

Review

Sialic acid derivatization for glycan analysis by mass spectrometry

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Abstract: Mass spectrometry (MS) is a well-accepted means for analyzing glycans. Before glycan analysis by MS, several chemical derivatizations are generally carried out. These are classified into three categories; (1) labeling of the reducing end of glycans, (2) permethylation, and (3) sialic acid derivatization. Because sialic acid residues are unstable, they are easily lost during pretreatment and during or after ionization in a mass spectrometer. Sialic acid derivatization can prevent the loss of this residue. Recently, new types of sialic acid derivatization techniques have been developed, which allow straight-forward sialic acid linkage analysis (α 2,3-/ α 2,6-linkages) as well as residue stabilization. This review summarizes the developments in sialic acid derivatization techniques, especially the varied methods of sialic acid linkage-specific derivatization.

Keywords: sialic acid, derivatization, glycan analysis, linkage analysis, mass spectrometry

1. Introduction

Protein glycosylation, which is one of the most common forms of co- or post-translational modifications, plays a crucial role in biological functions such as cellular localization, turnover, and protein quality control.^{1)–4)} Thus, protein glycosylation analysis is becoming increasingly important in the field of post-proteomics. Glycosylation can be classified into two categories, *N*-glycans and *O*-glycans. *N*-glycans are attached to asparagine (Asn, N) residues, whereas *O*-glycans are attached to serine (Ser, S) or threonine (Thr, T) residues. Additionally, several unique types of *O*-glycosylation have been identified, including *O*-fucose (Fuc),⁵⁾ glucose (Glc),^{6),7)} *N*-acetylglucosamine (GlcNAc),^{8),9)} and mannose (Man)^{10),11)} glycans.

The use of mass spectrometry (MS) in detecting glycans is a relatively new approach; however, MS has now become an indispensable tool for analyzing protein glycosylation. MS is a technique where the mass of ions is determined in the gas phase, and it is necessary to both charge the glycans and then

transfer them from the condensed phase to the gas phase *via* evaporation.^{12),13)} This step is termed “ionization” in MS. Both electrospray ionization (ESI)¹⁴⁾ and matrix-assisted laser desorption/ionization (MALDI)^{15),16)} have been mainly used to ionize fragile glycoconjugates, including released glycans, glycopeptides, and glycoproteins. Structural characterization using MS mainly relies on tandem MS (MS/MS). In this technique, analyte ions of interest are selected as precursors and broken down, and the mass of fragment ions are measured. Because the mass of fragment ions reflects the structure of unfragmented original ions, their molecular structure can be estimated. Although a wide variety of ion fragmentation techniques have been developed,¹⁷⁾ the collision-induced dissociation technique in which ions are fragmented by collision with an inert gas is the most common, especially in glycan analysis. MS itself may be inherently unsuitable for analyzing isomer-rich glycans, which cannot be distinguished by MS alone. To compensate for the shortcomings of MS in isomeric glycan analysis, ESI-MS is often combined with various chromatographic techniques to separate the glycan isomers.

Although the MALDI technique cannot be directly combined with a chromatographic separation technique, MALDI-MS offers several advantages over ESI-MS. In particular, MALDI-MS facilitates: (i) relatively simple spectral interpretation of the

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production of singly charged ions; (ii) high-throughput measurements, and (iii) repeated measurements of the same sample. Thus, MALDI-MS has been widely used in a variety of applications for the analysis of glycans and glycopeptides, especially for high-throughput, multi-analyte measurements. By using a disposable MALDI sample plate, the risk of carryover can also be eliminated. However, glycosylation analysis by MS still remains a challenging task because of the low ionization efficiency, the labile nature of residues, and the complicated branched structures involving various linkage isomers. These can be attributed to the highly hydrophilic property of glycans, which potentially disturbs effective desorption from the condensed phase into the gas phase. Enhancing ionization efficiency is an important field of study in achieving highly sensitive MS-based glycosylation analysis.

The presence of sialic acid residues on glycans/glycopeptides offers other analytical difficulties. Sialyl bonds are highly unstable compared with other glycosidic bonds, leading to instantaneous loss of the residues during MS analysis. Strong negative charge retention on the residues also causes quantitative difficulties. The presence of sialyl linkage isomers increases the difficulty of analysis of sialylated glycans.

To facilitate glycan analysis by MS, chemical derivatization is often carried out. The derivatization can mainly be categorized into three types; (1) glycan reducing end labeling, (2) permethylation, and (3) sialic acid derivatization. It is also possible to carry

out two or more types of chemical derivatization in a single sample.

The standard method for glycan chemical labeling is reductive amination for the reducing end, in which the glycans are labeled by aromatic hydrocarbons with amine groups in the presence of reductive reagents. This labeling has been developed for high-performance liquid chromatography (HPLC) analysis with ultraviolet or fluorescence detectors. Most of the labeling reagents have an aromatic structure, which increases the hydrophobicity of the glycans; therefore, the reducing end labeling typically enhances ionization efficiency in MS.¹⁸⁾

Permethylation is a reaction in which hydrogens of hydroxyl, amine, and carboxyl groups are replaced by methyl groups. Permethylation can improve sensitivity of MS by increasing ionization efficiency. This may be due to the increased hydrophobicity caused by the incorporation of a large number of methyl groups in a glycan molecule. There are several original and refined reports on the permethylation procedure.^{19)–23)}

The need for (3) sialic acid derivatization is described below.

2. Difficulties in analyzing sialylated glycans by mass spectrometry

Sialic acids, a family of acidic 9-carbon carbohydrates (Fig. 1), often exist on the non-reducing ends of *N*-glycans and *O*-glycans as capping residues of Gal and GalNAc residues *via* mainly the α 2,3-, α 2,6-linkages. α 2,3-linked sialic acids can be further

