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Sialic acid linkage-specific permethylation for improved profiling of protein glycosylation by MALDI-TOF MS

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Abstract

Protein glycosylation mediates a wide range of cellular processes, affecting development and disease in mammals. Deciphering the “glycocode” requires rapid, sensitive and in-depth characterization of diverse glycan structures derived from biological samples. In this study, we described a two-step derivatization strategy termed linkage-specific sialic acid permethylation (SSAP) consisting of dimethylamination and permethylation for the improved profiling of glycosylation by matrix-assisted laser desorption/ionization (MALDI) time-of-flight (TOF) mass spectrometry (MS). High linkage-specificity (~99%) of SSAP to both the two most common forms of sialic acid, N-acetylneuraminic acid (Neu5Ac) and N-glycolylneuraminic acid (Neu5Gc), permitting direct discrimination of α 2,3- and α 2,6-linked sialic acids in MALDI-TOF MS. The enhanced intensity (>10-fold) and increased detection limit (>10-fold) of derivatized glycans were valued for sensitive glycomics. Moreover, the good compatibility and reaction efficiency of the two steps of SSAP allowed rapid sample preparation (<2 h), benefiting robust analysis of glycans in a high-throughput manner. The SSAP strategy was further applied to investigate the protein glycosylation of human serum associated with rheumatoid arthritis (RA). It was demonstrated that the relative abundances of individual glycans were different in RA negative and RA positive samples, and meanwhile the RA patient/control ratios of both α 2,3- and α 2,6-sialylated glycans tended to elevate accompanied with the increase of sialylation. Those findings of the glycosylation changes occurred in human serum protein may contribute to the diagnosis of RA. Herein, SSAP derivatization combined with MALDI-TOF MS exhibits unique advantages for glycomic analysis and shows potential in glycosylation profiling of therapeutic proteins and clinical glycan biomarker discovery.

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Notes

The authors declare that they have no conflict of interest.

Graphical Abstract

Keywords

Glycosylation; Glycans; Sialic acid linkage-specific permethylation; MALDI-TOF MS; Rheumatoid arthritis

1. Introduction

As one of the most widely observed protein post-translational modification (PTM), glycosylation contributes in almost all biological regulatory pathways [1,2]. The study of protein glycosylation in biological fluids and tissues has substantial medical importance, as changes in glycan structures have now been associated with a number of diseases [3,4]. However, reading “glycocodes” is still a demanding task due to the staggering heterogeneity of glycan arising from their complex nontemplate-driven biosynthesis and frequent abundances at low concentration. For systemic understanding of the biological roles of glycosylation, the advancement of analytical methodologies has, thus, been a key area of research in glycomics, allowing the sensitive, in-depth and high-throughput determination of glycan structures.

Benefiting from the generation of simply charged ions and high tolerance against salts and buffers, matrix-assisted laser desorption/ionization (MALDI) time-of-flight (TOF) mass spectrometry (MS) provides high throughput capability for the profiling of oligosaccharides derived from complex biological samples [5]. However, the sensitive and unbiased analysis of protein glycosylation using MALDI-TOF MS is significantly restricted by the low ionization efficiency of glycans and the labile property of sialic acid glycosidic bonds in vacuum MALDI source [6]. Additionally, the existence of multiple charged forms of individual sialylated glycan also complicates the interpretation of mass spectra.

With the advantages of stabilization of sialic acids, enhanced sensitivity, and easier tandem mass spectrometry interpretation, permethylation of glycans prior to MS characterization has become the most widely used derivatization method facilitating glycan analysis [7–10]. More recently, automated permethylation and annotation of MALDI-TOF-MS spectra were introduced to structural glycomics [11–13]. Although permethylation combined with MALDI-TOF MS provides a good snapshot of the most likely glycan compositions, the exact structures of glycans in terms of the explicit linkages are difficult to assign, particularly those of sialylated glycans with higher molecular weights. Since α 2,3- and

α 2,6-sialic acid linkages are different in stereostructures, with the treatment of appropriate activators towards carboxylic acids, several methods described that α 2,3-sialic acids formed intramolecular lactones with the penultimate galactose of glycans, while α 2,6-sialic acids were modified with nucleophiles [14–18]. Linkage-specific derivatization allows isomeric analysis of sialylated glycans by MALDI-TOF MS. However, sensitive analysis of neutral and neutralized glycans remains challenging due to the inherently low ionization efficiency of oligosaccharides. Moreover, the formed lactones of α 2,3-sialic acid linkages were unstable in water solutions, resulting in poor reproducibility in glycomic analysis [18,19]. Given that, the introduction of additional derivatization step of permethylation is therefore meritorious, since permethylation can stabilize α 2,3-sialic acids and afford enhanced MS sensitivity. For the methods of linkage-specific sialic acid derivatization, esterification is incompatible with permethylation, and amidation is limited by the time-consuming reaction and tedious sample purification [16,19]. In comparison, the selective labeling of α 2,3- and α 2,6- sialic acids by dimethylamidation is fastest. Moreover, permethylation shares the same reaction solvent with dimethylamine, which is useful for the simplification of sample preparation by eliminating the purification step during reactions.

