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Characterizing the Microstructure of Heparin and Heparan Sulfate using *N*-sulfoglucosamine ¹H and ¹⁵N NMR Chemical Shift Analysis

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Abstract

Heparin and heparan sulfate (HS) are members of a biologically important group of highly anionic linear polysaccharides called glycosaminoglycans (GAGs). Because of their structural complexity, the molecular-level characterization of heparin and HS continues to be a challenge. The work presented herein describes an emerging approach for the analysis of unfractionated and low molecular weight heparins as well as porcine and human-derived HS. This approach utilizes the untapped potential of ¹⁵N NMR to characterize these preparations through detection of the NH resonances of *N*-sulfo-glucosamine residues. The sulfamate group ¹H and ¹⁵N chemical shifts of six GAG microenvironments were assigned based on the critical comparison of selectively modified heparin derivatives, NMR measurements for a library of heparin-derived oligosaccharide standards, and an in-depth NMR analysis of the low molecular weight heparin enoxaparin through systematic investigation of the chemical exchange properties of NH resonances and residue-specific assignments using the [¹H, ¹⁵N] HSQC-TOCSY experiment. The sulfamate microenvironments characterized in this study include GlcNS(6S)-UA(2S), ΔUA(2S)-GlcNS(6S), *GlcNS(3S)(6S)-UA(2S)*, GlcNS-UA, GlcNS(6S)-red_α, and 1,6-anhydro GlcNS demonstrate the utility of [¹H, ¹⁵N] HSQC NMR spectra to provide a spectroscopic fingerprint reflecting the composition of intact GAGs and low molecular weight heparin preparations.

Introduction

Heparin and heparan sulfate (HS) are members of the glycosaminoglycan (GAG) family of polysaccharides. Heparin is sequestered in the secretory granules of mast cells following biosynthesis and is a potent and pharmaceutically important anticoagulant.¹ Heparin is used clinically in both its unfractionated and low molecular weight forms.² The structurally related GAG HS is found associated with the cell surface where it is a key component of membrane-bound proteoglycans, and in the extracellular matrix following heparanase cleavage.³ Both heparin and HS are involved in many critical biological processes including cell differentiation,⁴ development,^{5, 6} inflammation,^{7–10} as well as pathological conditions including cancer^{11–14} and viral infections.^{15–17} The myriad of biological activities associated with heparin and HS is largely attributed to the structural microheterogeneity of these biopolymers.

Heparin and HS are structurally diverse sulfated linear polysaccharides consisting of 1→4 linked repeating disaccharide subunits containing α-L-iduronic (IdoA) or β-D-glucuronic acid (GlcA) and glucosamine (GlcN). The uronic acid can be modified by sulfation at the 2-*O*-position, while the glucosamine residue can be unmodified, *N*-sulfated (GlcNS) or *N*-acetylated (GlcNAc), and can contain variable patterns of *O*-sulfation at the 3-*O* and 6-*O* positions.¹⁸ The anticoagulant activity of heparin is associated with a well-characterized

pentasaccharide sequence containing a 3-*O*-sulfated GlcNS in between glucuronic acid and 2-*O*-sulfated IdoA residues. A recent report suggests that additional GlcNS(3S) residues beyond this pentasaccharide further contribute to high affinity binding.¹⁹ This anticoagulant motif is only found in 1 in 3 heparin chains making it a minor component.^{20, 21} Heparin is highly sulfated containing on average 1.8 – 2.6 sulfate groups per disaccharide²² with the most abundant disaccharide subunit being the trisulfated L-IdoA(2S)-D-GlcNS(6S). Although HS contains fewer sulfate groups per disaccharide (0.8 – 1.8),²² its structure has greater variability with regions of high and low sulfation and a greater percentage of GlcNAc and GlcA residues.

Low molecular weight heparins (LMWH) have entered into wide-spread clinical use as they have increased bioavailability²³ with a reduced risk of heparin-induced thrombocytopenia²⁴ and osteoporosis²⁵ compared to unfractionated heparin (UFH). LMWH are prepared from UFH by means of enzymatic or chemical depolymerization. Aside from molecular weight, LMWH differs from unfractionated heparin by structural modifications resulting from the depolymerization process. This process introduces additional structural heterogeneity through the formation of non-native saccharide structures.²⁶ Enzymatic cleavage of heparin produces an unsaturated uronic acid (Δ UA) through introduction of a double bond between the C4 and C5 carbons of the newly formed non-reducing end. Cleavage of heparin by chemical β -elimination also introduces an unsaturated bond at the cleavage site as well as additional structural modifications including introduction of a 1,6-anhydro moiety at the reducing end, epimerization of the reducing end GlcN to manosamine and the formation of galacturonic acid.^{23, 27}

NMR has proven to be an invaluable method for characterizing GAGs such as heparin, HS, chondroitin sulfate and hyaluronan as well as for the analysis of UFH and LMWH pharmaceuticals.^{28–30} The utility of NMR for GAG characterization was aptly demonstrated by the 2008 contamination of the pharmaceutical heparin supply chain with oversulfated chondroitin sulfate. This event resulted in numerous reported deaths and highlighted the need for improved quality control methods. The identity of the contaminant was elucidated using one-dimensional ¹H and ¹³C NMR spectra as well as the two-dimensional [¹H, ¹³C] heteronuclear single quantum coherence (HSQC) NMR experiment which correlates ¹H and ¹³C chemical shifts.³¹ [¹H, ¹³C] HSQC NMR spectra provide useful information about heparin structural features and substitution patterns and can be used to identify and quantify modifications such as those found in LMWH.²³

