Automated sample preparation facilitated by PhyNexus MEA purification system for oligosaccharide mapping of glycoproteins

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Abstract

A reproducible high-throughput sample cleanup method for fluorescent oligosaccharide mapping of glycoproteins is described. Oligosaccharides are released from glycoproteins using PNGase F and labeled with 2-aminobenzoic acid (anthranilic acid, AA). A PhyNexus MEA system was adapted for automated isolation of the fluorescently labeled oligosaccharides from the reaction mixture prior to mapping by HPLC. The oligosaccharide purification uses a normal-phase polyamide resin (DPA-6S) in custom-made pipette tips. The resin volume, wash, and elution steps involved were optimized to obtain high recovery of oligosaccharides with the least amount of contaminating free fluorescent dye in the shortest amount of time. The automated protocol for sample cleanup eliminated all manual manipulations with a recycle time of 23 min. We have reduced the amount of excess AA by 150-fold, allowing quantitative oligosaccharide mapping from as little as 500 ng digested recombinant immunoglobulin G (rIgG). This low sample requirement allows early selection of a cell line with desired characteristics (e.g., oligosaccharide profile and high specific productivity) for the production of glycoprotein drugs. In addition, the use of Tecan or another robotic platform in conjunction with this method should allow the cleanup of 96 samples in 23 min, a significant decrease in the amount of time currently required to process such a large number of samples.

Keywords: N-linked oligosaccharide; Glycan profiling; Recombinant glycoprotein

Current analytical methods for characterization of well-known N- and O-linked carbohydrates involve the initial release of the oligosaccharides followed by derivatization with a suitable fluorescence tag, the cleanup of the sample to remove precipitated protein and excess tag, and finally the analytical separation by either liquid chromatography [1–3] or capillary electrophoresis [4,5]. A number of fluorescent tags have been introduced for the characterization of N-linked oligosaccharides [6–11]; however, 2-aminobenzoic acid (2-AA [anthranilic acid, AA])2 provides a single derivatization chemistry for the analysis of monosaccharides, oligosaccharides, and polysaccharides [12]. Most of the fluorescence detection methods are based on precolumn derivatization of carbohydrates by reductive amination via Schiff base in the presence of excess tag. In nearly all cases, there is a need for isolation of labeled oligosaccharides from the reaction mixture.

The procedures to remove the proteins, detergents, salts, excess reagent and its impurities, and the artifacts that are formed during the labeling process include gel filtration, extraction by organic solvents, ion exchange, and reversed-phase and paper chromatography. Normal-phase polyamide was introduced for isolation of labeled oligosaccharides, and the matrices for this purpose include mini sample preparation columns and filters (e.g., Spe-ed Amide-2/Polyamid 6S [Discovery DPA-6S]) and nylon filter [12]. The fastest and most convenient way to isolate the labeled oligosaccharides at the nanomolar level with
good recovery was to use a nylon syringe filter as a capture step in a gravity flow setup. The derivatized oligosaccharides are captured under high-acetonitrile (MeCN) conditions (~95–96%) and eluted with low MeCN (20%). However, this method is user-intensive and time-consuming when the number of samples being tested exceeds 10–15.

In this study, we have developed a process using a novel miniaturized purification technology to automate the sample preparation step, minimizing the amount of manual manipulation required while at the same time allowing the preparation of up to 96 samples in as short a time as 23 min. An added benefit of the methodology was a 10-fold reduction in the amount of glycoprotein required for analysis. Such procedures are likely to be found useful in the rapid screening of recombinant cells and optimization of their culture conditions for the production of cost-effective, efficacious therapeutic glycoproteins.

Materials and methods

Oligosaccharide release and labeling

N-linked oligosaccharides were cleaved and labeled in a manner similar to that described previously [13]; however, detergents were not used in the case of recombinant immunoglobulin G (rIgG). In brief, samples of the glycoprotein, either humanized IgG1 monoclonal antibody (in-house) or porcine thyroglobulin (Sigma), were taken and diluted to 48 l l with H2O. After this dilution, 2 l l of PNGase F (Prozyme) was added and the reaction mixture was incubated overnight at 37 °C. A 30-mg/ml solution of AA (Sigma) was prepared in an acetate–borate-buffered methanol solution (4% [w/v] sodium acetate trihydrate, 2% [w/v] boric acid). Following cleavage of the N-linked oligosaccharide with PNGase F, 100 l l of the 30 mg/ml AA solution and 50 l l of 1 M NaBH3CN in tetrahydrofuran (THF, Sigma) were added to the reaction tube and the mixture was incubated at 80 °C for 60 min. Samples were then allowed to cool to room temperature and diluted to 1 ml with 95% MeCN prior to purification of the labeled oligosaccharides.

