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Negative-Ion Mode Capillary Isoelectric Focusing Mass Spectrometry for Charge-Based Separation of Acidic Oligosaccharides

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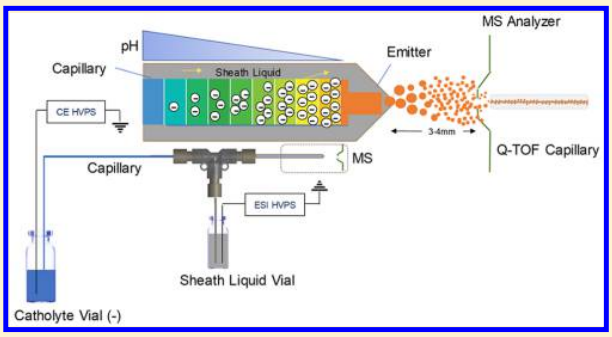
Supporting Information

ABSTRACT: Glycosaminoglycans (GAGs) are biologically and pharmacologically important linear, anionic polysaccharides containing various repeating disaccharides sequences. The analysis of these polysaccharides generally relies on their chemical or enzymatic breakdown to disaccharide units that are separated, by chromatography or electrophoresis, and detected, by UV, fluorescence, or mass spectrometry (MS). Isoelectric focusing (IEF) is an important analytical technique with high resolving power for the separation of analytes exhibiting differences in isoelectric points. One format of IEF, the capillary isoelectric focusing (cIEF), is an attractive approach in that it can be coupled with mass spectrometry (cIEF-MS) to provide online focusing and detection of complex mixtures. In the past three decades, numerous studies have applied cIEF-MS methods to the analysis of protein and peptide mixtures by positive-ion mode mass spectrometry. However, polysaccharide chemists largely rely on negative-ion mode mass spectrometry for the analysis of highly sulfated GAGs. The current study reports a negative-ion mode cIEF-MS method using an electrokinetically pumped sheath liquid nanospray capillary electrophoresis–mass spectrometry (CE-MS) coupling technology. The feasibility of this negative-ion cIEF-MS method and its potential applications are demonstrated using chondroitin sulfate and heparan sulfate oligosaccharides mixtures.



INTRODUCTION

In cell biology, the biological and physical interactions among cells are achieved through communications involving the extracellular matrix (ECM).[1] A major class of the biomolecules comprising the ECM are glycosaminoglycans (GAGs).[2] Most GAGs are glycosidically linked to core proteins in the form of glycoconjugates called proteoglycans.[3] GAGs play various biological roles in protein recognition, binding and activity modulation, cellular signaling and communication, and control of growth and developmental biology through interaction with large number of growth factors.[4−6] Despite their importance, a more complete understanding of GAG structure, composition, and sequence remains challenging because of difficulties associated with their sensitive analysis.[7]

One major analytical challenge in GAG analysis comes from their structural heterogeneity.[7] Intact GAGs are either difficult to separate into individual chains or currently nearly impossible to structurally characterize or sequence.[7−9] GAGs are linear, anionic polysaccharides containing various repeating disaccharides sequences.[7−9] These disaccharides are generally comprised of an uronic acid residue, which is D-glucuronic acid (GlcA) or L-iduronic acid (IdoA), and a hexosamine residue, which is *N*-acetyl or *N*-sulfo-D-glucosamine (GlcNAc or GlcNS) or *N*-acetyl-D-galactosamine (GalNAc). The hydroxyl groups on each of these disaccharide residues may be substituted with *O*-sulfo groups, giving rise to high degree of structural variability. There are several families of GAGs such as hyaluronan (HA), keratan sulfate (KS), chondroitin sulfate (CS), and heparan sulfate (HS), each of which has unique disaccharide compositions. These disaccharide compositions can be determined by treatment with polysaccharide lyases that break down these anionic polysaccharides into their various

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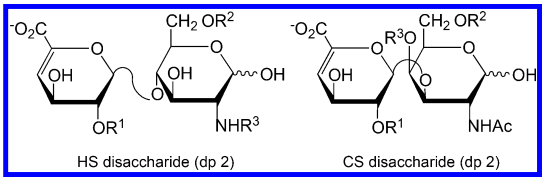
repeating disaccharide units.[10,11] HS and CS each typically contains eight types of unsaturated (ΔUA) disaccharides (Table 1).

Table 1. Structures of 16 Disaccharide Standards[a]



HS dp2	CS dp2	R^1	R^2	R^3
0S	0S	H	H	H
2S	2S	SO_3H	H	H
6S	6S	H	SO_3H	H
NS	4S	H	H	SO_3H
2S6S	2S6S	SO_3H	SO_3H	H
NS6S	4S6S	H	SO_3H	SO_3H
NS2S	2S4S	SO_3H	H	SO_3H
2S6SNS	2S4S6S	SO_3H	SO_3H	SO_3H

[a]The 0S HS and CS disaccharides are ΔUA(1 → 4)GlcNAc and ΔUA(1 → 3)GalNAc, respectively, where ΔUA is 4-deoxy-α-L-threo-hex-4-enopyranosyluronic acid.

