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Recent advances in the mass spectrometric analysis of glycosphingolipidome – A review

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Abstract

Aberrant expression of glycosphingolipids has been implicated in a myriad of diseases, but our understanding of the structural diversity, spatial distribution, and biological function of this class of biomolecules remains limited. These challenges partly stem from a lack of sensitive tools that can detect, identify, and quantify glycosphingolipids at the molecular level. Mass spectrometry has emerged as a powerful tool poised to address most of these challenges. Here, we review the recent developments in analytical glycosphingolipidomics with an emphasis on sample preparation, mass spectrometry and tandem mass spectrometry-based structural characterization, label-free and labeling-based quantification. We also discuss the nomenclature of glycosphingolipids, and emerging technologies like ion mobility spectrometry in differentiation of glycosphingolipid isomers. The intrinsic advantages and shortcomings of each method are carefully critiqued in line with an individual's research goals. Finally, future perspectives on analytical sphingolipidomics are stated, including a need for novel and more sensitive methods in isomer separation, low abundance species detection, and profiling the spatial distribution of glycosphingolipid molecular species in cells and tissues using imaging mass spectrometry.

Graphical Abstract

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Declaration of interests

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1. Introduction

Glycosphingolipids are ubiquitous component of the cell membranes and membrane-bound subcellular organelles [1–3]. Large differences in physicochemical properties between the glycan head and the lipid tail — two substructures within glycosphingolipids make it challenging to analyze this class of lipids, as the methods developed for either the glycan or the lipid portion oftentimes do not work well at the whole molecular level. However, the biological functions of glycosphingolipids are determined by the lipid and the glycan collectively [4–6], which requires these molecules to be analyzed in their intact form.

Mass spectrometry has been instrumental in structural elucidation and quantitation of glycosphingolipids. However, prior to the appearance of current mass spectrometric techniques, glycosphingolipids are typically being analyzed by mass spectrometry with the glycan and lipid portion characterized separately [2, 7–11]. Innovations in mass spectrometry and chromatography have fueled analysis of glycosphingolipid into development of glycosphingolipidomics [1, 2, 12–15], where the glycosphingolipids can be analyzed intact while providing the sequence of the glycan and the composition of the lipid portion simultaneously.

This paper reviews the methods used in the contemporary mass spectrometric analysis of glycosphingolipids. We discuss the principles, advantages, and limitations of each analytical approach. Current challenges associated with the analysis of intact glycosphingolipids are also emphasized.

1.1 Structure

Glycosphingolipids are amphiphilic molecules composed of a ceramide backbone (hydrophobic) and a glycan head (hydrophilic) (Fig. 1A). The ceramide contains amino alcohol that is amide-linked to a fatty acyl chain, which can be very different from the fatty acyl compositions in glycerophospholipids. The fatty acyls in glycosphingolipids generally are long ($C_{16} - C_{26}$) monounsaturated or saturated, while those in phospholipids often have varied lengths, and either saturated or polyunsaturated. The amino alcohol is also called long-chain base or sphingoid base, a common one is d18:1, where the *d* stands for ‘*di*’ (two)

hydroxyl groups (one used in glycosidic linkage and the other as free hydroxyl group), 18 for the number of carbons, and 1 for the number of carbon-carbon double bond. Other sphingoid bases like d18:0, d20:0, d16:1, and d20:1 are also present in eukaryotes [1, 12, 13]. *Lyso*-glycosphingolipids are characterized by a lack of the *N*-fatty acyl chain [16], oftentimes as a result of degradation of glycosphingolipids by glycosidases [17].

Glycosphingolipids are classified as neutral (cerebrosides and globosides) or acidic (gangliosides and sulfatides) based on the glycan composition. Cerebrosides are composed of a hexose sugar, which can be either glucose (Glc) or galactose (Gal); globosides may have more than one neutral, monosaccharide units; gangliosides contain sialic acid, and sulfatides may have one or more carbohydrate-sulfate ester groups [18]. Two forms of sialic acid exist in humans, namely, *N*-acetylneuraminic (Neu5Ac) and *N*-glycolylneuraminic (Neu5Gc)[19]. The latter is present only in trace amounts and may originate from the diet [20].

Chemical modifications can also occur in the glycan and lipid moieties to further increase the structural diversity of glycosphingolipids. Acetylation and lactonization may occur on the glycans, and hydroxylation and unsaturation (carbon-carbon double bonds) on the *N*-fatty acyls [2, 21, 22]. Considering the high propensity of glycans to form different linkage isomers, along with the variation of the position and stereochemistry of double bonds in the lipid backbone, it is likely that the > 3,000 unique glycosphingolipids listed in the Lipid MAPS database [23] are an underestimate, as many glycosphingolipids remain undetected or uncharacterized [5].

1.2 Nomenclature

Symbols and abbreviations are traditionally being used for naming the most commonly occurring glycosphingolipids. For example, the ganglioside nomenclature proposed by Svennerholm in the 1960s remains widely used till today [24]. Here, gangliosides are abbreviated by two letters followed by a number, *e.g.*, in GM3 the *G* stands for gangliosides; M stands for '*mono*' which means 1 sialic acid; and the number 3 referring to the elution sequence in thin layer chromatography (TLC)[24]. Each abbreviation represents a clearly defined glycan structure, *i.e.* GM3 stands for the structure of Neu5Ac α 3Gal β 4GlcCer. However, the discovery of many more novel glycosphingolipids quickly overwhelms this system. To this end, IUPAC-IUB Joint Commission on Biochemical Nomenclature recommended in 1997 to use a name that includes linkage information of sugars and any modification present in the glycan headgroup. This nomenclature uses a set of pre-defined core structures – which detail the glycan composition and linkage – to serve as a base name [25]. For example, for neutral glycosphingolipids, one of the core structures is GalNAc β 1-4Gal β 1-4Glc β 1-1', also called Gg₃ (base name). A glycosphingolipid with this composition: GalNAc β 1-4Gal β 1-4Glc β 1-1' Cer d18:1/18:0, is named Gg₃Cer d18:1/18:0. If the glycosphingolipid contains sugars that extend beyond this core structure, they are put as a prefix to this name. The prefix contains: 1) Roman numeral to indicate the sugar where modification occurs; 2) a superscript on the Roman numeral to denote the linkage position; 3) the configuration of the linkage; and 4) the abbreviated name of the attached monosaccharide unit. In the same example, if sialic acid (Neu5Ac) is connected to the galactose position 3 with an α linkage configuration, the new sequence is: GalNAc β 1-

4(Neu5Ac α 1-3)Gal β 1-4Glc β 1-1Cer d18:1/18:0. The systematic name of this sequence becomes: II³- α -Neu5Ac-Gg₃Cer d18:1/18:0. In this notation, the Roman numeral indicates which glycan the Neu5Ac is connected to (second monosaccharide, *i.e.* Roman numeral II), the superscript and the Greek letter α indicate what type of linkage was used, in this case, α (1-3) linkage (*i.e.*, 3 and α , respectively, in II³- α -). The use of this system eliminates ambiguity in the chemical literature, especially when dealing with many glycosphingolipid isomers. However, this system does not provide a guideline on how to name the lipid part.

In 2005, the Lipid MAPS consortium proposed a comprehensive classification system and developed a structural database for biologically relevant lipids [23, 26, 27]. Both the system name and the common name are listed for each glycosphingolipid species, including the known long-chain bases and *N*-linked fatty acyls. As different mass spectrometric approaches provide different levels of molecular structural details, Liebisch *et al.* [28] in 2013 proposed a shorthand notation to practically annotate lipid structures derived from mass spectrometry data. Notably, for glycosphingolipids, the number of hydroxyl groups in the long-chain base should be listed, and if it is unknown, the sum of the long-chain base and *N*-linked fatty acyl should be given as the total number of carbons: total number of double bonds.