Purification with PhyTip columns

PhyTip columns packed with DPA-6S resin (Sigma) were kindly supplied by Jeremy Lambert of PhyNexus (San Jose, CA, USA) for use with the PhyNexus MEA Purification System. Resin bed volumes of 5, 10, 20, 40, 80, and 160 l l were packed between the frits at the tip in a common 1-ml disposable plastic pipette tip for their performance evaluation. Following dilution with 95% MeCN, the oligosaccharide labeling mixture was transferred to a 96-deep-well plate for purification on the MEA system. An example plate map is shown in Fig. 1. In this description, one cycle represents one intake and one expulsion of solution over the DPA-6S resin. The operating conditions included a flow rate of 2 ml/min with a delay time of 1 s between each pipetting segment. Following elution of the labeled oligosaccharide from the PhyTip column, the samples were transferred to low-volume HPLC injection vials prior to analysis by HPLC.

Purification with nylon filters

Low-hold-up volume Fisherbrand nylon syringe filters (0.45 μm, 25 mm diameter, cat. no. 09–719–5, Fisher) were...
prewet and connected to a 3-ml syringe, essentially making the assembly a gravity flow column [14]. The filter was washed with an additional 2 ml of 95% MeCN. Next, the mixture containing the labeled oligosaccharide was allowed to pass through the filter and then washed with 2 × 1 ml of 95% MeCN. The bound oligosaccharides were then eluted directly into HPLC injection vials with 2 × 500 µl of 20% MeCN for analysis by HPLC with a 100-µl injection.

Normal-phase high-performance anion exchange chromatography

Chromatography was performed as described previously [14]. Briefly, an Agilent 1100 HPLC system with fluorescent detection (excitation 360 nm, emission 425 nm) was used for separation of oligosaccharides on a 4.6 × 250-mm Asahipak NH2-P50 column (5 µm, Phenomenex) at 50 °C with a flow rate of 1 ml/min. Mobile phase A consisted of 97% MeCN, 2% acetic acid, and 1% THF. Mobile phase B consisted of 5% acetic acid, 3% triethylamine (TEA), and 1% THF in water. The injection volumes ranged from 20 to 100 µl for samples eluted from the tip. Oligosaccharides were separated using the following gradient: step gradient of 30% mobile phase B for 2 min, linear gradient to 86.6% mobile phase B at 70 min, step gradient of 95% mobile phase B for 15 min, and reequilibration in 30% mobile phase B for 5 min.

Results

Optimization of PhyTip column method

For the initial experiment, duplicate samples of rIgG (50 µg) were treated with PNGase F and labeled as described above. One of the samples was processed using the nylon filter-based method, and the other was processed using a 160-µl PhyTip column. The processing time for the method was approximately 48 min with a 0.5-ml/min flow rate and a 20-s hold. The resulting chromatogram from this proof-of-concept experiment is shown in Fig. 2. The peak distribution obtained for both preparation methods was similar. The peak heights from the PhyTip samples were noticeably greater than those of the nylon filter samples. This was expected because the PhyTip samples were prepared in roughly half of the final volume (0.5 ml) of the nylon filter samples (1.0 ml). However, the PhyTip sample showed significant peak fronting and a loss of resolution of peaks when compared with the nylon filter sample.

Several modifications were made to improve the method performance. The flow rate was increased to 2 ml/min, and the delay time was reduced to 1 s. A prewash of the tips with 500 µl of 20% MeCN to elute any bound contaminants was inserted as the initial step. Also, a third wash of the bound oligosaccharides with 95% MeCN was added.
to reduce the amount of excess AA. In total, these modifications reduced the process time from 48 to 23 min.