High performance liquid chromatography−mass spectrometry (HPLC-MS) has become a widely used method for the analysis of GAG disaccharide composition as it provides confirmation of disaccharide identity based on both retention time and molecular mass.[12,13] A number of LC separation methods compatible with MS analysis have been applied to separate GAG disaccharides. Hydrophilic interaction chromatography (HILIC), which was first developed by Zaia and coworkers,[14] can provide excellent separation of underivatized GAG disaccharides.[15−18] Alternatively, reverse-phase (RP) separation of ion-paired[19] or derivatized GAG disaccharides on a C18 column affords excellent separation of mixtures containing up to 17 different GAG disaccharides.[20,21]

Capillary electrophoresis (CE)-MS has been recently applied to analyze the structure of GAG oligosaccharides.[22−25] The online CE-laser-induced fluorescence (LIF)-MS analysis of neutral glycans labeled with negatively charged fluorescent tags, such as 2-aminoacridone, has also been reported.[26−30] Over the past three decades, a number of CE-MS interfaces have been developed. The classic sheath-flow CE-MS interface was first reported in 1988.[31] This interface has subsequently been commercialized, and the sheath-flow CE-MS interface design has been widely accepted. In addition, sheathless and microfluidic CE-MS interfaces have been developed in the past decade.[32,33] Despite the commercial implementation of these interfaces, limitations on sensitivity and robustness constrains the applications of these CE-MS technologies. As a result, most CE separations of GAG oligosaccharides performed to date used capillary zone electrophoresis (CZE) or micellar electrokinetic chromatography (MEKC) system with UV detectors instead of mass spectrometers.[34−36] These CE separations are primarily based on the rate of analyte movement through the capillary.

A novel capillary isoelectric focusing (cIEF)-MS method has been developed that requires high voltage and analytes moving based on their net charge. Instead of direct movement under the influence of an electric field, these analytes are first focused within a pH gradient generated by carrier ampholytes into a certain pH region having the same pH as the analyte's isoelectric point (p*I*). At a pH equal to its p*I* value, an analyte has a net neutral charge. Proteins, with p*I* values ranging from 3 to 10, have been separated by cIEF to study their posttranslational modifications, such as glycosylation, phosphorylation, and lipidation.[37] cIEF-MS is potentially a powerful tool for distinguishing the charge heterogeneity of complex mixtures, such as GAG oligosaccharides, caused by different numbers of the anionic groups on the chain. Analyte separation in cIEF differs from CZE and MEKC as it is an equilibrium method. However, so far only positive-ion mode cIEF-MS methods have been developed for the analysis of protein and peptide mixtures.[38,39] There is a need for a negative-ion mode cIEF-MS method for highly sulfated glycans. We report a novel cIEF-MS workflow (Figure 1) implemented on an electrokinetically pumped sheath liquid nanospray CE-MS interface with a methanol-based anolyte and catholyte solvent system and demonstrate its feasibility and potential applications using chondroitin and heparin disaccharides.

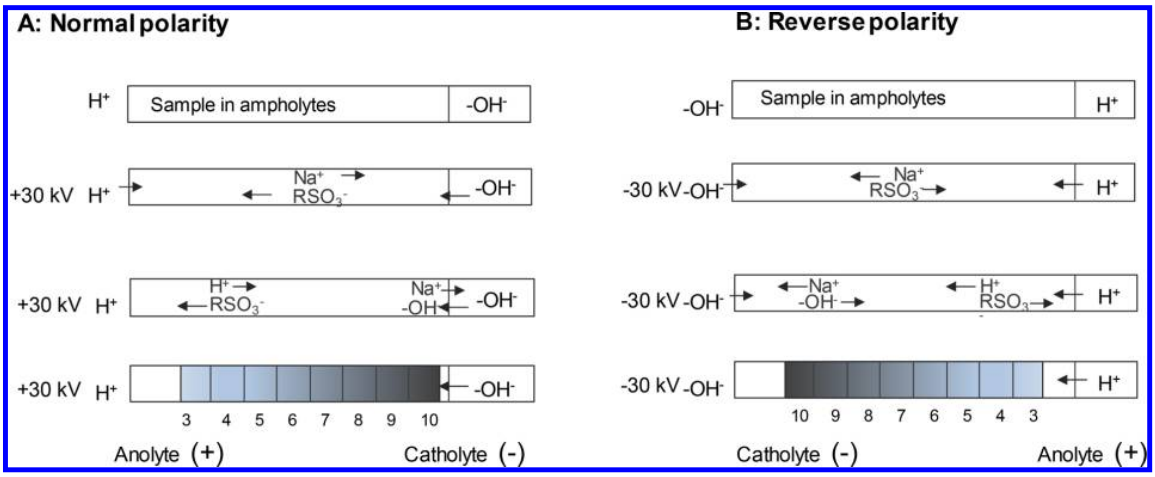


Figure 1. Schematic of negative-ion mode cIEF-MS workflow showing both (A) normal polarity and (B) reverse polarity separations.