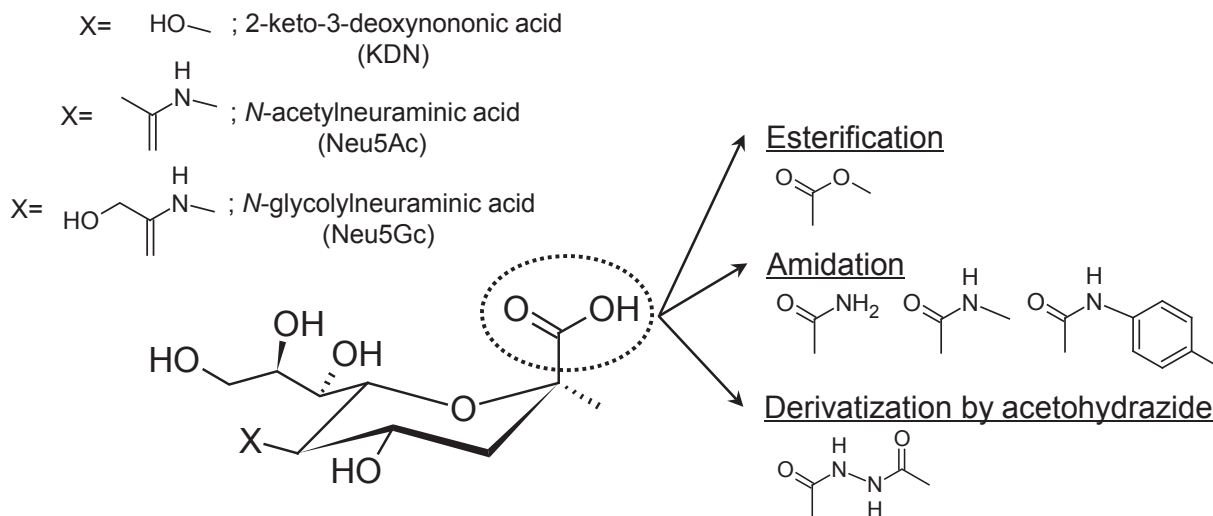


Fig. 1. Sialic acid families and derivatization scheme (linkage-nonspecific).^{31)–34),36),37)}

modified by α 2,8- and α 2,9-linked sialic acid residues. Sialylated glycans play important roles in various biological processes including viral infection²⁴⁾ and cancer development.²⁵⁾ Serum sialylation changes associated with various types of cancer have been investigated as a potential tumor marker for early and accurate detection.²⁶⁾ This indicates the importance of elucidating glycan structures including sialylation patterns (*i.e.*, the number of sialic acid residues and their linkage types).

The presence of sialic acid residues on glycans, however, causes various analytical problems in MS. In many cases, glycan analysis by MS is conducted in positive-ion mode analysis due to their good affinity with alkali-metal cations to form $[M + Na]^+$ or $[M + K]^+$ readily. The presence of strong negative charges on sialic acids generally decrease their ionization efficiency, biasing mass spectrometric quantification. In addition, due to the instability of the residues, sialic acids are readily lost in mass spectrometric steps (*i.e.*, during and/or after the ionization event) as well as in pre-treatment stages. Unfortunately, the loss of sialic acid residues by MALDI-MS is more pronounced than that of ESI-MS, probably due to the higher internal energy of the ions formed by MALDI events compared with the ions formed by ESI.

In MALDI, analyte molecules are mixed with large amounts of “matrix” molecules and then ionized using laser irradiation. The MALDI phenomenon is not fully understood yet; however, the function of the matrix is considered to be as follows; (1) absorption of irradiated laser energy; (2) the main actor causes desorption phenomenon by converting photon energy into kinetic energy; (3) ionizing reagents for analyte molecules (*e.g.*, proton donor/acceptor). Of note, the nature of the matrix molecules impacts the degree of analyte fragmentation, because the internal energy deposited on the analyte molecules depends on the nature of the matrix used.

To prevent the loss of sialic acid residues in MALDI-MS, several “cold” matrices, which induce less fragmentation of glycan ions during MS analysis, have been investigated;^{27)–30)} however, this has not been a satisfactory approach because it is difficult to completely stabilize the residues. Furthermore, the presence of carboxyl groups ($-COOH$) in sialic acids often leads to multiple salt formations ($-COONa$, $-COOK$, $-COONH_4$ *etc.*), depending on the experimental conditions. These salt structures have different masses, thereby one acidic glycan appears as multiple peaks in the mass spectrum. They not only complicate mass spectral interpretation but also reduce sensitivity of acidic glycan analysis.

The use of negative-ion mode might be an alternative approach for sialylated glycan MS analysis; however, the above analytical problems have not been solved. The loss of sialic acid residues and unfavorable salt formation are generally suppressed in the negative-ion mode analysis, but is not completely eliminated. Ionization bias between sialylated and neutral glycans cannot be eliminated, because acidic glycans readily form negatively charged ions (*i.e.*, $[M - H]^-$) compared with neutral glycans.

3. Linkage-nonspecific derivatization

Sialic acid derivatization provides a fundamental solution to the above problems. By neutralizing carboxyl groups, the loss of sialic acid and multiple alkali metal adduction are suppressed. Moreover, sialic acid derivatization inhibits preferential negative ion formation, allowing acidic glycans to possess similar ionization efficiencies as the neutral glycans. For this purpose, various types of derivatization methods have been reported. The established methods are summarized in Table 1 and Fig. 1.

The most conventional derivatization method is simple esterification. Powell *et al.* first introduced methyl esterification for stabilizing sialic acids in *N*-

Table 1. Linkage-nonspecific sialic acid derivatization

Nucleophile	Condensing reagents or main reagents	Sialic acid derivative	Reaction condition	Publication
—	CH ₃ I, DMSO	Methylester	Room Temp., 2 hours	Powell <i>et al.</i> , 1996 ³¹⁾
Ammonium chloride (NH ₄ Cl)	DMT-MM	Amide	50 °C, 24 hours	Sekiya <i>et al.</i> , 2005 ³³⁾
—	MTT	Methylester	50 °C, 1~2 hours	Miura <i>et al.</i> , 2007 ³²⁾
Acetohydrazide	EDC (pH 2.5)	Amidation by acetohydrazide	Room Temp., 2 hours	Toyoda <i>et al.</i> , 2008 ³⁴⁾
Methylamine chloride	PyAOP, NMM	Methylamide	Room Temp., 30 minutes	Liu <i>et al.</i> , 2010 ³⁷⁾
<i>p</i> -Toluidine	EDC (pH 4.5)	Amidation by <i>p</i> -toluidine	Room Temp., 4 hours	Shah <i>et al.</i> , 2013 ³⁶⁾

glycans and gangliosides.³¹⁾ They used methyl iodide for esterifying the carboxyl groups on sialic acids. The sialic acid residue is stable after methyl esterification, irrespective of its linkage to the adjacent monosaccharide residue. Miura *et al.* presented unique methyl esterification for derivatizing glycans attached to a solid support.³²⁾ In general, solid-phase esterification is more difficult than reactions in solution. They employed a triazene derivative, 3-methyl-1-*p*-tolyltriazene, for efficient and facile methyl esterification of glycans immobilized onto hydrazide beads.

