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Heterogeneity of depolymerized heparin SEC fractions: to pool or not to pool?

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ABSTRACT

In the structural analysis of heparin and heparan sulfate, it is customary to combine or pool like-sized fractions obtained by size-exclusion chromatography (SEC) of enzymatically derived heparin oligosaccharides. In this study, we examine the heterogeneity of preparative-scale SEC fractions obtained from enzymatic digests of porcine intestinal mucosa heparin. Each fraction was profiled by capillary electrophoresis with UV detection (CE–UV) using a 60 mM formic acid running buffer at pH 3.43. Differences in the composition and relative concentration of components of the SEC fractions were observed for disaccharides and larger oligosaccharides. The heterogeneity of the fractions becomes more pronounced when heparin is digested using a heparin lyase cocktail. The heterogeneity of preparative SEC fractions was further investigated by reversed-phase ion-pairing ultraperformance liquid chromatography coupled with mass spectrometry (RPIP–UPLC–MS) using the ion-pairing reagent, tributylamine (Bu_3N). Our results suggest that preliminary profiling of preparative SEC fractions prior to pooling may simplify efforts to identify and/or isolate rare structures.

1. Introduction

The glycosaminoglycans (GAGs) heparin and heparan sulfate (HS) are linear polysaccharides primarily located in the extracellular space of connective tissues and associated with cell surfaces. They are involved in many biological processes related to human health, and their structures can be pathologically altered through mechanisms that are not well understood.[1–3] Pharmaceutical heparin is extracted from the tissues of animals used for consumption (i.e., porcine intestine, bovine lung), purified, and administered as an anticoagulant. Heparin inhibits blood coagulation by binding to antithrombin III through a specific pentasaccharide sequence, and has become one of the top-selling anticoagulant drugs world-wide with yearly sales reaching nearly four billion dollars.[4,5] The recent health crisis resulting from contamination of lots of pharmaceutical heparin with chemically modified chondroitin sulfate points to the need for sensitive, selective, and robust methods for profiling the composition of glycosaminoglycans, especially those used for therapeutic purposes.[6,7]

HS is structurally related to heparin, but contains fewer sulfate groups and has a more varied structure. HS proteoglycans are important components of mammalian cell surfaces and basement membranes.[2,8] HS mediates a number of important biological processes including cell adhesion, tumor metastasis, angiogenesis, and viral recognition.[9,10] In addition to the current importance of heparin as a complex bio-derived anticoagulant therapeutic agent, heparin and heparan sulfate-like structures are important potential sources of new therapeutics for use in cancer therapy and in the treatment of viral infections.[1,11–13] Therefore, a complete understanding of their microstructure is crucial for designing new heparin- and HS-inspired drugs.

The structural complexity of heparin and HS is related to their sequence heterogeneity, conformation, molecular weight, charge density, and chain flexibility. These intricacies can be considered at various biological stages, beginning with their biosynthesis. Both heparin and HS are biosynthesized as proteoglycans and are assembled from the same disaccharide building blocks.[1,14] These building blocks form linear polysaccharide chains containing repeating disaccharide units of multi-substituted (1→4)-linked pyranosyluronic acid and glucosamine residues.[1] Following biosynthesis, the diversity of heparin and HS polymers is increased through enzymatic remodeling reactions that introduce varying degrees of sulfation, acetylation, and epimerization. In HS, D-glucuronic acid is more prevalent than the L-iduronic acid form, and its polysaccharide chains are generally longer (30 kDa) and more varied in structure than heparin chains.[15] In heparin, these chains are attached to the various serine residues of heparin's core protein, serglycin, via a specific tetrasaccharide linkage. Tissue proteases then act on this core protein yielding peptidoglycan heparin, a small peptide attached to a single long polysaccharide chain (100 kDa). Finally, this peptidoglycan is processed by the enzyme, β-endoglucuronidase, to produce several smaller polysaccharide chains (~15 kDa) known as GAG heparin. Heparin contains an

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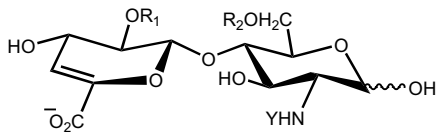
average of 2.7 sulfate residues per disaccharide unit.[15] It has the highest negative charge density of any known biological macromolecule due to the *O*- and *N*-sulfate groups as well as the iduronic acid carboxylate moiety.[15,16] The major building block of heparin is the trisulfated disaccharide, IdoA(2S)-(1→4)-GlcNS(6S), labeled as IS in Table 1. In contrast to heparin, HS GAG chains remain attached to their core proteins and are substituted with fewer sulfate groups, containing an average of only one sulfate group per disaccharide. It is important to note that the disaccharides shown in Table 1 are based on an α-(1→4)-linked D-glucosamine residue. Heparin and HS chains rarely also contain β-(1→4) linkages that can be important for protein recognition and binding; however, analysis of these oligosaccharides is beyond the scope of this work.[17,18]

Due to the difficulty of analyzing intact heparin, structural analysis begins by depolymerization of the isolated material into smaller oligosaccharide fragments using heparin lyases or chemical methods.[19,20] Exhaustive enzymatic digestion reduces the biopolymers predominantly to their disaccharide building blocks given in Table 1, although significant levels of larger enzyme resistant oligosaccharides remain even after 'exhaustive' enzymatic reaction.[21,22] During the enzymatic depolymerization process, a double bond is introduced in the C-4–C-5 bond of the uronic acid residue allowing UV detection at 232 nm. This provides a practical way to monitor the enzymatic depolymerization process and detect the relative abundance of different oligosaccharides in subsequent separations.