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EXPERIMENTAL SECTION

Materials. Sixteen unsaturated heparin/HS and CS disaccharide standards purchased from Iduron, Cheshire, UK (see Table 1 for structures). Recombinant *Flavobacterium heparinum* heparin lyases I, II, and III, *Escherichia coli*, and *Proteus vulgaris* chondroitin lyase ABC were expressed and purified in our laboratory as previously described.[40,41] HS tetrasaccharides were prepared from porcine intestinal HS (Celsus Laboratories, Cincinnati, OH) by partial treatment by heparin lyase II.[42] CS tetrasaccharides and hexsaccharides were prepared from bovine tracheal CS purchased from Celsus Laboratories by partial treatment by chondroitin lyase ABC (Supporting Information (SI), Figures S1−S2). Detailed experimental conditions and structure information are provided in the Supporting Information.

The carrier ampholytes, pharmalytes having pH range 2.5−5.0 and 3.0−10.0, were obtained from GE Healthcare Life Sciences (USP, Pittsburgh, PA). LC-MS grade reagents, including water, formic acid, ammonium hydroxide, acetonitrile, methanol, and p*I* markers (glutamic acid, aspartic acid, and iminodiacetic acid) were also obtained from Millipore-Sigma (Burlington, MA).

Online Capillary Isoelectric Focusing-Mass Spectrometry (cIEF-MS). Online cIEF-MS was performed on an Agilent 7100 CE system (Agilent Technologies, Santa Clara, CA) coupled to an Agilent 6545 quadrupole-time-of-flight (Q-TOF) mass spectrometer using EMASS-II CE-MS ion source (CMP Scientific Corp, Brooklyn, NY) (see abstract graphic). Cation coated electrospray emitters for online cIEF-MS analysis (1.0 mm o.d., 0.75 mm i.d., 15−25 μm tip size) and neutral coating PS1 capillaries (360 μm o.d. and 50 μm i.d.) for separation were from CMP Scientific. The catholyte was 0.2 N ammonium hydroxide, 50−70% methanol (v/v). The anolyte was 0.1−1% formic acid in 50−70% methanol (v/v). A negative electrospray ionization voltage (between −1.5 and −2.0 kV) was supplied by an external benchtop high voltage power supply that comes with the EMASS-II ion source. The sheath liquid was 10 mM ammonium acetate, 80% methanol (v/v). The capillary voltage (Vcap) on the Agilent 6545 Q-TOF was set at zero volts. A single bore inline nanospray shield was used to divert the drying gas out of the mass spectrometer in order to minimize interference with the nanospray out of the EMASS-II ion source. The distance from the emitter tip to the mass spectrometer was adjusted to be between 3 and 4 mm, as measured by the microscope camera. Samples (2−5 ppm) were prepared in 0.5% ampholyte 2.5−5 or 3−10, 50−70% methanol.

Normal and Reverse Polarity Modes. For normal polarity mode, sample solution injection time was set as 60 s following 180 s of the catholyte solution injection under 950 mbar in a 75 cm long capillary (50 μm i.d.). The minimum capillary length compatible with the instruments used in the current study was 50 cm. Capillaries of 50, 60, 70, and 75 cm were examined and the 75 cm long capillary afforded the best resolution. The total time for filling the 75 cm capillary, at a pressure of 950 mbar, was 80 s. A 180 s catholyte injection rinse represents about twice of the capillary volume and was sufficient for reconditioning the capillary after each run. A sample injection time of 60 s corresponds to filling 75% of the capillary length with sample. A positive CE separation voltage (+30 kV) was then applied at the capillary inlet end to initiate electric focusing. A constant pressure (10−100 mbar) was applied at the capillary inlet to generate hydrodynamic flow in order to shorten analysis time. For reverse polarity mode, the anolyte and catholyte injection sequences were the opposite. The separation voltage was set at −30 kV. A schematic workflow of the cIEF-MS is shown in Figure 1.

MS Parameters. The capillary voltage of Q-TOF was set at 0 V. Drying gas was 2 L/min at 350 °C. Fragmentor voltage was 100 V. Acquisition range was m/z 200−1700. Acquisition was 1 spectrum per second at negative-ion mode. The CE-MS method setup, data acquisition, and analysis were performed using Agilent MassHunter Workstation software (v B.08).