As another type of derivatization, amidation of sialic acid residues using a condensing reagent was introduced by Sekiya *et al.*³³⁾ Condensing reagents couple sialic acid with nucleophiles such as amines and alcohols. They used 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride (DMT-MM) as a condensing reagent to couple the carboxyl group on the sialic acid with ammonium chloride, forming a simple amide bond. An advantage of amidation over esterification is the stability of the derivative. Ester bonds can decompose in the MS/MS stages of structural analysis, resulting in the neutral loss of alcohol molecules. On the other hand, amides do not decompose during the collisional activation step. By using a stable isotope labeled with ¹⁵NH₄Cl instead of normal NH₄Cl, the *m/z* change of sialoglycans before and after derivatization could be preserved to 0.0013 Da.

For these "linkage-nonspecific" derivatizations, the difference in derivatization efficiency between α 2,3- and α 2,6-linked sialic acids is an important issue. Incomplete derivatization weakens quantification accuracy, reducing analytical usefulness. Toyoda *et al.* pointed out that the modification of α 2,3-linked sialic acids proceeds less efficiently than those at α 2,6-linkages, resulting in incomplete derivatization of α 2,3-linked sialic acids. To address this issue, they introduced quantitative derivatization for both α 2,3- and α 2,6-linked sialic acids.³⁴⁾ Amidation by acetohydrazide using 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) hydrochloride in acidic conditions (pH 2.5) successfully converted the carboxyl groups on sialic acids to the corresponding acetohydrazide derivatives regardless of linkage type. Quantitative amidation with acetohydrazide has been used in one-pot sialic acid derivatization of glycoproteins on a 96-well format 10K molecular weight cut-off membrane.³⁵⁾ Shah *et al.* extended the acidic reaction conditions to derivatize sialic acids by an aromatic amine, *p*-toluidine.³⁶⁾ The derivatization of sialic

acids with *p*-toluidine made the glycans hydrophobic, allowed stronger retention on C18 columns, and improved ionization of glycans. They also presented an example of relative quantification using both normal and heavy (D9) *p*-toluidine. Other facial amidation by methylamine (MA) was introduced by Liu *et al.*³⁷⁾ The methylamidation could derivatize both α 2,3- and α 2,6-linked sialic acids, probably owing to the use of a powerful condensing reagent, PyAOP. Because the reaction conditions were mild, unstable modifications such as *O*-acetylations on sialic acids were preserved.³⁸⁾

4. Linkage-specific derivatization

Although the derivatization methods mentioned above can effectively stabilize sialic acid residues, sialyl linkage isomers cannot be distinguished by these methods. The linkage isomers have identical mass; therefore, mass spectrometric distinction still remains a great challenge, even after the introduction of "linkage-nonspecific" derivatization methods.

The difference in sialyl linkages can be associated with various biological processes. The most famous example is the influenza virus infection.^{39),40)} Avian influenza hemagglutinin preferentially recognizes α 2,3-linked sialic acids, whereas human influenza hemagglutinin preferentially recognizes α 2,6-linked sialic acids. The recognition process is the first step in animal influenza virus infection. However, a virus variant of avian origin with an altered binding specificity for α 2,6-linked sialic acids capped glycans may acquire transmutability in humans, increasing the risk of a pandemic. The epithelial cells of the pig trachea have both α 2,3- and α 2,6-linked sialic acids, making them a fortuitous intermediate host for viral avian-to-human transmission. Aberrant glycosylation has also been recognized to be one of the signatures of cancer, because glycans participate in many cancer-associated events. Cancer-associated glycosylation changes often involve specific sialic acid linkages.^{26),41),42)}

There are two main approaches in the differentiation of α 2,3-/ α 2,6-linkages. The use of α 2,3-sialidases is a traditional approach. Although this method has proven successful, specificity of discrimination directly depends on the specificity of enzymes for the α 2,3-linked sialic acid residues. Therefore, the enzymatic reaction conditions need to be precisely controlled. In some cases, not all of the α 2,3-linked sialic acids are detached by the enzyme.⁴³⁾ Some sialidase-resistant structures including Sd^a antigen, Cad antigen, glycolipid GM1 and GM2 structure

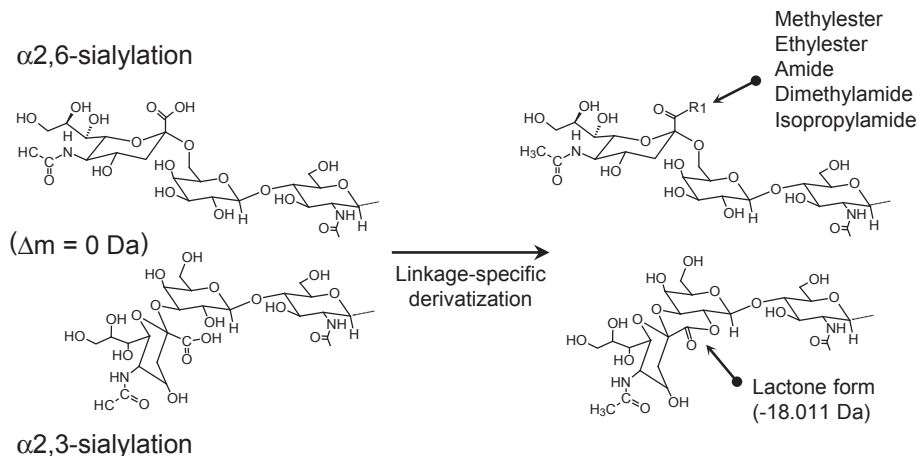


Fig. 2. Derivatization scheme of sialic acid linkage-specific derivatization.^{48)–52)}

limit their application. Nevertheless, linkage-specific sialidase treatment followed by MS has been used in the combination with various separation methods such as hydrophilic-interaction liquid chromatography (HILIC)^{44),45)} and ion mobility (IM) spectrometry.⁴⁶⁾ In MALDI-MS, “on-target” exoglycosidase digestion can be performed. Colangelo *et al.* optimized “on-target” exoglycosidase digestion conditions so that the digestion completes within 30 minutes at room temperature.⁴⁷⁾ After digestion, MS can be directly acquired by adding MALDI matrix onto the target.

Another approach is by using chromatographic separation followed by retention-time matching. Because retention times are influenced by the linkage type, we can identify the linkage types by comparing with the retention time of a previously analyzed glycan standard. It is also possible to estimate retention time of a new glycan structure; however, not all cases are successful, especially for multiply-sialylated large glycans.