Although [¹H, ¹³C] HSQC is routinely applied for the characterization and quality assurance of pharmaceutical heparins, ¹⁵N NMR has been underutilized for GAG characterization. Recently, we reported solution conditions that facilitate detection of the prominent heparin NHSO_3^- or sulfamate groups and allow the determination of ¹H and ¹⁵N chemical shifts using the [¹H, ¹⁵N] HSQC experiment.³² The rate of chemical exchange of the sulfamate proton with water is minimized in the basic pH regime, and is affected by the local negative charge density.^{32, 33} The determination of the NH exchange rates for a set of structurally defined heparin-derived oligosaccharides led to the characterization of the first reported intramolecular hydrogen bond involving the sulfamate NH group adjacent to the 3-*O*-sulfate in the pentasaccharide Arixtra.³³

The work described herein focuses on the application of ¹H and ¹⁵N chemical shift analysis for the characterization of heparin and HS biopolymers and their derivatives.^{32–34} The study was initiated by the systematic measurement of ¹H and ¹⁵N chemical shifts of GlcNS sulfamate groups in a variety of microenvironments using a library of oligosaccharide standards. Comparison of [¹H, ¹⁵N] HSQC spectra of enzymatically and chemically modified heparins provided further evidence for residue-specific assignments. Measurement

of [^1H , ^{15}N] HSQC spectra of the LMWH enoxaparin as a function of pH probed the chemical exchange characteristics of the sulfamate protons, reinforcing initial assignments. Finally, the [^1H , ^{15}N] HSQC-TOCSY spectrum of enoxaparin was used to assign specific NH correlations through TOCSY cross peaks to sugar ring protons with chemical shifts characteristic of specific GlcNS microenvironments. Enoxaparin was chosen for this study as it reflects a more diverse mixture of oligosaccharides than other LMWHs produced through β -elimination, such as tinzaparin, and does not suffer the loss of sulfamate groups in the formation of 2,5 anhydromannitol residue as in the reductive deamination process used to manufacture dalteparin.

EXPERIMENTAL SECTION

Materials and Reagents

HPLC-grade water used for NMR sample preparation was from Burdick and Jackson (Morristown, NJ), Deuterium oxide (99%), L-glutamine (^{15}N amide 98%), and ethylenediaminetetraacetic- d_6 (EDTA) were purchased from Cambridge Isotope Laboratories (Andover, MA). Dibasic sodium phosphate, sodium hydroxide, and sodium chloride were purchased from Fisher Scientific (Pittsburgh, PA). DSS- d_6 (3-(Trimethylsilyl)-1-propanesulfonic acid sodium salt) was purchased from Isotech (Miamisburg, OH). Calcium acetate, Tris-HCl, magnesium chloride, the trisulfated heparin-derived disaccharide (IS), porcine intestinal mucosa heparin and benzonase were purchased from Sigma-Aldrich (St. Louis, MO). enoxaparin was obtained from the U.S. Pharmacopeia (Rockville, MD) while porcine heparan sulfate was purchased from Celsus labs (Cincinnati OH). Bovine lung heparin was obtained from Upjohn Co. (Kalamazoo, MI) De-2-*O*-sulfated porcine mucosal heparin was prepared as previously reported.³⁵ The enzymes heparinase-I and heparinase-III were purchased from IBEX (Montreal, Quebec). Arixtra was provided by the University Pharmacy and Department of Pharmacy Administration of Semmelweis University. DMEM media without L-glutamine and fetal bovine serum were purchased from Mediatech (Manassas, VA). Pronase was obtained from Roche (San Francisco, CA) and Triton X-100 was purchased from Alpha Aesar (Ward Hill, MA).

Heparinase-I digestion—A 1 g sample of porcine mucosal heparin was dissolved in 50 mL of 100 mM Tris buffer pH 6.8 with 2.5 mM calcium acetate. An aliquot of 0.5 IU of heparinase-I was added to the warmed solution and the reaction allowed to progress for 66 h at 28 °C. The reaction was quenched by placing the solution in boiling water for 5 min.

Heparinase-III digestion—A 1 g sample of porcine mucosal heparin was dissolved in 50 mL of 20 mM Tris buffer pH 7.5 with 50 mM NaCl. An aliquot of 0.5 IU of heparinase-III was added to the warmed solution and allowed to react overnight at 35 °C. This reaction was quenched by placing the solution in boiling water for 5 min.

Isolation of Oligosaccharide Standards—Oligosaccharides containing the 1,6-anhydro structure at the reducing end were isolated from enoxaparin, while the remainder were isolated from the heparinase-I digestion of porcine mucosal heparin described above. These samples were subjected to size fractionation by size-exclusion chromatography (SEC) followed by charge fractionation using strong anion chromatography (SAX). SEC separations were performed as previously described using a 3.0×200 cm column packed with Bio-Rad P-10 superfine gel (Bio-Rad Laboratories Hercules, CA) at 0.08 mL/min.²⁸ Fractions of 4 mL were collected and the UV absorbance measured at 232 nm.

