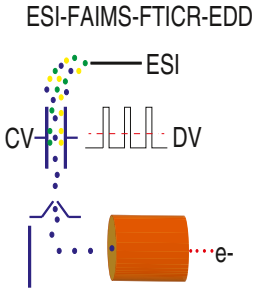
DOI: 10.1007/s13361-013-0771-1

RESEARCH ARTICLE

High-Field Asymmetric-Waveform Ion Mobility Spectrometry and Electron Detachment Dissociation of Isobaric Mixtures of Glycosaminoglycans

Muchena J. Kailemia,[1] Melvin Park,[2] Desmond A. Kaplan,[2] Andre Venot,[3] Geert-Jan Boons,[3] Lingyun Li,[4] Robert J. Linhardt,[4] I. Jonathan Amster[1]

[1]Department of Chemistry, University of Georgia, Athens, GA 30602, USA
[2]Bruker Daltonics, Billerica, MA, USA
[3]Complex Carbohydrate Research Center, University of Georgia, Athens, GA, USA
[4]Department of Chemistry and Chemical Biology, Chemical and Biological Engineering, and Biology, Rensselaer Polytechnic Institute, Troy, NY 12180, USA



Abstract. High-field asymmetric waveform ion mobility spectrometry (FAIMS) is shown to be capable of resolving isomeric and isobaric glycosaminoglycan negative ions and to have great utility for the analysis of this class of molecules when combined with Fourier transform ion cyclotron resonance mass spectrometry (FTICR-MS) and tandem mass spectrometry. Electron detachment dissociation (EDD) and other ion activation methods for tandem mass spectrometry can be used to determine the sites of labile sulfate modifications and for assigning the stereochemistry of hexuronic acid residues of glycosaminoglycans (GAGs). However, mixtures with overlapping mass-to-charge values present a challenge, as their precursor species cannot be resolved by a mass analyzer prior to ion activation. FAIMS is shown to resolve two types of mass-to-charge overlaps. A mixture of chondroitin sulfate A (CSA) oligomers with 4–10 saccharides units produces ions of a single mass-to-charge by electrospray ionization, as the charge state increases in direct proportion to the degree of polymerization for these sulfated carbohydrates. FAIMS is shown to resolve the overlapping charge. A more challenging type of mass-to-charge overlap occurs for mixtures of diastereomers. FAIMS is shown to separate two sets of epimeric GAG tetramers. For the epimer pairs, the complexity of the separation is reduced when the reducing end is alkylated, suggesting that anomers are also resolved by FAIMS. The resolved components were activated by EDD and the fragment ions were analyzed by FTICR-MS. The resulting tandem mass spectra were able to distinguish the two epimers from each other.

Key words: FAIMS, Electron detachment dissociation, Glycosaminoglycans, Fourier transform ion cyclotron resonance mass spectrometry, Differential mobility spectrometry, Fourier transform mass spectrometry, Carbohydrates

Received: 2 August 2013/Revised: 9 October 2013/Accepted: 9 October 2013/Published online: 20 November 2013

Introduction

Glycosaminoglycan (GAGs) are a linear, anionic, structurally diverse, and ubiquitous family of polysaccharides composed of a hexuronic acid and a hexosamine repeating disaccharide unit [1–3]. The identity of the monosaccharide residues, the linkage position of glycosidic bonds between the monosaccharides, as well as the degree and location of sulfate modifications distinguish the various classes of GAGs [2, 4]. Research has shown that modifications of the residues, specifically O-sulfation, N-deacetylation/sulfation, or epimerization of uronic acid residues impacts their biological activity. GAGs are present on all animal cell surfaces and in the extracellular matrix (ECM) within which they regulate many cellular and physiological processes [1–5]. They are known to bind and regulate a number of distinct proteins including small

Electronic supplementary material The online version of this article (doi:10.1007/s13361-013-0771-1) contains supplementary material, which is available to authorized users.

Correspondence to: I. Jonathan Amster; *e-mail:* jamster@uga.edu

secreted proteins (chemokines) [6], small secreted cell signaling proteins (cytokines) [7], growth factors like morphogens [5], enzymes, and adhesion molecules [4], GAGs also modulate cell–cell and cell–extracellular matrix communication, angiogenesis, axonal growth, tumor progression, metastasis, and anti-coagulation [2, 5]. Although numerous functions of GAGs are known, there are only a few function specific sequences whose structures have been fully elucidated [8]. To advance the study of the biological functions of GAGs, sophisticated analytical methods are needed to identify and characterize biologically important motifs within their basic sequences [9]. The structural complexity of GAG molecules, especially heparin and heparan sulfate, makes their structural determination more challenging than other biomolecules. The complexity is a consequence of the non-template nature of their biosynthesis, and the vast number of modifications that occur post-synthesis, including de-acetylation, sulfation, and epimerization, giving rise to a considerable polydispersity and heterogeneity.

Mass spectrometry (MS) using negative mode electrospray ionization (ESI) is widely used for the structural elucidation of GAGs [10, 11]. In addition to retention of sulfo groups during MS analysis and the production of multiply charged ions, ESI can be coupled with liquid chromatography or capillary electrophoresis separation methods that enhance sensitivity and selectivity of the GAG analysis [12]. In order to obtain detailed information about monosaccharide connectivity and their unique modifications, several tandem mass spectrometry (MS/MS) techniques have been examined and refined for the structural analysis of GAGs, including collision induced dissociation (CID[13–18], electron detachment dissociation (EDD) [19–23], and negative electron transfer dissociation (NETD)[24, 25]. These methods have been effective on relatively small oligomers produced by enzymatic digestion of full-length glycans. Recently, full length chains from a proteoglycan have been characterized by Fourier transform ion cyclotron resonance tandem mass spectrometry (FTICR-MS/MS) [8]. Despite a propensity for decomposition of sulfo modifications via SO_3 loss during MS/MS, careful control of the ion activation conditions lead to glycosidic and cross-ring cleavages, which can be used to assign the location of sites of sulfation at both the monosaccharide level and the ring position in GAG oligosaccharides [16, 17, 19, 20, 24–30].

Despite the versatility of mass spectrometry for characterizing GAGs, the complex mixtures produced by enzymatic de-polymerization and the isomeric nature of the resulting sequences require separation steps before they are introduced to the mass spectrometer [31–34]. Chromatographic separation methods such as normal phase and reverse phase high performance liquid chromatograph (HPLC), porous graphite carbon liquid chromatograph (PGC-LC), and hydrophilic interaction liquid chromatography (HILIC) have been used for separation of GAGs [32, 33, 35]. Ion exchange methods such as high-performance anion-exchange chromatography (HPAEC) [36] have also been used for GAG separations. However, these present challenges when combined with MS analysis, requiring desalting or removal of ion pairing reagents prior to introduction to the mass spectrometer [37]. Capillary electrophoresis (CE) methods have also been used for separating and characterizing GAGs [31]. The short time for LC or CE peak elution does not match the long analysis times required for high resolution analysis by FTICR-MS, particularly for ion activation by EDD [21].

As an alternative to liquid separations, there has been considerable recent interest in gas-phase separation of ions prior to mass spectrometry analysis, using ion mobility spectrometry (IMS). IMS separates gas phase ions on the basis of their charge state, overall shape, and the ionic collision cross-section while under a weak, time-invariant electric field [38, 39] and can achieve separation of structural and positional isomers [39–42]. Previously, others have used a combination of ion mobility, mass spectrometry, and proton NMR to characterize iduronic and glucuronic acid containing heparan sulfate hexasaccharides [34] and, recently, IMS was used for separation and analysis of positional isomers of heparin octasaccharides [43]. However, the peak width for a component in an IMS separation is on the order of microseconds, which is far too short to be used for FTICR-MS.

