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High-throughput profiling of nucleotides and nucleotide sugars to evaluate their impact on antibody *N*-glycosylation

Thomas K. Villiger[a,1], Robert F. Steinhoff[b,1], Marija Ivarsson[a], Thomas Solacroup[c], Matthieu Stettler[c], Hervé Broly[c], Jasmin Krismer[b], Martin Pabst[b], Renato Zenobi[b], Massimo Morbidelli[a], Miroslav Soos[a,d,*]

[a] *Institute for Chemical and Bioengineering, Department of Chemistry and Applied Biosciences, ETH Zurich, CH- 8093 Zurich, Switzerland*
[b] *Laboratory of Organic Chemistry, Department of Chemistry and Applied Biosciences, ETH Zurich, CH-8093 Zurich, Switzerland*
[c] *Merck Serono SA, Corsier-sur-Vevey, Biotech Process Sciences, ZI B, CH-1809 Fenil-sur-Corsier, Switzerland*
[d] *Department of Chemical Engineering, University of Chemistry and Technology, Technicka 5, 166 28 Prague, Czech Republic*

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ABSTRACT

Recent advances in miniaturized cell culture systems have facilitated the screening of media additives on productivity and protein quality attributes of mammalian cell cultures. However, intracellular components are not routinely measured due to the limited throughput of available analytical techniques. In this work, time profiling of intracellular nucleotides and nucleotide sugars of CHO-S cell fed-batch processes in a micro-scale bioreactor system was carried out using a recently developed high-throughput method based on matrix-assisted laser desorption/ionization (MALDI) time-of-flight mass spectrometry (TOF-MS). Supplementation of various media additives significantly altered the intracellular nucleotides and nucleotide sugars that are inextricably linked to the process of glycosylation. The results revealed that UDP-Gal synthesis appeared to be particularly limiting whereas the impact of elevated UDP-GlcNAc and GDP-Fuc levels on the final glycosylation patterns was only marginally important. In contrast, manganese and asparagine supplementation altered the glycan profiles without affecting intracellular components. The combination of miniaturized cell cultures and high-throughput analytical techniques serves therefore as a useful tool for future quality driven media optimization studies.

Glycosylation nomenclature

M9	$Man_9GlcNAc_2$
M8	$Man_8GlcNAc_2$
M7	$Man_7GlcNAc_2$
M6	$Man_6GlcNAc_2$
M5	$Man_5GlcNAc_2$
A2	$GlcNAc_2Man_3GlcNAc_2$
FA2	$GlcNAc_2Man_3GlcNAc_2Fuc$
FA2G1	$GalGlcNAc_2Man_3GlcNAc_2Fuc$
FA2G2	$Gal_2GlcNAc_2Man_3GlcNAc_2Fuc$
SIA	$SiaGal_2GlcNAc_2Man_3GlcNAc_2Fuc$

1. Introduction

Chinese hamster ovary (CHO) cells are the predominant host for the production of many complex therapeutic proteins mainly due to their robustness, high productivity and most importantly their

Abbreviations: 9-AA, 9-aminoacridine; ADP, adenosine diphosphate; AMP, adenosine monophosphate; ATP, adenosine triphosphate; CDP, cytidine diphosphate; CDP, cytidine diphosphate; CMP, cytidine monophosphate; CMP-Neu5Ac, cytidine diphosphate *N*-acetylneuraminic acid; CTP, cytidine triphosphate; DWP, deep well plate; GDP, guanosine diphosphate; GDP-Fuc, guanosine diphosphate fucose; GDP-Hex, guanosine diphosphate hexose; GDP-Man, guanosine diphosphate mannose; GMP, guanosine monophosphate; GTP, guanosine triphosphate; HPAEC, high pressure anion exchange chromatography; MAMS, microarray for mass spectrometry; MALDI, matrix-assisted laser desorption/ionization; PCA, principle component analysis; TOF-MS, time-of-flight mass spectrometry; UDP, uridine diphospate; UDP-Hex, uridine diphospate hexose; UDP-HexNAc, uridine diphospate *N*-acetylhexosamine; UDP-Gal, uridine diphospate galactose; UDP-GalNAc, uridine diphospate *N*-acetylgalactosamine; UDP-Glc, uridine diphospate glucose; UDP-GlcNAc, uridine diphospate *N*-acetylglucosamine; UDP-Xyl, uridine diphospate xylose; UDP-GlcA, uridine diphospate glucoronic acid; UMP, uridine monophospate; UTP, uridine triphosphate; VCD, viable cell density.

