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Ion Mobility–Mass Spectrometry of Complex Carbohydrates: Collision Cross Sections of Sodiated N-linked Glycans

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Supporting Information

ABSTRACT: Currently, the vast majority of complex carbohydrates are characterized using mass spectrometry (MS)-based techniques. Measuring the molecular mass of a sugar, however, immediately poses a fundamental problem: entire classes of the constituting monosaccharide building blocks exhibit an identical atomic composition and, consequently, also an identical mass. Therefore, carbohydrate MS data can be highly ambiguous and often it is simply not possible to clearly assign a particular molecular structure. A promising approach to overcome the above-mentioned limitation is to implement an additional gas-phase separation dimension using ion mobility spectrometry (IMS), which is a method in which molecules of identical mass and structure but different structure can be separated according to their shape and collision cross section (CCS). With the emergence of commercially available hybrid ion mobility–mass spectrometry (IM-MS) instruments in 2006, IMS technology became readily available. Because of the nonhomogeneous, traveling wave (TW) field utilized in these instruments, however, CCS values currently cannot be determined directly from the drift times measured. Instead, an external calibration using compounds of known CCS and similar molecular identity is required. Here, we report a calibration protocol for TW IMS instruments using a series of sodiated *N*-glycans that were released from commercially available glycoproteins using an easy-to-follow protocol. The underlying CCS values were determined using a modified Synapt HDMS instrument with a linear drift tube, which was described in detail previously. Our data indicate that, under in-source fragmentation conditions, only a few glycans are required to obtain a TW IMS calibration of sufficient quality. In this context, however, the type of glycan was shown to be of tremendous importance. Furthermore, our data clearly demonstrate that carbohydrate isomers with identical mass but different conformation can be distinguished based on their CCS when all the associated errors are taken into account.

Glycosylation, i.e., the covalent attachment of carbohydrates to amino acid side chains, is one of the most common post-translational modifications found in proteins. Although often overlooked, the involved oligosaccharides play important roles in a multitude of biological processes, ranging from fertilization and immune response to cell–cell recognition and inflammation.[1] Typically, the carbohydrates are assembled onto the protein in a consecutive manner in the endoplasmatic reticulum (ER) and the Golgi apparatus.[2] The connection of the sugar to the protein chain occurs either at the side chain amide group of asparagine residues, as so-called "*N*-glycans", or at the hydroxyl group of serine, threonine, or hydroxylysine, as so-called "*O*-linked glycans".[3]

Despite their biological relevance, glycans are still difficult to characterize, as the result of their extraordinary structural complexity. In contrast to linearly assembled biological macromolecules such as oligonucleotides and proteins, carbohydrates form branched structures from many isobaric monosaccharide constituents, often resulting in a multitude of isomers.

Many carbohydrate analyses use mass spectrometry (MS)-based techniques, either directly or in conjunction with chromatographic or electrophoretic separations.[3] However, the structural complexity of glycans and the shortage of comprehensive databases often limits the amount of structural information that can be obtained directly from MS experiments. Therefore, an additional step that helps to distinguish between different isomers would be highly desirable.

A method that provides such an additional dimension of structural information is ion mobility spectrometry (IMS), which is a technique that involves separation by shape as well as by the m/z ratio. This technique has recently become generally

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available, following the development of the first commercial hybrid quadrupole/IMS/oa-ToF instruments: the Synapt (2006) and later the Synapt G2 (2009) and G2-S (2011) instruments (Waters Corporation, Manchester, U.K.).[4]

The drift times measured in IMS are usually converted into collision cross sections (CCS), which are values that are independent of specific instrument parameters and, thus, are generally comparable.[5,6] Furthermore, IMS information can be complemented by theoretical CCS values, which are calculated using software tools such as Mobcal[7] or Sigma.[8] Measuring CCS values on the Synapt IM-MS instruments remains challenging. In contrast to typical home-built drift tube (DT) IMS instruments, which utilize a uniform electric field to propel the ions through the IMS cell, these instruments make use of a traveling wave (TW) to transport the ions. This configuration has several advantages, with respect to sensitivity, speed, and general usability, which enable ion mobility−mass spectrometry (IM-MS) analyses of complex biological samples to be performed on a routine basis. However, since the correlation between the residence time of the ions in a TW IMS cell and the mobility is not fully understood to date, it is not possible to determine CCS values from Synapt data directly. Instead, an external calibration using samples with known CCS is usually required.[5,6,9−13] In this context, it is crucial that the utilized calibrants are of the same molecular class as the analyte, with similar mass and mobilities.[6] For oligonucleotides,[14,15] peptides and proteins,[11,16] protein complexes,[6] small synthetic sugars,[17] and polymers,[18] such calibration frameworks are available. For native *N*- or *O*-linked glycans and milk sugars, however, reference data for calibrant CCS values are still largely lacking. Here, we close this gap by reporting a comprehensive database for the calibration of TW IMS instruments using *N*-linked glycans that are released from naturally occurring and commercially available glycoproteins. A problem with calibration with *N*-glycans is a complex and generally nonlinear relationship between structure and CCS.[17,19] It is shown here that this problem can be overcome by use of a sufficiently large number of calibration points obtained from both the glycans and their fragments. Absolute CCS values for the cleaved glycans and the descending fragments generated by collision-induced dissociation (CID) are reported for the drift gases He and N_2.

