

Rapid Sample Preparation of Biologics to Support High-throughput and High-resolution Glycan Analysis by Capillary Electrophoresis

Andras Guttman, Zoltan Szabo, Barry Karger, Barnett Institute, Boston, MA USA

Jo Wegstein, Craig Nishida, ProZyme, Inc., Hayward, CA USA

Jeff Chapman, Hans Dewald, Beckman Coulter, Inc., Brea, CA USA

Stefan Mittermayr, Jonathan Bones, Margaret Doherty, Pauline Rudd, National Institute for Bioprocessing Research and Training, Dublin, Ireland



Abstract

Structural characterization of the glycan moieties of biologics, especially recombinant monoclonal antibody therapeutics (rMAB), is critical during clone selection, cell-culture optimization and product characterization. High-throughput screening methods are required in order to return results promptly to allow multiple iterations for selection of optimal candidates. This poster gives an overview of a state-of-the-art screening protocol with rapid sample preparation coupled with capillary electrophoresis (CE)-based glycan analysis that includes: automatable sample preparation with optional purification modules to allow direct screening of cell-culture samples; glycan labeling for laser-induced fluorescence (LIF) detection; clean-up to reduce excess reagent peaks and desalting followed by oligosaccharide CE profiling and/or carbohydrate sequencing and glucose unit (GU) value-based structural prediction.

Glycans released from subnanomolar amounts of rMABs are quickly and accurately prepared and analyzed, e.g., rapid (<7 min) CE separation of twelve, key, IgG glycans can be obtained along with 96-well format operation for convenient overnight processing. Particular attention is paid to full separation of core-fucosylated and afucosylated forms, as the presence or absence of this modification is of high importance in regulating the effector function of rMABs. The excellent relative migration time reproducibility of the optimized CE separation method (RSD <0.09%) facilitates high-fidelity peak assignments for the individual components in the glycan pools, and consequently allows high-precision, structural predictions using GU-value databases. Exoglycosidase, array-based, sequence verification of the predicted glycans is also presented.

Introduction

The distribution of N-linked glycans plays a critical role in the efficacy, immunogenicity and pharmacokinetics of therapeutic proteins. Historically multi-step, multi-day sample preparation has limited N-linked glycan analysis to only samples that represent critical decision points. N-glycan analysis for the large number of samples needed for strain selection or cell-culture optimization are seen as highly desirable.

Figure 1 shows the proposed workflow that includes ProZyme's GlykoPrep™ Sample Preparation, automated on Beckman Coulter's Biomek® laboratory automation systems, and rapid analysis and interpretation with a CE/LIF (Beckman Coulter's PA 800 plus Pharmaceutical Analysis System). The platform dramatically streamlines sample preparation; from the optional rMAB purification

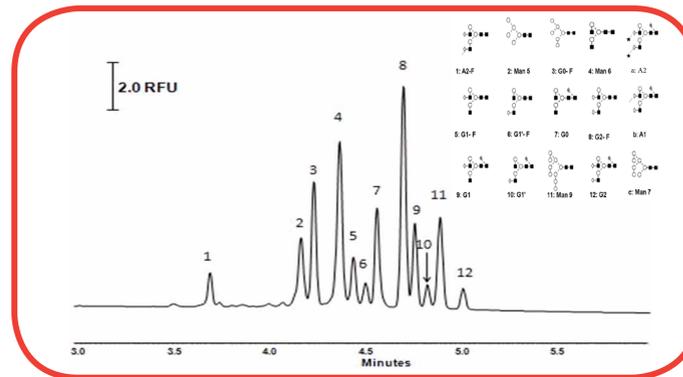


Figure 2 - Rapid Separation of the Glycan Test Mixture at 35°C

Conditions: Capillary: bilayer coated (50 µm i.d.), (total length: 50 cm, effective length: 40), Applied electric field: 600 V/cm; Injection: 10 sec/0.5 psi, 25 mM boric acid and 1.5% LPA (Mw 10,000) in 7.5 mM NH₄Ac buffer (pH 9); Temperature: 35°C.