Structural characterization of heparin and HS requires determination of the identity of each disaccharide subunit, including the orientation of the glycosidic linkage and the sequence in which the disaccharide building blocks are assembled in the polysaccharide chain. This sequence information can be obtained by analysis of more complex mixtures of larger oligomers resulting from incomplete enzymatic depolymerization. Resolution of mixture components is typically accomplished by separation first according to size and second by the degree of negative charge using strong anion-exchange high-performance liquid chromatography (SAX-HPLC)[23,24] or capillary electrophoresis (CE).[25,26] The size separation involves size-exclusion chromatography (SEC) and can be conducted at the analytical or preparative scale. Fractionation of microgram quantities of heparin or heparan sulfate demands the use of high-pressure analytical-scale SEC to avoid dilution effects inherent in low-pressure methods.[22] Preparative-scale SEC is a low-pressure method used to separate bulk quantities of GAGs such as heparin extracted from animal tissue. In preparative-scale SEC, fractions are collected and UV absorbance is measured as a means to follow the separation, allowing the investigator to identify the fractions containing the disaccharides, tetrasaccharides, hexasaccharides, and larger oligosaccharides.

It has been customary in GAG analysis to combine or pool like-sized preparative SEC fractions due to the limited amount of oligosaccharides contained in each fraction.[27–31] Ziegler and Zaia have recently demonstrated the chromatographic resolution of the individual components IS, IIS, IVS, and IVA comprising the disaccharide peak obtained by analysis of a heparin digest with high-pressure analytical-scale SEC.[22] In this paper, we examine whether partial fractionation also occurs in preparative-scale SEC of heparin digests and explore the possibility that the practice of pooling fractions prior to analysis may complicate efforts to identify and/or isolate rare structures.

Table 1
Legend for the structures of commercially available heparin disaccharide standards; IA–IVA, IS–IVS, and IH–IVH



Disaccharide	R^1	R^2	Y
IS	SO_3^-	SO_3^-	SO_3^-
IIS	H	SO_3^-	SO_3^-
IIIS	SO_3^-	H	SO_3^-
IVS	H	H	SO_3^-
IA	SO_3^-	SO_3^-	$COCH_3$
IIA	H	SO_3^-	$COCH_3$
IIIA	SO_3^-	H	$COCH_3$
IVA	H	H	$COCH_3$
IH	SO_3^-	SO_3^-	H
IIH	H	SO_3^-	H
IIIH	SO_3^-	H	H
IVH	H	H	H

2. Results and discussion

2.1. Enzymatic depolymerization and SEC separation of porcine heparin

The heterogeneity of preparative-scale SEC fractions isolated from two enzymatic depolymerizations of intestinal porcine heparin, ED1 and ED2, was examined. Heparinase I was used in ED1 to selectively cleave highly sulfated polysaccharide chains containing (1→4) linkages between hexosamine and 2-O-sulfated iduronic acid residues via a β-elimination reaction.[19] In ED2, a mixture of heparinases I, II, and III was used. Heparinase II has less specificity than heparinase I, cleaving the glycosidic linkage of either 2-O-sulfated or 2-OH-containing uronic acids.[32] Heparinase III demonstrates a high selectivity for linkages in unsulfated regions of heparin that contain 2-OH-D-glucuronic acids and, to a lesser degree, 2-OH-L-iduronic acids.[14,32] Enzymatic cleavage introduces an unsaturated C-4–C-5 bond on the non-reducing end of the terminating uronic acid residue, producing a characteristic absorbance maximum at 232 nm. The reported molar extinction coefficient of a monounsaturated disaccharide at 232 nm is approximately 5500 M^{-1} cm^{-1}.[24] This chromophore can be used to monitor the progression of the enzymatic reaction through UV absorbance measurements and to assess the oligosaccharide content of each fraction collected during the subsequent SEC size separation. For ED1, the final absorbance corresponded to cleavage of roughly one-third of the available disaccharide bonds based on the nominal mass of an average heparin disaccharide.

Preparative-scale SEC was performed following ED1 and ED2. The progress of the size separation was monitored by plotting the UV absorbance versus SEC fraction number, as shown for ED1 in Figure 1. In this chromatogram, the peak with the greatest absorbance (fractions 194–246) corresponds to the disaccharide components (dp 2) produced by complete enzymatic digestion. The preceding peaks correspond to larger oligosaccharides including tetrasaccharides (dp 4), and hexasaccharides (dp 6). The resolution of this SEC–UV chromatogram is poorer than could be obtained with high-pressure SEC, but this compromise is necessary for the efficient fractionation of larger masses of material.[15] A similar UV–SEC chromatogram was also constructed for ED2, but the peak with the greatest absorbance (fractions 46–79) corresponded to the tetrasaccharide components.

