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Quantitative characterization of intact sialylated O-glycans with MALDI-MS for protein biotherapeutics

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Abstract—For validating O-glycosylation of protein biotherapeutics, we presented a quantitative O-glycomics method which is based on the neutralization of sialic acids, the specific release of O-glycans, and the introduction of permanent positive charge followed by quantitative MALDI-MS analysis. This method shows excellent technical reproducibility, linearity and sensitivity. In addition, it enables the quantification of intact O-glycans with minimal degradation or loss of sialic acids on these glycans compared to a conventional HPLC-based method. We then applied this method to quantitatively characterize O-glycans present on Etanercept. The analysis showed the relative abundances of mono- and di-sialylated core 1 O-glycans - were 79.3±0.8% and 17.3±1.4%, respectively. This glycomics technology could allow for the reliable quantitative analysis of intact O-glycans from glycoproteins and may contribute to validation of O-glycosylation protein biotherapeutics in the pharmaceutical industry.

Keywords: Chemical Derivatization, MALDI-MS, O-glycan, Protein Biotherapeutics, Quantitative Analysis

INTRODUCTION

Protein glycosylation is an important part of drug development because protein-based drugs may have suboptimal pharmacological potencies [1-4]. Development and validation of effective protein biotherapeutics, therefore, depend on quantitative and qualitative analysis of protein glycosylation. One of the most abundant types of protein glycosylation is mucin-type O-glycosylation, but there have been only a few methodological studies of quantitative mass spectrometry (MS) analysis of O-glycans. Methods based on permethylation of O-glycans have been widely used to quantify O-glycans [5-7], but current methods still require labor-intensive and complicated procedures. Recently, Wang et al. developed a one-pot method allowing for the simultaneous non-reductive release and quantitative derivatization of O-glycans with 1-phenyl-3-methyl-5-pyrazolone [8]. However, this method is limited by unreliable quantification of both neutral and sialylated glycans using MS due to the different ionization efficiency between them.

In this study, we described a sensitive and reliable method to quantify mucin-type O-glycans, including sialoglycans. It was based on the neutralization of sialic acids using amidation in mildly acidic conditions, the specific release of O-glycans *via* non-reductive β-elimination, and chemical derivation of the O-glycans using Girard's regent P (GP). The O-glycans were then identified and quantified by MALDI-MS. We first validated both the reproducibility and the linearity of this method using model glycoproteins and bovine submaxillary gland mucin (BSM). Next, to assess the quality of our analysis, we compared the O-glycan profiling of BSM with that from the conventional UPLC-based approach. Finally, we applied this method to the analysis of O-glycans present in a fusion biotherapeutic protein, Etanercept.

MATERIALS AND METHODS

1. Neutralization of Sialic Acids

This procedure was performed following previous methods [9, 10]. 100 μL of 10 mg/mL glycoprotein sample in water was denatured by heating at 95 °C for 3 min, and was applied to a 10-kDa molecular weight cutoff (MWCO) membrane filter (Millipore, USA). Next, 50 μL of 2 M acetohydrazide (Ah) in water, 20 μL of 1 N HCl, and 20 μL of 2 M N-(3-dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride (EDC) in water were mixed with the sample in the filter. After incubation at room temperature for 12 h, the amidated glycoproteins were washed with 300 μL of water five times by centrifugation (14,000 ×g, 10 min, 15 °C).

2. Chemical Release and Purification of O-glycans

The chemical release of O-glycans involved using a GlycoProfile β-elimination kit (Sigma-Aldrich, St. Louis, MO, USA) according to the manufacturer's protocol. The amidated glycoprotein sample was mixed with 40 μL of the β-elimination reagent mixture and was incubated at 4 °C for 18 h. After the reaction was completed, the

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release O-glycans were passed through a microcon centrifugal filter (14,000 ×g, 10 min, 15 °C), were purified using a porous graphite carbon (PGC) cartridge [11], and completely dried.

3. Derivatization of O-glycans with GP

This procedure was adopted from a previous report [12]. The purified O-glycans were dissolved in 10 μL of 50% (v/v) methanol/water and were mixed with 100 μL of 5 mM GP in 99% (v/v) methanol/acetic acid. After incubation at room temperature for 4 h, the GP-labeled glycans were dried with a centrifugal evaporator, and stored at −20 °C for further MS analysis.