Here, taking advantages of dimethylamidation and permethylation, we describe a simple and speedy approach of linkage-specific sialic acid permethylation (SSAP) combined with MALDI-TOF MS for rapid, isomeric, sensitive and robust analysis of sialylated glycans. Dimethylamidation was used to selectively derivatize α 2,6-sialic acids, and meanwhile α 2,3-sialic acids were lactonized. Permethylation was for the intensity enhancement of neutralized and neutral glycans as well as the stabilization of the lactonized α 2,3-sialic acids. With the two-step derivatization, a mass difference of 13 Da between α 2,3-sialic acid and α 2,6-sialic acid can be obtained, facilitating the isomeric assignment of sialylated glycans. The feasibility of this method was evaluated by using standard isomers of sialylated glycans and sialylated glycans derived from glycoproteins.

The final set of N-glycans carried by proteins is the result of various intracellular events. Changes in N-glycosylation are therefore an excellent parameter for probing cell-type-specific or tissue-specific dysfunction [20–22]. Rheumatoid arthritis (RA) is a chronic disease affecting as much as 1% of the worldwide population [23]. Aberrant glycosylation happens on several important serum glycoproteins, such as IgG, haptoglobin (Hp), and alpha-1-acid glycoprotein (AGP) in RA patients [24–26]. Previous studies investigated the N-glycan patterns in the serum of RA patients [27–29]. However, the comparison of glycans between RA patients and healthy controls in detail has not yet been investigated, since the isomeric glycans were difficult to be identified and annotated. We therefore applied SSAP strategy to determine the N-glycans derived from the serum of RA patients and controls, especially sialylated glycan isomers, demonstrating the glycosylation alterations in RA development.

2. Experimental section

2.1. Materials

Oligosaccharide standards with Neu5Ac were synthesized according to our previous reports [30,31]. Oligosaccharide derivatives with Neu5Gc were obtained from Chemilly

Glycoscience (Atlanta, GA, USA). Dimethylamine solution (2 M in methanol), dimethyl sulfoxide (DMSO), 1-hydroxybenzotriazole (HOBt) hydrate, 1-ethyl-3-(3-(dimethylamino)propyl)-carbodiimide (EDC) hydrochloride, sodium hydroxide (NaOH), sodium dodecyl sulfate (SDS), 2,5-dihydroxybenzoic acid (2,5-DHB), bovine fetuin, human serum immunoglobulin G (IgG) and rat serum IgG were from Sigma-Aldrich (St. Louis, MO, USA). Optima™ LC/MS grade water, acetonitrile (ACN), trifluoroacetic acid (TFA), dithiothreitol (DTT) and Nonidet P-40 (NP-40) were from Fisher Scientific (Fair Lawn, NJ, USA). Peptide-N-glycosidase F (PNGase F) was from New England BioLabs (Beverly, MA, USA). SepPak C18 SPE columns (100 mg/ 1 mL) were from Waters (Milford, MA, USA). Non-porous graphitized carbon (NPGC, Carbograph) SPE cartridges (150 mg/4 mL) were from Alltech Associates (Deerfield, IL, USA).

2.2. Patients and samples

Peripheral blood samples were collected from 20 RA patients and 20 healthy individuals at Peking University People's Hospital in Beijing, China according to an institutional review board approved clinical trial, and all participants gave written informed consent. Serum samples were collected at 6 a.m. and processed within 18 h of collection, and each of them was split into aliquots and stored at -80°C until use. All patients met the American College of Rheumatology (ACR) 1987 revised criteria for RA classification and had active arthritis at the time of sampling. Detailed demographic and clinical characteristics were presented in Table S1.

2.3. N-glycan release and purification

For N-glycan release, 50 μg of bovine fetuin, human IgG, rat IgG, and 5 μL of serum were dissolved in 180 μL of denaturing buffer and denatured at 90°C for 10 min, respectively. After the addition of 20 μL of 10% NP40 (v/v) and 100 U of PNGase F to each sample, the reaction mixture was incubated for overnight at 37°C . Prior to purification, 5 μL of PNGase F digestion from each of RA control and RA patient was mixed, respectively. To remove the deglycosylated proteins, the digestion was loaded onto a SepPak C18 SPE column pre-conditioned with 5 mL of acetonitrile (ACN) and 5 mL of 5% ACN/95% H_2O in 0.1% TFA. Glycans were eluted with 4 mL of 5% ACN/95% H_2O in 0.1% TFA. The eluent from SepPak C18 SPE was loaded onto and desalted by a NPGC column which was preconditioned with 5 mL of ACN and equilibrated with 5 mL of 5% ACN/95% H_2O in 0.1% TFA. The sample was washed with 10 mL of 5% ACN/95% H_2O in 0.1% TFA, eluted with 4 mL of 25% ACN/95% H_2O in 0.1% TFA, and lyophilized.

