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De Novo Sequencing of Heparan Sulfate Oligosaccharides by Electron-Activated Dissociation

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Abstract

Structural characterization of highly sulfated glycosaminoglycans (GAGs) by collisionally activated dissociation (CAD) is challenging because of the extensive sulfate losses mediated by free protons. While removal of the free protons may be achieved through the use of derivatization, metal cation adducts, and/or electrospray supercharging reagents, these steps add complexity to the experimental workflow. It is therefore desirable to develop an analytical approach for GAG sequencing that does not require derivatization or addition of reagents to the electrospray solution. Electron detachment dissociation (EDD) can produce extensive and informative fragmentation for GAGs without the need to remove free protons from the precursor ions. However, EDD is an inefficient process, often requiring consumption of large sample quantities (typically several micrograms), particularly for highly sulfated GAG ions. Here, we report that with improved instrumentation, optimization of the ionization and ion transfer parameters, and enhanced EDD efficiency, it is possible to generate highly informative EDD spectra of highly sulfated GAGs on the liquid chromatography (LC) time-scale, with consumption of only a few nanograms of sample. We further show that negative electron transfer dissociation (NETD) is an even more effective fragmentation technique for GAG sequencing, producing fewer sulfate losses while consuming smaller amount of samples. Finally, a simple algorithm was developed for *de novo* HS sequencing based on their high resolution tandem mass spectra. These results demonstrate the potential of EDD and NETD as sensitive analytical tools for detailed, high-throughput, *de novo* structural analyses of highly sulfated GAGs.

Introduction

Heparin (Hep) and structurally related heparan sulfate (HS) constitute a subset of the glycosaminoglycan family of acidic polysaccharides. Heparin and HS are of particular interest as they bind proteins, participate in growth factor sequestration and act as growth factor co-receptors at the cell surface.^{1,2} These binding activities are related to anticoagulation, cell proliferation, angiogenesis and tumor metastasis.³ In order to gain and then exploit an understanding of Hep/HS properties, it is necessary to correlate function with fine structure. However, due to the heterogeneous nature of Hep/HS resulting from their non-template-driven biosynthesis, the structures of Hep/HS are extremely polydisperse and therefore pose serious analytical challenges.⁴ The ideal analytical technique should be able to determine the sulfate and acetate positions on each residue, as well as the occurrence and position(s) of uronic acid epimers, and work on a time-scale compatible with on-line chromatographic separations.

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Among the current methodologies for Hep/HS characterization, tandem mass spectrometry (MS/MS) using electrospray ionization (ESI) has demonstrated great potential, as it offers high sensitivity, accuracy and throughput.⁵⁻⁷ In a recent landmark study, Linhardt and coworkers used collisionally activated dissociation (CAD) tandem MS analysis to successfully sequence the bikunin peptidoglycosaminoglycan, a simple proteoglycan containing a single lowly-sulfated chondroitin sulfate chain.⁸ However, when applied to the analysis of the highly sulfated Hep/HS, CAD often failed to produce sufficient sequence information due to dominant SO₃ losses mediated by free protons.⁹ To preserve the information on the number and location of sulfate groups during MS/MS, complete deprotonation is the key to observation of abundant informative backbone dissociation using CAD tandem MS.¹⁰ Efficient deprotonation can be achieved by supercharging the saccharide ions in the negative ionization mode by infusion of selected chemicals such as sulfolane. Such methods only work for HS with relatively low degrees of sulfation and will eventually fail for highly sulfated heparin, due to the occurrence of charge-charge repulsion in a single molecule as the charge density increases.^{11,12} Chemical derivatization to replace *N*-sulfate groups with *N*-acetate-*d*₃ reduces the number of potential free proton sites,¹³ however, as with any chemical derivatization procedure, it adds complexity to the analytical workflow. An alternative strategy for removal of the free protons is their replacement with metal cations.^{9,14} Recent studies have shown that, when 1 mM NaOH is added into the ESI solution, most of the free protons on the highly sulfated heparin drug, Arixtra, can be replaced by Na⁺. Upon CAD, the selected sodium-adducted precursor ion underwent extensive fragmentation with decreased sulfate loss, demonstrating the potential for complete structural characterization of highly sulfated heparin by MS/MS.¹⁵ However, this technique exhibited reduced sensitivity, due to the generation of a broad distribution of precursor ions with various Na⁺/H⁺ combinations. A rational selection of precursor ions with specific Na⁺/H⁺ combinations that produce backbone dissociation without sulfate losses is necessary in such a method.^{15,16} Furthermore, analysis of the tandem mass spectra of such precursor ions is challenging, due to the Na⁺/H⁺ heterogeneity in the product ions.¹⁷ Additionally, the use of high concentration of NaOH results in buildup of salts on the mass spectrometer ion source optics and represents a potential risk for most silica based capillaries that are widely used in small scale liquid chromatography (LC) separation. Thus, it is not likely that this approach will be compatible with high throughput on-line LC-MS/MS analysis.

