



Lectin bio-layer interferometry for assessing product quality of Fc-glycosylated immunoglobulin G

Authors:

Jakob Wallner^{1*}, Bernhard Sissolak^{2*}, Wolfgang Sommeregger², Nico Lingg¹, Gerald Striedner¹, Karola Vorauer-Uhl¹

1 Department of Biotechnology, University of Natural Resources and Life Sciences, Muthgasse 11, Vienna, Austria

2 Bilfinger Industrietechnik Salzburg GmbH, Salzburg Austria

[°]Corresponding Author:

Jakob Wallner, Department of Biotechnology, University of Natural Resources and Life Sciences, Muthgasse 11, A-1190 Vienna, Austria.

E-mail: Jakob.Wallner@boku.ac.at

* Both authors contributed equally to this work

The authors declare no commercial or financial conflict of interest.

This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1002/btpr.2864

© 2019 American Institute of Chemical Engineers

Received: Apr 18, 2019; Revised: May 28, 2019; Accepted: June 06, 2019

Abstract

Glycosylation, as the most prominent post-translational modification, is recognized as an important quality attribute of monoclonal antibodies affected by various bioprocess parameters and cellular physiology. A method of lectin-based bio-layer interferometry (LBLI) to relatively rank galactosylation and fucosylation levels was developed. For this purpose, Fc-glycosylated immunoglobulin G (IgG) was recombinantly produced with varying bioprocess conditions in 15 L bioreactor and accumulated IgG was harvested. The reliability, the robustness and the applicability of LBLI to different samples has been proven. Data obtained from LC-MS analysis served as reference and were compared to the LBLI results. The introduced method is based on non-fluidic bio-layer interferometry (BLI), which become recently a standard tool for determining biomolecular interactions in a label-free, real-time and high-throughput manner. For the intended purpose, biotinylated lectins were immobilized on disposable optical fibre streptavidin (SA) biosensor tips. *Aleuria aurantia* lectin (AAL) was used to detect the core fucose and *Ricinus communis* agglutinin 120 (RCA120) to determine galactosylation levels. In our case study it could be shown that fucosylation was not affected by variations in glucose feed concentration and cultivation temperature. However, the galactosylation could be correlated with the ratio of mean specific productivity (q_P) and ammonium ($q_{NH_4^+}$) but was unrelated to the ratio of mean q_P and the specific glucose consumption (q_{gluc}). This presented method strengthens the applicability of the BLI platform, which already enables measurement of several product related characteristics, such as product quantity as well as kinetic rates (k_d , k_{on}) and affinity constants (k_D) analysis.

Keywords: bio-layer interferometry, CHO cell culture, glycosylation, galactosylation, fucosylation

Introduction

Monoclonal antibodies (mAbs) continue to dominate biopharmaceutical approvals and constitute about 40% of biotherapeutics that are available on the market and thus form a major class of molecules produced and developed by the biopharmaceutical industry¹. Product quality and quantity are important measures in antibody discovery and in process development. These requirements are driven by a large number of influential factors such as process performance and cell physiology. The complexity of such a multifactorial system makes it difficult to identify the parameters with relevant influence on product quality. Thus, direct control of product quality in the individual bioprocess, a central goal of the Quality by Design approach, is severely limited². Consequently, it is crucial to get a deeper understanding of the biological system, the process, the product and the interdependencies amongst each other.

In respect of product quality, the glycosylation is among others, a relevant characteristic, which can profoundly affect protein stability and the functions which are relevant to their therapeutic application. Recently, the correlation between the glycoform profiles and the safety and efficacy of a drug, in particular, has achieved significant attention of researchers worldwide³. The glycan structure is affected by the enzymatic machinery of the host cell, transit time in the Golgi bodies, environmental factors and the availability within the sugar nucleotide pool^{4–6}. With respect to process conditions, several publications have shown that the osmolality level (in combination with pH)⁷, availability of glucose^{8,9}, ammonium production rates¹⁰ or dissolved CO₂ level^{7,11} can lead to alternations in glycosylation patterns. In order to study such mechanisms in more detail and to measure product quantity and relevant quality parameter, appropriate analytical assays are required.