RESULTS AND DISCUSSION

Carrier Ampholytes. The carrier ampholytes used in this study are Pharmalytes from GE Healthcare, which are a proprietary complex mixture of low molecular weight compounds.[43] During reversed polarity isoelectric focusing, protons from anolyte solution (formic acid) move toward CE electrode (with the opposite charge) and hydroxides in the catholyte solution (ammonium hydroxide) move away from the CE electrode (toward the mass spectrometer). Under the impact of surrounding solvent pH, each of the ampholytes can carry positive, negative, or zero net charge. An ampholyte compound stops moving when it arrives at a point where the pH equals its p*I* value.[44] The analytes, which are premixed with ampholytes, will move toward anolyte if their p*I* values are higher than the surrounding pH, toward catholyte if their p*I* values are lower than the surrounding pH. With the assistance of this pH gradient formed by the ampholytes, the analytes then condense into focused zones at their characteristic p*I* values. In the absence of ampholytes, there was no separation between the sulfo group-containing HS disaccharides (SI, Figure S3).

Commercial ampholytes, Pharmalyte, are available at pH ranges of pH 2.5−5.0, 5.0−8.0, and 3.0−10.0. We first tested the Pharmalyte with a pH range 3.0−10.0 for the normal polarity cIEF-MS analysis but soon realized that the acidic sulfated oligosaccharides were retained strongly at the CE electrode, fouling the mobilization process. In subsequent reverse polarity cIEF-MS experiments, we tested Pharmalyte with pH range 2.5−5.0 or a mixture of ampholytes with pH ranges 2.5−5.0 and 5.0−8.0 (data not shown). The best results were obtained using pH range 2.5−5 under the reverse polarity mode.

Comparing Normal and Reverse Polarity cIEF for the Separation of HS Disaccharides. In normal polarity cIEF, the injection order begins with the catholyte solution, followed by sample solution (in ampholytes) and anolyte solution (Figure 1A). The catholyte solution (ammonium hydroxide) is injected first into the capillary to form a high pH barrier on the cathode side. This ammonium hydroxide plug is pushed close to the capillary outlet end by the following sample and ampholyte mix injection, sitting next to the electrospray emitter tip, which points toward the mass spectrometer. After sample injection, the anolyte solution (formic acid) vial was engaged onto the capillary inlet and CE electrode, serving as the low pH barrier on the anode side. When a positive 30 kV was applied at the CE electrode, positively charged ions moved toward the cathode and negatively charged ions moved toward the anode under the influence of the electric field force. Eventually, continuous pH gradient zones were formed inside the capillary. Ampholytes as well as each analyte were then focused into pH zones where the surrounding pH equaled

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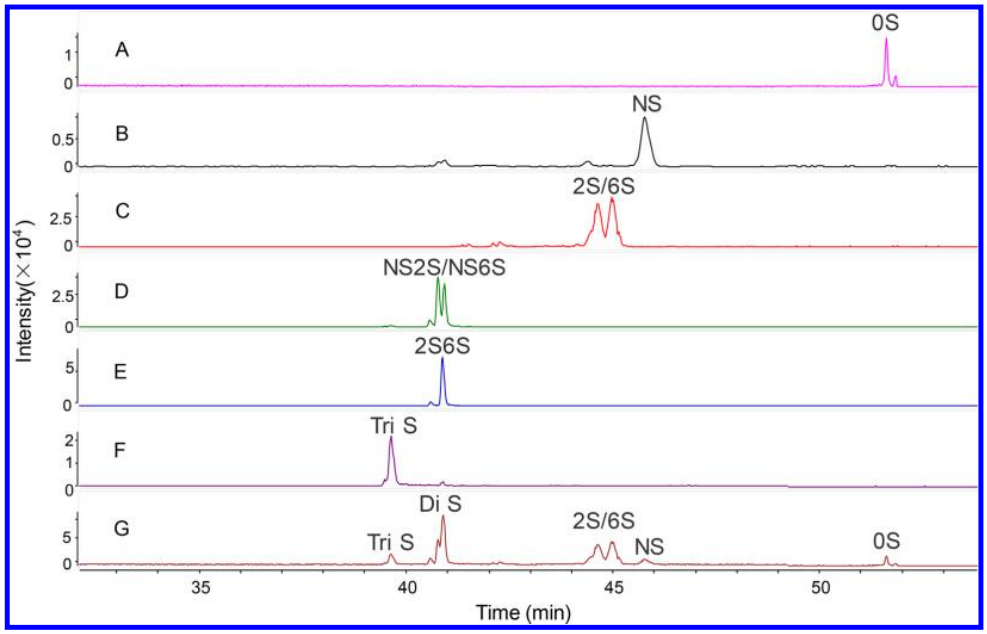

Figure 2. Reverse polarity cIEF-MS of 8 HS dp2 standards using pH 2.5−5.0 ampholytes. (A−F) Extracted-ion electropherograms of individual dp2 masses, (G) of all dp2 masses. The peak shapes for single component analytes, i.e., 0S and TriS, are nearly ideal, while those of multicomponent analytes, i.e., Di S and Mono S are not. The separation window between TriS and NS is 7 min.

