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Relative quantitation of neutral and sialylated *N*-glycans using stable isotopic labeled d0/d5-benzoyl chloride by MALDI-MS

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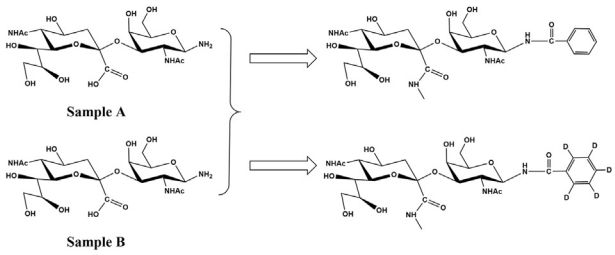
HIGHLIGHTS

- The analytical procedure can be achieved in 4 h by microwave-assisted deglycosylation.
- Both neutral and sialylated *N*-glycans can be quantified simultaneously by a dual-labeling strategy.
- A good linearity across 100-fold dynamic range for relative quantitation of *N*-glycans.

GRAPHICAL ABSTRACT



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ABSTRACT

Quantitative analysis of glycans is an emerging field in glycomic research. Herein we present a rapid and effective dual-labeling strategy, in the combination of isotopic derivatization of *N*-glycosylamine-based glycans by d0/d5-benzoyl chloride and methylamidation of sialic acids, to relatively quantify both neutral and sialylated *N*-glycans simultaneously by MALDI-MS. The derivatization efficiencies were increased by microwave-accelerated deglycosylation which not only largely reduce the time of glycoprotein deglycosylation but also inhibit the hydrolysis of intermediate glycosylamines produced by PNGase F digestion. Three model glycoproteins, including RNase B, bovine fetuin and IgG from human serum, were applied to validate this technique. Results showed that the glycans from microgram level of glycoprotein can be successfully quantified with high reproducibility and the whole time of analytical procedure was shortened to 4 h. Furthermore, this proposed method was applied for the comparative analysis of *N*-glycans from serum of healthy donors and multiple myeloma patients. It was found that five *N*-glycans may be as the potential biomarkers for rapid detection of early multiple myeloma, indicating the feasibility of this strategy in monitoring subtle quantitative differences of serum glycomics.

1. Introduction

As one of the most complex and widespread post-translational modifications, protein glycosylation plays an important role in various biological processes such as cell signaling, adhesion and communication [1–4]. Numerous studies have demonstrated that many mammalian diseases such as immune deficiencies [5],

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different types of cancers [6–8], inflammation [9] and cardiovascular diseases [10], are associated with the aberrant glycosylation of proteins. Due to the biological significance of glycosylation, quantitative glycomics strategies allowing to effectively profile and quantify these aberrant changes in glycosylation are indeed required.

Various approaches have been employed to monitor the subtle changes of glycans, including capillary electrophoresis, liquid chromatography and mass spectrometry. CE-LIF of APTS-labeled glycans has been utilized to assess the differences of liver diseases [11,12]. LC detection quantitative glycomic strategies with multiple fluorophores, such as 2-aminobenzamide (2-AB), 2-amino pyridine (PA), and 2-aminobenzoic acid (2-AA), have also been used to quantify changes of glycans [13–15]. However, the requirement of defined pure internal or external standards and the decrease of quantitation reliability caused by increased sample complexity may limit these spectroscopy-based quantitative methods. To date, numerous MS-based quantitative glycomics strategies have been developed [16–18]. However, deficiencies such as signal bias to different mass and time as well as differences in ionization efficiency may influence the quantitative reliability.

Recently, the incorporation of powerful MS tools and stable isotopic reagents appears to be one of the most viable strategy in quantitative glycomics. Relative quantification with stable-isotope species onto chemically similar glycans can not only effectively eliminate the divergence in ionization efficiency but also allow simultaneous MS analysis of multiple samples with a distinguishable mass difference. By comparing peak intensities of isotopically labeled analytes, relative abundances of glycans with same chemical characteristics were obtained, reducing the run-to-run variation of glycan analysis.

