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ELECTRON DETACHMENT DISSOCIATION OF SYNTHETIC HEPARAN SULFATE GLYCOSAMINOGLYCAN TETRASACCHARIDES VARYING IN DEGREE OF SULFATION AND HEXURONIC ACID STEREOCHEMISTRY

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Abstract

Glycosaminoglycan (GAG) carbohydrates provide a challenging analytical target for structural determination due to their polydisperse nature, non-template biosynthesis, and labile sulfate modifications. The resultant structures, although heterogeneous, contain domains which indicate a sulfation pattern or code that correlates to specific function. Mass spectrometry, in particular electron detachment dissociation Fourier transform ion cyclotron resonance (EDD FT-ICR MS), provides a highly sensitive platform for GAG structural analysis by providing cross-ring cleavages for sulfation location and product ions specific to hexuronic acid stereochemistry. To investigate the effect of sulfation pattern and variations in stereochemistry on EDD spectra, a series of synthetic heparan sulfate (HS) tetrasaccharides are examined. Whereas previous studies have focused on lowly sulfated compounds (0.5–1 sulfate groups per disaccharide), the current work extends the application of EDD to more highly sulfated tetrasaccharides (1–2 sulfate groups per disaccharide) and presents the first EDD of a tetrasaccharide containing a sulfated hexuronic acid. For these more highly sulfated HS oligomers, alternative strategies are shown to be effective for extracting full structural details. These strategies include sodium cation replacement of protons, for determining the sites of sulfation, and desulfation of the oligosaccharides for the generation of product ions for assigning uronic acid stereochemistry.

Keywords

Glycosaminoglycan; electron detachment dissociation; EDD; FT-ICR; desulfation; heparan sulfate

INTRODUCTION

Glycosaminoglycan (GAG) carbohydrates are sulfated linear biopolymers that are typically covalently associated with a protein core as proteoglycans. These polydisperse biomolecules are involved in the regulation of biochemical pathways [1–5] and disease progression [6–8] and are largely found in the extracellular matrix and cell surface [9, 10]. The associated GAG chains can be categorized based upon constituent hexose residues, glycosidic linkage between hexoses, extent of O- or N-sulfation and N-acetylation, and hexuronic acid stereochemistry into specific glycoform classes i.e. chondroitin sulfate, heparan sulfate,

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hyaluronan, etc. The heparinoid (heparan sulfate (HS) and heparin) glycosaminoglycans represent one of the most analytically challenging classes of biomolecules based on the high degree of variability in oligomer length, extent and location of sites of sulfation, and hexuronic acid stereochemistry.

The relationship between variability in heparinoid sulfation and epimerization and cellular function [11, 12] has led to increased interest in the characterization of domain structure [13] and sulfation patterns [14]. The most notable of these domains is the five residue heparin sequence for binding to antithrombin [5, 15]. The binding motif consists of eight sulfate groups specifically distributed along the pentasaccharide, of which the tri-sulfated disaccharide (IdoA2S-GlcNS6S) is a key element in protein recognition [16]. Additional variation of domain structure has been observed based upon organism age [17] as well as specificity to organ-type [18]. To fully understand these patterns and variations, there has been increased interest in the full characterization of structure-function relationships, generally known as glycomics [19].

The inherent heterogeneity of these acidic biomolecules has encouraged the application of a variety of analytical techniques for their structural determination. Mass spectrometry has recently been demonstrated as a highly sensitive method for the characterization of GAGs, [20–22] based on the combination of accurate mass measurement of intact oligomers, [23–25] as the capability to determine the locations of labile sulfate modifications, [26–37] and the remarkable ability to assign the hexuronic acid C-5 stereochemistry [28, 38, 39].

Recently developed synthetic methods have provided a unique opportunity to systematically examine the effect of sulfate position and hexuronic acid stereochemistry on electron detachment dissociation (EDD) spectra in a controlled fashion at the tetrasaccharide level [40]. We show here that as the positions and extent of sulfation are varied, appropriate precursor ion selection is required to produce bond cleavages that precisely locate the sites of sulfation as well as to generate product ions that can assign C-5 stereoisomerism in hexuronic acid residues. Specifically, the use of sodium cationization enhances the ability to determine sites of sulfation, and chemical desulfation aids the assignment of uronic acid stereochemistry.