Because binding capacity of the resin was not an issue given that small amounts of material were used (1 μg labeled oligosaccharide), the bed volume of resin could potentially be reduced to 20 μl. To evaluate the effects of these changes, 3 x 50 μg material was digested and labeled as before. One aliquot was processed using the nylon filter method to serve as a control. A second aliquot was processed using the original PhyTip method (160 μl of bed volume with elution in 500 μl of 20% MeCN). The third aliquot was processed using a modification of the original PhyTip method using a 20-μl bed volume column and elution in 100 μl of 20% MeCN. The injection volume was 100 μl for the nylon filter sample as well as for the 500-μl PhyTip eluted sample. The injection volume was reduced to 20 μl for the 100-μl PhyTip eluted sample. The resulting chromatograms are shown in Fig. 3. The 100-μl injection from the PhyTip column preparation again showed significant peak fronting. This was not observed in the 20-μl injection sample. The cause for the peak fronting does not appear to be due to mass overloading given that the mass loads for both PhyTip preparations were roughly equivalent (~200 ng labeled oligosaccharide injected). In addition, volume overloading does not appear to be the cause given that the injection volume for the control sample (nylon filter preparation) was also 100 μl with no evidence of peak fronting.

**Determination of optimal PhyTip bed volume**

To evaluate the performance of different PhyTip bed columns on the chromatographic profile, a set of experiments was performed spanning a range of resin volumes from 5 to 160 μl. For the experiment, 50-μg samples of rIgG were digested with PNGase F and processed using the optimized PhyTips wash elution steps with bed volumes of 20, 40, 80, and 160 μl. A constant injection volume of 20 μl was used for all samples. The resulting chromatograms are shown in the inset of Fig. 4. The amount of excess AA present in the samples increased with increasing resin bed volumes. The relative amounts of AA remaining in each sample in comparison with the 20-μl bed volume column were 1.5, 6.0, 10.5 x for the 40-, 80-, and 160-μl PhyTip columns, respectively, and 10.3 x for the nylon filter sample. There was no appreciable difference in peak area for the excess AA peak between the standard nylon filter preparation and the 160-μl PhyTip column. Subsequently, PhyTip resin bed volumes of 10 and 5 μl were evaluated. The 10-μl PhyTips showed additional reduction in AA removal of more than 100-fold over the nylon filter at similar amounts of oligosaccharide (Fig. 4). Additional experiments showed this reduction in AA to vary from 70- to 150-fold. PhyTip columns with 5-μl bed volumes demonstrated problems with fouling due to the precipitated protein present in the sample after dilution with 95% MeCN.
Effect of rIgG concentration

The more efficient removal of excess labeling reagent allowed a rather rapid return of the AA peak to baseline and an anticipated reduction in the amount of sample required for quantitation of the oligosaccharide peaks. To verify this, a titration experiment was performed testing rIgG digests from 50 μg to 10 ng. Samples were cleaved and labeled as described previously and were processed using either the nylon filter method or the optimal PhyTip method. Injection volumes for normal-phase high-performance anion exchange chromatography (NP–HPAEC) were 100 μl for the nylon filter samples and 20 μl for the PhyTip samples. These injection volumes were chosen to give comparable oligosaccharide mass on the column. Chromatograms of selected runs are shown in Fig. 5. Both methods proved to be adequate down to 5 μg digested rIgG. At the next dilution, 1 μg, the peak corresponding to Man5, a high-mannose structure accounting for approximately 1.5% of the total oligosaccharide, no longer was distinguishable from background in the nylon filter sample. This peak remained clearly identifiable in the PhyTip column samples down to 500 ng. At 100 ng, the Man5 peak as well as the G4 peak (biotennary with an additional galactose residue in α1–3 linkage on each branch) no longer was distinguishable from background. By the 10-ng level, no peaks were discernible from background (data not shown). However, greater sensitivity can be achieved with a small/narrow bore HPLC column than with the standard 4.6-mm column used in this study.

Furthermore, the linear range of the method was determined. Digests of 500, 50, 5, 1, and 0.5 μg rIgG were prepared and analyzed. The sum of the peak areas for glycoforms G0, G1, G2, G3, and G4 were totaled and plotted as a function of the mass of rIgG digested (data not shown). The 500-μg digest resulted in fouling of the 10-μl PhyTip column and, therefore, was excluded from the response curve. Linear regression analysis of both sample preparation methods showed a good correlation (R² > 0.999).

