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N and O-glycosylation Studies with Ion Mobility Mass Spectrometry (IM–MS) : an Overview

Bharath Sampath Kumar*

Independent Researcher, Chennai, TamilNadu, India 600061

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Abstract : Proteoform diversity is greatly increased by glycosylation, the primary post-translational modification of proteins. Glycans, also known as oligosaccharides, are molecules that are essential to almost all living things. They can affect protein folding and functionality, modulate cell-cell interactions, and support the proliferation of numerous diseases when they are found on cell surfaces or bound to proteins. A thorough understanding of their fundamental structure is necessary to gain insight into their characteristics and functions. But a major obstacle is the structural intricacy of glycans by design. The stereochemistry and regiochemistry of carbohydrates vary and are frequently branched. Because of its superior sensitivity and the abundance of fragmentation information it can provide, mass spectrometry is now the method of choice for glycan and glycopeptide analysis. Differentiating between the structures of isomeric and isobaric glycopeptides, however, presents a difficulty for MS-based characterization. Ion mobility plus mass spectrometry (IM–MS) has become a very promising new method for glycan research in recent years. Recent developments in the growing discipline of glycosylation analysis utilizing IM-MS are outlined in this review, with a focus on the MS methodology and its ability to resolve isomeric glycans.

Keywords : ion mobility spectrometry, glycosylation, glycoproteomics, mass spectrometry, N-glycans, O-glycans

Introduction

Glycosylation

Multiple techniques are used by biological systems to adapt and modify their structures; these strategies can be generally categorized as responses to genetic processes and environmental stimuli. Biological systems attain structural variety through several methods, with posttranslational modification (PTM) being one of the most important ones.[1-5] One type of PTM that affects many physiological processes, including ligand-receptor recognition and intercellular communication, is glycosylation.[6-10] There are other subclasses of protein glycosylation, but the two most studied subclasses are (1) O-glycosylation on a Ser or Thr residue and (2) N-glycosylation on an asparagine (Asn) residue in the sequence structure Asn-XSer/Thr (X = any amino acid but proline (Figure 1).[11-13] For instance, two proteins may have identical glycans attached, but their stereochemistry may change, producing different proteoforms.[14] Glycans bind to proteins during the highly regulated process of glycosylation, which takes place at locations throughout the protein chain. The kind of glycosidic connections that form between the glycans and the protein backbone, however, as well as their attachment sites, can differ. The resultant proteoform exhibits a notable level of diversity due to the variations in glycan attachment. Moreover, altering linkages between glycans with identical stereochemistry and the protein might change the proteoform's overall structure and characteristics.[15-17] Differentiated proteoforms can also arise from the site of glycan attachment on the protein. Attaching identical glycans with the same stereochemistry and connectivity to different amino acid residues might result in diverse proteoforms with different biological activities, even though the glycans themselves are the same.[18-20]

The process of glycan biosynthesis is not template driven. Rather, glycans bind together through an intricate series of end-block attachments and successive de- and reglycosylation processes.[21] As a result, the resultant oligosaccharides have an array of structural variations that are influenced by several factors. The types of linked building blocks are described by the composition of monosaccharides.[22] The simplified symbol nomenclature for glycans (SNFG), or chemical structures, can be used to represent monosaccharides. Only the following monosaccharides are

*Reprint requests to Bharath Sampath Kumar
https://orcid.org/0000-0002-3594-7881
E-mail: bskumar80@gmail.com

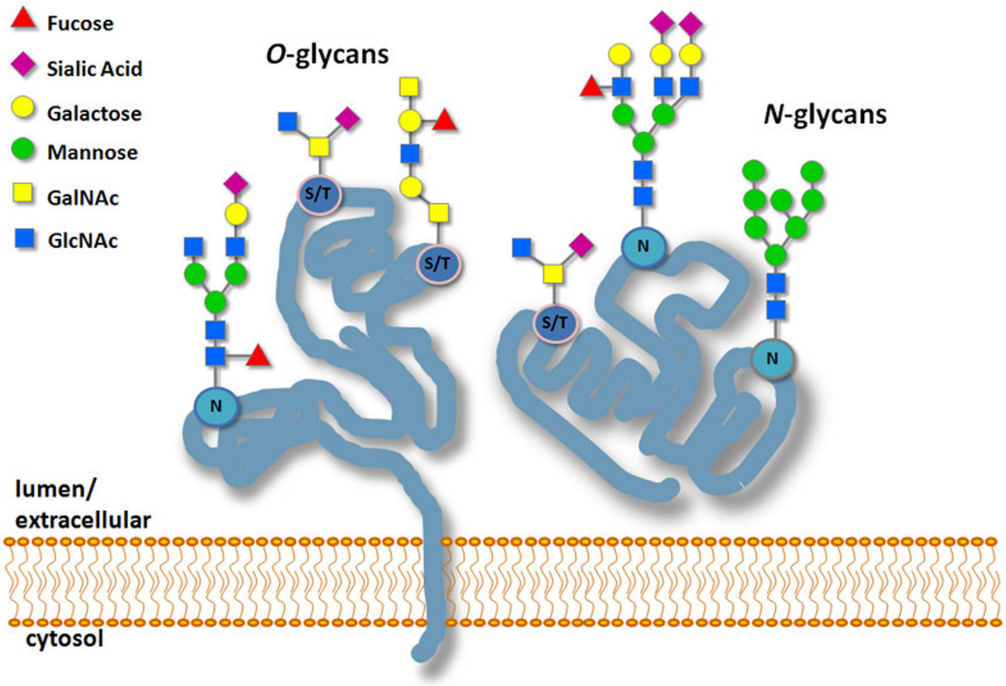

Figure 1. Glycosylation of proteins.

found in vertebrates: mannose (Man), glucose (Glu), N-acetyl- glucuronic acid (GlcA), iduronic acid (IdoA), fucose (Fuc), galactose (Gal), xylose (Xyl), N-acetyl-D-glucosamine (GlcNAc), D-galactosamine (GalNAc), D-glucuronic acid (GlcA), and N-acetyl-D-neuraminic acid (Neu5Ac).[22] On the other hand, this number is far higher in invertebrates, which greatly enhances structural complexity.[22] The connection, or the biochemistry of the glycosidic bond that joins the separate building units, is another crucial factor. Unlike proteins and oligonucleotides, monosaccharides have several glycosidic OH groups, which might all be linking sites.

At the linking site, a new stereocenter with two potential anomeric configurations arises upon the condensation of two monosaccharides. Finally, glycans can have one or more branching points in addition to their usual strictly linear assembly. [22]

Glycomics and Glycoproteomics

Studies aimed at characterizing the entire repertoire of glycans produced by a tissue, cell, or organism in particular times, places, and environmental conditions are referred to as glycomics. This glycome's appearance over the cellular proteome is referred to as "glycoproteomics." Glycoproteomics identifies the glycosylated regions on every glycoprotein in a cell and, ideally, quantifies the diverse glycan structures present at each site. Several methods are usually employed in tandem to determine tissue expression and build a repertoire of specific cell-type glycans, as there is currently no single technique that can identify every component of the glycoproteome or glycome. The glycoproteome and glycome must be examined directly because the transcriptome, proteome, and genome are unable to reliably forecast the dynamically conveyed protein-linked glycans. To characterize, for instance, the structures of glycolipids against glycoproteins, O-glycans versus N-glycans, etc., different methods and procedures are needed. Glycomic analyses can be broken down into three fundamental procedures according to the degree of information needed: glycan class characterization, glycoprofiling, and comprehensive structural examination of the glycans produced from protein(s). Ideally, the extent of detail will depend on the specific topic being addressed.

1. Glycoprofiling: In glycoprofiling, a complex combination of glycans is separated, yielding a signature or fingerprint that provides a quick summary or image of the glycans in the sample.[23]
2. Glycan class characterization: The method allows glycan mixtures to be divided into different categories of glycans according to their structural characteristics. For example, di-, mono-, and non-galactosylated IgG glycan separations using mass spectrometry. This method provides relative quantification of the various glycan classes and is a useful technique to emphasize defined, important traits.[23]
3. Comprehensive structural analysis: determining the sequence and any alterations to the branch points, anomericity, glycosidic connections, and monosac-

charides of the glycans in a glycome are necessary for structural analysis. Orthogonal technologies are typically needed in this thorough study, initially for the assignment of initial structures and then for the confirmation of those assignments. In addition, absolute or relative quantification of the assigned glycan structures—such as a particular structure binding to a bacterial protein—can be included in a full structural analysis. Glycans present a challenge for full structural investigation because multiple structures might have the same mass, coelute often on separation techniques, and necessitate meticulous transcription of MS/MS spectra. Glycans typically receive a first structural designation from one technology, which is subsequently verified by more than one orthogonal technology.[23]

Mass spectrometry

In glycan analysis, MS-based methods continue to be among the most used techniques. But even with advanced tandem MS^{24} or MS^{25} methods, it is frequently unable to completely untangle the structure of a particular glycan. Because natural glycans frequently exist in complex matrices, they must first be isolated and purified before being subjected to MS analysis. For example, N- and O-glycans must be separated from glycoproteins using chemical or enzymatic techniques to be subjected to further glycan analysis. Strategies for glycan enrichment and purification are essential preconditions for high-quality MS studies.[26] Chromatography, electrophoresis, liquid-phase extraction, and solid-phase extraction are frequently used techniques for glycan enrichment and purification.[27,28] Reversed-phase high-performance liquid chromatography (RP-HPLC], hydrophilic interaction liquid chromatography (HILIC), capillary electrophoresis (CE), porous graphitized carbon (PGC) chromatography, and strong anion exchange (SAX) chromatography are among the chromatographic and electrophoretic techniques frequently used for glycan separation.[29-31] Glycans are frequently derivatized after being extracted from natural sources to improve separation, ionization, and detectability.[32-34] The range and precision of structural data that can be obtained by MS-based techniques have greatly expanded because of the creation of new ion activation techniques, the successful commercialization of ion mobility mass spectrometry (IM-MS), and the application of gas-phase ion spectroscopy in the glycomics discipline.

