. Vann WF, Schmidt MA, Jann B, Jann K. *Eur. J. Biochem* 1981;116:359–64. [PubMed: 7018909]
22. Babu P, Kuberan B. *Anal. Biochem.* 2009 doi:10.1016/j.ab.2009.08.036.
23. Minamisawa T, Suzuki K, Kajimoto N, Iida M, Maeda H, Hirabayashi J. *Carbohydr. Res* 2006;341:230–7. [PubMed: 16330006]
24. Kuberan B, Lech M, Zhang L, Wu ZL, Beeler DL, Rosenberg RD. *J. Am. Chem. Soc* 2002;124:8707–18. [PubMed: 12121115]
25. Fritz TA, Gabb MM, Wei G, Esko JD. *J. Biol. Chem* 1994;269:28809–14. [PubMed: 7961837]

Figure 1.

Preparation of [^{15}N]*N*-acetylheparosan oligosaccharides of various sizes from the polysaccharides by digestion with heparitinase I.

Figure 2.

500-MHz ^1H NMR spectra of $[\text{^{15}\text{N}}]\text{N-acetylheparosan}$. (A) One-dimensional ^1H NMR spectrum of $[\text{^{15}\text{N}}]\text{N-acetylheparosan}$ with waltz decoupling of ^{15}N during acquisition. The spectrum reveals a single peak centered at 8.21 ppm that is otherwise split by ^{15}N when recorded in the absence of ^{15}N decoupling (B). The measured one-bond scalar coupling ($^1J_{\text{NH}}$) is 91.7 Hz, consistent with the expected value of amides. A single asterisk (*) indicates the signal arising from a small population for amide protons attached to ^{14}N , indicating incomplete ^{15}N incorporation, and is furthermore consistent with the 94% isotopic purity of $[\text{^{15}\text{N}}]\text{N-acetylheparosan}$ calculated by ESIMS. The double asterisks (**) indicates the residual water signal. (C) Two-dimensional (^{15}N , ^1H) HSQC shows a single correlation at ^1H , 8.21 and ^{15}N , 123.3 ppm.

Figure 3. ESIMS spectra of *N*-acetylheparosan disaccharide: [^{15}N]*N*-acetylheparosan isolated from *E. coli* grown in minimal media containing $^{15}\text{NH}_4\text{Cl}$ (A) and [^{14}N]*N*-acetylheparosan isolated from *E. coli* grown in LB culture broth (B).

Figure 4. Anion-exchange HPLC chromatogram of partially digested [^{15}N]*N*-acetylheparosan by heparitinase I during semipreparative scale purification.

Figure 5. ESI-TOFMS spectra of [^{15}N]N-acetylheparosan oligosaccharides: DP 2 $^{1-}$ (A), DP 4 $^{1-}$ (B), DP 6 $^{1-}$ (C), DP 8 $^{2-}$ (D), DP 12 $^{3-}$ (E), DP 14 $^{3-}$ (F), DP 16 $^{4-}$ (G), and DP 18 $^{3-}$ (H).

Figure 6. Comparison of ESI-TOFMS spectra of ^{14}N and ^{15}N -labeled DP 10 and DP 20 *N*-acetylheparosan oligosaccharides: ^{14}N -DP 10 $^{2-}$ (A), ^{14}N -DP 20 $^{4-}$ (B), ^{15}N -DP 10 $^{2-}$ (C), and ^{15}N -DP 20 $^{4-}$ (D).

Table 1

Summary of the results of ESI-TOFMS measurements of size-defined [^{15}N]N-acetylheparosan oligosaccharides.

Oligosaccharide	ESI-TOFMS $[\text{M} - n\text{H}]^n - (m/z)$		
Size	Charge state (n)	Calculated	Found
2-mer	-1	379.10	379.09
4-mer	-1	759.21	759.20
6-mer	-1	1139.32	1139.34
8-mer	-2	759.22	759.22
10-mer	-2	949.27	949.27
12-mer	-3	759.22	759.20
14-mer	-3	885.92	885.90
16-mer	-4	759.22	759.47
18-mer	-3	1139.33	1139.65
20-mer	-4	949.27	949.52