EXPERIMENTAL METHODS

Heparan Sulfate Oligosaccharide Preparation

Heparan sulfate tetrasaccharides were synthesized by a modular approach and purified by silica gel column chromatography [40]. Prepared structures were confirmed by ^1H NMR and accurate mass measurement by FT-ICR MS. Compounds were prepared as tetrasaccharides with varying degrees and positions of sulfation as well as hexuronic acid stereochemistry are depicted in Scheme 1 as structures: 1A – GlcA-GlcNAc6S-GlcA-GlcNAc6S-(CH₂)₅NH₂ 1B – GlcA-GlcNAc6S-IdoA-GlcNAc6S-(CH₂)₅NH₂; 2 – GlcA-GlcNAc6S-IdoA2S-GlcNAc6S-(CH₂)₅NH₂; 3A – GlcA-GlcNS6S-GlcA-GlcNS6S-(CH₂)₅NH₂ 3B – GlcA-GlcNS6S-IdoA-GlcNS6S-(CH₂)₅NH₂.

Tetrasaccharide Desulfation

The pyridinium salts of structures 1A/B were dissolved in 10 % aqueous MeOH and heated for 6 hrs at 60° C to produce the desulfated tetrasaccharides GlcA-GlcNAc-GlcA-GlcNAc-(CH₂)₅NH₂ and GlcA-GlcNAc-IdoA-GlcNAc-(CH₂)₅NH₂ respectively [41]. The mass-selective quadrupole of the FT-ICR MS instrument was employed to isolate the desulfated compounds in lieu of additional laboratory based purification.

Mass Spectrometry Analysis

Experiments were performed with a 9.4 T Bruker Apex Ultra QeFTMS (Billerica, MA) fitted with an MTP dual ion source, 25 W CO₂ laser (Synrad model J48-2, Mukilteo, WA) for IRMPD, and an indirectly heated hollow cathode (HeatWave, Watsonville, CA) to generate electrons for EDD. The sample solutions were infused at a rate of 120 μ L/hour and ionized by electrospray using a metal capillary (Agilent Technologies, Santa Clara, CA, #G2427A) or at 10 μ L/hour and ionized by nanospray (pulled fused silica tip model FS360-75-15-D-20; New Objective, Woburn, MA, USA). Based on the extent of sulfation and desired charge state or degree of sodiation, the ESI solvent was varied. Solutions of each di-sulfated oligosaccharide were introduced at a concentration of 0.1 mg/mL in 50:50:0.1 methanol:H₂O:FA (Sigma, St. Louis, MO) to generate doubly deprotonated ions and 0.2 mg/mL in 50:50 methanol:H₂O with 1% 100 μ M NaOH to generate triply deprotonated ions and sodium adduct ions. To achieve quadruply deprotonated precursor ions for the tetra-sulfated tetrasaccharides, 105 mM sulfolane in acetonitrile was introduced to the ESI line via a line splitter [42]. A secondary syringe pump controlled the relative amount of sulfolane introduced to the ESI solvent. All HS oligosaccharides were examined in negative ion mode.

