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## High-resolution preparative separation of glycosaminoglycan oligosaccharides by polyacrylamide gel electrophoresis

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### ABSTRACT

Separation of milligram amounts of heparin oligosaccharides ranging in degree of polymerization from 4 to 32 is achieved within 6 h using continuous elution polyacrylamide gel electrophoresis (CE–PAGE) on commercially available equipment. The purity and structural integrity of CE–PAGE-separated oligosaccharides are confirmed by strong anion exchange high-pressure liquid chromatography, electrospray ionization Fourier transform mass spectrometry, and two-dimensional nuclear magnetic resonance spectroscopy. The described method is straightforward and time-efficient, affording size-homogeneous oligosaccharides that can be used in sequencing, protein binding, and other structure–function relationship studies.

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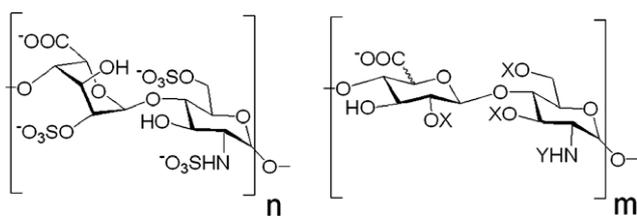
Heparin is a linear sulfated glycosaminoglycan (GAG)<sup>1</sup> consisting of 1 → 4-linked iduronic or glucuronic acid (IdoA or GlcA) and glucosamine (GlcN) disaccharide building blocks with an average of 2.7 sulfo groups per disaccharide repeat (Fig. 1). Heparin mediates a number of biological pathways through its interactions with various regulatory proteins such as growth factors, chemokines, cytokines, and protease inhibitors [1,2]. Structure–function relationship studies involving heparin–protein interactions often require purified heparin oligosaccharides of various sizes and sulfation states obtained through enzymatic depolymerization of heparin polysaccharide. The ability to prepare high-purity oligosaccharides in sufficient amounts for the X-ray crystallography, protein–GAG binding studies, and domain sequencing is critical for the development of new drug targets and potential GAG-based therapeutics.

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<sup>1</sup> Abbreviations used: GAG, glycosaminoglycan; IdoA, iduronic acid; GlcA, glucuronic acid; GlcN, glucosamine; SAX, strong anion exchange; IP–RP HPLC, ion pairing reverse phase high-pressure liquid chromatography; SEC, size exclusion chromatography; PAGE, polyacrylamide gel electrophoresis; NMR, nuclear magnetic resonance; CE–PAGE, continuous elution PAGE; LC, liquid chromatography; BLH, bovine lung heparin; MW, molecular weights; <sup>1</sup>H NMR, proton NMR; ESI–MS, electrospray ionization mass spectrometry; APS, ammonium persulfate; TEMED, N,N,N',N'-tetramethylethylenediamine; disodium EDTA, disodium salt of ethylenediaminetetraacetic acid; T, total acrylamide; dp, degree of polymerization; MWCO, molecular weight cutoff; UV–VIS, ultraviolet–visible; ESI–FTMS, ESI–Fourier transform mass spectrometry; <sup>2</sup>H<sub>2</sub>O, deuterium oxide; %T, total acrylamide monomer concentration; 2D COSY, two-dimensional correlation spectroscopy; 2D HMQC, 2D heteronuclear multiple quantum coherence.

Fractionation and purification of heparin oligosaccharides can be achieved in several ways using strong anion exchange (SAX) chromatography [3–5], ion pairing reverse phase high-pressure liquid chromatography (IP–RP HPLC) [6–8], size exclusion chromatography (SEC) [9], and micropreparative polyacrylamide gel electrophoresis (PAGE) [10–12] in combination or alone. Under low-pH conditions, SAX chromatography separates GAG-derived oligosaccharides according to the number of sulfo groups. Bound oligosaccharides are eluted with an increasing salt gradient and must be desalted for most downstream applications. During the IP–RP HPLC separation, an alkyl ammonium mobile phase modifier interacts with the negatively charged carboxy and sulfo groups in heparin through the positively charged ammonium moiety. The alkyl chain of the modifier interacts with the alkyl chains of the reverse phase support, imparting longer retention times to the more highly sulfated oligosaccharides, which are eluted with an increasing organic solvent gradient. SEC separates the oligosaccharides by size and may be used with a volatile buffer such as 30 to 50 mM ammonium formate [13]. Native discontinuous PAGE affords unmatched resolution for separation of GAG-derived oligosaccharides and provides an additional analytical dimension during the process of GAG characterization [11,12]. On a micropreparative scale, native PAGE in a slab format has been used in our laboratory for the separation of heparin-derived oligosaccharides [10] and intact chondroitin sulfate polysaccharides from a proteoglycan bikunin [14]. Recovery of sample by soaking a cut portion of a fixed and stained gel containing a band is difficult. Instead, a narrow strip of the gel is stained to serve as a guide for the localization of bands in the unstained portion of the gel from which a sample is