Recently, electron-activated dissociation (ExD) methods have emerged as a set of powerful MS/MS tools for glycan analysis.¹⁷⁻²⁶ Among these techniques, electron detachment dissociation (EDD),^{22,24} electron-induced dissociation (EID),²³ and negative electron transfer dissociation (NETD)^{25,27} have been implemented for the characterization of GAGs. EID and EDD involve irradiation of GAG precursor anions with ~19 eV electrons which results in both electronic excitation and electron ejection, producing extensive and informative product ion patterns, even for precursor ions in which potentially mobile protons are present. Despite their advantages, these fragmentation techniques were only reported to work well for HS samples with relatively low degrees of sulfation.^{28,29} As the number of potential deprotonation sites per residue increases in highly sulfated HS, the precursor ion charge density also increases, leading to reduced anion/electron collision cross-section due to increased columbic repulsion. Consequently, electron detachment becomes less efficient and the tandem mass spectra are less useful for sequencing. On the other hand, unlike EDD, which involves interaction between like-charge ions, NETD is initiated by the much more efficient electron transfer process between oppositely charged particles from the multiply charged analyte anion to the reagent radical cation. NETD has shown great promise in structural analysis of GAGs with low to medium levels of sulfation. Although both EDD and NETD have been applied to analyze highly sulfated Hep/HS oligosaccharides, partial Na⁺-adduction was often needed for complete deprotonation. As discussed above, such a strategy

would lead to reduced sensitivity and difficulty in spectral interpretation, and is generally not amenable to the on-line LC-MS/MS workflow.^{17,27}

In the present contribution, we demonstrate improved EDD and NETD methods that allow acquisition of highly informative tandem mass spectra of Hep/HS oligosaccharides on a chromatographic time scale. We further present a simple informatics approach that, in principle, permits *de novo* structural determination of Hep/HS oligosaccharides based on their ExD spectra.

Experimental Methods

Materials

Arixtra (GlcNS6S-GlcA-GlcNS3S6S-IdoA2S-GlcNS6S) purchased from Organon Sanofi-Synthelabo LLC (West Orange, NJ) was dialyzed using a 100 Da molecular weight cutoff membrane filter before LC-MS analysis. Hex7S (Δ HexA2S-GlcNS6S-HexA-GlcNAc6S-GlcA-GlcNS3S6S) and Hex6S (Δ HexA2S-GlcNS6S-HexA-GlcNAc6S-GlcA-GlcNS6S) were purchased from New England BioLabs (Ipswich, MA). The synthetic HS tetrasaccharide was generously provided by Professor Geert-Jan Boons from the Complex Carbohydrate Research Center at the University of Georgia.

Mass Spectrometry Analysis

All experiments were performed on a 12-T solariXTM hybrid Qh-Fourier transform ion cyclotron resonance (FTICR) mass spectrometer (Bruker Daltonics, Bremen, Germany). Each HS oligosaccharide standard was dissolved in 5% isopropanol, 0.2% ammonia solution to a concentration of 5 pmol/ μ L, and directly infused into the mass spectrometer using an Apollo II nanoESI source.³⁰ The instrument was operated in the negative ion mode and the instrument parameters were optimized for efficient ion transfer and minimum sulfo group losses. Precursor ions of interest were isolated by the mass-filtering quadrupole and externally accumulated in the hexapole collision cell before tandem MS analysis either in the collision cell (NETD) or in the ICR cell (EDD). In a typical EDD experiment, the cathode heating current was set to 1.6 A, the cathode bias -20 V, the extraction lens potential -19.8 V, and the irradiation time between 0.5 and 1 s. For NETD experiments, fluoranthene cation radicals were generated in the chemical ionization source in the presence of argon. A 500-ms reagent accumulation time and a 500-ms reaction time were typically used to ensure efficient NETD. For all experiments, one analyzer fill was utilized per scan. Each transient was acquired with 1 M data points. Each tandem mass spectrum was acquired by signal averaging up to 100 transients for improved S/N ratio, or 4 acquisitions for the sensitivity test. The instrument was externally calibrated before use with sodium-TFA clusters.

