Chapter 19

High-Throughput *N*-Glycan Analysis with Rapid Magnetic Bead-Based Sample Preparation

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Abstract

N-glycan profiling of therapeutic glycoproteins is essential to ensure the activity and efficacy of these promising new-generation drugs. The N-linked glycan moieties of these entities highly affect circulation half-life, immunogenicity and receptor-binding activity as well as physicochemical and thermal stability properties. In addition, more than half of the biopharmaceuticals are glycoproteins representing multibillion dollar worldwide business, further emphasizing the importance of their analysis. In the biomedical field, on the other hand, revealing disease-related glycan structure alterations holds the promise of the discovery of new biomarkers for early diagnostics. Therefore, there is a great demand for widely applicable, high-throughput sample preparation and analysis methods for N-glycan profiling of glycoproteins. One of the newest exciting developments of the field is the magnetic bead based glycoprotein sample preparation technique. A detailed protocol of this method is given in this chapter in conjunction with rapid capillary electrophoresis analysis of the prepared samples by laser induced fluorescence detection (CE-LIF). N-glycans are digested by the endoglycosidase PNGase F and the released carbohydrates are labeled with the charged fluorophore dye of aminopyrenetrisulfonate (APTS). Effective glycan capture by magnetic microparticles enabled fast, easily automated sample preparation both in individual (single vial) and 96-well plate formats, including excess dye removal. Rapid separation of APTS labeled IgG glycans is also shown utilizing an optimized CE-LIF protocol.

Key words Magnetic bead, N-glycan analysis, Fluorophore labeling, Capillary electrophoresis

Abbreviations

PNGase F	Peptide-N4-(N-acetyl-beta-glucosaminyl) asparagine-amidase
APTS	8-aminopyrene-1,3,6-trisulfonic acid
CE-LIF	Capillary electrophoresis with laser induced fluorescence detection
IgG	Immunoglobulin G

1 Introduction

N-glycosylation analysis is one of the fastest growing areas in the biopharmaceutical and biomedical fields [1, 2]. Glycosylation of the expressed polypeptide chains, e.g., in humans it is mediated by

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more than 600 enzymes during complex co-translational and posttranslational modification processes [3] resulting extensive micro- and macro-heterogeneity [4]. Actually, the true variability of glycan structures comes from the fact that posttranslational modifications are highly dependent on the surrounding environment, state of health, presence of inhibitors and suppressors, etc. Therefore, changes may occur frequently and rapidly. Thus, the analysis of disease-associated structural changes such as core and antennary fucosylation is key and may lead to glycan based biomarker development and personalized treatments [5]. As a matter of fact, precisely defining the position of this particular sugar residue is not easy, e.g., MS based methods are prone to in-source fucosylation rearrangement [6]. The biopharmaceutical industry represents another important segment that needs large scale N-glycosylation analysis methods. In 2015, glycosylated monoclonal antibodies represented almost half of the biotherapeutic drugs on the market with an estimated $70 billion dollar business worldwide [7]. It has been recognized that structural changes in the sugar moiety of therapeutic glycoproteins may significantly modify their activity and efficacy [8], highlighting the importance of their characterization during the entire manufacturing process. Large scale automated glycosylation analysis is of particular interest in early stages of protein drug development, e.g., during clone selection where transfection can result in numerous expressed variants.

One of the most frequently used high resolution glycan analysis techniques is capillary electrophoresis (CE) with laser induced fluorescence detection (LIF). CE separation is capable to differentiate glycans based on their molecular shape, even if their mass to charge ratio are exactly the same [9]. Due to the high sensitivity of the selective LIF detection method (only fluorophore labeled species are detected), injection of picomolar sample amounts are usually sufficient to acquire reliable data, and microliter sample volumes are enough for dozens or even hundreds of analyses [10].