**Fig. 1.** Structure of bovine lung heparin. An average chain contains major trisulfated disaccharide repeating units ( $n \sim 27$ ) and minor disaccharide repeating units ( $m \sim 3$ ), where  $X = H$  or  $SO_3^-$  and  $Y = SO_3^-$  and occasionally  $COCH_3$  or  $H$ .

recovered. This approach results in a reduction of both the purity and amount of sample recovered. Alternatively, following PAGE, oligosaccharide bands can be electrotransferred onto positively charged membranes, excised, and eluted from the membrane strips with 2 M sodium chloride. Electrotransfer from an unstained membrane is similarly guided only by a narrow vertical strip cut from the membrane and visualized with a dye. In both soaking and electrotransfer, band curving during PAGE (smiling effect) can result in the contamination of a band of interest by neighboring bands. The recovered oligosaccharide also contains oligo- and polyacrylamide, and these contaminating substances are difficult to remove, making it hard to obtain sample of sufficient purity to afford clean nuclear magnetic resonance (NMR) and mass spectral data required for structural assignment. Native continuous elution (CE) PAGE (CE-PAGE) has been used by a number of researchers for the micropreparative separation of proteins, *N*-glycans, lipopolysaccharides, and nucleic acids [15]. CE-PAGE combines the convenience of a liquid chromatography (LC) system with the many advantages of PAGE, especially the possibility of customizing the experimental conditions for a particular sample mixture without the high additional cost.

Here we report a high-resolution separation of milligram amounts of oligosaccharide mixture obtained by enzymatic depolymerization of bovine lung heparin (BLH) using native discontinuous CE-PAGE. The method described here permits simultaneous purification of a broad range of heparin oligosaccharides with molecular weights (MWs) of 1155 to 8660 Da within 6 h using a commercial electrophoresis cell. Purity and structural integrity of PAGE-separated oligosaccharides were verified using proton NMR ( $^1H$  NMR) spectroscopy, SAX-HPLC, and electrospray ionization mass spectrometry (ESI-MS).

## Materials and methods

### Chemicals

Electrophoresis-grade acrylamide, *N,N'*-methylene-bis-acrylamide, sucrose, glycine, ammonium persulfate (APS), *N,N,N',N'*-tetramethylethylenediamine (TEMED), and bromophenol blue were obtained from Bio-Rad (Hercules, CA, USA). Boric acid, disodium salt of ethylenediaminetetraacetic acid (disodium EDTA), phenol red, Azure A, and Alcian blue were obtained from Fisher (Pittsburgh, PA, USA). Heparin lyases I, II, and III (EC 4.2.2.7 and EC 4.2.2.8) were obtained from Cape Cod Associates (Seikagaku America, East Falmouth, MA, USA). All solvents were HPLC grade, and all other chemicals were molecular biology grade.

### Preparation of heparin oligosaccharides

BLH (Celsus Laboratories, Cincinnati, OH, USA) was partially depolymerized using a mixture of heparinases I, II, and III. The enzymes were denatured by boiling and removed by centrifugation. The supernatant containing the oligosaccharide mixture was collected, and the disaccharides and buffer salts were removed

using size exclusion resin (Bio-Gel P-4, Bio-Rad) and double-distilled water mobile phase. The resulting BLH oligosaccharide ladder was lyophilized and stored at  $-80^\circ C$ .

