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Ion Mobility Mass Spectrometry-Based Disaccharide Analysis of Glycosaminoglycans

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Glycosaminoglycans (GAGs) are linear and acidic polysaccharides. They are ubiquitous molecules, which are involved in a wide range of biological processes. Despite being structurally simple at first glance, with a repeating backbone of alternating hexuronic acid and hexosamine dimers, GAGs display a highly complex structure, which predominantly results from their heterogeneous sulfation patterns. The commonly applied method for compositional analysis of all GAGs is "disaccharide analysis." In this process, GAGs are enzymatically depolymerized into disaccharides, derivatized with a fluorescent label, and then analysed through liquid chromatography. The limiting factor in the high throughput analysis of GAG disaccharides is the time-consuming liquid chromatography. To address this limitation, we here utilized trapped ion mobility-mass spectrometry (TIM-MS) for the separation of isomeric GAG disaccharides, which reduces the measurement time from hours to a few minutes. A full set of disaccharides comprises twelve structures, with eight possessing isomers. Most disaccharides cannot be differentiated by TIM-MS in underivatized form. Therefore, we developed chemical modifications to reduce sample complexity and enhance differentiability. Quantification is performed using stable isotope labelled standards, which are easily available due to the nature of the performed modifications.

Introduction

Glycans, also known as carbohydrates or saccharides, are indispensable biomolecules found in all living organisms, playing critical roles in various biological processes. With diverse structures that range from simple monosaccharides to complex branched polysaccharides, glycans serve as essential components of cell membranes, energy storage molecules, and mediators of cellular communication.[1] Among them, glycosaminoglycans (GAGs) stand out due to their therapeutic applications, placing their analysis at the forefront of glycomics. GAGs are linear, often heavily sulfated polysaccharides consisting of repeating disaccharide units, predominantly composed of a uronic acid and a hexosamine.[2] They are ubiquitous in mammalian tissues and fluids, exerting essential functions in processes such as blood coagulation, inflammation, and cell signaling.[3] Heparin and heparan sulfate (HS) form the most prominent group of GAGs. Heparin, primarily found in mast cells and basophils, is renowned for its anticoagulant properties.[4] In contrast, HS is located on cell surfaces, bound to proteoglycans in the extracellular matrix, and plays a role in various biological activities such as cell adhesion, growth factor signaling, and morphogenesis.[3,5] However, the direct relationships between the structure Heparin/HS and its biological activity remains elusive because of the underlying structural complexity. While the composition is fairly simple, the complexity arises from diverse patterns of sulfation, backbone isomerization and N-acetylation. Possible sulfation sites are the *2O*-position of the uronic acid and the *6O*-, *N*- and rare *3O*-position of the glucosamine. Further variability along the Hep/HS chains can stem from the acetylation of the glucosamine or the epimerization of the uronic acid between *L*-iduronic or *D*-glucuronic acid.[6] This structural complexity yields significant challenges in their analysis. Given that their biological activity is intricately linked to their structural features, it is crucial to elucidate the fine structural details of heparin/HS in order to understand their functions and promote glycan-based therapeutic interventions. Consequently, analytical methods capable of characterizing heparin/HS structures with high precision and sensitivity are indispensable in glycomics research.

In addition to conventional condensed phase techniques such as nuclear magnetic resonance[7] and infrared spectroscopy,[8] mass spectrometry (MS)-based techniques play a pivotal role in in GAG characterisation. Sequencing is typically achieved through MS/MS-based approaches, with proof-of-concept studies available for common activation techniques such as collision-induced dissociation,[9] ultraviolet photo

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dissociation,[10] negative electron transfer dissociation[11] and electron detachment dissociation.[12] However, despite these efforts, high-throughput application remains a challenge, primarily due to the intricate fragmentation patterns of GAGs. The combination of MS/MS experiments with databases is a promising avenue, particularly when coupled with techniques such as ion mobility spectrometry (IMS), which facilitates simpler structural assignments.[13]

Given the complex nature of GAG sequencing, a more straightforward approach is the compositional disaccharide analysis, which can already serve as a biomarker in disease progression. The typical workflow in heparin/HS disaccharide analysis involves an enzymatic depolymerisation followed by chromatographic separation and detection. Enzymatic depolymerisation with the specific GAG lyases heparinase I, II and III generates disaccharide fragments from heparin/HS chains (figure 1a).[14]

During the depolymerisation the stereocenter at C5 of the uronic acid is lost and a 4-5-unsaturated 4-deoxy-hex-4-enopyarnuronic acid is formed. This unsaturated uronic acid can be detected via UV absorption at 232 nm due its conjugated 1–4 Michael system. Since the UV absorption is very low and the sample scale is usually in the sub-microgram region, a fluorophore is most often attached to the reducing end of the disaccharides to promote their detection.