To demonstrate Liebisch *et al.*'s proposed nomenclature [28], consider the glycosphingolipid shown in Fig. 1A. This glycosphingolipid can be annotated in different ways (Note: Hex₂Cer should be used instead of LacCer if the monosaccharide identity is not known): 1) LacCer 36:2 (if double bond positions and stereochemistry are unknown and assuming a sphingoid base with two hydroxyl groups); 2) LacCer d36:2 (if long-chain base hydroxyl is known but double bond position, whether in the long-chain base or fatty acyl chain is unknown); 3) LacCer d18:1/18:1 (if the double bond can be ascertained to be in the long-chain base or fatty acyl chain); 4) LacCer d18:1(4E)/18:1(9Z) (if both position and stereochemistry or double bonds are known). This widely accepted proposal only covers the oligosaccharide headgroups of up to two monosaccharide units, the more complex glycosphingolipids were not covered. Given the simplicity of the Svennerholm system and the abbreviations in naming glycan headgroups and the practicality of shorthand notation to describe the lipid portion, a combination of these two would be convenient to annotate structures of complex glycosphingolipids derived from mass spectrometry data.

1.3 Biological significance

Glycosphingolipids can form high molecular weight micelles, segregate within the plasma membrane, and form lipid rafts and microdomains with cholesterol, sphingomyelin, and proteins [29]. In these domains, glycosphingolipids interact with receptors and modulate signal transduction. Their hydrophilic heads form a cluster on the cell surface called "glycosynapse" [29].

Table 1 summarizes some of the known functions of the headgroup and the lipid moiety. The glycan headgroup assumes many functions in biology [30, 31]. For example, it can serve as a receptor of viruses and bacteria [4, 32], or as an antigen in some autoimmune diseases [33]. The structure and composition of the glycan also play vital roles in protein-protein interactions, receptor regulation, adhesion, initiation/modulation of signal transduction,

trafficking, cell recognition and differentiation [4, 29, 32, 34, 35]. To name a few: a change in glycan structure perturbs antigenicity [36] and embryonic development [4, 32]; a high degree of sialylation of glycosphingolipids is also crucial in cancer and other diseases [37]; the negatively charged sulfate group in sulfatides is vital in the kidneys, the central nervous system [38], and in cardiovascular processes, as reviewed elsewhere [18].

The ceramide serves as an anchor to the cell membrane and modulates glycan traits [39, 40]. Desaturases introduce double bonds at specific positions in the fatty acyl chains [41]. The presence of double bonds and hydroxyls in ceramide can impact biological processes. For example, in the study of verotoxins (toxin from *E. coli*), the presence or absence of a carbon-carbon double bond affected the toxin binding to the Gb3 receptor, with the one containing the double bond having higher affinity than the one without [4, 42]. Lipid double bonds can also influence membrane order and signal transduction [4]. Both double bonds and length of the fatty acyl chain may affect spermatogenesis [43, 44] and retinal function [45]. The hydroxylation of fatty acyls enhances hydrogen bonding, which makes more rigid lipid rafts [46]. Interestingly, recent metabolic tracer studies showed that turnover rates of glycosphingolipids are related to the lipid backbone composition [47]. Taken together, this body of literature emphasizes that intact structure analysis can help advance our understanding of the role of glycosphingolipids in biology.

1.4 Glycosphingolipids as disease biomarkers and therapeutic targets

Glycosphingolipids play a role in many pathological conditions [3–6, 30, 48]. The composition or quantity of glycans may fluctuate in the course of disease progression and serve as potential disease biomarkers [49]. Many brain diseases have dysregulated metabolism of gangliosides and sulfatides [38, 50–52]. In lysosomal storage diseases, glycosphingolipids accumulate in cells and tissues because enzymes needed to metabolize them are either absent or weak [6]. Cancerous cells could be potentially differentiated from the healthy ones by their different glycosphingolipid profiles [53]. Dysregulation of glycosphingolipids have also been implicated in autoimmune, metabolic, and other complex diseases [53, 54].

Glycosphingolipids are also promising targets for immunotherapy because they can induce therapeutic cytokines and cell-mediated responses [55]. One such types of cells, invariant natural killer T cells (iNKT cells) recognize glycosphingolipids and release cytokines that kill cancer or virally-infected cells, making this a potential modality to treat cancer [56, 57] and infectious diseases [55]. Knowing the exact molecular structures and the involved pathways would further advance therapeutic applications of glycosphingolipids.

2. Analytical challenges

Within biological samples, glycosphingolipids co-present with phospholipids and are less abundant [58]. Higher levels of phospholipids interference with sample extraction and compete for ionization in mass spectrometric detection [59], which poses notable challenges for analysis of glycosphingolipids. For example in extraction, the Folch method uses methanol (CH₃OH) and chloroform (CHCl₃) (1:3, vol/vol) as the extraction solvent, which is effective in extracting the less complex global lipidome, but it is not effective for the more

complex glycosphingolipids like gangliosides, as the latter requires higher aqueous proportion. In general, no single extraction method can recover all glycosphingolipid classes completely. As such, the current global lipidomics pipeline that relies on a single extraction method (*e.g.*, Folch's) may fail to detect and interrogate these molecules.

Glycosphingolipids are also problematic for detection using either optical or mass spectrometric techniques. The lack of chromophores makes them difficult to detect using optical methods. Such chromophores could be introduced by derivatization (*e.g.*, 2-Aminobenzamide derivatization) to enable glycosphingolipid analysis using visible and fluorescence spectrophotometry. In mass spectrometry, heavily glycosylated glycosphingolipids suffer from low ionization efficiency, partly because of the decreased hydrophobicity and volatility [60, 61]. If present, labile groups such as sialic acid or fucose, further exacerbate this issue. These structures may be cleaved off during ionization and may be overlooked if the method is not tailored to these labile groups.

The presence of many structural variants often yields ambiguous results if not considered early in the method development pipeline. It is important to distinguish isomers because they may have distinct biological functions. Structural variants may result from the headgroup and the lipid tail. Headgroup variants involve molecules that differ in connectivity (positional isomers) or orientation in space (stereoisomers). In gangliosides, positional isomers may result from varied placement of sialic acid. Examples include: disialogangliosides (GD1a, GD1b, and GD1c) (Fig. 2A); monosialogangliosides (GM1a and GM1b); and trisialogangliosides (GT1a, GT1b, and GT1c) [21]. Positional isomers can also result from varying connectivity in the hexosyl ring. The following are some notable examples: 1) Gb3 and iGb3 isomers differ in the connectivity of terminal Gal to the second hexosyl [62] (Fig. 2B); 2) the sialic acid linkage position on the hexosyl ring can also be either α 2–3 or α 2–6 (Fig. 2C)[63]. These positional isomers are very important in virology, mounting evidence suggested that different sialic linkages in protein-linked glycans could dictate the degree of viral entry to host [64, 65]. A method dubbed as sialic acid linkage specific alkylamidation (SALSA) derivatization has been recently developed to distinguish different sialic linkages in glycosphingolipids [66]. Stereoisomerism in the glycan can result from a difference in the configuration of bonds in the hexosyl ring. The best example of stereoisomers is GlcCer and GalCer, which only differ in the orientation of the hydroxyl group at the C-4 position (Fig. 1A).

On the other hand, the presence of a hydroxyl group and carbon-carbon double bonds in *N*-fatty acyls yields isomeric and isobaric species (Fig. 2D). For example, the following species are both isobaric and isomeric: GalCer d18:1(*n*-14)/23:1(*n*-9)(OH), GalCer d18:1(*n*-14)/23:1(*n*-7)(OH), and GalCer d18:1/24:0, *i.e.*, all have a nominal mass of 811 Da; two of them only differ in the double bond position of *n*-7 *vs.* *n*-9 [67]. The identification of these variants is difficult using traditional mass spectrometry techniques. Newer methods attempted to address these issues [66, 68–72], but their wide adoption remains limited.

Finally, a lack of commercially available standards obstructs the analysis of glycosphingolipids. To date, a few standards derived from natural sources (bovine or porcine brain) are available for gangliosides (GM1, GM2, GM3, GD1a, GD1b, GD2, GT1, and

GQ1b), and neutral glycosphingolipids like cerebrosides and globosides (up to four sugar units). Most isotopically labeled standards are deuterated. However, the ^{13}C -labeled standards [73] are more desirable for LC-MS based analysis because it ensures coelution of internal standards and target analytes in reversed-phase chromatography. No standards are available for the *O*-acetylated, lactonized, and sulfated complex glycosphingolipids that could greatly facilitate studies of these rare molecules.

