Published in final edited form as:

J Am Soc Mass Spectrom. 2017 September ; 28(9): 1844–1854. doi:10.1007/s13361-017-1709-9.

Negative Electron Transfer Dissociation Sequencing Of Increasingly Sulfated Glycosaminoglycan Oligosaccharides On An Orbitrap Mass Spectrometer

Franklin E. Leach III¹, Nicholas M. Riley^{2,3}, Michael S. Westphall², Joshua J. Coon^{2,3,4,5}, and I. Jonathan Amster^{1,*}

¹University of Georgia, Athens, GA

²Genome Center of Wisconsin, University of Wisconsin-Madison, Madison, WI, 53706, USA

³Department of Chemistry, University of Wisconsin-Madison, Madison, WI, 53706, USA

⁴Department of Biomolecular Chemistry, University of Wisconsin-Madison, Madison, WI, 53706, USA

⁵Morgridge Institute for Research, Madison, Wisconsin, USA

Abstract

The structural characterization of sulfated glycosaminoglycan (GAG) carbohydrates remains an important target for analytical chemists due to challenges introduced by the natural complexity of these mixtures and the defined need for molecular-level details to elucidate biological structure-function relationships. Tandem mass spectrometry has proven the most powerful technique for this purpose. Previously, electron detachment dissociation (EDD), in comparison to other methods of ion activation, has been shown to provide the largest number of useful cleavages for *de novo* sequencing of GAG oligosaccharides, but such experiments are restricted to Fourier transform ion cyclotron resonance mass spectrometers (FTICR-MS). Negative electron transfer dissociation (NETD) provides similar fragmentation results, and can be achieved on any mass spectrometry platform that is designed to accommodate ion-ion reactions. Here, we examine for the first time the effectiveness of NETD-Orbitrap mass spectrometry for the structural analysis of GAG oligosaccharides. Compounds ranging in size from tetrasaccharides to decasaccharide were dissociated by NETD, producing both glycosidic and cross-ring cleavages that enabled the location of sulfate modifications. The highly-sulfated, heparin-like synthetic GAG, ArixtraTM, was also successfully sequenced by NETD. In comparison to other efforts to sequence GAG chains without fully ionized sulfate constituents, the occurrence of sulfate loss peaks is minimized by judicious precursor ion selection. The results compare quite favorably to prior results with electron detachment dissociation (EDD). Significantly, the duty cycle of the NETD experiment is sufficiently short to make it an effective tool for on-line separations, presenting a straightforward path for selective, high-throughput analysis of GAG mixtures.

Graphical Abstract

* Address for correspondence: I. Jonathan Amster, Department of Chemistry, University of Georgia Athens, GA 30602, Phone: (706) 542-2726, Fax: (706) 542-9454, jamster@uga.edu.

INTRODUCTION

Sulfated glycosaminoglycan (GAG) carbohydrates have presented a persistent challenge to the analytical community based on their enzymatically driven biosynthesis which leads to high variability in the degree of polymerization, hexuronic acid stereochemistry, and location of the labile sulfate half-ester modification. The assignment of sulfate positions in a GAG chain is a significant need as this pattern determines the GAG-protein binding relationship and influences biological processes ranging from cell signaling, infection, and inflammation to metastasis and tumor growth [1–5]. Although the resultant highly heterogeneous GAG chain mixtures have historically defied efforts at full structural characterization, the advent of modern biological mass spectrometry, in particular soft ionization by electrospray (ESI) [6], coupled to advances in isolation, purification, and separation have led to an increasing understanding of GAG structure and functional relationships. The most recent discovery that the structures of GAG polysaccharides in the smallest proteoglycan, bikunin, are far from random, and in fact have a large degree of conservation in their sulfation pattern, demonstrates that sequencing intact GAG chains by mass spectrometry is a tractable analytical problem [7].

Due to the acidic nature of the sulfate and carboxyl groups that decorate GAG chains, they are readily ionized in negative mode MS by ESI. Accurate mass measurement of intact molecular species with high mass resolution yields a chemical composition, from which the degree of polymerization, the extent of O-sulfation of the chain, and N-acetylation or N-sulfation of the hexosamine units can be determined [8, 9]. To define the exact sulfate location in the hexose ring of each monosaccharide constituent, a variety of tandem mass spectrometry approaches including threshold [10] and electron-based [11, 12] methods in conjunction with statistical and computational approaches [13–15] have been applied.

Fourier transform ion cyclotron resonance mass spectrometry (FTICR-MS) provides a powerful platform for GAG structural analysis due to its high mass accuracy and resolution in addition to the capability to perform a variety of ion activation approaches for tandem mass spectrometry (MS/MS) [16]. Collisional, photo-initiated and electron-based dissociation techniques have been utilized to successfully sequence GAG chains ranging from intact polysaccharides (dp27 to dp43) [7] to their depolymerized constituents typically varying from disaccharide (dp2) to decasaccharide (dp10) [10, 17–21]. Regardless of the activation method, the lability of the sulfate half ester modification remains a key analytical concern and appropriate precursor ion selection is mandated to avoid preliminary loss during ionization, ion manipulation, or during activation. If the sulfate remains protonated, a low-energy, facile rearrangement will result in loss of the SO₃, leaving behind a hydroxyl or amino group, and eradicating any information about the location of this modification in the GAG chain. To obtain informative tandem mass spectra of highly sulfated GAGs during threshold activation such as CID, it has been recently demonstrated that either deprotonation or hydrogen/sodium ion exchange can significantly stabilize GAGs against sulfate decomposition [22, 23]. Failure to do so results in additional complexity to the spectrum and induces uncertainty in the assignment of the sulfate location, especially in *de novo* sequencing efforts [24]. Similar approaches have been demonstrated in electron detachment dissociation (EDD) and have enabled sequencing of oligosaccharides shorter than dp10 [25–27], but EDD typically requires activation periods greater than 0.5 s [28] and the addition of excess sodium ions hampers fragmentation channels as the remaining acidic protons are hypothesized to participate in radical rearrangement reactions that lead to fragment ion production [26].