Ionization sources

The two primary ionization methods used to transport intact glycans into the gas phase are ESI and matrix-assisted laser desorption/ionization (MALDI).[35-37] Whereas ESI permits glycan ionization from solution and is consequently conducive to (LC) liquid chromatography, MALDI is frequently employed to ionize glycans directly from tissue specimens. Even-electron molecular ions—which are usually protonated or metal adducts in positive ion mode—are produced by ESI. Deprotonated or anion-adducted species are seen in negative ion mode. ESI frequently exhibits several charge states, particularly in big compounds. The ESI parameters, such as pH and the existence of salts or detergents, affect the charge state. Because they are hydrophilic and lack protonable basic sites, glycans often do not produce a large amount of ion signals. Derivatization techniques like permethylation, peracetylation, or tagging at the reducing end can raise the ion yield.[38] Permethylation stabilizes labile moieties like sialic acid in addition to improving ionization efficiency. Glycans can fragment in a specific manner upon activation once they are in the gas phase. Glycosidic and cross-ring cleavage are two essentially distinct forms of cleavage that can happen in polysaccharides. Typically, the fragments that are produced are named using the Domino-Costello nomenclature.[39] Components that keep the reducing end are denoted by the alphabets X-, Y-, and Z-ions, whereas the alphabets A, B, and C stand for fragments that contain the nonreducing end. The other ions are created by glycosidic cleavage involving two monosaccharides on both sides of the glycosidic oxygen, whereas A- and X-fragments are the product of cross-ring cleavage within a monosaccharide's sugar ring. Superscript numbers on cross-ring fragments represent the bonds that are cleaved within the corresponding monosaccharide unit, whereas subscript numbers within the glycan chain express the location of cleavage. The subscript numerals that represent the number of cleaved glycosidic bonds vary depending upon whether the fragments include the reducing or nonreducing end: for A, B, and C-type fragments, the cleavage position leading to X, Y, and Z-type fragments is added up from the reducing end, while the glycosidic bonds are considered from the nonreducing end, starting with 1.

Ion activation techniques

Different ion activation techniques have different activation times and total amounts of energy delivered to the precursor ion. As such, distinct techniques result in essentially distinct, frequently mutually reinforcing fragmentation mechanisms. Isolated precursor ions in tandem mass spectrometry can generally dissociate through collision-, photon-, or electron-mediated activation. The key ion activation techniques for the structural investigation of glycans and glycoconjugates will be discussed in brief and contrasted with one another in the sections that follow.

When using collision energy in the eV range, collision-induced dissociation (CID)[40] typically permits extended activation durations, allowing for several collisions between the precursor ion and the target gas. Fast ion activation and one or a few collisions are the outcomes of higher-energy collisional dissociation (HCD), which utilizes collision energies in the keV range.[41] Although less well-established, the method can produce many fragments from direct bond breakage that are not accessible by CID,

such as a significant amount of fragmentation from the peptide backbone and glycan in glycopeptides.[41]

Ion activation is induced by photon-mediated fragmentation, which involves photons from various regions of the electromagnetic spectrum being irradiated. A gradual heating technique called infrared multiple photon dissociation (IRMPD) uses the successive absorption of infrared photons, which are typically produced by a CO2 laser.[42] Intramolecular vibrational redistribution (IVR) redistributes the rise in internal energy in the preliminary ion over all vibrational modes following each photon absorption event. The dissociation cutoff is achieved after several absorption IVR cycles, and the weakest bonds cleave as in CID. IRMPD can be utilized for collecting gas-phase IR spectra by using an adjustable light source as opposed to a fixed wavelength.[43] UV photons between 10 and 400 nm in wavelength are used in ultraviolet photodissociation (UVPD)[44] because they are intense enough for single-photon dissociation. UVPD is a quick activation technique that uses electronic stimulation of the parent ion. Both site-specific and cross-ring fragmentation on sialic acids result in a profusion of fragmentation, including A- and X-fragments.[45,46]

Electron-mediated ion activation techniques (ExDs) are categorized as rapid activation techniques that cause distinct bond cleavage and are especially significant for free glycan and glycoconjugate structure research.[47] These consist of electron detachment dissociation (EDD),[48] electron transfer dissociation (ETD),[49] electron capture dissociation (ECD),[50] electron induced dissociation (EID), and electronic excitation dissociation (EED)[51]. These techniques, which induce charge state reduction by adding or removing an electron, are restricted to multiply charged cations (ECD and ETD) or anions (EDD), except for EID and EED. ECD uses an electron source with minimal energy and needs an FTICR cell's magnetic fields to trap electrons. Because trapped electrons are not used for ion activation, ETD is also appropriate with linear and 3D quadrupole ion traps. On the other hand, stable odd-electron anions produced at a different ion source mediate the electron transfer. ECD and ETD are widely used in the study of labile PTMs in O-glycosylation,[52] as well as in the sequence analysis of oligosaccharides, which, upon electron capture, can produce useful cross-ring fragments.[53,54] Electron detachment (EDD) uses electrons with moderate kinetic energy to convert multiple charged anions into radical anions. The technique is especially helpful for analyzing sulfated oligosaccharides, such as GAGs, since it produces informative fragments from cleavages that identify sulfation sites.[55] EED is suitable for studying small glycoconjugates, such as glycopeptides and lipids, that are only detectable as singly charged species since it may be applied to singly charged positive or negative ions.[56] EED has a great deal of potential for linkage analysis since it causes widespread cross-ring cleavage.[57] Many ExD techniques have low fragmentation efficiency, which means that to achieve suitably intense fragment ion peaks, lengthy reaction and interaction times are required. The simpler online coupling of several ExD methods to chromatographic and electrophoretic separations has been hampered by ion-electron interaction times that vary between hundreds of milliseconds and seconds, which are much longer than those used in CID.

While short activation times induce immediate bond cleavage prior to the energy being redistributed across the entire molecule, long activation times generally result in fragmentation based on bond dissociation energies. Every ion activation technique has a specific use, and when combined, they can provide a more complete understanding of intricate glycan and glycoconjugate structures.

Enrichment methods in glycoproteomics

Because of their low abundance, the microheterogeneity between protein glycosylation locations, the intricate nature of glycan structures, and their lower ionization efficiency compared to unmodified peptides, glycoproteins in diverse samples remain difficult to analyze directly using MS-based approaches. Thus, for the purpose of glycoproteomics research, the isolation and enrichment of glycopeptides contained within biological substances are crucial. Research on glycoproteomics has effectively employed several enrichment techniques that have been developed over the last 10 years.

Because lectins selectively recognize glycan motifs in glycoconjugates, they are frequently utilized as affinity labels to effectively detect glycans in complicated biological media. Glycopeptide enrichment has been successfully achieved by immobilizing lectins on magnetic beads, or a resin-based substance.[58,59] Van der Waals forces and hydrogen bonding are two of the interactions that give rise to a lectin's selectivity towards a target. Owing to the different ways in which lectins bind carbohydrates, it is necessary to use a variety of lectins with different glycan recognition specificities to completely capture proteins that are glycosylated from biological mixtures.

By utilizing a protein engineering strategy to enhance the intrinsic features of Fbs1, Chen et al. demonstrated the selective extraction of N-linked glycopeptides from complicated peptide mixtures. Fbs1, a lectin-like protein found in eukaryotes, usually works through ubiquitin to remove N-glycosylated proteins that are misfolded.[60] Mutant Fbs1 can bind to many kinds of N-linked glycoproteins, while wild-type Fbs1 prefers to bind glycans that contain high mannose.[60] The reliable enrichment of N-linked glycopeptides present in human serum was accomplished with success using the mutant type Fbs1. Additionally, the incorporation of a hydroxyl group at position 173, which results in the formation of tyrosine from phenylalanine, had a favorable effect on sugar ring binding.[60]

Hydrazide-based enrichment is not as selective as lectin techniques, yet it remains capable of detecting a wide range of glycopeptides. However, the dissociation of glycopep-

tides with unmodified glycan structures is impossible because the covalent link that exists between the glycopeptide and support is irreversible. Using FASP, hydrazide-infused PAMAM dendrimers were developed to enrich N-linked glycopeptides effectively and selectively from a human serum specimen.[61] When it came to the enrichment of glycopeptides produced from fetuin samples, hydrazide-functionalized PAMAM dendrimers performed better than commercial hydrazide beads.

