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Glycomic analysis using glycoprotein immobilization for glycan extraction

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Abstract

Glycosylation is one of the most common protein modifications and is involved in many functions of glycoproteins. Investigating aberrant protein glycosylation associated with diseases is useful in improving disease diagnostics. Due to the non-template nature of glycan biosynthesis, the glycans attached to glycoproteins are enormously complex; thus, a method for comprehensive analysis of glycans from biological or clinical samples is needed. Here, we describe a novel method for glycomic analysis using glycoprotein Immobilization for glycan extraction (GIG). Proteins or peptides from complex samples were first immobilized on solid support, and other non-conjugated molecules were removed. Glycans were enzymatically or chemically modified on solid-phase before releasing from glycoproteins/glycopeptides for mass spectrometry analysis. The method was applied to the glycomic analysis of both N- and O-glycans.

INTRODUCTION

Protein glycosylation is involved in many biological pathways including cell-cell signaling, protein stability, protein solubility, and interactions of ligands and receptors¹. Aberrant glycosylation plays a pivotal role in a multitude of pathological states including cancer², immunity^{3,4}, and arthritis⁵. Disease-associated alterations in glycosylation can be exploited for diagnosis or targeted treatment of diseases either independently or in combination with protein abundance⁶. The glycan profile is also critical for therapeutic glycoproteins such as antibodies because glycosylation can impact efficiency and safety of the glycoprotein drugs^{7,8}.

Glycoproteins are formed through covalent linkages such as glycosidic bond from the glycans to proteins. The resultant N-linked and O-linked glycosylated proteins carry remarkable complexity in their oligosaccharide chains. Complex N-linked and O-linked glycosylation on proteins is expressed on the surface of the plasma membrane and on secreted proteins. N-glycans are conjugated to proteins through asparagine residues in the consensus tri-peptide sequence *Asn-X-Ser/Thr* (where *X* is any amino acid except proline). These glycans can be released from proteins enzymatically using peptide-N-glycosidases (PNGases)⁹. The O-glycosylation of proteins occurs on both serine and threonine residues, and there is no specific enzyme comparable to PNGase that removes the intact O-linked glycans. Therefore, chemical methods such as β -elimination are generally used to release certain type of O-glycans from peptides or proteins¹⁰.

To identify and quantify glycans from glycoproteins in complex biological samples, glycoproteins are traditionally purified from complex mixtures using methods such as chromatography or gel electrophoresis. Glycans are then released and isolated from proteins

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and other chemicals in the mixture, and their structures are usually characterized by orthogonal approaches utilizing chromatography, capillary electrophoresis, or/and mass spectrometry (MS)^{11,12}. One of the predominant chromatographic techniques used for glycan analysis is hydrophilic-interaction chromatography (HILIC)^{13–15}. Other methods that are often used include conventional porous graphitic carbon (PGC) reverse-phase liquid chromatography (RPLC) or high-pH anion exchange high performance liquid chromatography (HPLC)^{16–18}. Fluorescently labeled glycans can also be separated and profiled by capillary zone electrophoresis^{19,20}. To analyze the glycans with MS, a PGC cartridge is typically employed to purify glycans from proteins/peptides and other contaminants prior to MS analysis^{21–24}. Currently, high-throughput analyses of glycans are challenging due to the enormous complexity of glycans and lack of efficient methods for isolation of glycans from complex biological samples.

To achieve the aforementioned needs, we described the development of a high-throughput method for profiling of glycans using glycoprotein immobilization for glycan extraction (GIG). First, proteins or peptides were conjugated to a solid support and the un-conjugated molecules were washed away. In an optional second step, the glycans on immobilized glycoproteins/glycopeptides were modified enzymatically or by chemical reactions. In the third step, the glycans were released from the solid support and analyzed. The method was applied to the glycomic analysis of *N*-linked glycans from human sera and *O*-linked glycans from mucin.

EXPERIMENTAL PROCEDURES

Materials and Reagents

Sialylglycopeptide (SGP) was purchased from Fushimi Pharmaceutical Co., Ltd. (Marugame, Kagawa, Japan). Spin columns (snap cap) and AminoLink resin were from Pierce (Thermo Fisher Scientific Inc.; Rockford, IL); Carbograph was purchased from Grace (Deerfield, IL). Peptide-*N*-glycosidase F (PNGase F), denaturing buffer and neuraminidase ($\alpha(2-3)$, $\alpha(2-6)$, $\alpha(2-8)$) were from New England BioLabs (Ipswich, MA); ribonuclease *B* (RNase *B*) from bovine pancreas, mucin from porcine stomach, 2,5-dihydroxybenzoic acid (DHB), and *N,N*-dimethylaniline (DMA) were purchased from Sigma-Aldrich (St. Louis, MO); μ -Focus MALDI plate and its holder were from Hudson Surface Technology (Forte Lee, NJ); Axima Resonance MALDI QIT-TOF mass spectrometry was from Shimadzu Biotech (Columbia, MD). Serum was collected from healthy men with the approval of the Institutional Review Board of Johns Hopkins University and pooled for use. All other chemicals were purchased from Sigma unless specified.