In a typical heparin analysis utilizing SEC fractionation of oligosaccharide components, the next step is to pool like-sized fractions. This is done with the goal of increasing the oligosaccharide content of each size fraction prior to further fractionation using an orthogonal separation method such as anion-exchange chromatography. The underlying assumption inherent in the pooling of

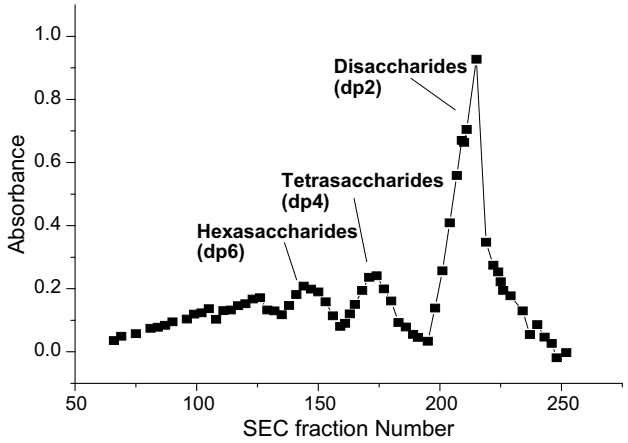


Figure 1. The preparative SEC-UV chromatogram showing relative absorbance versus fraction number for the separation of ED1. Fractions 194–246 contain mainly disaccharide components, while the preceding peaks correspond to larger, less abundant oligosaccharides.

SEC fractions is that a peak containing like-sized oligosaccharides, for example, dp 2, is fairly homogeneous across all of the fractions collected. However, if partial fractionation occurs within a SEC peak, the pooling of fractions may complicate efforts to identify and/or isolate rare structures since the individual fractions would not be homogeneous in their composition. In this case, pooling would dilute minor components that might be present in only a few of the collected fractions.

2.2. CE–UV analysis of SEC fractions

Testing whether indiscriminant pooling of SEC fractions can hinder the isolation of minor components requires an analytical method that can resolve the size-selected components based on another property of the oligosaccharides, such as electrophoretic mobility. CE–UV is an ideal tool for profiling size-selected SEC fractions due to its high resolving power and low sample consumption.[33] Because heparin disaccharides are commercially available, we initiated these experiments using the SEC fractions from the dp 2 peak of ED1. The electropherogram shown in Figure 2 demonstrates the separation of a mixture containing 11 commercially available disaccharide standards. The majority of the disaccharides in the mixture were completely resolved, even two of the three isomeric sets (IIS/IIIS/IH and IIA/IIIA). The IIH/IIIH isomeric pair was not as well resolved, in part, because these compounds have only a fractional negative charge under the separation conditions. In addition, the IIH and IIIH peaks may be broadened due to incomplete separation of the glucosamine anomers.[34] For example, heparin disaccharide IH produced a split peak resulting from the partial separation of its glucosamine anomers. Although heparin disaccharide standard IVH was present in the mixture, it is not detected due to its cationic behavior under these conditions. This electropherogram of the disaccharide standards was used to identify the components in each of the dp 2 SEC fractions of ED1. The identity of individual components was confirmed in several electropherograms by spiking with authentic standards. A representative example of the CE electropherograms obtained in the spiking experiments is reported in the Supplementary data Figure S1 for fraction 216 from the preparative SEC separation of ED1.

The CE electropherograms of the dp 2 fractions for ED1 revealed that although the components are structurally similar disaccharides, there can be differences in the abundance of various components across the series of SEC fractions. This seems reasonable, since all the disaccharides shown in Table 1 do not have the same nominal mass. In addition, the more highly sulfated disaccharides may behave as though they have a larger effective size than anticipated from their molecular formula due to hydrodynamic drag from associated cations. The CE electropherogram (Fig. 3A) of fraction 201, taken near the beginning of the SEC dp 2 peak, contains a single component corresponding to disaccharide IS. Later in the SEC separation, but also included in the dp 2 peak, fraction 219 was collected and analyzed (Fig. 3B). This fraction contained several other peaks in addition to the IS peak. Spiking confirmed that the peaks migrating at 11.52 and 11.93 min were heparin disaccharides IIIS and IIS, respectively. The earlier peaks in the electropherogram, migrating prior to IS, are unknown. However, it can be inferred from the migration order that they have a greater charge-to-mass ratio than the disaccharides. It is possible that these components are larger oligosaccharides, perhaps trisaccharides.[22,35] CE analysis of all the SEC fractions revealed that overall the most