Glycan cis-diol groups and boronic acids generate reversible cyclic boronate esters, which can be utilized to isolate and capture glycopeptides.[62,63] The primary chemistry-related distinction between hydrazide and boronic acid is their reversibility. While the chemistry of boronic acid can be readily reversed in an acidic environment without changing the glycan structure, the chemistry of hydrozides necessitates irrevocable and detrimental glycan oxidation to produce aldehyde groups. Glycopeptides present in human serum samples were enriched using a boronic acid-functionalized framework, which made it possible to identify N-glycosylation peptides produced from 89 distinct glycoproteins.[63]

The goal of chemical biology techniques via glycopeptide enrichment is to target glycans with nonendogenous chemical handles to selectively distinguish glycosylated molecules from nonmodified peptides.[64,65] Frequently, these techniques employ bioorthogonal reactions, which are chemical processes that take place within living systems without influencing or interacting with the systems' natural metabolic processes.[66] Using chemically functionalized monosaccharides, bioorthogonal handles can be metabolically infused into glycans. Because they are minuscule, stable in vivo, unreactive with endogenous biological components, and simple to incorporate into synthetic sugars, azides are commonly selected to serve as bioorthogonal handles.[67,68] Then, azido-sugars can be selectively labeled by Staudinger ligations, strain-promoted azide-alkyne cycloadditions, or azide-alkyne cycloadditions catalyzed by copper.[69-74] Moreover, alkynes can serve to act as bioorthogonal handle for the integration of artificial monosaccharides, which are then tagged with azide tags.[66-68] Selectivity is by far the greatest benefit of modern chemical biology tools, particularly since bioorthogonal processes may be used to enrich materials under strict conditions that reduce nonspecific interactions.

Strategies for enrichment are summarized in Figure 2. There isn't a single, all-encompassing enrichment technique for glycoproteomics, and this needs to be emphasized again. Researchers must be practical when planning experiments because different strategies can be utilized among glycan classes of interest.

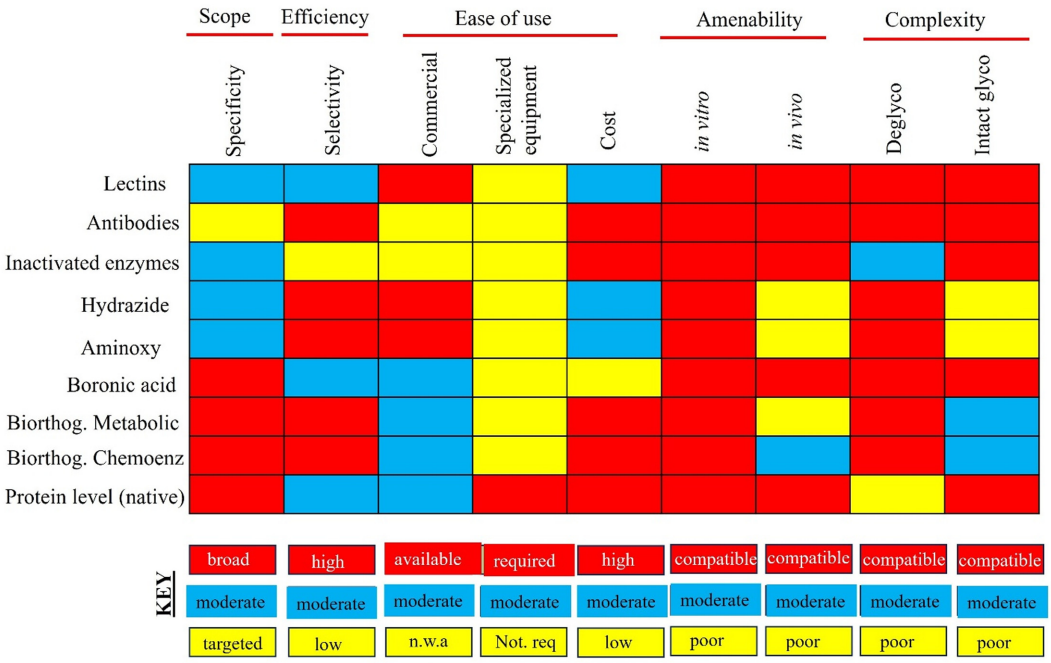

Figure 2. Considerations when choosing a glycopeptide enrichment approach. Success of various glycoproteomic enrichment strategies depends on several experimental conditions. Selection of the appropriate enrichment tool must be evaluated based on criteria that include scope of the experiment (broad versus targeted glycan class specificity), enrichment efficiency (i.e., selectivity), the ease of use and implementation, amenability to various biological systems, and the complexity of the analytes to be analyzed (deglycopeptides versus intact glycopeptides). The abbreviation "n. w. a." stands for "not widely available," and "poorly" is short for "poorly suited." Adapted and modified from the reference 28.

Derivatization

Enhancing the ionization capacity of glycopeptides is critical, even though low abundant glycoproteins might be enriched by various techniques.[75] To adequately capture the biological data pertaining to protein glycosylation, a direct study of intact glycoproteins is necessary. To improve MS ionization and regulate acidic glycans, many chemical derivatization approaches have been utilized to alter glycopeptide structures.

Disease onset and progression are influenced by sialylation.[76-78] Due to the in-source and metastable degradation of sialic acids, it is challenging to evaluate glycopeptides that have been sialylated. Sialic acid has been stabilized using methylamine and dimethylamine amides.[79,80] In the realm of glycomics, esterification is a frequently employed derivatization approach in addition to amidation.[81,82] It neutralizes carboxyl groups in sialic acids and enhances ionization efficiencies while stabilizing sialic acid motifs to increase sialylation quantification. Without the requirement for separate procedures, esterification can also discriminate between sialic acid linkage isomers.

Several benefits come with permethylation, which replaces hydroxyl and amino groups in glycans and the peptide backbone with methoxy groups. These benefits include heightened detection sensitivity and the elimination of in-source fragmentation.[83] This method requires a glycopeptide with 3-6 amino acid residues to identify positive glycosylation sites and provide simple data interpretation.

Analytical challenges related to N and O- glycans structural analysis

Chromatography combined with MS is widely employed as an analytical methodology for the study of N-glycans. It is possible to compare experimental retention times to values retained in databases.[84] While this method has been shown to be dependable, it doesn't always offer enough structural details about the analytes. Fast-atom bombardment (FAB)-MS may ionize the N-glycopeptide counterparts of N-glycans; however, underivatized N-glycans cannot be effectively ionized using this method. An enhanced sensitivity toward both native and derivatized glycans was seen upon the integration of ESI and MALDI as ionization sources.[85-87] Since the molecular masses of monosaccharide units, such as Man and Gal or GlcNAc and GalNAc, are identical, MS alone is typically unable to distinguish between them. Furthermore, it is unable to get details regarding glycosidic bond arrangement and branching. To address the previously mentioned problems, multidimensional information can be obtained by combining MS with other fragmentation techniques, IMS, and spectroscopic approaches.

In mucins, extensive O-glycosylation can account for 50–80% of the glycoprotein's total mass.[88] This frequently has a profound effect on the molecule's overall characteristics. Oligosaccharides are typically more hydrophilic and, depending on their terminal alterations, frequently negatively charged as compared to the protein backbone. O-glycans are therefore typically greatly solvated by water and salt ions, leading the mucus to have a greater degree of viscosity and gel-like structure.[88] O-glycosylation does not require specific sequons, in contrast to N-glycosylation. Furthermore, the process of biosynthesis is sequential and does not involve the cleavage of specific residues by glycosidases in the later stages of processing. O-glycans are therefore usually smaller than N-glycans, but the fundamental structural space is very large and has several sources of isomerism.

In general, O-glycosylation analysis is more difficult than N-glycosylation analysis. For this reason, identifying O-glycosylation locations within a protein is far more difficult than identifying N-linkages. There are no specific enzymes that directly and preferentially hydrolyze the glycosidic link to the protein, like PNGaseF does with N-glycans. Usually, severe chemical processes are used instead.[89-91]

Top-down and bottom-up approaches

While bottom-up glycoproteomics initially uses proteolytic enzymes to breakdown the glycoproteins before characterizing the glycopeptides, top-down glycoproteomics concentrates on the direct study of intact glycoproteins. The information obtained on the entire amino acid sequence, including all post-translational modifications, is the benefit of top-down glycoproteomics. With little sample preparation, each precursor's charge state and isotopic distribution represent an incredibly intricate yet precise set of information about the glycoprotein structure. Very high glycoprotein precursor *m/z* are the product of low charge states, and deciphering the data requires sophisticated detection methods or appropriate high-mass equipment with exceptional resolution.[92] Nevertheless, high charge states result in low precursor *m/z* with many charge states, which are easily identified by many MS. The broad range of charge states, nevertheless, can severely reduce signal sensitivity and cause several glycoprotein precursors to overlap, making identification difficult. The variety of glycosylation sites on both a macro- and micro-level exacerbates this issue.