### PAGE

Heparin oligosaccharides were separated by the native CE-PAGE using a Mini Prep cell with a 7-mm internal diameter gel tube or a Model 491 Prep cell with a 28-mm internal diameter gel tube (Bio-Rad) and a discontinuous buffer system [11]. The electrode running buffer was 1 M glycine and 0.2 M Tris (pH 8.8, achieved on dissolution), and the resolving gel buffer was 0.1 M boric acid, 0.1 M Tris, and 0.01 M disodium EDTA (pH 8.3). A 15% total acrylamide (T) monomer solution contained 14.08% (w/v) acrylamide, 0.92% (w/v) *N,N'*-methylene-bis-acrylamide, and 5% (w/v) sucrose in the resolving gel buffer. A 10-cm  $\times$  7-mm diameter resolving gel column was cast from 4 ml of 15% T monomer solution, to which 4  $\mu$ l of TEMED and 12  $\mu$ l of 10% APS were added to catalyze polymerization. A 10-cm  $\times$  28-mm diameter resolving gel column was cast from 40 ml of 15% T monomer solution with 20  $\mu$ l of TEMED and 100  $\mu$ l of 10% APS. Resolving gel was allowed to polymerize overnight, after which a 5% T stacking gel was cast. The stacking gel monomer solution was prepared in the resolving gel buffer with pH adjusted to 6.3 using HCl and contained 4.75% (w/v) acrylamide and 0.25% (w/v) *N,N'*-methylene-bis-acrylamide. The volume of the stacking gel was twice that of the sample and contained 1  $\mu$ l of TEMED and 30  $\mu$ l of 10% APS per 1 ml of monomer solution.

The BLH oligosaccharide mixture was dissolved in the running buffer and mixed with an equal volume of 50% (w/v) sucrose containing 10  $\mu$ g/ml phenol red, a tracking dye. The upper buffer chamber was filled with the electrode running buffer, and the elution buffer reservoir and lower buffer chamber were filled with the resolving gel buffer. A peristaltic pump (Econo-Pump, Bio-Rad) was set to a flow rate of 80  $\mu$ l/min (Mini Prep cell) or 500  $\mu$ l/min (Model 491 Prep cell) and was connected to a fraction collector (Model 2110, Bio-Rad) set to collect 3-min fractions. Electrophoresis was carried out at a constant power of 1 W (Mini Prep cell) or 5 W (Model 491 Prep cell) for a total of 6 h, and fractions were collected after approximately 2 h when the tracking dye was 1 cm above the bottom of the gel column. Collected fractions were analyzed by analytical PAGE on 0.75-mm  $\times$  6.8-cm  $\times$  8.6-cm minigels cast in-house using 15% or 22% T resolving gel monomer solution and 5% T stacking gel monomer solution. The minigels were subjected to electrophoresis at constant 200 V using the discontinuous buffer system described above, stained in 0.5% (w/v) Alcian blue containing 2% (v/v) aqueous acetic acid solution for 30 min, and destained in water.

### Sample purification

Prior to MS and NMR analyses, Tris-HCl and disodium EDTA were removed from the fractions collected during CE-PAGE using SAX spin columns (High-Capacity Mini-Q or Maxi-Q, Sartorius, Göttingen, Germany). To each fraction, a one-half volume of 225 mM NaCl solution was added, and the resulting sample was loaded onto the spin column preequilibrated with 150 mM NaCl. The sample was washed twice with the 150 mM NaCl, eluted with 300  $\mu$ l (Mini-Q) or 1 ml (Maxi-Q) of 2 M NaCl, and desalted using a centrifugal filter (Microcon YM-3 for degree of polymerization 4 [dp4], Microcon YM-10 for dp6 or higher, or Amicon Ultra, 3000 molecular weight cutoff [MWCO], Millipore, Billerica, MA, USA).