3. Sample preparation

Various types of samples have been used for analyses of glycosphingolipids depending on the analytical goal and sample availability. Because of the uniqueness of each tissue or biofluid and the different sample matrixes associated with it, various methods have been developed to extract glycosphingolipids using either liquid-liquid or solid phase extraction (SPE) approaches or to enrich from complex sample matrix utilizing chemical or enzymatic modifications.

3.1 Type of samples

Glycosphingolipids exist in all tissue, organ and biofluid [3]. The tissues obtained from biopsy or post-mortem samples are widely used for immunohistochemical staining to identify the distribution of glycosphingolipids [74]. The rat brain tissue [20, 22, 71, 75–81] is often used as a model to demonstrate new analytical methods [20, 82, 83]. An appreciable amount of glycosphingolipids have been found in the following: lung [84], heart [85], intestines [86–88], kidneys [74, 89, 90], retina [91], ovarian cells [92] [93], breast cancer stem cells [94–97], fetal tissues [98, 99], and cerebrospinal fluids [100–102]. Blood and urine are suitable for biomarker studies because 1) they can be obtained quite easily in a less invasive procedure, and 2) during pathologic states, some of the glycosphingolipids could be shed-off from the surface of diseased tissues or cells and end up in blood circulation [103]. Urine could also reflect the pathologic state of specific organs such as the kidneys [104]. Recently, dried blood spots have also been used, especially in the screening of metabolic errors in infants [105].

3.2 Extraction

In biological matrix, glycosphingolipids co-exist with major lipids and biopolymers (proteins and nucleic acids). These molecules can suppress ionization of glycosphingolipids in mass spectrometric analysis and therefore must be removed during sample preparation [106]. Extraction is a common procedure to remove the interfering matrix molecules. Prior to extraction, homogenization is performed for tissues or cells; in the case of biological fluids, this step is not necessary, but the removal of suspended particles is needed (Fig. 3). Sample amounts are study dependent, and the following starting quantities are commonly used: 1.0 to 500 mg for tissues, at least 10^6 counts for cells, and 1.0 to 100 μL for biological fluids.

In lipidomics studies, total lipids are typically extracted using $\text{CHCl}_3/\text{CH}_3\text{OH}$ (2:1) in the Folch method [107], $\text{CHCl}_3/\text{CH}_3\text{OH}$ (1:2) in the Bligh and Dyer method [108], or 1-butanol/ CH_3OH (3:1) in the butanol-methanol (BUME) method [109]. Addition of CH_3OH

disrupts the interactions between lipids and biopolymers so the lipids can be dissolved in the organic phase more effectively. However, these methods are not very effective in recovery of the amphiphilic glycosphingolipids. Alternatively, the lower phase of the two-layer isopropanol: hexane: H₂O (55:25:20, v/v) solvent system is used before and after CHCl₃/CH₃OH (1:1, v/v) extractions to further increase the recovery of complex glycosphingolipids [110]. The crude extract is dried and partitioned with CHCl₃/CH₃OH/H₂O (30:60:8). The resultant CHCl₃ lower phase contains phospholipids, cholesterol, and the more hydrophobic glycosphingolipids, *i.e.*, cerebroside and sulfatides. The upper phase contains complex neutral glycosphingolipids and gangliosides. Proteins and other debris remain in the interface. Cerebroside and sulfatides can be recovered from the lower layer *via* optional peracetylation, followed by silicic acid column chromatography and deacetylation (Fig. 3b) [111, 112]. The impurities can be removed efficiently using acetylation/deacetylation, but this step destroys *O*-acetyl groups [111]. In silicic acid chromatography, phospholipids are retained on the column, while acetylated glycosphingolipids are eluted out using CHCl₃/CH₃OH gradient. Acidic and neutral glycosphingolipids can be separated using a weak anion exchange column, diethylaminoethane (DEAE)-Sephadex A25. Resultant glycosphingolipids fractions are purified by C18 or C8 SPE or dialysis using a membrane with 3 kDa molecular weight cutoff [112]. SPE cartridges of aminopropyl and silica gel packing materials can also be used to fractionate crude lipid extracts. Aminopropyl SPE cartridge has been used to isolate cerebroside and globoside using acetone – methanol (9:1.35 v/v) solvent system [113]. However, this approach cannot separate sulfatides from sphingosine-1-phosphate and ceramide-1-phosphate. Silica gel SPE cartridge has been used to enrich sulfatides from crude extracts [114]. Titanium dioxide (TiO₂) is also useful for glycosphingolipids [115]. More recently, selective phospholipid removal during ganglioside extraction was accomplished using Phree™ columns [116].

Gangliosides and sulfatides can be selectively extracted using simple procedures. Svennerholm and Fredman's method [117] can be used for gangliosides. This method uses a higher proportion of polar solvents (methanol and water) to increase extraction efficiency since complex gangliosides are relatively more soluble in water. Svennerholm and Thorin's [118] and Hara and Radin's [119] methods are useful for sulfatides; the latter is CHCl₃-free, which makes it a safer alternative. For dried blood spot samples, sulfatides can be efficiently extracted using methanol or isopropanol after being incubated with water [120]. Methods involving non-ionic detergent called hexaethyleneglycol mono-*n*-tetradecyl ether [121] and monophasic extraction [106] have also been described. Alternatively, preparative thin-layer chromatography (TLC) can be used to isolate specific classes of glycosphingolipids [122, 123]. Isolation is done by developing a TLC plate in an appropriate solvent system, followed by detection using reversible stains such as primuline, and then scraping the spots of interest prior to extraction using CHCl₃/CH₃OH.

On the other hand, glycosphingolipids are relatively resistant to alkaline hydrolysis compared to other ester-linked lipids. As such, alkaline hydrolysis can be used to eliminate major lipids such as phospholipids, and glycerides using dilute sodium hydroxide or potassium hydroxide in the presence of methanol [12, 124]. The resultant lower analytical background significantly improved the sensitivity of LC-MS/MS analysis of permethylated glycosphingolipids [125].

3.3 Novel enrichment strategies

The common enrichment strategies generally require biomolecules having functional groups/ reactive sites, such as aldehyde, ketone, or free amine. These groups are lacking in glycosphingolipids but could be selectively generated by chemical or enzymatic modifications (Fig. 4). For chemical-based oxidation, solution-phase ozonolysis can be used to cleave the carbon-carbon double bond on the sphingosine backbone to introduce an aldehyde in the lipid. This method also cleaves any carbon-carbon double bonds in the *N*-fatty acyl, if present, providing more than one reactive site [126]. Another very selective way to introduce an aldehyde is through oxidation of the trihydroxy functional group in sialic acids using dilute sodium meta periodate (NaIO_4) [127]. Other chemical oxidation approaches include oxidation of the sphingosine hydroxy group to allyl ketone using 2,3-dichloro-5,6-dicyano-1,4-benzoquinone (DDQ) [128]. More recently, the oxidative release of the natural glycans (ORNG) method was used to oxidize the ceramide backbone of glycosphingolipids using sodium hypochlorite (NaOCl) [129]. The resultant nitrile can be reduced to an amine and subsequently used for other chemistries.

Enzymes such as endoglycoceramidase (EGCase) [103, 130, 131], or sphingolipid ceramide *N*-deacylase (SCDase) [132, 133] can also be used to add reactive sites to glycosphingolipids. EGCase cleaves the glycosidic bond between the ceramide and the glycan moiety to form the oligosaccharide with a free reducing end (aldehyde form). SCDase cleaves the amide bond releasing the fatty acyl chain while leaving behind a free amino group.

