

Glycan Analysis by Ion Mobility-Mass Spectrometry

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Abstract: Carbohydrates form one of the major classes of biological macromolecules in living organisms. To investigate their properties and function, an in-depth knowledge of their underlying structure is essential. However, the inherent structural complexity of glycans represents a major challenge. Carbohydrates are often branched and exhibit diverse regio- and stereochemistry. This in turn leads to a vast number of possible isomers, which are difficult to distinguish using established analytical tools. In the last decade, ion mobility-mass spectrometry, a technique that separates ions based on their mass, charge, size and shape, emerged as a powerful alternative for isomer distinction. This review highlights recent advances in ion mobility-mass spectrometry of complex carbohydrates and discusses its role in future analysis workflows.

1. Introduction

Oligosaccharides—often referred to as glycans—play a vital role in virtually all living organisms. Present on cell surfaces or attached to proteins, they can influence protein folding and function, mediate cell-cell interactions, and play a role in cancer progression.^[1] The key for understanding their biological role is the ability to characterize a glycan's structure in all its details. To do so, a variety of techniques are available today, with the majority deriving directly from technologies developed in genomics and proteomics. In contrast to DNA or proteins, however, oligosaccharides do not have a linear sequence and are not synthesized in a template-driven fashion. Instead, they are often branched and have complex regio- and stereochemistry, which complicates their analysis tremendously.

Oligosaccharides are composed of monosaccharide building blocks. These are often isomers that only differ in the stereochemistry at a single carbon atom as for example in glucose and galactose (Figure 1a). The position at which two monosaccharides are linked to each other determines their connectivity. Due to the presence of multiple hydroxyl groups, regioisomers as well as branched structures can occur. In addition, a new stereocenter emerges when a glycosidic bond is formed, which gives rise to two configurations termed α and β .

The three above-mentioned types of isomerism lead to an immense structural diversity that represents a great challenge for almost all branches of the glycosciences. They also have consequences for how glycan structures can be depicted. Using the precise chemical structure is certainly the most accurate way. However, minute structural differences can be easily overlooked, and for larger, more complex glycans this is often not practical. To solve this problem, a simplified

symbol nomenclature for glycans (SNFG) was developed.^[2] Here, each monosaccharide is depicted by a specific symbol, and the regio- and stereochemistry of the glycosidic bond is represented by the angle and the type of the connecting lines, respectively (Figure 1b-c).

The isomerism in glycans often leads to structures of identical atomic composition and mass. Surprisingly, mass spectrometry based techniques (MS) are still amongst the most widely utilized tools in glycan analysis. However, even when sophisticated tandem MS^[3] or MS^{n[4]} techniques are used, it is often not possible to fully disentangle the structure of a given glycan. Further sample derivatization techniques^[5] or treatment with exoglycosidases^[6] can improve the glycan identification but are time consuming and expensive. In many cases hyphenation to liquid chromatography (LC) techniques is used to provide an additional dimension of separation.^[7] Due to the high polarity of glycans, however, they are not ideally suited for reversed-phase separations and other stationary phases have to be employed. Alternatively, nuclear magnetic resonance (NMR) spectroscopy can be used to obtain detailed structural information. However, the analysis requires larger amounts of sample compared to MS, and complex mixtures or samples with a complex matrix cannot be investigated.

In the last years, a combination of ion mobility spectrometry and mass spectrometry (IM-MS) emerged as a very promising new tool in glycan analysis.^[8] Here, not only the ions mass-to-charge (m/z) is measured, as in conventional MS, but also the time these ions need to traverse a cell filled with inert neutral gas under the influence of a weak electric field. This provides additional information on the size and shape, which can help to distinguish isomers.^[9]

Similar to a retention time obtained from chromatography, the drift time of an ion is dependent on a multitude of experimental parameters, which makes a direct comparison difficult if not impossible. The advantage of IM-MS, however, is that the drift time can be used to calculate the rotationally-averaged collision cross section (CCS) of an ion in a specific drift gas. This CCS is a molecular property, which under controlled conditions is independent of instrument parameters and is correlated to the shape of an ion. As such, CCSs can be used as an additional identification parameter, which can be stored in databases, allowing an easier and more reliable structural assignment.

