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Recent Advances in Sialic Acid-Focused Glycomics

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Abstract

Recent emergences of glycobiology, glycotecology and glycomics have been clarifying enormous roles of carbohydrates in biological recognition systems. For example, cell surface carbohydrates existing as glycoconjugates (glycolipids, glycoproteins and proteoglycans) play crucial roles in cell-cell communication, cell proliferation and differentiation, tumor metastasis, inflammatory response or viral infection. In particular, sialic acids (SAs) existing as terminal residues in carbohydrate chains on cell surface are involved in signal recognition and adhesion to ligands, antibodies, enzymes and microbes. In addition, plasma free SAs and sialoglycans have shown great potential for disease biomarker discovery. Therefore, development of efficient analytical methods for structural and functional studies of SAs and sialylglycans are very important and highly demanded. The problems of SAs and sialylglycans analysis are vanishingly small sample amount, complicated and unstable structures, and complex mixtures. Nevertheless, in the past decade, mass spectrometry in combination with chemical derivatization and modern separation methodologies has become a powerful and versatile technique for structural analysis of SAs and sialylglycans. This review summarizes these recent advances in glycomic studies on SAs and sialylglycans. Specially, derivatization and capturing of SAs and sialylglycans combined with mass spectrometry analysis are highlighted.

I. Introduction

Glycosylation is a common post-translational modification that plays important roles in both physiological and pathological pathways. The glycan moieties of extracellular glycoproteins may stabilize the conformation of proteins and confer proteolytic resistance and influence protein turnover, and are involved in receptor-ligand interactions, cell-cell signaling, and adhesion, however, molecular mechanisms in many instances are still unknown [1]. Detailed knowledge of protein glycosylation at the proteomics level involving structural information of both the glycan microheterogeneity and the backbone peptide sequence, known as glycoproteomics, will facilitate mechanism investigation of glycoprotein and is of growing importance in postgenomic science and clinical research [2]. In addition, abnormal protein glycosylation is highly involved in disease development [3]. Glycoproteomic studying uncovers new clinical glycoprotein biomarkers, which can be used for both disease diagnosis and monitoring and evaluating therapeutic efficiency, and even for personal

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medicine purpose. On the other hand, there has been a rapid increase in the number of glycoproteins approved as biopharmaceuticals [4]. Glycan analysis is an important quality parameter of biopharmaceuticals with regard to drug stability, clinical activity, and immunogenicity [5, 6].

Sialic acids (SAs), a family of 9-carbon containing acidic monosaccharides, often terminate the glycan structures of cell surface and secreted glycoconjugates such as glycoproteins and glycolipids. They are found on both *N*- and *O*-linked glycans, being attached to either galactose (Gal), or *N*-acetylgalactosamine (GalNAc) units *via* α 2,3- or α 2,6-linkages, or to SA *via* α 2,8- or α 2,9-linkages, whose syntheses are catalyzed by specific enzymes [7]. In addition, various substituents present on carbon 4-, 5-, 7-, 8- and 9-positions generate more than 50 SA species (Figure 1). SAs play crucial roles in cell surface interactions [8], protect cells from membrane proteolysis [9], help in cell adhesion and have been shown to determine the half-life of glycoproteins in blood [10]. It has been demonstrated previously that cancers and cancer staging may be associated with a significant overrepresentation of SAs on the surface glycoproteins of cancer cells compared with normal cells [11–18]. Also, it is well known that the amount of free SAs and lipid- and protein-bound SAs are elevated in plasma from cancer patients compared with healthy individuals [19–22]. In addition, glycosylation microheterogeneity in the form of different branching patterns (where the number of sialic acid moieties reflects the glycan branching structure) is linked with acute phase condition and chronic disease [23, 24], possibly indicating that SAs or SA-containing glycoproteins/peptides could be good biomarker candidates for cancer diseases. Therefore, the ability to detect and monitor changes in the sialylated glycans could be an important aid in the diagnosis of cancer at an earlier stage, thus improving the patient's prognosis. Both glycomics and glycoproteomics data defined a significant change in sialylation as the most prominent feature associated with serum acute phase glycoproteins from mice bearing tumors [25].

Mass spectrometry (MS) has become a central tool for monitoring, identifying and characterizing biomolecules in bioscience. With an increasing ability to correctly characterize miniscule quantities and more complex mixtures of proteins and peptides, MS has been quickly becoming a key tool in the glycomics recently [26]. It provides many advantages over traditional analytical methods, such as low sample consumption and high sensitivity. Particularly, the developments of matrix-assisted laser desorption/ionization (MALDI) and electrospray ionization (ESI) mass spectrometry have greatly accelerated the use of MS-based technology in structural analysis of carbohydrates [27]. MALDI MS in conjunction with enzymatic digests and post source decay has been widely used for identification and structural determination of the glycans. ESI and collision induced dissociation (CID) has been proved to be powerful and versatile techniques to obtain detailed structural and linkage information on complex carbohydrates, especially with the MSⁿ capability of the ion trap. The gain in measurement sensitivity achieved with glycans during the past decade has been highly significant. However, it should be noted that SA-containing acidic oligosaccharides had not yet fully benefited from the usefulness of mass spectrometry in the early stage. This is mostly due to the instability of the α -glycosidic bond between SA and the adjacent sugar residue under general conditions for ionization/detection processes during mass measurement, giving rise to several inevitable difficulties in the analysis. It has been well documented that various sialylated oligosaccharides, when analyzed by mass spectrometry in either positive or negative ion modes, lose SAs as a result of the presence of the free carboxyl group. In addition, oligosaccharides containing multiple SA residues usually give complicated mass spectra resulting from mixtures of cation adducts when analyzed in positive ion mode. It therefore seems likely that structural heterogeneity due to the partial loss of SA residues has often been reduced by the complete removal of terminal SAs from the target oligosaccharides on treatment with some acidic solutions [28].

Therefore, the development of simple and versatile method for avoiding the risk of desialylation has been explored extensively. With the goals of addressing these issues in measurements of acidic oligosaccharides in mass spectrometry, as well as of enhancing the sensitivity and resolution, several approaches have been employed so far. One approach is chemical modifications of sialylated glycans, which stabilizes sialic acids and improve the ionization efficiency of the glycans for MS analysis. On the other hand, the inherent ionization bias of MALDI MS may lead to preferential detection of unmodified peptides and partial or complete suppression of glycopeptides. In such cases, a separation step for removal of peptides and enrichment of glycopeptides is needed prior to the MALDI MS detection. Various methods for enrichment and separation of SA-containing glycopeptides and SAs have been developed including using lectins. Herein, we summarize recent advances in glycomic analysis that focuses on SAs and sialylglycans, the most important human glycoforms. Specially, SA derivatization and SA capturing combined with mass spectrometry analysis are highlighted.