MATERIALS AND METHODS

A detailed description of the experimental methods and procedures can be found in the Supporting Information. Briefly, *N*-linked glycans were released with hydrazine[20,21] from the well-characterized glycoproteins ribonuclease B,[22] porcine thyroglobulin,[23,24] chicken ovalbumin,[25,26] and bovine fetuin[27] obtained from Sigma Chemical Co., Ltd. (Poole, Dorset, U.K.) and reacetylated. Sialic acids were removed from the thyroglobulin and fetuin samples by heating with 1% acetic acid for 1 h at 70 °C. For electrospray analysis, samples were dissolved in water:methanol (1:1, v:v) at ∼1 mg/mL.

Measurement of absolute collision cross sections (CCS) were performed on a modified Synapt HDMS[4] (Waters Corporation, Manchester, U.K.) quadrupole/IMS/oa-ToF MS instrument with a linear (not traveling wave (TW)) drift tube, which was described in detail previously.[6,28] The reported CCS represent averages of three (He) or two (N_2) replicates acquired in independent measurements, for which each data set was measured at eight different drift voltages. In addition, high-resolution TW IMS measurements were performed on an unmodified Synapt HDMS G2-S 8 kDa quadrupole/IMS/oa-ToF MS instrument (Waters Corporation, Manchester, U.K.). Based on these data, CCS values were estimated as reported previously.[5,6]

RESULTS

Within this study, sets of glycans released from different commercially available glycoproteins have been investigated. A detailed list of all obtained CCS values can be found in the Supporting Information. In addition, the CCS values of some of the most prominent native glycans are summarized in Table 1. For the sake of clarity, however, the glycans released from ribonuclease B are used as an example for most of the following discussion.

Table 1. Absolute CCS Values of Typical Glycans Measured in He and N_2[a]

Glycan	[M+Na]$^+$	CCS in He (Å)^2						CCS in N_2 (Å)^2					
		Fet	Fet DS	Ov	Rnase B	Thyr	Thyr DS	Fet	Fet DS	Ov	Rnase B	Thyr	Thyr DS
H_3N_2	933.3	205 (3)	200 (5)	212 (5)	210 (4)	209 (3)	208 (4)	285 (4)	286 (8)	298 (4)	292 (2)	298 (1)	294 (3)
M_5N_2	1257.4			261 (4)	265 (7)	264 (7)	259 (5)			358 (1)	349 (1)	357 (2)	356 (4)
H_4N_3	1298.5					259 (4)	259 (5)					355 (2)	347 (5)
H_4N_3	1298.5			251 (4)						348 (2)			
H_3N_4	1339.5			267 (4)						361 (2)			
M_6N_2	1419.5			281 (4)	275 (5)	280 (6)	279 (4)			376 (3)	379 (1)	382 (4)	373 (2)
H_3N_5	1542.6			299 (3)						395 (3)			
M_7N_2	1581.5				305 (3)	298 (4)	296 (4)				399 (2)	399 (2)	395 (1)
$H_4N_4F_1$	1647.6					313 (4)	310 (4)					413 (1)	409 (3)
H_5N_4	1663.6		297 (1)			301 (3)	298 (3)		401 (3)			398 (4)	407 (2)
H_5N_4	1663.6			319 (3)						423 (6)			
H_4N_5	1704.6			318 (3)						435 (1)			
M_8N_2	1743.6				332 (5)	325 (3)	323 (3)				425 (1)	426 (4)	428 (1)
H_3N_6	1745.6			327 (3)						443 (8)			
$H_5N_4F_1$	1809.6					333 (3)	331 (3)					435 (1)	429 (4)
H_5N_5	1866.7			342 (4)						468 (5)			
H_5N_5	1866.7					341 (2)	335 (5)					434 (3)	442 (13)
M_9N_2	1905.6				348 (2)	347 (3)	346 (3)				462 (5)	453 (1)	451 (8)
H_4N_6	1907.7			344 (6)						450 (5)			
H_6N_5	2028.7	348 (4)	346 (1)					441 (14)	451 (2)				

[a]The structure of glycans is shown using the UOXF nomenclature.[29] The acronyms indicate the composition, with H = hexose, M = mannose, F = fucose, and N = *N*-acetyl hexosamine. DS indicates desialylated glycan samples. Each of the reported CCS values represents an average of at least two (N_2) or three (He) independent measurements with the standard deviation given in parentheses.