4. Derivatization with 2-Aminobenzamide (2-AB) for UPLC-HILIC/FLD-MS Analysis

The purified O-glycans were incubated with 5 mL of the 2-AB labeling reagent (LudgerTagTM 2-AB glycan labeling kit) at 65 °C for 3 h and the excess reagent was removed using Whatman 3MM chromatography paper (Whatman, UK). The 2-AB labeled O-glycans solution was spotted on the 3MM paper, which was wetted with ACN. Next, the 2-AB labeled glycans spot were cut out, inserted into the syringe (Norm-Ject, Germany) and pushed through PTFE Millex-LCR filter (Millipore, USA). After incubation with 0.5 mL of water for 10 min, the labeled glycans were eluted twice and dried in a centrifugal vacuum concentrator (Hanil Research & Development, Korea).

5. HILIC/FLD Analysis

HILIC-UPLC was performed to separate the 2-AB-labeled glycans using the ACQUITY UPLC I-Class system (Waters, Bedford, MA, USA) in which a BEH glycan column (1.7 μm particle size, 2.1 i.d.×150 mm, Waters) and an ACQUITY UPLC BEH glycan HILIC column were used (Waters; 150 mm×2.1 mm×1.7 μm). Column temperature was set to 60 °C, and flow rate was 0.561 mL/min. Labeled glycans were mixed with 80% acetonitrile, and 5 μL was injected for the analysis. Buffer A was 50 mM ammonium formate (pH 4.4) in water and buffer B was 100% acetonitrile. Buffer A was maintained for 1.47 min, and then increased linearly to 47% over 47.62 min. The column was washed with 30% buffer B for 4.28 min and then returned to initial conditions for 7 min. The fluorescence detector setting was an excitation wavelength of 330 nm and an emission wavelength of 420 nm. UPLC/FLD data were acquired and analyzed using Empower 2 software (Millipore).

6. ESI Q-TOF/MS Analysis

A quadrupole-time of flight hybrid MS (Synapt G2, Waters, Manchester, UK) equipped with an ES source and lockspray was operated in positive mode (ES+). The MS was operated in resolution mode using a capillary voltage of 2.0 kV and sample cone voltage of 30 V. The cone and desolvation gas flow rates were 0 and 800 L/h, respectively. The source and desolvation gas temperatures were 110 and 250 °C. Mass range was 100-4,500 Da. The MS/MS spectra were obtained in MSE mode, and collision energy was 20-40 V for selected precursors. The MS and MS/MS data were acquired and analyzed using MassLynx software (Waters).

7. MALDI-MS Analysis

1 μL of the O-glycan sample was dissolved in 10 μL of 50% (v/v) methanol/water and was mixed with 1 μL of the matrix solution (50 mg/mL 2,5-dihydroxybenzoic (DHB, Sigma-Aldrich) in 70% (v/v) ACN/water). 1 μL of the mixed sample was spotted on a MALDI-plate and dried at room temperature. A quantitative analysis of GP-labeled O-glycan samples was performed using a Microflex LRF MALDI-TOF mass spectrometer (Bruker Daltonics, Bremen, Germany). MALDI spectra were obtained by scanning a total of 1000 shots from five different spots. The intensity of each ion was determined by summing the first, second, and third isotopic peak areas. Instrument control and processing was performed with FlexAnalysis software (ver. 3.3). The analysis parameters were as follows: positive mode, reflectron mode, acceleration voltage=20 kV, laser frequency=60 Hz, ion source 1 voltage=19 kV, ion source 2 voltage=16 kV, lens voltage=9.8 kV, detector acquisition=5.8 and