The most desirable fragmentation approach is one in which bond dissociation is performed via non-ergodic, low energy channels that would lessen the strict requirement to fully ionize all sulfate groups while requiring a short duration that would enable coupling to front end separations. The technique that satisfies these two requirements for anion tandem mass spectrometry is negative electron transfer dissociation (NETD) [29, 30], the negative-ion complement of electron transfer dissociation (ETD) [31]. While ETD is most useful for biomolecules that readily form polycations, such as peptides and proteins, NETD was developed for polyanionic species. Tandem MS analysis of GAGs by NETD was first demonstrated using an ion trap mass spectrometer, but the modest resolving power of this instrument limited the ability to identify high charge states, or to make mass measurements of sufficient accuracy to identify products [12]. NETD of GAGs was later demonstrated on an FT-ICR with ion-ion reaction capabilities [32] but initial reaction times ranging from 0.5 s to 1 s were no better in increasing experimental throughput than EDD [28]. In the presented work, we examine the suitability of NETD on a quadrupole linear ion trap-Orbitrap hybrid mass spectrometer to sequence biologically relevant GAG chains on a time scale amenable to capillary-based separation. Orbitrap NETD has been utilized to examine the acidic proteome [33–36], but no published reports of GAG analysis by Orbitrap NETD exist. Orbitrap systems have become the most prevalent FTMS platform in biological mass spectrometry and are capable of reagent ion accumulation and ion-ion reaction times on a scale of tens of milliseconds, especially at higher precursor ion charge states [37]. This increase in MS/MS duty cycle by roughly an order of magnitude compared to previous GAG

NETD studies coupled to the mass resolving power provided by Orbitrap FTMS presents a direct path towards a GAG sequencing approach on widely available MS instrumentation.

METHODS

Well characterized GAG standards were obtained from chemosynthesis or enzymatic depolymerization as detailed below with chemical structures for each compound included as figure insets found in Results and Discussion.

Preparation of synthetic heparan sulfate/heparin oligosaccharides

Heparan sulfate tetrasaccharide and hexasaccharide standards were prepared by chemical synthesis using a modular approach [38] and further purified by silica gel column chromatography. Synthetic product structures were confirmed by ^1H nuclear magnetic resonance (NMR) and accurate mass measurement by FT-ICR MS. The pharmaceutical ArixtraTM was received as a gift.

Preparation of chondroitin sulfate oligosaccharides

Chondroitin sulfate A (CS-A) oligosaccharides were prepared by partial enzymatic depolymerization of bovine trachea chondroitin sulfate (Celsus Laboratories, Cincinnati, OH, USA). A detailed protocol can be found in prior reports [39]. Briefly, the intact oligosaccharide mixture was depolymerized with chondroitin ABC lyase from *Proteus vulgaris*, EC4.2.2.4. (Associates of Cape Cod, E. Falmouth, MA, USA) and reaction progress monitored by UV absorbance at 232 nm. The oligosaccharide mixture was further fractionated by gel-permeation chromatography (GPC), desalted, and lyophilized. Additional fractionation was performed by strong anion exchange, SAX-HPLC. The resulting fractions containing individual oligosaccharides were desalted and characterized by PAGE, ESI-MS, and high-field NMR spectroscopy.

Mass Spectrometry

Mass spectrometry experiments were performed on a modified quadrupole linear ion trap-Orbitrap MS system (Orbitrap Elite, Thermo Scientific) that has been described elsewhere [33, 34, 40]. Additional modifications were made to the instrument control software to enable NETD. Each analyte was directly infused at a concentration of 0.05–0.1 mg/mL and ionized by electrospray. Multiply deprotonated anions were observed for each sample presenting a range of charge states and Na/H exchange for tandem mass spectrometry experiments. Precursor anions were mass selected in the Velos Pro dual linear ion trap and reacted with the radical cation of fluoranthene for 10–100 ms with 24–36 Orbitrap scans acquired at a specified resolution of 120 k and averaged to obtain a tandem mass spectrum for each sample. External calibration of mass spectra resulted in a mass accuracy of 10 ppm or better. Peaks were selected with at least S/N 3. Data analysis was performed manually and in a semi-automated manner with Glycoworkbench [41] or in-lab developed software. Fragment ions are assigned based on the Domon-Costello nomenclature [42] with graphical depictions based on an extension of this approach developed by Wolff-Amster [26].

RESULTS AND DISCUSSION

Previous work in our laboratory demonstrated the first applications of NETD to sequence GAG oligosaccharides. Initial efforts were performed on a quadrupole ion trap mass spectrometer, which had the advantage of facile simultaneous trapping of positive and negative ions. This platform was capable of sequencing tetrasaccharides presented as doubly-charged anions [12], but the resolving power was insufficient for the examination of longer, more highly sulfated and higher charge states. Since the latter are of greater biological relevance, we next attempted NETD on a FT-ICR mass spectrometer [32]. This subsequent work demonstrated a capability to analyze longer GAG chains and more complex MS/MS spectra [32], yet the duty cycle and the ion-ion reaction times (500–1000 ms per scan) for NETD in the prototype instrument precluded the direct coupling to on-line separation methods. In our current efforts, we have ported our NETD sequencing approach to a hybrid Orbitrap FTMS system that provides an efficient platform for ion-ion reactions with times below 100 ms, which is much more amenable to front-end separations.