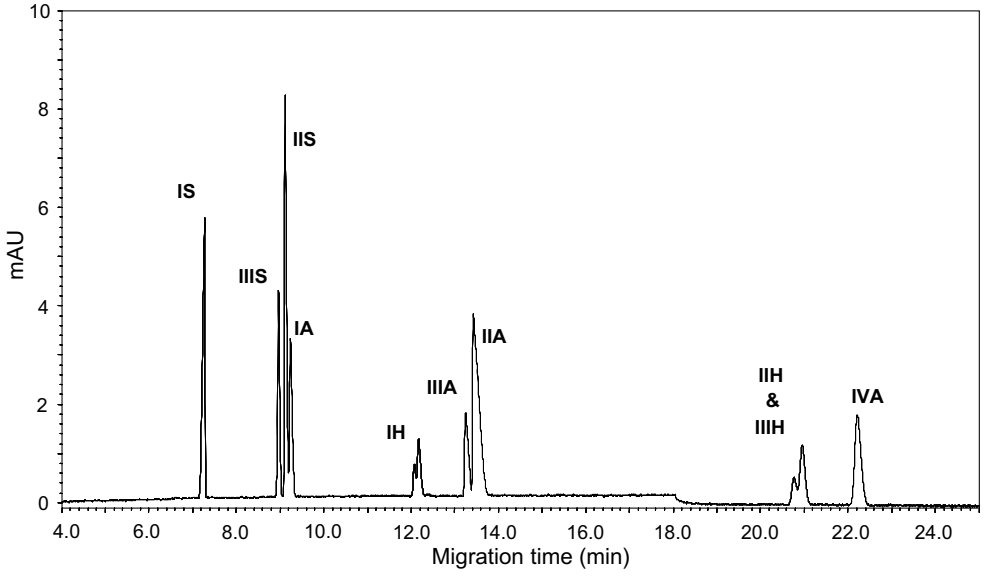


Figure 2. Electropherogram of the separation of 10 commercially available disaccharide standards from Table 1 in 60 mM HCO_2H buffer at pH 3.43. Although heparin disaccharide standard IVH was present in the mixture, it is not detected because it is cationic under these conditions.

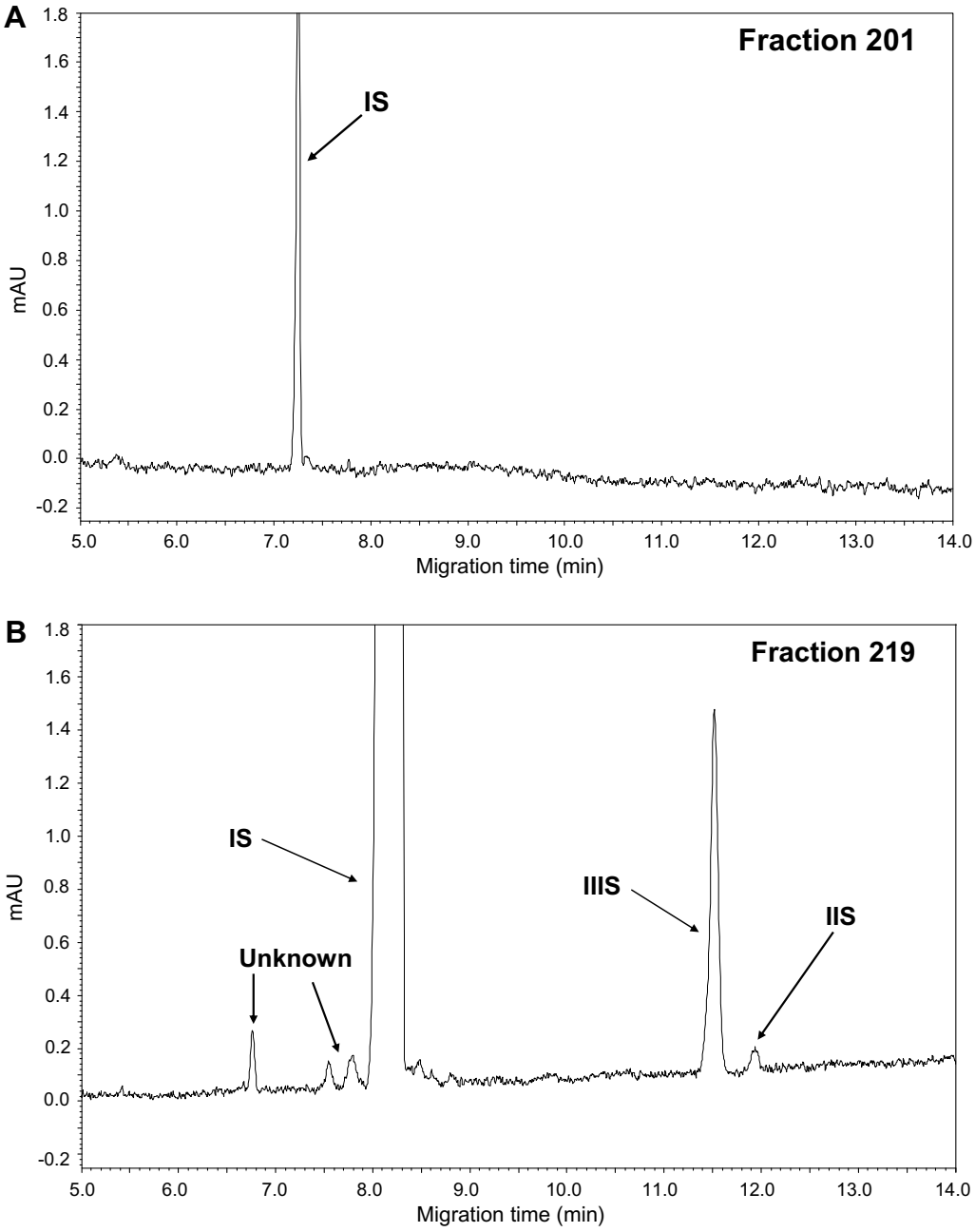


Figure 3. CE electropherograms of fraction 201 (A) and fraction 219 (B) from the dp 2 peak of the preparative SEC separation of ED1.

abundant component in each fraction comprising the dp 2 peak is heparin disaccharide IS. Disaccharide IIIS is the next most abundant component, however, as observed in Figure 3B, it is present at much lower amounts than IS. Disaccharides IIS and IA appear intermittently as minor components across the dp 2 peak.