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In-Source Fragmentation of *N*-Linked Glycans. In order to generate calibrants over a broad range of m/z and mobilities, in-source fragmentation conditions were used that simultaneously produced molecular ions from the native glycans, as well as their fragments. A typical mass spectrum for the glycans released from ribonuclease B is shown in Figure 1. In positive ionization mode, *N*-glycans preferentially form

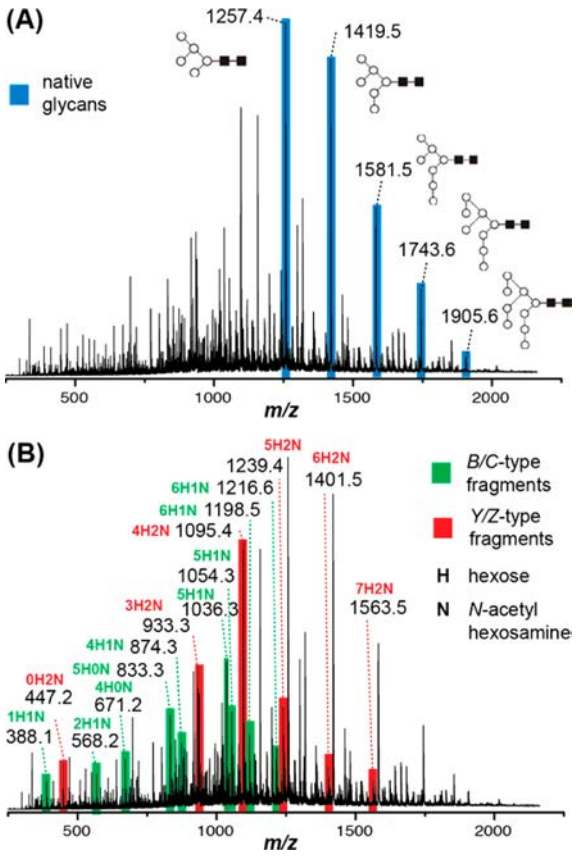

Figure 1. Typical spectra obtained for glycans released from ribonuclease B: (A) native glycans and (B) their fragments generated via collision-induced dissociation (CID). Under in-source fragmentation conditions, data for the native glycans and their fragments generated via CID can be acquired simultaneously. The structure of the native glycans in panel (A) is shown using the UOXF nomenclature, with open circles representing mannose residues (○, Man) and filled squares representing 2-*N*-acetyl glucosamine (■, GlcNAc). The angle of the bond indicates the linkage of the glycosidic bond (e.g., a horizontal line for a 1,4-linkage), while the anomeric configuration is depicted by the type of the connecting line (dashed line for α; solid line for β).[29] Red and green acronyms in panel (B) indicate the composition of each fragment with H being a hexose and N being *N*-acetyl hexosamine, respectively. Both spectra are identical and only singly charged, sodiated species are highlighted.

Na^+ adducts, particularly when Na^+ is added in small amounts as a salt. As a consequence, singly sodiated native glycans with masses ranging from m/z 1257.4 to m/z 1905.6 were the most abundant species in the spectrum in Figure 1. Their structure is illustrated using the UOXF nomenclature.[29]

The fragmentation of *N*-linked glycans in positive ionization mode via CID is dominated by glycosidic cleavages,[30] for which the terminology devised by Domon and Costello is used.[31] In Figure 1B, typical sodiated B/C and Y/Z-type fragments obtained from the ribonuclease B glycans are highlighted in green and red, respectively. Multiple glycosidic cleavages at different sites of the native glycan may lead to fragments of identical mass. Therefore, only their composition is given as acronyms, with H indicating a hexose (in this case mannose, Man) and N indicating an *N*-acetyl hexosamine (in this case, *N*-acetylglucosamine, GlcNAc).

CCS Measurements. For the native, sodiated glycans and their in-source CID fragments, absolute CCS values in He and N_2 were measured using a modified Waters Synapt HDMS instrument as described in detail previously.[6] The experimental CCS values obtained for singly charged glycans and fragments from ribonuclease B, as well as the associated errors, are shown in Figure 2 and the CCS values are listed in Table 1 and in Table S1 in the Supporting Information.