Prior work in GAG MS/MS has also demonstrated the necessity of judicious precursor selection to retain the labile sulfate half-ester modification [43]. After accurate mass measurement, it is trivial to assign the number of sulfate groups on a given precursor. Failure to choose an ion that presents all sulfates in a deprotonated state will lead to loss of the sulfate as SO_3 during threshold ion activation, with methods such as CID or IRMPD. Due to charge repulsion, it is unfavorable for all of the sulfate groups to carry a formal charge. The replacement of ionizable protons by sodium ions stabilizes sulfo-modifications, particularly as their density in a GAG chain increases.

The investigation of appropriate precursor ion selection in the electron based ion activation of GAGs is still ongoing. We have previously introduced the concept of an “ionized sulfate criteria (ISC)” [27] to determine the minimum ionized condition in GAGs that minimizes sulfate loss during dissociation where the total number of ionized sites (by deprotonation or sodium adduction) should equal or exceed the sulfate number. For chondroitin sulfate GAGs, it was determined that an ISC of 1 was optimal [26]. A systematic study of the appropriate ISC in heparin/heparan sulfate GAGs has yet to be conducted. Here we have selected precursors with an ISC of 0 or higher for fragmentation studies.

Although NETD induces fragmentation via radical pathways, sulfate loss is still observed, even when the optimal precursor is selected. Upon examination of the tandem mass spectrum for each analyte, multiple electron transfer events can be assigned, as well as a competing pathway, proton transfer. This gas phase protonation in turn leads to a facile path for sulfate loss upon mild ion activation which can occur in the high pressure regions of the dual linear ion trap during the NETD ion-ion reaction. Here we have structurally characterized highly sulfated chains that contain on average at least two sulfate groups per disaccharide, which represent a more realistic target for sequencing domains involved in protein binding, and obtained fragmentation consistent with prior NETD results while decreasing the reaction time ~ ten-fold.

Compound 1 – HS tetrasaccharide IdoA-GlcNS6S-IdoA-GlcNS6S-(CH₂)₅NH₂

The MS/MS spectrum shown in Figure 1 results from the NETD of the [M-4H]⁴⁻ precursor ion of the synthetic heparan sulfate (HS) tetrasulfated tetrasaccharide, IdoA-GlcNS6S-IdoA-GlcNS6S-(CH₂)₅NH₂ with an ion-ion reaction time of 40 ms. This species corresponds to the deprotonation of four of the six acidic residues on the tetramer. Based on the lower pK_a of the sulfate moiety, it could be assumed that the two carboxyl groups are protonated, but based on prior experimental observations that protons can become mobile in gas-phase GAG ions [44], the possibility of a protonated sulfate cannot be precluded. As noted previously, two electron transfer events are assigned leading to stable odd-electron [M-4H]^{3-•} and [M-4H]^{2-••} ions. Additionally, one proton transfer event is also observed, forming [M-3H]³⁻.

Depicted in the Figure 1 inset, glycosidic bond cleavages are assigned between all monosaccharide residues with the corresponding B/Y and C/Z ions assigned for each. A single sulfate decomposition (indicated by an open circle) is observed for all fragments except for Y₂. No sulfate groups are present on the non-reducing end (NRE) iduronic acid. The presence of the complete set of glycosidic bond fragments locates two sulfo-modifications on each glucosamine ring. By accurate mass measurement of the precursor alone, it can be determined that both glucosamine residues are *N-sulfo* modified by the difference in sulfo (79.9568 Da) vs acetyl (43.0184 Da). These assignments are further confirmed by the presence of the ^{1,5}A cross-ring cleavages on each hexosamine. The location of the *6-O*-sulfation on each ring can be assigned by the mass difference between the ^{3,5}A_n fragment and preceding B_(n-1) or Y_(n-1) bond cleavage. Although rare, it is possible that the *3-O* position of glucosamine residues may be sulfated, and the generation and observation of cross-ring cleavages (as demonstrated here) is required to differentiate *6-O* vs *3-O* sulfation when all O-sites on a hexose are not fully occupied by a sulfo group.

Compound 2 - HS hexasaccharide GlcA-GlcNS6S-IdoA-GlcNS6S-GlcA-GlcNS6S-(CH₂)₅NH₂

The synthetic heparan sulfate hexasaccharide, Compound 2, is longer than the previously sequenced tetramer by one disaccharide repeat unit, and increases the total number of sulfate groups to six. Although this molecule differs in the hexuronic acid stereochemistry when compared to Compound 1, epimeric variations were not investigated, and recent work has uncovered methods to distinguish some of these epimeric residues [13, 45]. The selected precursor for the presented spectrum in Figure 2 corresponds to 6 ionized sites (5 deprotonated and 1 sodium counter-ion) and was reacted for 25 ms. Similar to compound 1, both electron and proton transfer events are assigned in the mass spectrum with charge-reduced ions detected for [M-6H+1Na]^{4-•} and [M-6H+1Na]^{3-••} and the protonated [M-5H+1Na]⁴⁻.

Glycosidic bond cleavages are assigned for all possibilities as both non- and sodiated ions, except for B₁ which is not detected. As described for Compound 1, through a combination of accurate mass and glycosidic bond cleavage, two sulfate groups can be assigned to each amino sugar with one at the N- position on each residue. Variation is observed in the assigned cross-ring cleavages on the amino sugars, and the assignments utilized to deduce the site of O-sulfation vary slightly along the chain. For the NRE glucosamine, the

difference between the $^{1,5}X_4$ and $^{2,4}X_4$ cleavages supports the existence of 6-*O* sulfation. The central and reducing end sites are both elucidated as 6-*O* sulfates by the assignment of $^{3,5}A$ cleavages and the corresponding B or C glycosidic cleavage that occurs towards the NRE terminus.

