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Recent Advances in Ion Mobility-Mass Spectrometry for Improved Structural Characterization of Glycans and Glycoconjugates

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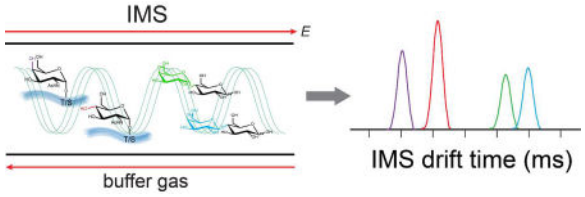
Abstract

Glycans and glycoconjugates are involved in regulating a vast array of cellular and molecular processes. Despite the importance of glycans in biology and disease, characterization of glycans remains difficult due to their structural complexity and diversity. Mass spectrometry (MS)-based techniques have emerged as the premier analytical tools for characterizing glycans. However, traditional MS-based strategies struggle to distinguish the large number of coexisting isomeric glycans that are indistinguishable by mass alone. Because of this, ion mobility spectrometry coupled to MS (IM-MS) has received considerable attention as an analytical tool for improving glycan characterization due to the capability of IM to resolve isomeric glycans prior to MS measurements. In this review, we present recent improvements in IM-MS instrumentation and methods for the structural characterization of isomeric glycans. In addition, we highlight recent applications of IM-MS that illustrate the enormous potential of this technology in a variety of research areas, including glycomics, glycoproteomics, and glycobiology.

Graphic abstract



Keywords

Ion mobility; Mass spectrometry; Glycans; Glycoconjugates; Isomers; Glycomics

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Introduction

As one of the most abundant and complex protein post-translational modifications (PTMs), glycosylation is associated with many key biological processes including cell adhesion, molecular trafficking, receptor activation, and signal transduction [1]. The analysis of glycans and glycoconjugates is challenging due to the large diversity of structures resulting from the non-template driven biosynthesis [2,3]. In addition, many of the monosaccharides that compose larger glycans are structural isomers, and they can be connected via either α- or β-stereochemistry at multiple linkage positions, resulting in many glycan isomers (Figure 1a). Isomeric glycans can also have a variety of connectivities to numerous sites on other classes of molecules such as proteins, contributing to their strucutral complexity (Figure 1b).

Separation and detailed structural characterization of glycan or glycoconjugate isomers is crucial for understanding their roles in various biological processes. Benefiting from speed and sensitivity of analysis, liquid chromatography (LC)-MS and capillary electrophoresis (CE)-MS have emerged as powerful techniques for glycan characterization [4–6]. Despite recent improvements to nearly all aspects of MS-based analyical workflows for glycan and glycoconjugate characterization, it remains challenging to achieve complete structural elucidation due to the complexity of glycans and lack of standard reference databases. Therefore, new techniques and methods that enhance the differentiation of glycan and glycoconjugate isomers would be highly desirable.

Although it has been two decades since IM-MS was orginally used to separate glycan isomers, recent technological advancements have sparked increased interest in IM-MS for glycan and glycoconjugate analysis [7]. Unlike other commonly used separation techniques such as LC and CE, IM-MS is a post-ionization gas-phase technique that separates ions based on differences in shape and charge as they travel through a buffer gas under the influence of an electric field [8,9]. The time it takes for an analyte ion to travel through the IM cell can be used to calculate rotationally averaged collision cross section (CCS) which provides an additional parameter that can be used to identify compounds as well as information about molecular conformation [8–10].

Initial applications of IM to carbohydrate analysis focused on distinguishing small isomeric carbohydrate standards [11,12]. Due to the advancement and commercialization of IM-MS instrumentation, a growing number of labs continue to demonstrate that IM-MS is a fast, sensitive, and effective method for resolving carbohydrate isomers. For example, IM-MS has been used to separate a variety of isomeric species, including connectivity and configurational isomers [13]. Furthermore, studies have been extended to more complex systems such as N- and O-glycans and intact glycopeptides [14–20]. Here, we discuss the latest developments in IM-MS methods and technology that have allowed for enhanced sepration and structural characterization of glycans and glycoconjugates and discuss advances necessary for IM to become more widely used in glycomics and glycoproteomics workflows.