### SAX chromatography

SAX-HPLC analysis of gel-eluted fractions was performed on a Shimadzu LC-10Ai LC system equipped with an SPD-20A ultraviolet

let-visible (UV–VIS) detector using a  $4.6 \times 250$ -mm Waters Spherisorb S5 SAX column. A two-segment gradient elution was achieved using mobile phase A (water, pH 3.4, adjusted with HCl) and mobile phase B (2 M aqueous NaCl, pH 3.4, adjusted with HCl) at a flow rate of 1 ml/min. The bound sample was washed with 0% to 40% B over 5 min and eluted with 40% to 75% B over 125 min. The elution was monitored at 232 nm, the  $\lambda_{\text{max}}$  for the 4,5-unsaturated uronic acid residue generated on the oligosaccharide non-reducing end by the heparin lyase treatment. For semipreparative separation, a  $20 \times 250$ -mm Waters Spherisorb S5 SAX column was used with the same gradient but at a flow rate of 4 ml/min.

#### MS

CE–PAGE fractions containing dp4 to dp14 were analyzed by ESI–Fourier transform mass spectrometry (ESI–FTMS) on an LTQ XL Orbitrap mass spectrometer (Thermo Fisher Scientific, San Jose, CA, USA). Mobile phase consisting of 0.1% formic acid in 50% aqueous methanol [16] was delivered by an Agilent 1200 nano-LC pump at a flow rate of 50  $\mu\text{l}/\text{min}$ . Desalted and lyophilized samples were dissolved in the mobile phase, and 2 to 8  $\mu\text{l}/\text{injection}$  was introduced by direct infusion through an Agilent 1200 autosampler. Mass spectra were acquired at a resolution of 30,000 in the negative ion mode under the following conditions: spray voltage, 3 kV; capillary temperature, 200  $^{\circ}\text{C}$ ; capillary voltage,  $-15$  V; tube lens,  $-100$  V; sheath and auxiliary gas flow rates, 20 and 5 U, respectively.

#### $^1\text{H}$ NMR analysis of gel-eluted heparin

Purity and structural integrity of gel-eluted heparin was assessed using  $^1\text{H}$  NMR. The heparin oligosaccharide mixture and oligosaccharides eluted from gel were purified by SAX spin chromatography as described above, desalted, and freeze-dried. Dry samples were dissolved in 0.5 ml of 99.996% deuterium oxide ( $^2\text{H}_2\text{O}$ , Sigma, St. Louis, MO, USA) and freeze-dried. The  $^2\text{H}_2\text{O}$  dissolution/freeze-drying was repeated three times before NMR analysis. NMR spectra were acquired in 99.996%  $^2\text{H}_2\text{O}$  on a Bruker Ultrashield 600-MHz (14.1-Tesla) NMR instrument equipped with an ultrasensitive HCN cryoprobe with a z-axis gradient. The spectra were acquired at a probe temperature of 298 K and an acquisition time of 2.6 s.

#### Azure A metachromatic assay

Recovery of oligosaccharides from CE–PAGE was estimated by measuring their metachromatic activity in an Azure A solution [17]. An assay working solution, 0.02 mg/ml Azure A, was prepared from a 1-mg/ml stock immediately before the assay. BLH ladder solutions for the standard curve were prepared in the lower chamber buffer (elution buffer), and the buffer was also used for the blank. The Azure A working solution (190  $\mu\text{l}$ ) was thoroughly mixed with 10  $\mu\text{l}$  of heparin solution, and the absorbance of this mixture at 620 nm was measured against the elution buffer blank. The decrease in absorbance at 620 nm was plotted as a function of heparin oligosaccharide concentration, and the amounts of recovered oligosaccharides were calculated using the resulting linear equation (see Supplementary Fig. 1 in supplementary material). The assay standards and samples were prepared in triplicate in a 96-well plate, and their absorbance was measured on a Spectra-Max M5 plate reader (Molecular Devices, Sunnyvale, CA, USA).

#### Results and discussion

The BLH oligosaccharide mixture used in the current study contained approximately 15 components with dp4 to dp32

monosaccharides. The difference in size between individual chains is due mainly to a disaccharide-repeating unit given that BLH has fairly uniform disaccharide composition (Fig. 1). As the chain length increases, the percentage difference in size between the chains decreases, making it difficult to separate large heparin oligosaccharides by SEC. Native discontinuous PAGE affords high-resolution separation over a wider range of MWs compared with SEC. The goal of this work was to assess the utility of the native CE–PAGE for the preparative separation of heparin oligosaccharides in the amounts and purity sufficient for downstream structure–function relationship studies and in a time-efficient way.