The three main steps in a typical bottom-up experiment workflow are preparing samples, sample enrichment, and MS assessment. Typically, sample preparation involves the glycoprotein's optional enrichment from its elaborate matrix and the enzyme-assisted proteolytic breakdown that follows. MS analysis of the proteolytic digestion mixture is possible, but low abundances of each glycopeptide are caused by the wide range of glycans bound to every glycan site and associated heterogeneous site occupancy. A bottom-up strategy comprises two typical analytical methods. One strategy is to use chemical or enzymatic techniques to liberate the glycans from the glycoproteins, after which the

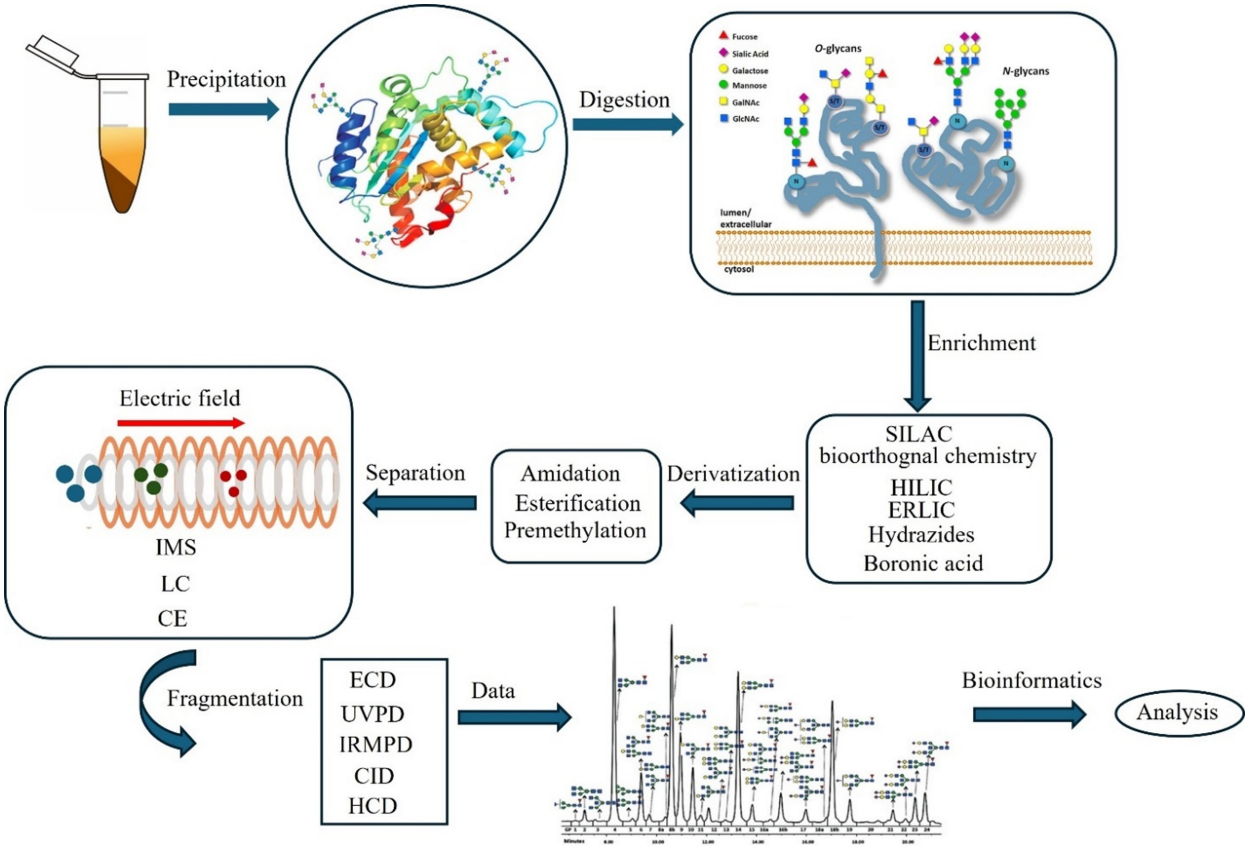

Figure 3. Generalized glycomics workflow.

proteins and carbohydrates are separated and examined, respectively. Typical N-glycanase (PNGase) enzymes, including PNGase F, are employed for N-linked glycoproteins.[93,94] When it comes to O-linked glycoproteins, chemical techniques like β-elimination are frequently used to liberate glycans.[95] Carbohydrate derivatization is frequently required to enhance the MS evaluation.[96-98] After being broken down by endoproteases like trypsin, the deglycosylated proteins are identified by MS. It is possible to extract protein sequences and glycan structures. However, it's possible that information on the carbohydrate binding sites of proteins will be lost. An alternative method involves the direct digestion of glycoproteins using endoprotease, followed by the mass spectrometry analysis of the digested glycopeptides.[99] One can ascertain the sites of glycosylation. These two methods work well together to identify glycoproteins. Figure 3 presents a typical bottom-up approach in glycoprotein analysis workflow.

Ion mobility spectrometry

The gas-phase electrophoretic separation technique known as ion mobility spectrometry (IMS) is frequently used as a stand-alone procedure for determining explosives, narcotics, and chemical warfare agents.[100-102] IMS segments ions according to variations in their gas-phase mobilities (K), a transport characteristic pertaining to the mass, charge, size, and form of the ions. The effective temperature at high fields and the kind and number density of the buffer gas also has an impact on K. Under the control of an electric field, ions travel through an appropriate gas-filled compartment, where they collide with neutral gas particles. When larger, longer species move electrophoretically, they collide more frequently and move at slower speeds than ions that are more compact. IMS can distinguish between isomers and conformers, which are chemical components with the same *m/z* ratios but different morphological characteristics.

$$K = \frac{3}{16}\left(\frac{2\pi}{K_B T}\right)^{\frac{1}{2}}\left(\frac{1}{m}+\frac{1}{M}\right)^{\frac{1}{2}}\frac{q}{\Omega} \quad (1)$$

K_B = Boltzmann Constant, T = temperature in Kelvin (K), m & M mass of buffer gas and ion respectively, q = ionic charge, Ω = collision cross section (CCS).

Fundamental low-field ion mobility is described by Mason−Schamp (equation 1). Ion-neutral collisions in the

Table 1. Key characteristics of the different ion mobility systems.

IM type	Manufacturer	Rm	DL	MSA	Pros	Cons	CCS data
DTIMS	Agilent	~50-60	5-100 cm	QTOF	High resolving power, Range of analytes	Ions are pulsed, Longer analysis time	Direct ccs measurement
FAIMS	Thermo Fisher	~30	~20-40 cm	Orbitrap	Greater selectivity, smaller sample size, and compatibility with MS	Narrow ion mobility resolution and sensitive to environmental parameters	CCS data not readily available
TWIMS	Waters	~30-40	Few square meters	QTOF	Higher mobility resolution, faster analysis time, and accommodate tandem MS	Limited dynamic range, lesser sensitivity	requires calibration from known CCS values
SLIM	PNNL	~230-315	13 m	QTOF	Higher mobility resolution, can accommodate ion trapping and collisional cooling	Limited commercial availability	requires calibration from known CCS values
cIM	Waters	~60-90	~97 cm	QTOF	Higher mobility resolution, and accommodate tandem MS	ion transmission reduces with increasing passes	requires calibration from known CCS values
TIMS	Bruker	~200-400	1 m	QTOF	Higher mobility resolution, accommodate different ionization sources, and can analyze complex analytes.	Longer analysis time, and limited sample capacity	requires calibration from known CCS values

Table 2. Empirical studies on Nand O-glycosylation with IM-MS. Rm = resolving power, DL = drift length, CCS = collision cross section, MSA = mass spectrometry analyzer.