Glycoblotting can be used to enrich glycans released from glycoproteins [134] and glycosphingolipids [103, 135] to improve sensitivity in mass spectrometry [126]. Here, an appropriate glycan release strategy introduces an aldehyde group (Fig. 4). The aldehyde forms a covalent bond *via* reduction with the amino groups on the beads, typically hydrazide. The excess hydrazide groups are blocked by esterification, and the glycans are then recovered by transamination, and collected as an aqueous solution (Fig. 5). Another approach is to immobilize the glycosphingolipids on the polyvinylidene difluoride (PVDF) membrane, followed by EGCase treatment [130]. The hydrophobic PVDF facilitates immobilization of glycosphingolipids through interaction between the PVDF surface and the ceramide backbone, so that the glycan portion is adequately exposed to EGCase. This technique was successfully applied to analysis of glycosphingolipids in ovarian cancer cells and tissues.

4. Common ionization methods

Mass spectrometry has emerged as an essential tool to structurally characterize and quantitatively profile glycosphingolipids [7, 10, 11, 136–138]. One of the keys to a successful mass spectrometric analysis is the choice of the appropriate ionization method, which is both sample and study dependent. In this section, we discuss the soft ionization techniques commonly used for glycosphingolipid analysis.

4.1 Electrospray ionization (ESI)

ESI is the most commonly used method for ionization of biomolecules, particularly when front end chromatographic separation is coupled online to mass spectrometric detection. In ESI, glycosphingolipids are ionized by electrospray (typically, 1–3 kV), and form $[M + \text{alkaline}]^+$, $[M + H]^+$, or $[M + \text{NH}_4]^+$ adducts in the positive mode, and $[M - H]^-$, $[M + \text{HCOOH} - H]^-$ or $[M + \text{halide}]^-$ in the negative mode, depending on the composition of the solvent used to dissolve the sample. The most common form is $[M + \text{Na}]^+$ because sodium ion is ubiquitous, and glycosylated molecules have a high affinity toward sodium ion. This is especially the case when sodium formate is used to calibrate mass spectrometers, and no lengthy flushing of the ESI source is made before infusing samples. When added in excess, acetic acid or formic acid overwhelms sodium ions and favors $[M + H]^+$ ions [139, 140]. In most cases, neutral glycosphingolipids are detected as $[M + \text{NH}_4]^+$, $[M + H]^+$, $[M + \text{Na}]^+$ or $[M + \text{Li}]^+$ (when a source of lithium ion, such as lithium chloride is introduced) while acidic glycosphingolipids are detected mostly as $[M - H]^-$, $[M + \text{HCOOH} - H]^-$ or $[M + \text{halide}]^-$ ions. Gangliosides with more than one sialic acids form multiply charged species as $[M - 2H]^{2-}$ and $[M - 3H]^{3-}$ [141]. Besides spiking the LC mobile phase with ammonium formate, sodium ion or lithium ions can be infused post-LC using a T-connector [142]. The use of different adducts could impact fragmentation behaviors. For example, fragmentation of $[M + \text{Na}]^+$ but not $[M + H]^+$, can be used to differentiate GlcCer and GalCer [72]. In ozone-induced dissociation (discussed in Section 5.6), $[M + H]^+$, $[M + \text{Na}]^+$, and $[M + \text{Li}]^+$ can reveal distinct structural information. These suggest that early on in an analytical method development, it is important to consider appropriate solvent and select appropriate adducts for downstream analysis.

4.2 Matrix-assisted laser desorption ionization (MALDI)

Besides ESI, MALDI is another commonly used soft ionization technique in biomolecular analysis, which unlike ESI, typically generates singly charged molecular ions. This ionization method starts with sample co-crystallized with an Ultraviolet (UV)-absorbing organic acid matrix on a metallic plate, and then irradiated by laser to generate ions [143]. MALDI was first applied to glycosphingolipids by Costello and colleagues (for a comprehensive review, see Ref. [144–147]). Common matrices used for glycosphingolipids include 2,5-Dihydroxybenzoic acid (DHB), 1,5-Diaminonaphthalene, 4-Hydrazinobenzoic acid, and 6-Aza-2-thiothymine [144]. Selective ionization of sulfatides can be achieved using 9-Aminoacridine (9-AA) [148]. MALDI can operate in positive and negative ionization modes to yield $[M + \text{Na}]^+$ and $[M - H]^-$ ions, respectively. The negative ions are more sensitive and provide richer fragmentation for gangliosides [144]. In contrast to ESI, MALDI is more tolerant to salts and can ionize even the higher mass glycosylated lipids, but they generally have lower ionization efficiency.

Poor choice of a matrix can result in potential post-source decay of glycosphingolipids, which results in prominent decarboxylation reactions and loss of sialic acid moiety, especially for gangliosides. Infrared lasers and collisional cooling have been introduced to address this issue [149, 150]. Alternatively, gangliosides are often permethylated [151] to increase the stability of sialic acids while improving their overall hydrophobicity, volatility, and ionization efficiency. MALDI-MS suffers from high background noise due to matrix

clusters that preclude the detection of low molecular weight glycosphingolipids [152]. Newer methods, although matrix-free, have not yet been used for glycosphingolipids [147]. Without prior off-line chromatographic fractionation, MALDI-MS cannot resolve isomers and is prone to ion suppression effects, especially in biological samples. To resolve this, MALDI-MS has been coupled to ion mobility spectrometry but not yet used for glycosphingolipids [153].

Sample spotting on the MALDI plate can be tricky, which may result in uneven sample deposits and variable ion intensities. This issue has been addressed using a normal phase nano-HPLC coupled with an automated robotic spotter; the developed method has been used to analyze a mixture of acidic and neutral glycosphingolipids [154]. Briefly, the samples were separated using normal phase nano-HPLC, and the eluting compounds were precisely collected on the MALDI plates and co-crystallized with matrix [154]. This method gave homogeneous spots; however, it is quite tedious and may be impractical for routine, high throughput applications.

The use of MALDI-MS for quantitation of glycosphingolipids remains challenging, until recently, when quantitative MALDI-TOF MS/MS was demonstrated [103]. After endoglycanase digestion and glycoblotting to prepare the sample, a total of 42 distinct glycosphingolipids were detected in human serum at a concentration of 12.1 to 21.4 μM ; this is lower than those of *N*-linked glycans which were 700 to 850 μM [103]. However, the distributions, both spatial and anatomical, of glycosphingolipids in tissues, are mostly unknown. MALDI-MS is an emerging tool for imaging (also called MALDI imaging mass spectrometry) that can provide both molecular and spatial distributions of analytes [75, 155–160]. The use of MALDI imaging mass spectrometry may unearth previously unknown biology of glycosphingolipids. The reader is referred to a comprehensive review on this subject [161] and we will not elaborate here.

5. MS/MS fragmentation

Tandem mass spectrometry (MS/MS) is essential in structural elucidation of glycosphingolipids [7], which is relying on multiple ion dissociation (fragmentation) techniques that can be broadly classified into four categories: 1) collision-induced; 2) electron-aided; 3) photoinduced; and 4) chemical-induced dissociations. Each dissociation method cleaves bonds at different locations of the molecule and the combined use of these methods can provide complementary and comprehensive structural details of glycosphingolipids. Based on the predictability of fragmentation pathways, structural databases could be constructed *in silico* to identify both the known and unknown glycosphingolipids by matching experimental MS/MS spectra with the database.

5.1 Nomenclature of fragments

The systematic naming of MS/MS fragments was proposed by Domon and Costello [162] and later modified by Ann and Adams to include more detailed lipid backbone fragments [163]. The fragments are labeled based on whether the fragment contains the non-reducing end (A, B, and C) or the reducing end (X, Y, and Z) (Fig. 6). Fragments B, C, Y, and Z correspond to glycosidic bond cleavages that determine carbohydrate sequence, while A and

X are cross-ring cleavages that mark linkage positions. The superscripts in cross-ring cleavages denote which bond is fragmented, for example, cleavage of the bonds between C1-C2 and C5-O forms the $^{1,5}X$ ion. The ceramide fragment ions include E, S, T, U, and O'' which result from the cleavage of glycosidic bond between the ceramide and the glycan. Among them, the major fragment ions are the O'' and E. The O'' fragment is diagnostic of the long-chain base composition (Fig. 6B).

5.2 Collision-induced dissociation

Collision-induced dissociation is the bread and butter of all dissociation techniques and the default fragmentation technique for MS/MS in modern mass spectrometers. It includes low energy collision-induced dissociation (CID) and higher-energy collisional dissociation (HCD), with the latter uniquely on orbitrap instruments. The CID fragmentation of neutral and acidic glycosphingolipids has been extensively reviewed [7].