Data Analysis

All mass spectra were recalibrated internally using a few fragment ion peaks assigned with high confidence, resulting in a typical mass accuracy of 1 ppm or less. DataAnalysisTM 4.1 software (Bruker Daltonics, Bremen, Germany) was used for spectral deconvolution, and the SNAPTM algorithm was used for peak picking with the averagine formula set to C1 O1.29 S0.15 H1.55 N0.08. Occasionally, SNAP failed to identify isotopic clusters containing only two isotopic peaks, in which case manual peak picking was performed in the low-mass region for peaks with S/N greater than 10 that appear to have the correct isotope distribution. Peak assignment was achieved using an in-house VBA Excel spreadsheet and the GlycoWorkBench software.^{31,32}

Results and Discussion

Improving the EDD Efficiency

The effect of key experimental parameters that influence the EDD fragmentation efficiency of anionic carbohydrates has been well documented.^{19,33,34} The electron kinetic energy threshold for inducing the EDD fragmentation has been reported to be ~16 eV, below which few odd-electron products characteristic of the radical EDD process were observed.³³ These high-energy electrons can be produced by decreasing the voltage bias of the cathode dispenser (to a more negative value). Meanwhile, the higher negative potential creates a stronger electric field at the emitter surface that lowers the surface barrier seen by the escaping electrons, leading to a higher emission current due to the Schottky effect. The resulting larger electron flux into the ICR cell produces a stronger radial repulsive field that pushes the precursor anions away from the electrons and reduces the anion/electron interaction.³³ It is thus necessary to set the extraction lens potential close to the dispenser bias to minimize the field-enhanced thermionic emission. The more negative extraction lens potential also helps to block electrons at the low-energy end of the electron kinetic energy distribution that contribute little to the EDD process. In addition, undesirable radial repulsion of the electron beam and analyte anions can be alleviated by employing a magnet of higher field strength, as the radial repulsive force can be counterbalanced by a stronger Lorentz force. Over the past few years, significant improvements have been made in commercial FTICR instruments. The solariXTM instrument used here employs ion funnels to more efficiently capture and introduce the electrosprayed ions into the mass spectrometer. In this instrument, the electrostatic lenses have been replaced with multipole ion guides to improve ion transfer into the ICR cell. Furthermore, the FTICR instrument with which the EDD experiments reported herein were performed is equipped with a 12-T magnet, and the higher field strength has led to significant improvement in EDD fragmentation efficiency compared with that reported in earlier studies.

Because EDD is inherently an inefficient process, a large quantity of precursor anions is needed for generation of a sufficient number of EDD fragment ions. Prior EDD studies often employed electrospray of GAG samples in high concentrations (~100 pmol/ μ L at an infusion rate of ~100 μ L/h), prolonged external ion accumulation, and/or multiple ICR cell fills.^{22,27} However, the large sample consumption and the extended experiment time in these studies are not compatible with high-throughput LC-MS/MS analysis of GAG mixtures that are available in limited quantities.

In the present study, a nanoelectrospray ionization (nanoESI) source was used to reduce the sample consumption. It was found that addition of 5% isopropanol and 0.2% ammonium hydroxide (NH₄OH) into the ESI solution can significantly improve the spray stability.³⁰ With the use of the nanoESI source, improved instrumentation, and careful tuning of the ion source and ion transfer parameters to favor production and transfer of labile HS oligosaccharide anions with low m/z values, we found it possible to acquire informative EDD spectra using a very small amount of sample.

Supporting Figure S-1 shows a four-scan EDD spectrum of the quadruply deprotonated Hex7S produced by nanoESI of a Hex7S solution in 5 pmol/ μ L concentration at a spray rate of ~5 μ L/h. Each scan took approximately 2.4 s, corresponding to the consumption of 17 fmol of Hex7S. This result illustrates the feasibility of online LC-MS/MS analysis of highly sulfated GAG samples with EDD as the fragmentation method.

EDD of Hep/HS Oligosaccharides

Three Hep/HS oligosaccharide standards, Arixtra, Hex6S, and Hex7S, were used as model systems to investigate the EDD fragmentation behavior of highly sulfated GAGs. The EDD spectrum of Arixtra in its most abundant charge state, $[M - 4H]^{4-}$, is shown in Figure 1, with its cleavage map in Figure 2a. Unlike its CAD spectrum (Supporting Figure S-2), which is dominated by sequential losses of sulfate groups, this spectrum shows that extensive glycosidic and cross-ring fragments were produced by EDD.

Fragment ions have been labeled using the Domon-Costello nomenclature,³⁵ with a slight modification to account for product ions observed with sulfate loss(es). In all spectra presented, sulfate losses are specified directly, for example, a B_3 ion with loss of one sulfate group is represented as $B_3 - SO_3$. Alternatively, the same ion can be labeled as a $B_3 (4S)$ ion, with the number in the parenthesis indicating the total number of sulfate groups contained in that fragment ion. The later convention allows fragment ion assignments even if the sulfate locations in the precursor ion are unknown. For known structures, a fragment ion without accompanying sulfate loss may also be labeled normally without succeeding parenthesis. For cleavage maps, a variation of the labeling scheme first introduced by Wolff and Amster was used, in which only the maximum sulfation state observed in ions of each cleavage type was indicated by a number next to that cleavage. A number in black suggests that at least some fragment ions were observed with no accompanying losses of sulfates for that cleavage, whereas a number in red flags a cleavage that was always accompanied by some degree of sulfate losses.