to glycan release, labeling and cleanup, the entire sample preparation for a large number of samples can be completed in <4 hours. Rapid analysis methods developed by the Barnett Institute were needed to match the GlykoPrep throughput and allow the analysis of up to 96 samples overnight.¹

Methods/Discussion

Figure 2 shows an electropherogram for known standards as a control. Exoglycosidase array digestions were performed on rMAB A to identify the peaks present in the UPLC-fluorescence and CE-LIF IgG N-glycan profiles. Subsequent profiling of the digestion products was conducted using both separation techniques. After desialylation, the resulting CE-LIF profiles exhibited similar selectivity to UPLC-fluorescence (data not shown), which facilitated structural annotation. Figure 3 displays the resulting electropherograms accompanied by the experimentally annotated oligosaccharide digestion pathway for the IgG N-glycan pool. Each electropherogram was aligned using the lower and upper bracketing standards; however, for better clarity, only the migration window between ten and fifteen minutes is displayed. The structural annotation of the peaks corresponding to APTS-labeled asialo N-glycans was consistent with that generated using UPLC-fluorescence.² For these APTS-labeled asialo N-glycans, structural annotation was performed using a bottom up approach, considering the knowledge that the peaks present in the lowermost trace corresponded to the biantennary N-glycan and its analogue carrying a bisecting GlcNAc residue.

The GU shifts following the consecutive removal of terminal sugar units when using UPLC-fluorescence were in agreement to those previously reported, i.e., removal of a β(1-4)-linked galactose residue caused a shift of ~0.8 GU and removal of an α(1-6)-linked fucose or a β-linked GlcNAc residue resulted in a shift of ~0.5 GU.³ A noteworthy observation in CE-LIF was that the GU shifts following digestion showed deviations based upon the presence of other structural components due to the resulting differences in the hydrodynamic volume. The removal of an α(1-6)-linked fucose from the core fucosylated biantennary bigalactosylated glycan FA2G2, resulted in a

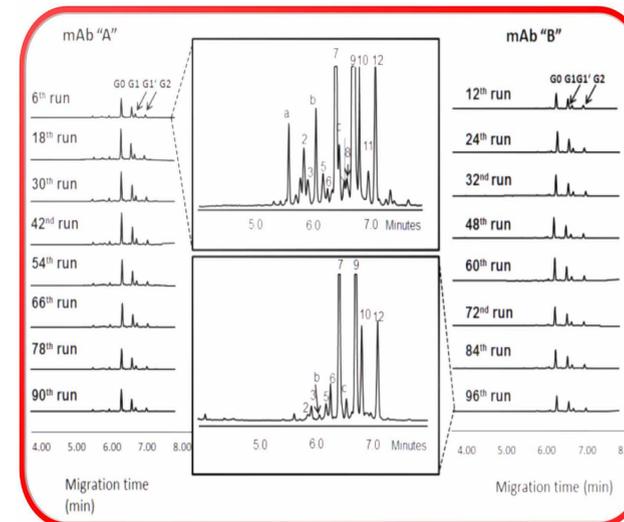


Figure 4 - 96-well Plate Operation of Glycosylation Pattern Analysis of Two Monoclonal Antibody Therapeutics (A and B).

The insets display the enlarged separation window showing all separated minor components. Conditions: 25 mM boric acid and 1.5% LPA (Mw 10,000) in 7.5 mM NH₄Ac buffer (pH 9); Temperature: 25°C; Capillary: bilayer coated (50 µm i.d.), (total length: 50 cm, effective length: 40 cm). Applied electric field: 600 V/cm. Injection: 10 sec/0.5 psi.