In this review, we summarize how and where IM-MS can be applied for structural identification and separation of carbohydrates and present recent methodological developments.

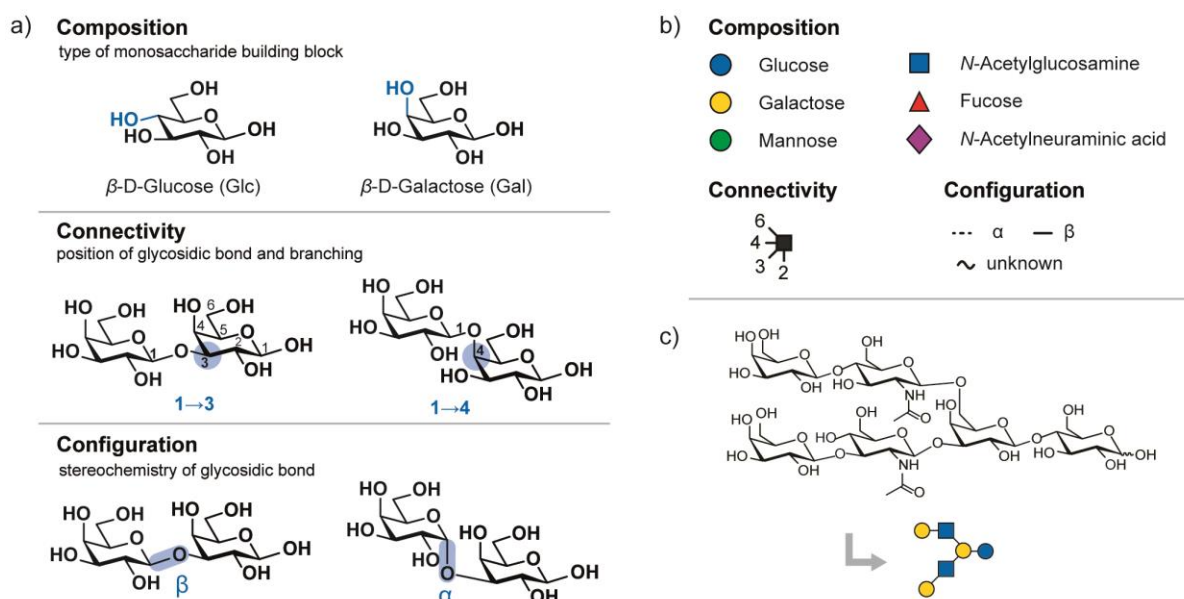


Figure 1. Structural determinants of glycans and how they are depicted using the symbol nomenclature for glycans (SNFG)^[2]. a) The structure of a carbohydrate is defined by its monosaccharide composition, the building block connectivity, and the configuration of the glycosidic bond. All three types of isomerism lead to species with an identical atomic composition and mass. b) To depict glycans in a simplified manner the SNFG was established. Monosaccharides are illustrated using symbols, while the glycosidic bond regiochemistry is described by the angle and the stereochemistry by type of the connecting line, respectively. c) Example how more complex oligosaccharides can be depicted using the SNFG system.

2. IM-MS of Carbohydrates

2.1. Instrumentation and First Applications

There are several types of IM-MS instruments, with the most common being drift tube (DT)^[8], travelling wave (TW)^[10], high-field asymmetric wave form ion mobility spectrometry (FAIMS)^[11], and recently trapped ion mobility spectrometry (TIMS)^[12]. The largest difference between these techniques is the nature of the electric field that is used to propel the ions through the IM cell. Briefly, DT designs utilize a homogenous electric field, which has the advantage that CCSs can be determined directly from the measured drift times.^[8, 13] In TW IM-MS on the other hand, traversing potential pulses are used, and CCSs can only be estimated using a calibration with substances of known CCSs.^[14] FAIMS uses alternating high- and low-electric fields and can be easily coupled to existing MS instrumentation. Despite its exceptionally high resolution, however, it does not provide direct CCS information. Detailed insights into IM-MS techniques and instrumentation can be found elsewhere.^[8, 9b, 15]