As shown in Figure 2a, complete sets of glycosidic bond cleavages were identified, including B_{1-4} , Y_{1-4} , C_{1-4} , and Z_{1-4} ions, the mass differences of which could be used to confirm the sulfate number on each residue. EDD also produced more extensive complementary cross-ring fragments, especially for the sulfated glucosamine residues on both termini, as compared to the reported CAD experiment that had been performed after the Na^+/H^+ exchange.¹⁵ In combination, the cross-ring cleavages produced by EDD and CAD can be interpreted to elucidate all sulfate positions on Arixtra. Meanwhile, the extensive EDD fragmentation also generated many isobaric fragment ions; these were labeled in green on the spectrum shown in Figure 1. The high resolving power offered by the FTICR mass analyzer helped to eliminate some of these ambiguities, but ambiguity in peak assignment can still arise due to the presence of isomeric fragment ions. These isomeric ions were labeled in the cleavage maps by cleavage lines in matching colors, *e.g.* $^{2,4}A_3$ and $^{1,5}X_2$ in purple in Figure 2a.

To explore the effects of the charge state on EDD fragmentation, EDD analysis was also carried out on the Arixtra precursor ions in different charge states under similar experimental conditions. For the $[M - 5H]^{5-}$ (Figure 2b) and $[M - 6H]^{6-}$ (Figure 2c) precursor ions, similar EDD fragmentation patterns were observed, but fragments with low signal intensities in the EDD spectrum of $[M - 4H]^{4-}$ were found to be below the confident detection threshold. It is possible that residues with higher local charge density can prevent electrons from approaching, resulting in the absence of certain product ions. To explore the effects of the overall charge state on sulfate losses from each residue, the I/z (intensity/charge state) of each glycosidic fragment and its various sulfate loss forms were plotted for the EDD spectra of Arixtra in the 4-, 5- and 6- charge states (Figure 3a). Whereas many fragment ions displayed a decrease in the extent of sulfate loss as the precursor ion charge state increased, a significant number of fragment ions showed an opposite trend, indicating that the degree of sulfate loss is dependent on both the charge state and the residue type. Wolff *et al.* reported that the sulfate loss in EDD can be reduced significantly when all sulfate groups are deprotonated in GAG oligosaccharides with low degrees of sulfation.¹⁶ However, such sulfate losses would be inevitable for Arixtra, because the necessary charge

density would be too high to reach full sulfate deprotonation. Nevertheless, even with the EDD spectrum of Arixtra in the lowest charge state (4-) studied, locations of nearly all sulfate groups can be assigned with certainty on the basis of fragment ions that exhibit the maximum number of sulfates for each residue. Moreover, most cleavages that did not produce fragment ions without sulfate losses should not lead to erroneous assignment of the sulfate locations. For example, although the observed maximum sulfation number for the B₃ ion is 4, the presence of its sulfation-site-equivalent C₃ ion in its fully sulfated state (5S) should override the use of the B₃ (4S) ion for sulfation site determination. The ^{3,5}A₃ (2S) ion should also be excluded, as the presence of Z₃ (6S) and C₃ (5S) ions already indicates that the third residue is fully sulfated.

EDD was further applied to analyze the heparin hexasaccharide standards, Hex6S and Hex7S, both of which contain a Δ -unsaturated uronic acid (Δ HexA) residue at the non-reducing end, resembling Hep/HS oligosaccharides produced by polysaccharide lyase digestion. The EDD cleavage maps of Hex6S and Hex7S are shown in Figure 4 and Figure S-3 (Supporting Information), respectively. Extensive fragmentation is observed across different charge states, with fragment ions in their maximum possible sulfation states observed for most cleavages, demonstrating the potential for wide application of EDD to structural analysis of highly sulfated Hep/HS oligosaccharides.

NETD of Hep/HS Oligosaccharides

As discussed above, the inherent inefficiency of EDD arises from the need to overcome Columbic repulsion between like-charge particles to initiate electron detachment. Even with improved instrumentation and EDD performance, only a small percentage of the precursor ions underwent fragmentation. In addition, irradiation with high-energy electrons can deposit a large amount of excess energy, leading to both electronic and vibrational excitations of precursor ions. Consequently, other processes, such as EID and electron impact excitation of ions from organics (EIEIO),³⁶ can also take place. These alternative fragmentation pathways are not initiated by electron detachment, and are generally not radical driven, and often lead to substantial sulfate loss. Finally, the requirement to use electrons in EDD restricts its implementation to the expensive and less frequently available FTICR mass spectrometers.

These limitations can be overcome by using radical cation reagents, rather than high-energy electrons, to induce electron detachment from the precursor ions, as implemented in NETD. Because a cationic radical has a much higher m/z value than an electron, it can be effectively trapped in an RF-only device, such as an ion trap or a multipole collision cell, permitting the implementation of NETD on a wider variety of mass spectrometers.²⁵ Moreover, the energy input in NETD is significantly lower than in EDD, and is largely limited by the electron affinity of the cation radical, and the vibrational excitation of precursor ions can be further reduced by collisional cooling in the higher-pressure ion trapping devices. Most importantly, the oppositely charged precursor anions and radical cation reagents are mutually attracted to one another; this feature should result in a large ion/ion interaction cross section and efficient electron transfer process.