Protein or Peptide Conjugation

Both glycoproteins and SGP glycopeptides were conjugated to beads using reductive amination. For peptides, 100 μ L of AminoLink resin (200 μ L of 50% slurry) was incubated with sample in 400 μ L buffer (pH 10.0) (40 mM sodium citrate and 20 mM sodium carbonate) at room temperature for 4 hrs with mixing. Then 40 μ L of 500 mM sodium cyanoborohydride (1x PBS) was added to incubate for another 4 hrs. After rinsing the resin with 400 μ L of 50 mM phosphate buffer (pH 7.4) twice, sample on the solid support were further reduced by adding 50 mM sodium cyanoborohydride (NaCNBH_3) in 50 mM phosphate buffer (pH 7.4) at room temperature for 4 hrs with mixing. After incubation, the beads were washed with 1 M Tris-HCl (400 μ L, pH 7.6) twice before addition of 400 μ L of 1 M Tris-HCl (pH 7.6) in the presence of 50 mM NaCNBH_3 to block the unreacted aldehyde sites on the bead surface for 0.5 hrs. For protein conjugation, 2 mg of mucin from porcine stomach, 400 μ g RNase B glycoprotein or 20 μ L of serum proteins were first denatured in 100 μ L solution consisting of 10 μ L denaturing buffer (10 $\times$) (New England Biolabs) and 70

μL buffer (pH 10.0) for 10 minutes at 100°C before following glycopeptides conjugation procedure. The proteins or peptides immobilized on beads were washed three times with 400 μL of 1 M NaCl and three times with H_2O .

Sialic Acid Modification

For additional exoglycosidase treatment, the neuraminidase (4 μL , New England Biolabs) was added to 116 μL of buffer which contains 12 μL G1 (10 $\times$, 50 mM sodium citrate; New England Biolabs) and 104 μL water. The reaction solution was mixed with protein or peptide-immobilized resin and incubated at 37°C for 2 hrs to remove sialic acid groups. The enzyme and chemical reagents were removed by sequentially washes using 400 μL of 1 M NaCl and H_2O . To protect the sialic acid groups, glycans on solid support were incubated with 465 μL of p-toluidine (Sigma) solution (pH 4–6), which consists of 400 μL p-toluidine, 25 μL 36–38% HCl, and 40 μL EDC (N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide; 5.6 M; Sigma). Reaction was preceded for 3 hrs at room temperature before the chemicals were washed off from the solid support with 400 μL of 1% formic acid (twice), 400 μL of 10% acetonitrile (twice), 400 μL of 1 M NaCl (twice) and finally H_2O (twice).

N-Glycan Release

After removing the wash buffer, two μL of PNGase F with 118 μL of 5 mM NH_4HCO_3 was added to the bead mixture and incubated at 37°C for 2 hrs to release N-glycans. The released N-glycans in the supernatant were collected and dried in vacuum. Additionally, the reaction buffer (G7; 50 mM sodium phosphate; pH 7.5) is recommended especially for glycoproteins immobilized on solid-phase. Twelve μL of 10 $\times$ G7 buffer (New England Biolabs) was added to 2 μL PNGase F and 106 μL water before 2-hr incubation. The eluted glycans were purified by Carbohydrate prior to dried by vacuum²⁵. The extracted N-glycans were resuspended in HPLC grade of water, 40 μL for 400 μg RNase B, 40 μL for each SGP sample, and 100 μL for 20 μL serum glycans respectively.

O-Glycan Release

The beads conjugated with mucin were divided into two equal amounts. The first sample was incubated in 400 μL of 26–28 % ammonium hydroxide (Sigma) at 45°C for 4, 8, 24, 48, 72, and 144 hrs. Supernatants were collected after incubation and fresh 400 μL ammonium hydroxide was added for further reaction. The second sample was directly digested with 1 μL of PNGase F in 12 μL of 10 $\times$ G7 and 107 μL of water at 37°C for 2 hrs. The pH of the collected supernatant after incubation in ammonium hydroxide was adjusted to 3–5 by gradually adding 250 μL of formic acid (100%). The samples were then cleaned by Carbohydrate²⁵. Released glycans from both samples were dried and dissolved in 100 μL of water for MS analysis.