The size-uniform oligosaccharide fractions isolated by SEC were subjected to SAX as previously described using a Dionex 500 ion chromatograph equipped with a CarboPac PA1 column (9 mm $\times$ 25 cm) purchased from Dionex (Sunnyvale, CA).²⁸ The separation was

carried out using two mobile phase buffers at a constant flow rate of 2.5 mL/min. Solution A consisted of 50 mM sodium phosphate pH 7.0 and solution B contained the same buffer as in A with 2.0 M sodium chloride. The gradient profile used was: 0 min: 100% A, 0% B; 10 min: 71% A, 29% B; 80 min: 39% A, 61% B, ending at 90 min. Individual SAX peaks were collected, pooled and desalted using a 1.6 cm $\times$ 70 cm Sephadex G10 superfine column (GE Healthcare, Pittsburgh, PA).

Oligosaccharides were identified as previously described based on ^1H NMR chemical shift assignments obtained from one-dimensional spectra, as well as the COSY, TOCSY, and ROESY spectra acquired in D_2O using a Bruker 600 MHz Avance spectrometer equipped with a BBI probe.^{36, 37} The TOCSY spectrum was used to identify each saccharide through measurements of the chemical shifts of the protons within each sugar ring. The DQF-COSY spectrum confirmed scalar connectivities while the ROESY spectrum revealed the oligosaccharide sequence through detection of through-space connectivities across glycosidic linkages.

In addition to NMR, reverse-phase ion-pairing ultra performance liquid chromatography (UPLC) mass spectrometry (MS) was used to confirm the oligosaccharide sequence. Isolated oligosaccharides were analyzed using a Waters Acquity UPLC with a Q-TOF MS detector running in negative mode.^{38, 39} The separations were performed at 40 °C using a 2.1 mm $\times$ 100 mm Waters BEH C18 column containing 1.7 μm particles. The mobile phase buffers consisted of 10 mM acetic acid with 10 mM dibutylamine (DBA) as the ion-pairing reagent. Solution A was composed of 90% H_2O /10% methanol and solution B was composed of 100% methanol. A linear elution gradient was used starting at 30% B and going to 55% B over 15 min. An equilibration time of 7 min was used between injections.

Isolation of HS from cell culture—HEK293T cells were grown to near confluence in 150 cm^2 vented flasks using 25 mL of DMEM without L-glutamine, 10% FBS and 3 mM of ^{15}N -labelled L-glutamine (^{15}N amide, 98%).²⁹ No antibacterial or antifungal supplements were used. Cells were lysed directly on the culture flask surface using 100 mM NaOH with 1% Triton X-100. The cell lysate and media were collected, combined and adjusted to neutral pH with concentrated HCl. CaCl_2 was added to make a final concentration of 2 mM and the sample treated with a final concentration of 0.5 mg/mL pronase and digested twice overnight at 50 °C. Following pronase digestion, the sample was adjusted to contain 2 mM MgCl_2 and incubated with benzonase at a final concentration of 25 mU/mL for 4 h at 37 °C. After enzymatic treatment the sample was twice passed through a 3 mL column of DEAE-Sephacel anion-exchange resin. The column was washed with 50 mL of wash solution (50 mM sodium acetate, 200 mM NaCl and 0.5% Triton X-100 at pH 6.0) and eluted with 6 mL of the wash solution containing 2 M NaCl. The eluent was treated with a solution containing 10% NaBH_4 in 2M NaOH at a ratio of 0.33 mL per 3 mL of column eluent and incubated overnight at -20 °C. The NaBH_4 was destroyed with enough acetic acid to neutralize the solution and the GAGs precipitated with 4 volumes of denatured ethanol. The solution was centrifuged at 9000 g and pellet collected. The pellet was hydrated in a minimal volume of water and dialyzed against HPLC-grade water using 3000 Da MWCO dialysis tubing from Spectrum labs (Rancho Dominguez, CA).

NMR Experimental Parameters

The [^1H , ^{15}N] HSQC spectra of enoxaparin, porcine mucosal heparin, bovine lung heparin, Celcus porcine HS and ^{15}N -enriched HEK293T-derived HS, shown in Fig. 1, and the heparinase-III digested porcine mucosal heparin in Fig. 2b were prepared by dissolving 75 mg of material in 600 μL of a solution containing 90% H_2O /10% D_2O with 10 mM sodium phosphate buffer at pH 8.2, 2 mM EDTA- d_6 , and 2 mM DSS- d_6 . Spectra were referenced to