Reproducibility of PhyTips

Different PhyTip columns from the same lot were used in the reproducibility studies with thyroglobulin, a glycoprotein with a much more complex glycosylation pattern, as a model. As such, 2 × 50 μg thyroglobulin was digested with PNGase F and labeled as before. One of the aliquots was then diluted to 5 ml with 95% MeCN and further divided into 5 × 1-ml aliquots. Three of these aliquots were processed on three different 10-μl PhyTip columns using the standard method. An additional 50-μg digest was processed using the nylon filter method for reference. Samples were analyzed by NP–HPAEC with 20-μl injections for all samples. The resulting chromatograms are shown in Fig. 6. The three samples prepared using the PhyTip columns...
yielded superimposable chromatograms and were quantitatively comparable to the nylon filter (Table 1). In addition, even with the much more complex mixture of glycoforms, there were no selectivity differences between the two methods, including the charged oligosaccharides.

**Discussion**

Glycosylation can play a critical role in the efficacy of therapeutic glycoproteins, for example, the profile of the Fc glycosylation in IgGs [15] and in erythropoietin [16]. Screening of potential clones for those with desirable oligosaccharide profiles earlier in the development of the glycoprotein drug can eliminate significant resources required for scale-up to a point where adequate quantities of material can be generated for further studies (including animals, humans, etc.). To define the glycoprofile at an early stage in development, a reproducible sample preparation method that is adaptable for use with high-throughput robotics and a small amount of glycoprotein is required.

The analysis of the glycosylation profile of therapeutic glycoproteins is a time-intensive effort with two potential bottlenecks: the time required to prepare labeled oligosaccharides for analysis and the time required to perform the analysis itself. Although the time requirements for chromatographic separation grow linearly with increasing numbers of samples, this time increase is more exponential for the preparation of samples. The use of this method allows a significant increase in the sample preparation bottleneck. Using a fully robotic system in conjunction with this method would allow the cleanup of 96 samples in less than 25 min with minimal manipulation required by a technician. Coupled with recent advances in the use of capillary electrophoresis (CE)-based separations using 2-AzA labels with cycle times of less than 30 min [4], total throughput times from enzymatic digestion to completion of analytical characterization of batch sequences containing 96 samples or more could be reduced by more than 50%. With advances in ultra-performance liquid chromatography (UPLC) continuing to be made, when appropriate UPLC

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![Fig. 5. Dilution series of rIgG digests prepared using either a 10-µl PhyTip column or a nylon filter: (A) 5-µg digest; (B) 1-µg digest; (C) 0.5-µg digest; (D) 0.1-µg digest. All injections from PhyTip column samples were 20 µl (representing 20% of total material), and all injections from nylon filter samples were 100 µl (representing 10% of total material). Man5 represents the Man5–Gn2 structure from PNGase F digestion. LU, luminescent units.](image-url)
columns become available, a similar reduction in chromato-
graphic time could be expected with UPLC-based
methods. In addition, laboratories using sample purifica-
tion methods with multiple solid-phase extraction (e.g.,
Carbograph, S or E cartridges) and evaporation steps, total
efficiency improvements of 75% or more can be expected
on batch preparations of 12 samples with an additional
cost reduction benefit on disposable reagents.

In summary, we have developed a sample preparation
method using PhyTip columns packed with readily avail-
able polyamide DPA-6S resin that is transferable to a
96-channel pipette platform with sample requirements of
only 500 ng of glycoprotein. Compared with the existing
method using a nylon syringe filter or other methods [1],
this procedure decreases the sample requirements by a min-
imum of 10-fold while at the same time increasing the speed
of sample preparation. The very low sample requirement
for oligosaccharide mapping was due mainly to a signifi-
cant reduction in the amount of excess AA visible in the
chromatogram. Following optimization of the operational
parameters, this method allows the preparation of up to 96
samples after labeling with AA in approximately 23 min.
Furthermore, it is conceivable that the entire process of oli-
gosaccharide mapping with PNGase F digestion, fluores-
cence labeling, sample cleanup, and HPLC could be fully
automated.

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