#### PAGE conditions

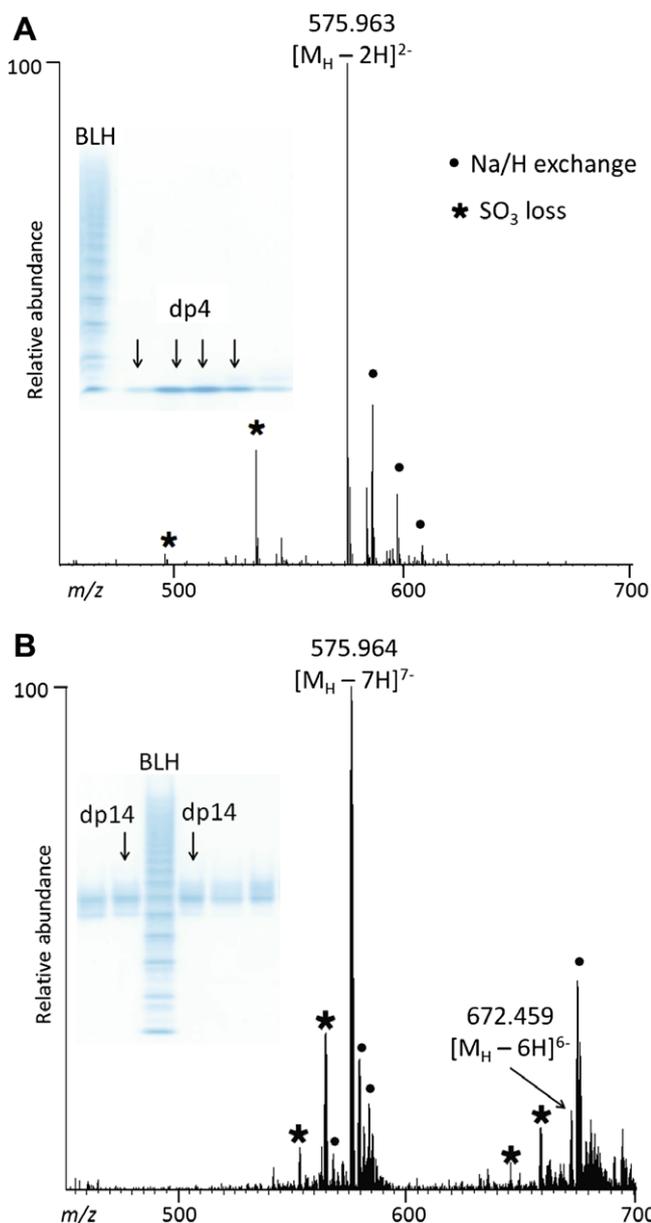
Taking into account the number and MW distribution of the BLH ladder components, the following PAGE parameters were optimized: (i) total acrylamide monomer concentration (%T), (ii) gel dimensions, and (iii) running conditions. During the %T optimization, 10  $\mu\text{g}$  of the BLH oligosaccharide mixture was loaded on 10, 15, and 22%T slab minigels and subjected to electrophoresis for 30 min at a constant 200 V. In the 22%T gel, a tetrasaccharide band migrated only half of the gel length during the 30-min run, making the separation time-inefficient. In the 10%T gel, a portion of the sample migrated off of the gel, causing the loss of smaller oligosaccharides. The 15%T afforded the best sample resolution and was selected for the preparative separation.

Next, the preparative gel column length was selected guided by the basic idea that although a longer gel column would improve separation, it would also require a longer running time than a shorter column. Using a Mini Prep cell with a 7-mm diameter gel tube, 5-, 7-, 9-, and 10-cm gel columns were compared in terms of separation achieved under the same experimental conditions: 1 mg of sample loading, 1 W constant power electrophoresis, 80  $\mu\text{l}/\text{min}$  elution buffer flow rate, and 240  $\mu\text{l}$  fraction volume. Predictably, there was a clear advantage in using the 10-cm gel length because the increase in the total running time was compensated by improved resolution.