DSS- d_6 per the IUPAC NMR referencing recommendation.⁴⁰ It is important to note that the sulfamate group NH resonance chemical shifts and line widths are temperature dependent.³³ and for the purpose of consistency all [^1H , ^{15}N] HSQC spectra in this work were acquired at 20 °C unless otherwise noted. Samples were placed into a 5 mm NMR tube and [^1H , ^{15}N] HSQC spectra measured using a Bruker 600 Avance spectrometer equipped with a BBI probe. These spectra were acquired in 4096 complex points in t_2 with 128 scans coadded for each of the 160 t_1 increments. The spectral window was 9 ppm in F2 centered on the HOD signal and 15 ppm in the F1 centered at 92 ppm. A $^1J_{\text{NH}}$ of 80 Hz was used with a 2.0 s relaxation delay. The spectra were linear predicted to 224 points in F1, zero-filled to 4096×512 points and apodized using the $\cos^2$ window function in both dimensions. Spectra were typically acquired as overnight experiments with 15 hours being a representative measurement time. Spectra were measured at 20 °C to minimize peak broadening due to chemical exchange and prevent the slight variation in chemical shifts that could result from temperature fluctuations. In the future, it may prove useful to acquire GAG [^1H , ^{15}N] HSQC spectra at even lower temperatures or using higher field cryoprobe-equipped spectrometers to facilitate detection of less abundant sulfamate group microenvironments. The [^1H , ^{15}N] HSQC spectra of de-2-*O*-sulfated heparin was acquired using a sample mass of 17 mg in 325 μL of phosphate buffer, as described above, in a 5 mm D_2O matched Shigemi tube (Shigemi Inc, Allison Park, PA). The acquisition parameters were identical other than having a total of 176 t_1 increments. The [^1H , ^{15}N] HSQC spectra of bovine lung heparin was acquired in a 3 mm NMR tube using a cryoprobe-equipped 500 MHz Bruker Avance spectrometer at the G. Ronzoni Institute.

To maximize the measurement sensitivity, [^1H , ^{15}N] HSQC spectra of the oligosaccharide standards were acquired using a 1.7 mm capillary insert with a sample volume of 35 μL centered in a 3 mm NMR tube (New Era Enterprises, Sevierville TN) using a Teflon holder. Sample buffer conditions mimicked those described above except that the sample was prepared in 100% H_2O with the spectrometer lock provided by 100% D_2O in the 3 mm tube's annular volume. Depending on the availability of the isolated oligosaccharides, samples were prepared using masses that ranging from 0.2 mg to 2 mg dissolved in 35 μL . Oligosaccharide [^1H , ^{15}N] HSQC spectra were measured using the aforementioned 600 MHz Bruker spectrometer for samples of 0.4 mg or more; compounds isolated in quantities of 0.2 mg or less were measured at the Complex Carbohydrate Research Center (CCRC) of the University of Georgia using a 600 MHz Varian spectrometer equipped with a 3 mm coldprobe. NMR parameters were similar to those describe above, with t_1 increments ranging from 128 to 320 and numbers of scans ranging 176 to 320 depending on the available mass.

The [^1H , ^{15}N] HSQC-TOCSY spectrum was measured using 200 mg of enoxaparin in 350 μL of 90% H_2O /10% D_2O buffer in a D_2O matched Shigemi tube (Shigemi Inc, Allison Park, PA). The spectrum was acquired in 4096 complex points in t_2 with 256 scans coadded for each of 148 t_1 increments. A spectral window of 7 ppm in F2 and 13 ppm in F1 were used with a relaxation delay of 2.0 s. A $^1J_{\text{NH}}$ value of 80 Hz was used with a TOCSY mixing time of 100 ms. The spectrum was zero filled to 8192×512 points and apodized using $\cos^2$ in both dimensions.

RESULTS AND DISCUSSION

Recent reports on the detection of the ^1H and ^{15}N NMR resonances of the *N*-sulfoglucosamine sulfamate groups suggest that this information could be useful for the structural characterization of complex GAGs including UFH, LMWH and HS. For example, the [^1H , ^{15}N] HSQC spectrum of the sulfamate region of the LMWH drug enoxaparin demonstrates that it is possible to detect discrete NH correlations suggesting regular

elements of structure despite the complexity of this polydisperse mixture of heterogeneous heparin oligosaccharides.³² The 600 MHz [¹H, ¹⁵N] HSQC spectra measured for enoxaparin, UFH from porcine intestinal mucosa, UFH from bovine lung, porcine HS, and HS derived from ¹⁵N enrichment of cultured HEK293T cells are presented in Fig. 1. The peaks observed in these spectra are designated using Roman numerals with the corresponding chemical shifts presented in Table 1. Figure 1 highlights the similarities and differences in the spectra measured for GAGs obtained from the various sources. The porcine mucosal heparin, bovine lung heparin and enoxaparin spectra all have peaks designated as I. In the enoxaparin spectrum, however, this correlation appears to be split into two distinct populations, with the correlation having a ¹⁵N chemical shift of 94.6 ppm labeled as II. Cross peak III is particularly sharp and is present in enoxaparin as well as both intact heparin samples, but is not detected in the HS spectra. Peak IV is consistent throughout all five spectra in Fig. 1 and is present in both heparin and HS samples. The LMWH enoxaparin also has a unique correlation designated as V. At higher levels of vertical scale than used in Fig. 1, a weak peak corresponding to V can also be observed in the porcine intestinal mucosa and bovine lung heparin spectra, but the signal is of low intensity and close to the noise floor. Additional unlabeled peaks were observed in the [¹H, ¹⁵N] HSQC spectrum of enoxaparin (Fig. 1a). These peaks were not identified in this work and will require further investigation to allow their assignment. It is also likely that additional, lower abundance peaks, are present below the noise floor of the spectra shown in Fig. 1 and the use of a more sensitive spectrometer would permit their detection.