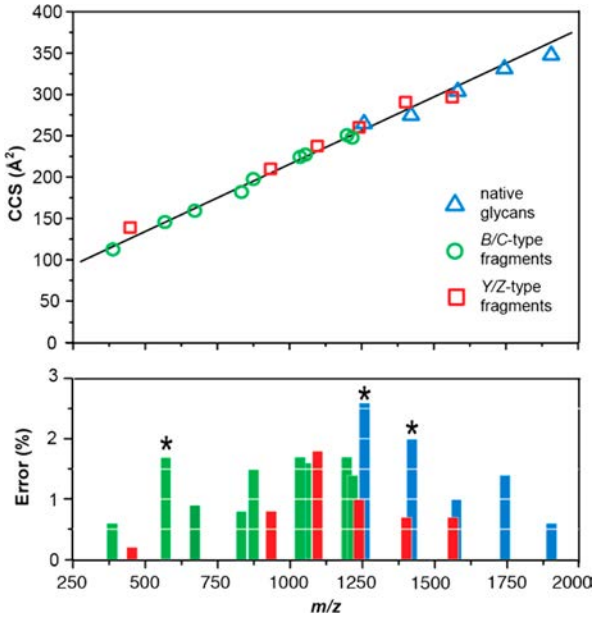

Figure 2. Collision cross section (CCS) values (top) and the corresponding errors (bottom) of selected *N*-linked glycans released from ribonuclease B and the resulting CID fragments. For repeat measurements deviations around and above 2% were typically observed for ions in which the ATD peak shape and width indicates the presence of multiple structural or positional isomers (indicated by an asterisk).

The CCS of small B/C and Y/Z-type fragments increases more or less linearly with increasing mass. For large native glycans, however, considerable deviations from this trend line are observed, which is likely the result of their complex and branched structures. The absolute errors associated to CCS measured on this instrument where estimated to be ~3%, while relative errors are considerably smaller and typically fall below 2% (Figure 2, lower panel). However, for certain glycans and fragments the variation increases considerably (indicated by an asterisk in Figure 2). The shape and width of the corresponding ATDs indicates the presence of multiple coexisting isomers or possibly conformers.

The Problem of Multiple Coexisting Isomers. A general problem with carbohydrates—not only in mass spectrometry—is caused by the fact that the constituting building blocks often exhibit an identical mass. As a result, a multitude of isomers with different composition (i.e., the type of building block), connectivity (i.e., the position of the glycosidic bond) or configuration (i.e., the stereochemistry at the anomeric center) can potentially occur at the same m/z value. While IM-MS has

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an enormous potential to solve this problem via separation of the different isoforms in the gas phase, this could also cause problems during calibration of TW IMS instruments, particularly when the coexisting species are not resolved.

Some of the glycoproteins investigated here contain isomeric glycans. A typical example is the $Man_3GlcNAc_3$ isomer pair found on ovalbumin in which the nonreducing GlcNAc residue is positioned either at the 3 (lower) or 6 (upper) antenna (Figure 3A). Using conventional mass spectrometry, both

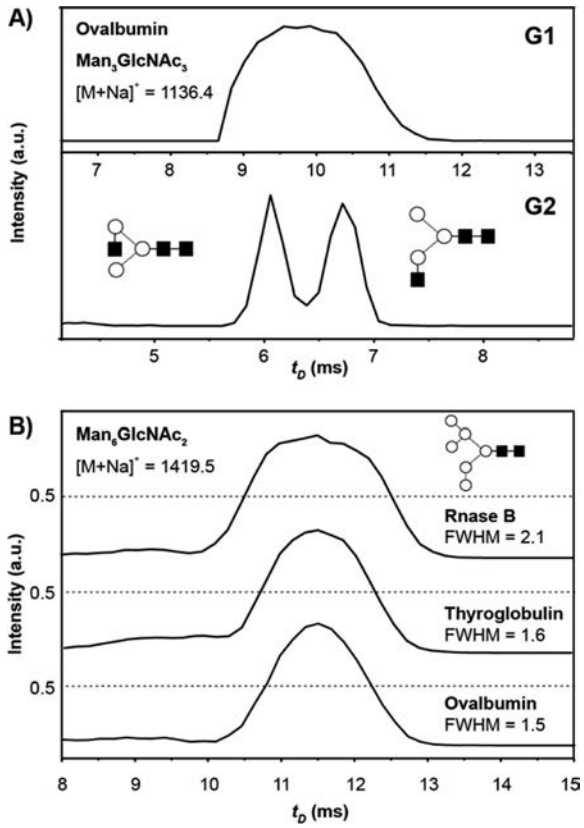

Figure 3. Glycan isomers and isomeric fragments. (A) ATDs of the natively occurring $Man_3GlcNAc_3$ isomers from ovalbumin measured with a low-resolution first-generation TW IMS (upper panel) and a higher-resolution second-generation (lower panel) instrument. (B) ATDs of the characteristic $Man_6GlcNAc_2$ glycan obtained from different sources. The shape of the arrival time distribution and the increased peak width, in the case of the ribonuclease B carbohydrates, suggests the presence of at least two isomers, conformers, decomposition products, or fragments.[35] Unlike that observed in panel (A), only the most abundant isomer in panel (B) is populated natively, while the other isobaric species were formed via the fragmentation of higher-order structures.