Both CID and HCD provide glycan and lipid structural information. The B and C type ions aid in elucidating the glycan sequence. Incremental mass differences of 162 Da and 204 Da indicate the loss of a hexose and *N*-acetylhexosamine, respectively. Sialic acid and sulfate groups can be confirmed using m/z 290 and m/z 97, respectively, in the negative mode. The lipid composition can be identified from the following in positive mode: m/z 266 (d18:0), m/z 264 (d18:1), m/z 236 (d16:1), and m/z 292 (d20:1) [9]. These ions are characteristic in targeted tandem mass spectrometric methods for the known and are also very useful in structural elucidation of novel glycosphingolipids when combined with accurate mass measurement [140, 164, 165].

One caveat of CID-MS/MS is that it cannot distinguish isomers with subtle structural differences even having chromatographic separation in the front end. Multi-stage collisional dissociation is more capable in this respect. For example, globo- and isoglobo-glycosphingolipids can be differentiated in cell extracts using MS⁵ experiments [62].

5.3 Electron-aided dissociation

Electron-aided dissociation involves two general steps: 1) precursor ions are converted to radical ions; and 2) the metastable radical ions dissociate. Thorough discussion on electron-aided dissociation is provided in a recent review [166]. While sharing similar strategy in ion dissociation, electron capture dissociation (ECD), electron detachment dissociation (EDD), and electron transfer dissociation (ETD) differ in how radical ions are formed. In ECD, multiply charged positive ions pass through a beam of low energy electrons, and capture the electrons to form radical cations. EDD operates just like ECD but applies to multiply charged negative precursors instead. Both have been implemented mainly in FTICR instruments, but attempts have been made to integrate them into the more accessible instruments such as orbitrap, which would allow greater access to the scientific community [167]. EDD is relatively inefficient for glycosphingolipids, while ECD provides extensive fragmentation that could aid structural elucidation.

In ETD, the precursor ions react with radical anions generated *in situ*, e.g., fluoranthene [166]. The reaction generates odd and even electron fragments, and ions with a lower charge state. This was used initially in peptide sequencing [168] but was shown to work equally

well for oligosaccharides. ETD yields ceramide backbone fragments and identifies acetylated glycans [169]. To our knowledge, ETD has not been applied to glycosphingolipids, probably because most of these molecules seldom form multiply charged positive ions, a prerequisite for using this technique.

5.4 Ultraviolet photodissociation

Photoinduced dissociation uses energetic photons to activate and fragment chemical bonds. Ultraviolet photodissociation (UVPD) uses UV light to irradiate precursor ions in a collision cell [170]. The typical wavelengths used are 266 nm, 213 nm, 193 nm, and 156 nm, depending on the application. The 193 nm has been shown to provide extensive fragmentation of both the glycan headgroup and the lipid backbone in gangliosides and neutral glycosphingolipids [171]. While CID and HCD produce only B/Y type and C/Z type ions (Fig. 7), UVPD provides more informative cross-ring fragments (A/X types) to differentiate isomeric species [171]. This approach can also provide some level of fragmentation adjacent to the carbon-carbon double bonds to pinpoint their location, albeit at lower efficiency [172]. Being the dissociation technique generated the most comprehensive, structurally-informative fragmentation ions for glycosphingolipid analysis, this method, however, lacks wide adoption by the community partly due to the lack of software for automated spectral annotation.

5.5 Radical directed dissociation

Radical directed dissociation (RDD) is unique in that it combines the principles of electron-aided activation, collisional dissociation, and photoinduced dissociation. RDD involves three steps: 1) derivatization to introduce chromophores in the molecule; 2) generation of radical ion using UV, and 3) fragmentation of the radical ion using CID or HCD. RDD requires attachment of chromophores with labile bonds, *e.g.*, carbon-iodine bonds that break *via* homolytic cleavage to form radicals. These radicals abstract protons from the analyte ions to form a radical cation before being fragmented using CID.

RDD was used by Pham and Julian to differentiate the glycosphingolipid epimers (GlcCer and GalCer) [69]. Briefly, *lyso*-glycosphingolipids were prepared from intact glycosphingolipids by removal of the N-fatty acyl chain using alkaline hydrolysis in aqueous solution. The product was non-covalently complexed with a molecule that contains a labile carbon-iodine bond [4-iodobenzoyl 18-crown-6 (IB18C6)], where the free amino group in the *lyso*- species served as an anchor for IB18C6. Photodissociation (PD) using 266 nm UV laser cleaved the carbon-iodine bond to form a radical ion. The radical ion was mass-selected for CID fragmentation to give the RDD (PD followed by CID) spectra (Fig. 8). The two epimers gave the same major fragment ions (*i.e.*, m/z 443 and m/z 250) but inverted abundance of the fragments. The difference in abundance was attributed to the differential ability of the epimers to lose water during fragmentation (neutral loss of water is easier in galactose than in glucose) which in turn depends on the configuration of the -OH group at the C-4 position. This approach could potentially be used to distinguish GalCer and GlcCer, but wide application to analysis of other glycosphingolipids would likely be limited due to the complicated sample preparation and ion dissociation processes involved.

5.6 Ozone-induced dissociation

Ozone induced dissociation (OzID) is based on alkene and ozone (O_3) reaction chemistry [173]. Historically, this approach was used to elucidate double bond locations in lipids using gas chromatography. In 2008, Blanksby's group implemented this reaction inside a mass spectrometer by replacing collision gas with O_3 and O_2 mixture [174]. In this reaction, O_3 forms ozonide through a cycloaddition reaction; the metastable ozonide spontaneously decays to the more stable Criegee and aldehyde product ions, which can indicate position of the double bond [173].

Recently, we implemented OzID in a high-resolution mass spectrometer with enhanced ozonolysis efficiency [175]. We studied unsaturated glycosphingolipids, for example, LacCer d18:1(4E)/18:1(9Z) (Fig. 9). OzID fragmentation of the $[M+Na]^+$ adduct gave two pairs of products (Criegee and aldehyde) corresponding to each double bond [67]. The mass difference between OzID products and the precursor ion was used to pinpoint the double bond position [173]. We were able to differentiate isobars and isomers in porcine brain galactocerebrosides mixture, which were non-distinguishable by CID alone [67]. The differential reactivity of the long-chain base double bond and the fatty acyl double bond was very useful in assigning which acyl chain contains the double bond. We also found that the OzID fragmentation behavior of glycosphingolipids could be dictated by the adduct type: $[M+Li]^+$ and $[M+Na]^+$ fragment mainly at the double bond position; $[M+H]^+$ undergoes dehydration followed by CID-like loss of the glycan head group [176].

Taken together, the various fragmentation techniques discussed here require different instrumental setup and provide distinct levels of structural information. Depending on the study aims, necessary information like glycan sequence, lipid composition can be obtained from CID and HCD. More detailed structural features like branching and linkage in the glycan and double bond position in the lipid can be determined using more specialized techniques (ECD, EDD, UVPD, RDD, and OzID).

6. Common operating modes of tandem mass spectrometry

CID-MS/MS generates fragment ions diagnostic of the glycan composition, long-chain base, or *N*-fatty acyls (see Section 5.2). For instance, m/z 264 is characteristic of the long-chain base d18:1 (sphingosine backbone) (Fig. 6b). Sulfatides and gangliosides yield m/z 97 and m/z 290 attributed to the sulfate and sialic acid moieties, respectively. When measured with high resolution mass spectrometers such as Fourier transform ion cyclotron resonance (FTICR), time-of-flight (TOF), or orbitrap that are capable of distinguishing isobaric species and allowing the assignment of elemental compositions [177], these characteristic fragment ions and the corresponding precursor ions permit identification of glycosphingolipids in the untargeted workflows and differentiation of ganglioside positional isomers. In this respect, high resolution untargeted MS/MS, when combined with HILIC-based separation, raises the total number of ganglioside species identified to 145 in a diverse set of tissues [178].