To better understand the structural characteristics or microenvironments that give rise to the observed correlations in the [¹H, ¹⁵N] HSQC spectra of the intact GAG polymers, a series of oligosaccharide standards were isolated by SEC and SAX from porcine mucosal heparin digested with heparinase-I and from enoxaparin. These oligosaccharides were structurally characterized by RPIP-UPLC-MS and one- and two-dimensional homonuclear NMR experiments.^{37, 41} The [¹H, ¹⁵N] HSQC spectrum of each standard was measured and the chemical shifts of the isolated di, tetra and hexasaccharides along with the Arixtra pentasaccharide, which is used clinically, are summarized in Table 2. Several preliminary peak assignments in the GAG [¹H, ¹⁵N]HSQC spectra shown in Fig. 1 can be made by comparison to the results in Table 2, as described below.

Chemical shifts of the Terminal GlcNS Residues

The α -anomer of the *N*-sulfoglucosamine residue of the reducing end of the GAG chains is the easiest peak to assign using the information in Table 2. The sulfamate proton resonance of the β -anomeric form of the reducing end GlcNS was not detected in any heparin or HS preparation, likely due to its lower abundance and the greater line widths observed in the spectra of the oligosaccharide standards. The ¹H and ¹⁵N chemical shifts of the disaccharide α -anomer along with those of the reducing ends of the isolated tetrasaccharides are consistent with the corresponding peak V in the enoxaparin HSQC spectrum. This peak is much less intense in spectra measured for preparations of the higher molecular weight UFH and is not observed in the HS samples, which contain a proportionally smaller fraction of reducing ends. As a result of the chemical depolymerization steps used in its preparation, the LMWH heparin enoxaparin has a lower average molecular weight resulting in more reducing ends per unit mass.

The peak arising from the non-reducing end of enoxaparin oligosaccharides can be assigned by comparison with the hexasaccharide chemical shifts in Table 2. This hexasaccharide has two internal GlcNS(6S) residues and one residue at the reducing end. Sulfamate chemical shifts of the GlcNS(6S) residue adjacent to the non-reducing end 2-*O*-sulfo unsaturated uronic acid (Δ UA(2S)), (5.27 and 92.7 ppm), and the internal residue linked to the

unmodified IdoA(2S) (5.32 and 92.4 ppm) are strikingly similar to the peaks II and I, respectively, in the enoxaparin spectrum. Assigning peak II as the GlcNS(6S) residue directly adjacent to the Δ UA(2S) is supported by the chemical shifts measured for the hexasaccharide. Peak I is tentatively assigned as an internal GlcNS residue as it is the most intense cross peak in both UFH samples (Fig. 1b and c) and closely matches the chemical shifts of the internal GlcNS(6S) within the hexasaccharide. These assignments are further supported by the fact that peak II is observed only in the enoxaparin spectrum (Fig. 1a), the only heparin sample examined in which Δ UA residues are expected at the non-reducing ends of the oligosaccharide chains resulting from the depolymerization process.

Effect of Uronic Acid 2-*O*-sulfation on GlcNS Chemical Shifts

The chemical shifts in Table 2 can also provide information regarding the effect of 2-*O*-sulfation of the neighboring uronic acid by comparing tetrasaccharides differing only by a single factor such as saccharide identity (IdoA or GlcA) or a single substitution. Comparing the chemical shifts of the sulfamate group of the internal GlcNS(6S) of Tetra-3 (5.79 and 93.3 ppm) to that of Tetra-5 (5.85 and 93.4 ppm) reveals only subtle differences in the ^1H and ^{15}N chemical shifts of a GlcNS(6S) residue linked to the H4 of an IdoA or a GlcA. This suggests that the sulfamate chemical shifts do not reflect the configuration of the neighboring uronic acid.

For some tetrasaccharides in Table 2, the sulfamate chemical shifts appear to reflect the influence of 2-*O*-sulfation of the adjacent uronic acid. For example, both the ^1H and ^{15}N chemical shifts of the internal GlcNS(6S) of Tetra-1 (5.39 and 92.6 ppm) and Tetra-3 (5.79 and 93.3 ppm) reflect whether the adjacent IdoA residue is 2-*O*-sulfated. A similar grouping is apparent in the [^1H , ^{15}N] HSQC spectra in Fig. 1 suggesting that the peak I may be due to sulfamate groups adjacent to a 2-*O*-sulfated uronic acid while peak IV arises from sulfamate groups linked to uronic acid residues that are unmodified at the 2-*O* position. The chemical shifts of the corresponding sulfamate group in the GlcA containing oligosaccharide Tetra-5 (5.85 and 93.4 ppm) support this hypothesis, however the trend appears to break down for Tetra-2 (5.17 and 92.8 ppm) which lacks 6-*O*-sulfation of the reducing end GlcNS. Removal of the sterically hindering 6-*O* sulfate group may increase the freedom of motion of the reducing end saccharide impacting the chemical shifts of the internal sulfamate NH group.

