Drug Glycosylation Analysis using Automated, High Throughput, Orthogonal Methods

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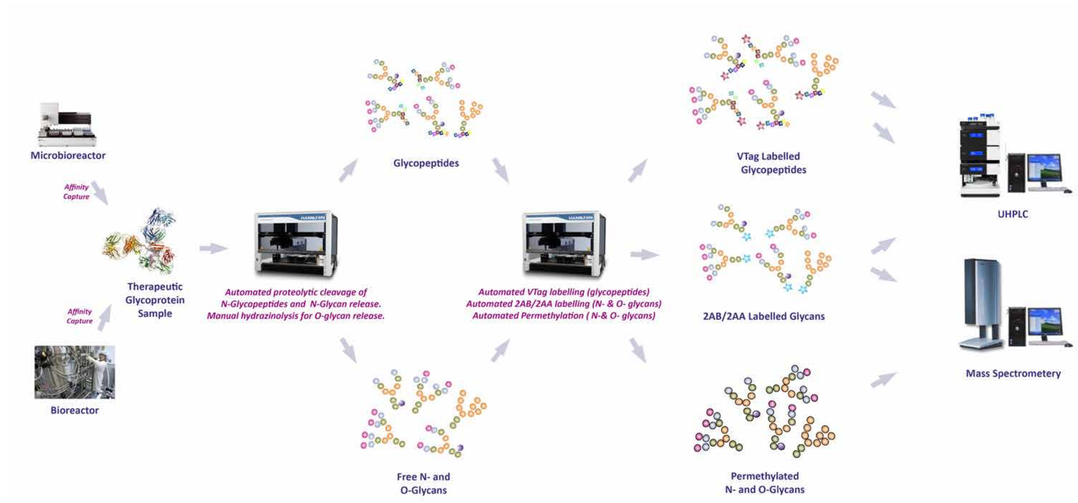
Introduction

Monitoring the glycosylation of biopharmaceuticals throughout the drug's life cycle is vital as changes in glycosylation of therapeutics can significantly affect their safety, efficacy and immunogenicity. Therapeutic glycoproteins need to be optimised with respect to their glycosylation and the Glycosylation Critical Quality Attributes (GCQAs) should be determined to ensure consistent drug quality.

Glycosylation analysis is often a major challenge due to its complexity and heterogeneity. Although there are a wide range of conventional methods available for the characterization of glycans, each has limitations with respect to speed and robustness, and not all of these methods are conducive for high-throughput sample analysis as they are labour intensive [1-3]. Here, using biopharmaceutical samples, we have adapted our work flow to a liquid handling robot for fast, reliable and robust N- and O-glycan analysis by using two orthogonal methods HILIC-UHPLC and MALDI-MS.

Methods

To demonstrate our methods we analysed N-glycans and N-glycopeptides from an IgG4 monoclonal antibody (mAb) and N- and O-glycans from recombinant human erythropoietin (rhEPO). The methods are outlined in Scheme 1.



Scheme 1: Workflow for drug samples from bioreactor to analysis by MS and UHPLC

Glycopeptide Analysis

IgG4 mAb glycosylation was analysed at the level of fluorescently labelled glycopeptides. A Ludger V-tag glycopeptide labelling kit (LT-VTAG-24) was used on a liquid handling robot. In short, IgG4 mAb was subjected to proteolytic cleavage. The resulting peptide/glycopeptide mixture was fluorescently labelled followed by enrichment of the labelled glycopeptides. Fluorescently labelled glycopeptides were then analysed by UHPLC and MALDI-MS.

Released N- and O-glycan Analysis

N-glycans from IgG4 mAb and rhEPO were released using Peptide N Glycosidase F enzyme (PNGase-F, E-PNG01 from Ludger) and cleaned up using a Ludger Clean™ Post-Exoglycosidase Clean-up Plate (LC-PBM-96). Both steps were performed on a liquid handling robot. A manual chemical release method (Hydrazinolysis) was used for release of O-glycans from rhEPO.

The released glycans were:

a. **Fluorescently labelled.** Glycans were labelled with 2-aminobenzamide (2AB) using the LudgerTag™ 2-AB labelling kit (LT-KAB-VP24) following by a clean up using LudgerClean™ T1 cartridges (LC-T1-A6). Both steps were performed on a liquid handling robot (Hamilton Starlet). 2AB-labelled glycan samples were analysed by UHPLC with fluorescence detection.
b. **Permethylated.** This is the process of derivatizing all the hydroxyl and N-acetyl groups with a methyl group. Permethylation also methyl esterifies the carboxy function on the sialic acid. Both these derivatizations lead to a stabilization of the sialic acids and to significantly enhance MS analysis. Glycans were permethylated using the prototype LudgerTag™ permethylation micro plate format kit (LT-PERMET-96). Both the permethylation and post-derivatisation sample clean up steps were performed on the robot and then analysed by MALDI-MS (Bruker Daltonics).