The plot shown in Figure 4 was constructed to illustrate both the variation of the IIIS/IS peak area ratio (□) and the relative disaccharide concentration (▲) across the ED1 dp 2 peak. This plot shows that the fractions containing the largest concentration of IIIS were collected in the latter half of the dp 2 peak. If the goal of this experiment was to preferentially isolate the IIIS disaccharide, fractions 230–245 should be used. With the usual practice of pooling all of the SEC fractions comprising dp 2, the concentration of IIIS would be diluted significantly, making its subsequent isolation more difficult.

Differences in the composition and relative concentration of components in the SEC fractions are also observed for larger oligosaccharides. The heterogeneity across the SEC dp 4 peak for ED1 (fractions 160–193) was also examined by CE. The CE electropherograms from fractions 162, 163, 168, and 176 are shown in Figure 5. Unfortunately, because of the lack of authentic tetrasaccharide standards, we could not positively identify the individual peaks in these electropherograms. The overall tetrasaccharide content for fraction 163 (Fig. 5B) is more than twice the concentration of the preceding fraction, 162 (Fig. 5A), although there seems to be little difference in the relative abundance of the mixture components. Comparison of fractions 163 (Fig. 5B), 168 (Fig. 5C), and 176 (Fig. 5D) reveals interesting trends in the relative abundance of the major components P1, P2, and P3. Assuming that the trends observed for the elution of the disaccharides holds for the tetrasac-

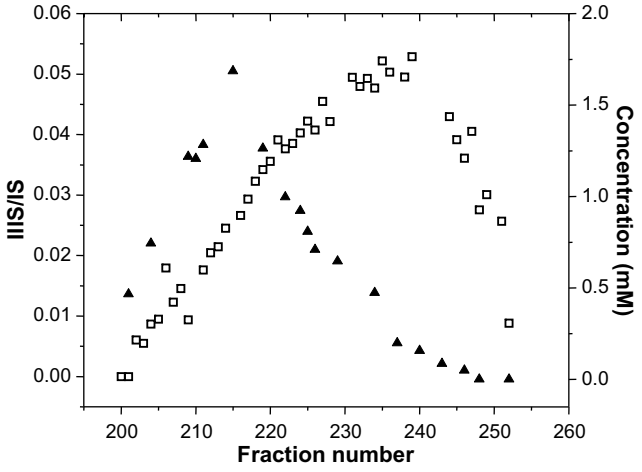


Figure 4. A plot of the peak area ratios of disaccharides IIIS and IS (□) and relative disaccharide concentration (▲) versus fraction number demonstrating the variability in the abundance of IS and IIIS across the dp 2 peak shown in Figure 1.

charides, we suspect that P1 is the most highly sulfated of the three major components, and is most likely the IS–IS tetrasaccharide. Various minor components that migrate after the three major components are also present in all four electropherograms, but are most abundant in fractions 163 and 176. Pooling of all the dp 4 fractions would clearly dilute and further complicate isolation of these minor components.

The heterogeneity of the preparative SEC fractions becomes even more pronounced when heparin is digested using a heparin lyase cocktail. The CE–UV profile (Fig. 6A) of SEC fraction 72 of the tetrasaccharide peak of ED2 has greater compositional diversity than observed in Figure 5 for the electropherograms derived from ED1. The electropherogram for fraction 72 shows two major components migrating between 4 and 5 min, as well as several additional components of intermediate and minor abundance. A major limitation in identification of the tetrasaccharide components in these electropherograms is the lack of structural information available from CE–UV. Furthermore, without standards there is no way to evaluate peak purity, which is important because peak overlap is a potential problem for complex mixtures. Although CE–MS could potentially provide important structural information about the separated components, such experiments are far from routine.

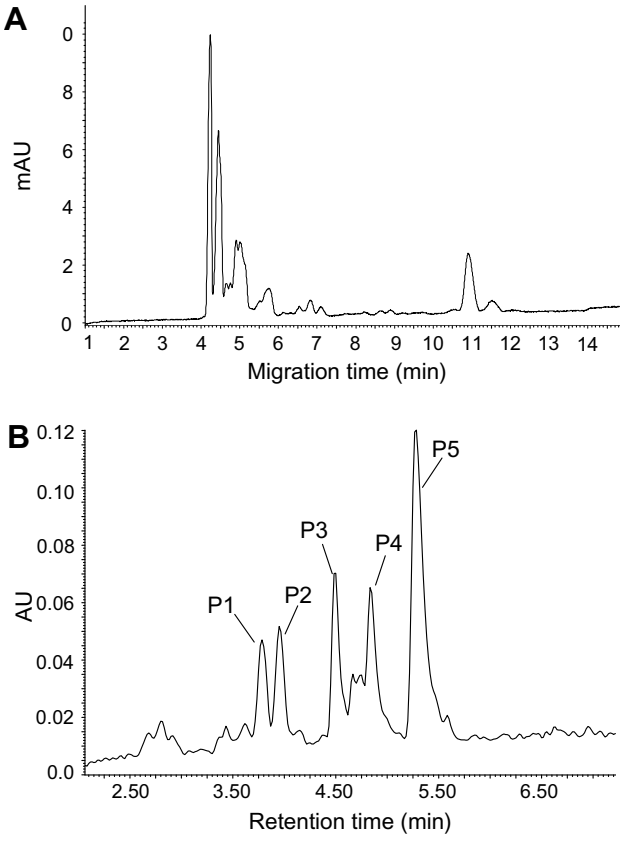


Figure 6. (A) CE–UV electropherogram of SEC fraction 72 of the dp 4 peak of ED2. (B) RPIP–UPLC–UV chromatogram of fraction 72 measured with UV detection at 232 nm. The major peaks are labeled P1-P5.