Improving IM-Based Isomer Separations

Although many proof-of-principle experiments have shown the potential of IM to separate glycan isomers, baseline separation of isomeric glycans is difficult as they often have minor differences in CCS. This is especially problematic as studies are expanded to larger glycans and glycoconjugates because minor changes in the glycan composition often result in subtle differences in the overall structure. Furthermore, improved glycan separation will be crucial for extending the applications of IM-MS technology to large-scale studies of glycans and glycoproteins in complex mixtures from biological systems (i.e., glycomics and glycoproteomics). Thus, various analytical workflows that include IM separation have been developed to enhance the separation of isomeric glycans.

One of major factors that has limited the utility of IM-MS for many applications is that many instrument platforms lack the mobility resolution necessary to resolve isomeric species that have minor differences in CCS. The development of high resolution IM instrumentation using structures for lossless ion manipulations (SLIM) technology has shown great potential to enable separations of a variety of isomeric species [21]. Recently, a novel instrument was developed capable of ultralong pathlength travelling wave ion mobility (TWIM) separations on a serpentine-shaped SLIM device that has a 30-fold increase in IM resolution compared to traditional drift tube IM and TWIM instruments [22]. In addition to providing baseline separation of isomers lacto-N-hexaose and lacto-N-neohexaose, high resolution SLIM IM-MS revealed a new conformation of lacto-N-neohexaose. This suggests that SLIM-based IM separations will provide a level of conformational information about glycans that was previously inaccessible.

An alternative approach to increase isomer separation by improving IM-MS instrumentation is to optimize the charge state or polarity of the glycan ions to yield optimal separation of isomeric species. Numerous studies have demonstrated that mobility separations of glycan isomers can be optimized by manipulating the ion charge state or charge polarity [23,24]. Because of this it is important to consider a variety of charge carriers, such as metal cations and anions, and ionization methods for improving mobility separations (Figure 2a) [25–29]. Furthermore, it was demonstrated that electron transfer reactions with group II metal-coordinated carbohydrates improve separation of isomeric species, suggesting the potential for ion-ion reactions in the gas-phase for differentiation of isomeric oligosaccharides [30].

As the detection and the characterization of glycans is often hindered by the lack of a chromophore and their poor ionization properties in either spectroscopic or mass-spectrometric detection, a wide variety of glycan labeling reagents have been developed, which provide the opportunity to manipulate the conformation of the glycans in the gas-phase. Although 1-phenyl-3-methyl-5-pyrazolone (PMP) was originally developed to enhance UV detection, a recent study showed increased separation of structural isomers after PMP derivatization [31]. Reacting with *cis*-diols on carbohydrates, boronic acid (BA) derivatization has also been shown to have great potential in improving isobaric carbohydrate differentiation as an ion mobility shift strategy [32]. Besides covalent binding, non-covalent binding such as crown ethers for peptides and metal cations for carbohydrates is another promising approach to enhance separations [24,33,34]. Recently, non-covalent

complexes between monosaccharides and combinations of metal cations, peptides, and amino acids were demonstrated to improve differentiation of 8 pairs of enantiomeric glucose isomers [35]. Although there is no universal strategy to use sample preparation or gas-phase chemistry to improve glycan isomer separation, the strategies discussed above are important considerations for achieving optimal separation.

Coupling IM with orthogonal separation techniques

In addition to improving IM-MS technology, it is important to consider the enhanced analytical capability by coupling IM-MS with a variety of orthogonal separation techniques. Because mobility separations typically occur on the order of milliseconds it is possible to couple IM between LC [36,37] or CE [38] and mass spectrometry for glycan analysis (Figure 2a). The combination of orthogonal separation methods has been demonstrated to offer improved characterization of isomeric glycans. For example, the combination of hydrophilic interaction chromatography (HILIC) and TWIM was used for separation of isomeric pectic oligosaccharides [37]. In addition, a straightforward reversed-phase LC-IM-MS platform was developed for integrated proteomic and glycomic studies [39]. Furthermore, the combination of CE with TWIM-CID-MS/MS provided improved separation and quantitation of aminoxy tandem mass tag (aminoxyTMT)-labeled human milk oligosaccharides (HMOs) (Figure 2b) [38].