A wide range of liquid chromatography (LC) techniques have been applied for the separation of GAG disaccharides including reversed phase,[15] ion pairing,[16] hydrophilic interaction,[17] size exclusion, and strong anion exchange chromatography.[18] However, a complete LC separation of HS disaccharides typically takes half an hour to an hour, depending on the method and the chosen disaccharide range. Additionally, calibration, regeneration, and blank runs have to be performed in-between sample injections to maintain column performance, which is often not accounted for in the total run time. Although the LC analysis time is relatively short compared to the total sample preparation time, the capacity to process large batches, comprising hundreds of samples at once during sample preparation, contrasts with the sequential nature of the LC separation, rendering it the time-limiting step.

IMS has emerged as a powerful technique for separating glycans in the gas phase. Several studies have demonstrated the similarity of both LC and IMS in terms of resolution and resolving power.[20] The separation time of glycans in IMS is decreased compared to LC by a factor of >1000 with most IMS instruments working in the sub-second region. The mobility of glycans in IMS experiments depend on their respective collision cross-section (CCS), a molecular descriptor primarily influenced by the size, shape, and charge of the analyte ion.[21] While IMS resolution is still lower compared to most LC experiments, instrumentation in IMS has significantly improved in recent years. With increasing IMS resolution, the potential for replacing common LC-MS workflows with faster and more sample-efficient IM-MS workflows becomes increasingly enticing.

Here, we introduce a trapped ion mobility spectrometry (TIMS)-based method to separate and characterize isomeric heparin/HS disaccharides from mixtures and biological samples. The entire analytical workflow takes only a few minutes, making it an attractive alternative to the comparatively slow established LC-MS/FLD methods.

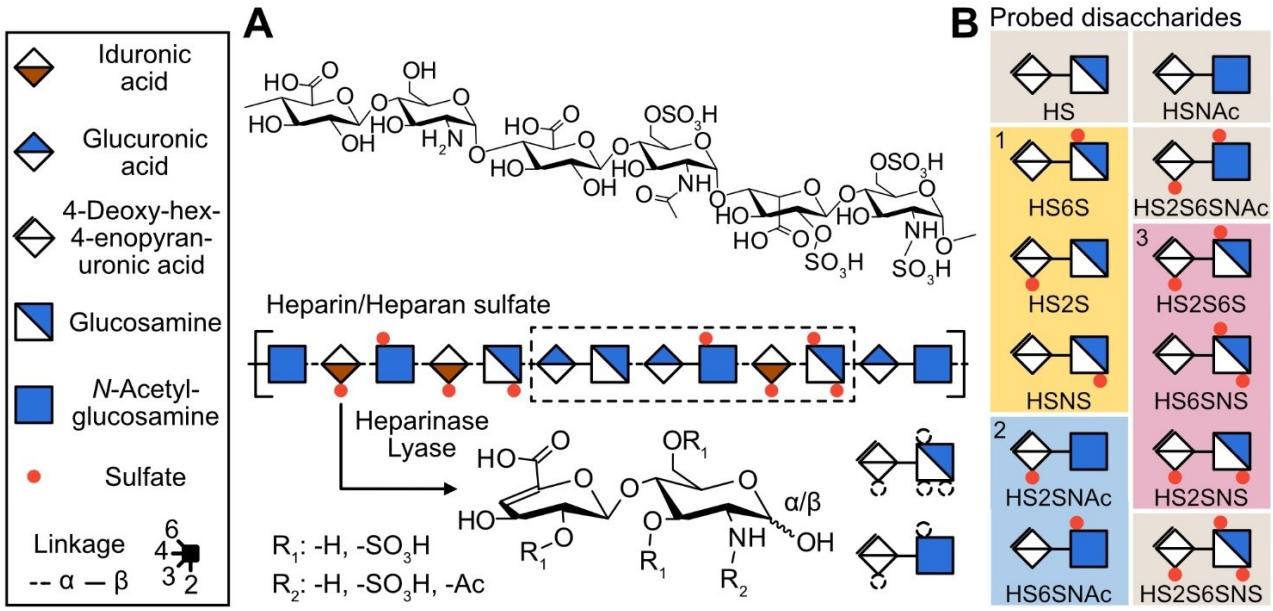

Figure 1. The glycosaminoglycans (GAGs) heparin/heparan sulfate (HS). A) HS as chemical structure and simplified based on the Symbol Nomenclature for Glycans (SNFG).[19] The example structure (top) can be seen as SNFG depiction in the doted box. B) List of probed disaccharides and their nomenclature. Three groups of isomeric disaccharides are present: group 1, monosulfated and non-acetylated (yellow); group 2, monosulfated and acetylated (blue); and group 3, disulfated and non-acetylated (red).