In this protocol a rapid magnetic bead based sample preparation workflow is introduced for high-throughput N-glycan analysis applications in conjunction with fast CE-LIF separation. The N-linked carbohydrates were released from the glycoproteins by PNGase F (peptide-N4-(N-acetyl-beta-glucosaminyl) asparagine-amidase), a reliable and specific cleavage enzyme that works under simple and mild conditions. PNGase F cleaves the bond between the carbohydrate holding asparagine residue at the conserved consensus sequence of Asn-X-Ser/Thr (X is any amino acid except proline, serine, and threonine) and the innermost N-acetyl glucosamine of the trimannosyl chitobiose core [11]. The released glycans are captured by magnetic microparticles and tagged in situ *on bead* via reductive amination with 8-aminopyrene-1,3,6-trisulfonic acid (APTS). The labeling reaction is specific to the reductive end of the sugars, therefore, only one APTS molecule binds to one

sugar molecule, allowing easy quantification. More importantly, since the sugar ring at the reducing end opens up during the derivatization step [12], no anomeric isomers are generated, such as might be the case in the so called instant labeling approaches. The excess fluorophore dye is removed by the same magnetic beads that were used in the earlier glycan capture step. Optimal separation parameters were also developed for rapid CE-LIF analysis of the released, fluorophore labeled and purified sugars.

2 Materials

HPLC grade water should be used (18 MΩ cm at 25 °C) in all buffer and reagent preparations. Some parts of sample preparation should be done under fume hood wearing powder-free nitrile gloves, lab coat, and eyeglasses. Only use new disposable vials that are free from possible environmental carbohydrate contaminations.

2.1 N-Glycan Release, APTS Labeling and Cleanup

1. 0.2 mL safety lock flat cap PCR tubes (actual total volume is 350 µL).
2. Thermostat block to provide stable temperatures at 37 °C and 65 °C.
3. Vortex mixer.
4. Automata pipettors and disposable pipette tips (2, 20, 200 and 1000 µL sizes).
5. Miscellaneous labware for buffers and reagents.
6. Neodymium magnet or magnetic stand or magnetic plate.
7. Agencourt CleanSEQ magnetic microparticles (Beckman Coulter, Part #A29151).
8. 50 mM dithiothreitol (DTT) in water. Prepare the solution fresh before use.
9. 250 mM iodoacetamide (IAA) in water. Prepare the solution fresh before use.
10. 5% sodium dodecyl sulfate (SDS) in water.
11. 0.5% Nonidet P-40 (NP-40) in water.
12. Peptide-N-glycosidase F enzyme (PNGase F) (ProZyme, Part #GKE5006B).
13. 20% acetic acid.
14. 8-aminopyrene-1,3,6-trisulfonic acid (APTS) (Beckman Coulter, Part #M501706).
15. 40 mM 8-aminopyrene-1,3,6-trisulfonic acid (APTS) in 20% acetic acid.
16. 1 M sodium cyanoborohydride ($NaBH_3CN$) in tetrahydrofuran (THF) (Sigma, Part #296813).
17. Acetonitrile (100%, HPLC grade).

2.2 CE-LIF Analysis of APTS Labeled N-Glycans

1. PA 800 plus Pharmaceutical Analysis System (SCIEX, Brea, CA) with Laser Induced Fluorescent (LIF) detector (488 nm excitation wavelength/520 nm emission filter) and 32 Karat software (version 8.0 or higher).
2. N-linked carbohydrate separation gel buffer (SCIEX, Part #477623).
3. N-CHO Capillary, 30 cm length (20 cm effective length), 50 μm ID (SCIEX, Part #M402370).
4. Capillary cartridge (SCIEX, Part #144738) with LIF aperture plug assembly (SCIEX, Part #721125) and probe guide (SCIEX, Part #721126).
5. Universal buffer vials (2 mL), vial caps, sample vials.

3 Methods

3.1 In Solution N-Glycan Release

1. Dissolve the dry glycoprotein sample in HPLC grade water. Preferred stock solution concentration is between 1 and 10 mg/mL.
2. Pipette 200 μL of Agencourt CleanSEQ magnetic bead suspension into a safety lock flat cap 0.2 mL PCR tube.
3. Place the tube with the beads on a magnetic stand and after the beads are pulled to the side of the vial (60 s), remove the storage solution by careful pipetting (*see* **Note 1**).
4. Add 10 μL of glycoprotein sample into the sample tube (1–10 mg/mL concentration).
5. Mix the sample with the beads using a vortex (10 s at maximum speed) (*see* **Note 2**).
6. Prepare the denaturation solution by mixing 2 μL each of 50 mM of DTT, 250 mM of IAA, 5% of SDS and 0.5% of NP-40 solutions.
7. Pipette 4 μL of denaturation solution into the sample containing tube and incubate at 65 °C for 10 min (denaturation step).
8. Pipette 16 μL of 0.5% of NP-40 and 1 μL of PNGase F enzyme (2.5 mU) to the sample and incubate at 50 °C for 1 h (deglycosylation step) (*see* **Note 3**).
9. After the denaturation step, mix the sample using a vortex (10 s at maximum speed).
10. Pipette 210 μL of acetonitrile into the reaction vial (this step will result in 87.5% final acetonitrile concentration in the vial) for glycan capture and vortex the sample (10 s at maximum speed).
11. Wait for 1 min at room temperature.