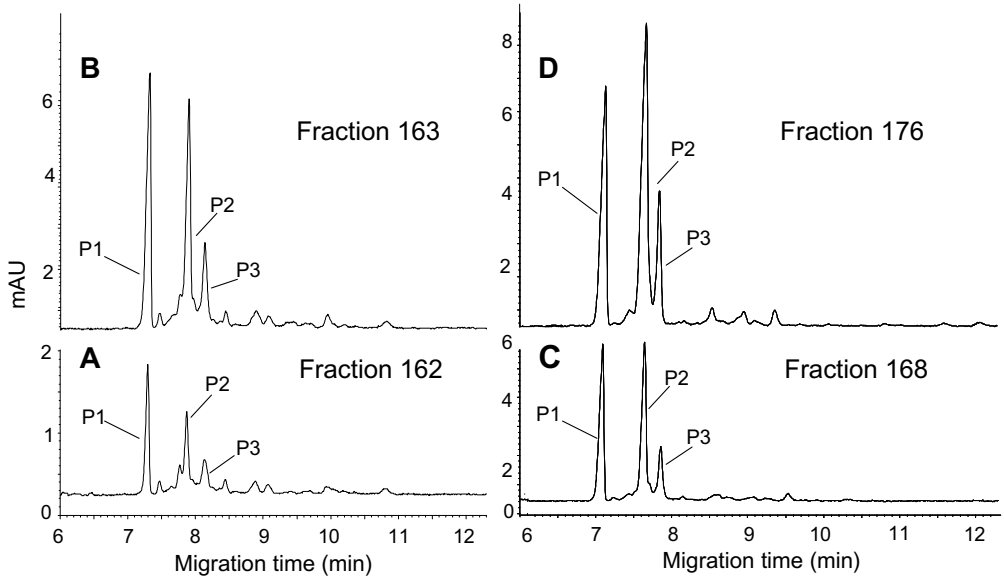


Figure 5. CE electropherograms of the preparative SEC dp 4 fractions 162 (A), 163 (B), 168 (C), and 176 (D) for ED1. The three major tetrasaccharide components are labeled P1, P2, and P3.

2.3. Reversed-phase ion-pairing-UPLC–UV–MS

To further investigate the composition of ED2 SEC fraction 72, RPIP–UPLC–UV–MS was employed using the volatile ion-pairing reagent, Bu_3N. We have previously utilized this approach to separate and detect the disaccharide standards in Table 1.[34] Because the reversed-phase ion-pairing separation mechanism relies on the relative strength of interactions between the negatively charged oligosaccharides and the cationic ion-pairing reagent, highly sulfated compounds are more highly retained by the C_{18} column. As a result, the elution order of the disaccharides in the RPIP–UPLC separation is roughly the inverse of the CE migration order shown in Figure 2.

The RPIP–UPLC chromatogram of fraction 72 measured with UV detection at 232 nm is shown in Figure 6B. The fully sulfated heparin tetrasaccharide IS–IS should be the most highly retained component in this separation. A portion of the mass spectrum extracted from the prominent peak (P5) is shown in Figure 7A. The doubly charged molecular ion, *m*/*z* of 575.9639, confirms the identity of this tetrasaccharide as IS–IS. The mass spectral analysis of heparin-derived oligosaccharides is complicated by the preponderance of isomers. As a result, it is not possible for us to unambiguously assign the remaining components from these data alone. The mass spectra for P2 (Fig. 7B) and P1 are very similar with a doubly charged molecular ion of *m*/*z* 517.0101, indicating that at least one of the disaccharide components contains an acetylated glucosamine. Similarly, P4 (Fig. 7C) and P3 have a doubly charged molecular ion *m*/*z* 535.9874, which could be produced by a number of different disaccharide combinations involving IS and IIS, IIIS, or IH. Although additional structural information could be derived for these components from MS/MS and NMR experiments, these are beyond the scope of the current study to probe the heterogeneity resulting during SEC fractionation of heparin enzymatic digests.[36–38]

3. Conclusions

The heterogeneous composition of peaks obtained by SEC separation of heparin digests was demonstrated using CE–UV profiling of collected fractions. Although this was most clearly observed for