A series of effective and convenient methods for MS-based stable isotope labeling in quantification of glycans have been developed, including permethylation [19], reductive amination [20–22] and hydrazone formation [23]. All these quantitative strategies are implemented by labeling glycans with ^{12}C and ^{13}C (or with ^{1}H and D) modified-isotopic labeling reagents. Permethylation was carried out by labeling glycans with heavy and light methyl iodide [24–27]. However, the mass differences between the heavy and light pattern of each glycan were varied by the numerous number of their methylation sites, causing difficulties in the interpretation of mass spectra [28]. Additionally, the extremely alkaline conditions for permethylation reaction may lead to the oxidative degradation of glycans, causing the irreversible damage to the relative quantitation [29,30]. The other methods for relative quantitation of glycomics are reductive amination and hydrazone formation, which specifically introduce a mass tag to the single aldehyde group on the reducing end of glycans. Recently, the use of stable isotope labels based on reductive amination for carbohydrate analysis has been well documented, such as d0/d6-2-aminopyridine, $^{12}C_6/^{13}C_6$-2-aminobenzoic acid and $^{12}C_6/^{13}C_6$-aniline [21,22,31]. In addition, a group of stable isotope mass tags for comparing multiplex samples in parallel have been reported [20,32]. Moreover, isotopic quantification of glycomics based on hydrazone chemistry was also carried out, allowing the direct analysis of LC-MS [23]. Unfortunately, both reductive amination and hydrazine formation requires acid catalyst at high temperature, causing the degradation of acid-labile groups such as sialic acid residues and N/O-sulfate groups [33,34]. Especially for the analysis of MALDI-MS, the damage of non-neutralized glycans caused by in- and/or post-source decay cannot be avoided. Recently, Zhou and Warren have reported a dual modifications strategy which can simultaneously quantify neutral and sialylated glycans within one sample without the degradation of acid-labile groups [28]. However, this strategy still need lengthy reaction time, which may limit the application in rapid quantitation of glycomics.

In order to overcome those deficiencies described above, an ideal method for relative quantitation of glycomics is indeed required. Recently, an analytical strategy based on domestic microwave rapid digestion strategy has been developed in our previous report, which can provide *N*-glycosylamines with a shorter incubation time [35]. Here we described a cheap, rapid and effective isotopic labeling strategy, in which the *N*-glycosylamine-based glycans are firstly labeled with d0/d5-benzoyl chloride (d0 denotes non-deuterium and d5 denotes deuterium) and then the sialic acid residues are derivatized with methylamine prior to the analysis of MALDI-MS (Fig. 1). The strategy is designed to be simple and time saving which can be achieved in 4 h. In addition, as sialic acid residues are neutralized, the relative quantitation of neutral and sialylated glycans within an individual can be achieved simultaneously without the degradation of acid residues. Moreover, benzoyl chloride has been commercial and is very cheap, reducing the experimental costs. Furthermore, this strategy has also been used to analyze glycans from standard glycoproteins and human serum, suggesting the capability of the strategy in quantitative glycomics.

2. Materials and methods

2.1. Chemicals

Peptide-*N*-glycosidase F (PNGase F) and endoglycosidase buffer kit were purchased from LCP Biomed (Lianyungang, China). Benzoyl chloride-d5, dimethyl sulfoxide (DMSO), (7-zabenzotriazol-1-yloxy) trispyrrolidinophosphonium hexafluorophosphate (PyAOP), methylamine hydrochloride, 4-methylmorpholine, 1-butanol, ethanol, microcrystalline cellulose (MCC), sodium hydroxide, bovine pancreas ribonuclease B (RNase B), bovine fetuin, human IgG from serum and 2,5-Dihydroxybenzoic acid (DHB) were obtained from Sigma-Aldrich (MO, U.S.A.). Benzoyl chloride were from Aladdin Industrial Inc. (Shanghai, China). Acetonitrile (ACN) were purchased from Merck KGaA (Darmstadt, Germany). Pure water were obtained from Thermo Fisher Scientific (MA, U.S.A.). The empty cartridges and frits were purchased from Tianjin Bonna-Agela Technologies Inc. (Tianjin, China).

2.2. Human serum specimens

Individual human serum samples in this study were acquired from donors of health (n = 15) and multiple myeloma (MM) patients (n = 15) with Stage II with informed consent in Tongji Hospital (Tongji Medical College, Huazhong University of Science and Technology), and the clinical metadata was listed in Table S1. It is noteworthy that in order to alleviate the effect of the individual variation in the following experimental process, the mixture of healthy serums was setted as the control group. The study was carried out in accordance with the Helsinki Declaration and informed consents were obtained from the participants in accordance with the study protocols approved by the Ethics Committee of Huazhong University of Science and Technology.

2.3. Rapid deglycosylation of glycoproteins

Rapid release of *N*-glycans followed by labeling with d0/d5-benzoyl chloride was implemented by a one-pot reaction. For the purpose of rapid deglycosylation, standard glycoproteins (10 μg) or human serum sample (5 μL) were dissolved in 20 μL reaction buffer solution containing 10 mM sodium phosphate (pH = 8.5), 0.13% dodecyl sulfate sodium and 10 mM dithiothreitol. The sample was denatured at 100 °C for 10 min prior to adding 2.4 μL of 10% octylphenoxypolyethoxyethanol (NP-40). Then 0.5 μL of PNGase F

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