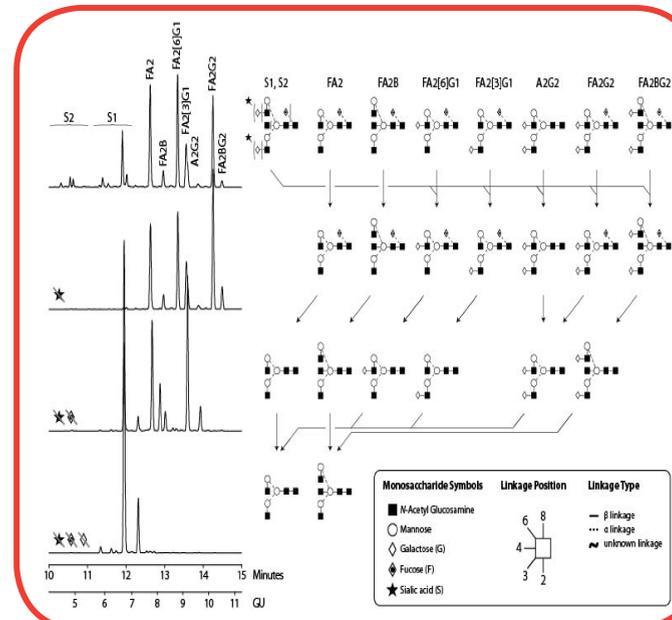


Figure 3 - Experimentally Annotated Exoglycosidase Digestion Pathway of the IgG N-glycan Pool Using CE-LIF for the Profiling of the Resulting Digest Products

GU shift of 1.01, whereas the removal from its structural analogue containing a bisecting GlcNAc residue resulted in a GU shift of 0.86. Previous *in silico*-based studies on the molecular dynamics and conformation of such N-glycans have revealed that removal of the fucose residue resulted in differential spacing of the antennae in the bisecting GlcNAc containing A2BG2 glycan compared to A2G2, due to a decrease in the distance between the antennary galactose residues following defucosylation.^{4,5} The galactose residues were also closer together in the absence of the bisecting GlcNAc. The information suggested by molecular modeling can be experimentally observed in the current data with discrete alterations in the GU shifts based upon the differences in the molecular volumes of the underlying oligosaccharide structures. Retention on the HILIC phase is dependent upon the number of hydrophilic groups presented.⁶ As the bisecting GlcNAc residue is contained within the cleft of the two antennae, it is only partially available for interaction resulting in a small increase in retention time and GU value.

Figure 4 shows representative traces of the glycosylation patterns from the two rMABs (A and B) generated using optimized conditions in the high-throughput 96-well operation.

An advantageous feature of CE-LIF was minimal sample consumption per injection; the injection volume is on average 3 orders of magnitude lower than that onto the UPLC column. For the characterization of rMABs, sample quantities are generally not limited; however, for the profiling of the glycosylation present on antibodies extracted from low volume clinical specimens, the reduced consumption of CE-LIF is an attractive feature. Accompanied with this is the ability to generate additional information from the same sample using CE-LIF due to the possibility of sequential digestion and reinjection.

Conclusion

1. The optimized separation parameters resulted in full separation of all selected rMAB N-glycans in less than 7 minutes at 25°C.
2. At 35°C, the separation time was decreased to 5 minutes, still featuring full resolution of the sample IgG N-glycans.
3. With the use of highly stable capillary coatings, faster separations are expected at higher separation temperatures. The optimal separation conditions at 25°C were applied to demonstrate high-throughput glycosylation profiling of two rMABs in a 96-well format for automated overnight operation.
4. With the help of a liquid handling robot, the entire glycan release, derivatization, sample cleanup and separation process can be fully automated.