Coupling IM with MS-based fragmentation and spectroscopic techniques

The development of instrumentation that couples IM with a variety of fragmentation techniques has been crucial for improving the identification of glycan and glycopeptide isomers. It is important to note that coupling IM with MS/MS is mutually beneficial for both techniques. That is, ion mobility separations can be used to deconvolute complex fragmentation spectra that arise from coeluting isomeric species (Figure 3a). In addition, fragmentation spectra can be used to deconvolute CCS distributions of partially resolved isomeric species (Figure 3a).

An increasing number of studies have demonstrated that it is beneficial to perform ion mobility experiments on both precursor and fragment ions of isomeric species (Figure 3b). For example, IM analysis of precursor and fragmentation ions was used to differentiate Lewis and blood group epitopes [40]. Furthermore, it was recently demonstrated that IM analysis of MS/MS derived fragments of glycans can be used to differentiate anomeric glycosidic linkages [41]. Another promising approach capable of performing mobility separation of precursor and fragment ions is tandem IM (IM-CID-IM) in which two mobility regions are separated by a region where ions can be mobility selected and collisionally activated [42]. IM-IM-MS was recently used to distinguish underivatized carbohydrate isomers based on differences in mobilities of fragments ions. By probing ion mobility profiles of product ions, IM was also used to distinguish α2,3 or α2,6 sialic-acid linkage [15,43].

In addition to coupling IM with collision-based fragmentation methods, IM has recently been combined with a variety of fragmentation and spectroscopic techniques, including

electron activated dissociation (ExD) [44], UV photodissociation [45,46], and cryogenic IR spectroscopy [47,48]. The combination of selected accumulation-trapped IM (SA-TIMS) with Fourier transform ion cyclotron resonance (FTICR) mass spectrometers makes it possible to perform ExD on mobility-selected ions [44]. It was recently demonstrated that coupling IM with cryogenic IR spectroscopy can be used to identify isomeric glycosaminoglycans that are partially resolved by IM [49].

Collision Cross Section Databases

Another benefit IM-MS provides is the ability to measure CCS values which can be implemented into databases and used as additional criteria for structural identification (Figure 3c) [43,50]. Because CCS values are an additional parameter to improve the identification of glycan and glycopeptides, several groups have compiled CCS databases of glycans and glycopeptides that have potential to aide in glycan identifications [25,28,51–53]. For example, GlycoMob is an online database of >900 CCS values of glycans and their fragments. In addition, a database containing glycopeptide CCS values was recently presented that has the potential to aide in identification of unique glycoforms [54].

Accurate glycan identification based on CCS values is still in its early stage with several challenges remaining before this approach could be used routinely for glycan identification. One of the challenges lies in the shortage of available CCS values resulting from the difficulty in synthesizing glycan standards especially for these complex N- or O-glycans [55]. Another limitation of CCS databases is that CCS values are not intrinsic properties of ions in the same way as *m/z* values. CCS values depend on a variety of factors such as ionization conditions, buffer gas, instrument parameters, and the calibration method if not measured in a linear drift tube. Because of this, the development of robust standard operating procedures, quality controls, and calibration methods is necessary for CCS databases to be used effectively. Despite the challenges listed above, implementation of CCS databases into analytical workflows to improve glycan and glycoconjugate identification will likely be a crucial step for expanding the role and utility of IM-MS in the glycosciences. With increasing amounts of IM-MS data acquired each day, especially after coupling with LC, powerful bioinformatics tools that enable easy data acquisition, analysis and processing in a high-throughput and rapid way is key to advancing the IM-MS enhanced glycomics workflow development. Continuous efforts into platform development to support the storage of IM-MS data is also highly needed to facilitate the database query for the glycoscience community. Such platform should have the capability to support the complexity of IM-MS data, including the precursor ion and fragment ion m/z information, CCS information and LC information such as PGC-LC retention time reference [56], as well as the connections among these important parameters and information. Furthermore, the advancement of instrumentation with higher mobility resolution and CCS measurement accuracy will certainly improve the effectiveness and accuracy of IM-MS assisted glycomics workflow in a substantial manner.