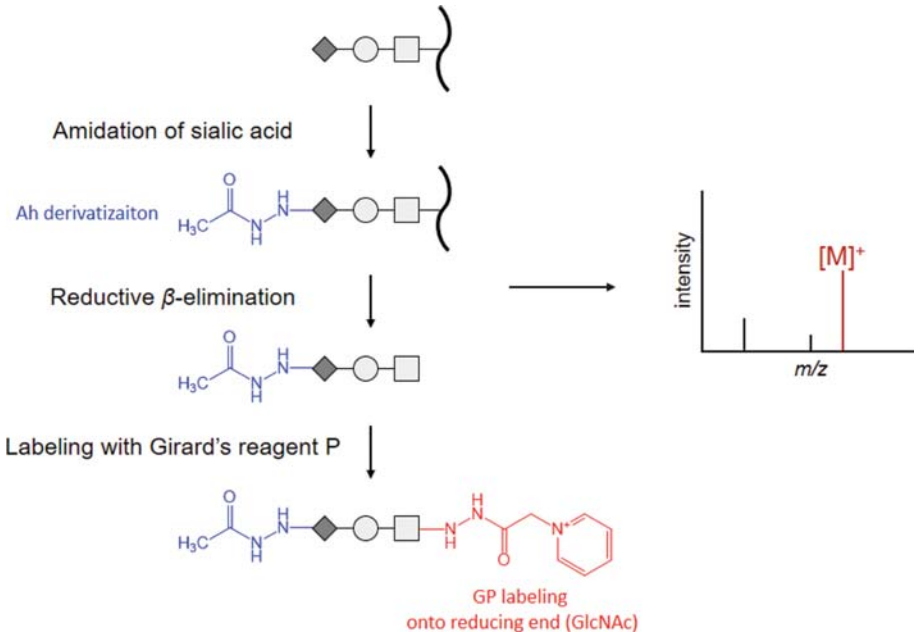


Fig. 1. A scheme for MALDI-MS-based quantitative analysis of O-glycans including sialoglycans derived from glycoproteins.

laser intensity=80-90%. Subsequently, MS^n analysis was performed using MALDI-QIT-TOF MS (Axima Resonance, Shimadzu, Manchester, UK). The ionization and desorption of O-glycans was conducted with the pulse (337 nm, 3 ns pulse width, max pulse rate 10 Hz). For multistage MS, ions were trapped in a 3-dimensional ion trap (2×10^{-5} to 2.5×10^{-5} mBar) and were fragmented with collision-induced dissociation values (between 100 to 120).

RESULTS AND DISCUSSION

1. Workflow of the Quantitative O-glycoprofiling Method

Fig. 1 shows a workflow for reliable preparation of intact O-glycans followed by MALDI-MS-based quantitative analysis. We first performed the neutralization of the sialic acid on O-glycans using amidation, which enables quantitative derivatization of sialic acids without their incomplete neutralization or degradation [9]. Next, we released the O-glycans from glycoproteins via non-reductive β-elimination, and carried out the purification of the released glycans using a PGC cartridge to remove salts and other contaminants [11]. Subsequently, the purified O-glycans were labeled with GP [12,13]. The derivatization with permanent positively charged substances such as GP improves sensitivity and quantitation in MALDI analysis by preventing analyte-adduct formation between analytes and alkali metal adducts (e.g., Li^+, Na^+, or K^+) [14,15]. Finally, the GP-labeled glycans were analyzed by MALDI-TOF MS without further purification steps. Two chemical derivatization techniques (neutralization of sialic acid and introduction of a positive charge moiety into the reducing end) enhanced homogeneous ionization efficiency on MALDI-MS, which enabled us to simultaneously quantify neutral and sialylated glycans [10,16]. However, although this combined method has been recently utilized for quantitative analysis of N-glycans [10,16], this is the first report on the reliable release and labeling of intact O-glycans followed by MS-based quantitative analysis.

2. Validation of the Quantitative O-glycoprofiling Method

To validate the reliability and reproducibility of this approach, this experiment was conducted in triplicates and four major peaks (m/z 704, 720, 907 and 923) from BSM were used for determination of the calibration curve (Fig. 2(a)). We determined the linearity of each corresponding O-glycan according to its abundance, demonstrating excellent technical reproducibility and linearity (R^2>0.99), as shown in Fig. 2(c). Moreover, the limit of detection (LOD) of O-glycans from BSM was less than 2.5 pmol in this study (data not shown).