Several techniques already exist to measure those quality specifications. The most commonly applied technique for the complete structural elucidation of glycoprotein oligosaccharides is the

Accepted Article

application of a combination of chemical, enzymatic and chromatographic techniques combined with mass spectrometry ^{12,13}. Another promising approach is the application of lectin-binding assays ¹⁴. Lectins are glycan-binding proteins that selectively recognize glycan epitopes of glycoproteins, which enables the specific monitoring of oligosaccharide structures. The interactions of lectins with glycan structures can be measured via the bio-layer interferometry (BLI), a well-established bio sensor technology ¹⁵. The BLI technology is performed in an open shaking micro-well plate format without any micro-fluidics and using disposable optical fibre biosensors. The physical principle of this technique is based on the correlation of the spectral shift $\Delta\lambda$ with a change in thickness (nm) on the biosensor surface. The platform also allows the measurement of biomolecular interactions, enabling full kinetic measurements and facilitate the quantitation of biomolecules ¹⁶. Accordingly, several product quality characteristics and the product quantity can be determined on a single platform.

A recent study showed the applicability of the BLI platform as a high-throughput technique for determining the sialylation of mAbs ¹⁷. This was done by measuring the binding rate of *Maackia amurensis* lectin II (MALII) to (α-2,3)-linked sialic acids of highly sialylated proteins bound in a native state to Protein A sensors. However, all oligosaccharides, except (α-2,3)-linked sialic acids are normally hidden located within the folded structure of immunoglobulin G (IgG) and are not accessible to related lectins. This induces the necessity for reduction of the disulphide bonds which lead to the opening of the tertiary and quaternary protein structure of IgG and enables lectins to bind to oligosaccharides attached in the Fc component ^{18, 19}.

In the present study a non-sialylated anti-tumour necrosis factor (anti-TNF-α) IgG1 (Figure 1) recombinantly produced in Chinese hamster ovary (CHO) cells in a 15 L pilot scale fed-batch process under varying conditions was used as a model protein. For this study the mAB was purified from the harvest of 13 fed batch cultivation runs with variations in feed media glucose concentration and cultivation temperature.

Ricinus communis agglutinin 120 (RCA120) and *Aleuria aurantia* lectin (AAL) were used to determine the terminal galactose and core fucose content of the pre-purified IgG¹⁴. The resulting galactosylation and fucosylation levels were compared with results obtained by the well-established analysis technique using MS detection²⁰. Since the harvest samples represent the accumulated product of the entire bioprocess, the glycosylation was further related to the overall process performance. To the best of our knowledge this study presents for the first time application of the BLI platform to determine the galactosylation and fucosylation levels of CHO culture samples.

Material and Methods

Bioprocess set up

As a model protein Fc-glycosylated anti-TNF- α IgG1 was used, produced by a recombinant monoclonal Chinese hamster ovary (CHO) cell line (Antibody Lab GmbH, Austria). Generation of the cell line was conducted by applying the Rosa26 bacterial artificial chromosome (BAC) expression strategy to a serum-free adapted host cell line derived from CHO-K1 (ATCC CCL-61)²¹.

A vial of the working cell bank (5×10^6 cells) was thawed in a chemically defined culture medium (Dynamis AGT, A26175, Thermo Fisher Scientific, USA) supplemented with 8 mM L-glutamine (25030081, Sigma Aldrich, Germany), 3 mL/L phenol red solution (RNBD642, Sigma Aldrich, Germany), 1:1000 Anti Clumping Agent (0010057DG, Thermo Fisher Scientific, USA) and 1 mg/mL G418 (G8168, Sigma Aldrich, Germany).

Every three to four days the cells were passaged with the aforementioned media but without anti-clumping agent and G418. Cultivation was performed in a humidified incubator (HeracellITM VIOs 160i, Thermo Scientific, USA) at 37 °C, 5% v/v CO₂ and 200 rpm (MaxQ 2000 CO₂ Plus, Thermo Scientific, USA).