As mentioned above, MS is inherently unsuited for isomer differentiation, including sialic acid linkage analysis. However, MS can differentiate glycan peaks, as long as they possess different mass. Recently, several unique derivatization methods for MS-based α 2,3-/ α 2,6-differentiation have been developed where α 2,3- and α 2,6-linked sialic acid residues have different masses.^{48)–52)} So far, all of the methods utilize the difference in reactivity of the carboxyl groups on the α 2,3- and α 2,6-linked sialic acid residues. They are based on a common approach: α 2,6-linked sialic acids react with external nucleophiles, whereas under the same reaction conditions, α 2,3-linked sialic

acids form lactones by intramolecular dehydration (Fig. 2). The resulting mass difference allows the direct discrimination of sialyl linkage isomers. For these types of reaction, the reaction conditions should be strictly controlled. In particular, the choice and combination of the condensing reagent(s) and nucleophiles are important factors in the successful distinction of sialyl linkages. The established methods are summarized in Table 2.

As an example, mass spectra of isomeric glycans after linkage-specific derivatization is shown in Fig. 3. Without derivatization, these isomeric glycans are detected in the mass spectra in identical m/z positions. By performing linkage-specific derivatization, isomeric glycans acquire an m/z change depending on the linkage types and their number.^{48)–52)} Figure 4 shows an example comparing the *N*-glycan mass spectra of bovine fetuin derivatized by linkage-nonspecific or linkage-specific derivatization. Linkage-nonspecific derivatization just stabilizes the sialic acid residues, whereas linkage-specific derivatization additionally provides linkage information by splitting a single peak into multiple peaks depending on the linkage type.

Originally, Wheeler *et al.* introduced linkage-specific methyl esterification using a condensing reagent DMT-MM and methanol as a nucleophile.⁴⁸⁾ In these derivatization conditions, α 2,6-linked sialic acids were converted to methyl esters by the reaction with methanol, whereas α 2,3-linked sialic acids formed lactones by intramolecular dehydration. The resulting mass difference obtained from the mass spectra allowed linkage-specific differentiation of the types of sialic acids.

Table 2. Linkage-specific sialic acid derivatization

(1st step) Linkage-specific derivatization			(2nd step) Lactone stabilization			
Nucleophile	Condensing reagents and reaction condition	Derivatives (α 2,6-/ α 2,3-)	Reaction condition for lactones originating from α 2,3-	Total reaction time for 2-step derivatization	Publication	Name
MeOH	DMT-MM, 80 °C, 1 hour	Methylester/ Lactone	—	—	Wheeler <i>et al.</i> , 2009 ⁴⁸⁾	
Ammonia	DMT-MM, 60 °C, 15 hours	Amide/ Lactone	Solid-phase permethylation, room temp., 0.5 hour	Over 15 hours	Alley <i>et al.</i> , 2010 ⁴⁹⁾	
EtOH	EDC+HOBt, 37 °C, 1~2 hours	Ethylester/ Lactone	—	—	Reiding <i>et al.</i> , 2014 ⁵⁰⁾	
			Lactone cleavage (pH 10, 1 hour) followed by methylamidation by PyAOP (room temp., 0.5 hour)	2.5 hours	Li <i>et al.</i> , 2016 ⁵⁴⁾	STSD
			Amidation by <i>p</i> -toluidine, room temp., 3 hours	5 hours	Yang <i>et al.</i> , 2017 ⁵⁷⁾	
			Amidation by ethylenediamine (EDA), 37 °C, 3 hours	5 hours	Yang <i>et al.</i> , 2018 ⁵⁸⁾	
Dimethylamine	EDC+HOBt in DMSO, 60 °C, 1~3 hours	Dimethylamide/ Lactone	—	—	de Haan <i>et al.</i> , 2015 ⁵¹⁾	
			Amidation by EDC+HOBt, 60 °C, 2 hours	3 hours	Holst <i>et al.</i> , 2016 ⁵⁵⁾	Double amidation
			Permethylation	2.5 hours	Jiang <i>et al.</i> , 2017 ⁵⁶⁾	SSAP
Isopropylamine	EDC+HOBt in DMSO, room temp., 1~2 hours	Isopropylamide/ Lactone	Methylamidation by PyAOP, room temp., 1 hour	2 hours	Nishikaze <i>et al.</i> , 2017 ⁵²⁾	SALSA
			Aminolysis by methylamine or ethylamine, room temp., Few seconds	1 hour	Hanamatsu <i>et al.</i> , 2018 ⁵⁹⁾	Aminolysis-SALSA
			Methylamidation by EDC+HOBt, 60 °C, 2 hours	4 hours	Suzuki <i>et al.</i> , 2018 ⁶⁰⁾	Modified-SALSA

Recently, Reiding *et al.* reported more practical linkage-specific esterification using a different condensing reagent mixture.⁵⁰⁾ They concluded that the use of a carbodiimide-based condensing reagent mixture (EDC and HOBt) and ethanol as a nucleophile results in ethyl esterification of α 2,6-linked sialic acids and lactonization of α 2,3-linked sialic acids. This type of reaction appears to be robust, allowing direct derivatization of PNGaseF-released glycan mixture including detergents, buffer, and deglycosylated proteins without a prior desalting

step. This linkage-specific ethyl esterification has been incorporated into automated platforms for glycomics analysis of large scale plasma *N*-glycans.⁵³⁾ Based on the Reiding's linkage-specific esterification condition, several amidation versions of linkage-specific derivatization methods have been developed using dimethylamine⁵¹⁾ or isopropylamine⁵²⁾ as a nucleophile.

Although the conversion from α 2,3-linked sialic acids into lactone forms actually stabilizes the sialic acid residues, the lactone forms are still unstable. In