their p*I* values. Only HS 0S disaccharide, ΔUAGlcNAc, having a single carboxyl group and no sulfo group (Table 1) was eluted under normal polarity cIEF mode using p*I* 3−10 ampholytes (SI, Figure S4). The ampholytes migrated out of the capillary between 20 and 25 min (SI, Figure S4-A). However, the HS 0S dp2 standard was well retained in the capillary until the pressure applied on capillary was increased from 25 mbar to 920 mbar, as was done at 100 min in SI, Figure S4-B, and Figure 2C (HS 0S dp2 standard, *m*/*z* 378). This resulted from the fact that the negatively charged carboxyl group in HS 0S exhibits a p*I* value leading to focusing near the anolyte side. In theory, chemical mobilization could work well alone but this approach is complicated by high viscosity and a high concentration of methanol. To be detected in MS within regular analysis time, the HS 0S needed to be flushed out of the capillary by increasing the applied pressure otherwise its migration time would be much longer than 100 min. Thus, we concluded that normal polarity cIEF-MS was unsuitable for the analysis of 0S dp2 or more highly charged GAG-derived oligosaccharides.

In contrast, in reverse polarity cIEF-MS, a negative voltage was applied (in place of the positive voltage as used in normal polarity) to the injection vial end (see abstract graphic), reversing the positions of the cathode and anode. As a result, reverse pH gradient was formed inside the CE separation capillary (Figure 1B). Analytes with the lowest p*I* values in this case moved closest to the capillary outlet during focusing, greatly shortening their migration time. This could be clearly observed using three p*I* standards (SI, Figure S5). Under this reverse polarity cIEF-MS condition, the eight HS disaccharides (Table 1), after focusing, left the capillary between 41 and 53 min and were separated based on their total number (−4 to −1 at pH 7) of negative charges (Figure 2G). The HS TriS (HS dp2, 2S6SNS with one carboxyl and three sulfo groups) have the highest number of negative charges, thus eluted the first at 41 min (Figure 2F). The peaks of HS 2S6S, NS2S, and NS6S followed the TriS peak (Figure 2D,E), they have nearly identical p*I* values as they all have one carboxyl and two sulfo groups at various positions on these HS disaccharides. HS disaccharides with only one carboxyl and one sulfo group, the 2S,6S and NS, migrated out of the capillary afterward (Figure 2B,C). The HS 2S and 6S disaccharides (Figure 2B), having identical molecular weights, both appeared at 45.9 min. The HS-NS peak migrated out of the capillary at 46.5 min. This very small difference in migration times may reflect the difference between the *O*-sulfo and *N*-sulfo p*I* values.[45] The HS 0S disaccharide showed the longest migration time due to a much higher p*I* because of the absence of a sulfo group (Figure 2A). Some of the peaks were slightly asymmetrical or split, presumably due to the presence of α- and β-anomeric forms.[23] In conclusion, under reverse polarity mode, HS disaccharides were focused between the cathode and the anode with the most negatively charged of the analytes, nearest the anode, migrated first from the emitter to be detected by a mass spectrometer. This reverse polarity cIEF method exhibited charge-based separation of HS disaccharides for MS detection. Although it is very challenging to separate analytes of nearly identical p*I* values, challenges in separating analytes with very similar properties (i.e., size, charge, hydrophobicity, etc.,) are also encountered in other separation methods such as size exclusion chromatography, ion exchange, or C18-based separations.[46−48] Thus, our approach is limited by being unable to separate analytes with the same p*I* values but it provides a new and different way to afford structural information on such analytes, which other methods cannot achieve.

Analysis of CS Disaccharides and the Linearity of Established pH Gradients in the Reverse Polarity Mode. The CS disaccharide standards (Table 1) contained a highly (−4 at pH 7) negative CS TriS, 2S4S6S, three isomeric disaccharides with two sulfo groups, CS 2S4S, 2S6S and 4S6S, three disaccharides containing one sulfo group, CS 2S, 4S and

