Automated High-Throughput Permethylation for Glycosylation Analysis Using MALDI-TOF-MS

Archana Shubhakar[a,b,c], Daniel I.R. Spencer[a], Manfred Wuhrer[b], Daryl L. Fernandes[a]

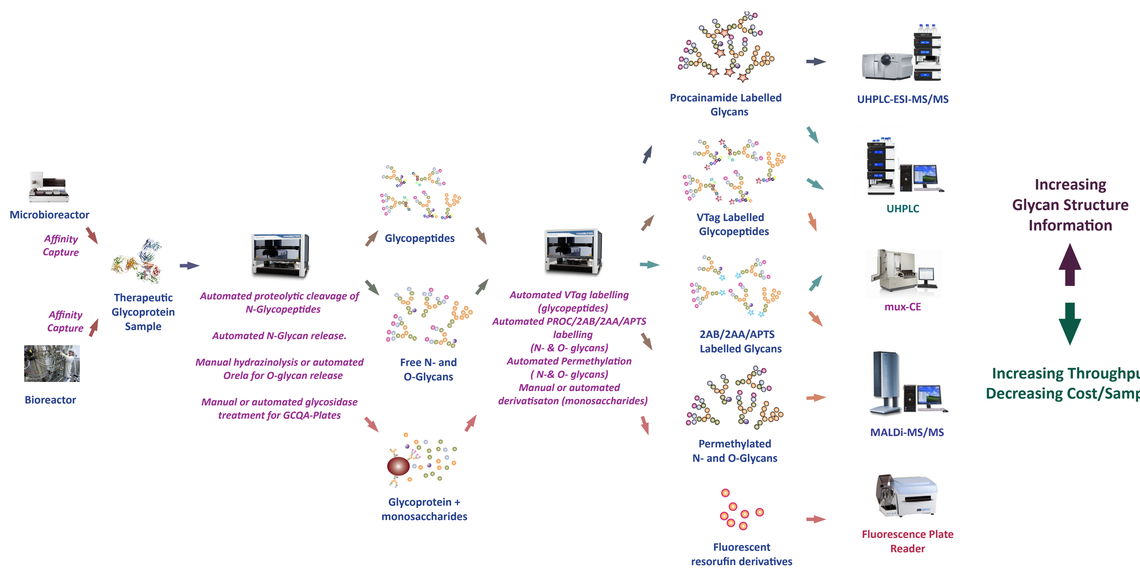
[a] *Ludger Ltd, Culham Science Centre, Abingdon, Oxfordshire, United Kingdom* [b] *Division of BioAnalytical Chemistry, VU University Amsterdam, The Netherlands* [c] *archana.shubhakar@ludger.com*

Abstract

For most therapeutic glycoproteins, the glycosylation patterns correlate strongly with the clinical safety and efficacy profiles. In biological tissues these patterns can also correlate with the state of health or disease of the individual. Given this, there is an increasing interest in accurately characterising changes in glycosylation — for example in Quality by Design (QbD) studies throughout biopharmaceutical development.

To perform these studies, reliable systems for high-throughput (HT) glycomics are needed. Despite many advances in glycosylation analysis there are still problems with current technologies, including high cost per sample, low sample throughput and high labour intensity.

This poster presents "LongBow" — a system developed at Ludger for reliable HT glycomics. The "LongBow" system is made up of flexible, modular technologies for semi-automated processing of glycans from a variety of clinical and bio-therapeutic samples as shown in Scheme 1. The emphasis of this poster will be on **Automated and HT Permethylation of N- and O-glycans analysed by Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS)**.



Scheme 1: Semi-automated HT LongBow glycomics workflow for glycoprotein sample preparation and analysis.