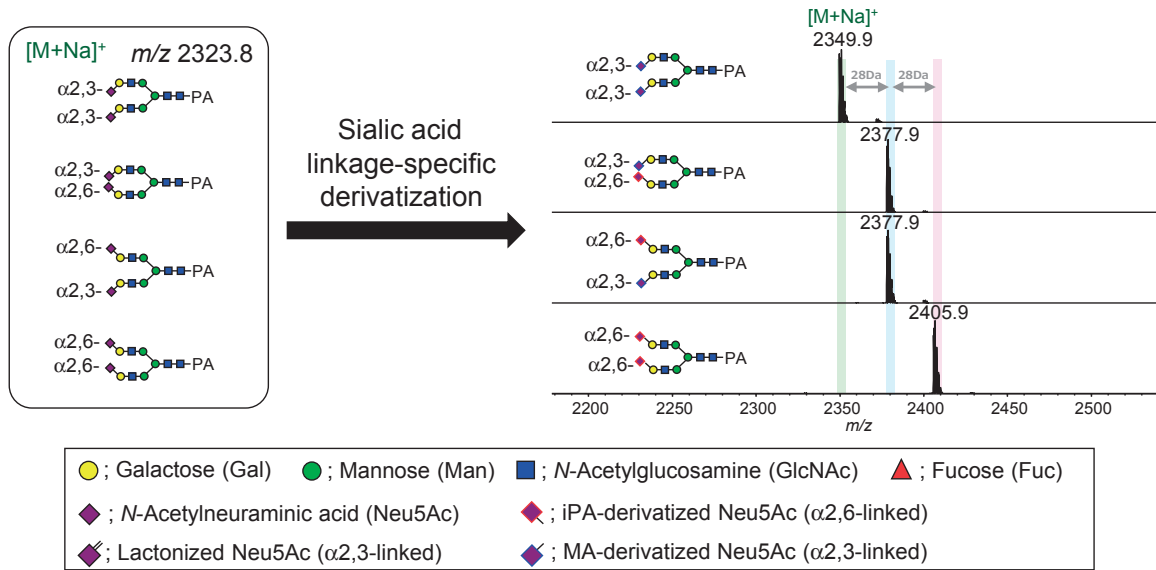


Fig. 3. Example of glycan mass spectra after sialic acid linkage-specific derivatization. All pyridylamine (PA)-labeled glycan standards have identical mass before derivatization. Here, sialic acid linkage-specific alkylamidation (SALSA) was adopted to derivatize sialic acid; therefore, the mass difference between α2,3-/α2,6- is 28 Da.⁵²⁾

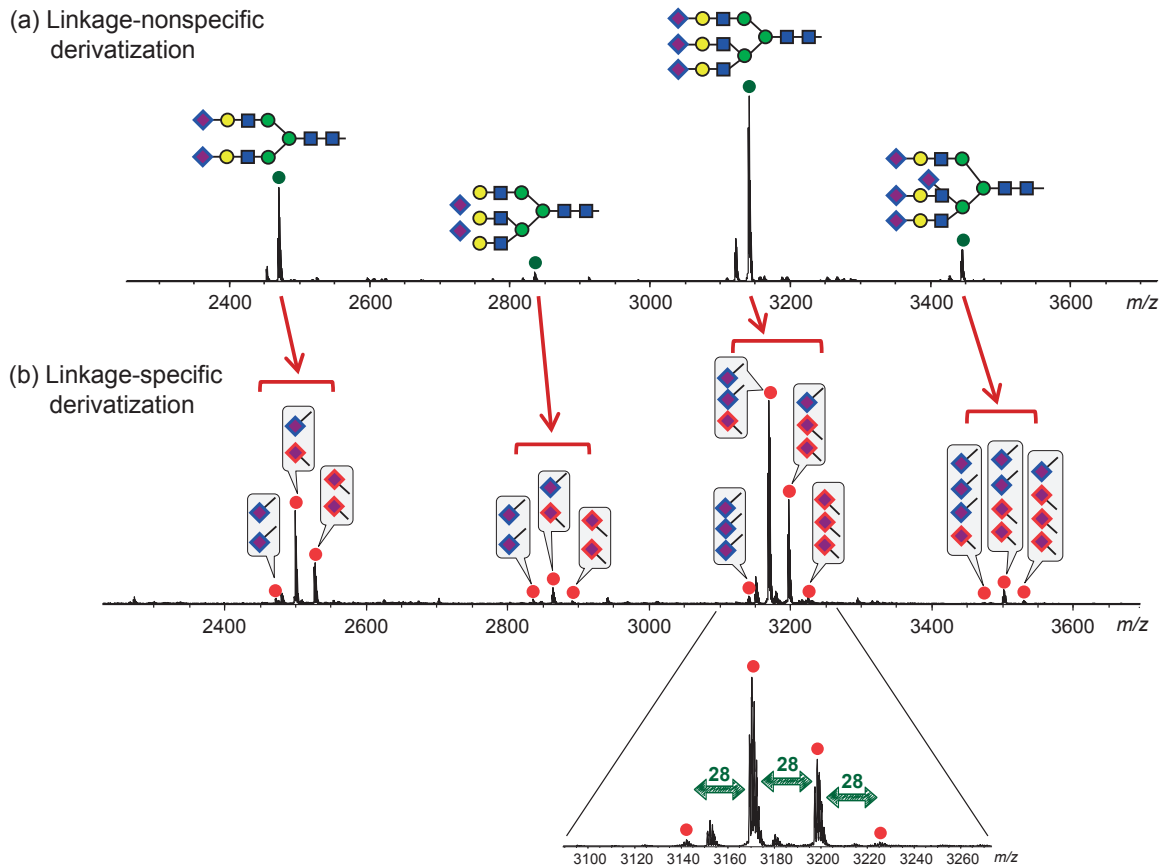


Fig. 4. Comparison of *N*-glycan mass spectra released from bovine Fetuin derivatized by (a) linkage-nonspecific methylamidation³⁷⁾ and (b) linkage-specific alkylamidation (SALSA).⁵²⁾

fact, Wheeler *et al.* pointed out that lactone can be completely degraded within 50 hours by just being dissolved in water.⁴⁸⁾ This instability can limit the downstream chemical and/or enzymatic treatments.^{54),55)} As pointed out by other researchers,⁵⁴⁾ lactone forms cannot survive enzymatic digestion for long, resulting in partial delactonization.

5. Linkage-specific derivatization with lactone stabilization

To overcome the limitations caused by lactone instability, two-step derivatization methods have been further developed recently.^{49),52),54)–60)} In a two-step reaction, α 2,3-/ α 2,6-discrimination specificity depends on the first reaction; the second step just converts the lactone forms into more stable forms. There are three methods for stabilizing the lactone form: permethylation, lactone cleavage followed by amidation, and direct aminolysis.

5-1. Linkage-specific derivatization + permethylation. In general, due to the harsh permethylation reaction conditions, ester bonds are easily cleaved. Therefore, linkage-specific esterification followed by permethylation is not suitable for linkage differentiation. Permethylation can only be combined with linkage-specific amidation (Fig. 5). Alley *et al.* developed linkage-specific amidation suitable for combination with the permethylation reaction.⁴⁹⁾ The newly developed linkage-specific amidation requires 15 hours incubation at 60 °C with ammonium chloride and DMT-MM. After this, amidated and/or lactonized glycans are permethylated in the solid-phase. By permethylation, amidated α 2,6-linked sialic acids are converted to dimethylamidated forms, whereas lactonized α 2,3-linked sialic acids

are converted to methylesterified forms, allowing m/z distinction by MS. More recently, Jiang *et al.* combined linkage-specific dimethylamidation with an in-solution permethylation technique. This linkage-specific sialic acid permethylation showed high derivatization specificity for α 2,3- and α 2,6-sialic acids.⁵⁶⁾ In the process, linkage-specific dimethylamidation using dimethylamine and EDC+HOBt was first carried out at 60 °C for 1 hour, and then the excess dimethylamine was evaporated by vacuum centrifugation for 20 min at 60 °C. A 10-minute permethylation was finally carried out by adding water, NaOH-dimethyl sulfoxide slurry, and methyl iodide at room temperature. Because the linkage-specific dimethylamidation shared the same reaction solvent of NaOH-dimethyl sulfoxide with permethylation, sample purification between the two steps could be avoided.