References

1. Szabo, Z.; Guttman, A.; Bones, J.; Karger, B. L., Rapid High Resolution Characterization of Functionally Important Monoclonal Antibody N-Glycans by Capillary Electrophoresis. *Analytical Chemistry* 2011, 83 (13), 5329-5336.
2. Mittermayr, S.; Bones, J.; Doherty, M.; Guttman, A.; Rudd, P.M., Multiplexed Analytical Glycomics: Rapid and Confident IgG N-Glycan Structural Elucidation. *Journal of Proteome Research* 2011, 10 (8), 3820-3829.
3. Royle, L.; Radcliffe, C. M.; Dwek, R. A.; Rudd, P. M., Detailed structural analysis of N-glycans released from glycoproteins in SDS-PAGE gel bands using HPLC combined with exoglycosidase array digestions. *Methods in Molecular Biology* (Totowa, NJ, United States) 2006, 347, (Glycobiology Protocols), 125-143.
4. Andre, S.; Kozar, T.; Kojima, S.; Unverzagt, C.; Gabius, H.-J., From structural to functional glycomics: Core substitutions as molecular switches for shape and lectin affinity of N-glycans. *Biological Chemistry* 2009, 390, (7), 557-565.
5. Andre, S.; Unverzagt, C.; Kojima, S.; Frank, M.; Seifert, J.; Fink, C.; Kayser, K.; von der Lieth, C.-W.; Gabius, H.-J., Determination of modulation of ligand properties of synthetic complex-type biantennary N-glycans by introduction of bisecting GlcNAc in silico, in vitro and in vivo. *European Journal of Biochemistry* 2004, 271, (1), 118-134.
6. Melmer, M.; Stangler, T.; Premstaller, A.; Lindner, W., Comparison of hydrophilic-interaction, reversed-phase and porous graphitic carbon chromatography for glycan analysis. *Journal of Chromatography, A* 2011, 1218, (1), 118-123.

Beckman Coulter® and Biomek® are registered trademarks of Beckman Coulter, Inc. Brea, CA USA
ProZyme® and GlykoPrep™ are trademarks of ProZyme, Inc., Hayward, CA USA

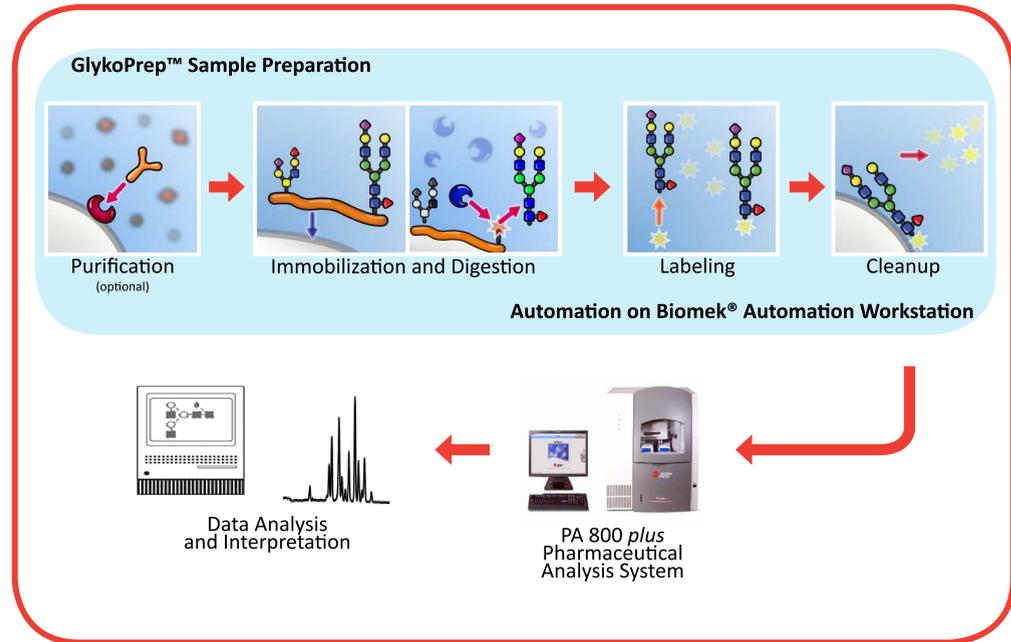


Figure 1: Proposed Workflow