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Online nanoliquid chromatography–mass spectrometry and nanofluorescence detection for high-resolution quantitative *N*-glycan analysis

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ABSTRACT

The characterization of the repertoire of glycans at the quantitative and qualitative levels on cells and glycoproteins is a necessary step to the understanding of glycan functions in biology. In addition, there is an increasing demand in the field of biotechnology for the monitoring of glycosylation of recombinant glycoproteins, an important issue with regard to their safety and biological activity. The enzymatic release followed by fluorescent derivatization of glycans and separation by normal phase high-performance liquid chromatography (HPLC) has proven for many years to be a powerful approach to the quantification of glycans. Characterization of glycans has classically been performed by mass spectrometry (MS) with external standardization. Here, we report a new method for the simultaneous quantification and characterization of the *N*-glycans on glycoproteins without the need for external standardization. This method, which we call glycan nanoprofiling, uses nanoLC-coupled electrospray ionization (ESI)–MS with an intercalated nanofluorescence reader and provides effective single glycan separation with subpicomolar sensitivity. The method relies on the isolation and coumaric derivatization of enzymatically released glycans collected by solid phase extraction with porous graphitized carbon and their separation over polyamide-based nano-HPLC prior to serial nanofluorescence and nanoelectrospray mass spectrometric analysis. Glycan nanoprofiling is a broadly applicable and powerful approach that is sufficient to identify and quantify many glycan oligomers in a single run. Glycan nanoprofiling was successfully applied to resolve the glycans of monoclonal antibodies, showing that this method is a fast and sensitive alternative to available methods.

One of the most common posttranslational modifications of proteins is glycosylation. More than 60% of all human cellular- and membrane-bound proteins are glycosylated, suggesting that carbohydrates are essential for protein function. Attachment of carbohydrates to proteins enables them to fulfill important roles in many biological events and has implications in pathological situations such as cancer and inflammation (for an extensive review, see Ref. [1]). To decipher these interactions, the characterization of the carbohydrate moiety is indispensable to establish a reliable correlation between its structure and function.

The variation in glycan composition affects both the number of glycans attached (macroheterogeneity) and the nature of these glycans (microheterogeneity). Importantly, glycan synthesis is a template-free process that depends on the relative composition of the glycosylation machinery, a set of enzymes and cofactors that is unique to every cell type and is subjected to tight regulation that is sensitive to many environmental cues and stimuli. Thus, glycoproteins usually exist as complex mixtures of glycosylated variants or glycoforms. To give an idea of how variable glycoforms can be, the whole pool of human erythrocyte CD59 glycoforms contains more than 130 different glycan structures on a single N-linked glycosylation site [2]. The large structural heterogeneity, the presence of numerous glycosylation sites, and the large number of possible isomers are aspects of glycan biology that make glycoprotein analysis significantly more difficult than protein analysis. Therefore, glycosylation analysis demands high-resolution separation techniques and highly sensitive detection methods. Full structural characterization of the glycan repertoire of a purified protein or a cell/tissue isolate requires the definition of branching, linkages, configurations, and the identification of same-mass sugar isomers. A complete analysis, therefore, will use not only modern soft ionization mass spectrometry (MS)[1] approaches

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[1] *Abbreviations used:* MS, mass spectrometry; SDS, sodium dodecyl sulfate; PNGase F, peptide *N*-glycosidase F; PGC, porous graphitized carbon; SPE, solid phase extraction; HPLC, high-performance liquid chromatography; 2AB, 2-aminobenzamide; DAP, diaminopyridine; AMC, 7-amino-4-methylcoumarin; 4AB, 4-aminobenzamidine; DMSO, dimethyl sulfoxide; DMF, dimethylformamide; ACN, acetonitrile; TFA, trifluoroacetic acid; ESI, electrospray ionization; MS/MS, tandem MS; HILIC, hydrophilic interaction chromatography; AMAC, 2-aminoacridone; HPAEC–PAD, high-performance anion exchange chromatography with pulsed amperometric detection.

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but also gas chromatography electron impact–MS of hydrolysates for composition and linkage analysis, together with biochemical methods such as exoglycosidase digestion, to define terminal sequences [3]. Because the glycosylation biosynthetic pathways are well known in higher organisms and the complete repertoire of known glycans is being catalogued, the combination of chromatographic separations of carbohydrates with MS serves as the "gold standard" for glycan analysis of biologically interesting tissues. The challenging aspect of this approach is to achieve high-resolution separation to accurately quantify minute amounts of highly similar glycoforms. However, poor chromatographic separation can lead to coelution of different oligosaccharides and, therefore, to incorrect quantification. Moreover, the physicochemical nature of carbohydrates cannot be used for sensitive and specific detection; thus, carbohydrates need to be specially derivatized with moieties that allow sensitive detection. The use of many different tags has been described, and certainly derivatization with a fluorophore offers the best choice for detection with very high sensitivity.

In the current study, we set out to develop an analytical method for the simultaneous characterization and quantification of *N*-glycans isolated from purified glycoproteins. The release and purification of *N*-glycans from glycoproteins is a well-established and reliable method, but there are several *N*-glycan derivatization protocols with differences in labeling efficiency and the *N*-glycan separation, quantification, and characterization methods require the combination of several techniques and instruments. Therefore, we aimed to achieve an optimal fluorescent derivatization of *N*-glycans and to be able to resolve *N*-glycans with both a highly sensitive quantification and a detailed characterization within a single chromatographic run.