Results and Discussion

The twelve most common GAG–disaccharides are available as standards. Of these, eight possess isomeric structures and are not distinguishable by mass spectrometry alone. These eight structures form three distinct isomeric groups (figure 1b). The first group contains three different monosulfated non-acetylated structures (yellow, 417 Da), the second group contains three different monosulfated acetylated structures (blue, 459 Da) and the third group is comprised of three disulfated non-acetylated structures (red, 597 Da). Other non-isomeric structures are the unmodified HS–disaccharide, the acetylated, non-sulfated disaccharide, a disulfated and acetylated species and finally the fully sulfated disaccharide variant.

Direct infusion TIMS analysis of the isomeric groups 1 and 3 revealed very complex mobilograms (figure 2a) which does not allow a clear distinction of the components. The presence of reducing-end anomers further complicates the analysis leading to the presence of at least six structures and conformers for each isomeric group.[22] Therefore, propionylation was performed to reduce the complexity of the sample. As both isomeric groups consist of at least one structure with a primary amine group on position 2 of the glucosamine, selective N-propionylation of the amine reduces the number of isomers for each group from three to two (figure 2b). The mobilograms of these isomeric groups demonstrate a significant reduction in complexity when compared to the non-modified variants (figure 2c). However, a clear annotation of structures from a mixture remains challenging, particularly evident in the case of HS–2SNS and HS–6SNS (red). If a study focuses on free amine-containing structures, the propionylation step should be included; otherwise, the distinction of sulfation positions remains ambiguous.

To further reduce complexity and enhance separation in TIMS, a label is attached to the reducing end of the disaccharides. Reductive amination removes the stereocenter at the anomeric carbon C1, which reduces complexity. However, the choice of the label is crucial, as different labels have been shown to either improve or worsen the separation of glycans using IMS.[23] A suitable label for the separation of GAGs is procainamide (ProA). Procainamide is often used as fluorescence label for LC-FLD glycan analysis but also finds use as a charge tag to improve the ionisation efficiency of glycans in positive ion mode MS.[24] However, due to their negative charge and the generally labile nature of sulfate groups in positively charged ions, IMS analysis is conducted in negative ion polarity, here. The use of procainamide as a label significantly enhances the separation of certain isomers, particularly when compared to the structurally related label procaine (see SI, Figure S1), which features an ester bond instead of an amide.

To evaluate the separation of a complete set of disaccharides, a model mixture containing all twelve standards was generated. This mixture was N-propionylated, labeled with procainamide, and then analyzed by direct infusion TIMS. Qualitatively, the constituents of the mixture can be readily identified by TIMS, with all disaccharides observable as singly charged species. Doubly charged species are mostly formed by the di- and trisulfated disaccharides, albeit at reduced abundance. For the TIMS analysis we focus on the singly charged species due to their better isomer separation. Mass spectrometric analysis reveals nine singly charged species. Six of these correspond to distinct disaccharides (*m/z* 597, 611, 635, 711, 757 and 795), while three signals (*m/z* 677, 691 and 715)