Improved characterization of intact glycoconjugates

Although most examples mentioned above illustrate the utility of IM-MS for separating free or released glycans, an emerging application of IM-MS is the structural characterization of glycans bound to other classes of molecules such as proteins, peptides, and lipids (i.e., glycoconjugates). The analysis of IM-MS can reveal information about the macro- and micro- heterogeneity of glycosylation of glycoconjugates. For example, IM can separate intact glycopeptides that differ only in the glycosylation sites [16]. Furthermore, IM-MS analysis of glycopeptide fragments was demonstrated to be an effective strategy to distinguish α2,3 versus α2,6 sialic acid linkages on intact glycopeptides [15,16]. In addition to glycopeptides, IM-MS was recently used to separate glycolipid isomers [57]. It is also important to note that as IM-MS technologies evolve, they have the potential to be extended to characterize glycosylation of larger systems such as intact glycoproteins, antibodies, and virus capsids. For example, IM-MS and collision induced unfolding was used to provide qualitative information about glycosylation levels for intact antibodies [58].

Improved characterization of biological samples

One of the most promising applications of IM-MS is the characterization of glycans and glycoconjugates in complex mixtures from biological systems. Several studies have shown the benefit of adding IM into MS-based workflows for the analysis of biological samples [20,59]. For example, incorporation of field asymmetric ion mobility spectrometry (FAIMS) into a bottom-up proteomic workflow increased the number of glycopeptides identified from flagellin from *Campylobacter jejuni* 11168 [20]. It is important to note that IM separation of isomeric and isobaric species prior to MS analysis has been shown to improve quantification accuracy of both peptides and glycans [38,60]. In addition to separating isomers, IM can also be used to extract glycan and glycopeptide regions from interference ions or other molecular species [39,61–63]. Being able to separate various molecular species, IM has the potential to aide multi-omics studies, which could greatly simplify sample preparation procedures and provide more detailed information about molecular and cellular processes [64,65]. Due to the recent improvements in technology and methods described here, the application of IM-MS will likely continue to be extended beyond proof-of-principle experiments performed on glycan standards to complex mixtures from a variety of biological samples.

Conclusions

IM-MS continues to emerge as a powerful method for characterizing the enormous structural diversity and complexity of glycans and glycoconjugates. Recent advances in IM-MS instrumentation and methods have positioned this technique to enable discoveries in the glycosciences. In the future, the development of hybrid methods that couples high-resolution IM with orthogonal separation techniques and MS-based fragmentation and spectroscopic techniques will likely provide the analytical capability to extend the application of IM-MS to more complex biological systems in order to unravel the role of glycans and glycoconjugates in biology and disease.

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Highlights

- IM-MS is a powerful tool for glycan and glycoconjugate isomer analysis.
- Coupling IM with orthogonal separation techniques along with gas-phase manipulations greatly enhance isomer separation.
- Coupling IM with MS-based fragmentation and spectroscopic techniques improves structure characterization.
- CCS database provides another dimension of information for confident structural identification.
- IM-MS contributes to improved characterization of complex biological samples.