To further assess the quality of our O-glycan analysis, we compared the O-glycan profile obtained via this MALDI-MS-based method to that from the conventional UPLC-HILIC/FLD-MS-based method, for which O-glycans were released using non-reductive β-elimination without a prior sialic acid derivatization step and were labeled with 2-aminobenzamide. Both MALDI-MS and UPLC-HILIC/FLD-MS analyses resulted in three types of BSM O-glycans: Tn-antigen, core 3 type and T-antigen O-glycans (Fig.

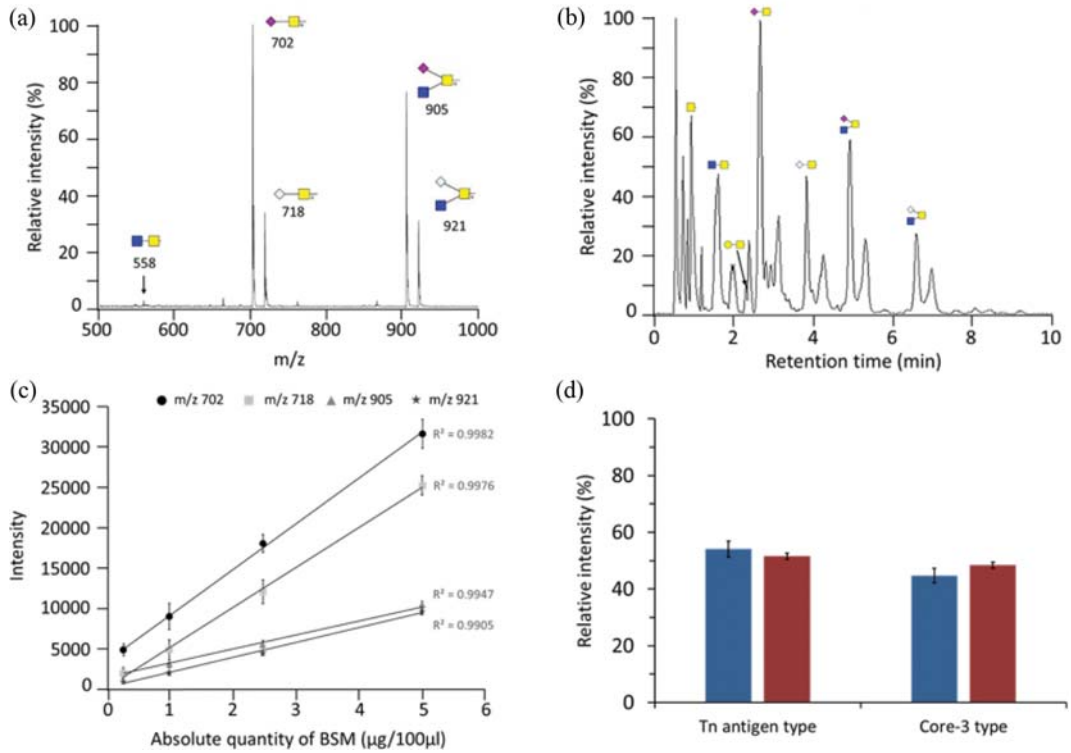


Fig. 2. (a) Positive-ion MALDI-MS spectrum of O-glycans from BSM; (b) UPLC-HILIC/FLD chromatogram of O-glycans labeled with 2-AB; (c) calibration curve of the O-glycans labeled with Ah and GP by MALDI-MS; (d) comparison between relative intensities of the O-glycans labeled with 2-AB by UPLC-MS (blue) and labeled with Ah and GP by MALDI-MS (red); yellow square, N-acetylgalactosamine; blue square, N-acetylglucosamine; purple diamond, N-acetylneuraminic acid; light blue diamond, N-glycolylneuraminic acid; yellow circle, galactose.