The characteristic fragment ions, however, are more oftenly used in the low resolution triple quadrupole mass spectrometers for targeted analysis of glycosphingolipids. This can be achieved through different operating modes of MS/MS, namely, precursor ion scan and

multiple reaction monitoring (MRM) (Fig. 10). In the precursor ion scan, all mass-selected ions from the source are fragmented, and a diagnostic ion is monitored. For example, the precursor ion scan has been used in ganglioside analysis, where all ions were scanned for the presence of m/z 290 fragment [179]. In MRM, specific precursor-product ion pairs are monitored for improved sensitivity and specificity. A sensitive MRM method has also been developed for comprehensive quantification of various classes of gangliosides based on the sialic acid-specific ion of m/z 290 and for further differentiation of isomeric glycosphingolipids based on diagnostic ions unique to the terminal regions of the sugar chains [141, 180]. Similar approach has been applied to measure Gb3 in plasma and urine of Fabry disease patients by monitoring the loss of m/z 162 [181].

One feature of MS/MS in orbitrap-based instruments is parallel reaction monitoring (PRM). Unlike MRM that uses only predefined MS/MS transitions without preserving information for all fragment ions in the MS² scan, PRM provides both precursor ion information (MS¹) and all generated ions during MS/MS, thus providing a wealth of data for identification and quantitation. This was applied for in-depth profiling of sphingolipids [165], and for quantitative analysis of gangliosides after isobaric labeling [140], which will be detailed below (section 8.3).

7. Absolute quantification

Omic level quantification of biomolecules is often conducted relatively, i.e. comparing the changes of a molecules in one group of samples (e.g. disease) relative to another group (e.g. controls). While this serves the purpose of molecular profiling or biomarker discovery studies, it is not adequate in the aspect of biological modeling or clinical diagnosis, where knowing the absolute amount of the analyte is critically important. Considering the complex biological matrices the glycosphingolipids typically reside in, internal standardization and standard addition are commonly used in absolute quantification by mass spectrometry, both requires standard calibration curves to interpolate the unknown concentration of analytes in a sample.

Internal standardization has the advantage of normalizing any variation introduced during sample preparation, chromatography, and mass spectrometry analysis, which requires compounds spiked into the sample mimic the physical and chemical traits of the analytes. Deuterated, ¹³C-, or ¹⁵N-labeled versions of the analyte are frequently used for such a purpose. The ¹³C and ¹⁵N-labeled standards are more desirable because unlike deuterium (²H), these isotopes do not perturb the overall physical and chemical properties of the analyte, for example, they coelute perfectly with endogenous analytes and have the same ionization efficiency [12]. Briefly, a known amount of internal standards is spiked into the samples prior to extraction. After extraction, samples, along with calibration standards are analyzed by LC-MS/MS [12]. Because glycosphingolipids are less abundant compared to other molecules in a biological sample, they are often measured by the more sensitive targeted mass spectrometry methods, typically using MRM scan mode on a triple quadrupole instrument. The ratios of the peak areas of analyte to the internal standards are plotted against the concentration of analytes to construct a linear calibration curve. The concentration of glycosphingolipids in the unknown sample is interpolated using this curve.

Internal standardization technique has been applied to quantify glycosphingolipids in samples derived from patients with lysosomal storage diseases in GM3 deficiency [182], determination of Gb3 and *I*iso-Gb3 in Fabry disease [183] and of sulfatides in dried-blood spots [105] as well as in tissues and biological fluids [114]. The same approach has also been applied to measure glucosylceramide, glucosylsphingosine and galactosylsphingosine in biological samples [184–186].

In the case where isotopically-labeled standards are not available or unaffordable, standard addition method may be a practical alternative. Here, unlabeled standards (i.e. analytes) of known amounts are spiked to the sample at increasing concentration and analyzed by LC-MS/MS. The peak areas are plotted against concentration of the spiked standards to create a linear calibration curve for each analyte. The value of *x*-intercept, i.e., *x* at *y* = 0, is the concentration of glycosphingolipids in the unknown sample. This approach has been used to quantify gangliosides in milk samples [187, 188].

8. Labeling approaches for qualitative and quantitative analysis

Glycosphingolipids are low abundant compared to other lipids and have low ionization efficiency, especially the heavily glycosylated ones. Chemical derivatization strategies are often used to improve the detectability and enable a more accurate and multiplexed quantification. In this section, we focus on the most common chemical labeling strategies in mass spectrometric analysis of glycosphingolipids.

8.1 Permethylation

The active protons (-OH and -NH) in glycans react with methyl iodide in the presence of sodium hydroxide and dimethylsulfoxide [189][190] (Fig. 11). This permethylation reaction enhances the volatility and sensitivity of the analytes. It has been used for qualitative analysis of glycans released from glycoproteins and some quantitative analysis using direct infusion ESI-MS or MALDI-MS [60, 191–193].

Permethylation can be performed using both the natural and isotope-labeled methyl iodide (¹³C or ²H atoms). When combined, the so-called differential isotope labeling technique is very useful in quantitative analysis [192, 193]. Here, individual samples are labeled with one version of the isotope and a reference sample is labeled with another version, (Fig. 11). The pooled mixture is then measured as a single sample for MS analysis, where the ratio of intensities serves as a surrogate of analyte concentrations. We recently implemented differential isotope labeling by permethylation and reversed-phase LC-MS/MS to quantify intact glycosphingolipids [125]. This method addressed the lack of commercially available ¹³C-labeled internal standards and the low sensitivity of glycosphingolipids in the presence of total lipids extract. Aliquots from individual samples were pooled to serve as a reference sample and permethylated with ¹³CH₃I; the individual samples were permethylated using ¹²CH₃I. After mixing, final samples (¹³CH₃I and ¹²CH₃I labeled) were analyzed by reversed-phase LC-MS/MS (Fig. 11). We observed up to 20-fold signal enhancement in the permethylated samples. We also found that highly alkaline conditions of permethylation depleted those ion-suppressing ester-linked lipids. The MS/MS fragments and retention time provided different levels but complementary information for compound identification. These

results were encouraging and illustrated the usefulness of permethylation for quantitative analysis of low-abundant glycosphingolipids in complex mixtures.

8.2 PAEA labeling

To improve the ionization efficiency of gangliosides, Huang and co-workers developed a labeling approach for monosialogangliosides (GM) using 2-(2-Pyridilamino)-ethylamine (PAEA) [194]. In this approach, GM species were derivatized using 4-(4,6-Dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride (DMTMM) and PAEA, with DMTMM as amidation reagent (Fig. 12) [194]. The PAEA was conjugated to the carboxylic acid group of sialic acid. The signal intensity was enhanced by 15-fold in positive mode compared to the underivatized analog. Coupled with MRM-based LC-MS/MS analysis, this derivatization method was successfully applied to determine the monosialoganglioside levels in plasma of patients with GM3 synthase deficiency [194].

8.3 Isobaric labeling

Isobaric labeling is a relatively new technique to glycosphingolipid analysis but has been used extensively in proteomics and glycomics [191, 195]. It provides quantitative information in a multiplexed fashion, where samples are labeled with isobaric tags that can fragment under CID or HCD to yield reporter ions as surrogate for the original sample (Fig. 13). The tag consists of the same molecule with a varying placement of heavy isotopes (*e.g.* ^{13}C and ^{15}N). Two versions of commercial isobaric tags have been applied to glycosphingolipids: iTRAQTM (isobaric tags for relative and absolute quantitation) and aminoxyTMTTM (aminoxy tandem mass tag) (Fig. 13)[133, 140, 196]. The iTRAQTM and aminoxyTMTTM differ in terms of reaction chemistry: iTRAQTM reacts with free amines; aminoxyTMTTM reacts with carbonyls (aldehyde and ketone). In terms of multiplexing, iTRAQTM can be used for up to eight samples while aminoxyTMTTM, up to six samples, currently.

Nabetani and co-workers used iTRAQTM to measure sphingolipids, which includes hexosylceramides (HexCer) in cells [133]. These analytes contain no free amines required by iTRAQTM. The *N*-fatty acyls were cleaved off from the HexCer using sphingolipid ceramide *N*-deacylase (SCDase), then, the resultant free amine was reacted with iTRAQTM. Improved sensitivity was observed after iTRAQTM labeling. One limitation of this method, however, is the complete disregard of the *N*-fatty acyl chain, which are structurally important and may have critical biological functions.