The first IM-MS experiments on carbohydrates were performed in the late 1990s using home-built DT instruments.^[16] Using a series of small oligosaccharides it was demonstrated for the first time that isomers can exhibit different drift times, which in turn enables their differentiation. However, after these proof-of-principle experiments, it took several years until glycans were investigated more

systematically. In 2003, Gabryelski and Froese were the first to look at different isomeric oligosaccharide mixtures using FAIMS.^[17] In particular, disaccharides differing in their regio- or stereochemistry or exhibiting site-specific derivatizations were analyzed. The data revealed that isomeric mixtures can be separated in positive as well as negative ion mode and that the attachment of different ions can significantly influence the quality of the separation.

The first promising results and the introduction of commercial instruments^[10a, 12b, 18] fueled the interest in using IM-MS more systematically for oligosaccharide analysis. Several studies reported different examples of carbohydrate analyses, but few of them went beyond the proof-of-principle level and were instead often focused on technical aspects.^[19] To improve the quality of a separation, Clemmer and co-workers for example extended the length of the IM cell up to three meters and developed a cyclotron IM-MS design.^[15a, 20] Furthermore, the Hill group showed that an increase of the drift gas pressure from a couple of mbar to atmospheric pressure significantly improves IM resolution.^[21] Until recently, however, very few examples for glycobiological applications existed, and a series of fundamental question remained open.

2.2. Intact Ions

The most obvious application of IM-MS is the analysis of intact glycan ions, either for synthesis control or for structural identifications. To establish the method, many investigations were focused on synthetic compounds or small biologically-relevant carbohydrates for which prior structural knowledge existed.^[22] The most prevalent examples of the latter are certainly N- and O-glycans. Connected to asparagine or serine/threonine side chains, respectively, they represent one of the most abundant forms of post-translational modifications on proteins.^[7c]

N-glycans can be chemically or enzymatically released from well-studied glycoproteins, such as ovalbumin or fetuin, and therefore represent ideal model systems to investigate the capability of IM-MS for structural analyses.^[23] Several studies showed that IM-MS is able to identify N-glycans and revealed the presence of isomers in complex mixtures.^[23-24] In this context, the ion's polarity (positive or negative) as well as adduct formation were shown to have a significant impact on the quality of the separation and therefore required further evaluation. Struwe and co-workers for example showed that deprotonated ions of high-mannose glycans can exhibit complex arrival time distributions (ATDs) with multiple features, which presumably result from multiple conformers. Protonated ions of the same glycans on the other hand led to ideal Gaussian-shaped ATDs, which indicate single, or multiple, structurally related conformers.^[24g] In a later study, a similar behavior was observed for phosphate adducts.^[24h] In this case, it turned out that the reducing end can lead to the formation of one or more larger conformers, which vanish upon reduction of the glycan.

The formation of non-covalent adducts, for example with metal ions, can induce alternative glycan conformations. First studies indicated that for certain adducts this can lead to an improved ion

mobility separation, and these effects were therefore studied in greater detail. In most cases alkaline and alkaline earth metal ions were used^[19a, 24g, 25], but also more exotic ions were tested such as silver, copper, lead,^[26] or transition metal ions (Mn^{2+} , Co^{2+} , Fe^{2+} , Ni^{2+})^[27].

Using this approach, typical milk sugar isomers have been investigated by several groups.^[19a, 25a, 25c, 28] Dodds and coworkers for example investigated two lacto-*N*-fucopentaose isomers and a series of other oligosaccharides to test the influence of five different cations on their CCSs.^[25a] For this set of molecules, significant CCS differences were observed using sodium adducts, whereas lithiated glycans exhibited very similar CCSs (Figure 2).