Analysis of Glycans Using MALDI-MS

Glycans (1 μL for each sample) extracted from SGP, RNase B, mucin, and serum using GIG were analyzed by Axima MALDI Resonance mass spectrometer (Shimadzu). We mixed 4 μL DMA in 200 μL DHB (100 $\mu\text{g}/\mu\text{L}$ in 50% acetonitrile, 0.1 mM NaCl) as MALDI matrix to increase the detection of sialylated glycans. The DHB-DMA spots formed uniform crystals and increased sialylated glycan stability by increasing laser power absorption and ionization efficiency¹³. The laser power was set to 200 for 2 shots each in 100 locations per spot. The average MS spectra (200 profiles) were used for glycan assignment by comparing to the database of glycans previously analyzed by MALDI-MS/MS in our lab. We confirm the assigned glycans from human serum established in literature^{26–28}.

RESULTS AND DISCUSSION

Glycoprotein immobilization for Glycan Extraction and Modifications

In order to allow high-throughput analysis of glycans, a solid-phase glycan extraction method was developed and followed by MS analysis. The procedure included the following steps (Figure 1): *i) Protein/peptide conjugation*: The proteins or peptides were coupled to aldehyde groups of a solid support through reductive amination of *N*-termini and/or lysine residues of proteins or peptides. After coupling, unreacted aldehydes on beads were blocked using Tris buffer via the same reductive amination reaction. Unconjugated molecules and reagents were washed away. *ii) Glycan modifications and release*: The glycans on conjugated glycoproteins were modified enzymatically or chemically, reaction buffer was exchanged and the glycans were released. *iii) Mass spectrometry analysis*: The released glycans were analyzed by mass spectrometry. Overall, the GIG method provides a rapid analysis platform for glycomic analysis of complex biological samples.

The critical step for immobilization of protein/peptide on solid-phase is dependent on the conjugation efficiency. We thus first conjugate protein/peptide to the resin at pH 10 to allow protein conjugation through amino groups from both *N*-termini and Lys of proteins. As shown in Figure 2, RNase B was quickly conjugated to resin where over 80% immobilization yield was observed at first 30-minute incubation. We typically conjugate proteins for 4 hrs to achieve over 95% protein coupling efficiency (Figure 2).

Modifications of Glycans on Beads

To develop the new method for glycan extraction and the solid-phase glycan modifications, we first used a sialylglycopeptide (SGP) with known *N*-glycan structure (Supplementary Figure 1). The SGP has a biantennary *N*-glycan linked to *Asn* residue of a peptide containing six amino acids (*Lys-Val-Ala-Asn-Lys-Thr*). The SGP was treated as described in supplementary and analyzed after each step. After conjugation to beads, samples on the solid support were aliquot in three spin columns. The first sample was directly digested by PNGase F without modification to release *N*-glycans from SGP (Figure 3 (i)); the second aliquot was treated with neuraminidase to remove sialic acids before PNGase F digestion (Figure 3 (ii)); the third aliquot was modified with p-toluidine to protect sialic acid groups (Figure 3 (iii))^{29,30}. The results showed that although SGP had single biantennary *N*-glycan, three peaks were observed when it was detected by MALDI without sialic acid stabilization. This is probably due to the labile nature of sialic acids in which loss of one or two sialic acids occur during MS analysis³⁰. Each sialic acid carries a sodium ion (0.1 mM NaCl is used in DHB-DMA matrix) (Figure 3 (i)) in MALDI-MS.

Immobilization of glycans on the solid support makes glycan modification convenient. Glycans on immobilized glycopeptides/glycoproteins could be treated with enzymes. After neuraminidase treatment of SGP glycan, we detected one single peak at *m/z* 1663.1 Da as showed in Figure 3 (ii). This indicates that removal of sialic acids is complete on solid-phase. On the other hand, derivatization of sialic acids by amidation showed one single peak at *m/z* 2424.3 Da (Figure 3 (iii)). The peaks losing one or two sialic acids were not observed after sialic acid modification. These results demonstrate that sialic acids after derivatization become stable. The purification and modifications of glycans are simplified by solid-phase glycan extraction, significantly minimizing sample loss.