High-field asymmetric waveform ion mobility spectrometry (FAIMS), also known as differential mobility spectrometry (DMS), separates gas-phase ions depending on the differences between their mobility in high and low electric fields and at atmospheric pressure [44, 45]. It is a particularly attractive choice for coupling to FTICR-MS, as ions are separated spatially rather than temporally [45, 46]. With FAIMS, ions are drawn by the flow of buffer gas into a mobility region consisting of two parallel or coaxial electrodes, located between an atmospheric pressure ion source and the inlet to the mass spectrometer. An asymmetric alternating potential, the peak amplitude of which is called the dispersion voltage (DV), is applied between the electrodes producing an electric field tangential to the direction of gas flow. The mobility of ions differ during the high-field and low-field portion of the waveform, leading to their lateral displacement. A small static voltage called the compensation voltage (CV) is applied to one electrode, to direct ions of a particular differential mobility onto the mass spectrometer inlet. In this fashion, ions that have been separated by FAIMS can be continuously admitted into the mass spectrometer, allowing this separation method to be coupled with slow analysis methods such as FTICR-MS. The FAIMS separation is orthogonal to both chromatographic and mass spectrometric techniques, thereby increasing selectivity and specificity of analysis when combined [45, 47].

ESI-FAIMS-MS has been used to effect gas phase separation and differentiation of anomers, linkage, and position isomers of disaccharides [48], glycopeptides [49], and other biomolecules [50–54]. In addition, FAIMS has been used to selectively introduce different gas-phase conformers of ubiquitin ions prior to FTICR-MS analysis, making it possible to resolve and identify different populations of conformers [55]. Here we show the utility of FAIMS as a separation tool

coupled to FTICR-MS and FTICR-MS/MS for the analysis of mixtures of glycosaminoglycans. A chondroitin sulfate A mixture of oligomers of degree of polymerization (dp) 4–10 produces isobaric ions because of the linear relationship between charge and length of the polymer. These cannot be resolved by a mass-to-charge separation, but are resolved by differential mobility spectrometry. A more demanding separation is demonstrated for a mixture of epimeric GAG tetramers, facilitating their MS/MS analysis. The effect of the reducing end anomericity and the behavior of sodiated adducts on the separation are examined. This is the first application of FAIMS separation to GAGs, with subsequent analysis of the separated isobaric molecular ions using EDD.

Experimental

Epimer Synthesis

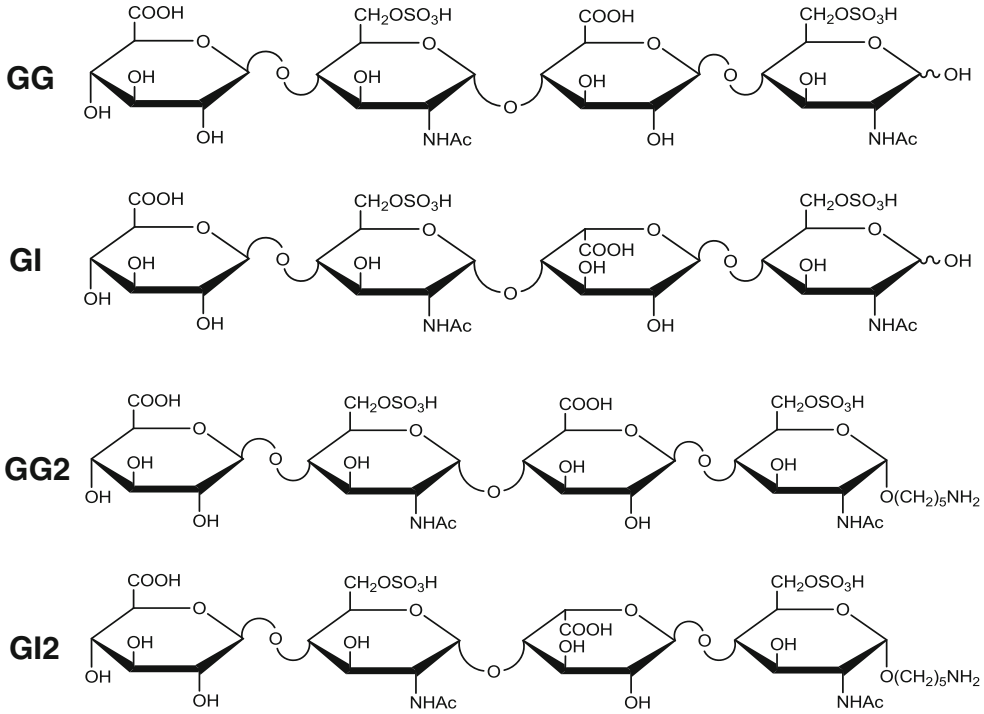
Heparan sulfate tetra-saccharides epimers GlcA-GlcNAc6S-IdoA-GlcNAc6S (GI) and GlcA-GlcNAc6S-GlcA-GlcNAc6S (GG) and GlcA-GlcNAc6S-GlcA-GlcNAc6S-(CH2)5-NH2 (GG2) and GlcA-GlcNAc6S-IdoA-GlcNAc6S-(CH2)5-NH2 (GI2) shown in Scheme 1, were synthesized and purified as described in literature [56]. Use of accurate mass measurement and ^{1}H NMR confirmed the structures of the tetrasaccharides.

Preparation of CS GAG Oligosaccharides

Chondroitin sulfate A (CS-A) oligosaccharides were prepared by partial enzymatic depolymerization of bovine trachea chondroitin sulfate A (Celsus Laboratories, Cincinnati, OH, USA) and purified as described in the previous paper [57]. High-field nuclear magnetic resonance (NMR), ESI-MS, and PAGE were used to characterize the final isolated individual oligosaccharide fractions [58].

ESI FAIMS

Ions were generated using negative mode ESI. Solutions for both tetrasaccharides were made at a concentration of 0.05 mg/mL in 50:50:0.1 MeOH:H_2O:HCOOH (Sigma, St. Louis, MO, USA) unless otherwise stated. All sample solutions were infused at a rate of 120 µL/h using ESI metal capillary (#G2427A; Agilent Technologies, Santa Clara, CA, USA) tip placed about 2–3 mm from the FAIMS curtain gas cap. Generated ions were passed through Bruker Daltonics planar FAIMS analyzer (Billerica, MA, USA) with electrode gap width of 0.5 mm. The FAIMS device was held in place by a cylindrical peek that facilitates proper interfacing to a 9.4 T Bruker Apex IV QeFTMS (Billerica, MA, USA). Ions were separated in FAIMS using 2.4 MHz bi-sinusoidal waveform between dispersion



Scheme 1. Structures for the HS tetrasaccharides used in this study; GG and GI have a free reducing end and they differ only at C5 stereochemistry of the uronic acid residue near the reducing end. GG2 and GI2 epimers have the same sequence like GG and GI but they are alkylated at their reducing end

voltages (DV) 1.50 and 1.95 kV, and the carrier gas used was air. No modifiers were added to the carrier gas.

ESI-FAIMS-EDD

The positive CV scan (0–30 V) was carried out (negative CV scan did not yield any ions) and the specific CV at which the ion of interest appears in the MS was noted and subsequently used to selectively and continuously transmit one ion at a time for EDD experiments. Indirectly heated hollow cathode was used for generating electrons for EDD experiments. Isolation refinement of the precursor was done in the external quadrupole where the ions were accumulated for 1–2.5 s before they enter the FTICR-MS analyzer cell. The isolation/cell fill was repeated up to three times. In-cell isolation with a coherent harmonic excitation frequency (CHEF) event was used for further ion selection. The precursor ions were irradiated with electrons for 1 s. For electron irradiation the cathode bias, ECD lens, and cathode heater were set at –19 V, –18.6 V, and 1.6 A respectively. The 36 acquisitions per mass spectrum were averaged and for each mass spectrum 512 k points were acquired, padded with one zero fill, and apodized using a sine-bell 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. All EDD products are reported using the Domon and Costello nomenclature [59].