5-2. Linkage-specific derivatization + amidation. Lactone stabilization can also be achieved by temporary lactone cleavage followed by amidation (Fig. 6). This type of two-step method has been mostly developed for solid-phase reactions (*i.e.*, glycans or glycoproteins bound to a solid support). The methods require different sequential reaction conditions; therefore, by adopting a solid-phase reaction the reaction solution can be changed easily without extensive purification steps such as solid-phase extraction.

Li *et al.* introduced two-step reactions for linkage-specific derivatization of sialic acids on glycoproteins fixed to a solid support.⁵⁴⁾ They mixed linkage-specific esterification reported by Reiding *et al.*⁵⁰⁾ with linkage-nonspecific methylamidation by PyAOP.³⁷⁾ In the first reaction, α 2,6-linked sialic

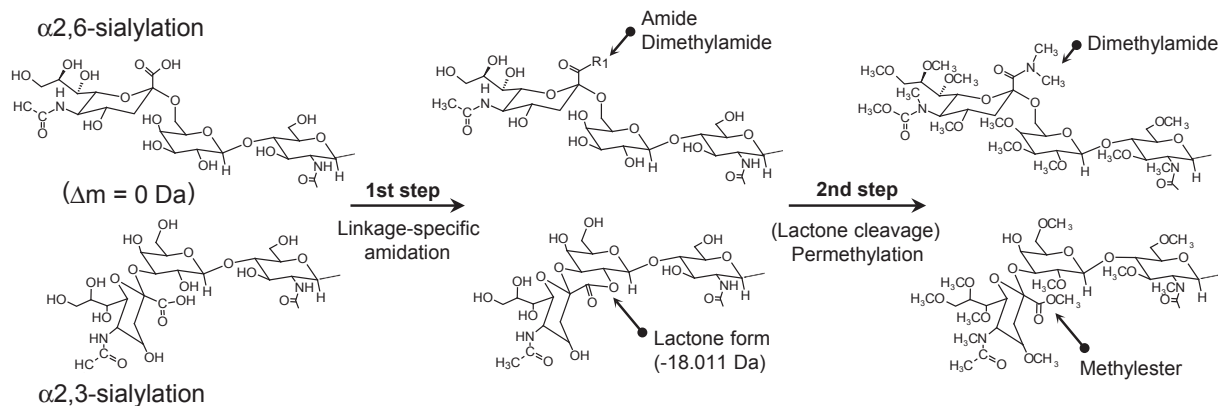


Fig. 5. Derivatization scheme of sialic acid linkage-specific derivatization combined with permethylation reaction as lactone stabilization step.^{49),56)}

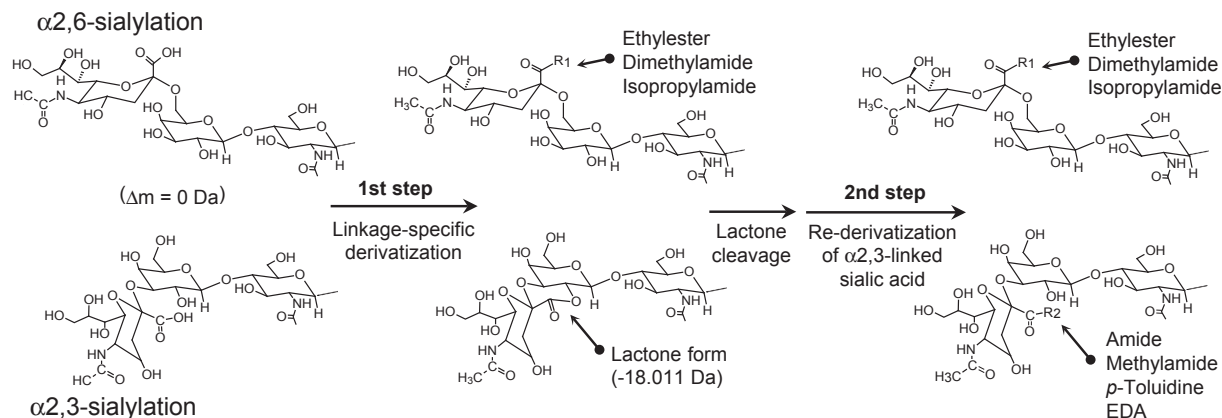


Fig. 6. Derivatization scheme of sialic acid linkage-specific derivatization combined with temporary lactone cleavage followed by re-derivatization of the carboxyl groups on $\alpha 2,3$ -linked sialic acids.^{52),54),55),57),58),60),69)}

acids were converted to ethylester whereas $\alpha 2,3$ -linked sialic acids were converted to lactones. The lactones were then cleaved at mild alkaline condition (pH 10 Tris-HCl solution, 1 hour incubation). Next, original carboxyl groups on $\alpha 2,3$ -linked sialic acids were converted to methylamide forms by the reaction with MA using the condensing reagent PyAOP. The final derivatives were as follows; $\alpha 2,3$ -linked sialic acid: methylamide, $\alpha 2,6$ -linked sialic acid: ethylester. This two-step derivatization (actually, three steps including the lactone cleavage process), requires completely different reaction conditions for each process. Therefore, the use of this two step derivatization method is in essence limited to the analyte derivatization on solid supports. Furthermore, alkali conditions can also partially cleave the ester forms originating from the $\alpha 2,6$ -linkages, therefore the lactone cleavage process needs to be strictly controlled. Recently, Yang *et al.* developed other two-step methods that employ linkage-specific esterification followed by amidation with *p*-toluidine.⁵⁷⁾ The final derivatives were as follows; $\alpha 2,3$ -linked sialic acid: *p*-toluidine derivative, $\alpha 2,6$ -linked sialic acid: ethylester. Unfortunately, there is an ionization bias between the *p*-toluidine derivatized form and the ethylester form, so an intensity correction is required for the quantitation using the signal intensities in mass spectra.