Compound 3 – Heparin pentasaccharide– GlcNS6S-GlcA-GlcNS3S6S-IdoA2S-GlcNS6S-OMe

Although the two previously discussed synthetic compounds correspond to chain lengths and sulfation levels that could occur in GAG-protein interaction domains and present realistic targets for method development, they contained a uniform disaccharide repeat leading to a homogeneous structure. We also examined the pharmaceutical product Arixtra™ to establish the suitability of Orbitrap NETD to sequence biologically relevant GAG oligomers with increased variability in sulfation pattern. With ten ionizable acidic sites (eight sulfate/two carboxyl) along the pentasaccharide, a variety of Arixtra™ precursor ions based on the combination of deprotonated and sodiated sites are generated. These data examine a series of increasingly sodiated, pentuply deprotonated ions, $[M-8H+3Na]^{5-}$, $[M-9H+4Na]^{5-}$, and $[M-10H+5Na]^{5-}$. Other recent work has examined the ability of NETD to sequence the $[M-4H]^{4-}$, $[M-5H]^{5-}$, and $[M-6H]^{6-}$ charge states in Arixtra™ [24]. Previously, we have examined an Arixtra™ series with eight to ten deprotonations by CID. At an ionized state of eight, only sulfate loss peaks were observed and no informative glycosidic or cross-ring were detected. It was only the fully deprotonated species of Arixtra™ (through a combination of ionization and sodium-proton exchange) that provided enough fragmentation to allow sequencing by collisional dissociation. In contrast, NETD is more tolerant of remaining ionizable protons.

For the tandem mass spectrum in Figure 3, the triply sodiated precursor ion, $[M-8H+3Na]^{5-}$ was reacted for 20 ms. Additional charge states were examined ranging from five to three minus with reaction times ranging from 15–20 ms to 50 ms, respectively. To maximize the duty cycle, the $[M-5H]^{5-}$ series was selected in the present work. Consistent with the prior heparan sulfate compounds, two electron transfer events are assigned at $[M-8H+3Na]^{4-\bullet}$ and $[M-8H+3Na]^{3-\bullet\bullet}$, as well as a single proton transfer leading to $[M-7H+3Na]^{4-}$. Glycosidic and cross-ring cleavages are assigned along the entirety of the GAG chain and enable the full characterization of all sulfate sites in a single MS/MS event. As the fragment length is increased, the number of possible sodium counter-ions (numerically annotated in the inset) is observed to increase and directly correlates to the number of sulfate groups available for ion-pairing. Sulfate losses are mainly assigned on glycosidic bond cleavages. These assignments are typically more intense than the cross-ring cleavages and the corresponding cross-ring sulfate loss peaks may be below the limit of detection. By accurate mass measurement of the precursor ion, it can be confirmed that eight sulfate groups are present on the pentasaccharide with three at the *N-sulfo* position. The remainder are *O-sulfo* sites and can be assigned as either 6-*O* or 3-*O* on the amino sugars and 2-*O* on the acidic sugars.

Starting from the NRE terminus, the first amino sugar is *N-sulfo* modified by accurate mass measurement of the following B₁ or C₁ glycosidic cleavage and 6-*O* sulfated by the difference in mass of the $^{2,4}X_4$ and $^{1,5}X_4$ cleavages. The second residue can be determined

as unsulfated by the monosaccharide mass deduced in the difference of the neighboring glycosidic bond cleavages. Due to the limited possible sites for O-sulfation on the amino sugar, glycosidic bond assignments we conclude that this residue is fully occupied and definitively locate the presence of 3-*O* and 6-*O* sulfation in addition to the *N-sulfo* group known from the precursor mass. These assignments are further validated by the difference in Y_3/Z_3 and $^{2,4}A_3$ which locates the 3-*O* site and difference in $^{1,5}X_2$ and Y_3/Z_3 . The 3-*O*-sulfation site is typically not observed but its presence in this sulfation pattern known to bind to Antithrombin III [46] and our ability to assign this site demonstrates the necessity of both accurate mass and a sufficient combination of glycosidic and cross-ring cleavages to confidently assign sulfate positions during GAG sequencing efforts. The adjacent hexuronic acid towards the reducing end contains one sulfate group by the increase in mass when the difference between the glycosidic bond cleavages on each side is measured. Similar to the unique assignment of 3-*O* sulfation on amino sugars [46], the only known O-sulfation site on acidic sugars is 2-*O* and is found on the iduronic acid epimers whereas the glucuronic acid isomer is not known to be sulfated [47]. Therefore, the determination of 2-*O* sulfation can be employed to infer the stereochemistry which has a direct effect on the three dimensional conformation of the GAG chain (and therefore protein binding), because the IdoA group can exist in either a chair or boat configuration while the GlcA ring is most stable in the chair configuration. The final O-sulfate group on the RE terminus is determined to be 6-*O* by the presence of $^{3,5}A_5$ and B_4/C_4 .

Increasing the number of ionized sites on the precursor ion beyond the number of sulfate groups by using sodium counter ions directly affects the observed tandem mass spectrum and number of assigned product ions. These changes are depicted in Figures 4 and 5. Although the absolute charge state remains the same, increasing the number of sodium ions reduces the ability of a deprotonated site to move through a mobile proton to a nearby sulfate or carboxyl group. We have previously hypothesized that the absence of mobile protons shunts radical rearrangement reactions that lead to fragment ion generation in gas-phase dermatan sulfate GAG ions [26] and this is the first observation of a similar effect in the electron-based activation of heparin GAG ions. In Figure 4, only one protonated site remains on the precursor ion. The number of assigned cleavages is reduced from 32 to 17 when compared to the triply-sodiated precursor. The number of detected sulfate loss peaks is also reduced with only 5 glycosidic bond cleavages accompanied by these assignments. This result is a direct consequence of the decrease in remaining protons from two to one further reducing the possibility for SO_3 loss by the well-known facile hydrogen rearrangement in sulfate half-esters.