Principal Component Analysis (PCA)

PLS toolbox (Eigenvector Research, Inc., Wenatchee, WA, USA) was used for the PCA. The intensity of 19 assigned fragment ions for each spectrum was used for PCA analysis. These fragment ions were selected based on relative intensity and then normalized using the intensity of the base peak from the background spectra. Each row of the data matrix consists of the mass spectra of each particular epimer sample, whereas each column contained the normalized peak intensities for the selected fragment ions. During the PCA analysis, the data was mean-centered and cross-validated. For each epimer peak, the spectra were acquired in quintuplicate starting with the same precursor intensity and using the same EDD experimental conditions.

Results and Discussion

As the MS/MS methods for GAGs have improved, there has been considerable interest in applying these approaches for the analysis of GAGs samples from natural sources, which are often heterogeneous mixtures. Particularly difficult is the characterization of isomeric oligosaccharides, which produce isobaric molecular ions in the MS step and generally identical MS/MS fragmentation patterns. In some cases, a mixture of different length GAGs may produce molecular ions with overlapping mass-to-charge values, resulting from longer oligomers having higher charge states. All these hurdles make complete characterization of GAG mixtures using mass spectrometry difficult. FAIMS separation is orthogonal to mass-to-charge separation in MS and can selectively and continuously transmit an ion of selected differential mobility into the mass spectrometer, thus making it possible to use multiple ion activation methods within a single experiment on an FT-ICR platform. Unlike LC and CE methods, FAIMS has the ability to continuously transmit selected ions, enabling the utilization of a host of ion activation methods for GAGs, some of which require a long activation time (seconds to tens of seconds), and/or significant signal-averaging, such as EDD.

Charge State Separation by ESI-FAIMS

Enzymatic depolymerization of chondroitin sulfate (CS) or dermatan sulfate (DS) yields oligomers of composition ΔHexA(GalNAc SO_3^--HexA)$_n$GalNAcSO_3^-) where n = 1, 2, 3, etc., as shown in the inset of Figure 1a. The mass spectrum in Figure 1a exhibits a peak at *m/z* 458.06, which corresponds to group of isobaric molecular ions $[M - mH]^{m-}$ for Δ-unsaturated CS/DS oligosaccharides of various lengths, with m = n + 1. Although the spacing of the isotope peaks above *m/z* 458.06 indicates the presence of a mixture of different charge states, it is not possible, using mass-to-charge discrimination, to isolate a length-selected component for MS/MS analysis by mass filtering. On the other hand, FAIMS is able to separate this mixture of gas phase ions $[M - mH]^{m-}$ for dp 4, 6, 8, and 10 for chondroitin sulfate (CSA) before the MS analysis.

The mixture that produced the data in Figure 1a was composed of oligomers dp4, dp6, dp8, and dp10. The dispersion voltage (DV) used was 1950 V with a compensation voltage (CV) scan from11 to 20 V, and a step size of 0.2 V. The extracted ion chromatogram (EIC) for ions of *m/z* 458.06 obtained from this scan is shown in Figure 1b, which shows the separation of the various oligomers in the mixture. The mass-to-charge separation of the isotope peaks at CV 12.0 V allows us to assign this peak to dp4 $[M - 2H]^{2-}$. The other charge states in the EIC profile are not baseline resolved but at specific CV only a single charge state is transmitted into the MS. The isotope profile at CV 14.5 V shows that only dp6 $[M - 3H]^{3-}$ is transmitted at that CV, while dp 10 $[M - 5H]^{5-}$ is the sole component passed at a CV of 16.2 V. Interestingly, the number of distinct FAIMS peaks observed is greater than the number of components in the mixture. Some components appear in more than one peak within the CV scan. A mixture of dp4 and dp 6 appear at CV 13.5 V, whereas dp8 $[M - 4H]^{4-}$ and dp10 ion appear at CV 15.4 V. Dp4 and dp6 appear as separate EIC peaks and as a mixture; the same is observed for the dp10 that appears as an individual peak and also as a mixture with dp8 peak. This suggests the existence of different stable conformers or anomers from individual molecular ions that are separated within the FAIMS device, with some having a unique differential mobility that distinguishes them from other charge states, whereas others have similar mobility to other charge states. The extracted ion chromatogram for the second peak in

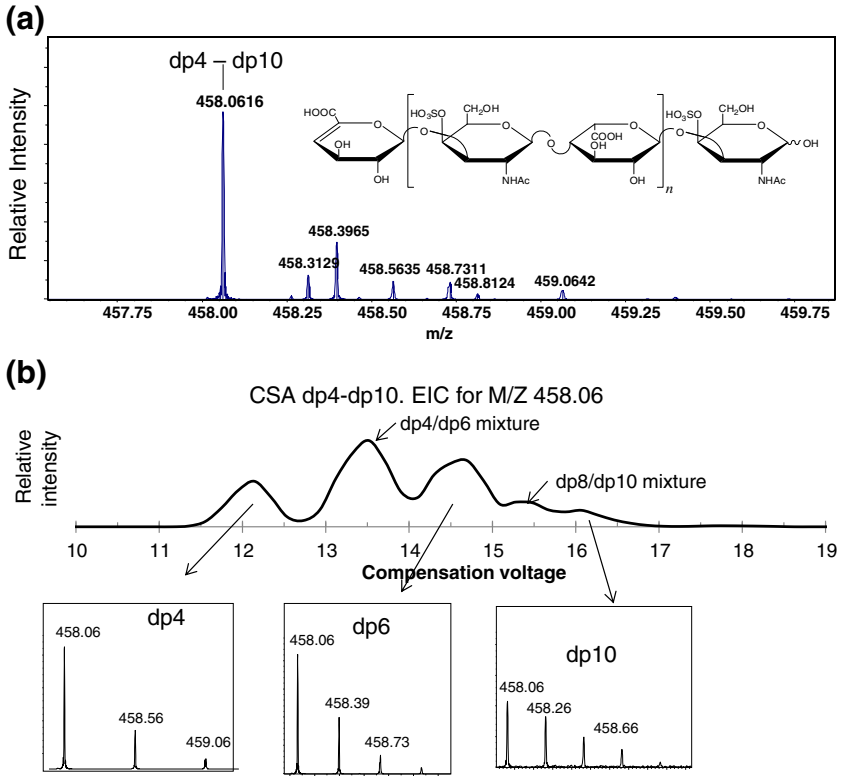


Figure 1. MS and the EIC of the mixture of dp4, dp6, dp8, and dp10 of chondroitin sulfate A (CSA). (**a**) The general structure of the dermatan sulfate (DS) and CS oligomers that ionize to produce an overlapping charge states at *m/z* 458.06 and the mass spectrum of the mixture showing the overlapping isotopic distributions of four different charge states. (**b**) FAIMS MS extracted ion chromatogram (EIC) for *m/z* 458.06 with the respective MS spectra at the indicated CV voltages for which various individual ions are transmitted into the FTICR

each charge state isotope distributions confirms this conclusion (Supplemental information SI, Figure 1).