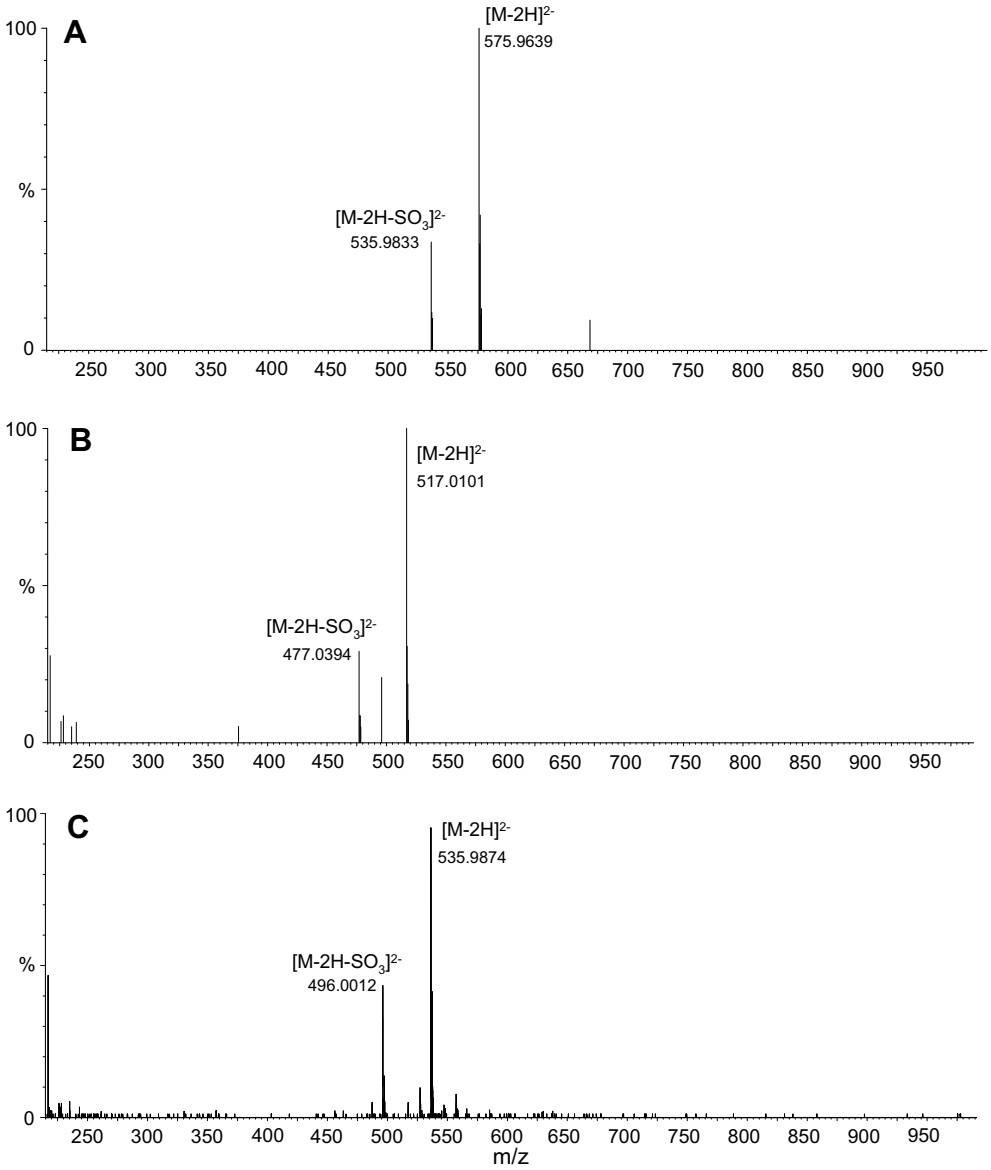


Figure 7. (A) Extracted mass spectrum of the most intense absorbance peak (P5) from the RPIP–UPLC–UV chromatogram shown in Figure 6B. The molecular ion, *m*/*z* 575.9639, confirms the identity of this tetrasaccharide as IS–IS. (B) Mass spectrum of P2 and (C) P4 from the same chromatogram.

the disaccharide SEC fractions, peak heterogeneity was also observed for the tetrasaccharide SEC peak. Examination of the preparative SEC fractions of larger oligosaccharides is complicated by their greater complexity and the limited availability of standards. Analysis using RPIP–UPLC–UV–MS can provide needed structural insights and should prove very useful in future studies. The results of these experiments suggest that if the goal of a heparin or HS digestion is the isolation of minor components, analysis of the SEC fractions prior to pooling can allow the intelligent combination of fractions containing an unknown species of interest.

4. Experimental

4.1. Chemicals

Heparin disaccharide standards I–IIIS, IA, I–IVH, and heparinase I (EC 4.2.2.7), heparinase II (EC number not yet assigned), heparinase III (EC 4.2.2.8) isolated from *Flavobacterium heparinum*, the sodium salt of porcine intestinal mucosal heparin, sodium acetate, calcium acetate, and tributylammonium acetate were purchased from Sigma–Aldrich, Inc. (St. Louis, MO). Heparin disaccharide standards IIA–IVA were purchased from V-Labs, Inc. (Covington, LA). HPLC-grade water was purchased from Burdick and Jackson (Muskegon, MI, USA). Formic acid, ammonium bicarbonate, acetonitrile, and sodium hydroxide were purchased from Fisher Scientific Co. (Fair Lawn, NJ).

4.2. Enzymatic digestion of heparin

In the first enzymatic depolymerization (ED1), 130 mg of porcine intestinal mucosal heparin was dissolved in a 4 mL buffer solution containing 20 mM $Ca(OAc)_2$ and 50 mM NaOAc and was digested with 50 U of heparinase I (Sigma units, where 1 U is defined as the quantity of enzyme that will form 0.1 μmol of unsaturated uronic acid product per hour at pH 7.5 and 25 °C).[39] The depolymerization reaction was performed at 37 °C at an initial pH of 7.5. The progression of the digestion was followed by performing UV measurements (232 nm) on 6 μL of the digest diluted to a total volume of 600 μL with buffer. All UV measurements were performed using a Varian Cary 50 Bio spectrophotometer (Palo Alto, CA, USA). Because the desired level of depolymerization was not reached during the initial reaction, after 30 h a second aliquot of heparinase I enzyme (50 U) was added. The total time for the depolymerization was 50 h. A second enzymatic depolymerization (ED2) was performed, following the above procedure, on 130 mg of porcine intestinal mucosa heparin using 50 U each of heparin lyases I, II, and III.