Figure 5 shows the NETD spectrum of the quadruply deprotonated Arixtra, with its cleavage map shown in Figure S-4a (Supporting Information). NETD appears to be a more efficient fragmentation method than EDD (Figure 1), producing fragment ions in much higher abundance. Meanwhile, the precursor ion abundance threshold for successful NETD is less than 5% of that required for EDD, once again demonstrating the higher sensitivity of the NETD method. Although the NETD fragmentation pattern resembles that of EDD (Figure 2a), it produces far fewer sulfate losses, with the result that only one type of fragment ion, B₃, is not observed in its maximum sulfation state. In comparison, EDD produced four

different types of ions with their highest observed sulfation number occurring at a value below their respective theoretical maxima. Figure 3 provides a semi-quantitative comparison of the extent of sulfate losses in glycosidic fragments produced by these two fragmentation processes. For some fragment ion types, *e.g.*, C₁, C₂, and Z₁, sulfate loss was observed in EDD, but not in NETD. Even for fragment ion types in which NETD did induce sulfate losses, the relative abundances of the sulfate loss products compared to their counterpart without sulfate loss are significantly lower in NETD spectra than in EDD spectra. The lower degree of sulfate loss in NETD, in conjunction with its better fragmentation efficiency, makes NETD the superior method for sulfation site determination.

Despite its lower fragmentation efficiency, EDD does exhibit some advantages over NETD. It is well known that ETD works poorly with doubly charged precursor ions, and supplementary collisional activation is often needed to achieve better ETD efficiency. Similarly, it was found that the NETD efficiency was generally lower for precursor ions in lower charge states. Unlike ETD, however, collisional activation cannot be applied here because it will result in extensive sulfate losses from these highly sulfated Hep/HS oligosaccharides. Consequently, for precursor ions in lower charge states, EDD can sometimes generate structurally informative fragment ions complementary to those produced by NETD. For example, confident determination of the number of sulfate groups on the first uronic acid residue in Hex6S requires the presence of a B₁ or a C₁ ion. Both were readily observed in the EDD spectra of Hex6S across all charge states, but were absent in the NETD spectrum of Hex6S in the 4- charge state. Although the C₁ ion was observed in the NETD spectrum of Hex6S in the 5- and 6- charge states, its abundance was quite low (Figure S-5). In addition, because NETD fragmentation and mass analysis occur in two different regions, transfer of NETD fragment ions having either very low and very high *m/z* values from the collision cell to the ICR cell can have poor efficiency due to the time-of-flight effect. EDD, on the other hand, takes place inside the mass analyzer, and all fragment ions can be detected with minimal loss. Thus, it can be advantageous to combine the results from the EDD and NETD analyses for structural characterization of GAG oligosaccharides.

The Informatics Approach

Tandem MS sequencing of GAG oligosaccharides relies on the mass difference between adjacent glycosidic fragments ions to deduce the proper assignment of each residue as well as its modification(s) (in this case, the number of sulfate groups on each residue). Further, the sulfate group(s) within each residue can be located if suitable cross-ring fragment(s) are observed. For example, for Hex6S with its EDD cleavage maps shown in Figure 2, the observation of C₁ (1S) and C₂ (3S) ions can be used to identify the second residue as a glucosamine (GlcN) with two sulfate modifications, and the 6-sulfation on this GlcN residue can be determined based on the presence of the ^{3,5}A₂ (2S) and C₁ (1S) ions. However, such a simplistic approach is flawed in two major aspects. First, it often fails to account for the possibility of isomeric fragment ions that can arise from a single structure. Second, it attempts to assign fragment ions based solely on the known structure of the compound used, without considering alternative interpretations that may be attributed to other possible isomeric structures. It is thus important to develop an unbiased, *de novo* informatics approach to address these limitations.

Complete HS sequencing requires the determination of oligosaccharide composition, sites of acetylation and sulfation, and the stereochemistry of uronic acid residues. Because differentiation of the uronic acid stereoisomers by tandem MS only works well for simple GAG standards with low degree of sulfation, and often involves multivariate statistical analysis of the fragmentation pattern, here, the discussion will focus on the first three tasks. HS composition is typically expressed in the format of [*n*_{ΔHexA}, *n*_{HexA}, *n*_{HexN}, *n*_{Ac}, *n*_S].