Holst *et al.* developed a linkage-specific double amidation method for obtaining sialyl linkage-specific *N*-glycan MS images from formalin-fixed, paraffin-embedded tissues.⁵⁵⁾ MS imaging of *N*-glycan requires sialic acid derivatization followed by glycan release by PNGaseF. Because lactone forms cannot survive this enzymatic treatment step, they introduced an

additional amidation step for stabilization of the lactone form. Their procedure was relatively simple; 1 hour linkage-specific amidation by dimethylamine followed by the direct addition of ammonia solution and 2 hour incubation at 60 °C. An additional ammonia adduction step initiated the hydrolysis of the lactones and the subsequent stable amidation of the $\alpha 2,3$ -linked sialic acids. They actually developed the method for on-tissue derivatization; however, the method is also suited to in-solution derivatization of sialic acids. Lageveen-Kammeijer *et al.* combined above lactone stabilization using ammonia with linkage-specific esterification using EDC + HOBt/ethanol. This two-step derivatization can be carried out as a one-pot reaction. Excess reagents can be removed using a single HILIC-solid-phase extraction clean-up step, offering simple handling over other two-step derivatization methods.⁶⁰⁾

Our group has developed a similar but more reliable amidation-based linkage-specific derivatization, named sialic acid linkage-specific alkylamidation (SALSA).⁵²⁾ SALSA converts carboxyl groups on sialic acid residues into alkylamide forms with different alkyl chain lengths in a linkage-specific manner, allowing direct discrimination of sialyl linkages based on the difference in *m/z* values. We employed isopropylamine (iPA) as the nucleophile for linkage-specific derivatization. The use of iPA offers selective conversion for distinguishing $\alpha 2,3$ - and $\alpha 2,6$ -linked sialic acids by converting them into lactone and isopropylamide forms. Lactone forms are then stabilized by amidation with MA. Methylamidation using a condensing reagent PyAOP appeared to directly convert lactonized $\alpha 2,3$ -sialic acids to their methylamide forms; however, employing a

lactone cleavage step before methylamidation improved conversion efficiency, especially for highly sialylated *N*-glycans containing α 2,3-linkages. The final derivatives were as follows; α 2,3-linked sialic acid: methylamide, α 2,6-linked sialic acid: isopropylamide. We combined derivatization and a one-pot glycan purification technique, glycoblotting.^{61)–63)}

5-3. Linkage-specific derivatization + ring-opening aminolysis. As described above, several researchers have tried to stabilize lactone forms by temporary lactone cleavage followed by amidation. We proposed a more straightforward approach. The second lactone stabilization process was replaced by direct amidation of the lactones, the so-called ring-opening aminolysis reaction (Fig. 7).⁵⁹⁾ It should be noted that the aminolysis of lactone is nearly instantaneous; therefore, no reaction time is required. Furthermore, this reaction does not require condensing reagents. Primary amine and hydrazine analogues can be used for ring-opening aminolysis, without causing unfavorable hydrolysis side-reaction.

We also demonstrated that the SALSA technique combined with ring-opening aminolysis (termed aminolysis-SALSA) can successfully distinguish serum glycosphingolipid (GLS)-glycan isomers in MS.⁵⁹⁾ The glycan structure in GLS is known to contain α 2,3-, α 2,6-, and additional α 2,8-linked sialic acid. The glycan moiety was first released from GLS by EGCCase I, and then the glycan moiety was captured by the hydrazide beads. Sialic acids were derivatized sequentially by aminolysis-SALSA on the beads. It was confirmed that α 2,8-linked sialic acids were derivatized by MA by aminolysis-SALSA. Although α 2,3- and α 2,8-linked sialic acid residues had identical mass even after aminolysis-SALSA,

α 2,8-linked sialic acid could be distinguished from α 2,3-linked sialic acids by MS/MS analysis. Because α 2,8-linked sialic acid residues are attached to other sialic acid residues to form polysialic acid structure, a characteristic neutral loss consisting of two or more sialic acid residues can be found in the MS/MS spectra. The characteristic neutral loss is less common in the MS/MS spectra of glycan with only α 2,3-linked sialic acid residues.⁵⁹⁾

6. Sialic acid derivatization on glycopeptides

Compared with the sialic acid derivatization of (released) glycans, the derivatization of glycopeptides remains a challenging task, mostly due to the difficulties in ensuring uniformity in the reactions occurring on the peptide moiety. At present, only a few reports are available, indicating that this is an ongoing research area.

Quantitative derivatization by acetohydrazide has been used to derivatize glycopeptides as well. Because the derivatization can cap carboxyl groups on both sialic acids and on the peptide backbone, other acidic groups such as sulfate groups can emerge, allowing the enrichment of sulfated glycopeptides.⁶⁴⁾ Methylamidation by PyAOP also shows highly effective and specific reactivity for carboxyl groups on glycopeptides. Methylamidation suppresses preferential loss of sialic acid residues during MS analysis and enhances important glycan fragments in negative-ion MS/MS conditions.⁶⁵⁾ Note that these derivatization techniques are linkage-nonspecific; therefore, sialyl linkages cannot be distinguished.

Linkage-specific sialic acid derivatization on glycopeptides is more challenging than the linkage-

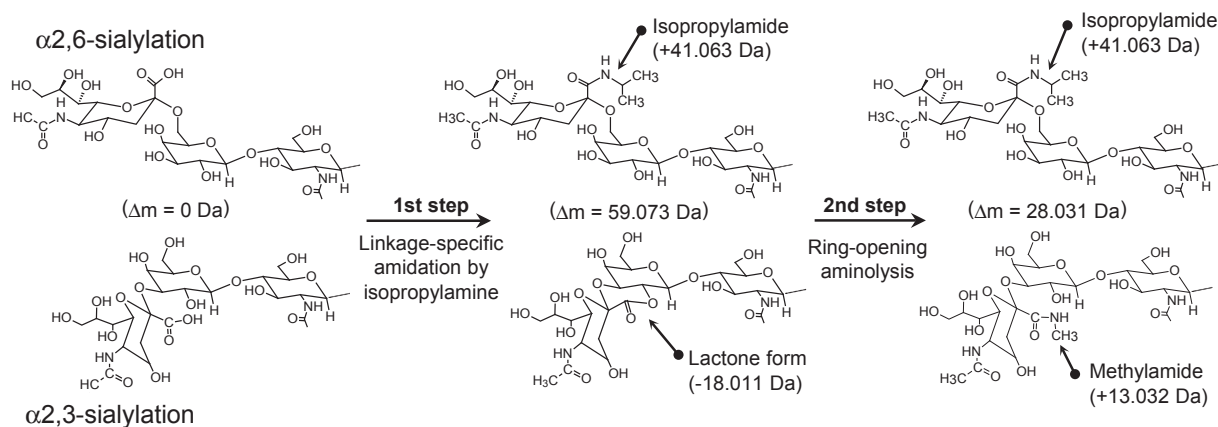


Fig. 7. Derivatization scheme of aminolysis-SALSA.⁵⁹⁾

nonspecific derivatization techniques mentioned above. de Haan *et al.* reported an application of linkage-specific derivatization using dimethylamine for IgG Fc glycopeptide derivatization.⁵¹⁾ The derivatization was successful, showing no side reactions affecting the peptide backbone. Because the report focused on only the derivatization of IgG Fc glycopeptides, other glycopeptide derivatizations were not shown. General use of linkage-specific dimethylamidation for the derivatization of a wide variety of glycopeptides needs to be optimized to ensure uniformity in the reactions occurring on the peptide moiety.

