Journal of Chromatography A, 1307 (2013) 201–206

Contents lists available at ScienceDirect

Journal of Chromatography A

journal homepage: www.elsevier.com/locate/chroma

Short communication

A cost-effective plate-based sample preparation for antibody *N*-glycan analysis

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ARTICLE INFO

Article history:
Received 29 June 2013
Received in revised form 27 July 2013
Accepted 30 July 2013
Available online 2 August 2013

Keywords:
Plate-based
High-throughput
Fluorescence labeling
Antibody glycosylation

ABSTRACT

During early cell line and process development of therapeutic antibodies, a cost-effective high-throughput approach to characterize the *N*-linked glycans is highly desired given that a large number of samples need to be analyzed. Using commercially available, low cost 96-well plates, we developed a practical procedure to prepare fluorescently labeled *N*-linked glycans for both qualitative and quantitative analysis by mass spectrometry (MS) and ultrahigh performance liquid chromatography (UPLC). Antibody samples were continuously denatured, reduced, and deglycosylated in a single 96-well hydrophobic membrane filter plate. Subsequently, released glycans were fluorescently labeled in a collection plate, and cleaned-up using a hydrophilic membrane filter plate. Carried out entirely in ready-to-use 96-well plates with simple buffer systems, this procedure requires less than 90 min to finish. We applied the optimized procedure to examine the *N*-linked glycosylation of trastuzumab and were able to quantify ten major *N*-linked glycans. The results from different amounts of starting materials (10–200 μg) were highly similar and showed the robustness of this procedure. Compared to other methods, this new procedure is simple to implement, economically more affordable, and could be very valuable for early screenings of antibody development.

1. Introduction

N-linked glycosylation is a prominent post-translational modification occurring on antibodies. In addition to its involvement in antibody binding of Fcγ receptors [1], recent literature suggest that sialylated *N*-linked glycans on the Fc play an essential role in the anti-inflammatory activity of intravenous immunoglobulin (IVIg) [2]. Throughout therapeutic antibody development, glycosylation analysis is frequently performed not only to profile their biochemical characteristics, but also to assess the stability of expression cell lines and robustness of downstream processes [3,4]. Furthermore, as an important structural and functional attribute of antibodies and related proteins, glycosylation is a critical aspect in comparing the biosimilar/biobetter monoclonal antibodies with the innovator's molecules as well as evaluating the Fc-fusion proteins [5–8]. Because a large number of samples need to be assayed in early cell line and process screening, a cost-effective high-throughput approach to prepare antibody *N*-glycans for both qualitative and quantitative analysis is highly desired.

Antibody *N*-glycosylation has been characterized in the form of either glycopeptides or free glycans [9]. Glycopeptide-based approaches rely on the observation that the amino acid sequences harboring Asn 297 in the CH2 domain are highly conserved among IgG subclasses, which allows reliable determination and quantification of glycan structures through liquid chromatography mass spectrometry (LC–MS). A recently reported method using MALDI-TOF, instead of LC–MS, to analyze the glycopeptides shortened the analysis substantially as the lengthy HPLC separation was avoided [10]. To generate glycopeptides in the high-throughput manner, protocols using the combination and/or excess of proteases to enable fast digestion of antibody samples have been explored [11,12]. The potential pitfalls for such approaches include the overlapping of glycopeptides with non-glycopeptides and the variation of digestion efficiency. For the analysis based on free glycans, released glycans are frequently fluorescently labeled prior to analysis on a fluorescence detector equipped HPLC (FLR-HPLC) system as the detection and quantification of free glycans are difficult [13–15]. Given the popularity of using ESI–MS to detect and quantify glycans, it is worthwhile to point out that cautions should be taken when only MS signal is used as labile glycans can fall off during the ionization process [16]. High-throughput procedures of preparing fluorescently labeled *N*-glycans have been reported previously. Royle et al. described a 96-well plate format using either SDS-PAGE gel or PVDF membrane for efficient glycan release, followed by 2-aminobenzamide (2AB) labeling and cleaning-up with microplates [17]. Even feasible for automation, this procedure is still tedious, labor intensive, and requires 2 or 3 days to execute. Ruhaak et al. demonstrated a much simplified approach, in which