isomers are indistinguishable, giving rise to a single peak at m/z 1136.4 when sodiated. The positive-ion tandem MS spectra of both isomers exhibit slight differences, which are, however, predominantly limited to variations in intensity. As a consequence, a clear distinction on the basis of exclusively MS information can only be made when the ions are subjected to a sequential fragmentation in an ion trap (MS^n)[32] or by the use of negative ion CID.[33]

Previous IM-MS experiments on a first-generation Synapt HDMS instrument revealed that a mixture of both $Man_3GlcNAc_3$ isomers results in an unusually broad ATD, which indicates different drift times of the components that, due to insufficient resolution, cannot be fully resolved.[33] The IMS resolution of the RF-confined drift tube used here is expected to be in the same range[6,11] and, consequently, a broad ATD was also observed for the isomer mixture in our experiments (see Figure 3A, upper panel). Second-generation Synapt G2 and G2-S instruments, on the other hand, provide a considerably increased resolution in which both species can be baseline-separated (Figure 3A lower panel).[34]

Besides native isomers, isomeric fragments can also be problematic. Figure 3B exemplarily shows ATDs for a $Man_6GlcNAc_2$ glycan (m/z 1419.5) released from ribonuclease B, thyroglobulin, and ovalbumin. The peak shape as well as the peak width in the ATD of the ribonuclease B sample indicates the presence of at least two structurally different isomers or conformers, while a single species can be expected for the thyroglobulin and ovalbumin glycans.[35]

In the particular case of ribonuclease B, however, it is very unlikely that multiple native isomers are present. The populated glycans have been thoroughly investigated previously,[22] and only one isoform was found to be present. As a consequence, the peak broadening observed here must arise either from fragments that are formed via (i) a decomposition of the sample prior to analysis, or (ii) in-source CID fragmentation during the experiment or by the presence of conformers. In order to test these possible scenarios, we further performed a series of tandem IM-MS measurements on a second-generation Synapt G2-S TW IM-MS instrument. Under conditions that do not facilitate in-source fragmentation, a clean and narrow ATD was obtained for the species at m/z 1419.5 when preselected in the quadrupole. This indicates that no decomposition products are present (in Figure S2 in the Supporting Information). However, when carbohydrates of higher mass such as the $Man_7GlcNAc_2$ or $Man_9GlcNAc_2$ glycans were isolated in the quadrupole and subsequently subjected to CID, a variety of fragments, including those at m/z 1419.5, were formed. In comparison to the native $Man_6GlcNAc_2$ glycan, which has an identical mass, however, the fragments exhibit broad and partially resolved ATDs indicating multiple different structures (see Figure S2 in the Supporting Information). Therefore, it is very likely that the unusually broad ATD of the $Man_6GlcNAc_2$ glycan from ribonuclease B is caused by CID fragments with identical mass and atomic composition but distinct regiochemistry and stereochemistry.

With respect to CCS calibration of TW IM-MS instruments, the coexistence of isomeric fragments has fundamental implications and can lead to faulty assignments. Avoiding peaks known to contain native isomers is relatively straightforward. For the glycans studied, such peaks are labeled in the table given in the Supporting Information. However, even though multiple isoforms may theoretically exist, most of the ATDs are still narrow and homogeneous enough to serve as calibration data points. Isomeric fragments, on the other hand, are more complicated to deal with. In principle they can always be formed from in-source CID fragmentation of larger species. In this work, acquisition of a relatively large number of ATDs from glycans and their fragments was used to average out these variations.

Correlation between He and N_2 CCSs. A crucial point when measuring CCS is the utilized drift gas. IM-MS measurements on TW IMS instruments are typically performed using N_2 as a drift gas. On the other hand, the majority of CCS values reported in the literature were measured in He. In addition, software tools used to compute theoretical CCSs

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usually yield He values.[7,8,36] Therefore, the question arises whether carbohydrate drift times measured in N_2 can be used to estimate He CCSs (CCS*), as previously shown for peptides, proteins, and protein complexes.[5,6,9−11]

In order to provide a measure for the correlation between He and N_2, we determined absolute CCSs in both drift gases for most of the carbohydrates reported here. In general, CCSs in N_2 are larger than values obtained in He. This effect is especially pronounced for ions of low CCS and has previously been explained to be predominant a result of the increased polarizability of the N_2 gas by ions with large charge density.[11] Figure 4A shows that (i) for most of the singly charged