Recently, Yang *et al.* developed a linkage-specific derivatization approach for glycoproteomics.⁵⁸⁾ Glycoproteins were first immobilized on a solid support and then the sialic acids (and side chains on acidic amino acid residues) were derivatized in a linkage-specific manner. They used Reiding's linkage-specific ethylesterification followed by lactone stabilization by amidation with ethylenediamine (EDA). Glycopeptides were released from the solid support by trypsin digestion. Derivatization by EDA enhanced ionization efficiency, achieving special suitability for the production of multiply charged ions. Although sialic acid derivatization before trypsin digestion can suppress the reaction complexity, the derivatization conditions seem to have room for improvement in suppressing cross derivatization (*i.e.* ethylester or EDA derivative) for side chains on acidic amino acid residues.

7. Sialic acid derivatization for liquid chromatography MS or capillary electrophoresis MS

Although the advantage of sialic acid derivatization is mostly emphasized in MALDI-MS analysis, the neutralization of sialic acids also has several benefits in chromatographic separation using liquid chromatography (LC) and capillary electrophoresis (CE). However, the number of reports is limited so far.

Tousi *et al.* reported the combination of linkage-specific methylesterification using DMT-MM and nano-HILIC-MS.⁶⁶⁾ Linkage-specific charge neutralization of sialic acids not only allows discrimination of α 2,3-/ α 2,6-sialyl linkages by MS, but also improves the preceding HILIC separation by increasing hydrophobicity and altering the selectivity of the glycan analytes. Linkage-nonspecific methylamidation has been applied to nano-LC analysis of sialoglycans.⁶⁷⁾ Improvements in separation and peak shape were achieved in porous carbon graphitic columns.

Khatri *et al.* used Reiding's linkage-specific ethyl esterification for capping sialic acids on released glycans and glycopeptides to develop a microfluidics-based CE-MS system.⁶⁸⁾ If sialic acids are esterified, migration in CE and peak shape are less affected by the negatively charged sialic acid residues. They also pointed out the detection of unesterified forms of glycans and multiple backbone modifications of glycopeptides. Recently, Lageveen-Kammeijer *et al.* developed a highly sensitive CE/MS glycan analysis platform adopting a sequential derivatization technique. The negative charges of sialic acids are first neutralized by two-step sialic acid linkage-specific derivatization. The reducing end of the glycan is then labeled by Girard reagent P to give permanent positive charges. As a result, all neutral and sialylated glycans have uniform charge, enabling efficient ionization in positive ion mode. By optimizing the ionization conditions, they achieved attomole level glycan analysis sensitivity with excellent reproducibility.⁶⁰⁾

Sialic acid linkage-specific alkylamidation has been applied and optimized for LC/MS analysis of PA-labeled glycans.⁶⁹⁾ Although the influence of sialic acid derivatization on the retention behavior in normal-phase and reverse-phase HPLC has not been mentioned, linkage-specific alkylamidation may accomplish identification of sialyl Lewis X structures in MS/MS structural analysis.

8. Application of linkage-specific sialic acid derivatization

Sialic acid derivatization was originally developed for glycan analysis by MALDI-MS, and a major application is in the field of high-throughput, multiple analyte glycan profiling measurements such as in cohort studies. Many large-scale serum/plasma *N*-glycan profiling studies have been conducted to find disease-related glycosylation changes.^{70)–73)} Using sialic acid linkage-specific derivatization, MALDI-MS based high-throughput analysis can identify new aspects for evaluation.

Other than high-throughput analyses, linkage-specific derivatization also offers the advantage of detailed analysis of glycans. Multi-dimensional HPLC analysis of PA-labeled glycans is recognized to be a powerful tool for full-characterization of the glycan structure.^{74),75)} However, these methods occasionally fail in determining sialyl linkages on multiply-sialylated large glycans. Nonomura *et al.* used linkage-specific derivatization to determine sialyl linkages on HPLC-separated PA-glycans from mouse

cochlea.⁷⁶⁾ Sialic acid linkage-specific chemical derivatization allows direct determination of sialyl linkages even in the absence of corresponding glycan standards.

9. Conclusions

MS has now become an essential device for post-translational modification analysis of proteins, including the analysis of glycosylation. To increase sensitivity and obtain more detailed structural information, several chemical modifications are generally carried out as pretreatment steps before analyzing glycans with MS. This review summarizes the methods for the modification of carboxylic acid groups on sialic acid residues. Sialic acid derivatization methods were originally developed to compensate for the disadvantages of MALDI-MS analysis of sialoglycans, such as ionization bias and sialic acid loss. However, recently developed sialic acid linkage-specific derivatization techniques have unlocked new possibilities for glycan analysis by MS. The value of sialic acid modification in LC/MS or CE/MS analysis is beginning to be recognized and their use will continue to expand.

Although glycan analysis by MS inherently involves difficulty in isomer differentiation, this is being overcome by many technical developments such as combinations with chromatographic techniques and the novel derivatization techniques mentioned above. These technical developments will contribute to the discovery of new disease biomarkers and functions of glycoproteins and glycolipids.

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Profile

Takashi Nishikaze was born in Kagawa prefecture in 1980. He graduated from Yokohama City University in 2003. His doctoral study was focused on the mass spectrometric analysis of biomolecules. After receiving his Ph.D. degree in 2008, he joined the laboratory of glycobiology in The Noguchi Institute as a researcher and entered the world of glycan analysis. In 2011, he moved to Shimadzu Corporation. He has been developing methods for highly sensitive and informative glycan analysis by mass spectrometry as a member of Koichi Tanaka Mass Spectrometry Research Laboratory in Shimadzu Corporation. He received MSSJ Research Award from Mass Spectrometry Society of Japan in 2017.