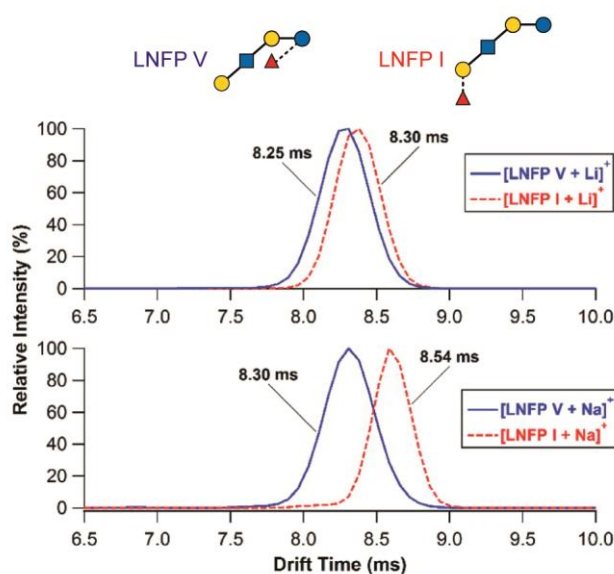


Figure 2. Arrival time distributions of lacto-*N*-fucopentaose I and V as different metal ion adducts. As lithium adducts both milk sugars have similar drift times while the addition of sodium allows for their differentiation. Adapted with permission from Ref. [25a]. Copyright 2013 American Chemical Society.

Furthermore, the impact of ion polarity was studied by several groups.^[29] Yamagaki and Sato for example investigated glucose tetra- and hexasaccharides that each contained solely α 1,4, α 1,6, β 1,3, or β 1,4 glycosidic bonds and observed large drift time differences in negative ion mode, whereas very similar drift times were observed for sodium adducts.^[29a] For other examples, however, the complete opposite result was obtained, *i.e.* a good separation of isomers as positively charged sodium adducts and a poor separation as deprotonated ions.^[29c]

With the advancement of automated solid-phase glycan assembly technology,^[30] it recently became possible to investigate the impact of adduct formation and ion polarity more systematically for the different types of isomerism. More specifically, a set of six trisaccharides, which only differed in the composition, regiochemistry, or stereochemistry of a particular glycosidic bond, was synthesized and evaluated using IM-MS.^[29b] Regardless of ion polarity and adduct formation, compositional isomers were shown to yield the least differences in their drift time and could not be differentiated in most cases. Regioisomers and anomers on the other hand, can be readily

distinguished as deprotonated ions. Even in mixtures, clear baseline-separation was demonstrated for both types of isomerism, and generally, a better separation was obtained for negative ions (deprotonated and chloride adducts).

Based on the currently existing data, it is very difficult to predict which ion polarity and/or adduct leads to the best separation of a given set of isomers, and a clear trend does not seem to exist. Unknown systems should therefore ideally be tested for various adducts and both ion polarities in order to achieve the best possible separation.