For EDD experiments, precursor ions were isolated in the external quadrupole and accumulated for 1–3 seconds in an RF only hexapole before injection into the FT-ICR MS cell. One or two quadrupole isolation/analyzer cell fills were utilized per scan. The selection of the precursor ion was further refined by using in-cell isolation with a coherent harmonic excitation frequency (CHEF) event [43]. For electron irradiation the cathode bias was set to -19 V. The extraction lens was set to -18.5 ± 0.1 V, and the cathode heater was set to 1.5 A. The precursor ions were then irradiated with electrons for 1 second. 24–36 acquisitions were signal averaged per mass spectrum. For each mass spectrum, 1M points were acquired, padded with one zero fill, and apodized using a sinebell window. Background spectra were acquired by leaving all parameters the same but setting the cathode bias to 0 V to ensure that no electrons reached the analyzer cell. External calibration of mass spectra produced mass accuracy of 5 ppm. Internal calibration was also performed using confidently assigned glycosidic bond cleavage products as internal calibrants, providing mass accuracy of <1 ppm. Due to the large number of low intensity products formed by EDD, only peaks with S/N > 10 are reported. Product ions were assigned using accurate mass measurement and Glycoworkbench [44]. All products are reported using the annotation proposed by Wolff and Amster [36], derived from the Domon and Costello nomenclature [45]. To account for additional sulfate loss observed during analysis of highly sulfated GAGs, a filled circle has been added to the annotation to indicate a loss of 2 or more sulfate groups as SO₃ (an open circle indicates the loss of one SO₃) from a sulfate half ester. In annotated spectra, the charge states of all ions are 1⁻ unless otherwise indicated.

RESULTS AND DISCUSSION

Electron detachment dissociation (EDD) has previously been applied to the structural characterization of enzymatically prepared heparan sulfate tetrasaccharides ranging in degree of sulfation from zero to two (1 per disaccharide repeat unit) [33, 34]. The examined synthetic heparan sulfate tetrasaccharides contain two to four sulfate modifications and a C-5 stereo-center on each hexuronic acid. Shown in Scheme 2, the synthetic compounds are also modified at the reducing end (RE) to provide targets for protein-GAG interaction screening assays. Although the RE modification is unnatural, it does provide a mass shift in fragments that contain the reducing end and enables the assignment of GAG product ions that would be otherwise be isobaric due to the symmetric nature of oligosaccharides with a constant repeat unit. Comparison with an EDD spectrum obtained from the synthetic GAG lacking the RE modification (data not shown) reveals that the linker has a minimal effect on the product ions that are observed. The synthetic oligosaccharide non-reducing end has a

chiral center at C-5, which is absent in the Δ -uronic acid residues produced by enzymatic cleavage of natural materials previously studied in our laboratory. In the current suite of examined tetrasaccharides, results are presented along the gradient of increasing sulfation from two to four groups. For the di- and tetra-sulfated compounds, epimer pairs are available and determination of the C-5 stereochemistry for the central hexuronic acid residue is treated tangentially during the discussion of sulfo-group location.

Consistent with previous findings for enzymatically prepared GAG oligosaccharides, EDD results in an information rich tandem mass spectrum containing glycosidic and cross-ring bond cleavages [33, 34] that provides more structural information than vibrational activation of the same precursor ion, shown in Figure 1. We previously reported that the application of EDD to HS tetramers produced ions specific to hexuronic acid stereochemistry [38]. For GlcA containing tetrasaccharides, these ions were assigned as $^{0,2}A_3B_3'$, and $B_3'-CO_2$ as well as $^{3,5}A_3$ when an N-sulfo group is present on the amino sugar to the non-reducing side of the acidic residue. The tetrasaccharides chosen for this prior study contained only one sulfate moiety. Selection of a doubly de-protonated precursor ion for EDD therefore resulted in an ionized sulfate and carboxyl, which provided a site for electron detachment and generated stereo-specific product ions based on a proposed radical mechanism. Increasing the degree of sulfation reduces the likelihood that a carboxyl will be ionized as the sulfate group is more acidic. To produce the desired product ions, it is necessary to select a precursor ion with an ionized state corresponding to one more than the number of sulfate groups.

EDD of di-sulfated tetrasaccharides [GlcA-GlcNAc6S-HexA-GlcNAc6S-(CH₂)₅NH₂]

EDD of the doubly de-protonated precursor ion for Structures 1A and 1B results in complete glycosidic bond cleavage as well as abundant cross-ring cleavages as shown in Figure 2. As these tetrasaccharides differ in only the stereochemistry of one hexuronic acid residue, it is not surprising that the fragmentation observed is nearly identical. In each case, the 6-O-sulfation in the GlcNAc residues can be assigned by the presence of cross-ring cleavages. For the reducing end hexosamine residue, the occurrence of $^{2,4}X_0$ and $^{0,2}A_4$ unambiguously locate the site of sulfation to C-6 instead of N. On the central hexosamine residue, the occurrence of five cross-ring cleavages, specifically $^{3,5}A_2$, enables the assignment of 6-O-sulfation.