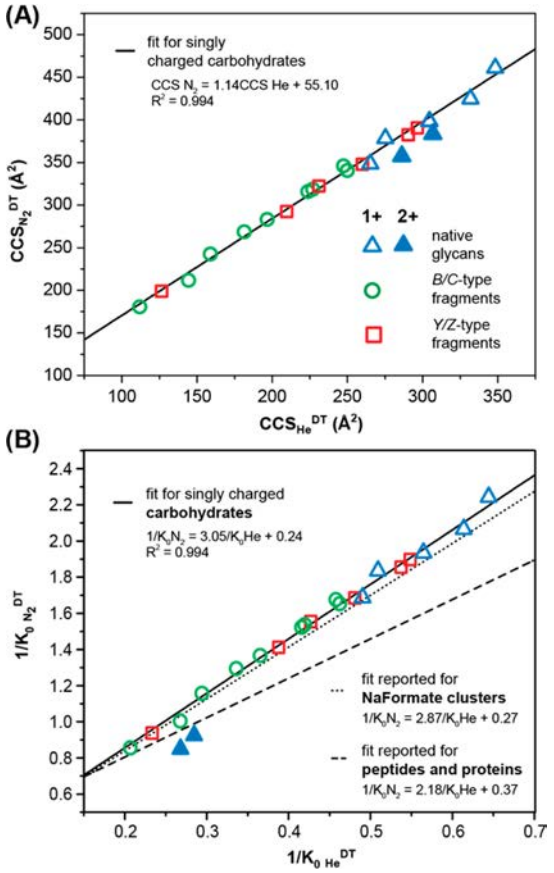

Figure 4. (A) Correlation between DT CCS measured in He and N_2. Data for the ribonuclease B glycans and their CID fragments are shown. The fit corresponds to the trendline observed for all singly charged carbohydrates investigated here. (B) Correlation between the reciprocal reduced motilities (K_0)[37] measured for ribonuclease B glycans and their fragments in He and N_2. The solid line fit corresponds to the trendline observed for all singly charged carbohydrates investigated here. It considerably deviates from the mobilities reported for native-like, multiply charged protein complexes (dashed line).[6]

carbohydrate ions, values measured in He and N_2 are linearly correlated and (ii) drift times measured in nitrogen can indeed be used to estimate helium cross sections. Especially noteworthy is the fact that the correlation between He and N_2 CCSs is similar, regardless of whether native glycans or their fragments are considered. This result indicates that a broad range of calibrants can be generated from the same glycan sample.

Doubly charged ions, on the other hand, considerably deviate from the trend line obtained for singly charged species (Figure 4A, filled triangles). Some of those deviations can be normalized with respect to charge state and reduced mass by comparing reciprocal reduced mobilities ($1/K_0$, directly proportional to CCS)[37] instead of CCS values. Figure 4B shows the reciprocal mobilities of the ribonuclease B glycans and their fragments measured in He and N_2. Similarly to CCS, there is a clear linear correlation between He and N_2 values for singly charged ions, while doubly charged ions considerably deviate from the best-fit trendline. Therefore, care must be taken when different charge states are compared, which is an approach that is very common for proteins[5,6] and other biomolecules.[15] Exclusive use of singly charged ions to calibrate for singly charged analytes and doubly charged ions for doubly charged analytes should, in the case of glycans, be used to minimize the error.

Comparison between the best-fit trendlines of carbohydrates in Figure 4B with those reported previously for peptides, proteins, and protein complexes furthermore support the necessity to calibrate TW IMS instruments with species of the same molecular identity. The dashed line in Figure 4B clearly indicates that there is no correlation between the reciprocal mobilities of multiply charged proteins and singly charged carbohydrates. As a consequence, a calibration with peptides and proteins would result in severely overestimated CCS values when applied to carbohydrates.

How Many Calibrants are Needed. In order to evaluate the quality of a carbohydrate calibration, test measurements where performed on a conventional TW-operated Synapt G2-S system. In our first analysis, we compared the correlation within each individual dataset. Generally, a good indication for the quality of a CCS calibration is given by the linear fit of the $\ln(t'_D)$ vs $\ln(CCS')$ plot that is used for calibration (for details, see the Supporting Information). For samples that contain a diverse, but relatively well-defined mixture of complex and hybrid glycans, such as those from fetuin, thyroglobulin, and ovalbumin, a good linear fit with R^2 values above 0.995 (0.9952 for fetuin, 0.9962 for ovalbumin, 0.9953 for thyroglobulin) is typically obtained. High mannose glycans, such as those released from ribonuclease B on the other hand, only poorly correlate, as indicated by an R^2 value of 0.9802. This poor relationship is a result of their inherent structural heterogeneity, which leads to a complex relationship between the branched structure and the drift times. Further details of this relationship will be published later. In addition, fragmentation of high mannose glycans may result in ATD peak broadening, as shown above. Consequently, we suggest avoiding the exclusive use of high mannose glycans as calibrants.