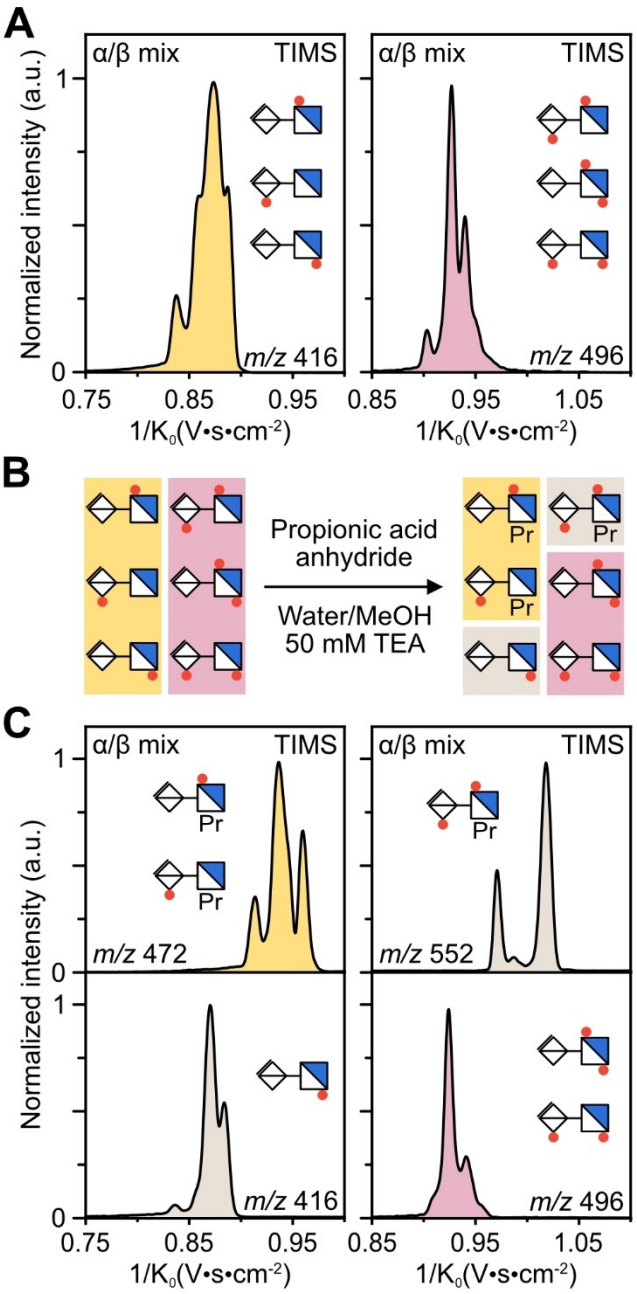

Figure 2. Chemical modification of the disaccharides to reduce complexity. a) TIMS mobilogram of the equimolar isomeric mixtures of monosulfated (HS–6S, HS–2S and HS–NS in yellow) and disulfated species (HS–2S6S, HS–6SNS and HS–2SNS in red). b) Propionylation selectively modifies primary amines of HS–2S, HS–6S and HS–2S6S resulting in a 56 Da shift which removes one of each isomer from the mixtures. c) TIMS mobilograms of the modified mixtures shows slightly improved separation and reduced complexity. However, multiple peaks are still visible due to the presence of α/β anomers at the reducing end.

correspond to pairs of sulfation isomers (figure 3a). Mixtures were compared to mobilities of single standards. The extracted TIMS mobilogram of HS–2SNAc and HS–6SNAc show a baseline separation of the species (figure 3b, blue). The structurally closely related HS–2SN and HS–6SN isomer pair also exhibits baseline separation, albeit with a slight shift towards lower mobilities (figure 3b, yellow). The disulfated species HS–2SNS and HS–6SNS can still be separated sufficiently, although no baseline separation is achieved (figure 3b, red) which is common in LC methods. A trend in the separation of the isomers is that *6O*-sulfated species generally have a lower mobility than their *2O*-sulfated counterparts, likely due to an interaction of the *6O*-sulfate with the procainamide label.

Procainamide labelling of the GAG disaccharides provides additional options for analysis. By utilizing a stable isotope-labelled version of procainamide, a heavy-labelled standard mixture with known concentrations for each disaccharide can be spiked into a sample for quantification. In principle, even absolute quantification could be achieved by this method. While also ^{13}C-labelled disaccharides are commercially available, they are very expensive, especially when considering that standards have to be spiked into each sample. Therefore, we here take advantage of the introduced reducing end label.

To prevent an overlap of isotopic peaks of the sample with the internal standard mixture, particularly for the more heavily sulfated species, a label with at least 5 Da difference should be selected. Due to its simple synthesis, we choose ProA-d_{10} as heavy label. A mass spectrum of ProA labelled HS disaccharide mixture spiked in with a ProA-d_{10} mixture is shown in figure 4a. By simply comparing the intensity of the ProA labelled sample with the 10 Da shifted ProA-d_{10} labelled internal standard a relative quantification of the sample can be achieved. For the isomeric disaccharides the previously discussed TIMS separation is used (figure 4b). Similar to liquid chromatography, the introduction of a heavy label results in a slight shift of mobilities. Since this shift is significantly smaller than the peak width (< 1 % shift in mobility) it does not affect the analysis. In general, we recommend comparing the TIMS 1/K_0-area of sample and internal standard for all disaccharides, not only for the isomeric structures. The TIMS separation aids accuracy by removing unrelated isobaric species e.g. ions with higher charge states from interfering with the quantification.

To benchmark the method, we tested the four mock mixtures 1–4 that represent various HS disaccharide ratios (figure 5). The relative abundance of the HS disaccharides ranges from 4.2 to 12.5 %. Propionylation and reducing end labelling was performed as described previously, but on the complete mixtures rather than the individual components. After the modifications the sample was dissolved in an ammonium acetate solution, and an equimolar ProA-d_{10} labelled standard