Although the doubly de-protonated precursor ion should possess two ionized sulfate sites and therefore preclude the possibility of SO₃ loss due to mechanisms known from collisional induced dissociation [22], SO₃ loss is nevertheless observed as minor products, most likely due to electronic excitation channels which are not eliminated by the presence of an ionized sulfate. SO₃ loss occurs largely from glycosidic bond fragments but also from the precursor ion, charge-reduced precursor, and selected cross-ring cleavages ($^{0,2}A_4$ and $^{1,5}X_3$). Additional losses of one or two hydrogen atoms are also observed from glycosidic bond cleavages as well as the loss of CO₂ from the charge reduced species and the Z₂ fragment ion. These products, which are often observed in EDD mass spectra [35], are not found by threshold methods of activation, such as IRMPD or CID, as shown in Figure 1.

Determination of Hexuronic Acid Stereochemistry—Contrary to previous EDD results specific to hexuronic acid stereochemistry in HS, the $^{0,2}A_3$ and B_3' ions are present in both the GlcA spectrum as well as the IdoA spectrum, possibly due to the presence of 6-O-sulfation which may introduce new fragmentation pathways. $B_3'-CO_2$ is not observed in either spectrum. These fragments could arise from a radical mechanism due to electron detachment or from electronic excitation of the precursor ion. To determine the responsible fragmentation mechanism, negative electron transfer dissociation (NETD) of the doubly de-

protonated precursor ion can be applied to assign products due to electron detachment [46], while electron induced dissociation (EID) of the singly de-protonated precursor ion produce fragments due only to electronic excitation [35]. NETD of the doubly de-protonated precursor ions for Structures 1A/B also produces the $^{0,2}A_3$ and B_3' ions in each epimer (data not shown) and indicates the existence of a detachment initiated mechanism not established in the original publication [38].

Selection of a charge state corresponding to the number of sulfates minimizes the loss of SO_3 during ion activation, but it does not lead to stereo-specific product ion formation because carboxyl groups are not ionized. To insure that the occurrence or absence of these product ions is due to a specific stereochemistry, a precursor ion must be selected where the charge state is 3^- (two ionized sulfates and one ionized carboxyl) or a proton has been exchanged for a sodium cation [36]. In the case of direct infusion, these two precursor ions can be generated by addition of NaOH to the ESI solvent.

Although both possibilities place an ionized site at a carboxyl, the resultant EDD spectra differ. The $[M-3H]^{3-}$ precursor for both epimers produces the $^{0,2}A_3$ and B_3' ions whereas $B_3'-CO_2$ is only observed for the GlcA epimer at low intensity (included as supplementary material). To generate product ions indicative of the hexuronic acid stereochemistry, the $[M-3H+1Na]^{2-}$ precursor must be selected. Shown in Figure 3a product ion distribution consistent with prior stereo-specific EDD results is obtained. For the GlcA containing epimer, $^{0,2}A_3+Na$ is observed while this ions is absent in the IdoA epimer ($^{0,2}A_3$ is also only observed in the GlcA epimer but is outside of depicted m/z region). Additionally, $B_3'+Na$ is more intense in the GlcA epimer relative to the B_3+Na intensity.