* Corresponding author at: Institute for Chemical and Bioengineering, Department of Chemistry and Applied Biosciences, ETH Zurich, CH-8093 Zurich, Switzerland.
E-mail address: miroslav.soos@chem.ethz.ch (M. Soos).
[1] Contributed equally to the paper.

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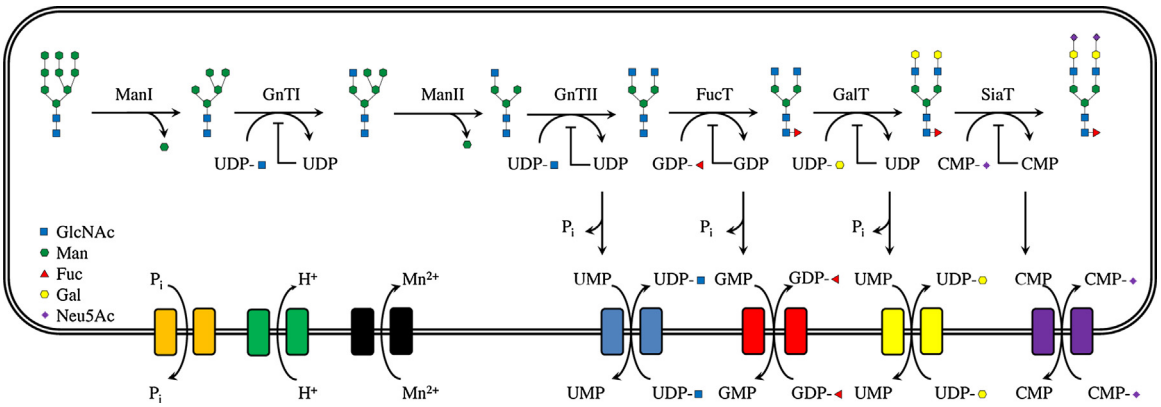

Fig. 1. Linear representation of the enzymatic pathway of mAb Fc N-linked glycosylation in the Golgi apparatus of mammalian cells with a simplified illustration of the most important enzymatic reactions, transports mechanism and utilization of nucleotides and nucleotide sugars regarding N-linked glycosylation of monoclonal antibodies.

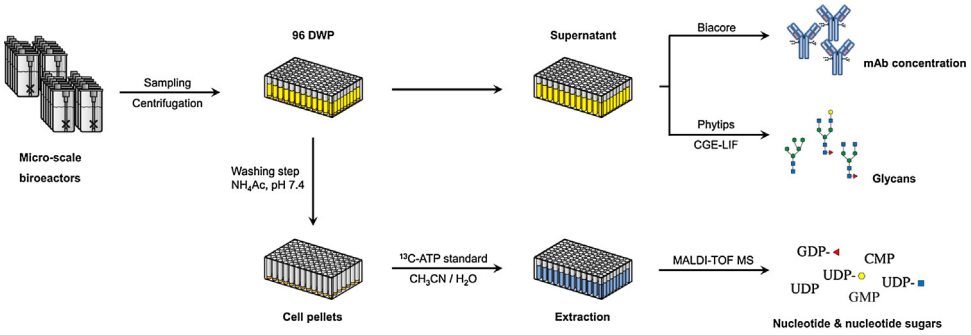

Fig. 2. Schematic workflow of the combination of micro-scale bioreactors and analytical techniques. The time-consuming steps such as sampling and extractions procedures are either automated or performed in multi-well plates.