2.4. Linkage-specific permethylation of sialylated oligosaccharides

Dried glycan sample (5 pmol–10 nmol) was mixed with 25 μL of 250 mM EDC, 500 mM HOBt and 250 mM dimethylamine in DMSO and incubated for 1 h at 60°C . The excess dimethylamine was evaporated in a Speedvac for 20 min at 60°C . To the resulting solution, 1 μL of water were added, and the sample was treated with 200 μL of NaOH-DMSO slurry and 100 μL of methyl iodide for 10 min with agitation at room temperature. The reaction was terminated by the adding 800 μL of water. Subsequently, 200 μL of chloroform was added, and the mixture was vortexed and centrifuged to facilitate partitioning. The top aqueous layer was removed, and the chloroform layer was washed at least six additional

times with 1 mL of water. The permethylated sample was dried, and dissolved in 50% ACN/50% H₂O for MS analysis.

2.5. MALDI-TOF MS analysis

One microliter of sample solution was deposited on the target plate (Bruker Daltonics, MTP 384 polished steel) with the same volume of DHB (10 mg/mL in 50% ACN/50% H₂O) by a dried-droplet method for MALDI-TOF-MS analysis. MS spectra were recorded on an ultrafleXtreme MALDI TOF-TOF mass spectrometer (Bruker Daltonics, Bremen, Germany) equipped with 1 kHz Smartbeam-II laser in reflectron mode, operated through an autoXecute method in flexControl (v3.4, Bruker Daltonics) and processed in flex-Analysis. External calibration was performed using maltodextrins. A total of 1000 shots were accumulated for each spectrum. Resulting spectra were interpreted manually assisted by the GlycoWorkbench software (Euro-Carbohydrate) [32].

3. Results and discussion

3.1. Linkage-specific permethylation of sialylated glycans

As proposed, the SSAP method consists of two steps of dimethylamidation and permethylation (Fig. 1). With the treatment of carboxylic acid activator EDC, the catalyst HOBt and the derivative reagent dimethylamine in DMSO, α 2,3-sialic acid linkages convert to lactone structure, while α 2,6-sialic acids are modified with dimethylamine, achieving a linkage-specific derivatization. Besides, the water-free reaction can prevent the loss of sialic acids from glycans. However, the formed lactones of α 2,3-sialic acid linkages are not stable during post-derivatization purification with water-containing solution (Fig. S1). Benefiting from following permethylation, the labile lactones of α 2,3-sialic acid linkages are recovered and stabilized with methyl groups, and α 2,6-sialic acids are permethylated with stable dimethylamide. Compared with previous methods, such as methylamidation (30 min) and amidation (2 h) to stabilize the labile lactones within α 2,3-sialylated glycans [18,19], the reaction of permethylation is faster (10 min). Since dimethylamidation shares the same reaction solvent of DMSO with permethylation, sample purification between the two step of SSAP can be avoided.

To study the feasibility of SSAP, standard trisaccharides of α 2,3- and α 2,6-Neu5Ac-LacNAc were used and were observed with the same m/z values in MALDI-TOF MS (Figs. S2A and B). After derivatization, the paired isomers were modified differently in the carboxyl group which was methylated in α 2,3-Neu5Ac-LacNAc while dimethylamidated in α 2,6-Neu5Ac-LacNAc, leading to a mass difference of 13 Da between them (Figs. S2C and D). Moreover, negligible non-specific products of the both trisaccharides were detected (Fig. S2E), showing an excellent selectivity (>99%) of this two-step derivatization. For MALDI-TOF-MS/MS analysis, the same fragment types of α 2,3- and α 2,6-Neu5Ac-LacNAc (B1 and Y2) were obtained (Fig. S3). B₁ fragments containing Neu5Ac moieties are distinct in the two isomers with a m/z difference of 13 (m/z 398.10, m/z 411.22).

To further confirm whether SSAP is applicable for N-glycan analysis. Four pairs of N-glycan isomers from our N-glycan isomer library were prepared, derivatized, and analyzed

by MALDI-TOF MS [30]. As presented in Fig. 2, each pair of isomers were only different in one sialic acid linkage. After derivatization, a m/z difference of 13 was observed for each paired glycans (N122/N123, m/z 2431.25/ 2444.28; N222/N223, m/z 2431.33/2444.33; N233/N003, m/z 2805.45/2818.61; N135/N145, m/z 2966.50/2979.54). N122/N123 and N222/N223 are pairs of N-glycans with a single Neu5Ac residue on their non-reducing end. The MALDI-TOF-MS data of this two pairs of isomers showed that the branch location of Neu5Ac does not affect the efficiency of linkage-specific derivatization (Fig. 2A, B, C and D). N233/N003 and N135/N145 are the paired isomers attached with Neu5Ac at both their branches, and N135/N145 are also fucosylated. Corresponding MS results indicated that the derivatization efficiency and selectivity of SSAP are still good, even though neighbouring Neu5Ac and/or fucose exist (Fig. 2E, F, G, and H).

Based on the relative intensities of MALDI-TOF MS, we evaluated the derivatization specificity of each pair of sialylated N-glycan isomers. The specificity of SSAP to N122, N123, N222 and N223 were all over 99% (Fig. 3). For N233, both branches of which are attached with sialic acids, and negligible amounts of its permethylated form without or with two dimethylamide were observed. The relative abundance of its permethylated product possessing one dimethylamide was 98.76 ± 1.03 , presenting the high specificity of SSAP to glycans with two neighbouring sialic acids. Similarly, the specificity of N003 terminated with two α 2,6-sialic acids was 99.06 ± 0.75 , N135 with two α 2,3- sialic acids and a fucose was 99.45 ± 0.82 , and N145 with neighbouring α 2,3- sialic acid and α 2,6-sialic acid and fucose was 98.93 ± 0.97 .