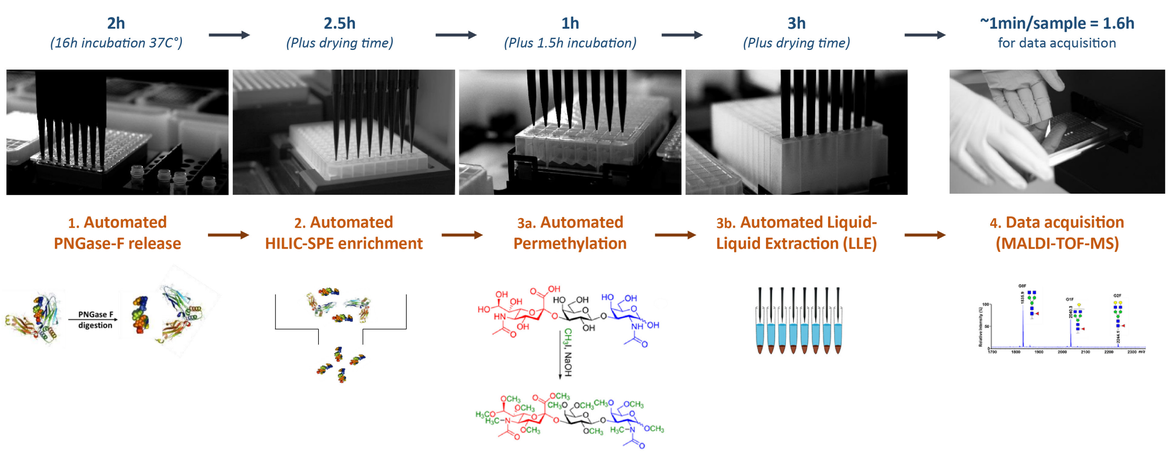
Introduction

Permethylation is the most popular derivatization of carbohydrates for MS detection, as it enhances ionization efficiency and stabilizes the sialic acids. This modification is the addition of methyl substituent to all of the hydroxyl and *N*-acetyl groups. Permethylation also methyl esterifies the carboxy function on the sialic acid.

The conventional in-solution technique is labour intensive with long turn around times, which prompted us to develop a microplate based permethylation kit which delivers a cost effective, automated and HT method for QbD and biomarker studies. (See Scheme 2)

However, the reliability of glycan quantitation using this method is challenging and to improve the reliability we have :

a) automated sample preparation and clean-up,
b) automated HT permethylation for glycan identification,
c) automated data acquisition on MALDI-TOF-MS and perform semi-automated data analysis
d) use a heavy isotope ^{13}C permethylated IgG standard for calibration and relative quantitation of IgG samples (Fig-1).



Scheme 2: LongBow system tuned for a largely automated and HT glycoprotein sample preparation workflow for (1) N-glycan release, (2) hydrophilic liquid interaction chromatography (HILIC)-solid phase extraction (SPE) of glycans, (3a) permethylation of released glycans, (3b)liquid– liquid extraction (LLE) and (4) Data acquisition.

Methods

To demonstrate our automated and HT technology, we successfully applied it to various sample subsets and the methods for N- and O-glycan release and derivatization are outlined as follows:

Glycan release: N-glycans from IgG1 monoclonal Antibody (mAb) standard and recombinant human erythropoietin (rhEPO) were released using Peptide N Glycosidase F enzyme and cleaned up using a LudgerClean™ Pre-Permethylation Clean-up Plate (LC-PERMET-96). A manual chemical release method (Hydrazinolysis) was used for release of O-glycans from rhEPO.

Glycan derivatization: The released and purified N-glycans from IgG1 mAb standard (Fig-2) and rhEPO (Fig-3) were permethylated using LudgerTag™ permethylation microplate kit (LT-PERMET-96). Both the permethylation and post-derivatisation sample clean up steps were performed on the a liquid handling robot and then analysed by flexAnalysis software from Bruker.

The O-glycans released from rhEPO were split into two aliquots to show comparability and orthogonality between two techniques:
a) Ultrahigh Performance Liquid Chromatography (UHPLC) and
b) MALDI-TOF-MS.
Sample processing and preparation for both methods was performed using the liquid handling robot. Pool 1 samples were reductively aminated with LudgerTag 2-AB labelling kit (LT-KAB-VP24), cleaned up using LudgerClean T1 cartridges (LC-T1-A6) and analysed by UHPLC (Fig-4a). Pool 2 samples were permethylated using the LT-PERMET-96 kit and analysed by MALDI-TOF-MS (Fig-4b).