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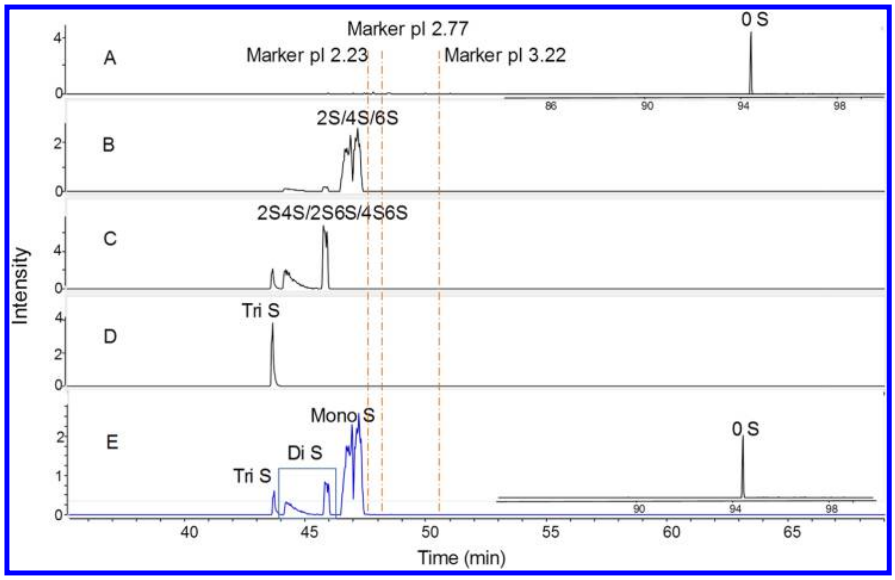
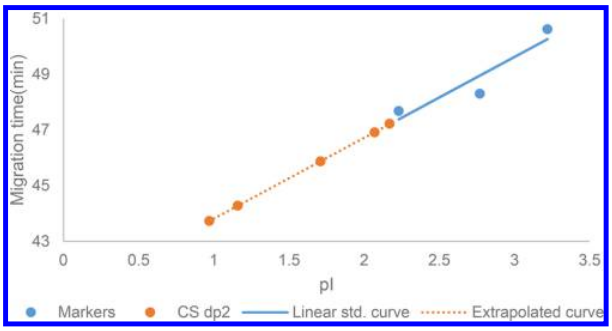


Figure 3. Reverse polarity cIEF-MS 8 CS dp2 standards and three known p*I* standards (positions shown by dashed lines) using pH 2.5−5.0 ampholytes. Extracted-ion electropherograms A, C, and D are at the intensity of 10^4, and B and E are at the intensity of 10^5. Some minor peaks (unlabeled) can also be observed due to sulfate loss. For example, the first small peak in C at 43.8 min corresponds to the loss of one sulfo group from the TriS. The separation window between TriS and Mono S is 4 min.

6S, and one nonsulfated disaccharide CS 0S. The results of cIEF-MS analysis of CS disaccharides (Figure 3) were similar to those obtained from the HS disaccharides (Figure 2). The CS disaccharides with higher negative charge (lower p*I*) displayed shorter migration times. In this experiment, three p*I* markers, iminodiacetic acid, aspartic acid, and glutamic acid (p*I* 2.23, 2.77, and 3.22, respectively), were also examined under the same conditions (SI, Figure S5). A linear regression analysis of the marker p*I* values and migration time was then used to calculate the observed p*I* values of CS disaccharides (Table 2). In terms of sensitivity, the sample loading amount was 0.5 ng for each disaccharide, affording an intensity of 10^4 using cIEF-MS method, which affords a sensitivity comparable to LC-MS and CE-MS methods currently used to analyze underivatized GAG-derived disaccharides prepared from biological samples.[24,25] It is difficult to comment on resolution because cIEF-MS resolves disaccharides based on their p*I* and other methods resolve molecules based on their net charge and size, but in general the resolution of these analytes is lower in cIEF because their apparent p*I* values are very similar. In terms of reproducibility, this is the first study using cIEF-MS so that this is difficult to assess and additional studies from various laboratories and using different CE instruments, capillaries, and ampholytes, mass spectrometers, and interfaces need to be compared.

Table 2. Migration Time of p*I* Markers in Reverse Polarity Mode ($y = 2.9096x + 40.894$, $r^2 = 0.8661$)



compound	p*I*	migration time (min)
glutamic acid	3.22	50.6
aspartic acid	2.77	48.3
iminodiactic acid	2.23	47.7
CS MonoS-2_{nd} peak	2.17	47.2
CS MonoS-1_{st} peak	2.07	46.9
CS DiS-2_{nd} peak	1.71	45.9
CS DiS-1_{st} peak	1.16	44.3
CS TriS	0.97	43.7

Analysis of Larger CS Oligosaccharides. With the established condition using commercial disaccharide standards, longer CS oligosaccharides (preparation procedure see SI; structural information, see SI, Figures S1 and S2) were analyzed by this negative-ion mode cIEF-MS method. A mixture of CS tetrasaccharides (dp4) containing, on average, one sulfo group on each of its two disaccharide units and CS hexasaccharide (dp6) containing, on average, one sulfo group on each of its three disaccharide units (Figure 4) were used for the testing. On the basis of our hypothesized p*I*-based separation mechanism, the larger CS dp4 and CS dp6 oligosaccharides should behave the same as the CS disaccharides with single sulfo groups. The CS dp4 and CS dp6 oligosaccharides migrated out of the capillary at nearly identical time (Figure 4C,D). CS dp4 and CS dp6 samples are mixtures of isomers, prepared from a CS sample containing a mixture of both 4-*O*- and 6-*O*- sulfo groups (SI, Figures S1 and S2), therefore each appeared as multiple peaks, which are indicative of four to eight potential positional isomers (Figure 4).