12. Place the sample tube on a magnetic stand and after the beads are pulled to the side of the vial (30 s), remove the supernatant by pipetting carefully (*see* **Notes 4** and **5**).

3.2 APTS Labeling

1. Add 9 μL of 40 mM APTS (fluorophore) in 20% acetic acid (catalyst) to the reaction vial containing the magnetic beads with the captured glycans from **step 12** in Subheading 3.1.
2. Pipette 1 μL of 1 M $NaBH_3CN$ in THF (reductive agent) and 1 μL of 0.5% NP-40 to the vial. Please note that the use of more $NaBH_3CN$ will not increase reaction speed.
3. Mix the sample using a vortex (10 s at maximum speed).
4. Incubate the reaction mixture at 37 °C for 2 h (labeling step).

3.3 Removal of the Excess APTS Dye

1. After the labeling reaction, pipette 9 μL of 0.5% NP-40 solution to the reaction tube and mix using a vortex (10 s at maximum speed) (*see* **Note 6**).
2. Add 150 μL of acetonitrile and vortex the sample at maximum speed for 10 s.
3. Wait 1 min at room temperature.
4. Place the sample tube on a magnetic stand and after the beads are pulled to the side of the vial (30 s), remove the supernatant by pipetting carefully.
5. Repeat **steps 1–4** three more times using 20 μL HPLC grade water and 150 μL acetonitrile in each step.
6. After the final supernatant removal, elute the labeled glycans from the beads by the addition of 50 μL of HPLC grade water and vortexing at maximum speed for 10 s.
7. Place the sample tube on a magnetic stand and after the beads are pulled to the side of the vial (30 s), transfer the supernatant (this contains the APTS labeled and purified sugars) into a CE sample vial by pipetting carefully.

3.4 CE-LIF Analysis of the APTS Labeled N-Glycans

3.4.1 Preparation of the Glucose Ladder and Setting Up the CE-LIF Equipment

1. Weight out 5 mg of maltooligosaccharide ladder standard (SCIEX, part #M405039).
2. Label the ladder standard similar as was described under Subheading 3.2. There is no need for excess APTS dye removal in this case due to the high sugar to dye ratio.
3. Stop the reaction by the addition of 90 μL of HPLC grade water.
4. Aliquot the APTS labeled maltooligosaccharide ladder sample into 10 μL portions and store at −20 °C.
5. Dilute the maltooligosaccharide ladder standard sample with HPLC grade water by 1000× before use in CE-LIF analysis.

6. Transfer the APTS labeled samples and APTS labeled maltooligosaccharide ladder standard into CE sample vials. Place the sample vials in universal buffer vials and cap them.
7. Fill universal buffer vials with the following reagents:
   (a) Three vials by 1.5 mL of HPLC grade water. Place two vials at home positions and one for capillary fill position.
   (b) 1.5 mL N-linked carbohydrate separation gel buffer into one vial (capillary refill) and 1.3 mL N-linked carbohydrate separation gel buffer into two vials (separation).
   (c) 0.5 mL of HPLC grade water into a waste vial (one universal buffer vial).
8. Separation/Detection parameters:
   (a) Detection: laser-induced fluorescence (488 nm excitation, 520 nm emission filter).
   (b) Data rate: 16 Hz.
   (c) Dynamic range: 100 RFU (relative fluorescence units).
   (d) Filter setting: normal
   (e) Peak width: 16–25.