Additional experiments were performed for two structurally modified heparins to probe potential end effects in the oligosaccharide data in Table 2 and to further test whether the sulfamate chemical shifts in larger saccharide chains systematically reflect 2-*O*-sulfation of the adjacent uronic acid. Figure 2a presents the [^1H , ^{15}N] HSQC spectrum of unfractionated porcine intestinal mucosa heparin as a reference for comparison of the effects of these structural modifications. The spectrum in Fig. 2b was measured for a porcine intestinal mucosa heparin sample digested using heparinase-III, which is reported to specifically cleave the heparin chain between GlcNS or GlcNAc and GlcA or IdoA residues that lack sulfation at the 2-*O* position.⁴² Digestion with heparinase-III should primarily affect the chemical shifts of GlcNS residues bonded to unsulfated uronic acid residues, as these are the locations where cleavage will occur. Comparison of the [^1H , ^{15}N] HSQC spectrum of heparinase-III digested heparin (Fig. 2b) with that of the intact polymer (Fig. 2a) reveals that only peak IV appears to be affected by enzymatic cleavage, consistent with its assignment to GlcNS residues adjacent to an unsulfated uronic acid. To confirm this assignment, a [^1H , ^{15}N] HSQC spectrum (Fig. 2c) was acquired for a heparin sample for which the uronic acid 2-*O* sulfate groups were chemically removed.⁴³ This modification should primarily affect the sulfamate group chemical shifts of GlcNS residues bonded to a 2-*O*-sulfated uronic acid. Comparison of Fig. 2c with the spectrum of unmodified heparin (Fig. 2a) indicates that the only peak not affected by 2-*O*-desulfation is the peak designated IV,

confirming its assignment as GlcNS-UA. The results in Figure 2 further suggest that the peaks I and III arise from internal GlcNS residues bonded to 2-*O*-sulfated uronic acid residues.

Effect of 3-*O*-sulfation on GlcNS Chemical Shifts

In the [^1H , ^{15}N] HSQC spectra in Fig. 1 the chemical shifts of peak III for enoxaparin (5.53 and 92.0 ppm), porcine mucosal heparin (5.54 and 92.0 ppm) and bovine lung heparin (5.58, 92.2 ppm) are similar to those of the GlcNS(3S)(6S) residue of Arixtra (Table 2, 5.60 and 92.0 ppm). This suggests that peak III corresponds to a 3-*O*-sulfated GlcNS residue and can be preliminarily assigned as GlcNS(3S)-UA(2S). The unique ^1H and ^{15}N chemical shifts of 3-*O*-sulfated GlcNS are likely due to the proximity of the 3-*O*-sulfate group and its reported intraresidue hydrogen bond involving the sulfamate group as the proton donor.³³ As a result of this stable hydrogen bond, the Arixtra GlcNS(3S)(6S) NH exchanges slowly with water therefore its resonance can be detected over a much wider pH range than the other sulfamate NH groups of Arixtra. This characteristic exchange profile can also be observed in the [^1H , ^{15}N] HSQC spectra of enoxaparin (Fig. 3) as peak III appears unchanged through the pH range of 7.00 to 9.42. The assignment of the most rapidly exchanging sulfamate groups of the reducing end GlcNS residues (peak V) is also confirmed by the results in Fig. 3. The reducing end GlcNS NH resonance has been reported to be detectable only over a narrow pH range (8.0 – 8.5) in oligomers larger than monosaccharides.³³ The enoxaparin peak V is observed in Fig. 3 only in the spectra measured at pH 7.85 and 8.20 closely matching the behavior of the reducing end GlcNS sulfamate group resonances described in our previous work.³³

HSQC-TOCSY Correlations Confirm Resonance Assignments

To further support the assignments of the [^1H , ^{15}N] HSQC spectra presented in Fig. 1, two-dimensional [^1H , ^{15}N] HSQC-TOCSY spectra were acquired (Fig. 4).³⁴ The HSQC-TOCSY experiment detects the protons within the GlcNS spin system and correlates them to the sulfamate group ^{15}N chemical shifts, reporting on the structural characteristics of the GlcNS residues responsible for the various peaks in the [^1H , ^{15}N] HSQC spectra.⁴⁴ While this experiment suffers from somewhat lower sensitivity than the simpler [^1H , ^{15}N] HSQC experiment, this is not a limitation for enoxaparin as it is readily available and highly soluble.

The [^1H , ^{15}N] HSQC-TOCSY spectrum in Fig. 4 shows all the sulfamate NH peaks observed in Fig. 1, with only peak IV observed at too low of intensity to give rise to detectable TOCSY correlations. The [^1H , ^{15}N] HSQC-TOCSY spectrum confirms the assignment of peak II, which is only observed in enoxaparin (Fig. 1a). The chemical shifts of the carbon-bound protons correlated to sulfamate peaks I and II have nearly identical chemical shifts with the exception of the H4 ring protons. The higher chemical shift of the H4 proton for sulfamate peak II supports its assignment to a GlcNS linked to a ΔUA residue.⁴⁵ Another observation in Fig. 4 of particular significance is the proton correlation at 4.39 ppm in F2 for NH peak III. In heparin this chemical shift is highly characteristic of the H3 proton of a 3-*O*-sulfated GlcNS.^{34, 46–48} Therefore the HSQC-TOCSY spectrum provides additional spectroscopic evidence for the assignment of peak III as GlcNS(3S)-UA(2S), a required sequence for heparin's antithrombotic activity.