II. Derivatization of sialylglycans for their mass spectrometry analysis

The labile nature of SA poses a challenge for MS characterization. In-source and post-source losses of SA often lead to inaccurate representation of the degree of sialylation. Sialylated glycans usually decompose by loss of SA when ionized by MALDI MS as the result of the labile carboxylic proton. In addition, ionic signal suppression is often a major issue in the analysis of sialylated glycans by MS, particularly due to the presence of a carboxyl group at the anomeric carbon, which is usually ionized at physiological pH, thus resulting in a negative charge above pH value of ~2.6. As the number of SA moieties increase on a particular glycan, it becomes inherently difficult to detect these glycans, which can lead to incomplete characterization of a given sample. To avoid these problems, sialylated glycans are typically characterized in the negative mode while asialoglycans are characterized in the positive mode. A variety of approaches have been reported to address the problems associated with SA dissociation. For example, the use of cool matrices [29], analysis by high-pressure MALDI MS [30]. An alternative approach is to chemically modify sialylated glycans, such that it stabilizes SAs for MS analysis. Additionally, the choice of chemical modification can also improve the ionization efficiency of the glycans. Indeed, these methods enabled detect sialylated glycans in positive-ion mode without a loss of sialic acid. So far, SAs have been converted to its ester, amide or derivatized with acetohydrazide and other small molecules for MS analysis (Figure 2 and Scheme 1).

II.1. Methyl ester formation of sialylglycans for their mass spectrometry analysis

Methyl ester formation is a common technique for achieving SA stabilization (Figure 2, compound 1). Diazomethane is a general choice for methyl ester formation in organic synthesis. However, attempted methyl ester formation of acidic carbohydrates with diazomethane in the presence of water has generally been unsuccessful. Nevertheless, glycolipids and glycoproteins are sufficiently lipophilic to allow organic solvents to be used with success. Thus, MacDonald et al. successfully formed methyl esters of gangliosides by reaction with diazomethane in methanol-ether [31]. To achieve a quantitative reaction, the gangliosides were first passed through a Dowex AG-50 (H⁺ form) column after which the reaction was rapid and could be completed in under an hour.

Methyl ester formation for achieving this stabilization in MS analysis was first introduced by Powell and Harvey for *N*-glycans and gangliosides [32]. Reaction was achieved by conversion of the acid to its sodium salt first with an AG-50 column that had been equilibrated with sodium hydroxide, followed by reaction with methyl iodide. The reaction produced a high yield of methyl esters that were stable under MALDI MS conditions. Particularly, methylation of the carboxyl group, that eliminates the labile proton, stabilizes

the sialic acid towards fragmentation and avoids multiple peaks formation caused by salt formation and results in a much cleaner signal in the MS spectrum. The methylation of sialic acid carboxyl group had subsequently been used by several investigators with improved methylation [33–36]. Li and coworkers [33] reported “one-pot” methylation method to esterify SAs and construct a permanent charge for *N*-linked glycan analysis, which combined complete nonspecific proteolytic digestion and methylation. In this study, a mixture of Asn-glycans prepared from Pronase E digestion of the glycoprotein was passed through a cation exchange column to convert carboxylic acids to the Na⁺ form before being methylated with methyl iodide. Derivatives were purified with a hydrophilic affinity chromatography cartridge. Mass spectrometry analysis was performed by MALDI-TOF MS and MALDI-TOF/TOF MS. The mass spectrometric data indicated that carboxylic acids were methylated in addition to the formation of a quaternary ammonium in the amino group of asparagine residues. This simple derivatization approach allows analyzing sialylated glycans without losing terminal sialic acid groups as well as improving analysis sensitivity. Three model glycoproteins, including ribonuclease B, ovalbumin, and transferrin, were employed to demonstrate the merits of this technique. The results showed that the stabilization of SA was achieved in addition to the formation of a permanent charge. Compared to the analysis of underivatized *N*-glycans, detection sensitivity improved 10-fold. This technique was further evaluated with glycan profiling of serum transferrin and proved to be a sensitive method for the characterizing protein glycosylation. Merits of this technique are simple methylation, stabilization of sialic acids and enhanced sensitivity as a result of introducing of a permanent charge.

Nishimura and co-workers [35] reported a solid-phase platform for quantitative methyl esterification of sialic acid residues. In this study, oligosaccharides from tryptic and/or PNGase F-digest mixtures of glycoproteins were adsorbed or covalently attached to the supporting materials and then were subjected to methyl esterification with triazene derivative, 3-methyl-1-*ptolyl*triazene, in a 96-well filter microplate. The recovered materials were directly applicable to subsequent characterization by MALDI-TOF MS for large scale glycomics of both neutral and sialylated oligosaccharides. On-bead derivatization of glycans containing sialic acids allowed rapid and quantitative glycoform profiling by MALDI-TOF MS with reflector and positive ion mode.

Recently, Harvey and co-workers [36] reported an alternative procedure for methyl ester formation that provides information on the sialic acid linkage directly from the MALDI spectrum. Briefly, the sugars were desalted, dissolved in methanol, and treated with 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride (DMT-MM). After removal of the solvent, the products were transferred directly to the MALDI target and examined from 2,5-dihydroxybenzoic acid. In this case, only α 2,6-linked SAs react in this way, while α 2,3-linked SAs formed lactones and, because the mass of these compounds is 32 units below that of the α 2,6-linked SAs, the linkage could easily be determined by MALDI MS. The method was applied to *N*-linked glycans released from bovine fetuin and porcine thyroglobulin.

II.2. Pyrenyl ester derivatives of sialylglycans for their mass spectrometry analysis

Due to the hydrophilic nature of the oligosaccharide moiety, glycopeptides ionize much less readily than nonglycosylated peptides. In order to enhance the ion yield of oligosaccharides, Pyrene derivatization method using 1-pyrenyldiazomethane has been explored for highly sensitive MALDI MS of glycopeptides. Intense and stable ionization in both positive and negative modes was achieved by derivatization with pyrene. Amano et al. [37] modified glycoproteins containing SA with 1-pyrenyldiazomethane (PDAM) (Figure 2, compound 2) and found greatly improved detection limits of the products in the presence of peptides. In