3.4.2 Performing Rapid CE-LIF Analysis

1. Set the temperature at 25 °C.
2. Rinse the capillary with the N-linked carbohydrate separation gel buffer for 1 min at 100 psi.
3. Sample injection (pressure or electrokinetic):
   (a) Pressure injection: 1 psi for 5 s.
   (b) Electrokinetic injection (field enhanced):
   - Inject water at 1 psi for 5 s (forward).
   - Inject sample at 2 kV for 2 s using the separation gel buffer at the outlet end of the capillary in reversed polarity mode (cathode at the injection side and the anode at the detection side).
   - Inject separation gel buffer at 1 psi for 5 s (forward).
4. Wash the tip of the capillary by moving the trays into home position (universal buffer vials filled with HPLC grade water).
5. Separation: Apply 30 kV for 3 min in reversed polarity mode (cathode at the injection side and the anode at the detection side) with 5 psi pressure applied at both sides of the capillary.
6. Set autozero at 0.5 min.
7. End method by moving the trays to home position.
8. A rapid separation example of APTS labeled glycans from human IgG (Sigma Aldrich, Part #I4506) is shown in Fig. 1 (*see* **Note** 7).

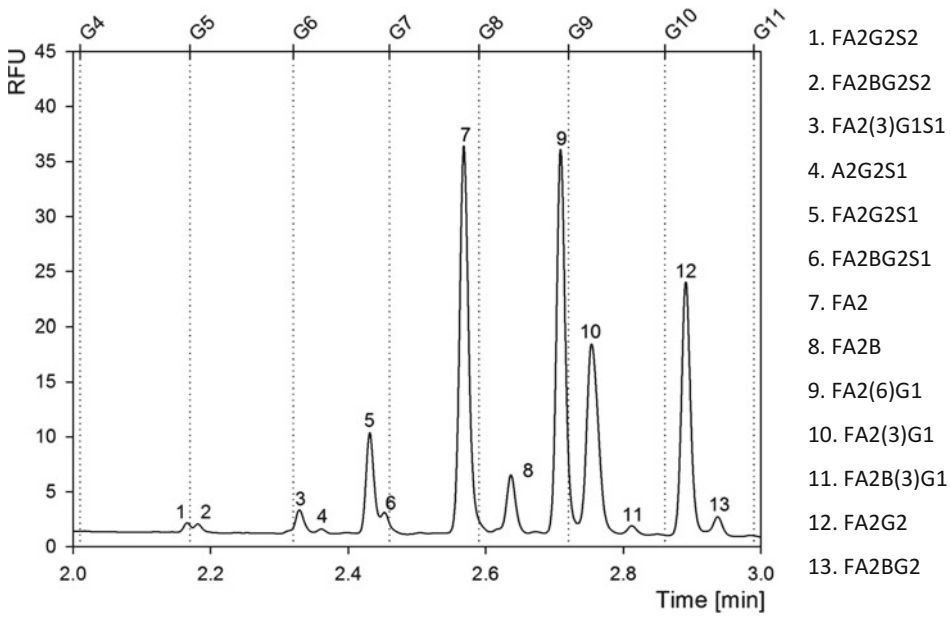

Fig. 1 CE-LIF separation of PNGase F released and APTS labeled human IgG N-glycans. Separation conditions: N-CHO capillary (total length 30 cm, 20 cm effective, 50 μm i.d.), N-linked carbohydrate separation gel buffer, $E = 30$ kV (reversed polarity), 25 °C temperature, Injection: enhanced electrokinetic by 1 psi/5 s water injection (reversed polarity) and, 2 kV/2 s sample injection, 1 psi/5 s buffer injection

4 Notes

1. Avoid touching the beads with the pipette tips to prevent bead/sample loss.
2. Beads are easy to resuspend in aqueous solutions but difficult in organics because of possible aggregation, e.g., during the acetonitrile mediated glycan capture step. Therefore, always vigorously resuspend the beads before acetonitrile addition.
3. No mixing is necessary during the deglycosylation and labeling steps.
4. Always pipette the supernatant from the bottom of the vials as in this case non-captured beads, which might be floating in the upper segment of the vials, will not be accidentally removed.
5. Use the magnets (or stand) only for the minimum time required during buffer exchange to avoid possible bead aggregation.
6. Max speed vortexing in manual sample preparation mode may push some of the sample up to the microvial cap. Applying a quick (few seconds) spin-down in a small microcentrifuge can solve the issue and prevents any potential sample loss.
7. GU unit calculation software is freely available at: www.gucal.hu.

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