With the reduction in assigned fragment ions, a combination of cleavage and inference based on known biology is required to identify the O-sulfation sites. As previously described, the *N-sulfo* sites are determined readily by precursor mass. The ability to identify the 6-*O* sulfation on both of the termini amino sugars is retained. For the NRE, the difference between $^{1,5}X_4$ and $^{2,4}X_4$, and the RE, the difference between $^{2,4}A_5$ and $^{1,5}A_5$, definitively assign the sulfation site. The remaining sites of 6-, 3-, and 2-*O* sulfation can only be determined by the population of all available sites by a sulfate.

Figure 5 depicts the NETD of Arixtra™ with all acidic sites ionized as $[M-10H+5Na]^{5-}$. Sulfate loss has been further reduced when compared to the two previous Arixtra™ spectra and only occurs due to proton transfer that leads to $[M-9H+5Na]^{4-}$. Without this gas phase protonation event no sulfate decomposition should be observed. Similar fragmentation is observed when compared to the previous NETD spectrum shown in Figure 4 with 17 assigned cleavages. The distributions vary between the two precursor ions, but NETD still generates informative glycosidic and cross-ring cleavages to determine the locations of all *O*-sulfo modifications. As previously described, the *N*-sulfo positions are determined by accurate mass measurement of the precursor ion in addition to the unique mass of each *N*-sulfated hexosamine. *6-O* sulfation can be determined by the mass difference in $^{1,5}X_4$ and $^{2,4}X_4$ on the NRE glucosamine, and on the RE glucosamine by the mass difference in $^{1,5}A_5$ and $^{2,4}A_5$. *2-O* sulfation can be determined on the iduronic acid residue by the occurrence of $^{0,2}X_1$ and the central glucosamine is fully sulfated by occupation of all *O* sites on the hexose.

Compound 4 – Chondroitin sulfate decasaccharide – 4,5 GlcA-(GalNAc4S-GlcA)₄-GalNAc4S

Prior work has demonstrated the utility of NETD to sequence a moderately sulfated (one per disaccharide) dermatan sulfate octamer [32]. Here we have extended this prior art to a chondroitin sulfate decamer presented in Figure 6. NETD sequencing of the $[M-5H]^{5-}$ precursor ion results in a rich mass spectrum whose interpretation benefits from the high resolving power provided by FTMS. The initial characterization of the epimeric octasaccharide required 100 scans with reaction times approaching 1 s. We obtained data of similar quality in 36 scans with reaction times reduced to 35 ms. Accurate mass measurement of the precursor ion indicates the presence of five sulfate groups, all of which are located at *O*-sites. The acquired spectrum contains glycosidic bond cleavages between all monosaccharide residues and enables the location of a single sulfate to each *N-acetyl*-galactosamine. Although cross-ring cleavages are assigned on each residue, none are sufficient to confirm the existence of *4-O* vs. *6-O* sulfation. This deficiency is consistent with prior results in the NETD and electron detachment dissociation (EDD) of chondroitin/dermatan sulfate as removal of an electron from the carboxylate is more energetically favorable [25].

Comparison to Prior EDD and NETD results

EDD has been applied to most sulfated GAG glycoform classes including chondroitin/dermatan sulfates, heparan sulfate, and heparin ranging from tetramers to decamers [11, 25, 27, 39, 48]. EDD consists of two simultaneous/coupled pathways that result in fragment ion production. One, the electron detachment path, leads to radical site based rearrangement mechanisms that produce both odd- and even-electron fragment ions and is shared by NETD. The other is an electronic excitation pathway that results in mostly even-electron fragment ions and is not accessed by NETD. We have previously employed this similarity between EDD and NETD to mechanistically examine the epimeric differentiation of chondroitin sulfate A and B (also denoted as dermatan sulfate) [39]. The low propensity for sulfate decomposition by NETD compared to EDD enables a broader range of ionized states for precursor ion selection. It should be noted that the minimum ISC of 0 has been selected

for this initial comparison of NETD and EDD. Only in the EDD of DS [26] has it been systematically determined that an ISC of 1 is optimal. The determination of the appropriate ISC for the EDD of heparin/heparan sulfate nor the NETD of GAGs has yet to be conducted. Shown in Figure 7 are annotated structures comparing the NETD and EDD of identically ionized and sulfated heparan sulfate tetrasaccharides. Nearly identical fragment ions are assigned in each case with the notable reduction in sulfate loss during NETD depicted in the fragment ion distribution. EDD also requires electron irradiation pulse lengths of approximately 0.5 to 1 s, which makes it less useful for coupling with capillary separation, whereas the demonstrated NETD results in the current work ranged from 10–50 ms and would enable the collection of multiple scans over a separation peak.

Regarding a comparison of NETD on various mass spectrometry platforms, there are only a few published examples of NETD of GAGs using FT-ICR with which to compare. From the limited data this we have examined, NETD on both the Orbitrap and the FT-ICR produce many low intensity fragment ions that are analytically significant, specifically cross-ring cleavages that enable the identification of sulfation positions. In this respect NETD behaves similarly to EDD. However, with the Orbitrap system, there is automated cutoff of low intensity signals, and the user cannot control or alter this setting during the processing of the data. This eliminates some important peaks, and also makes it difficult to make a direct comparison of the signal-to-noise levels for the two platforms.

