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A MALDI-MS-based quantitative glycoprofiling method on a 96-well plate platform

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ABSTRACT

Here, we developed a high-throughput MALDI-MS based quantitative targeted glycomics (HT MALDI-QTaG) method for analyzing total N-glycans. Although the chemical derivatization processes (i.e., neutralization of sialic acid and incorporation of a positively-charged moiety) were performed in a 96-well with built-in 10 kDa MWCO membrane filters, the quantitative linearity was still quite good ($R^2 > 0.99$) between varying amounts of the target glycoprotein and the MALDI peak intensities. In addition, we validated the relative quantitative reproducibilities in different well positions in a 96-well plate. As a proof-of-concept, the proposed HT MALDI-QTaG method was successfully used to analyze bovine fetuin, human serum and Enbrel®.

Introduction

In human glycoproteins, N-glycans are the major constituents of the outer cell-membranes and have a crucial role in biological and immunological systems [1–3]. Specifically, abnormal glycosylation changes are closely related to various human diseases and glycosylation profiles have been used as biomarkers for diagnosis and monitoring of different disorders [4–6]. In addition, an alteration in protein glycosylation can directly affect protein structure and function [7,8]. For instance, the glycosylation of recombinant biotherapeutic proteins determines and modulates their yield, in vivo stability and immunogenicity [2,9]. Therefore, the glycoforms of the therapeutic glycoproteins should be monitored during the development of cell expression system (i.e., cell lines), growth media and bioreactor conditions [10,11]. Gervais et al. has shown the high level of batch-to-batch reproducibility of recombinant gonadotrophins via glycoprofiling of more than 120 batch samples [12]. More recently, Planinc et al. analyzed batch-to-batch N-glycosylation of infliximab, trastuzumab and bevacizumab and regularly examined the consistency of the N-glycosylation of bevacizumab for 3 months [13]. In this respect, high-throughput glycan profiling platform is required to handle and analyze a large number of clinical and industrial specimens.

Over the last few decades, high-performance liquid chromatography combined with mass spectrometry (LC–MS) has been the gold standard method for quantitative or qualitative analysis of N-glycans [14–17]. Recently, improvements in N-glycan preparation could be achieved with ultra-performance liquid chromatography (UPLC) in terms of speed, sensitivity, selectivity, and peak efficiency [18–20]. Simply put, it is possible to separate and quantify isomeric glycan structures with UPLC. However, general challenges associated with LC-based methods are time-consuming chromatography runs and a requirement for highly-trained operators to collect reliable LC–MS data.

Alternatively, matrix assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-MS) has been highlighted as an alternative to LC–MS for analyzing N-glycans because of its high

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sensitivity, easy and fast analysis and high-throughput of analytes [21–23]. Several groups have reported MALD-MS-based quantitative methods for rapid screening of N-glycans using the following novel chemical derivatization techniques: (1) solid phase permethylation which can replace hydrogens on the –OH, $–NH_2$ and –COOH groups of total N-glycans with a methyl group ($–CH_3$) [24–26]; (2) methyl esterification and amidation of the carboxyl group on sialic acid [27–30]; (3) chemical derivatization using Girard's reagent T or P to introduce a permanent cationic charge to the reducing end of N-glycans [27,28,31,32]; and (4) stable isotopic labeling of N-glycans for relative quantitative glycan analysis [33–36]. Although new N-glycan derivatization techniques have dramatically improved the high-throughput MALDI-based workflow, identification and relative quantitation of isomeric N-glycan structures are ongoing challenges.

In this study, we present a high throughput MALDI-MS based quantitative targeted glycomics (HT MALDI-QTaG) method for analyzing total N-glycans. This method accomplishes N-glycan release, filtration and chemical derivatization in a 96-well 10-kDa filter plate (10-kDa cut-off). This robust and high-throughput MALDI-MS-based relative quantitative glycomics method can handle a large number of samples (e.g., clinical and biopharmaceutical products) with high accuracy and reproducibility. In addition, UNIFI solution-installed UPLC-fluorescence (UPLC-FLR) supports the HT MALDI-QTaG method to determine the relative quantities of isomeric N-glycans. We successfully applied the HT MALDI-QTaG method to the relative quantification analysis of total N-glycans from bovine fetuin, human serum and Enbrel®, etanercept.