Materials and methods

Materials

All glycoproteins (bovine pancreas ribonuclease B, bovine α_1-acid glycoprotein, human α_1-acid glycoprotein, type I calf serum asialo-fetuin, and calf serum fetuin) were purchased from Sigma–Aldrich. All preparations were of the highest purity. Sodium dodecyl sulfate (SDS) solution (20%, w/v), Tris–HCl (pH 8.0) ready-to-use powder, 10 M β-mercaptoethanol, and Igepal CA-630 were obtained from Sigma–Aldrich. Peptide *N*-glycosidase F (PNGase F) was obtained from Roche Diagnostics. Porous graphitized carbon (PGC, 100 mg) in 6-ml solid phase extraction (SPE) tubes (Supelco) and siliconized tubes (Aldrich) were obtained from Sigma–Aldrich. Detergent-out beads were purchased from Calbiochem. All solvents were high-performance liquid chromatography (HPLC) grade and obtained from Riedel de Haën. Sodium cyanoborohydride (Fluka), picoline borane (Aldrich), acetic acid (Aldrich), phosphorus pentoxide (Fluka), 2-aminobenzamide (2AB, Aldrich), diaminopyridine (DAP, Aldrich), 7-amino-4-methylcoumarin (AMC, Aldrich), 4-aminobenzamidine (4AB, Fluka), dimethyl sulfoxide (DMSO, anhydrous, Fluka), and dimethylformamide (DMF) were purchased from Sigma–Aldrich. Quartz microfiber filters were purchased from Whatman. A metal disk cutter was engineered with the same diameter as a 5-ml syringe tube.

Deglycosylation of glycoproteins

For the deglycosylation of glycoproteins, a denaturation buffer (2% SDS, 7 M urea, and 2 M thiourea in 0.25 M phosphate buffer, pH 8.5) and a neutralization buffer (15% Igepal CA-630 in 0.25 M phosphate buffer, pH 8.5) were prepared. Prior to use, 140 μl of β-mercaptoethanol was added to 2 ml of denaturation buffer to achieve a final concentration of 1 M in the denaturation buffer. First, lyophilized glycoproteins were denatured by incubating 100 nmol of glycoprotein in 50 μl of denaturation buffer for 1 h in a heated (40 °C) sonication bath. After 1 h, 50 μl of neutralization buffer was added to neutralize the inhibitory effect of SDS on PNGase F activity, and sonication was resumed for a further 15 min. Finally, 400 μl of phosphate buffer (pH 8.5) and 5 U of PNGase F were added. Samples were mixed thoroughly, and the mixtures were placed on a 37 °C shaker for 12 h. After 12 h, another 5 U of enzyme was added and incubation was prolonged for 12 h.

Isolation of oligosaccharides

Prior to use, PGC SPE columns were washed with 3 × 1 ml of 1 M NaOH, water, 80% acetonitrile (ACN) containing 0.1% trifluoroacetic acid (TFA), and again with water. After applying the protein mixture to the PGC SPE column [4], salts and detergents were washed out with 6 × 1 ml of water. Bound oligosaccharides were eluted with 3 × 0.4 ml of 25% ACN containing 0.1% TFA and collected in 10-ml glass tubes. The eluates were lyophilized in a rotary evaporator and redissolved in 3 ml of water. Then, 1 mg of detergent-out beads was added to this solution and incubated with shaking at room temperature for 30 min. This treatment removes any trace of detergents left in the solution and greatly enhances the MS signal. The beads were pelleted by a short centrifugation step, and the supernatant was recovered through an HPLC syringe filter. The filtrate was collected in a clean glass tube.

Derivatization of purified oligosaccharides

To standardize the derivatization method and minimize variation, ready-to-use stocks of all chemicals were prepared. Here, 1 g of 2AB or DAP was dissolved in 10 ml of methanol and aliquoted in 500-μl (50-mg) aliquots, 100 mg of AMC was dissolved in 500 μl of DMF and filled out in 10-μl (2-mg) aliquots, and 1 g of 4AB was dissolved in 10 ml of water and filled out in 500-μl (50-mg) aliquots. All aliquots were lyophilized in a rotary evaporator. Aliquots (25 mg) of sodium cyanoborohydride and picoline borane were weighted out in siliconized tubes. Together with the rotary evaporator dried fluorophores, all aliquots were placed in a vacuum desiccator, further dried in the presence of phosphorus pentoxide under vacuum for 48 h, and kept there until use.

Oligosaccharide solution (500 pmol) was lyophilized in a rotary evaporator. Prior to the derivatization reaction, 1 aliquot each of fluorophore (except AMC), sodium cyanoborohydride, and picoline borane were dissolved in 400 μl of DMSO. AMC was dissolved in 40 μl of DMSO to obtain the same concentration as the other fluorophores. Derivatization reaction was carried out by adding 5 μl of fluorophore, 5 μl of reductant, and 5 μl of acetic acid to the tubes containing the dried oligosaccharides and, after a brief centrifugation step, incubating in a heating block for 2 h at 65 °C.

Purification of derivatized oligosaccharides

Derivatized oligosaccharides were purified by paper chromatography. Briefly, two quartz microfiber filters were cut in a disk shape with the metal cutter and placed on top of each other inside an empty 5-ml syringe tube. After washing the quartz microfiber filters with 3 × 2 ml of 30% acetic acid, 2 ml of water, and 2 ml of 100% ACN each, the reaction mixture was applied equally on the filter surface. The assembly was left for 30 min at room temperature for optimal adsorption of derivatized oligosaccharide onto the filter. Unreacted material was washed away with 3 × 2 ml of 100% ACN and 6 × 2 ml of 96% ACN. Derivatized oligosaccharides were then eluted in 3 × 400 μl of water and collected in a