Desulfation Approach—A more direct approach to determine the hexuronic acid stereochemistry is to desulfate the oligosaccharide, which for a doubly de-protonated tetrasaccharide places an ionized site on each carboxyl and precludes the influence of sulfation on observed production ion distributions. This results in a two-step method for GAG oligosaccharide characterization. The first EDD experiment on the sulfate-intact oligosaccharide is used to determine the sites of sulfation while the second measurement, on desulfated oligosaccharides, provides the data needed to assign stereo-chemical information. Shown in Figure 4, the EDD spectra of the desulfated epimer pair results in unambiguous assignment based upon the occurrence/absence of diagnostic ions known from prior EDD experiments. $^{0,2}A_3$ is observed in the GlcA tetrasaccharide and B_3' is higher in intensity than the B_3 ion. Secondly, in the IdoA tetrasaccharide, C_3'' is more intense than C_3 which is consistent with prior report. Although this method is useful in the case of 6-O sulfation, caution is urged due to potential structural modifications inherent to the removal of N-sulfo groups.

EDD of a tri-sulfated tetrasaccharide [GlcA-GlcNAc6S-IdoA2S-GlcNAc6S-(CH₂)₅NH₂]

Previous EDD experiments on GAGs have examined the fragmentation behavior when sulfates are present on a hexosamine residue. The occurrence of 4/6-O-sulfation is typical for chondroitin sulfate glycoforms and N-sulfation or 6-O-sulfation is typical for heparan sulfate or heparin glycoforms. The 2-O-sulfation of hexuronic acid residues, particularly IdoA, is a common motif in heparin and has not been previously studied by EDD. Increasing the degree of sulfation introduces inherent heterogeneity to the mass spectrum based on sodium exchange and variation in charge state; therefore, multiple precursor ions exist for MS/MS analysis and proper selection is required. It has been previously demonstrated for both threshold and electron-based GAG activation that the precursor should have an ionized state corresponding to at least the number of sulfate groups or pairing sulfates with a metal counter ion to minimize SO_3 loss (In dermatan sulfate oligosaccharides, n+1 ionized sites,

where n is the number of sulfate groups, is typically required [36]). This precursor can be achieved in this case by selecting the $[M-3H]^{3-}$ or $[M-4H+2Na]^{2-}$ state.

EDD activation of the $[M-3H]^{3-}$ precursor ion results in the tandem mass spectrum shown in the accompanying supplementary material. Product ions are observed from charge states ranging from 3^- to 1^- . Charge-conserved fragments due to direct electronic excitation of the $[M-3H]^{3-}$ precursor are assigned as $^{1,5}X_3^{0,2}X_3$ and Y_3/Y_3-SO_3 . Similar to results obtained for DS oligosaccharides [33], the neutral loss of 1 or 2 SO_3 groups directly from the precursor ion is also observed even though the precursor should contain all ionized sulfates. This observation suggests the existence of electronic excitation channels for more highly sulfated HS oligosaccharides through which SO_3 loss can occur even though the presence of an ionized sulfate shunts this channel during threshold activation. Additionally, the loss of 2 SO_3 groups is observed from Y_2 and Y_3 . The first loss in each case occurs with charge conservation whereas the second occurs with charge reduction, e.g. $Y_3^{3-}(Y_3-SO_3)^{3-}$ and $(Y_3-2SO_3)^{2-}$.

The distribution of fragments based on charge state is largely due to the location of the sulfate groups and terminus (non-reducing end, NRE, or reducing end, RE) contained in the fragment. Due to the occurrence of 1 sulfate per residue in this tetrasaccharide, 1^- product ions are assigned as cleavages ranging in oligomer length from B_1 to $^{3,5}A_3$ from the NRE and Z_1/Y_1 from the RE. Increasing the fragment length to C_3 from the NRE or $^{0,2}X_1$ to Y_3 from the RE subsequently generates 2^- product ions due to the presence of two sulfates. Charge reduction by SO_3 loss from an ionized sulfate also generates several 2^- products such as $^{1,5}X_3-SO_3$ and $^{0,2}X_3-SO_3$.