Using the strategy of SSAP, sialylated glycans were stabilized, and α 2,3- and α 2,6-sialylated isomers were also discriminated and thereby easy to be assigned, because of their distinct m/z values observed in MALDI-TOF MS. Excess purification is not required prior to MS analysis, apart from the liquid-liquid extraction of final permethylated glycans. The rapid achievement of sample derivatization and purification within 2 h highlights SSAP as the speediest method for isomeric and robust analysis of sialylated glycans.

3.2. Enhanced MS detection and MS/MS analysis

Permethylation was utilized to stabilize the labile lactones of α 2,3-sialylated glycans formed in the first step of derivatization. Meanwhile, it is well known that permethylated glycans exhibit good ionization efficiency in MALDI-TOF MS [33,34]. Thus, Linkage-specific sialic acid derivatization combined with permethylation is an ideal strategy for isomeric and sensitive analysis of sialylated glycans. Alley and Novotny described a two-step method consisting of amidation/lactonization and permethylation, achieving an isomeric analysis of sialylated glycans by MALDI-TOF MS [16]. However, they chosen amidation with ammonium chloride to label α 2,6-linked sialic acids, resulting in quite long reaction time consumed (15 h) for complete derivatization. Besides, the step of purification before permethylation is also required, which may cause the hydrolysis of formed lactones of α 2,3-sialic acid linkages. Therefore, amidation/lactonization combined with permethylation is tedious, time-consuming and unstable. In contrast, SSAP is simpler and more rapid for isomeric and robust analysis of protein glycosylation.

To demonstrate the improved MALDI-TOF-MS detection of the sialylated glycans with SSAP derivatization, we employed N223 as a model. Two equal quantities of N223 (5 pmol) were separately derivatized using dimethylamidation and SSAP. As results, the MS intensity of SSAP derivatized glycans (Fig. 4A) was over 10-fold higher than their dimethylamidated forms (Fig. 4B). To explore the detection limitation of SSAP derivatized glycans, the two forms of derivatives were 10-fold and 100-fold diluted in series, respectively. Compared with dimethylamidated glycans, the SSAP derivatized glycans were detectable at as low as 50 fmol level and showed a good signal-to-noise ratio (>10) (Fig. 4, bottom spectra). As the results obtained from derivatized standard isomers, both signal intensity and detection limit of SSAP derivatized glycans were increased over 10-fold than non-permethyated glycans. Variation of sensitivity enhancement might be observed from different glycans with different compositions. Nevertheless, permethylation increased their signal intensity in MS, allowing samples in the low picomolar range to be detected. Compared with previously reported two-step methods which also exhibit good selectivity and stability for linkage specific derivatization between α 2,3- and α 2,6-sialic acids [18,19], SSAP provides a more sensitive approach for isomeric identification of sialylated glycans.

The emerging glycomics projects aim to characterize all glycan species from different sources. Tandem mass spectrometry (MS/MS) is the key experimental methodology to achieve that purpose. SSAP affords enhanced signals and diverse fragments of oligosaccharides analyzed by MALDI-TOF-MS/MS. Moreover, the sialic acid residue-containing fragments of α 2,3- and α 2,6-sialylated glycans (N122 and N123) generated in MS/MS showed 13 Da mass difference as expected (Fig. 5), facilitating structural determination of isomeric glycans. B-type and Y-type ions, designated according to the nomenclature proposed by Domon and Costello, are the dominating fragments of SSAP derivatized N122 and N123 [35]. Also, low abundance of C-type ions (m/z 259.00, m/z 258.99) cleaved from the non-reducing end of both derivatives were observed. $B_3\beta$ (N122, m/z 874.30; N123, m/z 860.26) and B_6 (N122, m/z 2154.13; N123, m/z 2176.04) ions showed a m/z difference of 13 in derivatized N122 and N123, which were used as diagnostic ions to identify the two isomers. The distinct fragments of B-type ions were also observed in other three pairs of sialylated glycans (data not shown), which indicates that MS/MS is useful for the structural elucidation of derivatized glycan isomers.

3.3. Structural analysis of glycans derived from human and rat IgG

To confirm the feasibility of SSAP for the characterization of glycans derived from biological samples, N-glycans released from bovine fetuin were prepared and derivatized. We utilized traditional permethylation combined with MALDI-TOF MS to identify the N-glycoforms of bovine fetuin, and 3 N-glycan compositions of H5N4S2 (m/z 2792.42), H6N5S3 (m/z 3602.85) and H6N5S4 (m/z 3964.08) were detected (Fig. S4A). Whereas, the exact structures of the glycans in terms of the explicit linkages were difficult to assign. With SSAP derivatization, 7 N-glycoforms including 2 isomers of H5N4S2 (m/z 2805.49, m/z 2818.54), 3 isomers of H6N5S3 (m/z 3615.90, m/z 3628.98, m/z 3642.03) and 2 isomers of H6N5S4 (m/z 3990.24, m/z 4003.30) were determined with linkage information (Fig. S4B).