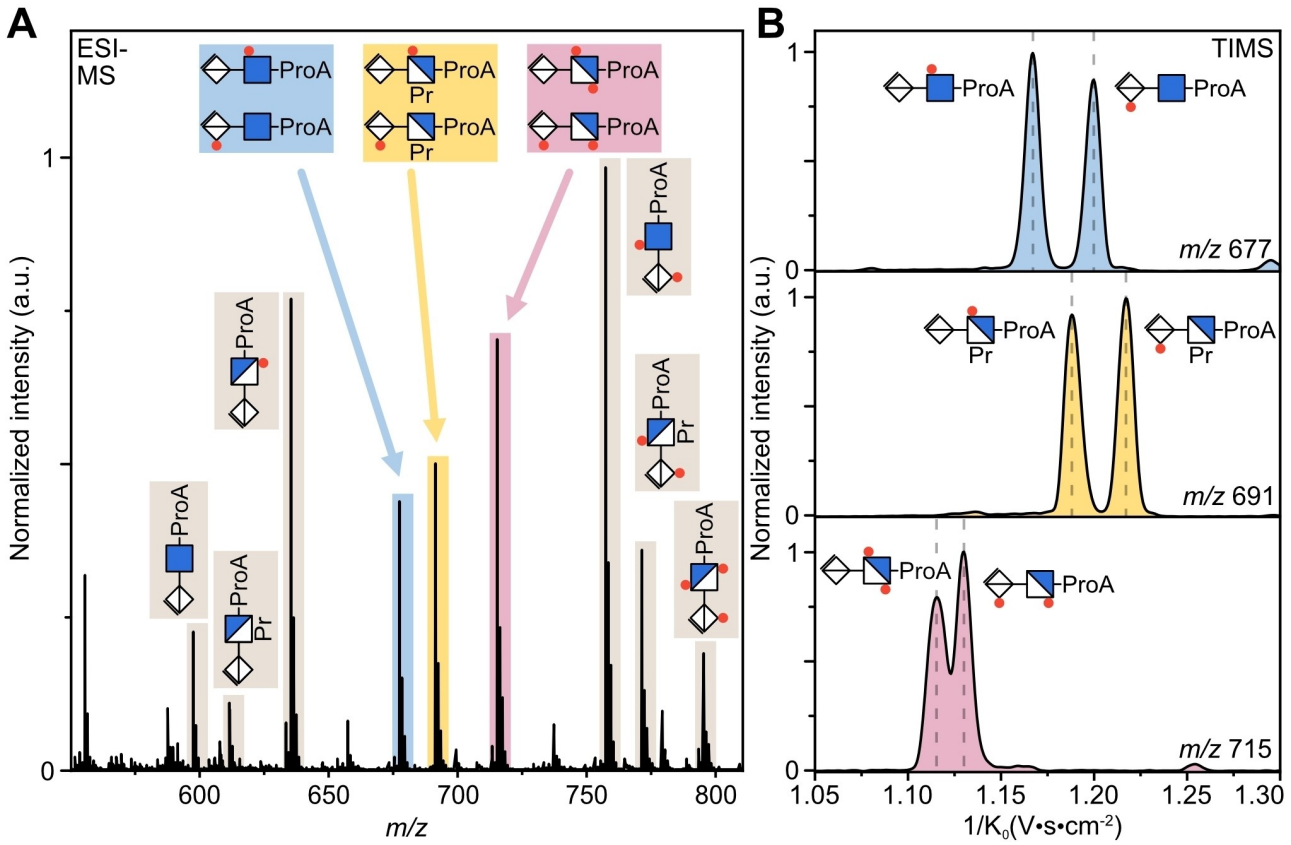

Figure 3. Separation of available standards a) Negative ion ESI mass spectrum of N–propionate, procainamide labelled standard mixture. Isomers are present in the coloured peaks. Blue corresponds to HS–2SNAc and HS–6SNAc (*m/z* 677), yellow to HS–2SN and HS–6SN (*m/z* 691); and red to HS–2SNS and HS–6SNS (*m/z* 715). Six non-isomeric disaccharides are labelled in grey and can be distinguished by their mass alone. b) Extracted TIMS mobilograms of the three isomeric pairs.