ability to perform human-like post-translational modifications (Jefferis, 2009). In particular, N-linked glycosylation is not only essential for the protein to fold correctly, but also influences several product-specific properties such as stability, immunogenicity, circulatory lifetime and therapeutic efficacy (Hossler et al., 2009). Glycosylation is therefore considered as a critical quality attribute and increasing attention is paid to obtain uniform and consistent glycan patterns during the development and manufacturing process (Hossler et al., 2009; Meuris et al., 2014). Growing awareness of the significance of glycosylation and the tremendous boost in upstream productivity during the last decades has pushed the focus in biopharmaceutical production from simple yield improvement towards a product quality driven multi-objective optimization.

The majority of recombinant glycoproteins are produced in fed-batch mode due to scalability, short development time and available facilities. During a fed-batch process, the environmental conditions vary considerably over time and affect the behavior of the cells which may also influence quality attributes including N-linked glycosylation (Pacis et al., 2011), charge variants (Luo et al., 2012) or even cause structural deterioration of the desired protein (Trexler-Schmidt et al., 2010). The reason for a changing environment is manifold, ranging from typical temperature and pH shifts to byproduct accumulation and fluctuating nutrient availability (Ivarsson et al., 2014). Such changes can induce differences in glycosylation gene expression and nucleotide sugar synthesis (Wong et al., 2010). Indeed, significant variations in intracellular concentration of nucleotides and nucleotide sugars have been observed during mammalian fed-batch cultures which consequently change the availability of sugar substrates for the glycosylation process (Fan et al., 2015; Kochanowski et al., 2008).

A simplified pathway of mAb N-linked glycosylation in mammalian cells is shown in Fig. 1. First, a large oligosaccharide $Glc_3Man_9GlcNAc_2$ is attached in the endoplasmic reticulum to a three amino acid sequence (ASN-X-Ser/Thr where X is any amino acid except proline) of the protein. This may occur on several locations, albeit most IgG-based antibodies show only *N*-glycosylation at the crystallizable region. After passing through a machinery of protein folding and quality homeostasis, the protein with its attached oligosaccharide structure $Man_9GlcNAc_2$ (M9) enters the Golgi apparatus (Helenius and Aebi, 2001). This polysaccharide is trimmed down and subsequently modified by a multitude of enzymatic steps which give rise to a broad range of different glycosylation structures. Glycosylation is a non-template process which is subject to changes during fed-batch cultures by disturbing the complex interaction of multiple components including metal cofactors, nucleotides, nucleotides sugars and the acceptor sugars itself (Hang et al., 2015). Manipulating these components using various cell culture supplementations is a cost- and time-effective way to modulate and fine-tune glycosylation, since the degree of glycosylation is mainly changed through a shift of intracellular compounds or glycosyltransferase activity while maintaining consistent process performance. Metal cofactors such as manganese have been applied to enhance the enzymatic activity leading to more processed glycan structures (Gramer et al., 2011; Pacis et al., 2011). Supplementing cell culture media with glucosamine (Baker et al., 2001), galactose (Hills et al., 2000) and *N*-acetylmannosamine (Baker et al., 2001) has been shown to increase the corresponding intracellular levels of nucleotide activated sugars uridine diphosphate *N*-acetylglucosamine (UDP-GlcNAc), uridine diphosphate galactose (UDP-Gal) or cytidine diphosphate *N*-acetylneuraminic acid (CMP-Neu5Ac), respectively. In contrast, the nucleotide pools may play an ambivalent role in terms of glycosylation since they can either increase the concentration of nucleotide sugar donors or act as enzyme inhibitors (Bernacki, 1975; Nishikawa