Results

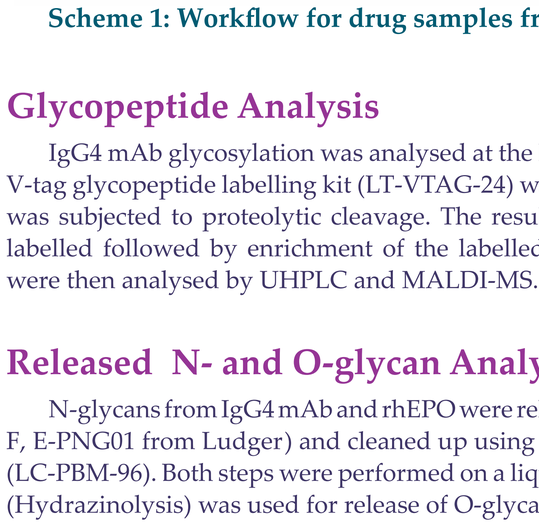


Figure 1a: UHPLC Chromatogram of IgG4 glycopeptides after pronase digestion and V-tag labelling by robot

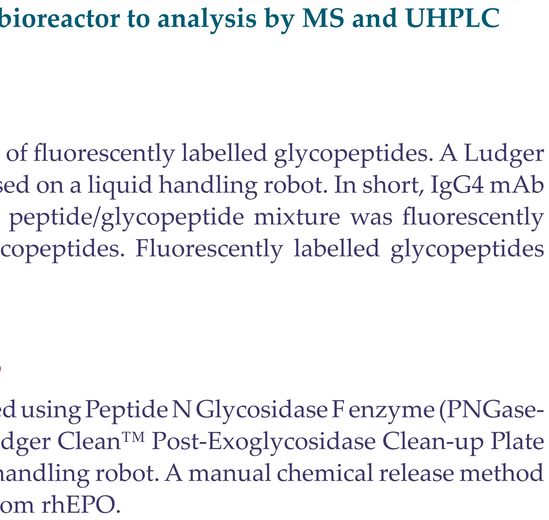
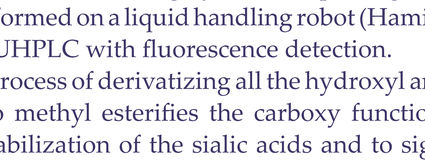


Figure 1b: Negative ion Mass Spectrum of IgG4 glycopeptides after pronase digestion and V-tag labelling by robot



Monosaccharide components of N- and O-glycan structures

Note: The isobaric structures are not distinguished in the MALDI-MS profiles

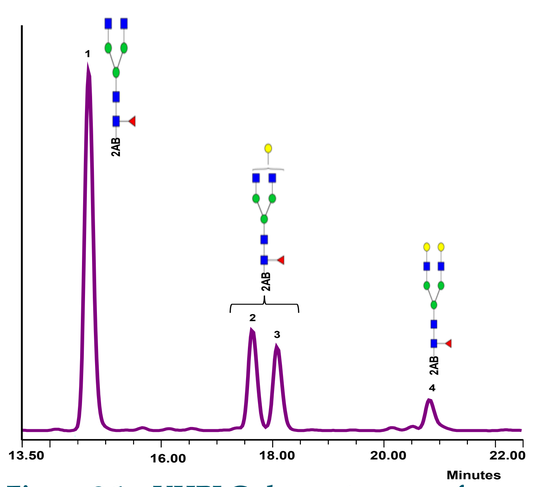


Figure 2.1a: UHPLC chromatogram of N-glycans from IgG4 mAb after PNGase F digestion and 2AB labelling by robot

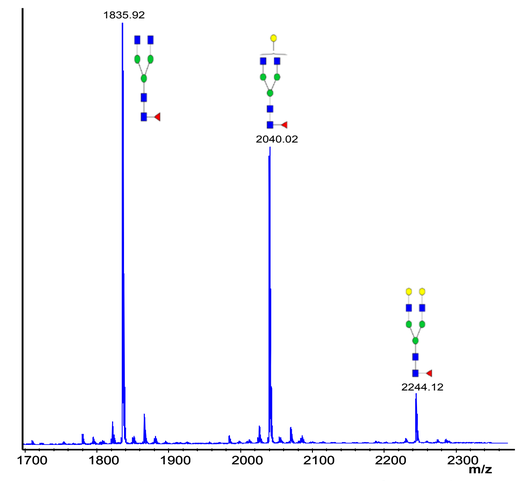


Figure 2.1b: Mass spectrum of N-Glycans from IgG4 mAb after PNGase F digestion and permethylation by robot