Epimeric Tetrasaccharides

Even more challenging to analyze is a mixture of diastereomers. The structures of two pairs of synthetically-produced diastereomeric heparan sulfate tetramers, GG, GI, GG2, and GI2, are shown in Scheme 1. The labels denote the type of uronic acid moiety in the first and the third residues, respectively, from the non-reducing end. GG indicates GlcA in the first and third residues from the nonreducing end, whereas GI has IdoA in the third residue, which differs from glucuronic acid only by the stereochemistry of C5. GG2 and GI2 epimers are alkylated at the reducing end, whereas GG and GI contain a free reducing end. In solution, the anomeric isomers of the reducing end sugar are in equilibrium with each other for the non-alkylated samples (GG and GI). In contrast, the alkylated samples (GG2 and GI2) have a single conformation of the anomeric carbon established during synthesis. In order to separate and characterize individual sets of epimeric tetramers, a mixture containing 0.05 mg/mL of each epimer was electro-sprayed using normal electrospray at the rate of 120 μL/h. The dispersion voltage within the electrode gap was 1800 V and four spectra were averaged at every 0.2 V. The molecular ions of interest appeared within a CV range of 9–22 V.

Figure 2 shows the CV scan obtained for the GG and GI molecular ion $[M - 2H]^{2-}$, *m/z* 467.06. The epimer containing two glucuronic acids (GG) produced only one main peak at CV 16.6 V, while epimer (GI) produced two main distinct peaks with the first and the second peak appearing at CV 15.0 V and 18.5 V respectively. There is also a small peak at CV 13.0 V that appears in both epimers. The assignment of the peaks from the two epimers was confirmed by introducing each individual epimer separately into the ESI-FAIMS-MS.

The appearance of multiple peaks for the GI epimer suggests a mixture of conformers or anomers. The reducing end sugar undergoes equilibration in solution, and so both anomers are expected in the sample that was analyzed. The presence of the acyclic form of the reducing end sugar could also introduce an additional component to the mixture, although this form is expected to be a minor component in solution. Finally, a mixture of conformers may also separate by FAIMS. Multiple peaks from a single reducing end monosaccharide residue have been observed in IMS experiments before [40]. Although GG and GI both have

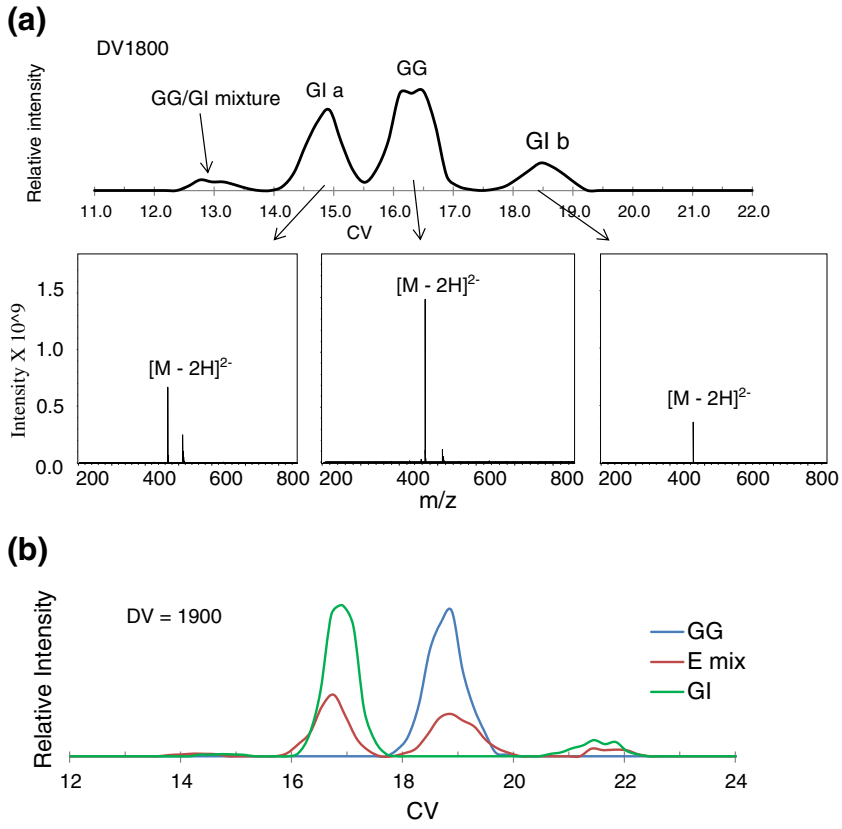


Figure 2. EIC for the epimeric mixture $[M - 2H]^{2-}$ m/z 467.06. (**a**) EIC for a mixture of GG and GI epimers with the MS at different peaks showing the separation of ions of the same mass-to-charge. (**b**) EIC for individual epimers overlayed on the EIC of the mixture. The GG molecular ion appears as a single peak while that of GI appear as two distinct peaks

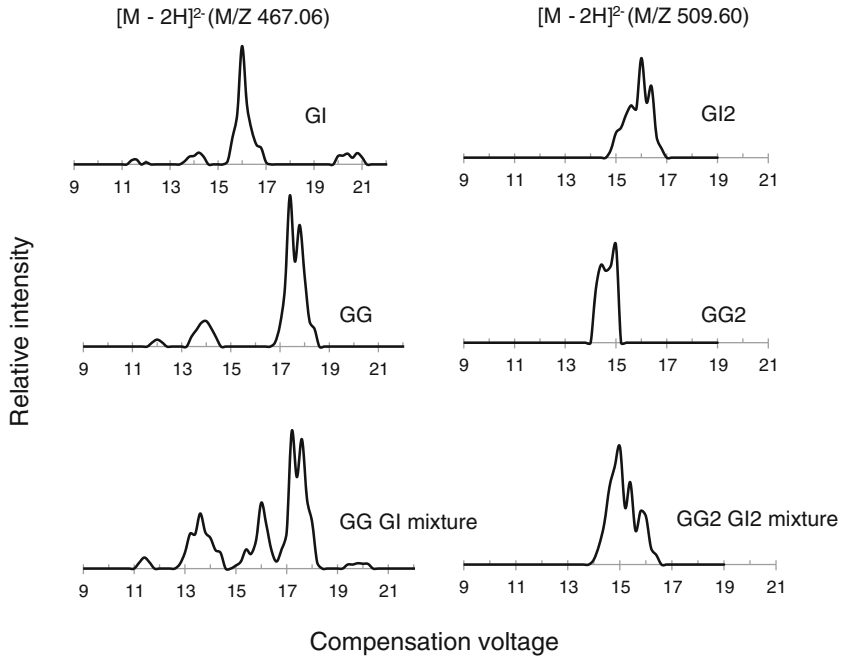


Figure 3. Comparison between the GG and GI $[M - 2H]^{2-}$ (m/z 467.06) EIC with the corresponding $[M - 2H]^{2-}$ (m/z 509.60) EIC obtained from GG2 and GI2 epimers. Multiple peaks are observed from the epimers with a free reducing end whereas only one main peak is observed for GG2 and GI2 epimers. In both cases, ions for each epimer can be separately passed into the FTICR for MS/MS analysis

equal anomeric and acyclic form possibilities, the flexibility of iduronic acid may be responsible for more peaks being observed in the EIC profile of the GI epimer. The appearance of a single major peak for epimer GG could suggest a single dominant conformer or anomer, although it could also indicate a mixture of unresolved components.

Iduronic acid is known to be more flexible than glucuronic acid and to exist as a mixture of different chair and skew conformations including $^{4}C_{1}$, $^{1}C_{4}$, and $^{2}S_{0}$ [60, 61]. This explains the appearance of more peaks for GI than GG $[M - 2H]^{2-}$ molecular ion, although we cannot discount the possibility that the peaks may consist of unresolved multiple gas phase conformers of the molecule originating from the ESI process. The small peak at 13.0 V could be conformers of both epimers having the same FAIMS behavior.

To explore the origin of the multiple peaks in the CV scan, the two epimers that are alkylated at the reducing end, GG2 and GI2, respectively, were selected for FAIMS-MS analysis. With these samples, the reducing end stereochemistry is locked into the α configuration, thus reducing the complexity of the sample. The CV scan obtained for the molecular ion $[M - 2H]^{2-}$ produces only one main peak for each individual epimer as shown in Figure 3, suggesting that some of the additional FAIMS peaks obtained for the GG and GI epimers with a free RE resulted from the anomeric α and β isomers.