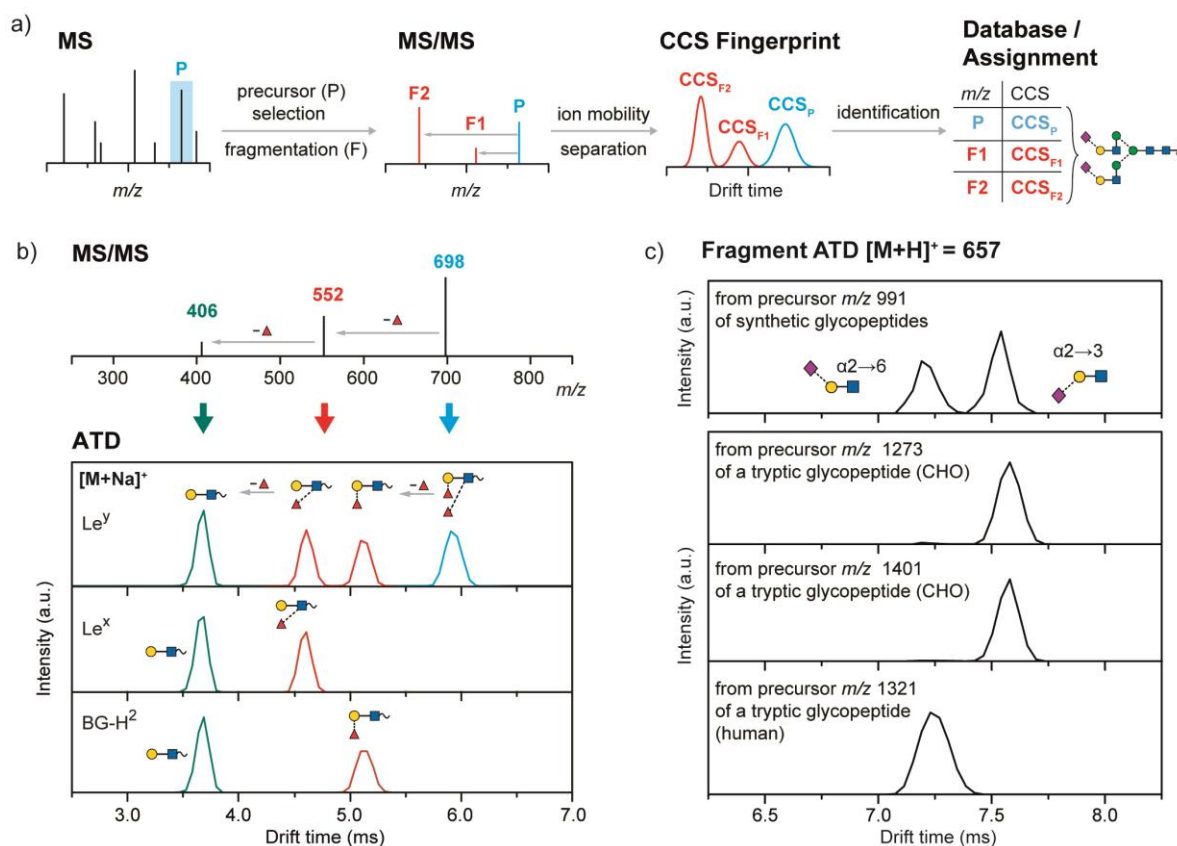


Figure 3. IM-MS analysis of carbohydrate fragments. a) Workflow of a tandem MS experiment followed by an ion mobility separation. The drift times of certain fragments (F) can be more characteristic than the intact precursor ions (P) and allow a structural identification. b) The fragmentation of tetrasaccharide Lewis y (Le^y) results in the formation of diagnostic fragments with the same structure as trisaccharides Le^x and blood group H type-2 (BG-H²).^[31] c) A characteristic trisaccharide fragment (m/z 657) can be used to differentiate *N*-acetylneuraminic acid linked via α 2,3 or α 2,6 glycosidic bonds to glycans. This fragment information is independent of the glycopeptide precursor as shown for tryptic digests of different glycosylation isoforms of α 1-proteinase inhibitor.^[32]

2.3. Carbohydrate Fragment Analysis

Not surprisingly, it becomes increasingly difficult to detect subtle structural differences in a macromolecule with increasing size of the molecule. As in other *omics* techniques, this inherent problem can be solved by generating more informative fragments of smaller size, either externally or directly within the mass spectrometer. In certain instruments, the fragmentation of m/z -selected

species can be performed before the actual IM separation step, and this feature can also be exploited successfully for the analysis of glycans (Figure 3a).^[29b]

In the context of oligosaccharides, smaller fragments composed of three to six monosaccharide building blocks generally appear to be more informative than larger intact ions.^[29b] Moreover, it was shown that fragments arising from cleavage of the glycosidic bond exhibit the same CCSs as smaller intact ions of the same structure.^[24d, 29b] As such, the diagnostic capabilities of fragments are independent of the precursor, which in turn enables the generation of fragment CCS databases on the basis of small synthetic molecules.

In a recent TW IM-MS study, several blood group and Lewis tri- and tetrasaccharides, which are common antibody epitopes, were investigated to find characteristic marker fragments. To do so, the CCSs of intact ions as well as their fragments were determined, and the resulting set of combined m/z and CCS information was used to generate carbohydrate fragment fingerprints (Figure 3b).^[31] Afterwards, larger glycans containing these epitopes were analyzed, and the CCSs of their fragments was estimated. By comparing the obtained values with the previously-determined CCS fingerprints, it was possible to unambiguously assign the presence of epitopes on large glycans.