Results

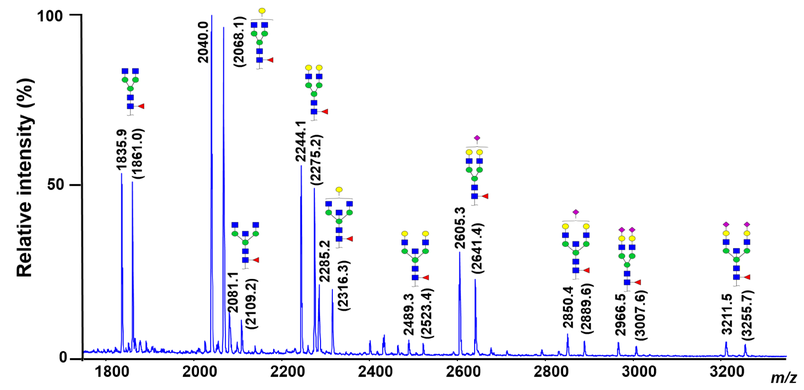


Fig-1: MALDI-TOF-MS spectrum of the ^{12}C and ^{13}C permethylated human-IgG N-glycan standards from Ludger (Cat# Cpm13C-IgG-01 and Cpm12C-IgG-01). ^{13}C was spiked with ^{12}C on the same sample spot to show the comparison of relative quantities of the major IgG N-glycans. The mass values shown in the spectra are $[M+Na]^+$ of permethylated glycans, with ^{13}C permethylated masses in parentheses.

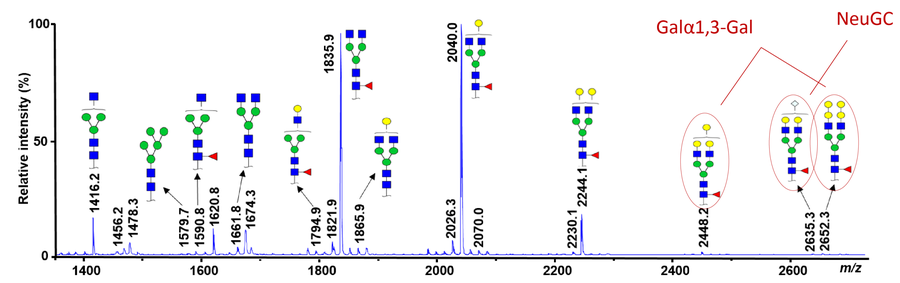


Fig-2: MALDI-TOF-MS spectrum of IgG1 mAb N-glycans permethylated on the liquid handling robot.
Note: Galα1, 3-Gal epitope and N-glycolylneuraminic acid (NeuGC) are non-human glycosylation features, reflecting possible critical quality attributes (CQAs) due to the potential immunogenic characteristics of the mAb identified after permethylation.

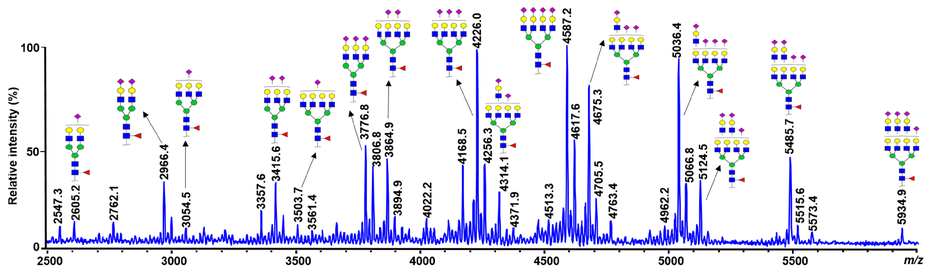


Fig-3: MALDI-TOF-MS spectrum of the permethylated N-glycans from rhEPO.