Author	Domain	Sample	Ionization & Fragmentation	IMS	MS analyzer	Ref #
Alagesan et al. (2022)	Protein glycosylation	Human serum	HCD	FAIMS	Orbitrap	169
Fang et al. (2021)	N-glycoproteomics	human lymphoma cells	ESI	FAIMS	Orbitrap	139
Gao et al. (2021)	Glycomics	Dairy beverages and juice	ESI	TIMS	Orbitrap	170
Pagel et al. (2023)	O-glycosylation	Porcine gastric mucin samples	nano-ESI	TWIMS & TIMS	Orbitrap & TOF	149
Williams et al. (2021)	Protein–Glycosaminoglycan Interactions	heparan sulfate	nano-ESI & CID	TWIMS		171
Rangel-Angarita et al. (2023)	Glycoprotein analysis	Podocalyxin	ESI & HCD	FAIMS	Orbitrap	172
Butler et al. (2022)	N-glycosylation	Human plasma samples	nano-ESI	DTIMS	QTOF	114
Vos et al. (2023)	Sialic acid O-acetylation	bovine submaxillary mucin	ESI	DTIMS	QTOF	123
Manz et al. (2022)	Sialic acid isomers	Human alpha-1-acid glycoprotein	nano-ESI & CID	TWIMS	Orbitrap	134
Quaranta et al. (2020)	N-glycosylation	Human serum samples	ESI	TWIMS	QTOF	173
Chandler et al. (2023)	Glycoprotein analysis	human alpha-1-acid glycoprotein (AGP)	ESI	FAIMS	Orbitrap	137
Veith et al. (2022)	O-glycosylation	Porphyromonas gingivalis	ESI & CID	FAIMS	Orbitrap	174
Bonnet et al. (2023)	Cyclic oligosaccharides	cyclodextrins (CD)-metals complexes	ESI	TIMS	TOF	175
Mukherjee et al. (2023)	Glycoproteomics	Proteolytic Digestion of Human Plasma	nano-ESI	TIMS	TOF	176

Table 2. Continued

Author	Domain	Sample	Ionization & Fragmentation	IMS	MS analyzer	Ref #
Torano et al. (2020)	N-glycosylation	Fetuin from bovine serum	ESI	DTIMS	QTOF	177
Wei et al. (2020)	N-glycosylation	ovalbumin	ESI, EED & CID	TIMS	TOF	148
Bansal et al. (2023)	N-glycosylation	lacto-N-difucohexaose II (LNDFH II), lacto-N-tetraose (LNT), and 3-fucosyllactose (3-FL)	nano-ESI & CID	TWIMS	TOF	135
Sarbu et al. (2022)	Glycosphingolipid	Cerebrospinal fluid (CSF)	nano-ESI & CID	DTIMS	QTOF	178
Manz et al. (2022)	N-glycosylation	Dextran	nano-ESI	DTIMS	QTOF	179
Harvey et al. (2021)	N-glycosylation	human embryonic kidney (HEK 293T) cells	nano-ESI & CID	TWIMS	QTOF	180

low-field range are basically heat, and K is largely independent of the electric field strength E. Collision cross sections (CCSs) depend on interaction potentials plus the size and form of the collision partners.[103-105] By representing momentum transfer among the colliding particles, they act as effective regions.[106] In IMS, the greater the degree of momentum transfer among the ions and the gas particles, the higher the CCS of an ion-neutral pair. CCSs make good molecular descriptors since they are unaffected by gas pressure and typically change less with temperature compared to mobilities. They make analyte identification easier and are compatible with many IMS platforms. They can also be easily stored in databases. Table 1 summarizes the resolving power of several ion mobility techniques in addition to their specific benefits and drawbacks. Table 2 summarizes empirical studies utilizing the various IMS systems.

Drift Tube Ion Mobility Spectrometer

The ion mobility analyzer in a Drift Tube Ion Mobility Spectrometer (DTIMS) system (Figure 4a) is a drift tube made up of many stacked-ring electrodes with an inert gas (helium or nitrogen) passing through the tube at all stages. The ions travel toward the detection zone when a constant electric field is applied along the drift tube. The mobility of the ions is determined by their collisions with the drift gas, which flows in the opposite direction as the ions do; smaller ions move quicker than augmented ones because they are subject to fewer collisions. As a result, the shape of the ions, especially when correlated with the CCS value, determines how long (drift time) it takes them to travel to the detector.[107,108] DTIMS is the lone analyzer that can accurately and directly estimate CCS values without the use of calibrating devices in rigorously monitored settings. For example, antiepileptic medication in human serum was identified using CCS values in conjunction with mass spectrometry data.[109] In addition, CCS measurements boost the identification confidence, while DTIMS offers an additional component to the LC separation.[109] In addition to being utilized for acquiring structural data, DTIMS has been applied to the isomer separation process. This is particularly true with bile acids, where liquid chromatography (LC) separation takes an extended amount of time and provides a low resolution for some isomeric molecules. When metal ions like copper and zinc were added to the sample, complexes with differential mobilities formed that could be separated using DTIMS.[110] Additionally, ion mobility has proven to be a viable choice for metabolite profiling—also known as "metabolomics"—by analyzing metabolites in biological samples.[111-114] The application of CCS determination in metabolomics has also been shown to be beneficial, as it can help with small molecule characterization during both targeted and untargeted metabolite evaluation.[114]

Application with DTIMS

Glycosylation abnormalities have been linked to several illnesses, including cancer and neurological diseases. However, because there are so many different glycosyl isomers, it can be challenging to comprehend how a glycan relates to its biological function. To investigate a variety of glycan standards as well as ones enzymatically generated from the glycoprotein's horseradish peroxidase, fetuin, and human plasma (Figure 5). Butler et al. designed a nanoflow LC-DTIMS-MS in conjunction with the INLIGHT^{TM} technique.[114] Furthermore, to expedite the identification of potential glycans in intricate biological samples, molecular trend lines dependent on the IMS and MS characteristics were examined for the INLIGHT^{TM} derivatized glycans.[115] Rapid detection and relative evaluation of putative glycans in complex biological samples can be achieved through the drift time agreement of the NAT and SIL derivatized glycans and their distinct isotopic distribution, as the incorporation of 13C does not significantly change the shape of the glycans. Because mobility-aligned NAT- and SIL-labeled glycans provide extra capabilities for isomer investigations, the combination of IMS with the natural (NAT) and stable-isotope label (SIL) in the INLIGHT^{TM} method provides additional confidence for each glycan identification.[115]

Sialic acids are nine-carbon monosaccharides with a neg-

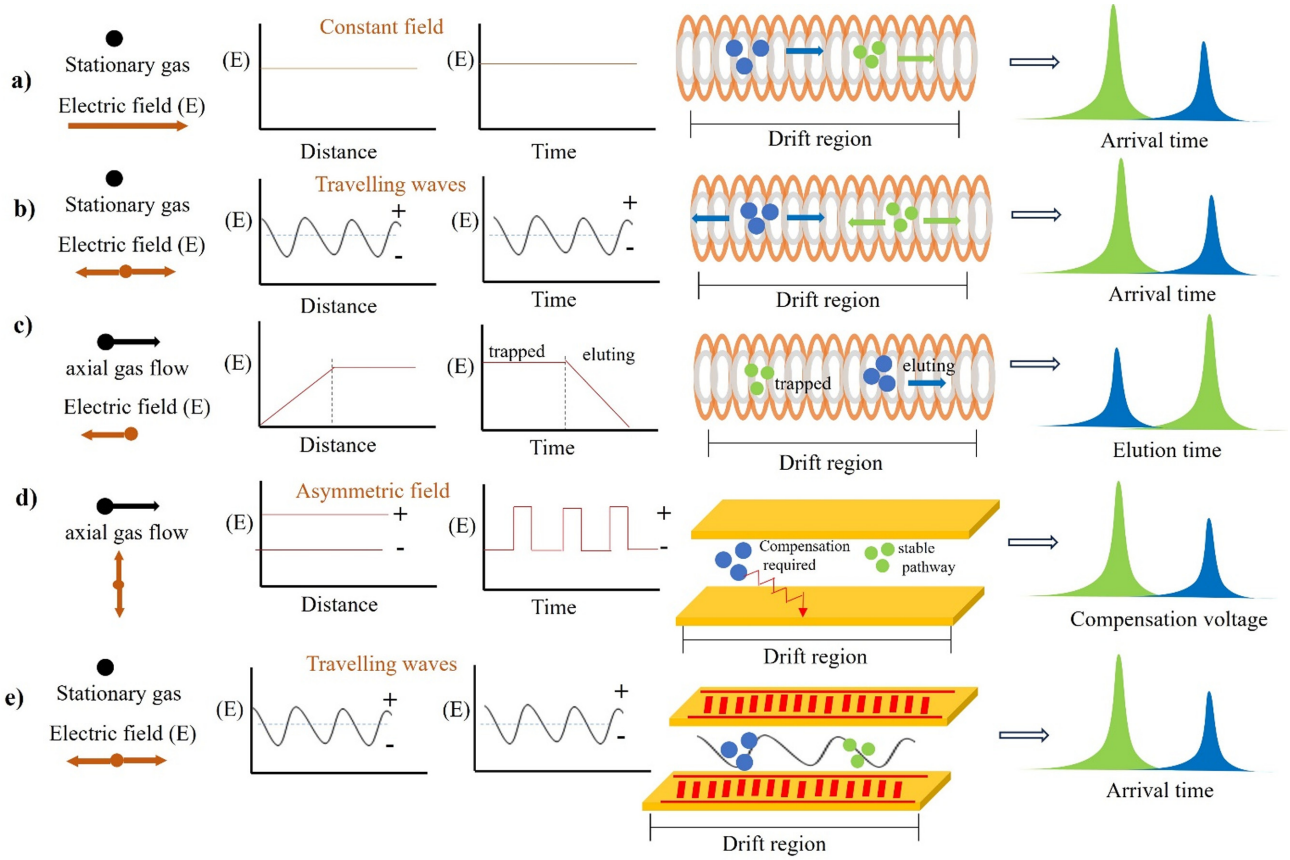

Figure 4. Schematic Illustration of a) DTIMS, b) TWIMS, c) TIMS, d) FAIMS, and e) SLIM systems.