N-Glycan Analysis Using GIG

To investigate the sensitivity of GIG for glycan analysis, different amount of SGP (0, 1, 5, 10, 50, and 100 μ g) was used in conjugation and release experiments, and the isolated glycan was analyzed by MS in triplicate (1 out of 10 μ L final sample was used for MS

detection). The limit of detection of the glycan was below 1 μg from SGP peptide. The S/N ratio was greater than 4000 for the sialylated biantennary *N*-glycan from 1 μg of SGP peptide (peak at 2424.3 Da, Supplementary Figure 2). To determine whether the isolation of glycan using GIG was reproducible, the peak areas in the MS spectra of sialylated biantennary *N*-glycan ($m/z = 2424.3$) isolated from 10 μg of SGP in triplicate were calculated. The peak areas were normalized to an internal standard (angiotensin). The CV of glycan analyses from triplicate isolations was 12.8%, which indicates that GIG is reproducible for the analysis of glycans.

To assess the potential of the GIG method for glycan analysis of glycoprotein, we then determined the specific glycan extraction from RNase *B*. RNase *B* is a glycoprotein with known five oligomannose structures^{14,31}, Man5, Man6, Man7, Man8, and Man9. All five of these previously reported glycans were detected by mass spectrometry after GIG procedure (Supplementary Figure 3). These results indicate that GIG can be used to isolate glycans from glycoproteins.

To apply the GIG method to the analysis of *N*-glycans from human serum, we included sialic acid modification in the GIG method^{29,32,33}. After coupling of 20 μL of serum proteins to the solid support, the beads were washed to remove unconjugated molecules present in the sample. Glycans from glycoproteins conjugated on the solid support were then reacted with p-toluidine. *N*-glycans were released from bead-bound glycoproteins by treating the beads with PNGase F. The glycan supernatant was dried and dissolved in 100 μL water. Of this, 1 μL (from 0.2 μL of serum) was analyzed by MALDI-MS and MS/MS. Without additional glycan separation, we were able to detect 66 unique *N*-glycan masses (Supplementary Table 1) and the proposed *N*-glycan assignment of up to 43 major peaks was shown in Figure 4 (A) and (B). Sixty-five of these glycans were previously reported in human serum *N*-glycan library^{26–28}. We performed MS/MS and MS/MS/MS on the unmatched glycan (m/z : 2285.9 Da), and potential composition is proposed in Supplementary Figure 4. Some of the *N*-glycans were further verified by MS/MS analyses of the serum sample (Supplementary Figure 5).

Mucin O-Glycan Analysis

O-glycans are usually released from glycoproteins by β -elimination. However, the released *O*-glycans are subjected to peeling at their reducing end. In mildly basic condition (pH 11), such as in ammonium hydroxide, peeling has been effectively prevented³⁴. After glycoprotein (mucin) was immobilized to the solid support, the beads were incubated in 26–28 % ammonium hydroxide for 4 hrs and the supernatant was collected; then another fresh ammonium hydroxide was added for extended treatment. *O*-glycans were efficiently released from mucin on solid-phase after 24 hrs incubation (Figure 5 (A)–(B)). We were able to match most of *O*-glycan peaks to those previously reported *O*-glycans³⁵. Further treatment on the immobilized mucin after 72 hrs showed that intact *N*-glycans were also released from solid-phase by β -elimination (Figure 5 (C)). This similar finding was also reported in other study³⁶. Figure 5 (D) is *N*-glycans released by PNGase F. By comparing *N*-glycans released in ammonium hydroxide (Figure 5 (C)) to the *N*-glycans released by PNGase F (Figure 5 (D)), it was approximately five-fold higher from PNGase F treatment than that from ammonia treatment. In addition, we also detected these *N*-glycans after 144 hrs ammonium hydroxide treatment, suggesting incomplete release of *N*-glycans after 72-hr incubation.

GIG Perspectives for Glycomics

This study established a novel method for glycan analysis using glycoprotein immobilization for glycan extraction (GIG). The GIG provides a chemoselective method for capture and

analysis of glycans from glycoproteins or glycopeptides directly from complex samples. The glycans on solid-phase can be readily treated by enzymes for glycan structural analysis as well as chemical modifications for sialic acid stabilization and quantification. GIG is also compatible with analytical platforms such as microfluidics, and released glycans can be further analyzed by liquid chromatography, electrophoresis, capillary electrophoresis, and MS detection.