this study, matrix 2,5-dihydroxybenzoic acid (DHB) caused loss of the ester group allowing the free SA to be observed whereas the intact ester was ionized with the ionic liquid matrix 3-aminoquinoline (3AQ)/ α -cyano-4-hydroxycinnamic acid (CHCA). Most recently, the same group developed a new approach for discriminating between α 2,3- and α 2,6-sialylation of glycopeptides by an onplate 1-pyrenyldiazomethane (PDAM) derivatization of sialyl glycopeptides followed by negative-ion MALDI MS² [38]. To stabilize the SAs, the carboxyl moiety on the SA as well as the C-terminus and side chain of the peptide backbone were derivatized using PDAM. The derivatization was performed on the target plate of MALDI MS, thereby avoiding complicated and time-consuming purification steps. After the on-plate PDAM derivatization, samples were subjected to negative-ion MALDI MS using 3AQ/CHCAv as a matrix. Deprotonated ions of the PDAM-derivatized form were detected as the predominant species without loss of SA. The negative-ion CID of PDAM-derivatized isomeric sialylglycopeptides, derived from hen egg yolk, showed characteristic spectral patterns. These data made it possible to discriminate α 2,3- and α 2,6-sialylation. In addition, sialyl isomers of a glycan with an asparagine could be discriminated based on their CID spectra. Interestingly, the approach was applicable to both the disialylated and monosialylated form. The unique fragmentation behavior was derived from the difference of pyrene-binding positions after ionization between α 2,3- and α 2,6-sialylated glycopeptides. Overall, pyrene derivatization gave superior mass spectra in terms of ion yield and S/N ratio in both the positive- and negative-ion modes. Particularly, hydrophobic derivatization with pyrene seems to improve both mixing with aromatic matrixes and the gasphase ion production of oligosaccharides, as compared with underivatized oligosaccharides.

II.3. Amide derivatization of sialylglycans for their mass spectrometry analysis

Amidation has also been explored for stabilizing the glycosidic bond with sialic acid and suppressed its preferential cleavage by in-source decay, postsource decay, or collision-induced dissociation. In addition, the suppressed dissociation considerably improved the ionization yield for structural analysis by MS/MS. Sekiya et al. reported that DMT-MM catalyst could be used to convert the COOH group of SAs to amides by use of ammonium hydroxide and measured positive ions [39] (Figure 2, compound 3). Although the SA is stabilized, the amide only produces a mass shift of one unit. The same group also tried to stabilize the moiety by amidation and found 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride to be a desirable condensing agent. Amidation stabilized the glycosidic bond with SA and suppressed its preferential cleavage by in-source decay, postsource decay, or CID. In addition, the suppressed dissociation considerably improved the yield of the B/Y type ions for structural analysis by MS/MS. In another study, Amano and coworkers labeled oligosaccharides with a pyrene derivative prior to negative-ion MALDI-QIT-TOF MSⁿ for the analysis of neutral oligosaccharides, such as fucosylated oligosaccharides containing blood group antigens H, Lea, and Lex. Moreover, sialylated oligosaccharide was converted to the corresponding neutral oligosaccharide by amidation, and the negative-ion spectrum was shown to be more informative than that of the original acidic oligosaccharide [40]. Structural determination of both fucosylated and sialylated isomers, such as sialylfucosyllacto-*N*-hexaose I and monosialyl monofucosyllacto-*N*-neohexaose, was succeeded because fragment ions bearing fucose or amidated sialic acid were obtained on negative-MSⁿ. These results demonstrated that amidation was an effective derivatization to reinforce the structural analysis of sialylated oligosaccharides by MALDI MS. In addition, amidation with ¹⁵N-labeled ammonium chloride decreases the mass shift from the acid to amide form to just 0.013, reducing the complexity of mass spectral interpretation and database searching.

Terabayashi and coworkers developed a method to modify sialic acid at the position of the carboxyl group of sialyl-compounds with a fluorescent reagent [41]. In this study, SA

(Neu5Ac) of 3-*O*-sialyllactose, 6-*O*-sialyllactose, and ganglioside GM3 were amidated with the fluorescent reagent, 2-(2-pyridylamino)ethylamine (PAEA) using the amidation reagent, DMTMM (Figure 2, compound 4). Since PAEA-amidation suppressed preferential cleavage by ISD, PSD, or CID by MALDI-TOF MS and MS/MS analyses, signal intensities of the molecular related ions on MS and of the B/Y type ions on MS/MS were enhanced. Furthermore, they found that PAEA-amidation enabled to analyze the complete structure of GM3. PAEA-amidation provided the following advantages: suppression of preferential cleavage of Neu5Ac; enhancement of molecular-related ion intensities; simplification of MS spectra; and finally, since PAEA-amidation did not cleave the linkage between sugar and aglycon of sialylglycoconjugate, MALDI-TOF MS and MS/MS analyses revealed the complete structure of the molecule.

Li and coworkers described another method of methylation of sialic acids of sialylated glycans in the presence of methylamine and (7-azabenzotriazol-1-yl)oxytrispyrrolidinophosphonium hexafluorophosphate (PyAOP) (Figure 2, compound 4) [42]. After methylation, sialylated glycans were analyzed by MALDI MS without loss of the sialic acid moiety. Both ESI MS and MALDI MS analysis of both 3'- and 6'-sialyllactose derivatives indicated that the quantitative conversion of sialic acids was achieved, regardless of their linkage types. This derivatization strategy was further validated with the *N*-glycans released from three standard glycoproteins (fetuin, human acid glycoprotein, and bovine acid glycoprotein) containing different types of complex glycans. Most interestingly, this derivatization method enabled the successful characterization of *N*-glycans of sera from different species (human, mouse, and rat) by MALDI MS. Because of the mild reaction conditions, the modification in SA residues can be retained. This improvement made it possible to detect sialylated glycans containing *O*-acetylated SA moieties using MALDI MS in positive ion mode.

II.4. Acetohydrazide derivatives of sialylglycans for their mass spectrometry analysis

Methyl esterification and amidation were tried to neutralize sialylated *N*-glycan as described above, however, these methods were problematic with incomplete modification for $\alpha(2\text{--}3)$ linked sialic acid and their byproducts. To solve this problem, Toyoda et al. [43] reported an alternative method of derivatizing the carboxyl group of SA with acetohydrazide (Figure 2, compound 6). Acetohydrazide was selected because it can be easily coupled with carboxylic acid and there is little steric hindrance for the amidation reaction. The reaction appeared to be quantitative with both $\alpha 2,3$ - and $\alpha 2,6$ -linked SAs as demonstrated with *N*-glycans released from bovine fetuin, a glycoprotein containing SAs in both linkages. The method was, however, not appropriate for glycans with an intact reducing terminus because the reagent also reacts with aldehydes of the reducing terminus. Therefore, the glycans had first to be derivatized at this terminus, in this case with 2-AP, after which the reaction worked well.