The above described fragment approach can be applied not only to isolated glycans, but also to larger glycoconjugates. As mentioned above, biologically relevant oligosaccharides are often connected to proteins or lipids, which make them highly relevant but also challenging analytical targets. In glycopeptides for example, a variety of distinct structural features such as the number and position of glycosylation sites, the peptide sequence, and the glycan structure have to be considered. In this context, it is of great advantage that different MS fragmentation techniques provide complementary information. Whereas collision-induced dissociation (CID) predominantly produces glycan fragments, electron transfer dissociation (ETD) can be applied to fragment the peptide backbone.^[33]

Similar to the analysis of intact glycans it was demonstrated that intact glycopeptide isomers can be differentiated using IM-MS.^[32, 34] More importantly however, also the previously described fragment approach can be used. Recently, glycopeptides that merely differed in the regiochemistry of a single glycosidic bond within the glycan were investigated.^[32, 35] More specifically, N-acetylneuraminic acid (NeuAc), which mediates cellular interactions, was attached to the sugar moiety of the glycopeptide either *via* an α 2,3 or α 2,6 linkage. CID of these glycopeptides resulted in the formation of characteristic trisaccharide fragments with highly diagnostic CCSs. Fragments containing 2,6 linked NeuAc exhibited a considerably shorter drift time and smaller CCS than their 2,3 linked analogs, which enabled a clear differentiation between the two NeuAc isoforms (Figure 3c). In order to test the robustness of the approach, similar experiments were performed on a tryptic digest of α 1-proteinase inhibitor from different sources.^[32] When recombinantly expressed in Chinese hamster ovary (CHO) cells α 1-proteinase inhibitor contains glycans with α 2,3 linked NeuAc, while the same protein isolated from human plasma is glycosylated with α 2,6 NeuAc. Remarkably, also

here it was possible to quickly determine the regiochemistry of the NeuAc linkage by tandem IM-MS. Since this assignment is purely based on diagnostic fragment CCSs, prior knowledge of the protein sequence is not necessary, which underlines the universal applicability of the method and its potential for high-throughput analyses.

2.4. Reducing Complexity and Multi-omics Approaches

IM-MS can be applied not only to identify specific isomers, but can also serve as a separation technique to elegantly reduce the complexity of acquired data. Due to their different chemical nature and the resulting difference in the interaction potential with the IM drift gas, distinct molecular classes exhibit a distinct mobility behavior.^[18a] In addition, the average packing density is different for each molecular class. May et al. for example showed that carbohydrates on average exhibit shorter drift times than peptides or lipids of the same m/z . This in turn enables a rapid classification based on trendlines in a plot of CCSs against m/z (Figure 4a).^[18a] Using a similar concept, Li and co-workers showed that even peptides and glycopeptides can be distinguished within one measurement and analyzed separately.^[36]

In addition, the charge-state dependence of the drift time can be exploited during data analysis.^[37] Singly-charged ions generally exhibit a considerably lower mobility than multiply charged ions, which results in the formation of signal groups that can be selectively extracted from the spectrum (Figure 4b).^[24a] In a study of released glycans from the envelope glycoprotein gp120, which is one of the most abundant proteins on the surface of the human immunodeficiency virus (HIV), Harvey et al. used this approach to extract glycan signals that overlapped with intense detergent signals deriving from sample preparation (Figure 4b-c).^[24a]

Another example for the application of this approach was demonstrated by Vakhrushev and co-workers, who selectively obtained mass spectra of singly-, doubly-, and triply-charged ions from IM-MS data of urine samples.^[24b] As a result, a significantly reduced signal-to-noise ratio and an easier identification of signals were achieved for these inherently complex samples. Also an application in the field of biopharmaceutical chemistry was recently presented. In particular, IM-MS in combination with LC-MS was used to characterize the glycan profile of a commercially available monoclonal antibody.^[24c] The results demonstrate that individual production batches exhibit considerable differences in glycan macroheterogeneity, which underlines the necessity of an efficient quality control of biopharmaceutical products.