Investigation of a higher charge state molecular ion $[M - 3H]^{3-}$ for both sets of epimers produced similar result where two or more peaks are observed for epimers with free reducing end (GG and GI) and only one peak is observed for each epimer GG2 and GI2 (S1, Figure 2). Another observation for the $[M - 3H]^{3-}$ is that there is less separation in the CV scan between the epimers, suggesting closer similarity in configuration when the molecular ion assumes an extended form due to charge-charge repulsion. Although the components are not baseline separated in their CV scan, GG2 and GI2 for $[M - 2H]^{2-}$ (509.60) are well-enough separated to enable selective introduction of an individual epimer ion for MS/MS analysis. Only $[M - 2H]^{2-}$ ion for GG2 ion is observed at CV 14.5 while only GI2 is present at CV 16.0 V. The separation of the alkylated GG2 and GI2 epimers is due to differences in the uronic acid C5 stereochemistry, since the reducing end is alkylated, locking in the stereochemistry of C_1 for these samples. The iduronic acid residue is known to be more

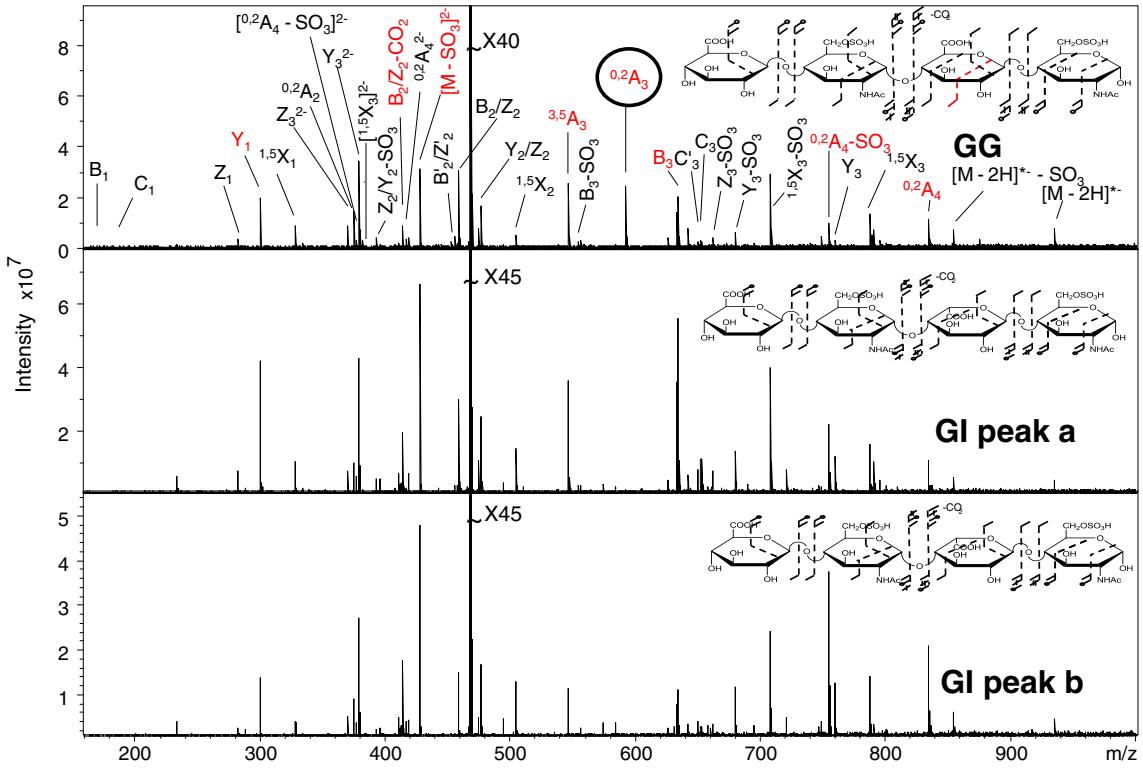


Figure 4. EDD spectra for the FAIMS separated GG and GI epimers, $[M - 2H]^{2-}$ molecular ions. A single GG peak and two GI peaks (GI peak a and b) were isolated from a mixture and subjected to EDD analysis. $^{0,2}A_3$ peak (encircled) is only contained in the GG spectra. EDD spectra from the two GI (GI a and GI b) peaks have many similarities, displaying similar fragmentation patterns, but with consistently different abundances. PCA identified ions that differ significantly within the spectra; these ions are colored in red

flexible that the glucuronic acid residue, causing differences in the overall shape of the molecules. Chemical reduction of the reducing end of GG and GI epimers using $NaBH_4$ utilizing the procedure described before [62] also reduced the number of peaks per single species to one, again suggesting that anomeric mixtures produce multiple peaks in the CV scan of the non-reduced samples (SI, Figure 3).

We also investigated the effect of Na^+ cationization on the FAIMS MS separation of the four epimers by extracting ion chromatograms of the molecular ions that contain Na^+. All four epimers generally produced single main peaks indicating that Na^+ alters the shape of the molecules and, thus, the mobilities within the FAIMS device. Na^+, being larger than the H^+, has been noted to bind multiple oxygen atoms within the GAG molecules, which leads to more stable gas phase ion conformations and, thus, a more defined shape [38]. In this particular case, the presence of Na^+ leads to gas-phase configurations with similar differential mobility. There is less separation for the GG and GI epimers compared with GG2 and GI2 sodiated molecular ions as seen in Supplemental Figure 5. Having a single peak per component during the analysis will be especially important when dealing with complex mixtures.

FAIMS-FTICR-EDD for the Epimers

To explore the potential of applying MS/MS on the separated molecular ions, CV separated GG and GI $[M - 2H]^{2-}$ were selected for EDD experiments. From a CV scan,