Complete glycosidic bond cleavage is observed which enables the location of two sites of sulfation, one each to an amino sugar, and the third to the central acidic sugar. The location on the acidic residue can be determined as the 2-O position by the occurrence of a $^{0,2}X_1$ cross-ring cleavage. On the hexosamine residues the sulfation site can be assigned as either 6-O- or 3-O- by the $^{0,2}A_2$ and $^{0,2}A_4$ cross-ring cleavages, which precludes the possibility of N-sulfation by fragment mass. Although the $[M-3H]^{3-}$ precursor ion does not provide conclusive evidence for 6-O-sulfation, activation of the $[M-3H+1Na]^{2-}$ precursor produces a $^{2,4}A_2+Na$ which eliminates the possibility of 3-O-sulfation on the central hexosamine and $^{3,5}A_4+Na$ which supports 6-O-sulfation on the reducing end hexosamine (data not shown.) The complete sulfation pattern is therefore –GlcNAc6S-HexA2S-GlcNAc6S.

The capability to locate sulfated residues by glycosidic bond cleavage, and to assign the site of sulfation in a hexose ring via cross-ring cleavage has been demonstrated, but the identification of hexuronic stereochemistry becomes more challenging as the sulfate density increases. To place an ionized site on a carboxyl group for subsequent electron detachment, the $[M-4H]^{4-}$ must be generated or a sufficient number of protons must be exchanged for sodium cations to produce either the $[M-4H+2Na]^{2-}$ or $[M-4H+1Na]^{3-}$ ion. EDD of the $[M-4H+2Na]^{2-}$ ion produces a more intense C_3^{2-} than C_3 . As previously discussed, the absence of $^{0,2}A_3$ and for the C_3^{2-} ion to be greater in intensity than C_3 are the diagnostic conditions for IdoA assignment. This requirement is satisfied, but the GlcA epimer was not available for analysis and subsequent comparison.

Increasing the number of sodium counter ions can provide a means to control the stereochemical-dependent fragmentation, as shown for the di-sulfated and tri-sulfated tetrasaccharides, but there also appears to be a direct effect on the degree of electronic excitation for each precursor state. Electronic excitation is inferred in EDD by the presence of charge conserved product ions, e.g. 2^- products generated by activation of the $[M-2H]^{2-}$ precursor ion. The number and relative intensity of the electronic excitation products

(indicated by circles in the accompanying supplemental material) decreases as the number of sodium counter ions is increased from zero to three. At the maximum number of sodium cations allowable for the 2- precursor, no doubly deprotonated products are observed. This result could be due to the suppression of electronic excitation channels by addition of counter ions. This hypothesis is supported by the reduction in the total number of product ions observed.

EDD of a Tetra-sulfated Tetrasaccharide [GlcA-GlcNS6S-HexA-GlcNS6S-(CH₂)₅NH₂]

The N-sulfo modification is also a common motif in heparinoid GAGs. Addition of N-sulfation to the previously examined di-6-O-sulfated tetrasaccharide produces a tetra-sulfated saccharide containing two sulfates on each amino sugar. Shown in Figure 5, the EDD spectra of the [M-4H]⁴⁻ precursor for the two epimeric possibilities of the central hexuronic acid residue, GlcA (top) and IdoA (bottom) further demonstrate the capability of EDD to generate glycosidic bond cleavage as well as cross-ring cleavage in the presence of a high degree of sulfation. As the sulfate density increases, the need for cross-ring cleavage to locate the sulfate position on the hexose ring is reduced as the possible sites for sulfation are occupied but still necessitated to fully confirm location unless all potential sites on a ring are sulfated.

In the case of the examined tetra-sulfated compounds, the observed glycosidic bond cleavages readily identify each amino sugar as di-sulfated. N-sulfo modification can be determined by the ^{0,2}X₀ cleavage for the reducing end hexosamine and by the difference in mass of the B₂ and ^{0,2}A₂ cleavages for the central amino residue. Alternatively, the extent of N-sulfation compared to N-acetylation can be determined by an accurate mass measurement of the precursor. In cases where there is a mixture of N-sulfo and N-acetyl, bond cleavage analysis is required to identify the residue location. Similar to the tri-sulfated tetrasaccharide, determination of 6-O-sulfation is also not absolute for the precursor containing all ionized sulfates. The addition of 1 sodium counter ion (data not shown) results in the required ^{2,4}A₂ cleavage which places the sulfate at 6-O instead of 3-O.