By taking the advantage that acetohydrazide amidation method is complete amidation of both $\alpha 2,3$ - and $\alpha 2,6$ -linked sialic acid and no byproduct, Van Cott and coworkers reported that amidation in mild acidic conditions could be used to neutralize acidic *N*-glycans still attached to the protein [44]. The amidation was performed on the carboxyl group of sialylated *N*-glycans using acetohydrazide and EDC as a coupling reagent in mild acidic conditions. Since the aldehyde of the reducing end of a free glycan is also likely reactive to hydrazide, therefore a permanent charge cannot be incorporated after the amidation reaction. To avoid this problem, they performed the amidation reaction on the sialylated *N*-glycan while it was still attached to the protein. The resulting amidated *N*-glycans could then be released from the protein using PNGase F, and then labeled with permanent charges on the reducing end to avoid any modification and the formation of metal adducts during MS.

analysis. Labeling with a permanent charge on the reducing end of the released sugar promotes homogeneous ionization efficiency on MS, particularly in MALDI-TOF MS. The *N*-glycan modification, digestion, and desalting steps were performed using a single-pot method that can be done in microcentrifuge tubes or 96-well microfilter plates, enabling high throughput glycan analysis. Using this method they were able to perform quantitative MALDI-TOF MS of a recombinant human glycoprotein to determine changes in fucosylation and changes in sialylation that were in very good agreement with a normal phase high-performance liquid chromatography (HPLC) oligosaccharide mapping method. As the authors noted, the mildly acidic conditions might cause problems with the analysis of glycoproteins having a low pI, as proteins tend to aggregate and precipitate near their pI. In this study, they did not observe precipitation of their sample protein tg-FIX that has PI in the range of 4–6. If aggregation or precipitation becomes a problem, addition of denaturants or chaotropes may be necessary and should not interfere. However, it was not explored if this method works with precipitating proteins.

II.5. Quinoxaline derivatives (1,2-Diamino-4,5-methylenedioxybenzene (DMB)) of sialylglycans for their mass spectrometry analysis

The α -keto-carboxyl group of SAs has been recognized as an unique structure to react with DMB (Scheme 1) in dilute acetic acid to form fluorescent (exc: 373 nm, em: 448 nm) quinoxaline derivatives [45], that have proved very useful for separations of different SAs by HPLC and for analysis of SAs by ESI MS and MALDI MS. Although SAs were known to migrate under certain conditions, the reaction did not appear to be susceptible to this problem. Using these derivatives, Klein et al. [46] have identified as many as 28 SAs by LC-ESI MS. Stehling et al. used DMB derivatization with MALDI MS analyzed *O*-acetylated SA [47]. Kakehi et al. employed LC-ESI MS after derivatization with DMB examined specific distribution of SAs in animal tissues [48]. Geyer et al. used MALDI-TOF MS combined with DMB derivatization and HPLC separation method analyzed mass spectrometric fragmentation of oligosialic and polysialic acids [49]. The same group later reported a method of quantification of nucleotide-activated SAs by LC-ESI MS combined DMB derivatization and HPLC separation [50]. The standard procedure for DMB labeling in acetic acid at 50 °C for 2 h results in significant hydrolysis of oligomers of α 2,8-linked-Neu5Ac. Changing the conditions to 10 °C for 48 h in 0.02 M TFA produced a compromise between the minimum degradation of the oligomers and a rapid rate of derivatization. It should be pointed out that DMB also reacts with other α -keto acids, such as α -keto glutaric acid, pyruvic acid, and phydroxyphenyl-pyruvic acid in the biological sample. Therefore, it should pay attentions when using DMB for direct derivatization of biological samples.

II.6. Lactone formation of oligosialic and polysialic acids for their mass spectrometry analysis

Oligosialic and polysialic acids play important roles in biological system and their structure characterization is highly demanded. So far, several methods have been reported for the characterization of oligosialic and polysialic acids, among which, derivatization with 1,2-diamino-4,5-methylenedioxybenzene (DMB) followed by HPLC and fluorometric detection. MALDI-TOF MS has been employed as an ultrasensitive method for the characterization of oligosialic and polysialic acids [49]. In addition, lactone formation was reported by Galuska et al. [51] to stabilize polysialic acids for their analysis by MALDI-TOF MS. Lactonization was achieved with TFA and *o*-phosphoric acid and, of several matrices tested, 6-aza-2-thiothymine (ATT) proved to be the most satisfactory with DHB being nearly as good. α 2,8-Linked sialic acids reacted readily under these conditions, but α 2,9-linked acids were much more reluctant to form lactones, thus providing a method for differentiating the two linkages. When SAs were α 2,8-linked, cyclization occurred with the hydroxyl group at C-9 position of an adjoining SA residue (Scheme 2A), whereas with α 2,9-linked polysialic

acids, cyclization was with the hydroxyl group at C-8 position (Scheme 2B). Good signal to noise ratios was obtained with masses as high as 10,000 Da and polymers with up to 100 residues could be examined. Geyer et al. used MALDI-TOF MS combined with lactone derivatization analyzed mass spectrometric fragmentation of oligosialic and polysialic acids [49]. Overall, MALDI-TOF MS/MS and ESI-IT-MSⁿ are universal techniques that are suitable for detailed structural characterization of oligoSia. Both techniques allow fragmentation analyses of individual oligosialic acids units, thus verifying the composition of the analyte. In general, sialic acid dimers, trimers, and tetramers are detected with higher efficiency using the electrospray approach, while fragmentation analyses of polysialic acids are possible using MALDI-TOF-MS/MS.

III. Capturing sialylglycans for their mass spectrometry analysis

Glycoprofiling of glycoproteins, glycopeptides and glycans is conveniently achieved by MALDI MS and MS/MS due to the high sensitivity, speed and robustness of this technique [51, 52]. However, the inherent ionization bias of MALDI MS may lead to preferential detection of unmodified peptides and partial or complete suppression of glycopeptides. In such cases, a separation step for removal of peptides and enrichment of glycopeptides is needed prior to the MALDI MS detection. Various methods for enrichment and separation of glycopeptides and glycans have been developed including lectins [54] graphitized carbon [55], titanium dioxide [56], hydrazide chemistry [57] and boronic acids [58] (see reviews for details [59, 60]). Furthermore, HILIC solid-phase extraction (SPE) is emerging as a very useful approach to enrich for glycopeptides [61]. This review summarizes recent capturing method for SAs and sialylglycans from complex peptide mixtures for the quantitative and qualitative assessment by mass spectrometry.

III.1. Lectin-affinity sialylglycan capturing for their mass spectrometry analysis

Lectins have been useful tools to capture, concentrate, and classify glycoconjugates, including glycoproteins and glycopeptides. Because non-reducing ends of naturally occurring glycans are limited to mannose, galactose, *N*-acetylglucosamine, SAs, and rarely *N*-acetylgalactosamine, a number of lectins with binding specificities to these glycans could be used to capture subsets of the glycoproteome. Using lectins such as SNA from *Sambucus nigra* or MAA from *Maackia amurensis*, SA-containing glycoproteins can be affinity captured. However, one of the disadvantages of lectins is their rather limited specificity: SNA and MAA are mainly restricted toward, respectively, α 2,6- and α 2,3-bound SA. Combining several lectins in a multi-lectin approach [62] or serial lectin affinity chromatography (SLAC) [63] may partially solve this problem. For a more comprehensive or systematic collection of glycopeptides from complex biological mixtures, multiple lectins with distinct binding specificities have been used in combination or in series [64–67].