ative charge that are frequently found in complex glycans found in higher animals.[116,117] An increasing amount of research links silica acid O-acetylation to several illnesses, such as infections, immunological problems, and cancer.[118-121] Additionally, it substantially decreases the rate at which certain endogenous human sialidases hydrolyze glycoconjugates, controlling turnover and degradation.[122] Furthermore, it can inhibit the function of bacterial sialidases, safeguarding the integrity of the epithelial mucus barrier in the process. It is still challenging to investigate the functions of different O-acetylated sialosides in health and disease, despite advancements.[122] One of the main obstacles is the absence of practical experimental methods to identify the precise structures of the sialic acid variations, such as the type of glycosidic bond and acetylation pattern. These molecules are difficult to isolate and characterize because they are chemically unstable and susceptible to acetyl ester movement and hydrolysis.[123] To clarify precise O-acetylation patterns and linkages between glycosidic types of sialosides isolated from complicated biological materials, Vos et al. (2023) employed the DTIMS technique.[123] The CCS measurements for diagnostic fragment ions were determined using a library of synthetic O-acetylated sialosides. O-acetylated sialosides from mucins and N-linked glycans from biologicals, as well as the nasal and tracheal tissues of horses, were all characterized using the CCS values.[124] The study revealed distinct sialic acid linkage patterns between acetylated and non-acetylated sialic acids and explained the sialic acid binding inclinations of H7 influenza A viruses that infect horses. The method can be applied to the structure analysis of N- and O-linked sialosides extracted from tissue samples and secreted mucins, as well as biotherapeutic examination for quality control. The discovery of sialylation sheds light on how this class of chemicals is biosynthesised.[124]

Travelling Wave Ion Mobility Spectrometry & Structures for lossless ion manipulations

Like DTIMS, Travelling Wave Ion Mobility Spectrometry (TWIMS) (Figure 4b) is a time-dispersive methodology. An additional component of the separation cell is a set of stacked-ring electrodes. Yet rather than using a linear electric field, TWIMS applies the electric field dynamically to achieve the creation of a moving wave, the ion separation of which is controlled by its magnitude and speed. More compact ions travel more quickly than extended ones because they experience less friction with the buffer gas, comparable to DTIMS.[125,126] TWIMS has generally been

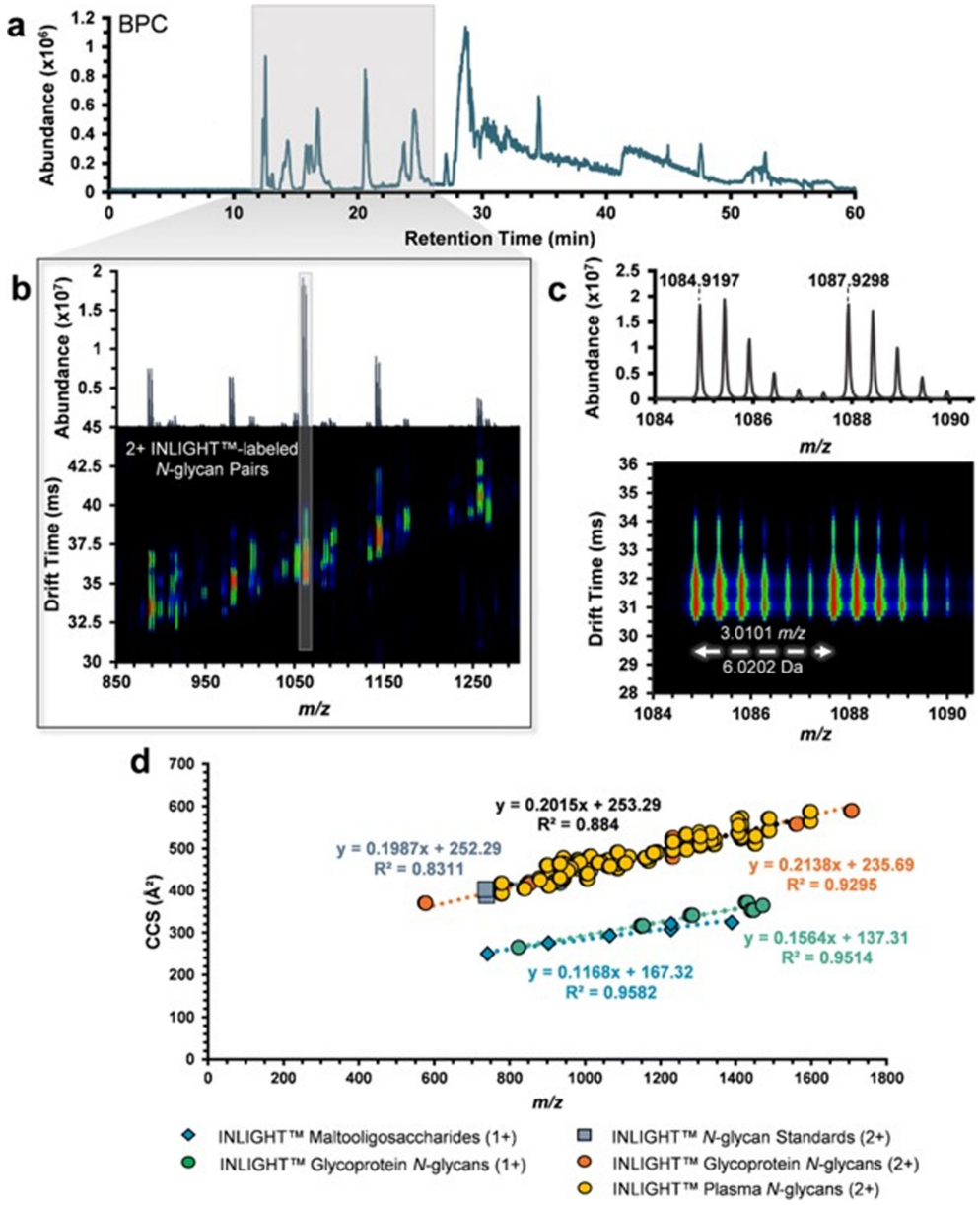

Figure 5. The base peak chromatogram for the nLC-IMS-MS analysis of INLIGHT™ derivatized N-linked glycans enzymatically released from human pooled plasma (**a**). Many drift-aligned peak pairs were observed between 11 and 26 min in the LC gradient (**b**). A zoomed-in view of one selected drift-aligned pair (**c**) illustrates a separation of 3.0101 m/z, consistent with a 2+ INLIGHT™ derivatized N-glycan. The identified features from plasma that displayed the distinctive isotopic distribution of being NAT- and SIL-labeled in an approximate 1:1 ratio and were drift time-aligned were then plotted on the m/z vs. Reproduced with permission at reference 114.

widely used as a separation method, but it can also be used for characterization because it can be used to determine CCS. However, in TWIMS, as opposed to DTIMS, the CCS computation requires calibration with reference standards. A procedure for the structural identification of drug metabolites using TWIMS was devised by Ross et al.[127] Through the examination of CCS and MS data, they were able to describe the metabolites of an array of medicines. They discovered that structural features of the parent medication also affect CCS alterations, in addition to the kind and location of the modification. TWIMS is also capable of achieving isomer separations. As an illustration, the successful separation of isomers of bile acid was accomplished.[127] A baseline separation cannot be achieved with the few variations in mobility among certain isomeric substances. To improve the resolution of ion mobility, Choui-

nard and colleagues employed the cyclodextrin adducts.[127]

While Structures for lossless ion manipulations (SLIM) also makes use of the traveling wave approach, it does so by drawing ions between parallel RF electrodes that are positioned in line with direct current guard electrodes along a predetermined extended path length (Figure 4e). With the disadvantage of reduced sensitivity, ions can travel several path lengths to improve drift resolution.[128,129]

Application with TWIMS & SLIM

It can be quite difficult to analyze sialylated glycans in depth, particularly when isomers need to be identified. Glycan analysis uses LC-MS and necessitates the glycans' enzymaticrrrrr release from a glycoprotein and converting it into a fluorescent tag derivative. Although LC-MS can distinguish and identify a significant number of common N-glycan structures, their exceedingly comparable MS/MS fragmentation patterns make it difficult and time-consuming to precisely assign α2,3- or α2,6-linkage isomers.[130,131] To determine the regiochemistry of the sialic acid bond, sequential digestion using several exoglycosidases can be used in addition to traditional LC-MS.[132,133] But this also results in a large increase in expenses and analysis time.