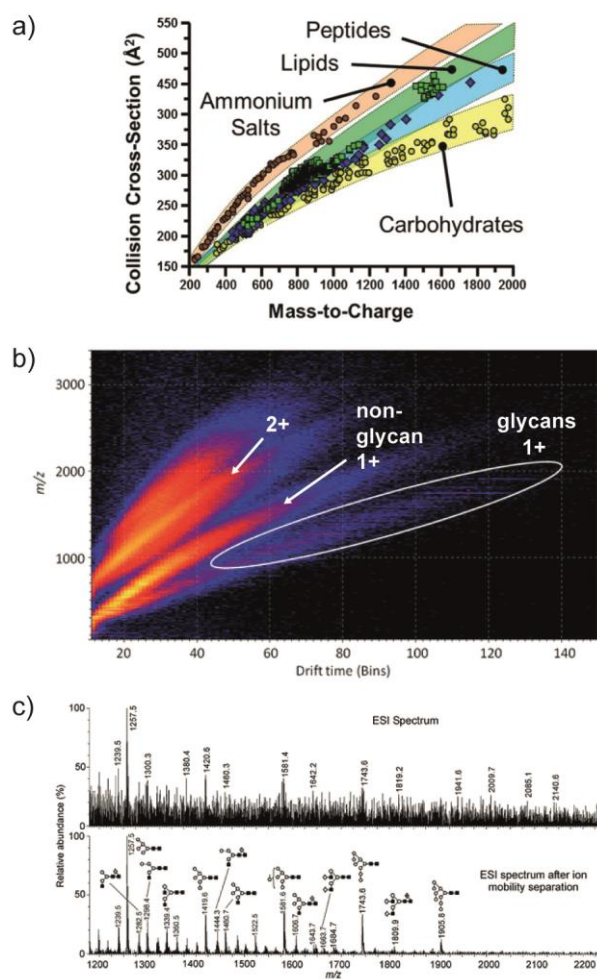


Figure 4. IM-MS to reduce spectral complexity. a) With increasing m/z the increase of the CCS is dependent on the class of the investigated ions. Modified from May et al. Modified with permission from Ref. [18a]. Copyright 2014 American Chemical Society. b) and c) IM-MS measurement of N-glycans released from the glycoprotein gp120 in positive ion mode. b) A plot of m/z against drift time shows a separation of the ions according to their charge and molecular class. c) The full mass spectrum is dominated by background signal, while the extraction of the highlighted region from b) reveals N-glycan signals. Adapted with permission from Ref. [24a] Copyright 2011 American Society for Mass Spectrometry.

3. Theoretical Calculations

The ability to theoretically predict ion mobility separations and CCSs would be beneficial for virtually all of the above-mentioned applications. Similarly to other biopolymers, however, the chemical structure of a glycan does not reflect the actual three-dimensional conformation adopted in the gas-phase environment of a mass spectrometer. Molecules which exhibit a very different chemical structure often show similar drift times in the experiment, whereas molecules with apparently minor differences can sometimes be separated with baseline resolution in IM-MS. As a result, sophisticated theoretical methods are the only suitable way to generate model structures from which CCSs can be predicted.

The calculation of CCSs itself is relatively straightforward and can be performed using various established methods.^[38] Generating meaningful and relevant structural candidates, however, is considerably more challenging, especially when glycans are considered. As recently highlighted by Agirre et al., there are very few theoretical methods that are capable of handling crystallography or solution data of glycans^[39] and for gas-phase data virtually no suitable tools exist. Certainly the most straightforward approach is to use force fields to generate a series of structural candidates. Unfortunately, however, there are currently no force fields available that provide satisfactory results for gas-phase ions of carbohydrates.^[40] Most of the existing glycan force fields are designed and optimized for the condensed phase and were developed on basis of experimental NMR data.^[41] Thus, these force fields will be biased towards condensed-phase conformers and in most of the cases an idealized chair conformation is assumed. This however, does not necessary reflect reality and additionally hinders the flexibility and structural space of the glycan.^[40] As a consequence, density functional theory (DFT) methods are often the only suitable option to obtain meaningful theoretical gas-phase structures for oligosaccharides. This however, leads to high computational costs, especially for larger molecules, where exploring the full structural space of a glycan with a large number of conformers can quickly turn into a theoreticians' nightmare.