4.3. SEC fractionation

Both heparin-derived oligosaccharide solutions were size fractionated separately on a column containing Bio-Rad Bio-Gel P-6 resin (3 cm × 47 cm) and eluted with 0.5 M NH_4HCO_3 at a flow rate of 0.5 mL/min. The total volume contained in each fraction for ED1 and ED2 was 0.5 mL and 1.0 mL, respectively. The progress of both separations was monitored by measurement of UV absorption off-line. Each fraction was lyophilized, reconstituted in 500 μL of HPLC-grade water, and then lyophilized again to reduce the NH_4HCO_3 content. The lyophilized powders were stored at −80 °C until further use.

4.4. Capillary electrophoresis

The CE separation method was optimized using a standard mixture of heparin disaccharides IA–IVA, IS–IIIS, and IH–IVH dissolved in 1.5 mL of 60 mM HCO_2H buffer (pH 3.43). The structures of the standards are defined in Table 1, and their concentrations ranged from 0.13 to 0.75 mM. CE separations were performed on a Beckman Coulter ProteomeLab™ PA800 instrument equipped with a diode array detector, fluid-cooled column cartridge, and automatic injector. The fused silica capillaries supplied by MicroSolv Technology Corporation (Eatontown, NJ) were 50 cm in length (length to detector 42.5 cm), 75 μm i.d., and 375 μm o.d. The detection window was formed by removal of ~1 mm of polyimide coating from the outside of the capillary using a MicroSolv Window Maker™. Capillaries were conditioned by rinsing with 0.1 M NaOH for 1 min, HPLC-grade water for 5 min, followed by a rinse with 60 mM HCO_2H buffer (pH 3.43) for 5 min, all at high pressure (20 psi). The capillary was equilibrated with the buffer by applying a separation voltage of 18 kV using reversed polarity for 7 min without pressure. Between experiments, the capillary was rinsed with HPLC-grade water for 2 min then with buffer for 3 min, both at a pressure of 50 psi. The separation voltage was 20 kV. Samples were pressure injected at 0.5 psi for 7 s. For the first 18 min of the separation a pressure of 0.1 psi was applied, after which the pressure was 0.5 psi for the duration of the run (12 min). Absorbance was monitored at 232 nm, and the data were collected and processed using the 32 KARAT software (Beckman Coulter, Inc., Fullerton, CA). The lyophilized, heparin-derived fractions were reconstituted in 600 μL of HPLC-grade water, and CE separations were performed on each fraction to investigate the oligosaccharide content and purity. During the development and optimization of the standard separation method, the interday reproducibility of migration times remained within 1%. Over the course of performing separations on the digested fractions, we observed occasional drift in the migration time of the components. This was probably due to the presence of residual enzyme or other matrix components that adsorbed to the capillary wall altering the electroosmotic flow. A neutral marker can be used to account for changes in electroosmotic flow and aid in the identification of analyte peaks.[36,37] However, because of the reduction in electroosmotic flow in reversed polarity CE experiments, the use of a neutral marker would require a significant increase in the total separation time. Therefore, spiking with standards was employed to confirm the identity of peaks in the electropherograms in lieu of a neutral marker (see example in Supplementary data).

4.5. Ultraperformance liquid chromatography (UPLC)–UV–MS

Chromatographic separations were performed on an AQUITY UPLC system (Waters Corp., Milford, MA) using a 2.1 × 100 mm Acquity UPLC BEH C_{18} column packed with 1.7-μm particles and a guard column containing the same material. Analytes were detected using both UV absorbance at 232 nm and negative-ion mass spectrometry. The column temperature was maintained at 40 °C, and the samples were eluted at 0.5 mL/min. A binary solvent system composed of 5% CH_3N (Buffer A) and 80% CH_3N (Buffer B) was used. Both buffers contained 2.5 mM tributylamine (Bu_3N) and 17.5 mM NH_4OAc buffer at pH 7.00. The separation was performed using a gradient of 69–31% A over 10 min. This was increased to 100% B over 1 min and held at 100% B for an additional 1 min before returning to the initial composition of 69% A over 1 min. This composition was retained for 2 min to allow for re-equilibration before the next injection.

Mass spectrometry was performed online using a Micromass quadrupole-time-of-flight (Q-TOF) instrument (Waters Corp., Milford, MA). Although Bu_3N is used as an ion-pairing reagent for the UPLC separation, prominent Bu_3N adduct ions were not detected in the mass spectra. The data acquisition software used was MASSLYNX NT, version 4.1. Mass spectra were obtained in the negative-ion mode. The capillary and cone voltages were set to 3000

and 20 V, respectively. The nebulization gas was set to 650 L/h at a temperature of 200 °C, the cone gas set to 10 L/h, and the source temperature was kept at 120 °C. These parameters were optimized using a mixture of heparin-derived tetrasaccharide fractions to minimize in source fragmentation and reduce ions resulting from loss of sulfate. The scan time was 0.5 s with a 0.1 s interscan delay. The mass-to-charge (m/z) range was set at 215–1000.

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Supplementary data

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