Automated Sample Preparation Facilitated by PhyNexus MEA Purification System for Oligosaccharide Mapping of Therapeutic Glycoproteins

Bradley D. Prater, Kalyan Anumula, Joseph M. Patti and Jeff T. Hutchins

Inhibitex, 9005 Westside Parkway, Alpharetta, GA 30004

ABSTRACT

Fast and reproducible oligosaccharide mapping methods are required for the rapid screening and selection of an appropriate cell line for the production of glycoproteins with the desired oligosaccharide profile. For example, Fc glycans play critical roles in therapeutic IgGs and can determine their success or failure in the clinic. Initial production levels are routinely monitored by isolation of the rIgGs using protein A packed pipette tips in a PhyNexus MEA Purification System. Oligosaccharides are then released using PNGase F and labeled with 2-aminobenzoic acid (AA) for high-sensitivity fluorescent high-resolution chromatography (Anumula, K. R (2006) Anal. Biochem. 350, 1-23). The Phynexus system was further adapted for automated isolation of fluorescently labeled oligosaccharides from the reaction mixture prior to mapping by HPLC. The oligosaccharide purification uses a normal-phase polyamide resin (DPA-6S, Supelco, 10µL bed volume) 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. All of the procedures were evaluated with rIgG and thyroglobulin serving as model glycoproteins. The automated protocol for sample cleanup described here eliminated all the manual manipulations with a recycle time of ~23 min. In addition to allowing automation of the process, we have also reduced the amount of excess AA by approximately 150-fold. This reduction in excess AA allowed for quantitation of oligosaccharides from as little as 50ng of glycoprotein. This low sample requirement allows for selection of clones based on their oligosaccharide profile at a much earlier point in the process. In addition, use of this method in conjunction with a fully automated TECAN robotics platform would allow for the cleanup of 96 samples in under 30 minutes.

MATERIALS AND METHODS

Oligosaccharide labeling. Samples of the glycoprotein (either porcine thyroglobulin from Sigma or humanized monoclonal antibody (in house)) were taken and diluted to 48µL with H_2O. PNGase F (2µL, Prozyme) was added and the mixture incubated overnight @ 37°C. Anthranilic acid (AA, 100µL of 30mg/mL in methanol-acetate-borate) and 50µL of 1M $NaBH_3CN$/THF (Sigma) were added and the mixture incubated @ 80°C for 60 minutes. Samples were allowed to cool to room temperature and diluted to 1mL with 95% MeCN.
Purification with Phynexus Tips. Samples were transferred to row C of a deep-well 96-well plates. Additional rows of 96-well plate contained the following: Row A: 500µL of 20% MeCN, Row B: 1mL of 95% MeCN, Row D: 1mL of 95% MeCN, Row E: 1mL of MeCN, Row G: 100µL of 20% MeCN. Tips packed with DPA-6S resin were kindly supplied by Phynexus. A method was written for the MEA Purification System (Phynexus) including the following steps: 1) A pre-wash with 500µL of 20% MeCN (2 cycles), 2) Reequilibration with 1mL of 95% MeCN (2 cycles), 3) Binding of labeled oligosaccharides (8 cycles), 4) Wash with 1mL of 95% MeCN (4 cycles), 5) Wash with 1mL of 95% MeCN (4 cycles), 6) Wash with 1mL of 95% MeCN (4 cycles) 7) Elute with 100µL of 20% MeCN (4 cycles). Eluant was then transferred to a low volume HPLC injection vial. Cycles in each step represent number of times the same solution passed over the resin.
Purification with Nylon Filters. Nylon syringe filters were pre-wet and connected to a 3mL syringe, essentially making the assembly a gravity flow affinity column. The filter was washed with an additional 2mL of 95% MeCN. Sample was allowed to pass through the filter and then washed with 2 x 1mL of 95% MeCN. The bound oligosaccharides were then eluted directly into HPLC injection vials with 2 x 0.5mL of 20% MeCN.
Normal-phase high–performance anion-exchange chromatography (NP-HPAEC). Oligosaccharides were separated using a 4.6mm x 250mm Asahipak NH2 P50, 5µm column (Phenomenex) connected to an Agilent 1100 HPLC with fluorescent detection. Mobile Phase A consisted of 97% MeCN, 2% acetic acid, 1% THF. Mobile Phase B consisted of 91% H2O, 5% acetic acid, 3% triethylamine (TEA), 1% THF. Injection volumes varied (see figure descriptions). Chromatography was performed using the following operating parameters: flow rate of 1mL/min, column temperature of 50°C, injection volume of 100µL. Oligosaccharides were separated using the following gradient: step gradient of 30% Mobile Phase B for 2 minutes, linear gradient to 86.6% Mobile Phase B at 70 minutes, step gradient of 95% Mobile Phase B for 15 minutes and reequilibration in 30% Mobile Phase B for 5 minutes.