Under the constraints of $n_{HexA} = 1$ to 8, $n_{HexN} = n_{HexA} - 1$ to $n_{HexA} + 1$, $n_{Ac} = 0$ to n_{HexN} , and $n_S = 0$ to $(n_{HexA} + 3 * n_{HexN} - n_{Ac})$, there exist 2692 possible HS compositions, none of which has the same mass. The smallest mass separation among these compositions is around 0.139 Da, a value which can be easily detected by an FTICR mass analyzer. With sufficiently high mass accuracy, the composition of an unknown HS oligosaccharide can be determined unambiguously based on its accurate mass measurement.

Once the composition is determined, the next step is location of the acetylation sites among the different HexN residues. Scheme 1 shows two possible acetylation site isomers for the composition [0, 2, 2, 1, 2] (HS composition is given as [Δ HexA, HexA, HexN, Ac, SO_3], representing the number of corresponding groups): G2H0G0A6, and G2A0G0H6, where the shorthand representation of the HS oligosaccharide structures follows the convention introduced by Rosenberg and Esko.³⁷ Intuitively, the acetylation site(s) can be determined based on the presence of diagnostic ion(s). For example, B_2 ions of the first and second isomers have different m/z values. However, due to the symmetry of the HS structure, the Z_2 ion of one isomer is isomeric to the B_2 ion of the other, which can lead to misinterpretation. Although the symmetry is broken for the B_3 and Z_3 ions, the cross-ring fragments, $^{0,2}A_3$ and $^{2,4}X_2$, of the second isomer have the same elemental composition thus the same m/z values as the B_3 and Z_3 ions of the first isomer, respectively. Therefore, it is imperative to consider all possible acetylation site isomers, and rank them based on the total number of observed fragment ions that can be matched to each structure, including fragment ions containing various number of sulfate groups, up to the number of potential sulfation sites or n_S , whichever is smaller.

Sulfation site analysis can then be carried out on the highest ranked acetylation site isomer. Because only ions without sulfate loss provide accurate information on the numbers and locations of sulfate groups on each residue, it is essential that at least some fragments from each cleavage type be observed in their fully sulfated states. The algorithm presented here assumes that this condition holds true for all glycosidic fragments, and indeed this is the case for the EDD and NETD spectra of all HS standards investigated in this study. Fragment ions containing equivalent sulfation sites, e.g., B_n^- , C_n^- , and $^{1,5}A_n^-$ ions, should be grouped together. For each group, the site occupancy number should be determined by the ion(s) containing the highest number of sulfate groups. As discussed above, the sulfation number on a given residue can be determined by the difference in the maximum number of sulfate groups in glycosidic fragments generated on either side of the residue, counting from either the non-reducing or the reducing end. For validation, the sulfation numbers of complementary glycosidic fragments should add up to n_S ; a smaller sum would suggest the absence of fragment ions without sulfate loss, and the sulfation site occupancy of the two residues next to the cleavage site should be deemed undetermined.

A Visual Basic for Applications (VBA) program was created based on the algorithm outlined above for *de novo* HS sequencing (the VBA code is available for download upon request). The VBA program takes as inputs the fragment ion peak list and the precursor ion mass, calculates its composition, ranks all acetylation site isomers, and determines the maximum number of sulfate groups observed in fragments resulting from either side of each glycosidic cleavage. Table 1 shows the output from the program when it was applied to analyze the ExD spectra of several HS standards. For Hex7S (EDD, 5-), the program correctly assigned the acetylation site to the fourth residue as the number of fragments interpretable by that structure is the highest among the three acetylation isomers. It also produced the correct residue-level sulfation pattern as {1, 2, 0, 1, 0, 3} using either the non-reducing end (NRE) or reducing end (RE) fragment ion series. For Hex6S (EDD, 4-), however, the program produced different sulfation patterns depending on whether the NRE or RE fragment ions were used. More surprisingly, the sulfation numbers of some pairs of

complementary glycosidic fragments, such as C₂ and Z₄, and C₄ and Z₂, added up to more than the total number of sulfate groups in the precursor ion. Such an unexpected result can be attributed to the symmetry of the Hex6S structure, which generates isomeric C- and Z-type glycosidic fragments if they contain the same even number of residues barring difference in their sulfation numbers. Consequently, for the native glycans, a C₄ (4S) ion can be misinterpreted as a Z₄ (4S) ion by the *de novo* approach, potentially leading to erroneous determination of the sulfation site occupancy.

As a remedy, the symmetry of the oligosaccharide can be broken by either reduction in D₂O, or reductive amination. Figure 6 shows the EDD and NETD spectra of the synthetic HS standard, where R = (CH₂)₅NH₂. This R-group has an elemental composition sufficiently different from those of typical HS fragments that it allows unambiguous differentiation of reducing-end fragments from nonreducing-end fragments. The modified sequencing algorithm takes an additional input, the mass of the reducing end modifier, and correctly deduces the structure of this modified HS oligosaccharide with no ambiguities.