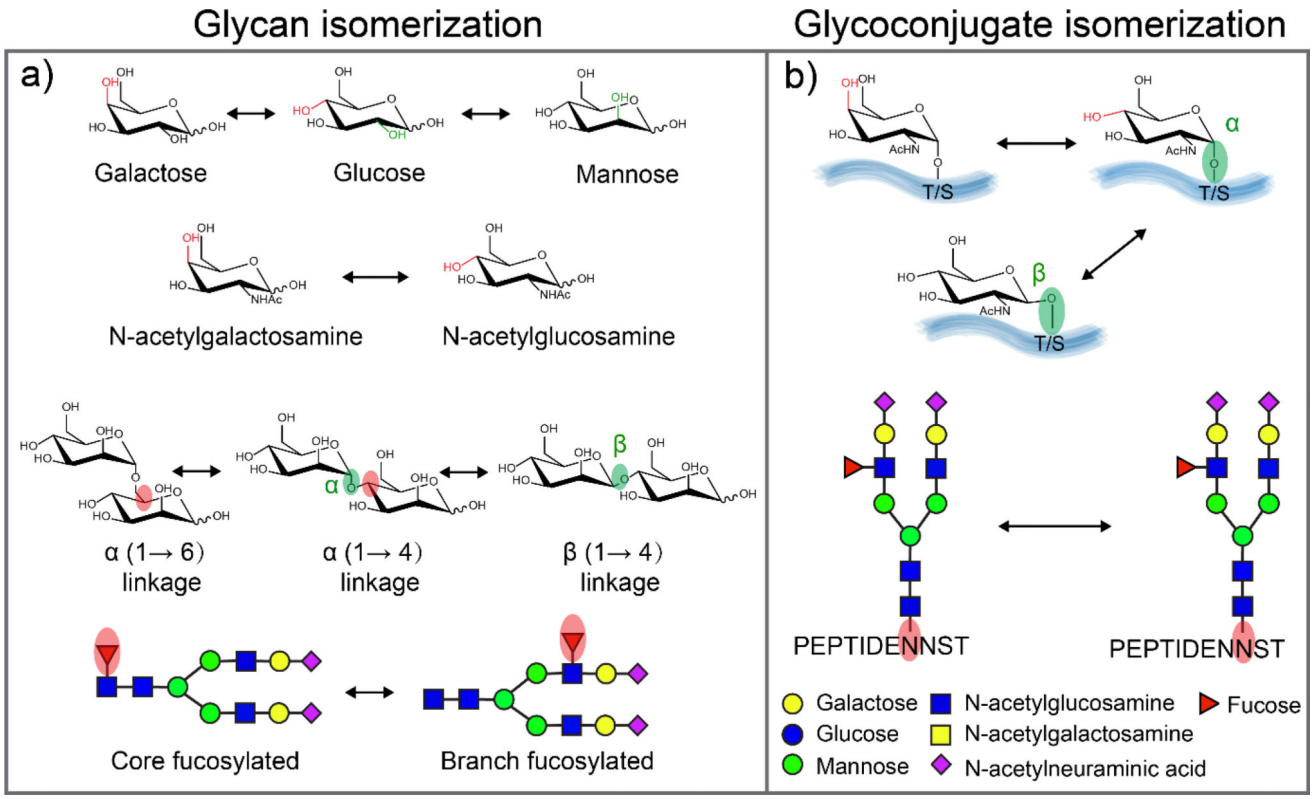

Figure 1.

The isomerization of glycan and glycoconjugates. **a)** The building blocks (monosaccharides) that compose larger glycans are structural isomers (Hexose: galactose, glucose, mannose, N-acetylhexosamine: N-acetylgalactosamine, N-acetylglucosamine); monosaccharides can be connected either α- or β-stereochemistry at multiple potential linkage position; fucose could be either attached to N-glycan core or branches. **b)** Epimeric glycoconjugates results from alternative configurations (α- or β-) at the anomeric linkages or the presence of epimeric glycan monomers (galactose or glucose), scheme modified from reference [43]; two isomeric N-glycopeptides differ in the site of N-glycan attachment.