CONCLUSIONS

Mass spectrometry based approaches for the gas phase sequencing of sulfated glycosaminoglycans continue to progress namely in the extended application of electron-based ion activation and informed precursor ion selection. The application of electron detachment dissociation (EDD) has resulted in an increase of structural information produced during a tandem mass spectrometry experiment, specifically the generation of cross-ring cleavages that enable the location of sulfate groups on each hexose. Although powerful, EDD conversion efficiencies are low and ion activation periods are typically on the order of 0.5–1.0 s per scan. To increase the experimental throughput of hyphenated separation-tandem mass spectrometry methodologies for GAG chain sequencing, ion activation must produce informative fragments in time durations that enable multiple spectra to be obtained during the elution time of a component. Collisional dissociation provides one means to attack this problem, but analyte ions produced with on-line separation do not always possess the appropriate degree of deprotonation to insure the retention of sulfate groups in the gas phase. We have demonstrated negative electron transfer dissociation (NETD) to be a more direct MS/MS solution for gas phase sequencing that can be performed on a millisecond time scale which can adequately sample a separation peak while at the same time be more tolerant of variations in sulfate group ionization states. NETD is observed to have most of the advantages of electron detachment dissociation efforts, particularly with respect to diminished sulfate loss, and NETD is readily coupled to the Orbitrap mass analyzer to provide the benefits of high resolution/accurate mass FTMS for GAG characterization.

Acknowledgments

FEL III and IJA gratefully acknowledge generous financial support from the National Institutes of Health, grants R21HL136271 and P41GM103390. J.J.C. gratefully acknowledges support from NIH grants R35 GM118110 and R21 GM118341, and N.M.R. was funded through an NIH Predoctoral to Postdoctoral Transition Award (F99 CA212454).

References

1. Wu ZL, Zhang L, Yabe T, Kuberan B, Beeler DL, Love A, Rosenberg RD. The Involvement of Heparan Sulfate (Hs) in Fgf1/Hs/Fgfr1 Signaling Complex. *Journal of Biological Chemistry*. 2003; 278:17121–17129. [PubMed: 12604602]
2. Chen Y, Maguire T, Hileman RE, Fromm JR, Esko JD, Linhardt RJ, Marks RM. Dengue Virus Infectivity Depends on Envelope Protein Binding to Target Cell Heparan Sulfate. *Nat Med*. 1997; 3:866–871. [PubMed: 9256277]
3. Liu DF, Shriver Z, Gi YW, Venkataraman G, Sasisekharan R. Dynamic Regulation of Tumor Growth and Metastasis by Heparan Sulfate Glycosaminoglycans. *Seminars in Thrombosis and Hemostasis*. 2002; 28:67–78. [PubMed: 11885027]
4. Williams RK, Straus SE. Specificity and Affinity of Binding of Herpes Simplex Virus Type 2 Glycoprotein B to Glycosaminoglycans. *J Virol*. 1997; 71:1375–1380. [PubMed: 8995662]
5. Fannon M, Forsten KE, Nugent MA. Potentiation and Inhibition of Bfgf Binding by Heparin: A Model for Regulation of Cellular Response. *Biochemistry*. 2000; 39:1434–1445. [PubMed: 10684625]
6. Fenn JB, Mann M, Meng CK, Wong SF, Whitehouse CM. Electrospray Ionization for Mass Spectrometry of Large Biomolecules. *Science*. 1989; 246:64–71. [PubMed: 2675315]
7. Ly M, Leach FE, Laremore TN, Toida T, Amster IJ, Linhardt RJ. The Proteoglycan Bikunin Has a Defined Sequence. *Nat Chem Biol*. 2011; 7:827–833. [PubMed: 21983600]
8. Chi LL, Amster J, Linhardt RJ. Mass Spectrometry for the Analysis of Highly Charged Sulfated Carbohydrates. *Current Analytical Chemistry*. 2005; 1:223–240.
9. Zaia J, Costello CE. Compositional Analysis of Glycosaminoglycans by Electrospray Mass Spectrometry. *Anal Chem*. 2001; 73:233–239. [PubMed: 11199971]
10. Zaia J, Costello CE. Tandem Mass Spectrometry of Sulfated Heparin-Like Glycosaminoglycan Oligosaccharides. *Analytical Chemistry*. 2003; 75:2445–2455. [PubMed: 12918989]
11. Wolff JJ, Amster IJ, Chi L, Linhardt RJ. Electron Detachment Dissociation of Glycosaminoglycan Tetrasaccharides. *Journal of the American Society for Mass Spectrometry*. 2007; 18:234–244. [PubMed: 17074503]
12. Wolff JJ, Leach FE, Laremore TN, Kaplan DA, Easterling ML, Linhardt RJ, Amster IJ. Negative Electron Transfer Dissociation of Glycosaminoglycans. *Analytical Chemistry*. 2010; 82:3460–3466. [PubMed: 20380445]
13. Oh HB, Leach FE III, Arungundram S, Al-Mafraji K, Venot A, Boons G-J, Amster IJ. Multivariate Analysis of Electron Detachment Dissociation Mass Spectra for Synthetic Heparan Sulfate Tetrasaccharides Differing Only in Hexuronic Acid Stereochemistry. 2010 Accepted to *J. Am. Soc. Mass Spectrom*.
14. Hu H, Mao Y, Huang Y, Lin C, Zaia J. Bioinformatics of Glycosaminoglycans. *Perspectives in Science*. 2016
15. Saad OM, Leary JA. Heparin Sequencing Using Enzymatic Digestion and ESI-MS with Host: A Heparin/Hs Oligosaccharide Sequencing Tool. *Analytical Chemistry*. 2005; 77:5902–5911. [PubMed: 16159120]
16. Laremore, TN., Leach, FE., III, Solakyildirim, K., Amster, IJ., Linhardt, RJ. *Methods in Enzymology*. 2010. Glycosaminoglycan Characterization by Electrospray Ionization Mass Spectrometry Including Fourier Transform Mass Spectrometry.
17. Zaia J, McClellan JE, Costello CE. Tandem Mass Spectrometric Determination of the 4s/6s Sulfation Sequence in Chondroitin Sulfate Oligosaccharides. *Analytical Chemistry*. 2001; 73:6030–6039. [PubMed: 11791576]