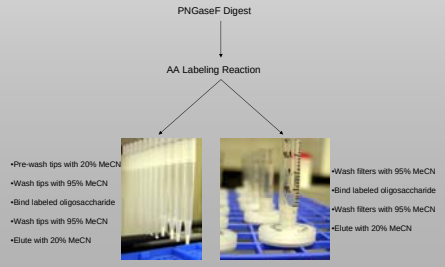
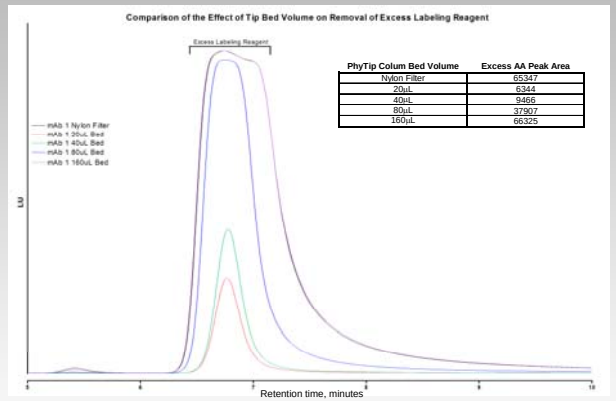


Figure 1. Schematic overview of the two oligosaccharide cleanup methods.

RESULTS



PhyTip Colum Bed Volume	Excess AA Peak Area
Nylon Filter	65347
20µL	6344
40µL	9466
80µL	37907
160µL	66325

Figure 2. Labeled oligosaccharides were purified using Phynexus tips packed with 160µL, 80µL, 40µL or 20µL of DPA-6S resin and compared to a standard preparation from a nylon syringe filter. Injection volumes were 100µL for all samples.

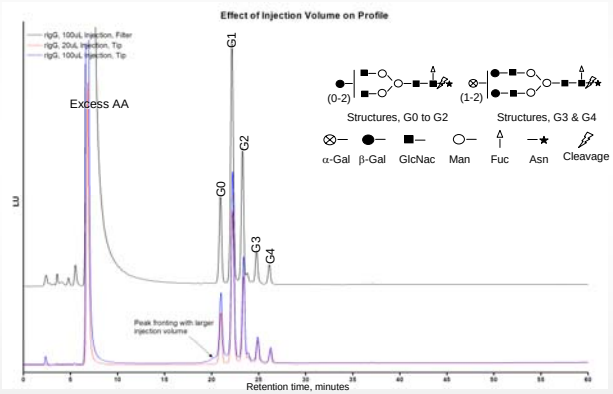
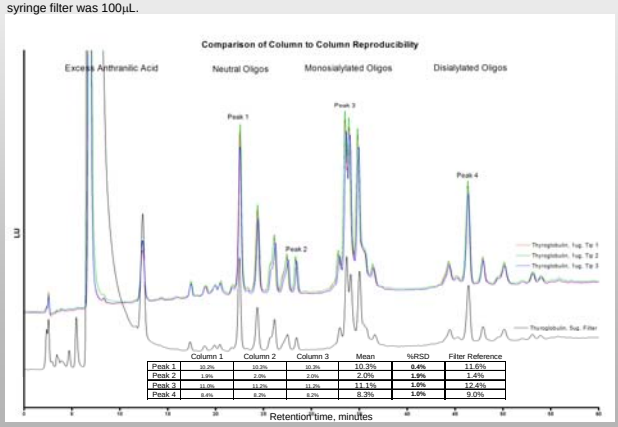


Figure 3. 5µg rIgG digest with purification on a 20µL Phynexus tip. Initial experiments were performed using a 100µL injection volume. This led to overloading of the column and peak fronting and loss of resolution of the neutral peaks. Reduction of the injection volume to 20µL eliminated this problem. This injection from the nylon syringe filter was 100µL.



