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Determination of *N*-glycans by high performance liquid chromatography using 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate as the glycosylamine labeling reagent

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ABSTRACT

Robust, efficient identification and accurate quantification of *N*-glycans are of great significance in *N*-glycomics analysis. Here, a simple and rapid derivatization method, based on the combination of microwave-assisted deglycosylation and 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate (AQC) labeling, was developed for the analysis of *N*-glycan by high performance liquid chromatography with fluorescence detection (HPLC-FLD). After optimizing various parameters affecting deglycosylation and derivatization by RNase B, the time for *N*-glycan labeling was shortened to 50 min with ~10-fold enhancement in detection sensitivity comparing to conventional 2-aminobenzoic acid (2-AA) labeling method. Additionally, the method showed good linearity (correlation coefficients > 0.991) and reproducibility (RSD < 8.7%). These advantages of the proposed method were further validated by the analysis of complex samples, including fetuin and human serum. Investigation of serum *N*-glycome for preliminary diagnosis of human lung cancer was conducted, where significant changes of several *N*-glycans corresponding to core-fucosylated, mono- and disialylated glycans have been evidenced by a series of statistical analysis.

1. Introduction

N-Glycosylation of proteins plays significant roles in many important biological processes, such as cell signaling, fertilization, proliferation and differentiation [1]. Alterations of protein *N*-glycans are associated with the pathogeneses of many diseases including cancers [2], diabetes [3] and heart failure [4]. Therefore, efficient identification and accurate quantification of *N*-glycans is necessary for a better understanding of related biological processes, as well as diagnostical glycan biomarkers discovery and therapeutic vaccine design.

Current methods such as mass spectrometry (MS) [5], liquid chromatography (LC) [6], capillary electrophoresis (CE) [7], and liquid chromatography coupled to mass spectrometry (LC–MS) [8] have been routinely applied for *N*-glycans analysis. Due to its high separation efficiency, prominent stability and good quantitative properties, HPLC-FLD has become a powerful tool to analyze *N*-glycans. However, due to their lack of fluorophores, *N*-glycans are required to undergo fluorescent derivatization before HPLC analysis. A variety of derivatization strategies have been developed, such as reductive amination and hydrazide labeling [9–11], among which the reductive amination of the reducing end of glycan using 2-aminobenzamide (2-AB) or 2-aminobenzamide acid (2-AA) are widely used in glycan analysis [12,13]. However, reductive amination can be interfered by detergents or salts during protein denaturation and some of which require extra purification steps before derivatization [14], leading to elongated analysis time and sample losses.

Compared to reduced glycans, glycosylamines can be acquired directly by PNGase F digestion, allowing alternative glycosylamine derivatization with various acylated reagents under aqueous condition [15,16] and significantly simplifying the sample preparation procedures. According to a previous work by Lauber et al.,

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glycosylamine could be easily hydrolyzed within hours after deglycosylation, and its hydrolysis tended to be slow at pH 8.0. Moreover, the released glycan present as glycosylamine, which has a half-life of approximately 2 h at 50 °C [17]. To improve the derivatization efficiency, tremendous efforts have been devoted to shorten the deglycosylation time for minimizing the hydrolysis of glycosylamine, including microwave assistance [18], use of a RG surfactant [17], and immobilization of PNGase F [19]. Microwave-assisted deglycosylation has been the simplest one since its ultrafast convection currents and thermal conductivity on a molecular level [20].

Additionally, the selection of an appropriate acylated reagent is important for achieving high derivatization efficiency of glycosylamines. It has been reported that *N*-hydroxysuccinimide (NHS)-activated carbamates and esters can efficiently derivatize glycosylamines [21,22]. Stöckmann et al. used a conventional fluorescent reagent, AQC, to derivatize *N*-glycosylamine-glycans [23] due to its high reactivity toward both primary and secondary amino acids [24]. Moreover, AQC labeling can proceed in aqueous solution without interference with salts or detergent, eliminating the requirement of additional time-consuming SPE purification prior to derivatization, and the buffer-exchange step between the deglycosylation and AQC derivatization because of their similar reaction buffer and pH ranges [23]. Recently, an MS enhancing reagent by modifying AQC was also applied to separate and identify glycan structures [17].

In this work, we developed a prominent glycosylamine labeling strategy that combined AQC as a fluorescent tag with the microwave-assisted deglycosylation in 20 min for HPLC-FLD analysis. It was optimized with RNase B and further validated by fetuin and human serum. Subsequently, the application of the proposed method shed light on the elementary determination of *N*-glycans biomarkers in human lung cancer samples by a series of statistical analysis such as ANOVA test, PCA and ROC (AUC) analysis.