Another aspect of method optimization was the maximum amount of sample that could be separated without compromising the resolution. In the mixture of 15 or more BLH oligosaccharides, the amounts of individual components decrease with the increasing size, as evidenced from the Alcian blue staining intensity of oligosaccharide bands (Supplementary Fig. 2 and Fig. 2). Thus, increasing the sample loading negatively impacts resolution of the smaller oligosaccharides to a greater extent than that of the larger ones. In this study, the maximum amount of the BLH oligosaccharide mixture that could be separated into its individual components on a 7-mm diameter, 10-cm 15%T gel was 2.3 mg, which afforded 20 to 40  $\mu\text{g}$  of purified oligosaccharide per fraction as estimated by the Azure A assay (Supplementary Fig. 1).

#### ESI–FTMS analysis

Fractions collected from a 7-mm diameter gel tube were analyzed by PAGE, and the like fractions were pooled. Oligosaccharides with dp4 through dp14 were analyzed by ESI–FTMS in the negative ion mode. Based on the MS data, the major component in each gel band consisted of trisulfated disaccharide building blocks (Figs. 1 and 2). Peaks corresponding to lower sulfation states are probably artifacts of MS analysis caused by the analyte fragmentation through a loss of  $\text{SO}_3$  given that their intensity is higher than expected based on the known composition of BLH. A major contaminant in the negative ion ESI–FTMS analyses of PAGE-eluted, desalted oligosaccharides was EDTA, which formed partially sodiated clusters at  $m/z$  605.1615 [ $2(\text{EDTA})+\text{Na}-2\text{H}$ ] $^-$ ,  $m/z$  627.1433 [ $2(\text{EDTA})+2\text{Na}-3\text{H}$ ] $^-$ , and  $m/z$  649.1252 [ $2(\text{EDTA})+3\text{Na}-4\text{H}$ ] $^-$ .



**Fig. 2.** PAGE and ESI-FTMS analysis of CE-PAGE-purified heparin oligosaccharides (insets): (A) tetrasaccharide, dp4; (B) tetradecasaccharide, dp14.

Apparently, EDTA interacts with heparin oligosaccharides and remains in the sample even after extensive desalting. We compared two methods for removing this contaminant: disrupting electrostatic interactions with 2 M NaCl and SAX spin column purification. The contaminant peaks were completely absent in the mass spectra acquired from samples purified by either method. Thus, for MS-based studies, the addition of 2 M NaCl followed by desalting is a fast and cost-efficient method for the EDTA removal.

#### SAX-HPLC analysis

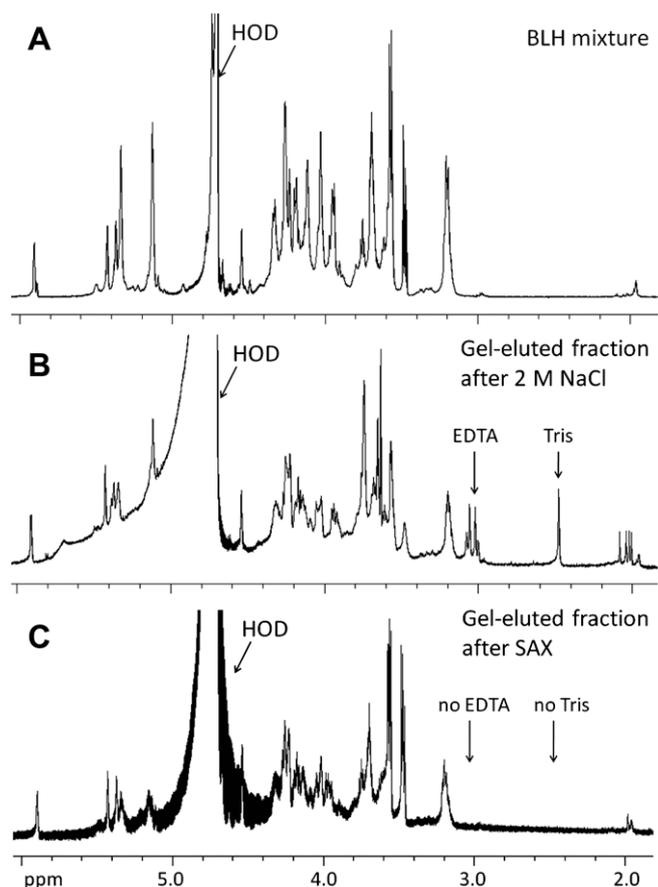
The detection of peaks corresponding to the loss of  $\text{SO}_3$  in the mass spectra of purified oligosaccharides could reflect the sulfation heterogeneity within the sample and/or could be a result of fragmentation during the MS analysis. To determine the sulfation states of major components in the gel-eluted oligosaccharide fractions, several purified oligosaccharide samples were analyzed by SAX-HPLC. Fractions eluted from two 7-mm diameter gel tubes