Likely due to the higher enoxaparin concentration used, the [^1H , ^{15}N] HSQC-TOCSY spectrum in Fig. 4 exhibits an additional sulfamate peak that was not observed in the HSQC spectrum in Fig. 1a. Labeled as peak VI, this correlation has a ^{15}N chemical shift of 96.1 ppm. Peak VI is correlated with two proton resonances at 5.38 and 5.60 ppm in Fig. 4. Comparing the chemical shifts for peak VI to the values in Table 2, the NH correlation at

5.38 and 96.1 ppm can be assigned to a reducing end 1,6-anhydro residue. The additional ^1H cross peak at 5.60 ppm is also consistent with the value measured for the H1 (5.602 ppm) of the 1,6-anhydro GlcNS terminated Tetra-6. Detection of this unique microenvironment and its assignment to a 1,6-anhydro GlcNS residue showcases the utility of the HSQC-TOCSY experiment as the anhydro structures of enoxaparin can include 1,6-anhydro glucosamine as well as 1,6-anhydro mannosamine. According to Mascellani et al., typically only 15–25% of the chains contain an anhydro structure at the reducing end corresponding to only about 1.4–2.3% of the total mass of the LMWH.⁴⁹

Effect of 6-*O*-sulfation on GlcNS Chemical Shifts

Limtiaco et al. reported that sulfamate group ^1H and ^{15}N chemical shifts are relatively insensitive to 6-*O*-sulfation in GlcNS monosaccharides. The results presented herein, however, provide evidence that sulfamate chemical shifts in larger oligosaccharides, such as those found in enoxaparin, may be sensitive to glucosamine 6-*O*-sulfation. While both the H5 and H6 protons can be used to report on substitution at the 6-*O* position, the H5 protons are fewer bonds away from the sulfamate group and thus give stronger signals in the [^1H , ^{15}N] HSQC-TOCSY spectrum. The [^1H , ^{15}N] HSQC-TOCSY spectrum in Fig. 4 shows correlations from each of the sulfamate peaks I, II, III, and V to carbon-bound sugar resonances at 4.01 ppm, 4.02 ppm, 4.12 ppm, and 4.14 ppm respectively. The sulfamate correlations for peaks I and II at 4.01 ppm and 4.02 ppm are consistent with published values for the H5 proton of GlcNS(6S). The resonance correlated to peak III also closely matches the H5 of the GlcNS(3S)(6S) found in Arixtra³⁴ and the resonance at 4.14 ppm is characteristic of the H5 proton of a GlcNS(6S) at the reducing end²⁸ which is consistent with the assignment of sulfamate peak V as the reducing end GlcNS.

Most glucosamine residues in heparin are sulfated at both the 6-*O* and *N* positions, and GlcNS residues lacking 6-*O* sulfation are expected to be relatively minor constituents of LMWH and UFH samples. To determine the impact of 6-*O* sulfation on GlcNS ^1H and ^{15}N NMR chemical shifts, the HSQC spectrum was measured for Tetra-8, a relatively minor component of the tetrasaccharide SEC fraction used for isolation of oligosaccharide standards. As shown in Table 2, the Tetra-1 and Tetra-8 oligosaccharides differ in their structures only by substitution at the 6-*O* position of the internal GlcNS residue. For Tetra-1 for which this GlcNS is 6-*O* sulfated, the ^1H and ^{15}N chemical shifts are 5.39 ppm and 92.6 ppm, respectively, while for Tetra-8 these values are 5.30 ppm and 92.8 ppm. This result demonstrates that the sulfamate chemical shifts of internal GlcNS residues are indeed sensitive to 6-*O* sulfation. Table 3 summarizes the ^1H and ^{15}N resonance assignments for the heparin and HS samples investigated.

Conclusions

This work provides the first characterization data for the GlcNS sulfamate ^1H and ^{15}N resonances of unfractionated and low molecular weight heparins, and heparan sulfate. Although measurements of isolated oligosaccharide standards were critical to the success of this study, the spectra of heparins modified structurally by 2-*O*-desulfation and heparinase-III digestion provided key insights leading to definitive resonance assignments. By assigning the identities of the microstructures responsible for the observed ^1H and ^{15}N correlations, these results pave the way for the analytical characterization of heparin and HS in terms of specific structural traits. This approach is intended to complement the use of [^1H , ^{13}C] HSQC NMR for GAG analysis and characterization. Low molecular weight heparin [^1H , ^{15}N] HSQC spectra are significantly less complicated than their ^{13}C analogs, while still reporting on the major structural components of the sample.

The [^1H , ^{15}N] HSQC-TOCSY spectrum of enoxaparin was invaluable in completing and confirming the resonance assignments of all the GAGs studied. The HSQC-TOCSY experiment shows considerable promise for quality assurance applications, including detection of unusual or unexpected structures. Although this experiment is somewhat less sensitive than the standard gradient HSQC pulse sequence, the sensitivity of these measurements could be improved through the use of a cryogenically-cooled probe or magnetic field higher than the 600 MHz instrument used in this study.

Relative quantitation of GlcNS(3S)(6S) [^1H , ^{15}N] HSQC correlations could perhaps be used to gauge potency of heparin products as anticoagulants and comparison of intensities of peaks I and IV could provide information about the overall extent of 2-*O*-sulfation in a sample. It may also be possible to quantify the extent of heparin depolymerization based on the relative intensities of sulfamate [^1H , ^{15}N] HSQC peaks at the reducing and non-reducing ends of the biopolymers. Additionally, depolymerization modifications like the 1,6-anhydro structures could be readily monitored in LMWH samples using [^1H , ^{15}N] [HSQC] experiments.