18. Zaia J, Li XQ, Chan SY, Costello CE. Tandem Mass Spectrometric Strategies for Determination of Sulfation Positions and Uronic Acid Epimerization in Chondroitin Sulfate Oligosaccharides. *Journal of the American Society for Mass Spectrometry*. 2003; 14:1270–1281. [PubMed: 14597117]
19. Desaire H, Leary JA. Detection and Quantification of the Sulfated Disaccharides in Chondroitin Sulfate by Electrospray Tandem Mass Spectrometry. *Journal of the American Society for Mass Spectrometry*. 2000; 11:916–920. [PubMed: 11014453]
20. Saad OM, Leary JA. Compositional Analysis and Quantification of Heparin and Heparan Sulfate by Electrospray Ionization Ion Trap Mass Spectrometry. *Anal Chem*. 2003; 75:2985–2995. [PubMed: 12964742]
21. Racaud A, Antoine R, Joly L, Mesplet N, Dugourd P, Lemoine J. Wavelength-Tunable Ultraviolet Photodissociation (Uvpd) of Heparin-Derived Disaccharides in a Linear Ion Trap. *Journal of the American Society for Mass Spectrometry*. 2009; 20:1645–1651. [PubMed: 19515575]
22. Kailemia MJ, Li L, Ly M, Linhardt RJ, Amster IJ. Complete Mass Spectral Characterization of a Synthetic Ultralow-Molecular-Weight Heparin Using Collision-Induced Dissociation. *Analytical Chemistry*. 2012; 84:5475–5478. [PubMed: 22715938]
23. Kailemia MJ, Li L, Xu Y, Liu J, Linhardt RJ, Amster IJ. Structurally Informative Tandem Mass Spectrometry of Highly Sulfated Natural and Chemoenzymatically Synthesized Heparin and Heparan Sulfate Glycosaminoglycans. *Molecular & Cellular Proteomics*. 2013; 12:979–990. [PubMed: 23429520]
24. Huang Y, Yu X, Mao Y, Costello CE, Zaia J, Lin C. De Novo Sequencing of Heparan Sulfate Oligosaccharides by Electron-Activated Dissociation. *Analytical Chemistry*. 2013; 85:11979–11986. [PubMed: 24224699]
25. Wolff JJ, Laremore TN, Busch AM, Linhardt RJ, Amster IJ. Electron Detachment Dissociation of Dermatan Sulfate Oligosaccharides. *Journal of the American Society for Mass Spectrometry*. 2008; 19:294–304. [PubMed: 18055211]
26. Wolff JJ, Laremore TN, Busch AM, Linhardt RJ, Amster IJ. Influence of Charge State and Sodium Cationization on the Electron Detachment Dissociation and Infrared Multiphoton Dissociation of Glycosaminoglycan Oligosaccharides. *Journal of the American Society for Mass Spectrometry*. 2008; 19:790–798. [PubMed: 18499037]
27. Leach FE III, Xiao Z, Laremore TN, Linhardt RJ, Amster IJ. Electron Detachment Dissociation and Infrared Multiphoton Dissociation of Heparin Tetrasaccharides. *International Journal of Mass Spectrometry*. 2011; 308:253–259. [PubMed: 22247649]
28. Leach FE III, Wolff JJ, Laremore TN, Linhardt RJ, Amster IJ. Evaluation of the Experimental Parameters Which Control Electron Detachment Dissociation, and Their Effect on the Fragmentation Efficiency of Glycosaminoglycan Carbohydrates. *International Journal of Mass Spectrometry*. 2008; 276:110–115. [PubMed: 19802340]
29. Coon JJ, Shabanowitz J, Hunt DF, Syka JEP. Electron Transfer Dissociation of Peptide Anions. *Journal of the American Society for Mass Spectrometry*. 2005; 16:880–882. [PubMed: 15907703]
30. Herron WJ, Goeringer DE, McLuckey SA. Gas-Phase Electron Transfer Reactions from Multiply-Charged Anions to Rare Gas Cations. *Journal of the American Chemical Society*. 1995; 117:11555–11562.
31. Syka JEP, Coon JJ, Schroeder MJ, Shabanowitz J, Hunt DF. Peptide and Protein Sequence Analysis by Electron Transfer Dissociation Mass Spectrometry. *Proc Natl Acad Sci U S A*. 2004; 101:9528–9533. [PubMed: 15210983]
32. Leach FE III, Wolff JJ, Xiao Z, Ly M, Laremore TN, Arungundram S, Al-Mafraji K, Venot A, Boons GJ, Linhardt RJ, Amster IJ. Negative Electron Transfer Dissociation Fourier Transform Mass Spectrometry of Glycosaminoglycan Carbohydrates. *European Journal of Mass Spectrometry*. 2011; 17:167–176. [PubMed: 21719917]
33. McAlister GC, Russell JD, Rumachik NG, Hebert AS, Syka JEP, Geer LY, Westphall MS, Pagliarini DJ, Coon JJ. Analysis of the Acidic Proteome with Negative Electron-Transfer Dissociation Mass Spectrometry. *Analytical Chemistry*. 2012; 84:2875–2882. [PubMed: 22335612]