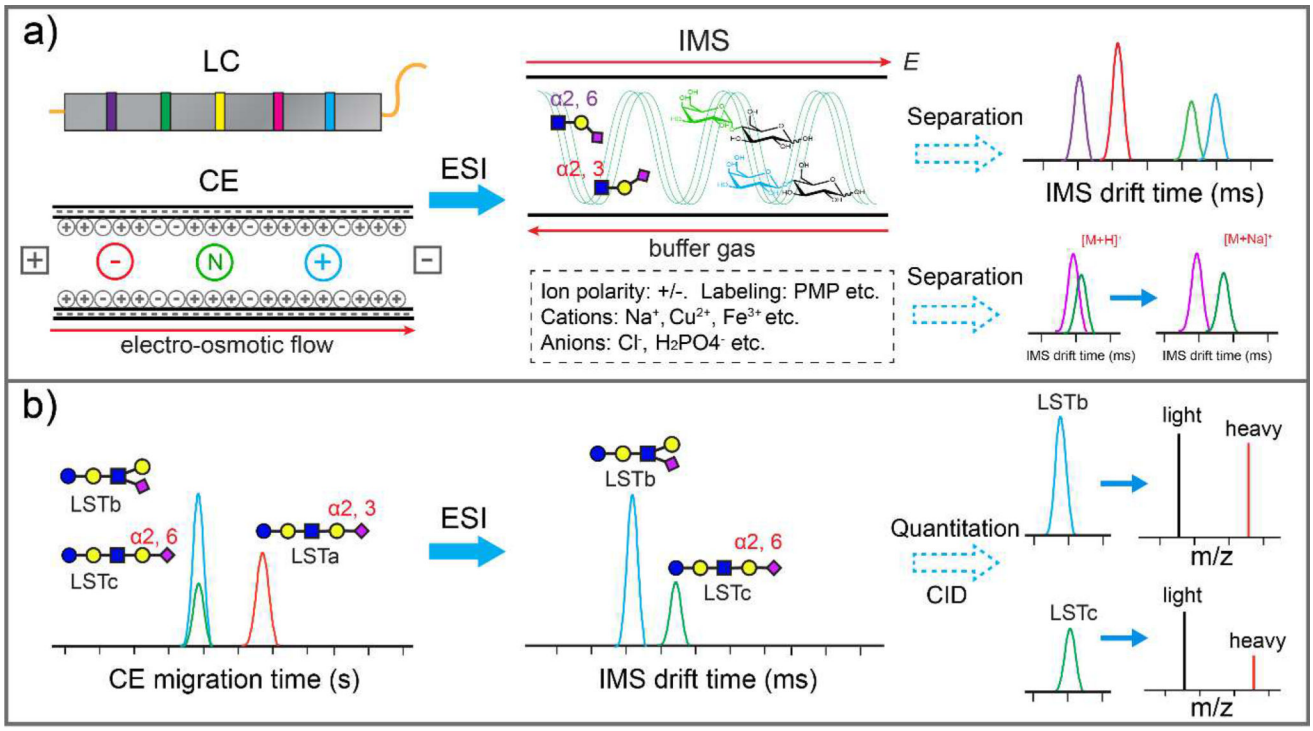

Figure 2.

Coupling IM with LC or CE. **a)** After LC or CE separation, analytes were ionized by ESI and subject to another dimension of separation afforded by IM based on their shape and charge through a buffer gas under a weak electric field (*E*). Sample preparation or gas-phase chemistry could be manipulated to improve glycan isomer separation. **b)** CE-ESI-TWIM-MS/MS analysis of a mixture of aminoxyTMT^6-128 (light) and aminoxyTMT^6-131 (heavy) differentially labeled sialyllacto-N-tetraose a, b, c (LSTa, LSTb, LSTc). CE was able to separate LSTa with LSTb/c, but was unable to resolve LSTb and LSTc. Benefiting from another dimension of separation afforded by TWIM, baseline separation between LSTb and LSTc was achieved, which enables quantitative analysis of each isomer following MS/MS [38].