With the fourth passage the cells were transferred into the 15 L bioreactor (LabQube, Bilfinger Industrietechnik GmbH, Austria) with a seeding concentration of 2.5×10^5 cells/mL and a starting volume of 10 L. The batch phase was kept identical for every experiment. The experimental setup included the variation of temperature, at 31 °C, 34 °C and 37 °C, in addition to variation in the amount of glucose (G7021, Sigma Aldrich, Germany), with the addition of 10, 20 or 30 g/L of glucose, and aspartate concentration with additions of 0 or 7 g/L aspartate, in the feed phase (CHO CD EfficientFeed A, A1442001, Thermo Fisher Scientific, USA). Additionally, the feed medium was also supplemented with 0.1% antifoam (A8011, Sigma Aldrich, Germany) to maintain a constant antifoam concentration during the process. A constant feed rate of 418 g/d was used during the feed-phase.

The process was controlled via process air mass flow (PA) and stirrer speed to maintain the dissolved oxygen level (DO) above 30% and CO₂ mass flow to keep the pH constant at 7.0. The total gas-flow range was kept within 0.01-0.1 vvm.

The experimental setup used is displayed in Table 1.

Off-line analyses

The total cell concentration (TCC) was determined by counting the cell nuclei using a Z2 particle counter (Beckman Coulter, USA). Therefore, an appropriate amount of cell suspension was centrifuged at 180 g for 10 minutes (min). The cell pellet was subsequently resuspended in a 0.1 M citric acid monohydrate and 2% (v/v) Triton X-100 buffer to lyse the cells for a minimum of one hour before measurement. Sample dilution was performed using a 0.9% NaCl solution.

Culture viability was assessed using a haemocytometer and trypan blue exclusion. The viable cell concentration (VCC) was determined by multiplying viability with the TCC.

Glucose was determined via ion exclusion chromatography (HPX 87H, 300 x 7.8 mm, #1250140, BioRad, USA) using an Agilent 1200 series (Agilent, USA) at 25 °C. The mobile

phase consisted of 5 mM sulphuric acid and the flowrate was set to 0.45 mL/min and measured via a Refractive Index Detector (35 °C). The calibration range for D(+)-glucose was 100-2000 mg/L. The chromatograms were evaluated using Chemstation software (revision B.04.01, Agilent, USA).

Ammonium was measured via an ion selective electrode (ISE, GZ-27512-00, Cole-Parmer, USA). Therefore the cell suspension was centrifuged at 180 rpm for 10 min and two times 3 mL of the supernatant were stored at -20 °C. Before each measurement the ISE was filled with the reference solution (0.1 M NH₄Cl, GZ-27503-71, Cole Palmer, USA) and calibrated (from 1-100 mM, GZ-27503-00, Cole Palmer, USA). 60 µL of 10 M sodium hydroxide solution (221465, Merck, Germany) were added to each sample and the solution was vortexed, shortly before measurement.

Osmolality was measured by using a freezing point osmometer (Osmomat 030-D, Gonotec, Germany). Thawed samples of the cell broth supernatant were measured in duplicates.

The product titre was determined by BLI using Protein A tips (Octet System, QK, ForteBio, USA)²².

Lectin Assay

Immunoglobulin G purification

The mAb was purified from clarified supernatants using Protein A affinity chromatography. The experiments were performed on an Äkta Pure system (GE Healthcare). A POROS A 20, 2.1 x 30 mm column was used for the stationary phase (Thermo Scientific). The column was equilibrated with phosphate buffered saline (PBS), pH 7.4 for 25 column volumes (CV). The column was loaded with 2 mL clarified supernatant at a residence time of 0.1 min (with the exception of sample ID 160714 and 180202 where only 1.5 mL was available). After sample

Accepted Article

application the column was washed with PBS for 20 CV. The mAb was eluted with 100 mM glycine pH 3.0 in a 10 CV step gradient elution and the collected fractions were immediately neutralized with 1 M Tris HCl pH 8.0. The column was cleaned with 6 M guanidine HCl, 50 mM Tris, pH 8.0 solution for two CV and immediately re-equilibrated with PBS. The outlet was monitored by measuring UV absorbance at 280 nm to detect eluting antibody. Due to tailing of the elution, only the main portion of the peak was collected, resulting in a loss of approximately 5% in the tail.