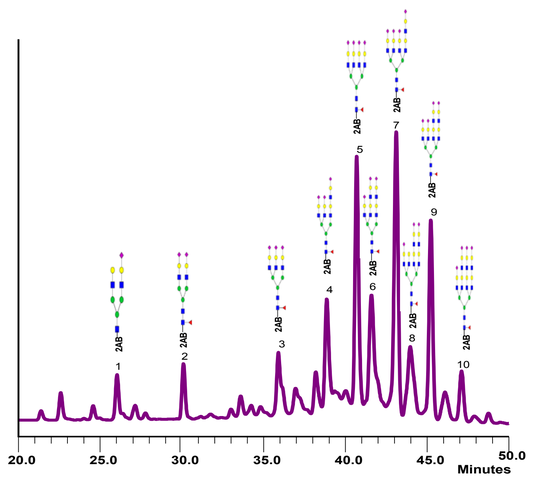


Figure 2.2a: UHPLC chromatogram of rhEPO N-glycans after PNGase F digestion and 2AB labelling by robot

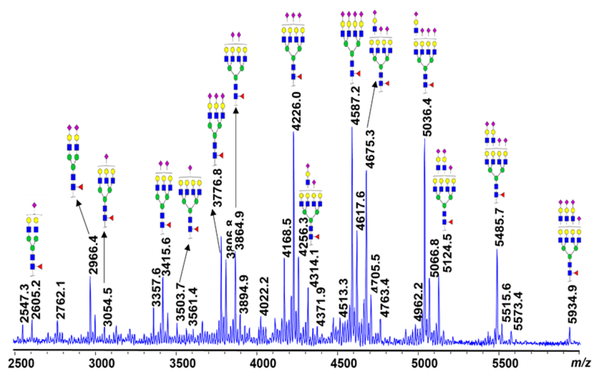


Figure 2.2b: Mass spectrum of rhEPO N-glycans after PNGase F digestion and permethylation by robot

Figure 2.3a: UHPLC chromatogram of rhEPO O-glycans 2AB labelled by robot after manual hydrazinolysis

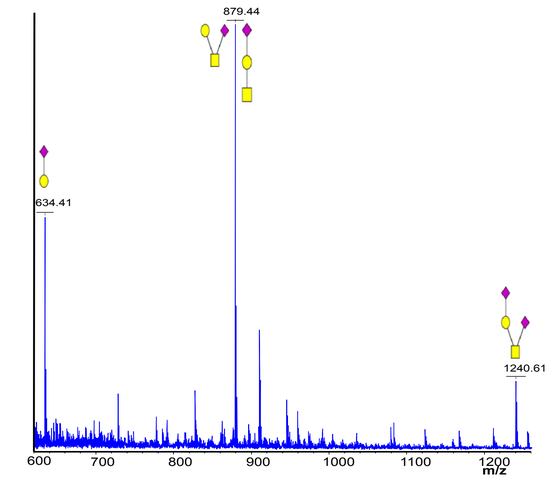


Figure 2.3b : Mass Spectrum of rhEPO O- glycans permethylated by robot after manual hydrazinolysis

Conclusions

We present different approaches to drug glycosylation analysis which exploit liquid handling robots to enable support higher-throughput glycan analysis of biopharmaceuticals. N-and O-glycan analysis were performed by two orthogonal methods; HILIC-UHPLC and MALDI-MS and our results demonstrate that these were in agreement with respect to identification and quantitation of glycan species.

The advantages of the UHPLC method for analyzing fluorescently labelled glycans are good sensitivity, isomer separation, accurate sample quantitation, repeatability and in-depth characterization of glycan species supported by exoglycosidase sequencing.

The advantages of the MALDI-MS method for permethylated glycan analysis are rapid, high throughput analyses, lower cost per sample, enhanced signal due to increased ionization efficiency, good sensitivity, and the possibility of getting additional structural information by MS/MS experiments.

The drug glycoprofiles from our high-throughput, semi-automated methods for UHPLC and MALDI-MS platforms have provided reliable and comparable results. This allows the flexibility to select the most suitable method at each stage of the product's life cycle depending on the requirements for analysis speed, sample throughput, cost per sample, and reliability of quantitation.

References

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Acknowledgments

We would like to thank Lonza Biologics, Slough, UK and University College London for generously providing Chinese hamster ovary (CHO) cell line (GS-CY01, IgG4 mA) for our study. We also want 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.

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