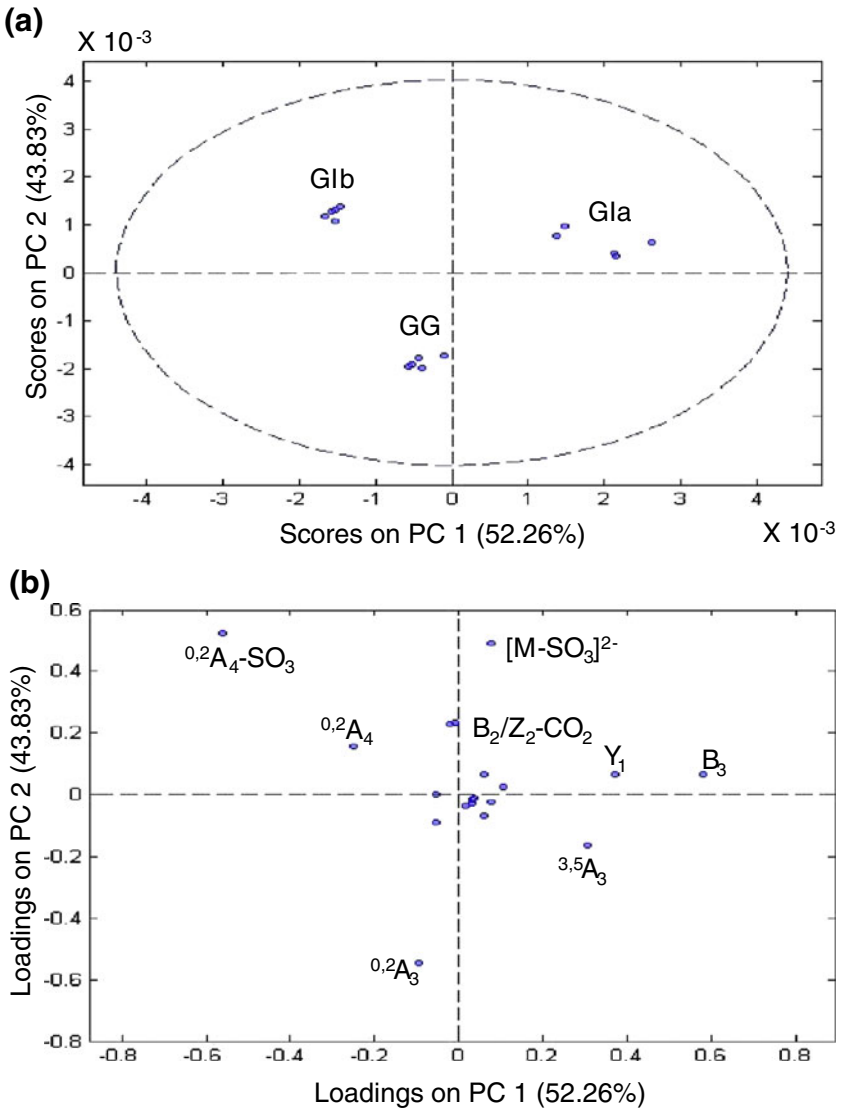


Figure 5. PCA results for the EDD spectra obtained from the three $[M - 2H]^{2-}$ FAIMS separated EIC peaks. (**a**) A plot of the first two principal components distinguishes the three sets of spectra obtained, with 95 % of the spectral differences accounted for by these two principal components. (**b**) The loading plot for the first two principal components reveals the ions whose intensities differentiate the three sets of EDD spectra. The further away the ion is from the center of the plot, the more significant it is in distinguishing the three sets of spectra

the respective value was obtained for the $[M - 2H]^{2-}$ of each epimer, GG and GI. The CV values were used to select a single epimer for the EDD experiments. Although there was a general reduction in signal intensity at high DV, the intensity of the two peaks emanating from the epimer GI differed with the DV voltage applied in a reverse order. The relative intensity of the first peak increased with the increase in the DV voltage, whereas that of the second peak decreased (SI, Figure 4). This behavior is observed for type A and C ions, respectively, with cylindrical FAIMS [45], but there is no focusing effect from inhomogeneous fields with planar FAIMS, so other mechanisms must be responsible, for example heating of the ions by the higher DV producing a change in their conformational equilibrium. DV 1800 V was used for the selection and EDD fragmentation and all other experimental conditions were identical and all the experiments were done the same day. The uniformity of the EDD precursor intensity was ensured for all the selected peaks, and each experiment was done in quintuplicate. Background spectra were acquired by leaving the other experimental conditions constant but changing the value of the ECD bias to zero to ensure no electrons reach the analyzer cell.

EDD spectra obtained from the three peaks are shown in Figure 4. EDD fragmentation patterns were similar to those observed in previous studies [19, 20]. The three spectra are rich in both glycosidic and cross-ring cleavages. The majority of peaks were glycosidic bond cleavages, and among the cross-ring fragments, there are more A type cleavages compared with the X type. Most of the assigned ion peaks are observed in the three spectra except the ion $^{0,2}A_3$ (encircled in Figure 5), which is only present in the GG epimer spectra, consistent with earlier findings on the EDD fragmentation of GlcA versus IdoA [20].

Principal Component Analysis of EDD Data

Multivariate analysis (MVA) of complex data has previously been used to distinguish closely related MS spectra [63, 64] and, more recently, PCA of the EDD data was used to differentiate four epimeric heparan sulfate tetrasaccharides [65]. To compare the fragment ion intensities for the EDD spectra obtained from the three CV values, principal component analysis was used. The data obtained from principal component analysis indicate that the three spectra differ in terms of the fragment ion intensities. As shown in Figure 5a, the first principal component (PC1) distinguishes the two GI peaks in the CV scan, whereas PC2 distinguishes GG spectra from both GI spectra. The two principal components account for 96 % of the spectral differences. A close observation of the loading plot in Figure 5b reveals the fragment ions that differ significantly between the spectra. These includes; $^{0,2}A_4$-SO_3, $^{0,2}A_4$, B_2/Z_2-CO_2, $[M\text{-}SO_3]^{2-}$, B_3, Y_1, $^{3,5}A_3$, and $^{0,2}A_3$ (they are shown in red in Figure 4). Some of these ions have been correlated with differences in the EDD behavior of GlcA versus IdoA [20]. The observed differences in the fragmentation intensities of the two GI peaks show that conformational differences have a significant effect on the dissociation behavior of the gas-phase ions.

Conclusion

EDD is known to be an important tool for the MS/MS characterization of GAGs because of its ability to produce spectra with high-information content, including both glycosidic and cross-ring cleavages. The results obtained in this study indicate that by combining FAIMS with FTICR-MS, it is possible to separate isomeric or isobaric GAG gas phase ions which have the same mass-to-charge, and therefore cannot be separated by a mass analyzer, including stereoisomers or conformers. Such GAG ions can then be characterized using EDD. The fragmentation patterns from EDD of different gas-phase conformations of GI were similar, but with consistently observed intensity differences, as revealed by PCA analysis. This gas-phase separation method can be used as an alternative to solution-phase separation methods such as HPLC to resolve GAGs isomer mixtures. The complexity introduced from single components producing multiple FAIMS peaks can be reduced by removing anomeric isomers from the mixture via alkylating the reducing end or by chemical reduction of the reducing end monosaccharide. A reduction of complexity in the CV scan can also be achieved by using sodium adduction. Sodiated molecular ions have been found to give very detailed structural information for highly sulfated GAGs [16, 28]. The work provides a step forward towards the characterization of these useful biomolecules from their natural sources.

Acknowledgments

M.J.K., L.L., R.J.L., and I.J.A. gratefully acknowledge financial support from the National Institutes of Health, R01-GM038060. M.J.K., A.V., G.J.B., and I.J.A. gratefully acknowledge financial support from the National Institutes of Health, P41-GM103390. M.J.K. personally acknowledges Dr. Franklin Leach for useful discussions about mass spectrometry analysis of GAGs.

References

1. Vynios, D.H., Karamanos, N.K., Tsiganos, C.P.: Advances in analysis of glycosaminoglycans: its application for the assessment of physiological and pathological states of connective tissues. J. Chromatogr. B **781**, 21–38 (2002)
2. Capila, I., Linhardt, R.J.: Heparin–protein interactions. Angew. Chem. Int. Ed. **41**, 390–412 (2002)
3. Hardingham, T., Fosang, A.: Proteoglycans: many forms and many functions. The FASEB J. **6**, 861–870 (1992)
4. Gandhi, N.S., Mancera, R.L.: The structure of glycosaminoglycans and their interactions with proteins. Chem. Biol. Drug Des **72**, 455–482 (2008)
5. Bernfield, M., Götte, M.; Park, P. W., Reizes, O., Fitzgerald, M. L., Lincecum, J., Zako, M.: Functions of cell surface heparan sulfate proteoglycans. Annu. Rev. Biochem. **68**, 729–777 (1999)