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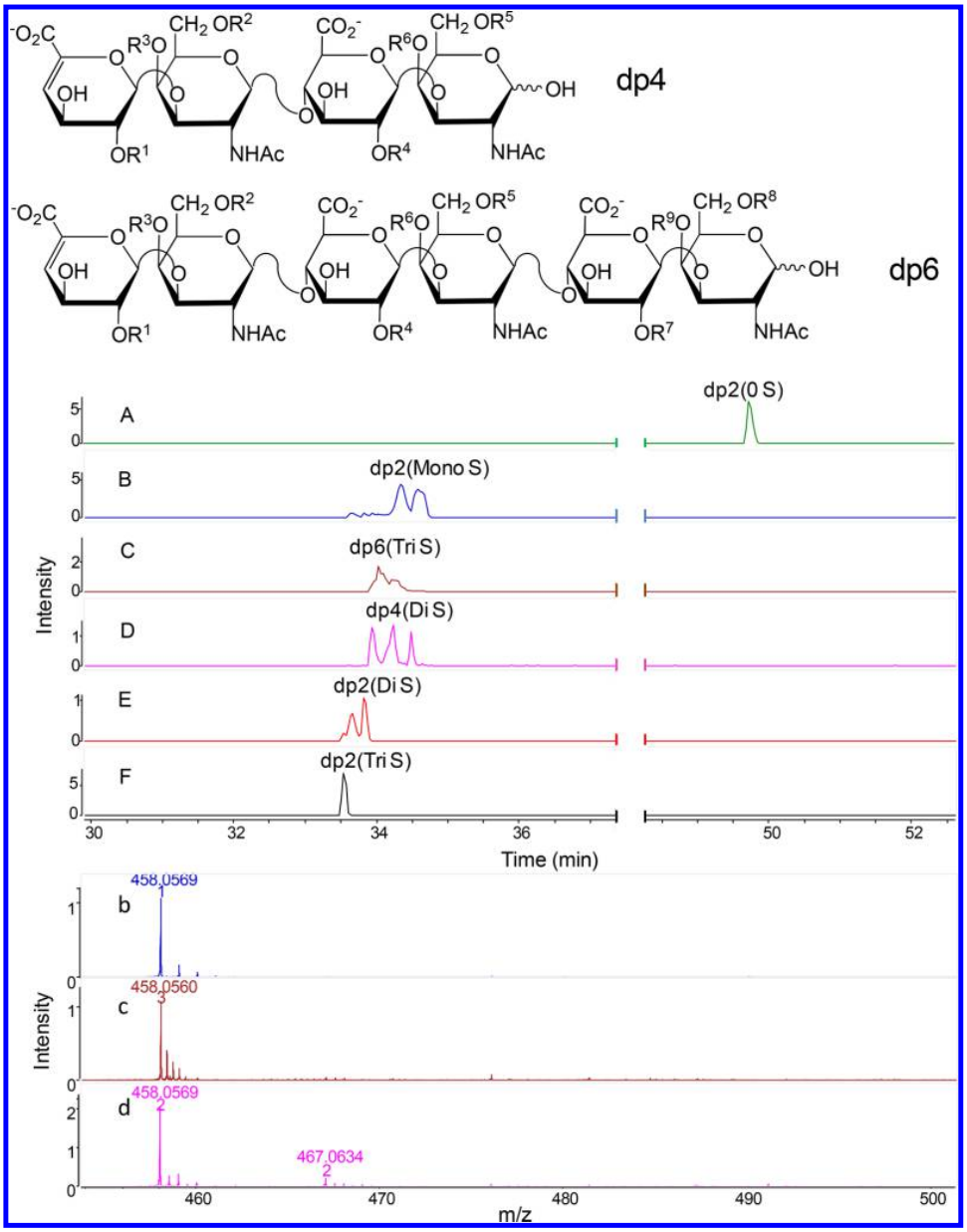

Figure 4. Reverse polarity cIEF-MS of CS dp2, dp4, and dp6 mixture using pH 2.5−5.0 ampholytes. A−F are extracted-ion electropherograma, and b,c, and d are the mass spectra of B, C, and D, respectively. A, C, D, E, c, and d are at intensity of 10^4, and B and b are at intensity of 10^5. The structure of the dp4, analyzed in D, is a mixture of 4S4S, 6S6S, 4S6S, and 6S4S corresponding to R^3R^6, R^2R^5, R^3R^5, and R^2R^6, respectively. The structure of the dp6, analyzed in C, is a mixture of 4S4S4S, 6S6S6S, 4S6S6S, 6S4S6S, 6S6S4S, 4S4S6S, 4S6S, 4S, and 6S4S4S, corresponding to $R^3R^6R^9$, $R^2R^5R^8$, $R^3R^5R^8$, $R^2R^6R^8$, $R^2R^5R^9$, $R^3R^6R^8$, $R^2R^6R^9$, and $R^3R^5R^9$, respectively.