In our second analysis, the ribonuclease B carbohydrates served as "unknowns" and fetuin, ovalbumin, and thyroglobulin were used as calibrants. Figure 5 shows the relative deviation between the TW IMS estimated CCS values ($CCS^*_{He}{}^{TW}$) and the values directly measured via DT IMS ($CCS_{He}{}^{DT}$) for the ribonuclease B carbohydrates, as a function of the utilized calibrants. All shown values are averages of five independent measurements at different wave velocities.

When singly charged ions were used to calibrate for singly charged analytes, average errors below 1.5% were typically obtained (see the green columns in Figure 5). These values are slightly better than those reported previously for the TW IMS calibration of peptides and proteins.[5,11] On the other hand, when singly and doubly charged ions were utilized, the average

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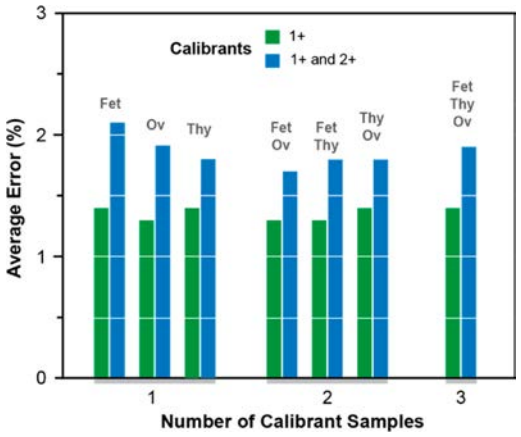

Figure 5. Average error of the estimated TW IMS CCSs of singly charged ribonuclease B carbohydrates as a function of the utilized calibrants. (Legend: Fet, fetuin; Ov, ovalbumin; Thy, thyroglobulin.) When singly charged glycans and their fragments were used to estimate CCSs of singly charged ions, average errors of <1.5% were typically observed (green columns). The quality of the calibration did not improve significantly when more than one sugar mixture was used for calibration. In contrast, the error of the estimated TW IMS CCSs increased to ~2% when singly and doubly charged ions were used as calibrants (blue columns). Average errors are calculated as $(CCS^{*}_{He}{}^{TW} - CCS_{He}{}^{DT})/CCS_{He}{}^{DT}$. Data points that are affected by the existence of multiple isomers are not considered.

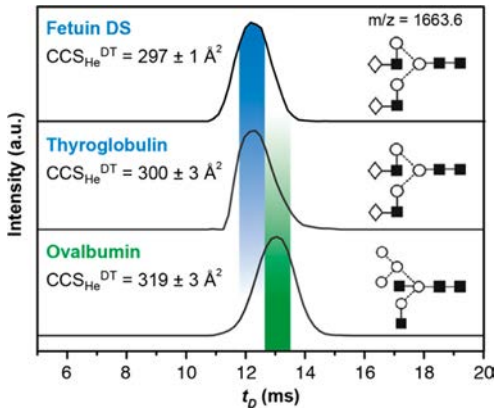

Figure 6. Distinction between structural isomers based on CCS. In desialylated (DS) fetuin as well as thyroglobulin, the same glycan is present at m/z 1663.6. The experimental CCS of this carbohydrate varies by ~1%, which is within the error of the method. In ovalbumin, on the other hand, a structurally different glycan, which exhibits a considerably higher CCS is present at the same mass. The difference between both CCS values is ~7%, which is far above the error of the method (~3%).

errors increase up to 2% (see the blue columns in Figure 5), even though the number of calibration data points increased by ~30%. This increase in deviation is very likely a result of the charge-state-dependent correlation of CCS values measured in He and N_2. For singly charged ions, a good linear correlation between the CCS values and the reciprocal mobilities measured in He and N_2 was observed (see Figure 4). However, doubly charged ions do not fall on the resulting trendline. As a result, considerable errors occur when different charge states are combined in the same calibration. This again emphasizes the necessity to use exclusively calibrants and analytes of the same charge state.

Surprisingly, the average deviation does not decrease significantly when more than one carbohydrate mixture is used for calibration. Similarly, there is almost no deviation between the individual calibrants. Both effects are likely a result of the large number of calibration data points that are obtained by spraying one single glycan sample. The absolute number of singly charged ions used here ranges from 18 in the case of fetuin to 56 in the case of thyroglobulin. However, the CCS range covered by one calibrant mixture remains essentially the same, with values between 100 and 400 Å^2. This similarity indicates that, in principle, one glycan sample (such as those from fetuin, ovalbumin, or thyroglobulin) with its resulting fragments would be sufficient to achieve a calibration of the required quality. However, in order to minimize the risk of systematic errors, we strongly suggest using at least two of the mentioned glycan mixtures measured independently during TW IMS calibration.