2. Experimental

2.1. Chemicals

Ribonuclease B (RNase B), fetuin, microcrystalline cellulose (MCC), ammonium formate, and 2-aminobenzonic acid (2-AA), and porous graphitic carbon (PGC) were obtained from Sigma-Aldrich (St. Louis, MO). 6-Aminoquinolyl-*N*-hydroxysuccinimidyl carbamate (AQC) was obtained from Finetech (Wuhan, China). 4-Fluoro-7-nitro-2,1,3-benzoxadiazole (NBD-F) and 9-Fluorenylmethl chloroformate (Fmoc-Cl) was purchased from TCI (Tokyo, Japan). The empty cartridges and frits were purchased from Tianjin BonnaAgela Technologies Inc. (Tianjin, China). *N*-Glycosidase F (PNGase F) was purchased from LCP Biomed (Lianyungang, China). ExpressPlusTM 8–16% Tris-Glycine Mini Protein Gel was purchased from GenScript Co., Ltd. (Nanjing, China). Acetonitrile (ACN), butanol, ethanol, methyl alcohol and formic acid (FA) were purchased from Merck KgaA (Darmstadt, Germany). Other reagents and solvents used in the present research were the reagent grade or HPLC grade obtained from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). All aqueous solutions were prepared using water purified by a Milli-Q purification system (Millipore, Bedford, MA).

2.2. Serum samples

Serums were obtained from Tongji Medical College of Huazhong University of Science and Technology. All samples were collected using vacuum tubes from subjects with empty stomach in the morning. The serums were isolated by centrifugation for 2 h (25 °C 1300*g* 15 min) and kept in cryogenic tubes at −80 °C. 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. N-*Deglycosylation*

Glycoproteins (10 μg) or human serum samples (5 μL) were mixed with buffer solution to 20 μL, containing 0.13% dodecyl sulfate sodium and 10 mM dithiothreitol. Then, the sample was denatured in water bath at 95 °C for 5 min. After denaturation, 2.4 μL of 10% octylphenoxypolyethoxyethanol (NP-40) was used for equilibration for 5 min. Next, the PNGase F (enzyme:substrate molar ratio of 1:100) was added. The microwave-assisted deglycosylation was irradiated using a Galanz household microwave oven (Model LRM1180W, China) at 119 W power for 20 min with samples in 500 mL water. The conventional deglycosylation was conducted in 37 °C water bath for overnight.

2.4. Glycans derivatization

2.4.1. Glycosylamine derivatization

After microwave-assisted deglycosylation, the glycosylamine labeling of NBD-F, Fmoc-Cl, and AQC was performed as the following: 20 μL of fresh NBD-F/Fmoc-Cl/AQC (150 mM in ACN) solution and 20 μL ammonium bicarbonate buffer were directly added. Mixtures of NBD-F, Fmoc-Cl, and AQC were vortexed and then incubated at 70 °C for 5 min, 37 °C for 1 h, and 40 °C for 20 min, respectively. The derivatives were purified by self-assembly microcrystalline cellulose solid-phase extraction (MCC SPE) prior to further analysis. Briefly, MCC cartridges were equilibrated by 3.0 mL of 1-butanol/ethanol/H_2O (4:1:1, v/v/v). Subsequently, the derivatives were loaded on MCCs and washed with equilibrium solution (3.0 mL). Finally, the sample was eluted by 1.0 mL of ethanol/H_2O (1:1, v/v), and dried under vacuum.

2.4.2. Derivatization using reductive amination of 2-AA

After conventional overnight deglycosylation, the sample was purified by self-assembly porous graphitic carbon solid-phase extraction (PGC SPE) prior to further labeling. Briefly, PGC cartridges were washed with 3.0 mL of 80% (v/v) ACN containing 0.1% TFA followed by 3.0 mL of water. The samples were loaded on PGC cartridges and then washed with water (3.0 mL) to remove buffer and salts. Glycans were eluted with 1.0 mL 40% ACN in 0.1% TFA. The fraction was collected and dried for further analysis or processing. The procedure of 2-AA labeling was given as the following: dried samples were mixed with 20 μL freshly prepared labeling solution (consisting of 0.35 M 2-AA and 1.0 M 2-picoline-boranein 3/7 (v/v) acetic acid/DMSO). The mixtures were vortexed and incubated at 65 °C for 2 h. After reaction, the glycan derivatives were purified according to the HILIC method as previously described prior to further analysis.

2.5. HPLC analysis with fluorescence detection

An analytical system (Shimadzu, Nakagyo-ku, Kyoto, Japan) of HPLC consisted of two LC-20AD pumps, a SIL-20AC auto sampler, a CBM-20A degasser, a CTO-20AC column oven, and a RF-10AXL fluorescence detector controlled with a LCMSstation® system.

The derivatized *N*-glycans was analyzed on an Amide80 column (Tosoh, Tokyo, Japan; 4.6 mm i.d., 250 mm) thermostated at 40 °C using a linear gradient. Separation of 2-AA and AQC labeled *N*-glycans used 50 mM ammonium formate (pH 4.4) as solvent A and ACN as solvent B at a flow rate of 1.0 mL/min. The column was initially equilibrated and eluted with 68% solvent B for 5 min. Then a mixing ratio of solvent B was decreased linearly to 43% over 55 min and then further decreased to 5% over 5 min. The

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