The GIG is a chemoenzymatic approach and has a number of advantages for glycan analysis. First, released glycans from solid phase can be directly analyzed without further purification. This reduces sample loss and results in high yields from the glycan isolation and increases sensitivity of detection, ultimately reducing time and recovering low abundance of glycans through elimination of multiple glycan purification steps such as C-18 and graphite columns²⁶. Second, the solid-phase capture method implements a platform for glycan modifications using enzymes or chemicals. In this study, we showed that exoglycosidase digestion was efficient while the glycosylated proteins were conjugated to the solid support; the sialic acids were efficiently modified for identification and quantification by mass spectrometry²⁹. The modifying enzymes and chemicals can be easily removed by washing steps, providing an efficient and rapid method for glycan modification or derivatization from complex samples. This will also help produce a platform for glycan sequencing or targeted glycan synthesis using combination of chemicals and enzymes with negligible sample loss. Third, the GIG procedure is reproducible and the isolated glycans are compatible with current downstream analytical platforms. We tested the glycan captured from SGP by GIG. The CV of the repeated analyses was 12.8%, indicating that the glycan isolation using GIG was reproducible. We are applying GIG in microfluidic platform to glycan enrichment and separation. Preliminary results show that glycan coverage from human sera can be significantly improved. Therefore, the GIG could be useful in glycan analysis from complex biological or clinical samples for better understanding on the roles of protein glycosylation in biological or disease-related studies.

Both *N*-linked and *O*-linked glycoproteins are conjugated to the solid support through reductive amination. In this study, we demonstrated that *N*-glycans were successfully released from the solid support by PNGase F. There is no specific enzyme comparable to PNGase F for removing the intact *O*-linked glycans. To successfully release *O*-linked oligosaccharides by *O*-glycosidase, it is necessary to sequentially remove monosaccharides by a panel of exoglycosidases until only the core 1 and core 3 *O*-linked disaccharides remain attached to the serine or threonine residue. The trimmed *O*-linked glycans can then be released by *O*-glycosidase. Since not all *O*-linked oligosaccharides contain these core structures, a chemical method such as β -elimination is effective for the release of *O*-linked glycans. We showed that *O*-glycans could be released from solid support by β -elimination using ammonium hydroxide. The GIG also dramatically simplifies procedures for *O*-glycan analysis.

CONCLUSION

In this study, we described the development of a solid-phase glycan extraction and modification method using glycoprotein immobilization for glycan extraction (GIG) and applied GIG method to the comprehensive analysis of *N*-glycans from human serum. We showed the detection of 66 *N*-linked glycan masses on a single spot by MALDI-MS and verification of 65 of these glycan masses by tandem MS. Glycans on the solid support can be modified by enzymes or chemicals with negligible sample loss by eliminating multiple chromatographic purification steps. GIG is a reproducible and sensitive method that dramatically increases our capability to analyze both *N*- and *O*-linked glycans present in complex biological samples. This method is useful for high-throughput glycan analysis from

complex samples for biological and clinical investigations. The immobilized glycoproteins on solid-phase after deglycosylation can be further digested for proteomic analysis. It could facilitate discovery of aberrant glycans associated with diseases and lead to the understanding of glycan functions in normal and pathological states.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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ABBREVIATIONS

GIG	Glycoprotein Immobilization for Glycan Extraction
MS/MS	Tandem Mass Spectrometry
HPLC	High Performance Liquid Chromatography
PGC	Porous Graphitized Carbon
SGP	Sialylglycopeptide

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Figure 1. Schematic diagram of glycan capture and release using glycoprotein immobilization for glycan extraction (GIG) method.

Figure 2.

Coupling of protein onto the solid support. Protein, ribonuclease B (RNase B, 400 μ g), was mixed with 200 μ L of Aminolink slurry after denatured in 100°C for 10 min. Protein concentration was determined after conjugation protein to beads for 0, 0.5, 1, 2, 4, 6, 24 hrs. Over 95% binding efficiency was observed after 4 hrs incubation in pH 10.0 buffer.

Figure 3.

Analysis of glycans from SGP using GIG and modification of glycan on solid support. The mass spectra for the glycans of SGP without any modification (i), desialylation by neuraminidase treatment (ii), and sialic acid stabilization (iii). Three glycans (1663.1 (1 Na⁺), 1976.1 (2 Na⁺) and 2289.1 (3 Na⁺)) were observed before sialic acid modification (i); only one glycan (1663.1) was detected after neuraminidase treatment (ii); only one glycan (2424.3) was detected after complete sialic acid derivatization (iii).

■ GlcNAc ● Mannose ● Galactose ▲ Fucose ◆ Neu5Ac

Figure 4.
N-Glycan analysis from human serum using GIG method in low mass range (A) and high mass range (B).

Figure 5. O-Glycan analysis from mucin using GIG method after incubation in ammonium hydroxide for 4 hrs (A), 24 hrs (B), and 72 hrs (C). N-glycan analysis from mucin using GIG by PNGase F (D).