To examine the glycan structures generated from human alpha-1-acid glycoprotein (hAGP), Manz et al.[134] used HILIC with IM-MS. The unprocessed blend composed of highly sialylated multi-antennary glycans is separated by HILIC, glycan composition is determined by MS, and α2,6- and α2,3-linked sialic acid isomers are identified and quantified using IMS based on distinctive fragments.[134] The integration of both methodologies demonstrated itself to be an efficient technique in the N-glycan characterization process. It fully resolves all sialic acid linkage isomers for every glycan separately and is entirely compatible with N-glycan analysis procedures currently in use.[134]

Recently, Bansal et al. created an IMS-CID-IMS approach for glycan analysis that combines cryogenic infrared (IR) spectroscopy with SLIM.[135] Even the slightest structural variations between isomeric glycans can be distinguished by gas-phase IR spectroscopy, especially at cryogenic temperatures.[136] Utilizing mobility-selected first-generation fragments, the researchers were able to create second-generation fragments, divide them again based on mobility, and then measure their infrared fingerprint. The investigation allowed the researchers to determine the anomericity of the first-generation pieces in addition to their isomericity.[135] When there isn't a database match for the first-generation fragments, the IR fingerprint database approach is especially helpful because it uses a database comparison to identify the fragments.[135] Reconstructing the unknown predecessor glycan structure and adding entries from newly identified structures to the IR database are the two uses of the (IMS)3-IR technique. Unknown glycans should be able to be quickly structurally identified in their exact isomeric form using such a method.[135]

Field Asymmetric Waveform Ion Mobility Spectrometry

Field asymmetric waveform ion mobility spectrometry (FAIMS) is a space dispersive technique where separation happens on a spatial scale as opposed to a temporal one (Figure 4d). Although the basic working concept of FAIMS and differential mobility spectrometry (DMS) is similar, there are a few minor variations between the two methods because of the electrode shape. The separation voltage (SV), an asymmetric radiofrequency (RF) used by FAIMS, is applied orthogonally to the direction of ion flow. Ions are swept into the device by a carrier gas, and as the SV is adjusted from low to high fields, they bounce up and down between two parallel electrodes, like a quadrupole. A compensation voltage (CV) is given to permit a particular ion to flow via the parallel electrodes after ion mobility toward one of the electrodes is filtered. In the device, the chosen SV determines the best CV for a certain ion. Drift time variables are neither determined in an FAIMS device nor comparable to CV, nor is CV comparable to CCS. The combined action of the SV and CV allows the path length to be extended without sacrificing sensitivity, which gives this device an advantage over SLIM.[137-139] The method's filtering ability offers definite advantages in terms of getting rid of interferences and cutting down on background noise, which may result in a sensitivity gain. Even though FAIMS had no capacity to estimate CCS values,compensating voltage — which is unique to each analyte under specific circumstances — might occasionally be applied to the identification of metabolites.[140] Lastly, it's important to note that while FAIMS (or DMS) has primarily been employed for targeted analysis, there are several uses for this method in metabolomic analysis.[141,142]

Application with FAIMS/DMS

The complexity and variability of glycosylation limit the scope of site-specific glycoproteomics investigations. There is evidence that FAIMS expands the application of bottom-up proteomics. Variations in FAIMS mobility resulting from N-glycopeptides with various properties, such as glycan type, precursor *m/z*, and glycan size, were extensively studied in a recent study by Fang et al.[137] without or with the tandem mass tag label (TMT), researchers adjusted the FAIMS parameters for N-glycopeptide identification. The detection of site-specific N-glycopeptides produced from human lymphoma cells was greatly enhanced by the optimized FAIMS technique. It's noteworthy that FAIMS enhanced multiplexed N-glycopeptide quantification using both the Glyco-SPS-MS3 method and the conventional MS2 acquisition method. The greatest level of quantitative precision and accuracy was obtained by combining the Glyco-SPS-MS3 and FAIMS techniques. The advantages of FAIMS for enhanced mass spectrometry-based quantitative as well as qualitative N-glycoproteomics are shown by the results.[137]

Porphyromonas gingivalis is an oral infection that is pri-

marily related to severe periodontal disease. It is also linked to dementia, rheumatoid arthritis, cardiovascular disease, and some types of cancer. In this phylum, O-glycosylation has been documented, revealing information about the O-glycosylation motif and the structure of the O-glycans in certain species. O-glycosylation in P. gingivalis has not, however, been verified yet.[143] By using glycoproteomics techniques, such as partial deglycosylation with trifluoromethanesulfonic acid and HILIC and FAIMS-based glycopeptide enrichment procedures, Veith et al. were able to identify 257 potential glycosylation sites in 145 glycoproteins.[143] Many proteins that were permeable to the periplasm were found not to be O-glycosylated, in contrast to those that were exposed to the surface.[143] The O-glycans are comprised of glycerol phosphate with 0–2 acetyl groups and seven monosaccharides. These glycans need to be considered when producing proteins in heterologous organisms, as they have the potential to stabilize the containing proteins.[143]

Trapped Ion Mobility Spectrometry

Trapped ion mobility spectrometry (TIMS) (Figure 4c) employs an electric field to keep ions static towards a circulating gas, trapping them inside the drift cell. In these circumstances, the ions are split apart depending on the size-to-charge ratio using the same separation techniques as DTIMS. Ions can be eluted from high to low size-to-charge ratios when the electric field is gradually reduced following the separation stage.[144,145] Outstanding resolving capabilities can be achieved in a compact device because of the high linear gas velocities in the tunnel, which can cause the effective route length covered by ions during their elution to be orders of magnitude higher than the physical size of the TIMS tunnel.[146] While the direct measurement of CCS values is theoretically possible using TIMS, it is quite challenging to measure the gas velocity and pressure accurately enough. It is therefore better in practice to create calibration curves with ions of known CCS.[147] Lastly, it is vital to consider ion heating during extended trapping intervals while investigating labile species because radial focusing is accomplished by utilizing radiofrequency fields within the TIMS tunnel.

Application with TIMS

The existence of several gas-phase conformations for a single structure might complicate IMS-based isomer analysis. This can lead to the misconstrual of conformers for isomers, in addition to making isomer separation more challenging. Using gated-trapped ion mobility spectrometry (G-TIMS), Wei et al. investigated the ion mobility properties of several sets of isomeric glycans, both in their reduced and nonreduced forms, as their permethylated derivatives.[148] Researchers investigated the possibility of employing multiple sets of isomeric glycan standards to distinguish isomers from conformers using G-TIMS EED MS/MS. By examining N-glycans from chicken ovalbumin, the G-TIMS EED MS/MS technique's value for precise isomer identification from complicated glycan mixtures is illustrated.[148] Following G-TIMS separation, EED MS/MS investigation of isomeric glycan mixtures demonstrated that EED can produce very similar tandem MS spectra for conformers while generating isomer-specific fragments for isomers, allowing for the confident determination of isomers with little to no proof of uncertainty brought about by the existence of conformers.[148] When N-linked glycans were produced from ovalbumin, G-TIMS EED MS/MS evaluation revealed that some mobility traits that were thought to be the product of different isomeric configurations were in fact conformers of the same structure. Ultimately, examination of ovalbumin N-glycans from various sources has shown that the G-TIMS EED MS/MS method can reliably identify each isomeric structure and precisely identify batch-to-batch differences in glycosylation characteristics at the isomer level.[148]

The protective qualities of the mucus hydrogel are mostly dependent on the extensive O-glycosylation of mucins. Glycosylation gone awry is frequently linked to inflammation and diseases like Crohn's disease, cancer, and COPD. Glycans' innate complexity and the variety of O-core structures represent important obstacles to the study of O-glycans of the mucin type. Multiple isomers coexist, necessitating the use of multidimensional processes like LC-MS.[149] PGC is frequently utilized as a stationary phase to extract highly polar carbohydrates.[148,157] Workflows for LC-MS, however, take a long time and are not repeatable. Pagel et al. created a rapid replacement based on TIMS for recognizing and separating O-glycans released from mucins.[149] According to the results, mobilograms produced by standalone TIMS technology with properly adjusted mobility parameters resemble LC chromatograms regarding informational content and resolution. Apart from its quick separation capability, IMS offers direct structural data as a kind of CCS that can be applied to structural assignment in intricate omics investigations. The study demonstrated that high-throughput examination of O-glycosylation characteristics in clinical samples is made possible by the combination of a fast analytical time and diagnostic CCSs.[149]

Software for data evaluation in IMMS experiments

For IM–MS investigations, data analysis considers several factors, including drift time, processing, visualization, glycopeptide identification, fragmentation analysis, and quantification. Distinct drift times will be displayed by glycopeptides that differ in size, shape, and glycan composition. Identification and mass-based quantification of glycopeptides are made possible by the mass spectrometer's detection of ions with a *m/z* value. By cross-referencing empirical mass-spectrum results with a glycan database, glycan annotation facilitates the identification of glycans

Table 3. Software tool for glycosylation.