Reduction of disulphide bonds

Purified IgG was diluted with PBS to a concentration of 100 µg/mL ($\pm$ 5 µg/mL), as determined by absorbance at 280 nm, using ϵ 1.43²³. Nine volumes of the diluted IgG sample were mixed with one volume of 2-mercaptoethanol (1M in PBS) Sigma (Vienna, Austria) and incubated for 2 hours (h) at 37 °C. To block thiol-groups, one volume of 0.2M iodoacetamide Sigma (Vienna, Austria) was added to the reduced sample (final concentration 0.1 M). Incubation was performed overnight, in the dark and at room temperature¹⁹.

Bio-layer interferometry for analysis of galactose and fucose content on IgG

Octet Red96e (ForteBio, Menlo Park, CA, USA) was used for lectin/carbohydrate binding studies. Typical assay performance is shown in Figure 2a. Samples were diluted in black 96 well plates (Nunc F96 MicroWellTMPlates, ThermoFisher Scientific, Langenselbold, Germany). Puffer (Sample diluent) contained PBS, 0,005% P20, Sigma (Vienna, Austria) and 0.1 mg/mL BSA. The total working volume for each step was 210 µL per well and the rpm setting for each baseline, loading, and association was 1000 rpm. The test was performed at 25 °C. Prior to each assay, streptavidin (SA) biosensor tips (ForteBio, Menlo Park, CA, USA) were pre-wetted in 210 µL sample diluent for at least 10 min followed by equilibration with sample diluent for 60 seconds (s). Afterwards, SA biosensor tips were non-covalently loaded with biotinylated *Ricinus*

communis agglutinin I (RCA I) or biotinylated *Aleuria aurantia* lectin (AAL), both obtained from Vector Labs, UK, in a sample diluent concentration of 0.83 $\mu\text{g/}$ for 120 s, followed by an additional equilibration step (60 s) with sample diluent. Prior to analysis, reduced samples were diluted three times in sample diluent, yielding an IgG concentration of 15 $\mu\text{g/mL}$ ($\pm$ 5 $\mu\text{g/mL}$). Association was carried out for 600 s. All measurements were performed in triplicate. Raw data, obtained with the Octet Software (Version 11.0, Menlo Park, CA, USA) were exported to Excel spreadsheets (Version 2003, Microsoft, Redmond, WA, USA). Raw data of the IgG association response were aligned to the individual association step. The individual specific response R at a defined time t (Rt) for each concentration was calculated as an average of three independent measurements. The reproducibility of (Rt) was determined with n = 8. The responses at 600 s (Rt) of the harvest 13 fed-batch experiments were compared with the outcomes of mass spectrometry measurements, using linear regression analysis. The computations were performed using Mathematica (Version 11.3 of 2018, Wolfram Research Inc., Urbana-Champaign, IL, USA). The statistical analysis was based on 95% significance. The quantitation limit (LOQ) for IgG association was assumed by measuring the minimum concentration at which the analyte can be reliably quantified. A typical signal-to-noise ratio is 10:1. The baseline noise was determined during the initial 60 s PBS buffer step (n = 8).

Monosaccharide analysis performed with mass spectrometry

The samples were digested in gel. The proteins were S-alkylated with iodoacetamide and digested with trypsin (Promega). The digested samples were loaded on a BioBasic C18 column (BioBasic-18, 150 x 0.32 mm, 5 μm , Thermo Scientific) using 65 mM ammonium formate buffer as the aqueous solvent. A gradient from 5% B (B: 100% ACCN) to 32% B in 35 min was applied, followed by a 15 min gradient from 32% B to 75% B that facilitates the elution of large peptides, at a flow rate of 6 $\mu\text{L/min}$. Detection was performed with QTOF MS (Bruker maXis 4G) equipped with the standard ESI source in positive ion, DDA mode (i.e., switching to MSMS

mode for eluting peaks). MS-scans were recorded (range: 150-2200 Da) and the three highest peaks were selected for fragmentation. Instrument calibration was performed using ESI calibration mixture (Agilent). Manual glycopeptide searches were made using DataAnalysis 4.0 (Bruker). For the quantification of the different glycoforms the peak areas of the extracted ion chromatograms (EICs) of the first four isotopic peaks were summed, using the quantification software Quant Analysis (Bruker). Note that MS of glycopetides only allows identifying the composition of the glycan and as a consequence only one possible isomer is annotated.^{20,24}