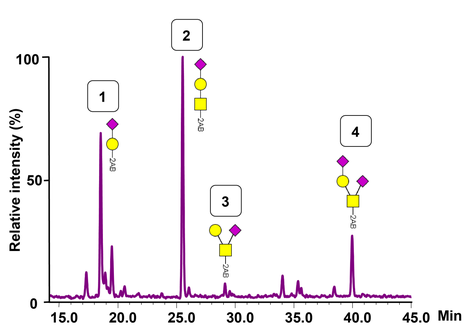


Fig-4a: Typical UHPLC profile of 2-AB labelled biosimilar rhEPO depicting the major O-glycans.
Note: Processing 96 samples on UHPLC *takes a minimum of 48 hours.*

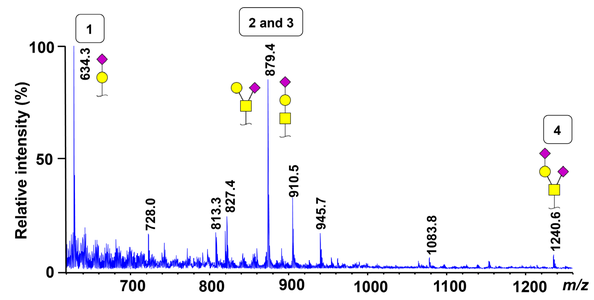


Fig-4b: MALDI-TOF-MS spectrum of the permethylated rhEPO, depicting the O-glycans.
Note: Automated data acquisition for 96 samples on MALDI-TOF-MS takes under 1 hour.

Glycan Key: ▲ **Fucose** ● **Galactose** ● **Mannose** ■ ***N*-Acetylglucosamine** ◆ ***N*-Acetylneuraminic acid** ◇ ***N*-Glycolylneuraminic acid**

The mass values shown in the MALDI-TOF spectra are $[M+Na]^+$ of permethylated glycans.

Conclusions

- We have developed and optimized a new permethylation technique which exploits the use of a liquid handling robot to enable higher throughput and automated glycosylation analysis of N- and O-glycans analysed by using MALDI-TOF-MS.
- This HT permethylation technique is comparable to UHPLC results (see Fig 4a and 4b) and it gives a reliable overview of the glycosylation profile in a short timespan.
- The advantages of permethylated glycan analysis using the MALDI-TOF-MS are: enhanced signal due to increased ionization efficiency, good sensitivity, and rapid analysis. You can process hundreds of samples per day i.e., on average one sample takes less than a minute to process, so 96 samples can be processed in under 1 hour on MALDI-TOF-MS compared to UHPLC which takes more than 48 hours to run 96 samples.
- Therefore we conclude that our microplate based, automated and HT permethylation technique delivers the most cost effective, fast and reliable method for QbD and biomarker studies.

References

- Fernandes DL, Biopharm Knowledge Publishing, Hampshire, UK, 2008, (A QbD approach to biopharmaceutical glycosylation).
- Wuhrer, M, Glycoconjugate journal, 2013, DOI: 10.1007/s10719-012-9376-3
- Shubhakar, A, et al , Chromatographia , 2014, DOI: 10.1007/s10337-014-2803-9
- Shubhakar, A.; et al , Anal. Chem. 2016, DOI: 10.1021/acs.analchem.6b01639

Acknowledgements

We would like to acknowledge and thank Raquel Montesino and Antonio Vallin from the Center for Genetic Engineering and Biotechnology, Cuba for kindly providing rhEPO for our studies. This work was supported by the European Union Seventh Framework Programmes HighGlycan (Grant Number 278535) and IBD-BIOM (Grant Number 305479).
and Biotechnology, Cuba for kindly providing rhEPO for our studies. This work was supported by the European Union (seventh Programme HighGlycan project, grant number 278535).

Contact for more information

Thank you for viewing my poster. If you'd like a copy or want to know more about our glycomics workflows then please email me: **archana.shubhakar@ludger.com**. Thanks, Archana.