Software	IMS compatibility	Description	OS	Website	Ref
Byonic	N	Supports the de novo sequencing and hybrid integrated database methods for glycosylation site detection.	N	http://www.proteinmetrics.com/products/byonic/	155,156
GlycoMod	N	GlycoMod calculates every conceivable glycan composition.	Y	web.expasy.org/glycomod/	157,158
Glycopep Grader	Y	Establishes the composition of N-linked glycopeptides. demands that the user define a set of hypothetical glycans as well as the protein sequence.	N	http://glycopro.chem.ku.edu/GPGHome.php	159
PGlyco	Y	An analytical technique for glycoproteomics. It has benefits such site localization, precise glycan composition determination, high-throughput processing, analysis that is particular to glycosylation, and integration with current workflows.	N	pGlyco (pfind.org)	160
MaxQuant	Y	for the analysis of enormous collections of high-resolution mass spectrometry data.	Y	http://www.maxquant.org	161
GlycoPeptide-Search	Y	makes it possible to analyze various glycosylation patterns and makes data analysis easier. However, it needs a lot of processing power and depends on perhaps inadequate databases for identification.	Y	https://edwardslab.bmcb.georgetown.edu/trac/GlycoPeptideSearch/	162
Skyline	Y	provides targeted proteomics and metabolomics with quantitative data processing. The main benefit is the openness of the data evaluation and conclusion.	Y	Start Page: /home/software/Skyline	163

bound to peptides. It can help with glycopeptide characterization by assigning glycan compositions, structural information, and modifications.[150] Glycopeptide heterogeneity, isotopic distributions, and the existence of many charge states can all result in extremely complicated glycopeptide spectra. Software applications make use of deconvolution techniques to increase the precision of mass assignments, predict charge states, and resolve overlapping peaks.[150, 158] Databases with glycopeptide sequences and related glycan compositions are integrated with software tools. The program may identify possible glycopeptides and offer statistical parameters for identification upon contrasting the experimental data with the database. Additionally, software techniques decipher glycan fragment patterns to help explain glycan structure and linkage data. Software programs that visualize glycopeptide data enable qualitative analysis of IM-MS data by researchers. Heatmaps, spectral overlays for comparison studies, and drift time in comparison mass-to-charge ratio plots are a few examples of visualizations.[150]

Manual glycopeptide identification and quantification is time-consuming due to the microheterogeneity of glycosylation, particularly when dealing with a large cohort of diverse biological samples. Isomers that interfere with peptides that have the same sequence, but a different order of glycosylation modifications can exacerbate this problem. Most of the software packages that are now available are designed for N-glycopeptide analysis. In the meantime, there is a lack of clarity in the literature on a specific method for O-glycan structural interpretation. In conclusion, specific software tools enable deconvolution, glycan annotation, identification, and glycopeptide data interpretation in IM-MS investigations. These resources facilitate the analytical process, aid in the comprehension of intricate glycopeptide data by investigators and offer insightful information on the interpretation of glycan structures. A list of frequently used software packages for glycopeptide analysis is shown in Table 3.

Glycosylation site prediction tools

Technically complex, the experimental identification of glycosylation sites makes precise prediction of unknown glycosylation sites extremely valuable. To this end, computational algorithms developed based on glycoproteomics site-dependent knowledge would be quite helpful. The accuracy of predicting glycosylation sites has increased over the past few years due to developments in machine-learning algorithms (Table 4). Two categories of prediction techniques exist: Using a protein's sequence as the only input, sequence-based predictors i) systematically evaluate the significance of predicted structural attributes alongside sequence information, and ii) structure-based predictors that systematically evaluate the significance of structural characteristics, as in solvent accessibility and local secondary structure, along with sequence information.[151] The significance of structural characteristics in glycosylation site

Table 4. Glycosylation site prediction tool.

Software	Type	Description	Glycosylation	Website	Ref
Glycomine	Sequence based	A complete approach for systematically identifying the human proteome's C-, N-, and O-linked glycosylation sites by in silico analysis.	N, O, and C-glycosylation	http://www.structbioinfor.org/Lab/GlycoMine/	164
Glycomine^{struct}	Structure based	improved modeling of human N- and O-linked glycosylation sites with the use of two-step feature selection techniques combined with structural and sequence characteristics in an integrated computational framework.	N, and O glycosylation	http://glycomine.erc.monash.edu/Lab/GlycoMine_Struct/	165
SPRINT-Gly	Sequence based	SPRINT-Gly is a machine learning technique that uses several predictive models that have been trained to identify glycosylation sites in both human and mouse proteins.	N, and O glycosylation	http://sparks-lab.org/server/SPRINT-Gly/	166
GlycoPP	Sequence based	GlycoPP is a web server that analyzes bacterial protein sequences to anticipate possible N- and O-glycosites.	N, and O glycosylation	http://crdd.osdd.net/raghava/glycopp/	167
NGlycPred	Structure based	Protein structural characteristics are incorporated by NGlycPred for N-linked glycosylation prediction. Only N-linked glycosylation-site prediction is supported by NGlycPred.	N-glycosylation	https://bioinformatics.niaid.nih.gov/nglycpred/	168
NetNGlyc	Sequence based	The NetNglyc server uses artificial neural networks to analyze the sequence context of Asn-Xaa-Ser/Thr sequons to predict N-Glycosylation sites in human proteins.	N-glycosylation	http://www.cbs.dtu.dk/services/NetNGlyc/	168
NetOGlyc	Sequence based	Mammalian protein mucin type GalNAc O-glycosylation sites are predicted by neural networks using the NetOglyc website.	O-glycosylation	http://www.cbs.dtu.dk/services/NetOGlyc/	168

determination is methodically evaluated by NGlycPred and GlycoMinestruct. The small number of structures that have been solved experimentally has led to a decrease in the number of structure-based predictors. SPRINT-GLY conducted a thorough comparison and found that sequence-based methods are generally more accurate than structure-based methods.[151] SPRINT-GLY outperforms structure-based approaches in terms of performance, which emphasizes the value of utilizing a bigger training dataset and newly predicted structural features. These prediction methods supplement mass spectrometry methodologies in identifying distinctive sites of glycosylation by offering a site prediction probability.[152]

Conclusions

Glycoproteomics' immense structural diversity and complexity can be effectively characterized by IM-MS, which is still showing promise as an efficient method. Recent developments in IM-MS techniques and instrumentation have put this technology in a position to support breakthroughs in the glycosciences. Nonetheless, there are several issues that must be resolved.

The first challenge is unquestionably the growing amount of information that can be collected in a very short period, especially when LC techniques and IM-MS are combined. To assess the data and systematize glycan identification, new and improved technologies are required, particularly for high-throughput screening. Public access to the available CCS data is necessary for this. Unfortunately, many IM-MS data for carbohydrates are only published in individual papers, making it challenging to obtain all relevant information and consistently monitor the release of new data.

Additionally, to deliver higher ion mobility resolution and more accurate CCS readings, additional technological advancements in ion mobility instruments are required. Currently, a CCS's usual error is about 1%, and TWIM-MS devices likely have much greater error rates.[153] Higher resolution, in conjunction with more accurate and exact CCS determination, will eventually allow for the differentiation of isomers that are currently undetectable, opening the door to the investigation of increasingly complex samples.

The comparative analysis of substantial glycoproteomic data from challenging diseases such as cancer is hampered by several issues that persist despite advances in glycopeptide identification. First, the scoring techniques used by the current algorithms are based on a restricted set of annotated MS/MS spectra of glycopeptides and glycans, and they might not translate well to additional glycopeptides. Second, there may have been an overestimation of the false discovery rate (FDR) in the results due to the lack of systematic validation of the methodologies employed to determine the FDR in glycopeptide identifications. Third, several diseases or control samples are frequently used in large-scale human glycoproteomic studies, which produce many spectra of the same glycans and glycopeptides at different abundances. Algorithms that can improve and speed up glycopeptide detection by leveraging the redundant data in this cohort-based and related research are lacking. Finally, there is still a chance that some glycan isomers' baseline separation and overlapping of IM peaks will cause issues.

HPLC can continue to use online separation in conjunction with IMS installation.[154] It is reasonable to assume that IMS research and development will continue to be heavily focused on tackling the structural difficulties of carbohydrates, given the growing spectrum of analytical challenges that IMS methods are being used to address. Glycoproteomic analysis-related research questions will, in fact, be both excellent testing grounds and potent accelerators for new developments in IMS.

Never has the area of glycomics experienced so many obstacles and unanswered questions, but now more than ever, analytical techniques[181] for glycosyl structure characterization are available. Examining diagnostic pieces that originate from larger macromolecules is very helpful in quickly analyzing the structural characteristics of more complicated samples. To fully utilize IM-MS and streamline regular analysis, linked CCS and *m/z* information must be implemented in publicly accessible databases in the future. Although IM-MS undoubtedly won't provide answers to every unanswered question in the glycosciences, it will be essential to improving our comprehension of the function of complex oligosaccharides in biology.

Conflicts of interest

"There are no conflicts to declare".

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