CONCLUSIONS

A novel online reverse polarity negative-ion mode cIEF-MS method has been developed for the separation and analysis of GAG-derived oligosaccharides. Both normal and reverse polarity cIEF were examined. Reverse polarity cIEF-MS was found to be most suitable for the GAG-derived oligosaccharides with low p*I* values. Mixtures of eight HS disaccharides and eight CS disaccharides were focused and separated by cIEF based on the number of sulfo groups. The neutral coating of the capillary to suppress electroosmotic flow is not completely stable in ammonium hydroxide, leading to some variability in migration time or the capillary lifetime that can be compensated by a normalization equation (see SI, Figure S9). This separation, however, showed that reasonable reproducibility and a linear nature of the pH gradient within the capillary was demonstrated using p*I* markers (Figure 5, and SI, S6−S8). Some cIEF separation was achieved between GAG disaccharides having the same number of *O*-sulfo groups that were positional isomers. In addition, *N*-sulfo and *O*-sulfo group substituted disaccharides migrated differently. Mixtures of larger oligosaccharides, dp4 and dp6, were also analyzed by cIEF-MS. Their migration in cIEF-MS correlated to their average number of sulfo groups per disaccharide unit. The

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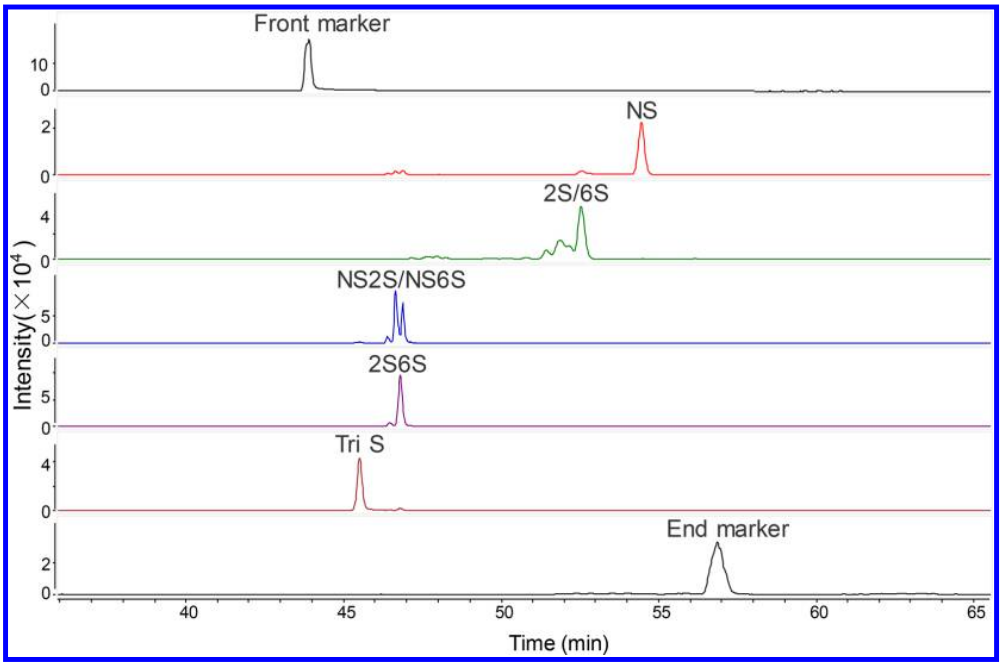

Figure 5. Reproducibility of reverse polarity cIEF-MS using eight heparan sulfate dp2 mixture. Extracted-ion electropherogram of eight component heparan sulfate dp2 mixture (second of three injections). The front marker is the first ampholyte peak eluting, and the end marker is the last ampholyte peak eluting.

observed p*I* values for these GAG-derived oligosaccharides were higher than previously reported,[49] as the separations were carried out in methanol-based anolyte and catholyte solvents suppressing carboxyl and sulfo group charge exposure,[50] their migration time, and their apparent p*I* values fit a linear regression model, supporting the underlying pH gradient formation and p*I*-based separation.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.analchem.8b03500.

CS tetrasaccharide (dp4) and hexasaccharide (dp6) preparation, dp4 and dp6 LC-MS data, CE separation in the absence of ampholyte, normal polarity cIEF of 8 HS disaccharides, extracted ion electropherogram and MS of HS 0S dp2, reverse polarity cIEF of pI standards, and reproducibility data of cIEF-MS (PDF)

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Notes

The authors declare the following competing financial interest(s): The authors Y.O., X.H., K.X., J.C., Z.Z. and R.J.L. declare no competing financial interest. Q.X. is an employee of CMP Scientific Corp. selling CEMS ion sources.

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