34. Riley NM, Rush MJP, Rose CM, Richards AL, Kwiecien NW, Bailey DJ, Hebert AS, Westphall MS, Coon JJ. The Negative Mode Proteome with Activated Ion Negative Electron Transfer Dissociation (Ai-Netd). *Molecular & Cellular Proteomics*. 2015; 14:2644–2660. [PubMed: 26193884]
35. Riley NM, Bern M, Westphall MS, Coon JJ. Full-Featured Search Algorithm for Negative Electron-Transfer Dissociation. *Journal of Proteome Research*. 2016; 15:2768–2776. [PubMed: 27402189]
36. Rush MJP, Riley NM, Westphall MS, Syka JEP, Coon JJ. Sulfur Pentafluoride Is a Preferred Reagent Cation for Negative Electron Transfer Dissociation. *Journal of The American Society for Mass Spectrometry*. 2017:1–9.
37. Liu J, McLuckey SA. Electron Transfer Dissociation: Effects of Cation Charge State on Product Partitioning in Ion/Ion Electron Transfer to Multiply Protonated Polypeptides. *International Journal of Mass Spectrometry*. 2012; 330–332:174–181.
38. Arungundram S, Al-Mafraji K, Asong J, Leach FE, Amster IJ, Venot A, Turnbull JE, Boons GJ. Modular Synthesis of Heparan Sulfate Oligosaccharides for Structure-Activity Relationship Studies. *Journal of the American Chemical Society*. 2009; 131:17394–17405. [PubMed: 19904943]
39. Leach F III, Ly M, Laremore T, Wolff J, Perlow J, Linhardt R, Amster IJ. Hexuronic Acid Stereochemistry Determination in Chondroitin Sulfate Glycosaminoglycan Oligosaccharides by Electron Detachment Dissociation. *Journal of the American Society for Mass Spectrometry*. 2012; 23:1488–1497. [PubMed: 22825742]
40. Rose CM, Russell JD, Ledvina AR, McAlister GC, Westphall MS, Griep-Raming J, Schwartz JC, Coon JJ, Syka JEP. Multipurpose Dissociation Cell for Enhanced Etd of Intact Protein Species. *Journal of the American Society for Mass Spectrometry*. 2013; 24:816–827. [PubMed: 23609185]
41. Ceroni A, Maass K, Geyer H, Geyer R, Dell A, Haslam SM. Glycoworkbench: A Tool for the Computer-Assisted Annotation of Mass Spectra of Glycans. *Journal of Proteome Research*. 2008; 7:1650–1659. [PubMed: 18311910]
42. Domon B, Costello CE. A Systematic Nomenclature for Carbohydrate Fragmentations in Fab-MS/Ms Spectra of Glycoconjugates. *Glycoconjugate Journal*. 1988; 5:397–409.
43. Zaia J. Principles of Mass Spectrometry of Glycosaminoglycans. *Journal of Biomolecular Mass Spectrometry*. 2005; 1:3–36.
44. Zaia J, Miller MJC, Seymour JL, Costello CE. The Role of Mobile Protons in Negative Ion CID of Oligosaccharides. *Journal of the American Society for Mass Spectrometry*. 2007; 18:952–960. [PubMed: 17383193]
45. Agyekum I, Zhong C, Boons GJ, Amster IJ. Single Stage Tandem Mass Spectrometry Assignment of the C-5 Uronic Acid Stereochemistry in Heparan Sulfate Tetrasaccharides Using Electron Detachment Dissociation. *Journal of the American Society for Mass Spectrometry Article*. 2017 In Press.
46. Lindahl U, Backstrom G, Thunberg L, Leder IG. Evidence for a 3-O-Sulfated D-Glucosamine Residue in the Antithrombin-Binding Sequence of Heparin. *Proc Natl Acad Sci U S A*. 1980; 77:6551–6555. [PubMed: 6935668]
47. Xiao Z, Zhao W, Yang B, Zhang Z, Guan H, Linhardt RJ. Heparinase 1 Selectivity for the 3,6-Di-O-Sulfo-2-Deoxy-2-Sulfamido- $\pm$ -D-Glucopyranose (1,4) 2-O-Sulfo- $\pm$ -L-Idopyranosyluronic Acid (Glcns3s6s-Idoa2s) Linkages. *Glycobiology*. 2011; 21:13–22. [PubMed: 20729345]
48. Leach FE III, Arungundram S, Al-Mafraji K, Venot A, Boons G-J, Amster IJ. Electron Detachment Dissociation of Synthetic Heparan Sulfate Glycosaminoglycan Tetrasaccharides Varying in Degree of Sulfation and Hexuronic Acid Stereochemistry. *International journal of mass spectrometry*. 2012; 330–332:152–159.

Figure 1. NETD spectrum of the $[M-4H]^4$ - precursor for a synthetic heparan sulfate tetramer with insets indicating assigned fragments and type distribution.

Figure 2. NETD spectrum of the $[M-6H+1Na]^{5-}$ precursor for a synthetic heparan sulfate hexamer with insets indicating assigned fragments and type distribution.

Figure 3.

NETD spectrum of the $[M-8H+3Na]^{5-}$ precursor for a synthetic heparin pentamer with insets indicating assigned fragments and type distribution.

Figure 4.

NETD spectrum of the $[M-9H+4Na]^{5-}$ precursor for a synthetic heparin pentamer with insets indicating assigned fragments and type distribution.

Figure 5.
NETD spectrum of the $[M-10H+5Na]^{5-}$ precursor for a synthetic heparin pentamer with insets indicating assigned fragments and type distribution.

Figure 6.

NETD spectrum of the $[M-5H]^{5-}$ precursor for a chondroitin sulfate A decamer with insets indicating assigned fragments and type distribution.

Figure 7. Annotated structures comparing the NETD and EDD electron-based ion activation for the [M-4H]⁴⁻ precursor in a tetrasulfated tetramer accompanied by a fragment type distribution for each. Note the reduction in sulfate loss from fragment ions during NETD.