Lubman and coworkers analyzed pancreatic cancer serum using SA-specific lectin affinity chromatography followed by fractionation using RP-HPLC and further separation by SDS-PAGE [68]. They used three different lectins (Wheat Germ Agglutinin (WGA), Elderberry lectin (SNA), *Maackia amurensis* lectin (MAL)) to extract sialylated glycoproteins from normal and cancer serum. The use of the different lectin columns allows them to monitor the distribution of α 2,3- and α 2,6-linkage type sialylation in cancer serum *vs* that in normal samples. Extracted glycoproteins were fractionated using NPS-RP-HPLC followed by SDS-PAGE. Targeted glycoproteins are characterized further using mass spectrometry to elucidate the glycan structure and glycosylation site. They applied this approach to the analysis of sialylated glycoproteins in pancreatic cancer serum. Approximately 130 sialylated glycoproteins were identified using LCMS/MS. Sialylated plasma protease C1 inhibitor was identified to be down-regulated in cancer serum. This strategy offers the

ability to quantitatively analyze changes in glycoprotein abundance and detect the extent of glycosylation alteration as well as the carbohydrate structure that correlate with cancer.

Kubota et al. demonstrated that the combination of lectin affinity chromatography and MALDI-TOF MS was a powerful tool to analyze complex mixtures of glycopeptides in a relatively rapid fashion [69]. Glycopeptides prepared from 1 nmol of a mixture of glycoproteins, transferrin, and ribonuclease B by lysylendopeptidase digestion were isolated by lectin and cellulose column chromatographies, and then were analyzed by MALDI-TOF MS and MALDI-quadrupole ion trap (QIT)-TOF MS, which enable the performance of MSⁿ analysis. The lectin affinity preparation of glycopeptides with *Sambucus nigra* agglutinin and concanavalin A provided the glycan structure outlines for the sialyl linkage and the core structure of *N*-glycans. Such structural estimation was confirmed by MALDI-TOF MS and MALDI-QIT-TOF MS/MS. Amino acid sequences and location of glycosylation sites were determined by MALDI-QIT-TOF MS/MS/MS. Taken together, the combination of lectin column chromatography, MALDI-TOF MS, and MALDI-QIT-TOF MSⁿ provides a way for the structural estimation of glycans and the rapid analysis of glycoproteomics.

Recently, Philip and coworkers developed a lectin-directed tandem labeling (LTL) quantitative proteomics strategy in which they enriched sialylated glycopeptides by SNA, labeled them at the *N*-terminus by acetic anhydride (1H6/2D6) reagents, enzymatically deglycosylated the differentially labeled peptides in the presence of heavy water (H₂¹⁸O), and performed LC-MS/MS analysis to identify glycopeptides [70]. They used fetuin as a model protein to test the feasibility of this LTL strategy not only to find true positive glycosylation sites but also to obtain accurate quantitative results on the glycosylation changes. Further, they implemented this method to investigate the sialylation changes in prostate cancer serum samples as compared to healthy controls. This study indicated that the LTL quantitative technique is a potentially useful method for obtaining simultaneous unambiguous identification and reliable quantification of *N*-linked glycopeptides.

III.2. Sialome using titanium dioxide chromatography and mass spectrometry analysis

Titanium dioxide-based affinity chromatography has proven to be a versatile tool in enrichment of various compounds such as phosphorylated biomolecules due to its unique ion and ligand exchange properties and high stability towards pH and temperature. Recently, titanium dioxide chromatography was also explored in purification of sialylated glycopeptides, thereby targeting the sialome, defined as the content of SA containing glycoproteins of a given cell, body fluid or tissue by Larsen and coworkers [56]. The method takes advantage of the extremely high affinity of titanium dioxide toward SA residues positioned in the non-reducing ends of glycans under specific buffer conditions. They confirmed the method in model experiments with bovine fetuin that the method was specific and complete for SA-containing glycopeptides. In addition, they showed for the first time a map of the sialome. Finally to illustrate the potential of the approach in biomarker discovery, it was used to compare the differences of the plasma sialome of a control individual and a patient with advanced bladder cancer. Besides being simple, fast, and efficient in enrichment of SA-containing glycopeptides from complex biofluids, this method was tolerant toward salts and other low molecular weight contaminants such as detergents. The same group combined the titanium dioxide enrichment procedure with HILIC as a means of pre-fractionating the deglycosylated SA-glycopeptides prior to LC-MS/MS analysis, to maximize the coverage of formerly sialylated glycopeptides in a given sample [71]. In this study, a total of 1632 unique formerly sialylated glycopeptides were identified from an enriched membrane fraction from less than 10⁷ HeLa cells. Most recently, by combining with TiO₂ chromatography, Wang et al. analyzed intact structures of the enriched sialylated glycopeptides of bovine fetuin by MALDI MS/MS [72]. They showed that the optimal loading buffer for titanium dioxide as matrix is 80% acetonitrile/2% TFA/100 mg/

mL DHB which is also compatible with MALDI MS spectrometric analysis. This study indicated that the improved enrichment approach combined with MALDI MS/MS was a powerful tool to analyze intact structures and components of the sialylated glycopeptides from complex peptide mixture.

III.3. Chemical ligation-based capturing of sialglycans for their mass spectrometry analysis

Based on the unique *cis*-diol structure feature, chemical derivatization combined with covalent conjugation strategy has been developed for glycan capture during the past decade. Zhang et al. developed a method to capture glycopeptides on a solid support by chemical coupling between aldehyde groups derived by oxidation of *cis*-diol groups of the glycan and hydrazide on the support [57]. The captured *N*-linked glycopeptides were then released from the resin by digestion with PNGase. Unlike lectin affinity chromatography, the method captures *N*-linked glycopeptides regardless of the glycan structure. Recently, several methods for glycopeptide characterization have been published including chemical ligation using hydrazine chemistry, which captures both SAs and neutral glycosylated peptides/proteins. Nishimura and coworkers developed a method for rapid enrichment analysis of peptides bearing sialylated *N*-glycans on the MALDI-TOF MS platform [73]. The method involves highly selective oxidation of SA residues of glycopeptides to elaborate terminal aldehyde group and subsequent enrichment by chemical ligation with a polymer reagent, namely, reverse glycoblotting technique (Scheme 3). This method based on the fact that periodate oxidation of carbohydrates could be controlled and afford different types of products according to the conditions employed [57]. In this method, the optimized conditions (1 mM sodium periodate, 0°C for 15 min) were chosen to assure rapid, selective, and quantitative oxidation of SA residues with terminal linear triols at the C7, C8, and C9 positions into the reactive derivatives with an aldehyde group at the C7 position. They reported that this chemoselective conversion proceeded quantitatively and specifically in all compounds bearing at least one SA residue. The highly reactive aldehyde groups generated on the SA residues could then be enriched quantitatively at 37°C for 2 h by chemical ligation with aminoxy-functionalized polyacrylamide [74], to form stable oxime bonds (Scheme 3). The sialylated glycopeptides captured covalently by the polymer were treated with 3% TFA aqueous solution at 100°C for 1 h to digest selectively at the α -glycosidic bond between the SA and adjacent galactose residues. α -L-Fucoside linkages were stable under the above conditions, while α -sialosides of glycopeptides were labile at a range from pH 2 to pH 3 and were selectively hydrolyzed under the conditions optimized in this study. They noted that hydrazide-carrying polymers could alternatively be used for this purpose.