and containing dp8 were pooled and divided, and one portion was separated on an analytical SAX column with UV detection at 232 nm (4,5-unsaturated uronic acid residue). Two major chromatographic peaks were collected and desalted, and their mass spectra were compared with those of PAGE-eluted dp8 before SAX-HPLC. The two mass spectra appeared to be virtually identical, including the abundance of ions corresponding to lower sulfation states (Supplementary Fig. 3).

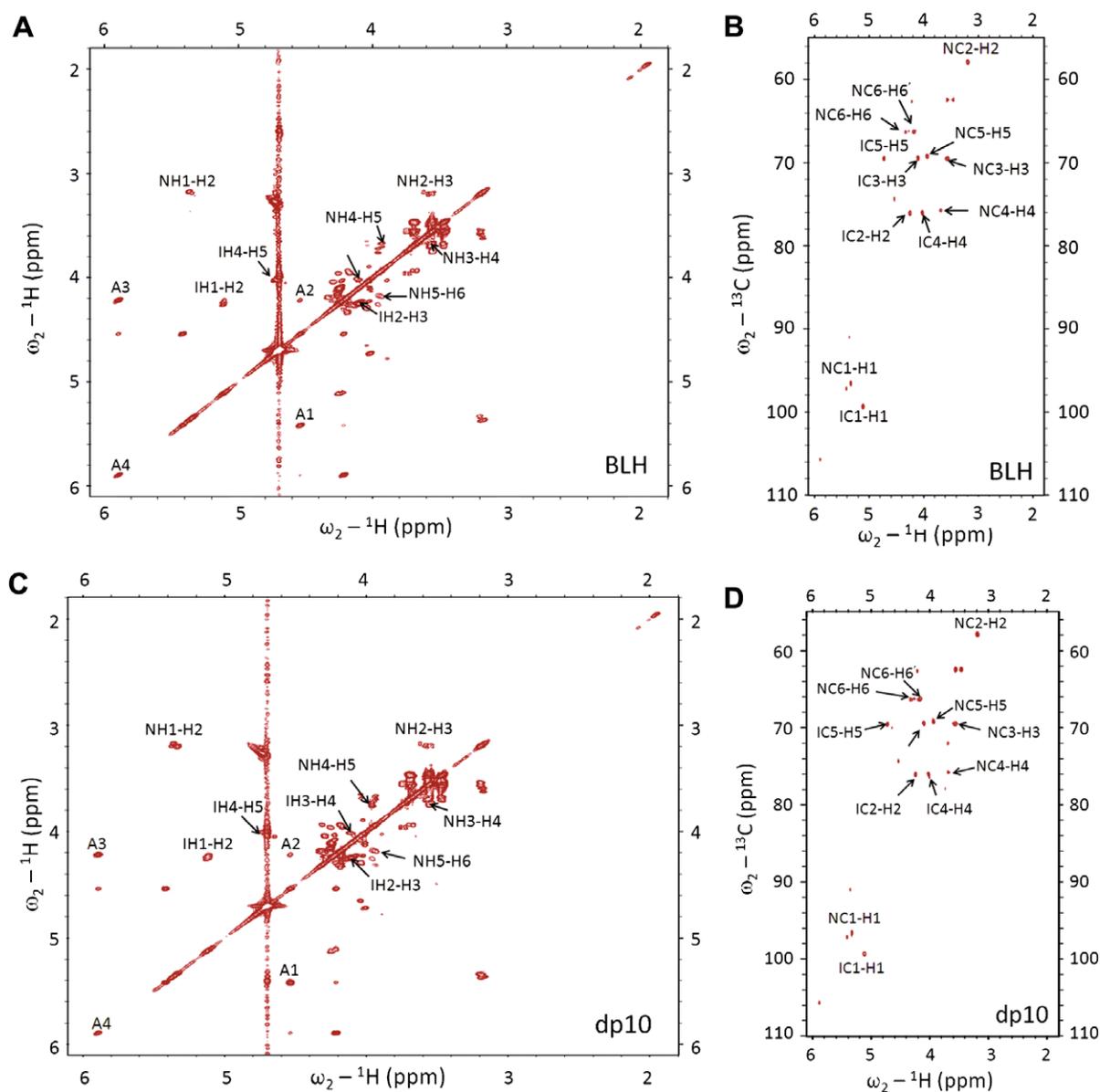
Fractions eluted from a 28-mm diameter gel tube and containing dp8, dp10, dp12, and dp14 were separated on a semipreparative SAX column, and in each case the largest chromatographic peak was collected, desalted, and analyzed by ESI-FTMS. As was the case with the dp8 fraction, the resulting mass spectra contained peaks corresponding to lower sulfation states of each oligosaccharide, and this can be attributed to the analyte fragmentation through the loss of  $\text{SO}_3$  during the MS analysis (Supplementary Fig. 4).