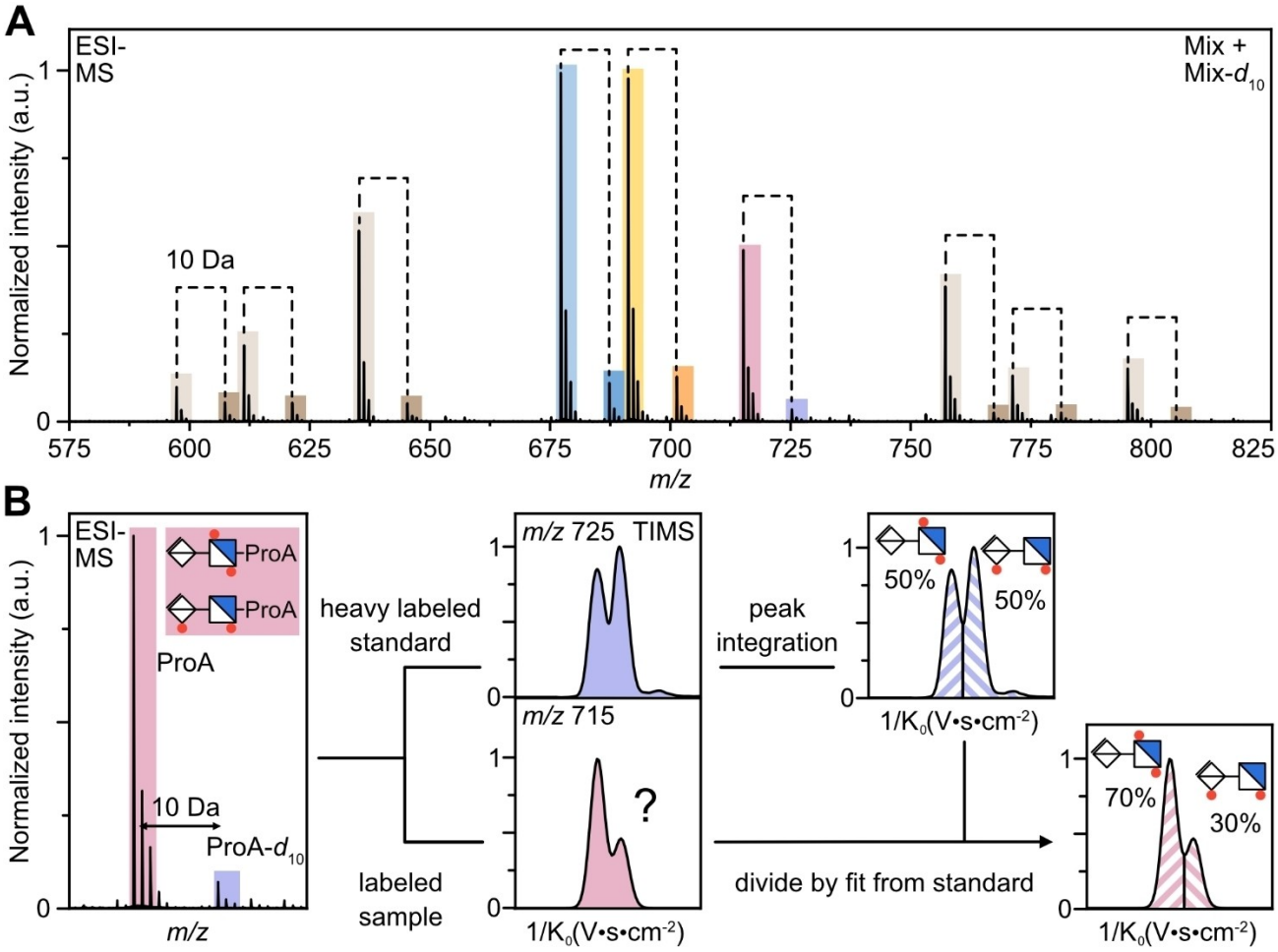

Figure 4. Quantification of isomeric disaccharide structures using stable isotope labelling and IMS. a) Mass spectrum of a ProA-labelled disaccharide mixture spiked with a ProA-d_{10} labelled equimolar disaccharide standard mixture. 10 Da shifts are indicated with a dotted line between sample and standard. b) TIMS based quantification approach for the three isomeric disaccharide pairs. Comparing the integrals of the heavy-labelled standard to those of the sample results in relative abundance of the respective disaccharide isomers.

disaccharide mix was spiked into the sample. The use of an ammonium-based additive is recommended, as it leads to a more favourable charge distribution towards lower charge states, especially for highly sulfated disaccharides. Quantification of all four mixes resulted in a very good agreement of theoretical and measured relative HS disaccharide content with an average relative error of 5.3%. A single outlier was observed in mix 2 for HS–2SN with a relative deviation of 33%. We exclude a systematic error in the propionylation or labelling reaction since the quantification of HS–2SN in the other three mixes is in very good agreement with the expected values. Omitting this value results in an average relative error of 4.7%. The remaining error can be attributed to non-linear detector-response rates during MS and coelution of unrelated isobaric species.

Finally, we applied the direct infusion TIM-MS based quantification method to a natural heparin sample from porcine intestinal mucosa. The heparin was digested by a heparinase I/II/III mixture, freeze-dried, and subsequently derivatized according to the previously described protocol.