One challenge to using [^1H , ^{15}N] HSQC spectra for quantitative determination of different GlcNS microstructures in complex heparin samples is the possibility that different local environments may give rise to differences in sulfamate group solvent exchange rates. Further study is required to evaluate the extent to which NH exchange rates of structurally different GlcNS residues vary under measurement conditions selected to minimize solvent exchange, and the impact of these differences on quantitative determinations.

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Fig. 1. $[^1\text{H}, ^{15}\text{N}]$ HSQC spectra of GAG samples: a) enoxaparin, b) porcine intestinal mucosa heparin, c) bovine lung heparin, d) Celcius porcine HS, and e) ^{15}N -enriched HEK293T-derived HS. Spectra were measured at 20° C in 90% $\text{H}_2\text{O}/10\%$ D_2O .

Figure 2. [^1H , ^{15}N] HSQC spectra of a) porcine intestinal mucosa heparin, b) porcine intestinal mucosa heparin treated with heparinase-III, c) 2-*O*-desulfated porcine intestinal mucosa heparin. All spectra were measured at 20° C.

Figure 3. $[\text{}^1\text{H}, \text{}^{15}\text{N}]$ HSQC spectra of enoxaparin measured as a function of pH. Sample pH is noted in each spectrum. Spectra were measured at 20°C.

Figure 4.
2D [^1H , ^{15}N] HSQC-TOCSY spectrum of enoxaparin measured at pH 8.2 at 20° C.
Sulfamate NH correlations are labeled with Roman numerals.

Table 1

Chemical shifts determined from the 600 MHz [^1H , ^{15}N] HSQC spectra shown in Fig. 1.

Sample	Designation	^1H (ppm)	^{15}N (ppm)
Enoxaparin	I	5.35	92.4
	II	5.33	92.6
	III	5.53	92.0
	IV	5.80	93.2
	V	5.38	94.0
	VI	5.38	96.1
Porcine mucosal heparin	I	5.35	92.4
	III	5.54	92.0
	IV	5.80	93.2
Bovine lung heparin	I	5.39	91.9
	III	5.58	92.2
	IV	5.83	93.0
Commercial HS	IV	5.81	93.2
HEK293T-derived HS	IV	5.81	93.2

Table 2

Summary of peak assignments and ^1H and ^{15}N chemical shifts determined for a series of oligosaccharide standards. Residue number is counted from the non-reducing end. Spectra were acquired at 20 °C for all compounds except Hexa, which was measured at 17 °C.

I.D.	Oligosaccharide Structure	Residue	^1H (ppm)	^{15}N (ppm)
Di	$\Delta\text{UA}2\text{S-GlcNS}6\text{S}$	II- α	5.44	94.2
Tetra-1	$\Delta\text{UA}2\text{S-GlcNS}6\text{S-IdoA}2\text{S-GlcNS}6\text{S}$	II	5.39	92.6
		IV- α	5.34	94.1
Tetra-2	$\Delta\text{UA}2\text{S-GlcNS}6\text{S-IdoA}2\text{S-GlcNS}$	II	5.17	92.8
		IV- α	5.36	94.0
Tetra-3	$\Delta\text{UA}2\text{S-GlcNS}6\text{S-IdoA-GlcNS}6\text{S}$	II	5.79	93.3
		IV- α	5.38	93.7
Tetra-4	$\Delta\text{UA}2\text{S-GlcNS}6\text{S-GlcA}2\text{S-GlcNS}$	II	5.78	92.9
		IV- α	5.42	94.0
Tetra-5	$\Delta\text{UA}2\text{S-GlcNS}6\text{S-GlcA-GlcNS}6\text{S}$	II	5.85	93.4
		IV- α	5.44	94.1
Tetra-6	$\Delta\text{UA}2\text{S-GlcNS}6\text{S-IdoA}2\text{S-GlcNS}(1,6\text{-anhydro})$	II	5.20	92.8
		IV	5.38	96.1
Tetra-7	$\Delta\text{UA}2\text{S-GlcNS}6\text{S-IdoA}2\text{S-ManNS}(1,6\text{-anhydro})$	II	5.21	92.8
		IV	5.75	95.9
Tetra-8	$\Delta\text{UA}2\text{S-GlcNS-IdoA}2\text{S-GlcNS}6\text{S}$	II	5.30	92.8
		IV	5.41	94.1
Arixtra	$\text{GlcNS}6\text{S-GlcA-GlcNS}3\text{S}6\text{S-IdoA}2\text{S-GlcNS}6\text{SOMe}$	I	5.88	92.8
		III	5.60	92.1
		V	5.41	93.9
Hexa	$\Delta\text{UA}2\text{S-GlcNS}6\text{S-IdoA}2\text{S-GlcNS}6\text{S-IdoA}2\text{S-GlcNS}6\text{S}$	II	5.27	92.7
		IV	5.32	92.4
		VI- α	5.37	94.2

Table 3

Summary of sulfamate peak of assignments for the [^1H , ^{15}N] HSQC spectra presented in Fig 1.

Sulfamate peak	Microenvironment
I	GlcNS(6S)-UA(2S)
II	Δ UA(2S)-GlcNS(6S)
III	GlcNS(3S)(6S)-UA(2S)
IV	GlcNS-UA
V	GlcNS(6S)-red _a
VI	1,6-anhydro GlcNS