#### NMR analysis

For NMR characterization, BLH oligosaccharides were separated on a larger scale using 28-mm gel tube (Model 491 Prep cell), which accommodates a 40-ml, 10-cm gel. Increasing the gel volume 10-fold permitted a 10-fold increase in sample loading. Following the PAGE analysis of fractions, like fractions were pooled, 1 volume of 4 M NaCl was added to each fraction, and the resulting solutions were desalted using 3000-MWCO centrifugal filters (Amicon Ultra). The  $^1\text{H}$  NMR spectra of gel-eluted fractions purified



**Fig. 3.**  $^1\text{H}$  NMR analysis of BLH oligosaccharide mixture (A), gel-eluted dp6 + dp8 fraction after desalting using a 3000-MWCO spin column (B), and gel-eluted dp6 + dp8 fraction after purification using SAX spin column (C). HOD, residual water.



**Fig. 4.** 2D NMR analysis of BLH oligosaccharide mixture (A,B) and CE-PAGE-purified BLH deca-saccharide, dp10 (C,D). The NMR peaks corresponding to H–H (COSY, panels A and C) or H–C (HMQC, panels B and D) correlations are labeled N for GlcN residue, I for IdoA residue, and A for nonreducing end unsaturated uronic acid residue.

by this method indicated that both Tris and EDTA were still present in the oligosaccharide sample. EDTA is an undesirable contaminant because it chelates  $\text{Ca}^{2+}$  and other cations, interfering with heparin–protein interactions and particularly  $\text{Ca}^{2+}$ -dependent protein interactions [18,19] and rendering the sample unusable for the protein interactions studies. A simple SAX spin column purification procedure of the gel-eluted oligosaccharides permitted complete removal of both EDTA and Tris according to the NMR data (Fig. 3). Structural integrity of gel-eluted oligosaccharides was assessed by two-dimensional correlation spectroscopy (2D COSY) and 2D heteronuclear multiple quantum coherence (2D HMQC) spectroscopy [20]. Comparison of the 2D COSY and 2D HMQC spectra of the BLH oligosaccharide mixture and gel-eluted oligosaccharides confirmed that the saccharide backbone structure remains unchanged after the CE-PAGE (Fig. 4).

In summary, the native CE-PAGE method for separation of the BLH oligosaccharide mixture described here affords high resolution of the mixture components over a broad MW range. After a simple buffer removal procedure, gel-eluted oligosaccharides are amena-

ble to structural characterization and/or protein binding studies. The amount of a purified oligosaccharide eluted from a 7-mm diameter gel tube was sufficient for SAX-HPLC and ESI-FTMS analysis; however, the NMR experiments required a 10-fold increase in sample amounts, and this was achieved using a 28-mm diameter gel tube and a larger electrophoresis cell. The results of PAGE, SAX-HPLC, ESI-FTMS, and NMR characterization of the gel-eluted oligosaccharides demonstrate that this method of separation does not introduce chemical artifacts or alter the oligosaccharide structure. To our knowledge, this is the first time that GAG oligosaccharides that are of sufficient purity for the spectral analysis of their structure have been recovered by PAGE.

#### Acknowledgments

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ab.2010.03.004.

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