Extension of C-5 hexuronic acid stereochemical determination is also possible for oligomers with this degree of sulfation and with both O- and N-sulfo groups. Introduction of a single sodium ion into the doubly charged precursor produces sodium attached product ions (B₃' + Na)⁻ and (^{0,2}A₃ + Na)⁻ products that are more intense for tetrasaccharides containing GlcA compared to IdoA, Figure 3. Desulfation of the tetrasaccharides increases the intensities of these diagnostic peaks. Previous EDD-based stereochemical determination has been achieved for a tetrasaccharide with N-sulfation. The introduction of the N-sulfo modification generated a more intense ^{3,5}A₃ product ion in GlcA compared to IdoA by a factor of six [38]. The intensity difference present in the current epimer pair (factor of four) is in agreement and consistent with prior results where a proposed mechanism based on an α-cleavage of an oxy-radical positioned at C3 or C4 generates the ^{3,5}A₃ cleavage with additional slow H-transfer for the cleavage to occur in IdoA with reduced intensity. Although the activated precursor ion charge state corresponded to the number of sulfate groups in this instance, it is possible that an ionized site has moved to the carboxylic acid group to reduce the unfavorable energy of having two sites of negative charge on a single residue.

CONCLUSIONS

The current study presents the first application of EDD to GAG tetrasaccharides containing 1–2 sulfate groups per disaccharide as well as a sulfated hexuronic acid. Consistent with threshold activation, selection of the appropriate precursor ion is necessary to minimize loss of the labile sulfate modification during EDD. The precursor ion typically possesses a

charge state corresponding to the number of sulfate groups. EDD activation of tetrasaccharides ranging in sulfation from two to four produces glycosidic and cross-ring cleavage necessary to locate the sulfate moiety. Unlike previous applications of EDD to GAGs, where the occurrence of an ionized carboxyl was typically assured due to multiple ionized sites and low numbers of the more highly acidic sulfate group, the current work enters a sulfation regime where the likelihood of electron detachment from the carboxylate is diminished. To generate stereo-specific cleavages for GlcA/IdoA determination, the addition of sodium counter ions is required to move an ionized site onto a carboxyl group. Sodium counter ions are also required to increase the abundance of cross-ring cleavages for sulfation location, in some instances, as noted for neutral oligosaccharides by Lebrilla and co-workers [47]. Chemical desulfation can be used to produce samples for which glucuronic acid can be unambiguously distinguished from iduronic acid. However, the desulfation of a highly sulfated oligosaccharides is inefficient and very challenging to accomplish. Other means for assigning uronic acid stereochemistry using complete replacement of ionizable protons by sodium ions are currently under investigation [48].

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Figure 1.
Comparison between threshold and electron-based activation of a di-sulfated synthetic GAG tetrasaccharide, structure 1A.

Figure 2.
EDD spectra of the epimer pair 1A/B. Only glycosidic bond cleavages are annotated.
Complete cleavage information is shown in the Figure 1 inset and is representative of both
epimers.

Figure 3. EDD spectra for the $[M-3H+1Na]^{2-}$ precursor for both 1A and 1B. The diagnostic ion, $^{0,2}A_3+Na$ is present in the GlcA epimer as well as increased fragmentation on the GlcA residue, shown in the structural inset.

Figure 4.

The stereo-specific region EDD spectra obtained from the $[M-2H]^{2-}$ precursor ion of the desulfated compounds for 1A and 1B. Intense $^{0,2}A_3$ and B_3' ions are assigned in the GlcA epimer, compared to IdoA, in addition an intense C_3'' ion in IdoA.

Figure 5.

EDD spectra for the tetra-sulfated epimer pair, structures 3A and 3B. Bracketed ions indicate molecular ions or loss of SO₃ from molecular ions. Parentheses indicate products ions with multiple charges. Products without explicit charge shown are singly-charged.