Similar analyses can also be performed to determine the locations of sulfate group(s) within each residue based on the sulfation number of relevant cross-ring fragments. However, definitive assignment of sulfation sites is often more difficult, because complementary cross-ring fragments are rather rare. An alternative, probability-based informatics approach is currently being developed for GAG sequencing based on their high-resolution ExD spectra, and the initial results show that the correct structure is consistently ranked the highest among all possible isomers. Detailed discussion of this approach is beyond the scope of this paper, and will be presented in a forthcoming publication.

Conclusions

In conclusion, with improved EDD and NETD efficiency, it is now possible to generate extensive, structurally informative fragments on highly sulfated Hep/HS oligosaccharides, even without complete deprotonation. The resulting high-resolution tandem mass spectra can be interpreted *de novo* to deduce the HS structure with the aid of new algorithms developed during these studies. EDD and NETD acquisition times can be short enough to allow measurements to be carried out on a time scale compatible with LC while still providing definitive structural information. Thus, several of the newly available ExD techniques show great promise as tools for structural characterization of highly sulfated HS/Hep oligosaccharides.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

1. Bishop JR, Schuksz M, Esko JD. *Nature*. 2007; 446:1030–1037. [PubMed: 17460664]
2. Capila I, Linhardt RJ. *Angew Chem Int Edit*. 2002; 41:391–412.
3. Esko JD, Selleck SB. *Annu Rev Biochem*. 2002; 71:435–471. [PubMed: 12045103]
4. Esko JD, Lindahl U. *J Clin Invest*. 2001; 108:169–173. [PubMed: 11457867]
5. Zaia J. *Mol Cell Proteomics*. 2013; 12:885–892. [PubMed: 23325770]

6. Chi LL, Amster J, Linhardt RJ. *Curr Anal Chem.* 2005; 1:223–240.
7. Zaia J. *Mass Spectrom Rev.* 2004; 23:161–227. [PubMed: 14966796]
8. Ly M, Leach FE, Laremore TN, Toida T, Amster IJ, Linhardt RJ. *Nat Chem Biol.* 2011; 7:827–833. [PubMed: 21983600]
9. Zaia J, Costello CE. *Anal Chem.* 2003; 75:2445–2455. [PubMed: 12918989]
10. Miller MJC, Costello CE, Malmstrom A, Zaia J. *Glycobiology.* 2006; 16:502–513. [PubMed: 16489125]
11. Zaia J, Miller MJC, Seymour JL, Costello CE. *J Am Soc Mass Spectrom.* 2007; 18:952–960. [PubMed: 17383193]
12. Huang Y, Shi XF, Yu X, Leymarie N, Staples GO, Yin HF, Killeen K, Zaia J. *Anal Chem.* 2011; 83:8222–8229. [PubMed: 21923145]
13. Shi XF, Huang Y, Mao Y, Naimy H, Zaia J. *J Am Soc Mass Spectrom.* 2012; 23:1498–1511. [PubMed: 22825743]
14. Kailemia MJ, Li LY, Xu YM, Liu J, Linhardt RJ, Amster IJ. *Mol Cell Proteomics.* 2013; 12:979–990. [PubMed: 23429520]
15. Kailemia MJ, Li LY, Ly M, Linhardt RJ, Amster IJ. *Anal Chem.* 2012; 84:5475–5478. [PubMed: 22715938]
16. Wolff JJ, Laremore TN, Busch AM, Linhardt RJ, Amster IJ. *J Am Soc Mass Spectrom.* 2008; 19:790–798. [PubMed: 18499037]
17. Wolff JJ, Laremore TN, Leach FE, Linhardt RJ, Amster IJ. *Eur J Mass Spectrom.* 2009; 15:275–281.
18. Yu X, Huang YQ, Lin C, Costello CE. *Anal Chem.* 2012; 84:7487–7494. [PubMed: 22881449]
19. Kornacki JR, Adamson JT, Hakansson K. *J Am Soc Mass Spectrom.* 2012; 23:2031–2042. [PubMed: 22911097]
20. Zhou W, Hakansson K. *Electrophoresis.* 2011; 32:3526–3535. [PubMed: 22120881]
21. Zhou W, Hakansson K. *Curr Proteomics.* 2011; 8:297–308. [PubMed: 22389641]
22. Wolff JJ, Amster IJ, Chi LL, Linhardt RJ. *J Am Soc Mass Spectrom.* 2007; 18:234–244. [PubMed: 17074503]
23. Wolff JJ, Laremore TN, Aslam H, Linhardt RJ, Amster IJ. *J Am Soc Mass Spectrom.* 2008; 19:1449–1458. [PubMed: 18657442]
24. Wolff JJ, Laremore TN, Busch AM, Linhardt RJ, Amster IJ. *J Am Soc Mass Spectrom.* 2008; 19:294–304. [PubMed: 18055211]
25. Wolff JJ, Leach FE, Laremore TN, Kaplan DA, Easterling ML, Linhardt RJ, Amster IJ. *Anal Chem.* 2010; 82:3460–3466. [PubMed: 20380445]
26. Yu X, Jiang Y, Chen Y, Huang Y, Costello CE, Lin C. *Anal Chem.* 2013; 85:10017–10021. [PubMed: 24080071]
27. Leach FE, Wolff JJ, Xiao ZP, Ly M, Laremore TN, Arungundram S, Al-Mafraji K, Venot A, Boons GJ, Linhardt RJ, Amster IJ. *Eur J Mass Spectrom.* 2011; 17:167–176.
28. Leach FE, Arungundram S, Al-Mafraji K, Venot A, Boons GJ, Amster IJ. *Int J Mass Spectrom.* 2012; 330:152–159. [PubMed: 23230388]
29. Leach FE, Xiao ZP, Laremore TN, Linhardt RJ, Amster IJ. *Int J Mass Spectrom.* 2011; 308:253–259. [PubMed: 22247649]
30. Naggar EF, Costello CE, Zaia J. *Glycobiology.* 2003; 13:847–848.
31. Damerell D, Ceroni A, Maass K, Ranzinger R, Dell A, Haslam SM. *Biol Chem.* 2012; 393:1357–1362. [PubMed: 23109548]
32. Ceroni A, Maass K, Geyer H, Geyer R, Dell A, Haslam SM. *J Proteome Res.* 2008; 7:1650–1659. [PubMed: 18311910]
33. Leach FE, Wolff JJ, Laremore TN, Linhardt RJ, Amster IJ. *Int J Mass Spectrom.* 2008; 276:110–115. [PubMed: 19802340]
34. Yang J, Hakansson K. *Int J Mass Spectrom.* 2008; 276:144–148.
35. Domon B, Costello CE. *Glycoconjugate J.* 1988; 5:397–409.
36. Cody RB, Freiser BS. *Anal Chem.* 1979; 51:547–551.