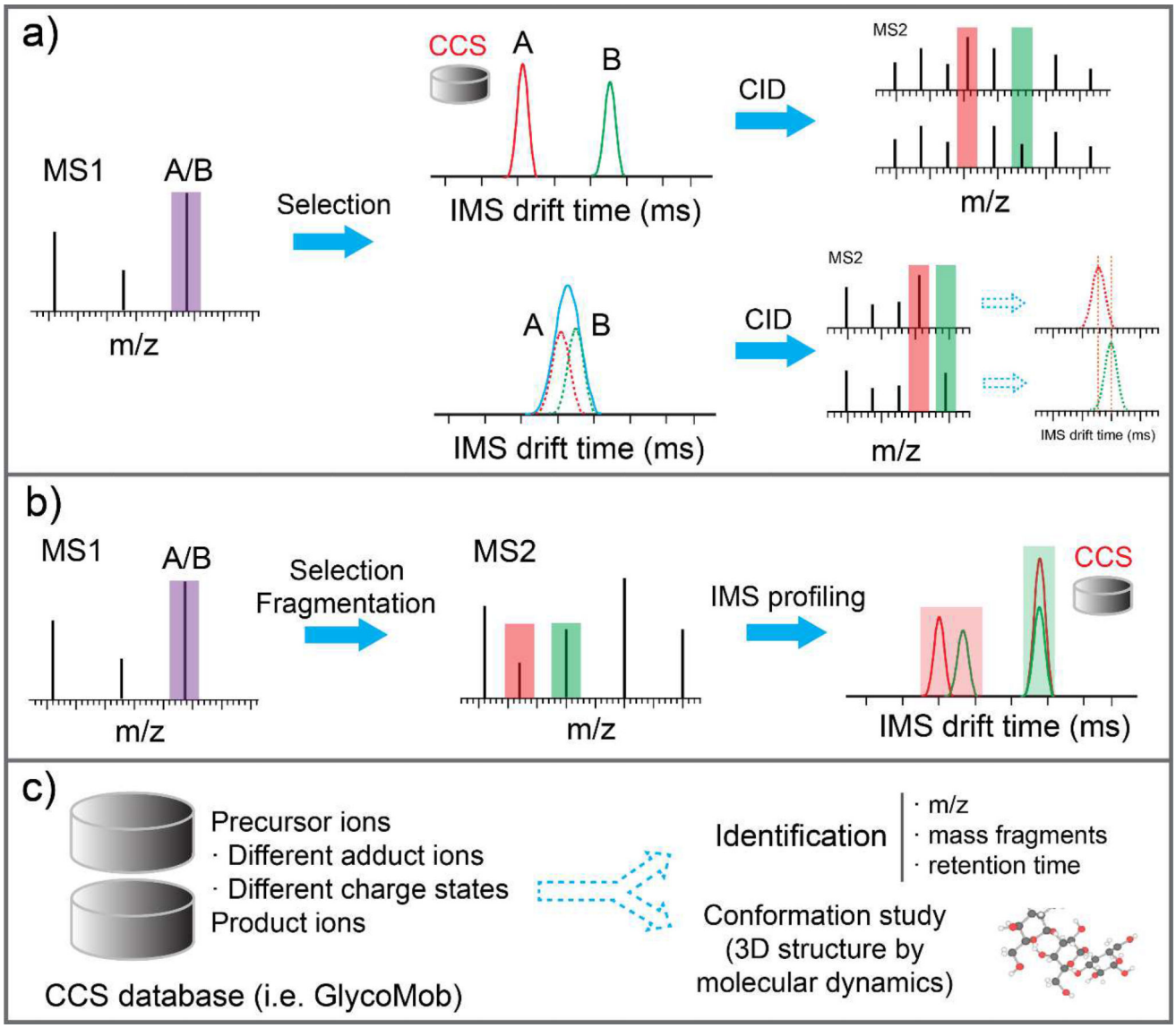

Figure 3.

IM-MS analysis of precursor ions and their fragments. **a)** Two scenarios exist for co-eluted isomers A/B for IM-MS analysis after being selected by quadrupole. Scenario one: A and B could be baseline-separated by IM. The mobility-selected ions could be subject to CID separately and signature product ions could be obtained for each †isomer. Scenario two: A and B could not be completely resolved by IM. Signature product ions were obtained for unresolved species. Drift time profiles of these signature products ions were extracted from total drift time profiles to differentiate A and B. **b)** Co-eluted isomers A/B were selected by quadrupole for MS/MS and the drift time profiles could be obtained for all product ions. Those product ions that are indicative of the isomeric structures of the analyte could be distinguished by IM and be used to differentiate the isomers. **c)** The CCS of both precursor ions and product ions could be measured and implemented into a CCS database. The CCS values could be used as an additional parameter for glycan identification besides the

commonly used m/z, mass fragments, and retention time. Furthermore, conformational study could be conducted by molecular dynamics to acquire the 3D structures of glycans.