Materials and methods

Chemicals and materials

Human serum samples from 9 healthy donors were obtained from Dongguk University. These blood specimens were collected in accordance with an institutional review board protocol. Acetohydrazide (Ah) was purchased from Tokyo Chemical Industry (Tokyo, Japan) and methanol (MeOH), acetonitrile (ACN) and acetic acid (AA) were obtained from Junsei (Tokyo, Japan). Fetuin from fetal bovine serum, *N*-(3-dimethylaminoprophyl)-*N*′-ethylcarbodiimide hydrochloride (EDC), sodium hydroxide, dimethyl sulfoxide (DMSO), carboxymethyl trimethylammonium hydrazide (Girard's reagent T, GT), ammonium formate, iodomathane, trifluoroacetic acid (TFA) and 2,5-dihydroxybenzoic acid (DHB) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Peptide N-glycosidase F (PNGase F) was purchased from Roche (Mannheim, Germany). The LudgerTag™ 2-AB glycan labeling kit and the 2-AB Dextran calibration ladder standard were purchased from Ludger (Oxfordshire, UK) and Waters (MA, USA) respectively. Enbrel® was obtained from Korea Pfizer (Seoul, Korea).

96. -well plate-based sialic acids amidation and enzyme digestion

Sialic acids amidation and enzyme digestion steps were as follows [31]: A 96-well molecular weight cutoff (MWCO) membrane filter (Harvard Apparatus, Holliston, MA) was prewashed with deionized water. Heat-denatured bovine fetuin (50 mg/mL), 20 μL of human serum and the biotherapeutic were loaded on a 96-well membrane filter. The samples were desalted with deionized water. Then, 100 μL of 1 M Ah, 20 μL of 1 N HCl, and 20 μL of 2 M EDC were added to carry out the sialic acid amidation reaction. The mixtures were incubated at RT for 4 h and washed with deionized water for desalting (5 × 300 μL with centrifugation at 4000 rpm). PNGase F (5 μL) was added to the sample and incubated at 37 °C for 16 h. After incubation, the 96-well plate was centrifuged to collect the N-glycan samples, and the loaded plate was dried under a speed vacuum. All steps were performed using the single 10-kDa MWCO membrane filter.

Girard's reagent T (GT) derivatization

The amidated N-glycans in the 96-well plate were reconstituted with 10 μL of 50% MeOH/50% water [v/v]. Girard's reagent T (GT) (100 μL of a 5 mM solution in 99% MeOH/1% AA [v/v]) was injected into the 96-well plate. This mixture was incubated at RT for 4 h [31]. The GT labeled N-glycan solution was subsequently dried in a centrifugal vacuum concentrator prior to MALDI analysis.

Single 10-kDa filter tube-based N-glycans release for permethylation and 2-AB labeling

Bovine fetuin and human serum samples were denatured for 3 min at 95 °C and were loaded on a single 10-kDa filter tube. Then, they were washed with deionized water (5 × 300 μL with centrifugation at 14000 × *g*). The samples were incubated with 5 μL of PNGase F at 37 °C for 16 h. After incubation, the N-glycan samples were eluted by centrifugation at 14,000 × *g* for 10 min.

Porous graphite carbon-based solid-phase extraction

The released N-glycans and standard oligosaccharides were loaded directly onto a porous graphitized carbon (PGC) cartridge (Thermo Scientific, Hudson, NH, USA) to remove the salts and other environmental contaminants. The solvent system was generated as follows: first, the well was prewashed with 200 μL of 30% AA/70% water followed by 200 μL of 0.1% [v/v] TFA in 50% ACN/50% water. The well was continually primed with 200 μL of 0.1% [v/v] TFA in 5% ACN/95% water, and then the N-glycans were loaded into the well. Subsequently, the well was continuously washed with 200 μL of water and 200 μL of 0.1% [v/v] TFA in 5% ACN/95% water. The N-glycans were eluted into a collection plate with 200 μL of 0.1% [v/v] TFA in 50% ACN/50% water. The eluent was transferred to a microtube and dried in a centrifugal vacuum concentrator.

Solid-phase permethylation

The spin column was packed with sodium hydroxide beads using a 1-mL syringe. A microspin column was filled with sodium hydroxide beads to approximately 1 cm below the top. The spin column containing 200 μL of ACN was then centrifuged at 5000 rpm for 30 s, and the ACN was discarded. The spin column containing 200 μL of DMSO was then centrifuged at 5000 rpm for 30 s and discarded. The sample containing 5.6 μL of water, 141.6 μL of DMSO, and 52.8 μL of iodomethane was added to the spin column, and the column was centrifuged at 1200 rpm for 20 s. This step was repeated seven more times. The permethylated sample was transferred to a new microtube. The spin column containing 200 μL of ACN was centrifuged at 1200 rpm for 1 s and the solution was transferred to the microtube. The spin column was centrifuged at 10,000 rpm for 1 min. The solution was transferred to the microtube. Chloroform and water (200 μL of each) were added to the permethylated sample, which was washed until the aqueous layer reached pH 7. Finally, the chloroform was removed under nitrogen gas.

Derivatization with 2-AB for UPLC analysis

The N-glycan samples were incubated with 5 μL of the 2-AB labeling reagent (LudgerTag™ 2-AB glycan labeling kit) at 65 °C for 3 h. Subsequently, the excess labeling reagent was removed using Whatman 3MM chromatography paper (Whatman, UK). The 3MM

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