N-glycans of IgG play significant roles in the bioactivity and pharmacokinetics [36]. Species-specific variation in glycosylation of human and rat IgG was found in previous study [37]. Of note, the sialic acid N-glycans accounts for less than 10% of total N-glycans but contributes to the anti-inflammatory properties of IgG [38]. Based on MALDI-TOF-MS analysis, 14 N-glycans including 7 Neu5Ac-glycans (GlcNAc₄Man₃Gal₁Fuc₁Neu5Ac₁, GlcNAc₄Man₃Gal₂Neu5Ac₁, GlcNAc₄Man₃Gal₂Fuc₁Neu5Ac₁, GlcNAc₅Man₃Gal₂Neu5Ac₁, GlcNAc₅Man₃Gal₂Fuc₁Neu5Ac₁, GlcNAc₄Man₃Gal₂Fuc₁Neu5Ac₂, GlcNAc₅Man₃Gal₂Fuc₁Neu5Ac₁) of human IgG were observed, while only 8 N-glycans of rat IgG were detected, including 2 Neu5Ac-glycans (GlcNAc₄Man₃Gal₁Neu5Ac₁, GlcNAc₅Man₃Gal₂Neu5Ac₁) and 2 Neu5Gc-glycans (GlcNAc₄Man₃Gal₂Fuc₁Neu5Gc₁, GlcNAc₄Man₃Gal₂Neu5Gc₂), and 6 neutral N-glycans existed in both human and rat IgGs (Fig. 6). Compared with human IgG, rat IgG contains very little amount of galactosylated glycans, resulting in fewer sialylated glycans (2 Neu5Ac-glycans and 2 Neu5Gc-glycans) observed in rat IgG. With respect to SSAP derivatization of Neu5Gc-glycans, the high specificity was demonstrated using α 2,3- and α 2,6-Neu5Gc-Lac derivatives as standards (Fig. S5). The dominating sialylated glycans derived from both human and rat IgG were assigned as α 2,6-linkages. The obtained results about the sialylation of human IgG are consistent with previous works [39], and the linkage information of rat IgG sialylation is here first presented. Recombinant IgG (rIgG) produced in different host cells are becoming major therapeutic agents to treat life threatening diseases such as cancer. We showed that the glycosylation of human and rat IgGs, particularly the sialylation of IgGs, was species-specific suggesting that a careful selection of host cell lines to produce rIgGs is necessary to avoid potentially immunogenic carbohydrate epitopes.

3.4. Analysis of glycans derived from blood serum of RA controls and patients

Most serum proteins are glycosylated, and the subtle changes of glycosylation are closely related to the development of disease [40]. To present the glycosylation variation of RA serum, we collected serum from 20 RA controls and 20 RA patients. The two groups of samples were derivatized by SSAP and analyzed with MALDI-TOF MS, respectively. The representative mass spectra were presented in Fig. S6, and the detail information of detected glycans was listed in Table S3. Totally, 59 N-glycans were observed in both groups including 5 neutral, 29 fucosylated, and 54 sialylated glycans. Forty-five α 2,3- and 34 α 2,6-sialylated glycans were detected, and 25 glycans were carried both α 2,3-sialic acid (S) and α 2,6-sialic acid (D) residues.

Base on MS intensity of each glycan, the relative abundances of serum N-glycans from RA patients were compared with the corresponding glycans in RA controls. Several individual glycans were found significantly different in the two sets of samples, of which H6N5S2 was 5-fold abundant in RA controls, while H6N5F1S1D2 was 3.34-fold abundant in RA patients (Fig. 7). The sialylated glycans were then grouped depending on the different compositions of α 2,3- and α 2,6-sialic acids. The N-glycans with a low degree of sialylation (S0D0, S1D0, S0D1 and S1D1) were relatively abundant in RA-control samples. While along with the increase of sialylation, the relative abundances of sialylated glycans from RA patients tended to increase compared with those glycans in RA controls (Fig. 8A). Both α 2,3- and α 2,6-

sialylated glycans with higher sialylation (D2 and D3; S3 and S4) have relatively high abundances in RA serum (Fig. 8B).

Numerous studies have shown the changes of glycosylation and their essential roles in RA development. It was reported that RA is associated with a marked increase in IgG glycoforms that lack galactose, and those terminal GlcNAc residues of which become accessible for MBP binding resulting in the activation of complement [24,41]. The tendency towards enhanced sialylation were also observed in the two extensively glycosylated serum proteins of Hp and AGP [25,26]. This study demonstrated the detailed structures including the linkage information and the abundance changes of sialylated glycans in RA serum. The differences of specific glycans and the significant increase of tri- and tetra-sialylated glycoforms as well as fucosylation was consistent with previous works [27]. These observed changes of glycosylation demonstrate the possibility of using glycans as biomarker for RA diagnosis in the future.