The isomer separation of the mobilograms for the heparin disaccharides match the mobilograms derived from the standards mixtures. The composition exhibits a high degree of sulfation with over 50% of disaccharides carrying at least two sulfate groups (Figure 5 – right). Non- and monosulfated structures are also observable, particularly HS–NAc with a large fraction of 16.8%. Free amine structures were not identified or only in very minute amounts. These results are in line with other disaccharide studies of heparin.[17]

Conclusions

The composition and sequence of glycosaminoglycans, particularly heparin and HS, vary among individuals and undergo significant changes during diseases.[25] A reliable high-throughput method for heparin/HS composition analysis could enable profiling these glycosaminoglycans as potential biomarkers for various diseases. Additionally, compositional analysis of heparin is crucial for quality control in heparin-based pharmaceuticals and would benefit from a reliable high-throughput method.

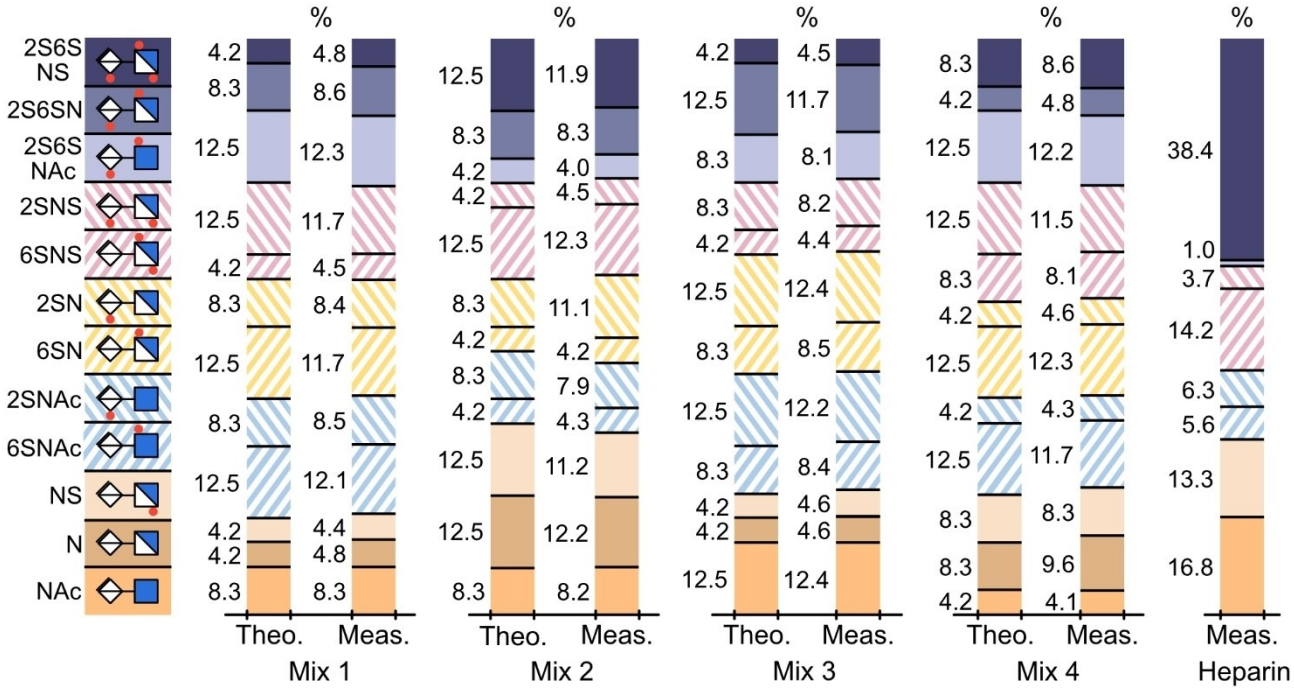

Figure 5. Relative quantification of four HS disaccharide mock mixtures and a porcine intestinal mucosa heparin sample using the ProA-d10 labelling of disaccharides. For each mixture, stacked bars for theoretical (left) and measured (right) values are shown; the numbers correspond to the relative content in percent. The twelve HS disaccharide structures are shown by their respective colour. Isomeric species are differentiated by the direction of the diagonal stripes. On the far right, the results obtained for a porcine intestinal mucosa heparin sample are shown, for which a high degree of sulfation was obtained.

Currently, compositional disaccharide analysis of HS and heparin involves enzymatic depolymerization, reducing end labeling, subsequent LC separation, and detection by FLD or MS. Here we present a TIM-MS-based method for the rapid separation and characterization of twelve partly isomeric heparin/HS disaccharides from complex mixtures and biological samples. The analytical workflow, taking only a few minutes, provides a compelling alternative to conventional LC approaches, offering faster and more sample-efficient analysis. The method involves a single additional chemical modification step, compared to common LC workflows, – propionylation combined with reducing end labeling using procainamide. This reduces the complexity of the mixture significantly, enabling clear identification and separation of isomeric structures during TIM-MS analysis.