To understand the distribution of glycosphingolipids at the subcellular level and the roles these lipids played in the biological processes, it is necessary to track and quantify the glycosphingolipid metabolism in cells simultaneously. In this respect, BODIPY-labelled LacCer – where the BODIPY dye is attached in place of *N*-fatty acyl – has been developed as a mimic to the endogenous LacCer and in this way its metabolism *in vivo* can be traced by fluorescence technique [197, 198]. To facilitate quantification, Son and co-workers [199] developed a strategy to add isobaric tags to the fluorophore using click chemistry. Briefly, an azide functional group was first introduced to BODIPY, which serves as an anchor for successful attachment of iTRAQTM. This novel bifunctional BODIPY-iTRAQ-LacCer was

used for metabolic tracing experiments in cells and facilitated quantitative multiplexed analysis of LacCer metabolism. This approach, similarly as the previous iTRAQ labeling method on sphingolipids [133], is not capable of providing information of endogenous *N*-fatty acyls.

Recently, we used aminoxyTMT™ for multiplexed analysis of gangliosides [140]. To keep the ganglioside molecules structurally intact, we introduced a reactive aldehyde in the terminal sialic acids by selective oxidation of the glycerol hydroxyls with dilute solution of sodium meta periodate (NaIO₄). The newly formed aldehydes were then labeled with aminoxyTMT™, six individual samples can be pooled and analyzed together by reversed phase LC-MS/MS (Fig. 14). Sensitivity was greatly improved in positive ionization mode (~40-fold). Characteristic fragment ions helped elucidate structure for both the glycan and the lipid components. For its sensitivity and specificity, this method is promising in analysis of gangliosides.

Taken together, the novel labeling approaches presented here greatly facilitated the LC-MS/MS based quantification of glycosphingolipids by enhancing the ionization efficiency and enabling multiplexed quantification. Their integration with fluorescence labeling also enabled multiplexed quantification and live cell imaging. It is expected that more specific labeling strategies will be developed to enrich and quantify specific type of glycosphingolipids.

9. Ion Mobility-MS

Ion mobility mass spectrometry is a gas-phase separation technique that is based on ions with different size, shape and charge having different mobility through a buffer gas under an applied electric field. Ion mobility has emerged as a useful tool for analysis of carbohydrates [200, 201]. This technique provides the collision cross-section (CCS), a measure of ion conformation in the gas phase. CCS is independent of instrument type and experimental measurement conditions; it can be used as another dimension for compound identification. In this respect, the ion mobility database for glycans and glycoproteins was developed (GlycoMob) [202].

The currently available ion mobility platforms are: drift tube ion mobility (DTIMS), traveling wave ion mobility (TWIMS), high-field asymmetric waveform ion mobility (FAIMS), and serpentine multi-pass structures for lossless ion manipulations (SLIMS) [203]. Except for SLIMS, these platforms are commercially available and are suitable for use in conjunction with most mass spectrometers. The simplest, DTIMS, uses a uniform weak electric field in a drift tube filled with buffer gas (*e.g.*, helium or nitrogen). Due to the uniform field strength, CCS values can be directly calculated using the Mason–Schamp equation. TWIMS uses alternating radiofrequency (rf) potentials on adjacent stacked ring ion guides (SRIGs) to confine the ions. When a transient direct current (DC) voltage from one end of the device to the other is applied, a ‘traveling wave’ is created, which carries the ions through based on their mobility differences. In contrast to DTIMS, CCS cannot be calculated directly in TWIMS, but can be obtained after a suitable calibration with ions having similar charge state and CCS. FAIMS, also called differential mobility spectrometry

(DMS), uses an asymmetric high electric field and a compensation voltage to effectively “filter” ions, so only ions with certain compensation voltage can exit the device without being neutralized on the electrode plates. FAIMS devices cannot be used to measure CCS values but they can achieve very high resolving power and thus typically being used for targeted analysis of specific ions. SLIMS operates on a similar principle as TWIMS, but instead of using SRIGs, it uses arrays of electrodes patterned on two parallel surfaces, thus greatly increases the flight path for very high resolving power ion mobility separation, and also functions as a versatile ion manipulation device. These techniques have been thoroughly reviewed [201, 203].

The application of ion mobility-MS in glycosphingolipids remains limited. The first use of ion mobility in gangliosides was reported by Jackson and colleagues [205]. Briefly, gangliosides were ionized in the positive mode as cesium adducts and analyzed by MALDI-TOF MS. The drift times varied with the number of sialic acid residues, but it failed to resolve GD1a and GD1b isomers. More recently, Sarbu and co-workers applied TWIMS for the first time in human fetal brain gangliosides using Q-TOF MS [98]. Ion mobility reduced chemical background noise and separated gangliosides based on glycan length and degree of sialylation. This resulted in at least 143 distinct gangliosides species identified, three times higher than the number previously reported. Separation of glycosylsphingosine epimers (Glc and Gal), and gangliosides (GD1a and GD1b) with the same lipid backbones was achieved using SLIMS [204] (Fig. 15). DMS, coupled with HILIC-MS, effectively resolved GlcCer and GalCer species from human plasma and cerebrospinal fluids [206]. Very recently, CCS databases for lipids, including a few glycosphingolipid species, were compiled from experimental measurement and computational prediction, which are expected to facilitate differentiation of lipid isomers in comprehensive lipidomics research [207, 208].

10. Conclusion and future perspectives

Our current understanding of glycosphingolipid biology remains scant. The presence of a vast repertoire of structural variants serves both as an analytical challenge and a research opportunity. Multiple experimental evidence suggest that the lipid backbone can influence the function of the glycan moiety, which underscores the importance of analyzing glycosphingolipids in their intact form for a holistic understanding of their biological functions. We reviewed the current state of the mass spectrometric analysis of glycosphingolipids, including sample preparation, structural characterization, and quantification. Differentiation of isomers that differ only on the position of carbon-carbon double bonds, linkage between sugars, and stereochemistry of those linkages in the headgroup are fertile areas for future investigation. In this respect, chemoselective derivatization, modern fragmentation methods (electron-aided fragmentation, photodissociation, and OzID-MS), and ion mobility all serve as promising tools. On the other hand, disease-relevant glycosphingolipids are extremely low abundant, which requires the development of more sensitive strategies to detect them. These include powerful separation methods before mass spectrometry, derivatization strategies to improve ionization efficiency or reduction of analytical background noise *via* the use of enrichment strategies or ion mobility spectrometry. Finally, while it is imperative to elucidate the structure of glycosphingolipids, it is equally important to know their spatial distribution. In this respect,

the use of imaging mass spectrometry tools can provide novel biological insights reflected by the differential distribution of glycosphingolipids in tissues, cells and subcellular structures. Coupling mass spectrometry imaging with novel structural analysis tools presented here, in our opinion, can greatly facilitate uncovering the roles of glycosphingolipids in biology and pathology.

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Highlights

- A critical review of recent advances in MS analysis of glycosphingolipidome
- Nomenclature and biological significance of glycosphingolipids are covered
- Comprehensive coverage of novel enrichment strategies for glycosphingolipids
- Extensive coverage of fragmentation techniques in glycosphingolipid analysis
- Labeling approaches for quantitation and perspectives on future directions

Figure 1. The general structure of glycosphingolipids and sialic acid.

A) Shown here is LacCer d18:1 (4E)/18:1(9Z), composed of a ceramide backbone with a long-chain sphingosine base (d18:1) and oleic acid connected via an amide bond. The double bonds are numbered x and the stereochemistry assigned using the E/Z system. **B)** The structure of *N*-acetylneuraminic (Neu5Ac) sialic acid (α -anomer). The numbering shown here will be used throughout this manuscript to address these positions in the structure of glycosphingolipids.

Figure 2. Representative structures that show the structural diversity of glycosphingolipids.