III.4. Chemical ligation and labeling of sialglycans for their mass spectrometry analysis

Glycoform-focused reverse glycoblotting technique greatly contributes to reducing the complexity of biological samples. Nishimura and coworkers demonstrated the feasibility of the reverse glycoblotting technique in quantitative analysis of the specific glycopeptides carrying SAs by combining with multiple reaction monitoring (MRM)-based mass spectrometry [75]. The method allows the identification and quantification of glycoproteins bearing sialylated oligosaccharides in a complex sample and the determination both of *N*-glycosylation sites and glycoforms concurrently. The improved reverse glycoblotting procedure made recovering enriched glycopeptides possible without loss of SA residues, and the terminal SA residues regenerated could be labeled directly by a fluorescence PA tag (Scheme 4). The advantage of PA-labeling with sialyl glycopeptides is that enhanced ionization potency by PA-derivatization should greatly facilitate the structural characterization of sialyl glycopeptides through ESI-MS and MS/MS analysis of ideal fragment ions. Moreover, it is well known that the PA-labeled glycopeptides can be monitored and quantified on HPLC by common fluorescence detector independent from the

peptide sequence. In this study, they considered that hydrazone linkage between glycopeptides and hydrazide-functionalized polymer supports appears to be much more sensitive to general conditions of acid hydrolysis than α 2,3/2,6 sialosides. Upon rapid, selective, and quantitative oxidation of the tryptic digests of whole glycoproteins derived from 50 μ l of mouse serum by treating with 30 mM NaIO₄ (final concentration) at 4 °C for 60 min, aldehydes at terminal SA residues were selectively captured at 37 °C for 2 h by chemical ligation with commercially available hydrazide modified polymer (Affi-Gel Hz). After thorough washing to remove nonspecifically bound molecules, glycopeptides covalently enriched were released by treating with ice-cold aqueous 1 M HCl as free aldehydes. One-pot reductive amination of the regenerated aldehydes with 2-AP was performed in the presence of 2-Pic-BH₃ (pH 7.0) at room temperature. This protocol greatly facilitated sensitive monitoring and accurate quantitation of mouse serum sialyl glycopeptides on LC-ESI MS. They identified 270 glycopeptides from 95 mouse serum glycoproteins as sialyl *N*-glycan carriers.

IV. Mass spectrometry analysis of sialylglycans

Mass spectrometry has become the practical analytical tool in glycomics and glycoproteomics. Two types of soft ionization techniques, MALDI and ESI, are generally used for the analysis of glycans. Four mass analyzer systems, time-of-flight (TOF), ion trap (IT), Fourier transform ion cyclotron resonance (FT-ICR), and Orbitrap are used in mass spectrometry for analysis of glycans. In addition, tandem MS methods such as TOF/TOF, QIT-TOF, LTQ FTICR, and LTQ-Orbitrap are used to analyze the sequence of glycans. MALDI-TOF MS is available for direct measurement of the mixtures of glycans samples. Although various matrix materials have been reported, DHB is most frequently used [76]. However, CHCA was employed for MS measurement of fully methylated glycans [77]. Detection sensitivity of ESIMS depends on the spray solvent. In positive-ion mode, water/methanol or acetonitrile containing a volatile acid is generally used to enhance ionization of glycans [78]. When various ions such as H⁺, Na⁺ and Li⁺ adduct ions are observed, Na salts are added in the spray solvent to suppress the formation of other ions. In negative-ion mode, water/acetonitrile containing ammonia or ammonium salts (ammonium bicarbonate or ammonium acetate) is often used. Water/acetonitrile without salt is also used in negative ion mode analysis. Selections of the solvents give distinctive effect on fragmentations of glycans in MS/MS analysis [79]. In contrast to MALDI MS which is used as a stand-alone technique, ESI MS is connected with separation techniques such as LC in the on-line manner. Especially, LC-ESI MS methods are often used for glycan analysis in clinical samples. Three separation modes of NP (normal phase), RP (reverse phase) and PGC (porous graphitized carbon) in HPLC are available in ESI MS. The suppression of sample diffusion and conditions for electronic spray are important to achieve sensitive detection in ESI MS analysis, and nano-LC-ESI MS methods are primarily used in microanalysis. In addition, chip-ESI MS method for glycan analysis has been developed [80]. In this section specific advances for both MALDI MS and ESI MS analysis of sialylglycans are described.

IV.1. MALDI MS analysis of sialylglycans

Under MALDI MS conditions, SAs are particularly labile to separate from sialylated glycans due to the instability of the α -glycosidic bond between SA and the adjacent sugar residue. Additionally, the presence of carboxylic acid moiety could often lead to multiple alkali metal adducts of sialylated glycans. Therefore, the spectra of sialoglycans often show multiple peaks as a result of ion formation from both the free acid and the corresponding salts. Furthermore, in-source and post-source losses of SA often lead to inaccurate representation of the degree of sialylation. Thus, it was significantly difficult to achieve accurate comparative analysis of sialylated glycans with MALDI MS previously. To

overcome the difficulties associated with MALDI MS analysis of sialylated glycans, instrumental improvements and inventions have been explored these years.