	Column 1	Column 2	Column 3	Mean	%RSD	Filter Reference
Peak 1	10.2%	10.2%	10.3%	10.3%	0.4%	11.6%
Peak 2	1.9%	2.0%	2.0%	2.0%	1.9%	1.4%
Peak 3	11.0%	11.2%	11.2%	11.1%	1.0%	12.4%
Peak 4	8.4%	8.2%	8.2%	8.3%	1.0%	9.0%

Figure 4. Thyroglobulin digests with purification on a 10µL Phynexus tip. All injections were 20µL. Sample preparations on three different tips gave super-imposable chromatograms. Peak area % observed were comparable to those obtained with nylon filter preparations with little variability from column to column.

RESULTS (continued)

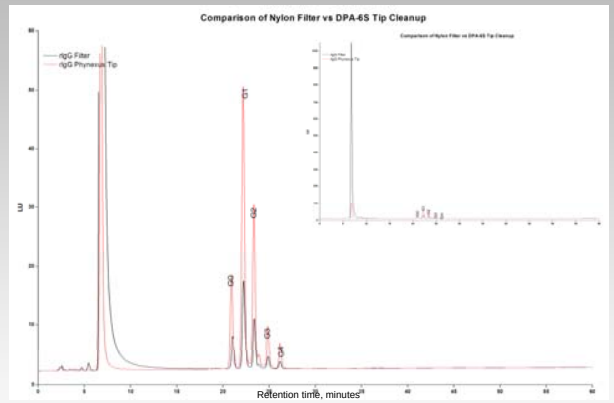


Figure 5. rIgG digests (5µg) with purification on either a 10µL Phynexus tip or a nylon syringe filter. Full scale chromatogram is shown as an inset. Both injections were 20µL (represents 20% for the PhyTip column and 2% for the nylon filter).

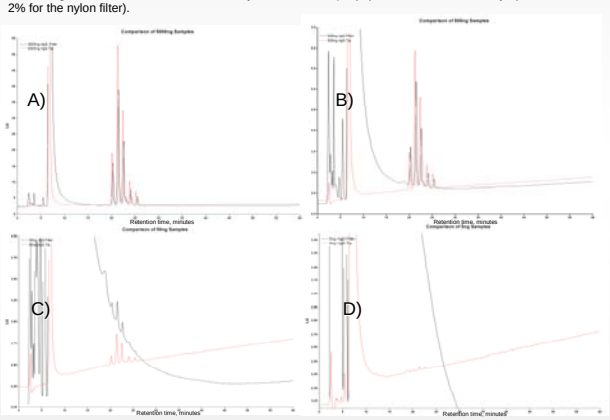


Figure 6. Panel A) 5000ng rIgG digest, Panel B) 500ng rIgG digest, Panel C) 50ng rIgG digest. Panel D) 5ng rIgG digest. The injection volume for all Phynexus preparations was 20µL. The injection volume for all filter preparations was 100µL. The 5000ng preparations showed no difference in the neutral oligosaccharide profile. However, even at 500ng, the effect of the excess AA can be seen in the nylon filter preparation. At 50ng, the nylon filter chromatogram is no longer usable. The Phynexus tip sample drops into the noise range at 5ng.

CONCLUSIONS

- We have developed a high-throughput method which allows for the automated preparation of labeled oligosaccharides in a rapid manner.
- The method was also able to reduce the amount of excess AA label by 50 to 150-fold based on the peak area in blank injections.
- The reduction in excess AA peak reduced the sample requirements for analysis by at least 10-fold (50ng of rIgG), which allows for screening of clones much earlier in the process development (96-well plate cultures as opposed to 24-well plate cultures).
- The more concentrated samples obtained using the tip-based method required reducing the sample injection volume to 20µL. However, this 20µL injection gave signal intensities equivalent to 100µL injections from the nylon filter method.
- The optimal bed volume of resin in the tips was found to be 10µL. Capacity of resin was not an issue as the amount of oligosaccharides bound was in the pg range. Instead, the focus was on optimizing the excess AA peak reduction. Bed volumes of 160µL showed no advantage over nylon filters. Bed volumes of 5µL began to incur problems with fouling due to precipitated protein.
- If used in conjunction with a fully robotic platform such as a TECAN, 96 samples could be prepared in less than 30 minutes.