4. Conclusions

A rapid and robust strategy termed SSAP for selective derivatization of α 2,3- and α 2,6-sialic acids were described in this study, to allow improved analysis of sialylated glycans by MALDI-TOF MS. The combination of dimethylamidation and permethylation shows good compatibility, high efficiency, high specificity and excellent reproducibility for linkage-specific sialic acid derivatization. Enhanced intensity and distinctive tandem mass spectrometry of isomeric sialylated glycans facilitated their structural interpretation. This method was utilized to characterize the N-glycans derived from IgGs and RA serum, showing its attractive application in glycomic analysis. Further investigation of SSAP to O-glycans may be necessary to establish a platform for in-depth and comprehensive structural elucidation of both the protein N-, and O-glycosylation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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References

1. Cummings RD, Pierce JM. The challenge and promise of glycomics. *Chem Biol*. 2014; 21:1–15. [PubMed: 24439204]
2. Ohtsubo K, Marth JD. Glycosylation in cellular mechanisms of health and disease. *Cell*. 2006; 126:855–867. [PubMed: 16959566]
3. Varki, A., Freeze, HH. Glycans in acquired human diseases. In: Varki, A.Cummings, RD.Esko, JD.Freeze, HH.Stanley, P.Bertozzi, CR.Hart, GW., Etzler, ME., editors. *Essentials of Glycobiology*. Cold Spring Harbor; NY: 2009.

4. Dube DH, Bertozzi CR. Glycans in cancer and inflammation—potential for therapeutics and diagnostics. *Nat Rev Drug Discov*. 2005; 4:477–488. [PubMed: 15931257]
5. Jiang K, Aloor A, Qu J, Xiao C, Wu Z, Ma C, Zhang L, Wang PG. Rapid and sensitive MALDI MS analysis of oligosaccharides by using 2-hydrazinopyrimidine as a derivative reagent and co-matrix. *Anal Bioanal Chem*. 2017; 409:421–429. [PubMed: 27314848]
6. Sekiya S, Wada Y, Tanaka K. Derivatization for stabilizing sialic acids in MALDI-MS. *Anal Chem*. 2005; 77:4962–4968. [PubMed: 16053310]
7. Mechref Y, Novotny MV, Krishnan C. Structural characterization of oligosaccharides using MALDI-TOF/TOF tandem mass spectrometry. *Anal Chem*. 2003; 75:4895–4903. [PubMed: 14674469]
8. Wührer M. Glycomics using mass spectrometry. *Glycoconj J*. 2013; 30:11–22. [PubMed: 22532006]
9. Lin Z, Lubman DM. Permethylated N-glycan analysis with mass spectrometry. *Methods Mol Biol*. 2013; 1007:289–300. [PubMed: 23666731]
10. Planinc A, Bones J, Dejaegher B, Van Antwerpen P, Delporte C. Glycan characterization of biopharmaceuticals: updates and perspectives. *Anal Chim Acta*. 2016; 921:13–27. [PubMed: 27126786]
11. Shubhakar A, Kozak RP, Reiding KR, Royle L, Spencer DI, Fernandes DL, Wührer M. Automated high-throughput permethylation for glycosylation analysis of biologics using MALDI-TOF-MS. *Anal Chem*. 2016; 88:8562–8569. [PubMed: 27479043]
12. Goldberg D, Sutton-Smith M, Paulson J, Dell A. Automatic annotation of matrix-assisted laser desorption/ionization N-glycan spectra. *Proteomics*. 2005; 5:865–875. [PubMed: 15693066]
13. Woodin CL, Maxon M, Desaire H. Software for automated interpretation of mass spectrometry data from glycans and glycopeptides. *Analyst*. 2013; 138:2793–2803. [PubMed: 23293784]
14. Reiding KR, Blank D, Kuijper DM, Deelder AM, Wührer M. High-throughput profiling of protein N-glycosylation by MALDI-TOF-MS employing linkage-specific sialic acid esterification. *Anal Chem*. 2014; 86:5784–5793. [PubMed: 24831253]
15. Wheeler SF, Domann P, Harvey DJ. Derivatization of sialic acids for stabilization in matrix-assisted laser desorption/ionization mass spectrometry and concomitant differentiation of alpha(2->3)- and alpha(2->6)-isomers. *Rapid Commun mass Spectrom RCM*. 2009; 23:303–312. [PubMed: 19089860]
16. Alley WR Jr, Novotny MV. Glycomic analysis of sialic acid linkages in glycans derived from blood serum glycoproteins. *J Proteome Res*. 2010; 9:3062–3072. [PubMed: 20345175]
17. Liu X, Qiu H, Lee RK, Chen W, Li J. Methylamidation for sialoglycomics by MALDI-MS: a facile derivatization strategy for both alpha2,3- and alpha2,6-linked sialic acids. *Anal Chem*. 2010; 82:8300–8306. [PubMed: 20831242]
18. Li H, Gao W, Feng X, Liu BF, Liu X. MALDI-MS analysis of sialylated N-glycan linkage isomers using solid-phase two step derivatization method. *Anal Chim Acta*. 2016; 924:77–85. [PubMed: 27181647]
19. Holst S, Heijs B, de Haan N, van Zeijl RJ, Briaired Bruijn IH, van Pelt GW, Mehta AS, Angel PM, Mesker WE, Tollenaar RA, Drake RR, Bovee JV, McDonnell LA, Wührer M. Linkage-specific in situ sialic acid derivatization for N-Glycan mass spectrometry imaging of formalin-fixed paraffin-embedded tissues. *Anal Chem*. 2016; 88:5904–5913. [PubMed: 27145236]
20. Adamczyk B, Tharmalingam T, Rudd PM. Glycans as cancer biomarkers. *Biochim Biophys Acta*. 2012; 1820:1347–1353. [PubMed: 22178561]
21. Dall'Olio F, Vanhooren V, Chen CC, Slagboom PE, Wührer M, Franceschi C. N-glycomic biomarkers of biological aging and longevity: a link with inflammaging. *Ageing Res Rev*. 2013; 12:685–698. [PubMed: 22353383]
22. Hart GW, Copeland RJ. Glycomics hits the big time. *Cell*. 2010; 143:672–676. [PubMed: 21111227]
23. Gibofsky A. Overview of epidemiology, pathophysiology, and diagnosis of rheumatoid arthritis. *Am J Manag care*. 2012; 18:S295–S302. [PubMed: 23327517]
24. Parekh RB, Dwek RA, Sutton BJ, Fernandes DL, Leung A, Stanworth D, Rademacher TW, Mizuochi T, Taniguchi T, Matsuta K, et al. Association of rheumatoid arthritis and primary