First, it is well known that the choice of the matrix is critical in MALDI MS. DHB is one of the most widely used matrices for glycan and glycopeptide analysis [76]. However, the application of DHB in sialylated glycans is very difficult because of the loss of SA groups or carboxylic groups leading to multiple peaks in the positive-ion mode. Therefore, some groups analyzed sialylated glycans in negative ion mode with an alternative matrix. Pitt et al. compared 2,6-dihydroxyacetophenone (DHAP) containing di-ammonium hydrogen citrate (DAHC), DHB and CHCA as matrices for sialylated glycopeptides, and then revealed that the DHAP/DAHC could afford higher quality spectra with superior resolution and less fragmentation [29]. Papac et al. reported that 2, 4, 6-trihydroxyacetophenone (THAP) was a preferred matrix for negative-ion spectra of pure mono-, di-, and trisialylated oligosaccharides in the linear mode [81]. THAP provided a lower limit of detection and gave less prompt fragmentation. Incorporation of ammonium citrate into the matrix, along with vacuum drying of the sample, was required in order to obtain maximum sensitivity with THAP. Wheeler et al. reported that the Darabinosazone was a more satisfactory MALDI matrix for sialylated glycans than CHCA and 2-(4-hydroxyphenylazo) benzoic acid (HABA) and DHB [82]. In addition, the DHAP and THAP showed as “cooler” matrices in that they did not cause prompt fragmentation to lose COOH. But they are still too “hot” to allow sialylated oligosaccharides to pass through the reflector without losing a significant percentage of SA. Sagi et al. compared the relative sensitivity of solid MALDI matrices 6-aza-2-thiothymine (ATT) with di-ammonium citrate (DAC); THAP; THAP with DAC; DHB for MALDI MS analysis of acidic *N*-glycans. The most favorable results for native acidic *N*-glycans were obtained with THAP/DAC (20 g/L/20 mM) as a matrix [83]. Snovida et al. demonstrated the application of DHB/*N,N*-dimethylaniline (DMA) matrix for analyzing the native glycan mixtures containing neutral and sialylated oligosaccharides by MALDI MS. With DHB/DMA the sialylated glycans may be automatically identified and quantitatively analyzed [84]. Recently, Colsch et al. reported a matrix DHAP/ammonium sulfate/heptafluorobutyric acid (HFBA) [85]. They found that the new matrix could maximize the detection of all sialylated sphingoglycolipids present in a tissue section.

Besides the solid matrices, the ionic liquid matrices (ILMs) were introduced by Armstrong et al. [86] to analyze the labile sulfated oligosaccharide [87, 88]. The essential point is that the ILMs consist of a conventional solid MALDI matrix and an organic base, e.g., tributylamine, pyridine, or 1-methylimidazole, which enables a relative state of “liquidity” under vacuum conditions [89]. Fukuyama et al. synthesized a new ILMs with 1,1,3,3-tetramethylguanidium (TMG) salt of pcoumaric acid (G3CA) [90]. They demonstrated that both sialylated and neutral oligosaccharides were detected with high sensitivity (e.g., 1 fmol) in the positive ion extraction mode and the dissociation of SAs was also particularly suppressed with G3CA. Chan et al. investigated an ionic liquid matrix ImCHCA (CHCA mixed with 1-methylimidazole) that offered excellent sensitivity for detection gangliosides (containing different numbers of SA residues) without significant loss of SA residues for mass spectrometry [91].

Furthermore, to decrease the metastable fragmentation, different pressures of MALDI MS were investigated to analyze the sialylated oligosaccharides. O'Connor et al. detected the fragile gangliosides without loss of the SA residue and decreased fragmentation significantly with a high-pressure (1–10 mbar) MALDI- FT-ICR MS [30]. Then von Seggern et al. first built an IR atmospheric pressure (AP)-MALDI-IT MS approach for the analysis of sialylated glycans utilizing liquid matrices including water, nitrobenzyl alcohol, and glycerol. And this “soft” ionization produced by AP-MALDI was effective in determining structural features-sequence, branching, linkages of intact and fully sialylated molecular

species [92]. Zhang et al. observed non-covalent complexes of sialylated glycans in minimized fragmentation for both positive and negative mode mass analyses with AP-MALDI-FT-ICR MS [93]. These developments would facilitate the analysis of sialylated glycoconjugates.

An alternative approach to analyze the sialylated glycans is to utilize the chemical derivatization such as permethylation and perbenzoylation before MALDI MS. These methods have proved to suppress the preferential cleavage of SA and enhance the MALDI MS intensity and sensitivity. Firstly, permethylation of acidic glycans is a commonly used method of derivatization to neutralize acidic glycans. Hakomori originally described that the DMSO was utilized to permethylate the glycans [94] and Ciucanu et al. introduced the addition of methyl iodide to DMSO containing powdered NaOH [95]. Kang et al. developed an on-line permethylation procedure for acidic oligosaccharides analysis by MALDI MS, leading to the use of capillary reactors packed with powdered NaOH [96]. Subsequently, they described another very useful high-throughput extension of the solid-phase methodology utilizing spin columns packed with NaOH beads [97]. Mechref et al. demonstrated the benefits of permethylation to differentiate structural isomers of sialylated glycans with MALDI-TOF/TOF MS [98]. However, permethylation is unsuitable for the analysis of biologically important sialoglycans containing partially *O*-acetylated SAs. This is because *O*-acetyl groups are easily decomposed under the harsh conditions used for permethylation, making it difficult to assess which positions were originally *O*-acetylated [43].

On the other hand, perbenzoylation [99] was also employed for the MALDI MS analysis of sialylated glycans. Chen et al. reported that perbenzoylation with benzoic anhydride could stabilize the sialyl residues for the MALDI MS measurements and differentiate terminal α 2,3 and α 2,6 sialogalactosylated linkages at subpicomole levels [100]. Arabinosazone appeared to be a suitable MALDI matrix for both the benzoylated sialoglycans. This study demonstrated that the benzoylation/ MALDI MS approach, when coupled with a use of selective neuraminidases, could also reveal sialyl linkage information on other multisialylated glycans. In addition, several chemical derivatization methods to modify the carboxylic acid of SAs such as methyl ester or amide formation, and acetohydrazidation have been employed for the MALDI MS analysis of sialylated glycans. All these were described in detail in the **Section II** above.

IV.2. ESI MS analysis of sialylglycans

ESI is one of the atmospheric pressure ionization (API) techniques and is well-suited to the analysis of polar molecules. In the late 1980's, Fenn and his co-workers successfully demonstrated the basic experimental principles and methodologies of the ESI technique, including soft ionization of involatile and thermally labile compounds, multiple charging of proteins and intact ionization of complexes [101, 102]. As an unusually gentle ionization method ESI could adjust conditions to evaluate "native molecules". In nanospray configuration ESI is more sensitive than MALDI because there is no matrix adduct peaks and the initial droplet is smaller, which lead to less concentration of suppressing molecules [26]. During the past decade, ESI has become a powerful tool for the detection of sialylated glycans and for acquiring structural information on them. Compared with MALDI-TOF MS, ESI tandem MS can select an ion easily and reanalyze it, which can give the information of the identical fragments, and sometimes is especially helpful in sialylated glycans identification when there is no any clue from MALDI MS.

Another major advantage of ESI is the ability to easily connect with liquid chromatography (LC). LC-ESI MS becomes a general method to simplify the complexity of the mixture, address this limitation and eliminate the suppression of salts or other sample components.