37. Lawrence R, Lu H, Rosenberg RD, Esko JD, Zhang LJ. Nat Methods. 2008; 5:291–292. [PubMed: 18376390]

Figure 1. EDD tandem mass spectrum of Arixtra, $[M - 4H]^{4-}$ (m/z 375.7306). Peaks with definitive assignments are labeled in blue, and peaks with multiple potential assignments are labeled in green. Detailed lists of all assigned peaks for Arixtra and all other structures identified in this study can be found in the supplementary materials (Tables S1-17).

Figure 2.

EDD cleavage maps of Arixtra in three charge states: a) $[M - 4H]^{4-}$, b) $[M - 5H]^{5-}$, and c) $[M - 6H]^{6-}$. The maximum sulfation state observed for a given fragment type is indicated by the number by that cleavage. Dotted lines with matching color are used to label isomeric fragment ions.

Figure 3. Percent of sulfate retention in glycosidic fragment ions generated by a) EDD and b) NETD of Arixtra in three charge states.

Figure 4.
EDD cleavage maps of Hex6S in three charge states, a) $[M - 4H]^{4-}$, b) $[M - 5H]^{5-}$, and c) $[M - 6H]^{6-}$.

Figure 5.
NETD tandem mass spectrum of Arixtra, $[M - 4H]^{4+}$ (m/z 375.7306).

Figure 6.

a) EDD and b) NETD tandem mass spectra and cleavage maps of a synthetic HS standard GlcA-GlcNS-IdoA2S-GlcNS6S-R (G0S0I2S6-R, R = (CH₂)₅NH₂), $[M - 4H]^{4-}$ (m/z 273.2748).

Scheme 1.

Presence of isomeric fragment ions from acetylation positional isomers can confound the acetylation site determination. Two isomers of HS oligosaccharide with the composition [0, 2, 2, 1, 2], G2H0G0A6 (A), and G2A0G0H6 (B), can produce fragment ions with the same elemental composition, as indicated by the dotted lines with the same color.

Table 1

Program output when the *de novo* sequencing algorithm was applied to analyze the ExD spectra of several HS oligosaccharide standards.

GAG	acetylation isomer	# matched fragments	cleaved bond index number	max # sulfation observed (NRE)	max # sulfation observed (RE)
Hex6S (EDD, 4-)	D0A0U0H0U0H0	34 <u>57</u> 39	1	1	5
	D0H0U0A0U0H0		2	3	4
	D0H0U0A0U0H0		3	3	3
	D0H0U0H0U0A0		4	4	3
	D0H0U0H0U0A0		5	4	2
Hex7S (EDD, 5-)	D0A0U0H0U0H0	42 <u>60</u> 36	1	1	6
	D0H0U0A0U0H0		2	3	4
	D0H0U0A0U0H0		3	3	4
	D0H0U0H0U0A0		4	4	3
	D0H0U0H0U0A0		5	4	3
G0S0I2S6-R (EDD, 5-)	<u>U0H0U0H0</u>	<u>38</u>	1	-	4
			2	1	3
			3	2	2