Table 1. Comparison between relative intensities of the O-glycans existing in BSM by amidation and by non-derivatization. The glycan compositions are given in terms of hexose (H), N-acetylhexosamine (N), fucose (F) and N-acetylneuraminic acid (S)

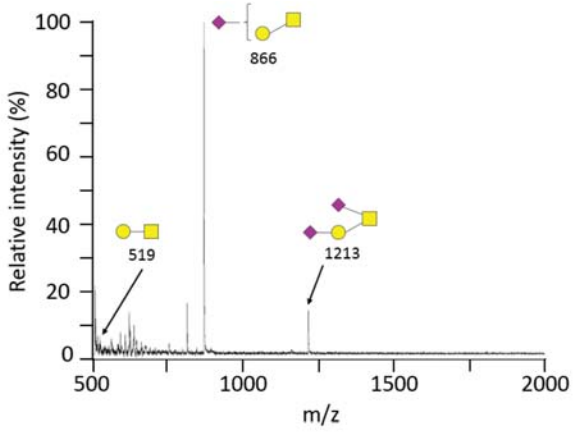
Type	No.	Sialic acid amidation+GP labeling			Non-derivatization+2-AB labeling			Proposed structure[c]	Composition
		Calculated mass[a]	Observed mass[a]	MALDI-TOF MS relative intensity (%)	Calculated mass[b]	Observed mass[b]	UPLC-HILIC/ FLD MS relative intensity (%)		
Tn antigen	1	357.3	N.D	N.D	342.2	342.1	9.5±2.6		N
	2	704.4	704	38.7±0.7	633.2	633.3	32.0±2.4		NS
	3	720.4	720	12.9±0.4	649.2	649.2	16.2±1.8		NS
Core-3	4	560.3	560	0.4±0.0	545.2	545.2	10.0±1.0		N_2
	5	907.5	907	34.7±0.8	836.3	836.3	20.9±0.4		N_2S
	6	923.5	923	13.3±0.3	852.3	852.3	10.3±3.4		N_2S
T antigen	7	519.3	N.D	N.D	504.2	504.2	1.1±0.1		NH

[a]Observed mass and calculated mass are the form of $[M]^+$

[b]Observed mass and calculated mass are the form of $[M+H]^+$

[c]Yellow square, N-acetylgalactosamine; blue square, N-acetylglucosamine; purple diamond, N-acetylneuraminic acid; light blue diamond, N-glycolylneuraminic acid; yellow circle, galactose

(a)



(b)

Peak no.	Sialic acid amidation + GP labeling: Calculated mass[a]	Observed mass[a]	MALDI-TOF MS Relative intensity(%)	Houel et al.[20]: GU value	UPLC-FLR Relative intensity(%)	Proposed structure	Composition
1	519.3	519	3.4 ± 0.7	1.77	7.31		NH
	663.3	N.D	N.D	1.97	33.56		HS (peeling product)
2	866.5	866	79.3 ± 0.8	3	48.99		NHS
3	1213.6	1213	17.3 ± 1.4	4.63	10.14		NHS_2

[a]Observed mass and calculated mass are the form of $[M]^+$.

Fig. 3. (a) Positive-ion MALDI-MS spectrum of Etanercept O-glycans, (b) comparison between relative intensities of the O-glycans by our method and by Houel's approach [20]; yellow square, N-acetylgalactosamine; purple diamond, N-glycolylneuraminic acid; yellow circle, galactose.

2(a) and 2(b)). Of Tn antigens, one O-glycan observed by UPLC-HILIC/FLD-MS-based method but not MALDI-MS was a monosaccharide (GalNAc-), and therefore was probably the residue lacking terminal sialylation. In addition, although three core 3 O-glycans were detected qualitatively by both methods, the relative abundance of one O-glycan (GlcNAc-GalNAc-) was much lower when using the MALDI-MS method compared to the UPLC-HILIC/FLD-MS method (0.4 vs. 10%), as shown in Table 1. These observations demonstrated that amidation of the carboxyl group on sialic acids may result in minimization of degradation or loss of sialic acid residues from the intact O-glycan under harsh reaction conditions [10]. This was supported by quantitative results, which showed that the total abundances of Tn antigen and core 3 O-glycan types measured by our method were similar to those in the UPLC-HILIC/FLD-MS (Fig. 2(d)).