A) Disialogangliosides as examples of positional isomers with varying connectivity of sialic acid. **B)** Globotriaosylceramide positional isomers where the terminal galactose is linked differently. **C)** Positional isomers that differ in the sialic acid linkage. **D)** Structural variants in the lipid tail that often result in isomeric and isobaric species.

Figure 3. General workflow to extract and isolate glycosphingolipids.

A) The tissue, cell culture or biological fluid is homogenized with CHCl₃/CH₃OH and partitioned with water. B) The top aqueous layer contains complex hydrophilic lipids such as gangliosides and complex neutral glycosphingolipids. Hydrophobic lipids including cerebroside and sulfatides are in the lower layer. Further purification uses DEAE cellulose chromatography to recover neutral and acidic glycosphingolipids. Silicic acid chromatography is used to separate most lipids from glycosphingolipids. C) Lipid extracts (crude, or enriched for a specific class) can be directly analyzed by MS *via* direct infusion, or LC-MS/MS.

Figure 4. Strategies to introduce reactive functional groups in glycosphingolipids.

Sphingoid *N*-deacylase (SCDase) is used to remove the fatty acyl chain leaving behind the sphingosine backbone. Endoglycoceramidase (EGCase) is used to remove the lipid part leaving the reducing end of the glycan intact. Oxidative release of natural glycans (ORNG) uses bleach (NaOCl) to convert the rest of the lipid tail to a nitrile group, which could be further reduced to form a primary amine group and used for other chemistries. Sodium meta periodate (NaIO₄) can selectively oxidize the trihydroxyfunctional group of sialic acid to aldehyde. Selective oxidation of the sphingosine double bond can be accomplished using 2,3-dichloro-5,6-dicyano-1,4-benzoquinone (DDQ). Ozonolysis cleaves the C=C on the sphingosine but could also cleave other C=C bonds if present.

Figure 5. Glycoblotting technique to enrich glycosphingolipids.

Glycoblotting involves the following steps: 1) Glycans with a free reducing end (*e.g.*, aldehyde) are mixed with amine-functionalized beads; 2) Reductive amination takes place to conjugate the glycans on the beads; 3) After washing, the glycans are released by transamination, typically using an aminoxy-functionalized compound, R-ONH_2 ; 4) The released glycans are purified by HILIC-SPE; and 5) Analyzed by LC-MS/MS.

Figure 6. Nomenclature of glycosphingolipid fragments generated in MS/MS.

A) The common fragments observed involving ceramide backbone (in red) and glycan linkage (in blue) are shown. **B)** The diagnostic fragment ions in MS/MS spectra. The *m/z* 290 and *m/z* 97 are observed in negative mode, while *m/z* 236, *m/z* 266, *m/z* 264, *m/z* 294, and 292 (O'' fragment ions) are in positive mode.

Figure 7. Fragmentation of ganglioside GD1 using different techniques.

(A) CID, (B) HCD, and (C) UVPD mass spectra of the doubly deprotonated porcine brain ganglioside GD1 18:1/18:0. The precursor ion in each spectrum is labeled with an asterisk. Fragment ions corresponding to GD1a 18:1/18:0 are labeled in black. Fragment ions corresponding to GD1b 18:1/18:0 are labeled in red. Reprinted with permission from O'Brien, J.P. & Brodbelt, J. *Anal. Chem.*, **85**, 10399–10407 (2013)[171] © 2013 American Chemical Society.

Figure 8. RDD spectrum of lyso-GalCer and lyso-GlcCer.

In RDD, *lyso*-glycosphingolipids were prepared from intact glycosphingolipids by alkaline hydrolysis, and then complexed via non-covalent bond with 4-iodobenzoyl 18-crown-6 (IB18C6). The complex was photoactivated using UV at $\lambda=266$ nm to cleave C-I bond and form a radical cation. The resultant radical cation was subjected to CID fragmentation to yield the RDD (PD followed by CID) spectra shown. Reprinted and modified with permission from Pham, H., and Julian, R. *Analyst*, **141**, 1273–1278 (2016) [69] © The Royal Society of Chemistry.

Figure 9. Gas-phase ozonolysis of LacCer d18:1/18:1(9Z) in a modified mass spectrometer.

A) Proposed reaction pathway showing selective cleavage of carbon-carbon double bonds producing Criegee and aldehyde ions. B) OzID-MS spectrum showing the Criegee and aldehyde product ions are depicted as (●) and (■), respectively. Open square (□) and circle (○) indicate ions generated through the elimination of $\text{H}_2\text{C}=\text{O}$ from the aldehyde ion and Criegee ion, respectively. Mass differences (neutral losses) between the precursor ion and Criegee/aldehyde ions are used to pinpoint the location of double bonds. Asterisk (*) indicates ions generated through secondary oxidation from $[\text{M}+\text{Na}+\text{O}_3]^+$. Colors represent different double bond locations. Reprinted with permission from Barrientos R.C. *et al. J. Am. Soc. Mass Spectrom.*, **28**, 2330–2343 (2017)[67] © American Society for Mass Spectrometry.

Figure 10. MS/MS scan modes used in the analysis of glycosphingolipids.

In full scan, all ions generated from the source are detected without fragmentation. In precursor ion scan, all ions generated from the source are isolated and fragmented while scanning to detect which precursor ion generating a predefined fragment ion. In multiple reaction monitoring (MRM), a fixed predefined precursor-product ion pair is monitored. In parallel reaction monitoring (PRM), a predefined ion is mass-selected and fragmented; all product ions are detected.

Figure 11. Labeling of glycosphingolipids by isotopic permethylation.

Shown here are the conversions of all active protons ($-\text{OH}$ and $-\text{NH}$) using isotopically labeled methyl iodide (MeI) in the form of ^{12}C , ^{13}C , or ^2H in the presence of alkali hydroxide (commonly, NaOH) and an aprotic solvent (dimethyl sulfoxide) under anhydrous conditions.

Figure 12. Labeling of intact, monosialoganglioside using PAEA.

The carboxylic acid group in Neu5Ac is activated by DMT-MM, followed by amidation using PAEA.

Figure 13. Principle of isobaric labeling and the chemical structures of mass tags used for glycosphingolipids.

A) Amine-reactive iTRAQ™, and carbonyl-reactive aminoxyTMT™. Both isobaric tags are commercially available and differ in their reaction chemistries. **B)** Workflow in isobaric labeling-based quantification of gangliosides. Briefly, samples are individually labeled with an isobaric tag. The labeled-samples are pooled and analyzed by LC-MS/MS. Sample clean-up maybe necessary in most cases, which can be accomplished using C18 SPE or other appropriate method. The isobaric tag fragments to yield the reporter ions, and the relative intensities of these ions serve as surrogates of analyte concentrations in the sample.

Figure 14. Isobaric labeling-based multiplexed quantification of gangliosides.

A) Labeling of BODIPY-modified LacCer with iTRAQ™. The BODIPY was functionalized with azide (-N₃) which reacts iTRAQ™ via click chemistry. B) Labeling of intact gangliosides using aminoxyTMT™. Gangliosides were chemoselectively oxidized at the trihydroxy functional group of sialic acid using NaIO₄ and the resulting aldehyde is labeled with aminoxyTMT™. Reprinted with permission from Barrientos, R.C. & Zhang, Q. *Anal. Chem.* **90**, 2578–2586 (2018)[140], © 2018 American Chemical Society.

Figure 15. Baseline resolution of isomeric GD1a and GD1b gangliosides. Samples were analyzed as $[M+2Na]^{2+}$ adduct using a serpentine multi-pass SLIM-IMS. Reprinted from Wojcik *et al. Int. J. Mol. Sci.*, **18**, 183 (2017)[204] © The Authors.

Table 1.

Key biological functions of the two components of glycosphingolipids.

Glycan headgroup	Ceramide backbone
<ul style="list-style-type: none">• Receptor• Antigen• Protein interaction• Receptor regulation• Mediator of cell adhesion• Initiator/modulator of signal transduction• Cell recognition and differentiation• Myelin sheath formation	<ul style="list-style-type: none">• Holds glycan on the plasma membrane• Modulates antigenicity of the glycan moiety• Influences clustering of glycan on the membrane surface• Second messenger in signal transduction