6. Lortat-Jacob, H., Grosdidier, A., Imberty, A.: Structural diversity of heparan sulfate binding domains in chemokines. Proc. Natl. Acad. Sci. **99**, 1229–1234 (2002)
7. Gupta, P., McCarthy, J., Verfaillie, C.: Stromal fibroblast heparan sulfate is required for cytokine-mediated ex vivo maintenance of human long-term culture-initiating cells. Blood **87**, 3229–3236 (1996)
8. Ly, M., Leach F.E. III, Laremore, T.N., Toida, T., Amster, I.J., Linhardt, R. J.: The proteoglycan bikunin has a defined sequence. Nat. Chem. Biol. **7**, 827-833 (2011)
9. Guerrini, M., Naggi, A., Guglieri, S., Santarsiero, R., Torri, G.: Complex glycosaminoglycans: profiling substitution patterns by two-dimensional nuclear magnetic resonance spectroscopy. Anal. Biochem. **337**, 35–47 (2005)
10. Oguma, T., Toyoda, H., Toida, T., Imanari, T.: Analytical method of chondroitin/dermatan sulfates using high performance liquid chromatography/turbo ionspray ionization mass spectrometry: application to analyses of the tumor tissue sections on glass slides. Biomed. Chromatogr. **15**, 356–362 (2001)
11. Zaia, J., Costello, C.E.: Compositional analysis of glycosaminoglycans by electrospray mass spectrometry. Anal. Chem. **73**, 233–239 (2000)
12. Zhang, Z., Linhardt, R.J.: Sequence analysis of native oligosaccharides using negative ESI tandem MS. Curr. Anal. Chem. **5**, 225–237 (2009)
13. Zaia, J., McClellan, J.E., Costello, C.E.: Tandem mass spectrometric determination of the 4s/6s sulfation sequence in chondroitin sulfate oligosaccharides. Anal. Chem. **73**, 6030–6039 (2001)
14. Zaia, J., Li, X.-Q., Chan, S.-Y., Costello, C.E.: Tandem mass spectrometric strategies for determination of sulfation positions and uronic acid epimerization in chondroitin sulfate oligosaccharides. J. Am. Soc. Mass Spectrom. **14**, 1270–1281 (2003)
15. Zaia, J., Costello, C.E.: Tandem mass spectrometry of sulfated heparin-like glycosaminoglycan oligosaccharides. Anal. Chem. **75**, 2445–2455 (2003)
16. Kailemia, M.J.; Li, L.; Ly, M.; Linhardt, R.J., Amster, I.J.: Complete mass spectral characterization of a synthetic ultralow-molecular-weight heparin using collision-induced dissociation. Anal. Chem. **84**, 5475–5478 (2012)
17. Saad, O.M., Leary, J.A.: Heparin sequencing using enzymatic digestion and ESI-MS^n with HOST:a heparin/HS oligosaccharide sequencing tool. Anal. Chem. **77**, 5902–5911 (2005)
18. Meissen, J.; Sweeney, M.; Girardi, M.; Lawrence, R.; Esko, J., Leary, J.: Differentiation of 3-O-sulfated heparin disaccharide isomers: identification of structural aspects of the heparin CCL2 binding motif. J. Am. Soc. Mass Spectrom. **20**, 652–657 (2009)
19. Wolff, J.J., Amster, I.J., Chi, L., Linhardt, R.J.: Electron detachment dissociation of glycosaminoglycan tetrasaccharides. J. Am. Soc. Mass Spectrom. **18**, 234–244 (2007)
20. Wolff, J.J., Chi, L., Linhardt, R.J., Amster, I.J.: Distinguishing glucuronic from iduronic acid in glycosaminoglycan tetrasaccharides by using electron detachment dissociation. Anal. Chem. **79**, 2015–2022 (2007)
21. Leach, F.E. III, Wolff, J.J., Laremore, T.N., Linhardt, R.J., Amster, I.J.: Evaluation of the experimental parameters which control electron detachment dissociation, and their effect on the fragmentation efficiency of glycosaminoglycan carbohydrates. Int. J. Mass Spectrom. **276**, 110–115 (2008)
22. Wolff, J.J., Laremore, T.N., Busch, A.M., Linhardt, R.J., Amster, I.J.: Electron detachment dissociation of dermatan sulfate oligosaccharides. J. Am. Soc. Mass Spectrom. **19**, 294–304 (2008)
23. Leach, F.E. III, Xiao, Z., Laremore, T.N., Linhardt, R.J., Amster, I.J.: Electron detachment dissociation and infrared multiphoton dissociation of heparin tetrasaccharides. Int. J. Mass Spectrom. **308**, 253–259 (2011)
24. Wolff, J.J., Leach, F.E., Laremore, T.N., Kaplan, D.A., Easterling, M.L., Linhardt, R.J., Amster, I.J.: Negative electron transfer dissociation of glycosaminoglycans. Anal. Chem. **82**, 3460–3466 (2010)
25. Leach, F.E. III, Wolff, J.J., Xiao, Z., Ly, M., Laremore, T.N., Arungundram, S., Al-Mafraji, K., Venot, A., Boons, G.-J., Linhardt, R.J.: Negative electron transfer dissociation Fourier transform mass spectrometry of glycosaminoglycan carbohydrates. Eur. J. Mass Spectrom. **17**, 167–176 (2011)
26. Zaia, J., Miller, M.J.C., Seymour, J.L., Costello, C.E.: The role of mobile protons in negative ion CID of oligosaccharides. J. Am. Soc. Mass Spectrom. **18**, 952–960 (2007)
27. Naggar, E.F., Costello, C.E., Zaia, J.: Competing fragmentation processes in tandem mass spectra of heparin-like glycosaminoglycans. J. Am. Soc. Mass Spectrom. **15**, 1534–1544 (2004)
28. Kailemia, M.J., Li, L., Xu, Y., Liu, J., Linhardt, R.J., Amster, I.J.: Structurally informative tandem mass spectrometry of highly sulfated natural and chemoenzymatically synthesized heparin and heparan sulfate glycosaminoglycans. Mol. Cell. Proteom. **12**, 979–990 (2013)
29. Desaire, H., Leary, J.A.: The effects of coordination number and ligand size on the gas phase dissociation and stereochemical differentiation of cobalt-coordinated monosaccharides. Int. J. Mass Spectrom. **209**, 171–184 (2001)
30. Wolff, J.J., Laremore, T.N., Aslam, H., Linhardt, R.J., Amster, I.J.: Electron-induced dissociation of glycosaminoglycan tetrasaccharides. J. Am. Soc. Mass Spectrom. **19**, 1449–1458 (2008)
31. Volpi, N., Maccari, F., Linhardt, R.J.: Capillary electrophoresis of complex natural polysaccharides. Electrophoresis **29**, 3095–3106 (2008)
32. Costell, C., Contado-Miller, J., Cipollo, J.: A glycomics platform for the analysis of permethylated oligosaccharide alditols. J. Am. Soc. Mass Spectrom. **18**, 1799–1812 (2007)
33. Hitchcock, A.M., Costello, C.E., Zaia, J.: Glycoform quantification of chondroitin/dermatan sulfate using a liquid chromatography-tandem mass spectrometry platform. Biochemistry (Mosc) **45**, 2350–2361 (2006)
34. Schenauer, M.R., Meissen, J.K., Seo, Y., Ames, J.B., Leary, J.A.: Heparan sulfate separation, sequencing, and isomeric differentiation: ion mobility spectrometry reveals specific iduronic and glucuronic acid-containing hexasaccharides. Anal. Chem. **81**, 10179–10185 (2009)
35. Staples, G.O., Bowman, M.J., Costello, C.E., Hitchcock, A.M., Lau, J.M., Leymarie, N., Miller, C., Naimy, H., Shi, X., Zaia, J.: A chip-based amide-HILIC LC/MS platform for glycosaminoglycan glycomics profiling. Proteomics **9**, 686–695 (2009)
36. Bruggink, C., Wuhrer, M., Koeleman, C.A.M., Barreto, V., Liu, Y., Pohl, C., Ingendoh, A., Hokke, C.H., Deelder, A.M.: Oligosaccharide analysis by capillary-scale high-pH anion-exchange chromatography with on-line ion-trap mass spectrometry. J. Chromatogr. B **829**, 136–143 (2005)
37. Maslen, S., Sadowski, P., Adam, A., Lilley, K., Stephens, E.: Differentiation of isomeric N-glycan structures by normal-phase liquid chromatography-MALDI-TOF/TOF tandem mass spectrometry. Anal. Chem. **78**, 8491–8498 (2006)
38. Jin, L., Barran, P.E., Deakin, J.A., Lyon, M., Uhrín, D.: Conformation of glycosaminoglycans by ion mobility mass spectrometry and molecular modelling. PCCP **7**, 3464–3471 (2005)
39. Fenn, L.S., McLean, J.A.: Structural resolution of carbohydrate positional and structural isomers based on gas-phase ion mobility-mass spectrometry. PCCP **13**, 2196–2205 (2011)
40. Dwivedi, P., Bendiak, B., Clowers, B.H., Hill, H. H. Jr.: Rapid resolution of carbohydrate isomers by electrospray ionization ambient pressure ion mobility spectrometry-time-of-flight mass spectrometry (ESI-APIMS-TOFMS). J. Am. Soc. Mass Spectrom. **18**, 1163–1175 (2007)
41. Clowers, B.H., Dwivedi, P., Steiner, W.E., Hill, J.H.H., Bendiak, B.: Separation of sodiated isobaric disaccharides and trisaccharides using electrospray ionization-atmospheric pressure ion mobility-time of flight mass spectrometry. J. Am. Soc. Mass Spectrom. **16**, 660–669 (2005)
42. Li, H., Giles, K., Bendiak, B., Kaplan, K., Siems, W.F., Hill, H.H. Jr.: Resolving structural isomers of monosaccharide methyl glycosides using drift tube and traveling wave ion mobility mass spectrometry. Anal. Chem. **84**, 3231–3239 (2012)
43. Seo, Y., Andaya, A., Leary, J.A.: Preparation, separation, and conformational analysis of differentially sulfated heparin octasaccharide isomers using ion mobility mass spectrometry. Anal. Chem. **84**, 2416–2423 (2012)
44. Schneider, B.B., Covey, T.R., Coy, S.L., Krylov, E.V., Nazarov, E.G.: Chemical effects in the separation process of a differential mobility/mass spectrometer system. Anal. Chem. **82**, 1867–1880 (2010)
45. Purves, R.W., Guevremont, R.: Electrospray ionization high-field asymmetric waveform ion mobility spectrometry-mass spectrometry. Anal. Chem. **71**, 2346–2357 (1999)
46. Barnett, D.A., Ells, B., Guevremont, R., Purves, R.W.: Separation of leucine and isoleucine by electrospray ionization-high field asymmetric waveform ion mobility spectrometry-mass spectrometry. J. Am. Soc. Mass Spectrom. **10**, 1279–1284 (1999)
47. Guevremont, R., Purves, R.W.: High field asymmetric waveform ion mobility spectrometry-mass spectrometry: an investigation of leucine