SAs do not need to be purified before analysis, and the derivatization reaction mixture including the individual derivated sialic acids is directly injected in the microbore LC column, and monitored by ESI MS. Morimoto et al. investigated the specific distributions of SAs in various tissues of rats and mice and found the LC-ESI MS technique revealed the significance of SA in tissues in detail [48]. Furthermore, isomer separation with LC in conjunction with ESI enhances the analysis of SAs. Valianpour et al. quantified the free SA in urine and demonstrated that the HPLC-ESI-tandem MS method for free SA in urine was more rapid, accurate, sensitive, selective, and robust and thus may serve as a candidate reference method for free SA in diagnosis of SSD [103]. Pan et al. reported that the α 2,3- and α 2,6-sialyl linkages could be different when analyzed using HPLC-ESI MS as the α 2,3- produced a prominent B2 (or B3 in disialyl lactose) mass fragment and this fragment was absent from mass spectra with α 2,6-linkages [104]. For very complex mixtures, it is often not possible to detect minor component SAs. But individual molecular species of SAs can be separately eluted in LC by their different sugar chain lengths, especially the number of SA residues. They can also be separately eluted by their fatty alcohol or *N*-acyl carbon chain lengths in gangliosides [105]. And 10–20 pmol of the compound with SA could obtain a complete spectrum, including molecular ions and CAD fragments [106].

Reverently, two kinds of ESI tandem MS, the triple quadrupole mass analyzer and ion-trap mass analyzer, were used to analyze the sialylated glycans. Wheeler et al. reported that negative ion electrospray quadrupole time-of-flight mass spectrometry (Q-TOF) could be used effectively for analysis of SA without derivatization [82]. Deguchi et al. found that it was especially useful for sialylated *N*-glycan structural analysis by using HPLC electrospray ionization linear ion trap time-of-flight mass spectrometry (ESI-LIT-TOF MS) and CID with helium as collision gas [107]. Chai et al. analyzed the chain and blood group type and branching pattern of sialylated glycans by Q-TOF. The main feature of this strategy is the unique double glycosidic cleavage induced by 3-glycosidic substitution that produces characteristic D-type fragments which can be used to distinguish the type 1 and type 2 chains, the blood group related Lewis determinants, and 3,6-disubstituted core branching patterns and to assign the structure details of each of the branches [108]. Wu et al. analyzed the enriched sialylated human milk oligosaccharides (SHMOs) from pooled milk sample by HPLC-Chip/QTOF MS. The instrument employed a microchip-based nano-LC column packed with porous graphitized carbon (PGC) to provide excellent isomer separation for SHMOs with highly reproducible retention time. A set of 30 SHMO structures with retention times, accurate masses, and MS/MS spectra was deduced and incorporated into the HMO library [109]. Compared with triple quadrupole, ion-trap tends to fragment the ions more thoroughly, i.e. higher efficiency in fragmenting. Casal et al. studied the fragmentation pattern of two couples of isomeric sialylated *O*-linked oligosaccharides by HPLC/ESI (–) MSⁿ using both an ion trap and a triple quadrupole mass spectrometer. They found that the ion traps seemed to be more appropriate than triple quadrupoles to develop a reliable analytical method to distinguish isomeric *O*-linked glycans [110].

Multiple reaction monitoring (MRM) is a mass detection by defined (or predicted) information and data gathering measurements on a target method. With highly selection, specificity and sensitivity, MRM has gradually attracted more attention of researchers for quantitative and qualitative analysis of sialylated glycans. Ikeda et al. developed an effective method for the targeted analysis of theoretically expected ganglioside molecular species, which are composed of different numbers of SA residues, by HPLC/ESI-quadrupole-linear ion trap hybrid mass spectrometer in combination with MRM. They found that the MRM detection specific for SA enabled them to analyze ganglioside standards at picomolar to femtomolar levels [108]. The same group later modified the method by combining the laser microdissection (LMD) with HILIC/ESI MS to analyze glycosphingolipids from mouse brain sections. Therefore, this method would be applicable to the local analysis of

pathological tissues in cancers and nervous system diseases, and thus specific biomarkers could be detectable as compared to normal tissues [109].

V. Conclusion

SAs are terminal residues in carbohydrate chains on cell surface and are involved in both physiological and pathological pathways. In addition, plasma free SA and sialoglycans have shown great potential for disease biomarker discovery. During the past decade, the developments of MALDI and ESI mass spectrometry have greatly accelerated the use of MS-based technology in structural analysis of sialoglycans. Particularly, simple and versatile method for avoiding the risk of desialylation during MS analysis has been explored. With the goals of addressing these issues in measurements of SA-containing oligosaccharides in mass spectrometry, as well as of enhancing the sensitivity and resolution, several approaches such as derivatization of SAs have been developed so far. On the other hand, the inherent ionization bias of MALDI MS may lead to preferential detection of unmodified peptides and partial or complete suppression of glycopeptides. In such cases, methods for removal of peptides and enrichment of sialoglycans by either lectin-affinity or chemical ligation method for the MALDI MS detection have been developed. These recent advances and continued development in SA-focused glycomics will contribute greatly to both biological research and biomedical practice in the future. Specifically it will play important roles for clarifying molecular mechanism of SA involved and discovering SA-based biomarkers which can be used for either disease diagnosis or monitoring therapeutic efficiency.

Highlights

Sialic acids existing on cell surface play important roles in biological system.

Plasma sialic acids show great potential for disease biomarker discovery.

Modification of sialic acids enhances their stability in mass spectrometry analysis.

Modification of sialic acids improves their ionization in mass spectrometry analysis.

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Figure 1.

Structural diversity of sialic acids (SAs) and their natural linkages. All SAs share the common feature of having nine carbons, a carboxylic acid residue at the 1-position, and a variety of linkages to the underlying sugar chain from the 2-position. Various types of substitutions at 5-position, and acetyl group at the 4-, 7-, 8- and 9-positions combine with the linkage variation to generate the diversity of SAs found in nature. Neu5Ac: 5-*N*-acetylneuraminic acid, Neu5Gc: 5-*N*-glycolylneuraminic acid, KDN: 2-keto-3-deoxy-D-glycero-D-galacto-nononic acid or deaminated neuraminic acid.

Figure 2.
Structures of SA derivatives for mass spectrometry analysis.

Scheme 1.

Scheme for the preparation of DMB derivatives of sialic acids.

Scheme 2.

Lactonization of oligosialic and polysialic acid: (A) α 2,8-linked oligosialic and polysialic acids and (B) α 2,9-linked oligosialic and polysialic acids.

Scheme 3.

Chemical ligation-based capture of sialylated glycopeptides. Copied from Ref. [73].

Scheme 4.

An improved protocol for reverse glycoblotting and labeling technique. Copied from Ref. [75].