Isomer Distinction Based on CCS. Previous studies showed that carbohydrate isomers can be separated according to their drift time via IMS.[17,33,38−41] Having established a robust calibration protocol, we assessed whether positional isomers could also be distinguished based on their CCS, when all the potential errors that are associated with the measurement are considered. In order to answer that question, we used isomeric glycans at $[M+Na]^+$ = 1663.6 Da with the composition $Hex_5HexNAc_4$ from ovalbumin and desialylated fetuin (Figure 6). Almost identical ATDs were obtained for both samples (see blue component in Figure 6). The corresponding CCS values of 297 and 300 Å^2 differ by merely 1%, which is well within the error of the method. On ovalbumin, on the other hand, a structurally different hybrid type glycan with a bisecting GlcNAc is present. In comparison to the ATDs of the complex glycans from fetuin and thyroglobulin, the ATD of the glycan from ovalbumin is shifted to longer drift times (see green component in Figure 6), which is reflected in a considerably increased CCS of 319 Å^2. This observation is in good agreement with previous measurements on permethylated glycans from ovalbumin, which showed that glycans with a bisecting GlcNAc tend to exhibit larger CCS values than their isomeric counterparts with antennary *N*-acetylated hexosamine.[41] More importantly, however, the difference in CCS between the complex glycan from fetuin/thyroglobulin and the bisecting structure from ovalbumin is almost 7%. This is far above the error of the method, which can be estimated to be ~3% or below. Therefore, it can be concluded that structural isomers of carbohydrates can indeed be distinguished based on their CCS, even when the associated errors are taken into account.[6]

■ CONCLUSION

Here, we report a comprehensive dataset for the collision cross section (CCS) calibration of traveling-wave ion mobility spectrometry (TW IMS) instruments using native, *N*-linked glycans. All utilized carbohydrates were released from naturally occurring and commercially available glycoproteins using an easy-to-follow protocol. Absolute CCSs of the intact glycans, as well their descending fragments, are reported for the drift gases He and N_2.

Our data indicate that calibration of TW IMS instruments with glycans is generally less time-consuming than previously reported for peptides and proteins.[6] This can predominantly be attributed to the fact that a large number of calibrants is

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generated by spraying a single set of glycans using in-source fragmentation conditions. As a consequence, fewer samples and less time is needed to generate a calibration of sufficient quality. Moreover, the released glycans are largely insensitive to degradation and can be stored for years at room temperature, or better in a freezer, as a powder (observations from this laboratory). This combination makes the CCS calibration with glycans very convenient and it is even conceivable to fully automate the entire procedure in the future using software tools such as Amphitrite.[13]

However, it also turned out that it is crucial for a successful calibration to use calibrants and analytes of the same charge state. The reciprocal mobilities of singly and doubly sodiated glycans in He and N_2 correlate differently and, as a result, a considerably increased error is obtained when both charge states are mixed within one calibration. Another potential source of error is the intrinsic structural heterogeneity—not only that of the glycans themselves, but also that of the fragments that are possibly originating from them during in-source collision-induced dissociation (CID). This heterogeneity, however, strongly depends on the nature of the utilized carbohydrates. High-mannose glycans such as those occurring in ribonuclease B, for example, consist of a chitiobiose core ($GlcNAc_2$) to which between 5 and 9 mannose residues are coupled at various positions and orientations.[22] Even though the composition is simple, these glycans are often inherently heterogeneous with, in some cases, several positional isomers. This multiplicity of compounds is further complicated by the fact that CID products often overlap with masses of native glycans. Therefore, high-mannose glycans are only of limited use and should be avoided as CCS calibrants. Complex and hybrid glycans, as occurring in fetuin, thyroglobulin,[42] and ovalbumin on the other hand consist of a diverse mixture of GlcNAc and various hexoses, as well as fucose and sialic acid.[23,24] This increased compositional complexity is often accompanied by a more heterogeneous distribution of isomers, which makes them much better-suited calibrants for a CCS estimation. In this regard, especially glycans released from fetuin turned out to be highly suitable.

In addition, we demonstrate here that glycan isomers can be distinguished not only on the basis of their drift time, but also based on their absolute CSS values, even when all the potential errors are taken into account. Our data show that the structural differences between glycan isomers can lead to distinctly different CCSs. From a broader perspective, these findings highlight the potential to implement IMS-derived CCS information as an additional search parameter for database analyses of complex carbohydrates. Because of the high compatibility of ion mobility-mass spectrometry (IM-MS) with other separation techniques such as high-pressure liquid chromatography (HPLC), it is even conceivable to implement CCS information in already existing databases such as GlycoBase[43] and databases listed in the review by Frank and Schloissnig.[44] In the future, such a multidimensional dataset might help to significantly improve the quality of carbohydrate structural assignments, not only for glycans but also other complex carbohydrates such as milk sugars.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information contains details about the utilized experimental methods, including details about the calculation and estimation of CCSs. Additionally, an Excel table with collision cross sections of all glycans and their fragments can be found. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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