The developed method allows quantification of disaccharides through the addition of an easily accessible, stable isotope-labeled internal standard, providing a reliable approach for assessing relative abundances. Benchmarking against mock mixtures showed an excellent agreement between theoretical and measured values. Furthermore, the application of the method to a natural heparin sample from porcine intestinal mucosa highlights its utility in the analysis real biological samples. The here developed method is also likely applicable to other high resolution IMS techniques and instruments, but care should be taken for unwanted ion activation and resulting fragmentation of the sulfate groups.

Experimental Section

Chemicals

All chemicals and solvents were purchased from Sigma-Aldrich (St. Louis, USA) and used without further purification. Heparin sodium from porcine intestinal mucosa was purchased from ABCR (Karlsruhe, Germany); glycan disaccharide standards from Iduron (Cheshire, UK). Heparinase enzymes were expressed in-house. HPLC-grade solvents were used throughout.

Heparin Digestion

Heparin sodium (100 μg) was suspended in 20 mM Tris/5 mM $CaCl_2$/200 mM NaCl pH 7.0 and incubated at 37 °C. A Heparinase mixture (10 mU for each Heparinase I, II and III) was added in a total volume of 40 μL and incubated overnight. After incubation, the sample was freeze-dried, dissolved in 500 μL MiliQ water and used without further purification.

Selective Amine Propionylation

For the propionylation of the mock mixtures and standards, 2 μL of a 1 mM glycan solution was mixed with 8 μL of a freshly prepared 5 vol% propionic acid anhydride in water:methanol:triethylamine (1:1:50 v:v:mM) solution. After one minute reaction time at room temperature, the mixture was frozen in liquid nitrogen and freeze dried. For the heparin digest 5 μL stock solution (~1 μg heparin disaccharides) was used.

Glycan Labelling

Glycans were labelled with procainamide according to established protocols (as per Ludger ProA labelling kit).[24] The freeze-dried samples were redissolved in 10 μL water and 20 μL of labelling solution consisting of 16 mg procainamide and 16.4 mg 2-picoline borane reductant dissolved in 150 μL AcOH:DMSO (3:7 v:v) was added. After 3 hours at 50 °C, 250 μL water was added and the samples were freeze-dried and used without further purification. Labelling for the d_{10} standard was carried out in a similar manner but with procainamide-d_{10}.

IM-MS Measurements

For IM-MS analysis samples were dissolved prior to use with water: methanol:ammoniumacetate (1:1:50 v:v:mM) to yield 5–10 μM analyte solutions. The measurements were performed on a Bruker timsTOF Pro in MS negative mode. For ionisation an in-house build nESI source was used, which was described in detail elsewhere[26] per sample ~5 μL were infused. Settings were optimized to prevent unwanted fragmentation with a capillary voltage of 1300 V, end plate offset of −500 V, quadrupole ion energy of 2.5 V, CID voltage of 7 V, collision gas flow rate 65%, prepulse storage time 10 μs, transfer time 100 μs. The TIMS parameters are D1: 20 V, D2: 30 V, D3: −100 V, D4: −130 V, D5: 0 V, D6: 50 V. An accumulation time of 20 ms was used. IMS ramping was performed between 0.47–1.61 V*s/cm^2 with a ramping time of 1000 ms. For $1/K_0$ measurements the instrument was calibrated using the Agilent ESI tune mix. For quantification Bruker DataAnalysis 5.3 was used to extract $1/K_0$ area values of the given disaccharides and heavy labelled disaccharide standard.

Preparation of Procainamide-D_{10}

N-Boc-ethylenediamine (100 mg, 0.62 mmol, 1 eq.) was dissolved in 2 mL of acetonitrile. After addition of DIPEA (272 μL, 1.56 mmol, 2.5 eq.), bromoethane-d_5 (103 μL, 1.4 mmol, 2.2 eq.) was added. The reaction was left to stir at room temperature overnight. After evaporation of the solvents, 1 mL of 30% TFA in DCM was added to the crude product at 0 °C. After addition, the mixture was allowed to warm up to room temperature and subsequently stirred for two hours. The solvent was evaporated, and the product redissolved in 6 mL methanol. *N*-Boc-4-aminobenzoic acid (150 mg, 0.63 mmol, 1.1 eq.) and DMT–MM (250 mg, 0.90 mmol, 1.43 eq.) were added and the reaction was stirred at room temperature overnight. The solvent was evaporated and the Boc-deprotection was carried out again in 1 mL of 30% TFA in DCM at 0 °C and stirred at room temperature for 2 hours. The crude product then was dried and purified a with Biogel P2 gel column (yield ~12%).

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Conflict of Interests

The authors declare no conflict of interest.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Keywords: Glycosaminoglycans · Heparin · Disaccharide analysis · TIMS · Quantification

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