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http://dx.doi.org/10.1016/j.chroma.2013.07.104

the denaturation, deglycosylation of glycoproteins, and subsequent 2-aminobenzoic acid (2AA) labeling were continuously performed in a single 96 deep-well plate [18]. Requiring no cleaning-up steps prior to the labeling makes this method very attractive. However, inclusion of detergent, surfactant, proteins, and other salts in the PNGase F digestion and labeling reaction could impact the efficiency of deglycosylation and glycan labeling, as well as subsequent solid phase extraction (SPE) purification of labeled glycans. Kim et al. introduced using porous graphitic carbon containing 96-well plate to separate free glycans from PNGase F digestion reaction prior to derivatization [19]. This method worked well for neutral glycans as the quantification is based on MALDI-TOF analysis of Girard's reagent T derivatized glycans. However the quantification of sialylated glycans is not reliable due to the instability of sialylated glycans under the employed labeling conditions and the negative charge introduced by the sialic acid. Cook et al. examined a rapid glycan labeling system from ProZyme [20]. While demonstrated the benefits of the speed and sensitivity, it is expensive and could be cost-inhibitive during early screening.

We recently reported a plate-based approach to isotopically label *N*-linked glycans for quantitative analysis using LC-MS [21]. It has several important beneficial attributes such as the speed, simplicity, and accuracy, however, the high cost of currently available isotopic labeling reagents prevents it from being applied to a large number of samples. In current work, we developed a cost-effective approach by using the inexpensive, commercially available 96-well plates and fluorescent dye. Different from other reported methods, this procedure was carried out entirely in ready-to-use 96-well plates and required ~90 min to finish. While optimized using palivizumab and demonstrated with trastuzumab, this procedure is readily applicable to many other antibodies and glycoproteins.

2. Material and methods

2.1. Reagents

8 M Guanidine chloride (GuHCl), 1 M Triethylammonium bicarbonate (TEAB) pH 8.5, and dimethyl sulfoxide (DMSO) were from Sigma Aldrich (St. Louis, MO). 0.5 M Tris (2-carboxyethyl) phosphine (TCEP) was from Pierce (Rockford, IL). PNGase F (500 kU/mL) was from New England Biolabs (Ipswich, MA). Palivizumab manufactured by MedImmune and trastuzumab by Genentech were purchased from Myoderm (Norristown, PA). InstantAB was from ProZyme (Hayward, CA). HPLC grade acetonitrile and water were from Fisher Scientific (Pittsburgh, PA). 10 kDa MW cutoff spin tubes, MultiScreen®$_{HTS}$ filter plates with hydrophobic Immobilon-P PVDF membrane, hydrophilic mixed cellulose esters (MCE) membrane, and hydrophilic Durapore PVDF membrane were from Millipore (Billerica, MA). Wizard® SV 96 Lysate clearing plate was from Promega (Madison, WI). AcroPrep™ Advance GHP®, Supor®, and DNA binding 96 Multi-well filter plates were from Pall Life Sciences (Ann Arbor, MI). 96-well collection plate was from Axygen Scientific (Union City, CA). DHB MALDI matrix was from Protea Biosciences (Morgantown, WV). The Acquity UPLC BEH glycan separation technology column was from Waters (Milford, MA). Prevail™ Carbohydrate ES HPLC column was from Grace Davison Discovery Sciences (Deerfield, IL).

2.2. N-glycan release

For PNGase F digestion of antibodies performed in Eppendorf tubes, palivizumab was buffer exchanged to 50 mM TCEP pH 8.5 buffer through 10 kDa MW cutoff spin tubes. 1 μL PNGase F and 2 μL 0.5 M TCEP were added to 100 μL 1 mg/mL Palivizumab. They were incubated at 25 °C, 37 °C, 50 °C, and 60 °C separately. 10 μL from each digestion was taken out at 30, 60, and 120 min and analyzed on an Agilent 6520 Q-TOF mass spectrometer.