3. Quantitative O-glycosylation of Etanercept

Finally, we applied this method to the analysis of O-glycosylation in a protein biotherapeutic, Etanercept, which has been approved for the treatment of patients with five chronic diseases including rheumatoid arthritis. This protein is a human tumor necrosis factor receptor 2 (TNFR2) -Fc fusion protein, with abundant expression of O-glycans on the TNFR2 domain [17,18]. DiPaola et al. previously identified core 1 O-glycan structures with one or two sialic acids on Etanercept, but did not perform quantification of O-glycans [19]. Houel et al. measured the relative abundance of O-glycans using HILIC-FLR. However, a peeled product was abundantly detected, which makes it difficult to precisely determine the quantity of O-glycans [20]. Our method resulted in three different O-glycans (*m/z* 519, 866 and 1213) present on Etanercept corresponding to neutral, monosialylated and disialylated core 1 O-glycans (Fig. 3). These glycan structures were then confirmed by MALDI-MS^n (Fig. 4). Our quantitative analysis showed the relative abundance of mono- and di-sialylated core 1 O-glycans was 79.3±0.8% and 17.3±1.4%, respectively. Thus, these outcomes were higher relative to those reported in Houels' results (49.0% and 10.1%, respectively). Considering that the loss of monosaccharides from the reducing terminus of O-glycans occurred via peeling reactions, which was observed in their study, the quantification result could indicate that mono-sialylated core 1 O-glycans would be the most abundant mucin-type O-glycans expressed on Etanercept. In addition, the higher relative abundance of di-sialylated core 1 O-glycans from our analysis demonstrated that amidation of sialic acids may prevent the degradation or loss of sialic acid residues under harsh reaction conditions.

CONCLUSION

MALDI-MS-based quantitative analysis combined with derivation reactions can be used to measure the relative abundance of intact O-glycans. This method was successfully applied to the identification and quantitation of O-glycosylation of Etanercept. Thus, this could represent an improved strategy that allows for simultaneous quantitation of both neutral and sialylated O-glycans present in a biotherapeutic protein. Furthermore, when combined with a 96-well plate purification platform, this method could be used for high-throughput analysis of protein O-glycosylation in the biotherapeutic industry.

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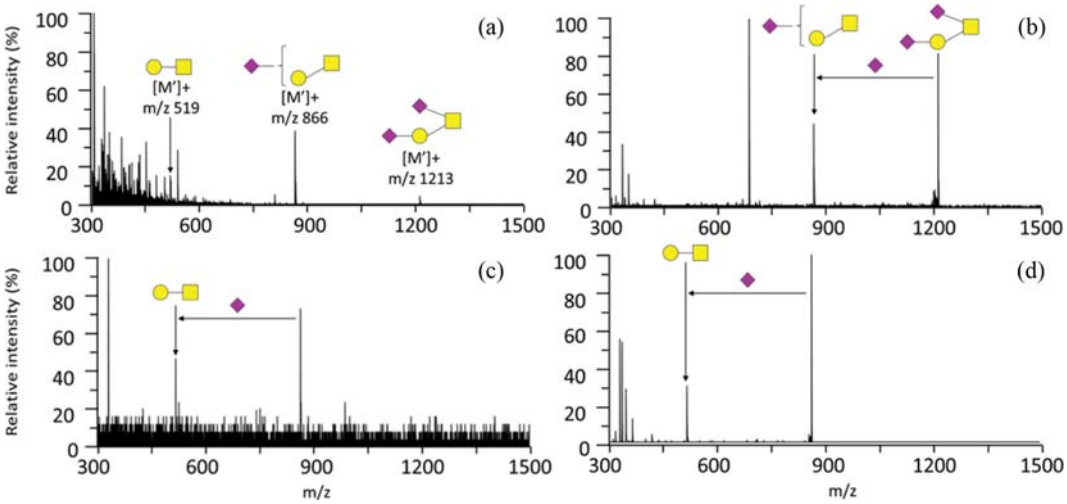

Fig. 4. Identification of O-glycans structure on Etanercept via MALDI-QIT-TOF MS^n analysis. (a) MS of total O-glycans on Etanercept; (b) MS/MS of 1213 ion; (c) MS^3 of 866 ion in MS/MS stage of 1213 ion; (d) MS/MS of 866 ion; yellow square, N-acetylgalactosamine; purple diamond, N-glycolylneuraminic acid; yellow circle, galactose.

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