- osteoarthritis with changes in the glycosylation pattern of total serum IgG. *Nature*. 1985; 316:452–457. [PubMed: 3927174]
25. Thompson S, Dargan E, Griffiths ID, Kelly CA, Turner GA. The glycosylation of haptoglobin in rheumatoid arthritis. *Clin Chim Acta; Int J Clin Chem*. 1993; 220:107–114.
26. Elliott MA, Elliott HG, Gallagher K, McGuire J, Field M, Smith KD. Investigation into the concanavalin A reactivity, fucosylation and oligosaccharide microheterogeneity of alpha 1-acid glycoprotein expressed in the sera of patients with rheumatoid arthritis. *J Chromatogr B, Biomed Sci Appl*. 1997; 688:229–237. [PubMed: 9061460]
27. Nakagawa H, Hato M, Takegawa Y, Deguchi K, Ito H, Takahata M, Iwasaki N, Minami A, Nishimura S. Detection of altered N-glycan profiles in whole serum from rheumatoid arthritis patients. *J Chromatogr B, Anal Technol Biomed Life Sci*. 2007; 853:133–137.
28. Royle L, Campbell MP, Radcliffe CM, White DM, Harvey DJ, Abrahams JL, Kim YG, Henry GW, Shadick NA, Weinblatt ME, Lee DM, Rudd PM, Dwek RA. HPLC-based analysis of serum N-glycans on a 96-well plate platform with dedicated database software. *Anal Biochem*. 2008; 376:1–12. [PubMed: 18194658]
29. Frisch E, Kaup M, Egerer K, Weimann A, Tauber R, Berger M, Blanchard V. Profiling of Endo H-released serum N-glycans using CE-LIF and MALDI-TOF-MS—application to rheumatoid arthritis. *Electrophoresis*. 2011; 32:3510–3515. [PubMed: 22180205]
30. Li L, Liu Y, Ma C, Qu J, Calderon AD, Wu B, Wei N, Wang X, Guo Y, Xiao Z, Song J, Sugiarto G, Li Y, Yu H, Chen X, Wang PG. Efficient chemoenzymatic synthesis of an N-glycan isomer library. *Chem Sci*. 2015; 6:5652–5661. [PubMed: 26417422]
31. Jiang K, Zhu H, Xiao C, Liu D, Edmunds G, Wen L, Ma C, Li J, Wang PG. Solid-phase reductive amination for glycomic analysis. *Anal Chim Acta*. 2017; 962:32–40. [PubMed: 28231878]
32. Ceroni A, Maass K, Geyer H, Geyer R, Dell A, Haslam SM. GlycoWorkbench: a tool for the computer-assisted annotation of mass spectra of glycans†. *J Proteome Res*. 2008; 7:1650–1659. [PubMed: 18311910]
33. Kang P, Mechref Y, Novotny MV. High-throughput solid-phase permethylation of glycans prior to mass spectrometry. *Rapid Commun Mass Spectrom RCM*. 2008; 22:721–734. [PubMed: 18265433]
34. Mechref Y, Novotny MV. Structural investigations of glycoconjugates at high sensitivity. *Chem Rev*. 2002; 102:321–369. [PubMed: 11841246]
35. Domon B, Costello CE. A systematic nomenclature for carbohydrate fragmentations in FAB-MS/MS spectra of glycoconjugates. *Glycoconj J*. 1988; 5:397–409.
36. Li H, d'Anjou M. Pharmacological significance of glycosylation in therapeutic proteins. *Curr Opin Biotechnol*. 2009; 20:678–684. [PubMed: 19892545]
37. Raju TS, Briggs JB, Borge SM, Jones AJ. Species-specific variation in glycosylation of IgG: evidence for the species-specific sialylation and branch-specific galactosylation and importance for engineering recombinant glycoprotein therapeutics. *Glycobiology*. 2000; 10:477–486. [PubMed: 10764836]
38. Kaneko Y, Nimmerjahn F, Ravetch JV. Anti-inflammatory activity of immunoglobulin G resulting from Fc sialylation. *Science*. 2006; 313:670–673. [PubMed: 16888140]
39. Wada Y, Azadi P, Costello CE, Dell A, Dwek RA, Geyer H, Geyer R, Kakehi K, Karlsson NG, Kato K, Kawasaki N, Khoo KH, Kim S, Kondo A, Lattova E, Mechref Y, Miyoshi E, Nakamura K, Narimatsu H, Novotny MV, Packer NH, Perreault H, Peter-Katalinic J, Pohlentz G, Reinhold VN, Rudd PM, Suzuki A, Taniguchi N. Comparison of the methods for profiling glycoprotein glycans—HUPO Human Disease Glycomics/Proteome Initiative multi-institutional study. *Glycobiology*. 2007; 17:411–422. [PubMed: 17223647]
40. Turner G. N-glycosylation of serum proteins in disease and its investigation using lectins. *Clin Chim acta*. 1992; 208:149–171. [PubMed: 1499135]
41. Malhotra R, Wormald MR, Rudd PM, Fischer PB, Dwek RA, Sim RB. Glycosylation changes of IgG associated with rheumatoid arthritis can activate complement via the mannose-binding protein. *Nat Med*. 1995; 1:237–243. [PubMed: 7585040]

Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.aca.2017.05.029>.

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HIGHLIGHTS

- Linkage-specific sialic acid permethylation (SSAP) shows high specificity to derivatize α 2,3- and α 2,6-sialic acids.
- SSAP combined with MALDI-TOF MS is suitable to analyze both Neu5Ac- and Neu5Gc-glycan isomers.
- SSAP characterizes by MS sensitivity enhancement (>10-fold) and distinct MS/MS spectra to the derivatized glycans.
- SSAP is the simplest and speediest method for isomeric, sensitive and robust analysis of sialylated glycans.
- SSAP applies to in-depth profiling of N-glycans derived from IgGs and human serum associated with rheumatoid arthritis.

Fig. 1.

Reaction scheme for the linkage-specific permethylation of N-acetylneuraminic and N-glycolylneuraminic acid (Neu5Ac and Neu5Gc) glycans. (A) $\alpha 2,3$ -linked sialic acid reacts with the penultimate galactose forming a lactone which is subsequently recovered and permethylated. (B) $\alpha 2,6$ -linked sialic acid is dimethylamidated and permethylated.

Fig. 2.

SSAP derivatization and MALDI-TOF-MS analysis of sialylated N-glycan isomers in reflectron positive mode. (A, B) and (C, D) isomeric glycan terminated with a single Neu5Ac residue. (E, F) isomeric glycan terminated with two neighbouring Neu5Ac residues. (G, H) isomeric glycan attached with fucose and terminated with two Neu5Ac residues. All the MS peaks were detected as $[M + Na]^+$ species. The nomenclature of used glycans refers to literature [30].

Fig. 3. Specificity analysis of SSAP derivatization based on the MALDI-TOF-MS intensity of four pairs of sialylated glycan isomers. The derivatization specificity of each glycan was calculated from three replications and showed as mean $\pm$ standard deviation.

Fig. 4. MALDI-TOF-MS analysis of (A) sialylated glycan derivatized via dimethylamidation and (B) sialylated glycan derivatized via SSAP in reflectron positive mode. All the MS peaks were detected as $[M + Na]^+$ species.

Fig. 5. MALDI-TOF-MS/MS analysis of sialylated glycan isomers derivatized by SSAP. (A) MALDI-TOF-MS/MS analysis of derivatized N122 ($[M + Na]^+$ precursor, m/z 2431.25). (B) MALDI-TOF-MS/MS analysis of derivatized N123 ($[M + Na]^+$ precursor, m/z 2444.28).

Fig. 6. MALDI-TOF-MS analysis of SSAP derivatized N-glycans from (A) 5 µg of human IgG and (B) 5 µg of rat IgG in reflectron positive mode. All the MS peaks were detected as $[M + Na]^+$ species.

Fig. 7.

Bar charts representing the relative intensities of the MALDI-TOF-MS signals for the different N-glycan species detected in the serum of RA controls and RA patients. The experiment was performed three times on separate days. The relative intensities of glycan species were normalized to the overall sum of intensities of corresponding samples and shown as mean + standard deviation. Abbreviations used are hexose (H), N-acetylhexosamine (N), fucose (F) and N-acetylneuraminic acid with α 2,3-linkage (S), and N-acetylneuraminic acid with α 2,6-linkage (D).

Fig. 8.

Bar chart demonstrating the changes in patient/control ratios of different types of sialylated N-glycans derived from RA serum. (A) Patient/control ratios of grouped SnDm types of glycans. (B) Patient/control ratios of grouped Sx and Dy types of glycans. S and D represent α 2,3-linked sialic acid and α 2,6-linked sialic acid, respectively, and n, m, x, and y indicate the numbers of sialic acid residues. The results were obtained from three independent experiments.