Another aspect that further complicates theoretical calculations is the location of the charge. Metal ion adducts are relatively straightforward because the glycan structure is largely dictated by the solvation of the cation. For deprotonated ions on the other hand, multiple potential deprotonation sites exist, all of which are chemically more or less identical. A clear prediction of where the negative charge is located is therefore rather difficult. To make matters worse, it was recently shown that the position of the charge in deprotonated glycan ions is by no means static. Instead the charge migrates rapidly between different OH groups that are temporarily brought in close spatial proximity by a dynamic structural reorganization.^[28]

4. Summary and Outlook

Within the last decade, IM-MS quickly developed from an exotic technique utilized only by specialists into a ready-to-use and commercially available technique that can be applied for routine analysis of synthetic and biological glycans. There are, however, several challenges that need to be addressed in the near future.

The first obstacle is certainly the increasing volume of data that can be obtained within a relatively short amount of time, particularly when LC methods and IM-MS are coupled. New and optimized tools are necessary to analyze the data and automate the glycan identification, especially for high-throughput screenings. This requires public access to available CCS information. However, most IM-MS data of carbohydrates are only reported in individual publications, and it is thus difficult to access all available information and continuously keep track of new data. As an overview of the

currently existing data, we provide two tables in the Supporting Information. Table S1 lists all publications containing carbohydrate CCSs and provides details about the analyzed samples, and Table S2 tabulates the reported CCS values. Already in these tables it is apparent that the comparison of data can be very complex as the same molecule is often investigated by different groups utilizing distinct chemical modifications or adduct ions. Such complexity emphasizes the necessity of a structured and well-curated database that also provides search tools to enable straightforward access to connected data of similar structures. These features will furthermore enable the implementation of fragment data, which are necessary for a marker-based structural identification.

One of the few platforms that is suitable for the integration and connection of MS, HPLC, and IM-MS data is unicarbKB.^[42] However, even in this case there is no universally accepted format to store and share acquired data, and further development is clearly needed. Common standards for data reporting should be established to enable a clear identification of the experimental conditions under which for example a CCS is obtained. It was for instance proposed to report CCS values in the form of $^{\text{instrument}}\text{CCS}_{\text{driftgas}}$ in which the utilized type of instrument and drift gas is indicated (e.g. $^{\text{DT}}\text{CCS}_{\text{He}}$ values were obtained from a DT instrument using helium as a drift gas). This nomenclature is already used by several groups and enables an easy and unambiguous reporting of CCSs.

In addition, further technological developments of ion mobility instruments are needed to provide higher ion mobility resolution and more precise CCS values. At the moment, the typical error of a CCS is around 1 % and probably even higher for TW IM-MS instruments. A more accurate and precise CCS determination combined with higher resolution will in the future enable the differentiation of isomers that currently cannot be distinguished and will therefore pave the way for the analysis of more complex samples.

Although there remain many challenges and open questions in the field of glycomics, there has never been a time that provided more analytical tools for the structural characterization of glycans. The combined analysis of intact glycan ions and their fragments to obtain characteristic CCS fingerprints is in our opinion essential for the structural identification of glycans and glycoconjugates. Especially the investigation of diagnostic fragments arising from larger macromolecules is of exceptional use for the rapid *feature analysis* of more complex samples. The future implementation of connected m/z and CCS information in publicly available databases is key to exploit the full potential of IM-MS and to simplify routine analyses. Certainly IM-MS will not answer all open questions in the glycosciences, however, we are confident that it will be crucial in developing a better understanding of the biological role of complex oligosaccharides.

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