enkephalin ions produced by electrospray ionization. J. Am. Soc. Mass Spectrom. **10**, 492–501 (1999)
48. Gabryelski, W., Froese, K.L.: Rapid and sensitive differentiation of anomers, linkage, and position isomers of disaccharides using high-hield asymmetric waveform ion mobility spectrometry (FAIMS). J. Am. Soc. Mass Spectrom. **14**, 265–277 (2003)
49. Creese, A.J., Cooper, H.J.: Separation and identification of isomeric glycopeptides by high field asymmetric waveform ion mobility spectrometry. Anal. Chem. **84**, 2597–2601 (2012)
50. Robinson, E.W., Garcia, D.E., Leib, R.D., Williams, E.R.: Enhanced mixture analysis of poly(ethylene glycol) using high-field asymmetric waveform ion mobility spectrometry combined with fourier transform ion cyclotron resonance mass spectrometry. Anal. Chem. **78**, 2190–2198 (2006)
51. Levin, D.S., Vouros, P., Miller, R.A., Nazarov, E.G.: Using a nanoelectrospray-differential mobility spectrometer-mass spectrometer system for the analysis of oligosaccharides with solvent selected control over ESI aggregate ion formation. J. Am. Soc. Mass Spectrom. **18**, 502–511 (2007)
52. Kolakowski, B.M., Mester, Z.: Review of applications of high-field asymmetric waveform ion mobility spectrometry (FAIMS) and differential mobility spectrometry (DMS). Analyst **132**, 842–864 (2007)
53. Shvartsburg, A.A., Bryskiewicz, T., Purves, R.W., Tang, K., Guevremont, R., Smith, R.D.: Field asymmetric waveform ion mobility spectrometry studies of proteins:dipole alignment in ion mobility spectrometry? J. Phys. Chem. B **110**, 21966–21980 (2006)
54. Shvartsburg, A.A., Creese, A.J., Smith, R.D., Cooper, H.J.: Separation of peptide isomers with variant modified sites by high-resolution differential ion mobility spectrometry. Anal. Chem. **82**, 8327–8334 (2010)
55. Robinson, E.W., Williams, E.R.: Multidimensional separations of ubiquitin conformers in the gas phase: relating ion cross sections to h/d exchange measurements. J. Am. Soc. Mass Spectrom. **16**, 1427–1437 (2005)
56. Arungundram, S., Al-Mafraji, K., Asong, J., Leach, F.E., Amster, I.J., Venot, A., Turnbull, J.E., \Boons, G.-J.: Modular synthesis of heparan sulfate oligosaccharides for structure−activity relationship studies. J. Am. Chem. Soc. **131**, 17394–17405 (2009)
57. Leach, F. E. III, Ly, M., Laremore, T.N., Wolff, J.J., Perlow, J., Linhardt, R.J., Amster, I.J.: Hexuronic acid stereochemistry determination in chondroitin sulfate glycosaminoglycan oligosaccharides by electron detachment dissociation. J. Am. Soc. Mass Spectrom. **23**, 1488–1497 (2012)
58. Muñoz, E., Xu, D., Avci, F., Kemp, M., Liu, J., Linhardt, R.J.: Enzymatic synthesis of heparin related polysaccharides on sensor chips: rapid screening of heparin-protein interactions. Biochem. Biophys. Res. Commun. **339**, 597–602 (2006)
59. Domon, B., Costello, C.E.: A systematic nomenclature for carbohydrate fragmentations in fab-ms ms spectra of glycoconjugates. Glycoconj. J. **5**, 397–409 (1988)
60. Remko, M., von der Lieth, C.-W.: Gas-phase and solution conformations of the α-l-iduronic acid structural unit of heparin. J. Chem. Inf. Model. **46**, 1194–1200 (2006)
61. Mulloy, B., Forster, M.J.: Conformation and dynamics of heparin and heparan sulfate. Glycobiology **10**, 1147–1156 (2000)
62. Pomin, V.H., Sharp, J.S., Li, X., Wang, L., Prestegard, J. H.: Characterization of glycosaminoglycans by 15N NMR spectroscopy and in vivo isotopic labeling. Anal. Chem. **82**, 4078–4088 (2010)
63. Berman, E.S.F., Kulp, K.S., Knize, M.G., Wu, L., Nelson, E.J., Nelson, D.O., Wu, K.J.: Distinguishing monosaccharide stereo- and structural isomers with TOF-SIMS and multivariate statistical analysis. Anal. Chem. **78**, 6497–6503 (2006)
64. Fängmark, I., Jansson, A., Nilsson, B.: Determination of linkage position and anomeric configuration in glucose-containing disaccharide alditols by multivariate analysis of data from mass spectrometry. Anal. Chem. **71**, 1105–1110 (1999)
65. Oh, H., Leach, F., Arungundram, S., Al-Mafraji, K., Venot, A., Boons, G.-J., Amster, I.: Multivariate analysis of electron detachment dissociation and infrared multiphoton dissociation mass spectra of heparan sulfate tetrasaccharides differing only in hexuronic acid stereochemistry. J. Am. Soc. Mass Spectrom. **22**, 582–590 (2011)