For the 96-well plate based PNGase F digestion, 50 μL 2 mg/mL palivizumab (For trastuzumab, 4 mg/mL, 2 mg/mL, 1 mg/mL, 0.2 mg/mL) was mixed with 150 μL 8 M GuHCl, 4 μL 0.5 M TCEP, and loaded onto a MultiScreen®$_{HTS}$ filter plate with hydrophobic Immobilon-P PVDF membrane. They were incubated at 50 °C for 10 min. The plate was then washed twice with 200 μL 100 mM TEAB pH 8.5 buffer (spun at 500 g for 1 min). 30 μL 100 mM TEAB pH 8.5 buffer containing 1 μL PNGase F was added to each sample. The PNGase F digestion was carried out in a moisturized Tupperware at 50 °C for 30 min. Released *N*-glycans were collected in a clean 96-well collection plate (centrifugation at 1000 × *g* 1 min) and used for derivatization with the fluorescent dye immediately.

2.3. N-glycan labeling and cleaning-up

One ampule of 18.5 mg instantAB dye was reconstituted in 200 μL DMSO. 5 μL dye solution was added to the glycans and incubated at 37 °C for 5 min. Meanwhile 96-well hydrophilic filter plates were prepared by washing with 200 μL 70% ethanol, water, and 100% acetonitrile sequentially (spun at 500 × *g* 1 min). 300 μL of 100% acetonitrile was added to the labeling reaction and the whole solution was loaded to the filter plates. The plates were spun at 50 × *g* for 1 min to allow the glycans to bind to the plates. Samples were then washed three times with 200 μL 96% acetonitrile (spun at 500 × *g* 1 min). The residual solvent was removed by spinning the plates at 1000 × *g* for 1 min. 30 μL HPLC grade water was then applied to elute the labeled glycans (spun at 1000 × *g* for 1 min).

2.4. Quantitative N-glycan analysis by HPLC/UPLC

For the analysis using the Carbohydrate ES column (250 × 4.6 mm) on an Agilent 1200 HPLC system, 10 μL of labeled glycans was mixed with 1 μL labeled glycan standards and 40 μL 100% acetonitrile. We injected 30 μL for each analysis. Buffer A was 100 mM formate pH 4.6 and buffer B was acetonitrile. The flow rate and column temperature were set at 1.3 mL/min and 45 °C throughout the experiments, respectively. The HPLC gradient was: 30–44% buffer A from 0 to 20 min, ramped to 100% A in 15 min, and maintained at 100% A for 3 min. The column was equilibrated with 30% buffer A for 7 min. For the fluorescence detector, the excitation/emission wavelengths were 278 nm and 344 nm, respectively.

For the analysis performed on an Acquity UPLC system with the Acquity BEH glycan column (2.1 × 100 mm), 5 μL of labeled glycans was mixed with 20 μL 100% acetonitrile. 10 μL was injected. The analyses were performed under the following conditions: column compartment temperature: 60 °C; sample manager temperature: 10 °C; flow rate: 0.4 mL/min; buffer A was 100 mM formate pH 4.6, buffer B was acetonitrile. The gradient was: 30% buffer A from 0 to 1 min, then to 40% buffer A in 15 min. The settings for the FLR were: excitation at 278 nm, emission at 344 nm, PTM gain at 1, data rate at 80 pts/s.

2.5. Antibody and N-glycan analysis by LC–MS and MALDI-TOF

PNGase F-digested antibody was analyzed on an Agilent 6520 Q-TOF mass spectrometer coupled with an Agilent 1200 HPLC system. The HPLC settings were: buffer A was water with 0.1% (v/v) formic acid and buffer B was 10% (v/v) water, 90% (v/v) acetonitrile with 0.1% (v/v) formic acid. The gradient was: 100% buffer A at 2 mL/min from 0 to 1 min, followed by 0.8 mL/min with 100% B for half minute. The flow rate dropped to 0.5 mL/min for the remaining of 2 and half min during MS analysis. The settings for Q-TOF were: gas temp: 350 °C; drying gas: 13 L/min; nebulizer: 45 psig;