Results and Discussion

Development of an appropriate bio-layer interferometry method for the determination of galactose and fucose

Selected IgG samples of defined bioprocesses were purified using Protein A affinity chromatography to remove potential interfering host cell proteins. For this approach, in principle also other procedures (e.g., immunoprecipitation, etc.) could be applied as long as the glycosylation of IgGs is not affected. After the purification procedure, IgG was diluted to a concentration of 100 µg/mL determined by absorbance at 280 nm. In a first step lectin binding to non-reduced IgG was tested. As expected, binding of non-reduced IgG to the immobilized lectins could not be achieved (see supplementary information Figure I). These results are in excellent agreement with published data and provide additional evidence that reduction of IgG is a prerequisite to obtain freely accessible carbohydrate structures attached to the Fc part of the IgG.^{18, 19, 25} Consequently, IgG was reduced with β-mercaptoethanol followed by carboxymethylation of cysteines with iodoacetamide to avoid reformation of the disulphide linkages²⁶. For the BLI assay, the lectin and the IgG concentration was adjusted to gain an optimized test performance as described in the materials and methods section. Finally, SA biosensor tips were captured to saturation with biotinylated lectins (data not shown). An

Accepted Article

additional equilibration step with buffer was applied to remove the excess of biotinylated lectins and to obtain a constant loading baseline. The equilibration time was 60 s to achieve a sufficient baseline signal. Moreover, the lectin-coated SA biosensor tips were incubated with the purified, reduced IgG to measure the corresponding association profiles. In preliminary experiments, different IgG concentrations were tested and finally optimized. After reduction the samples contained 0.1 M β -mercaptoethanol and 0.1 M iodoacidamid. During the test optimization it became evident that these concentrations are already too high to accurately associate the IgG to the lectins. A simple threefold dilution step with sample diluent was sufficient to obtain reliable signals. Non-specific interactions of the reduced IgG with the biosensor were eliminated by introducing 0.005% P20 and 0.1 mg/ml BSA to the sample diluent. No unspecific interaction of samples with uncoated SA biosensor tips occurred, which confirms the selectivity of the procedure. Finally, a standardized IgG concentration of 15 μ g/mL was used. Dose-response linearity was demonstrated with a serial dilution experiment (Figure 2b). Therefore, one sample (No 12) was diluted to 15, 7.5, 3.75 and 1.875 μ g/mL in sample diluent, attached to the pre-coated AAL lectin sensor and measured. A linear signal curve resulted in a correlation coefficient of 0.9986. The coefficient of variation for triplicate determinations of each concentration was < 10 %. The assay sequence begins with the equilibration of the SA biosensor tips with the PBS buffer in order to measure the baseline signal for calculation of the LOQ, which is defined as the lowest concentration at which the analyte can not only be reliably detected but at which some predefined goals for bias and imprecision are met. To obtain LOQ datasets for the evaluation of significant/reliable measurements, the average baseline noise of SA biosensor tips in PBS was determined and the LOQ assumed to be a signal-to-noise ratio of 10:1²⁷. The baseline noise of the initial 60 s was 0.004 nm (n = 8). Thus, the LOQ was calculated as 0.04 nm. From the dose-response linearity measurements, as explained above, it was found that the mean R_t of the lowest used IgG concentrations (1.875 μ g/mL) towards the pre-coated RCA120 lectin sensor was 0.068. Prior reduction IgG was diluted to a concentration

of 100 µg/mL. However, dose-response linearity experiments and estimation of LOQ demonstrate that significant lower initial IgG concentration can be applied. For both, galactose and fucose, it could be demonstrated that the lectin-BLI (LBLI) is a promising tool when the accessibility of the protected sugar moieties can be achieved.

Determination of fucosylation of IgG in different mammalian cell culture processes

In a next step the LBLI method was compared to a widely used standard approach for determining glycan structures. Therefore, the selected 13 harvest samples were purified and glycosylation levels were measured via LC-MS and the LBLI method as described above (data is shown in supplementary information Table I). In general, the fucosylated forms of the harvest product were constant within the experimental setup. With both methods a similar fucosylation level (Figure 3a) was determined. The level range was found to be 82.3% $\pm$ 1.2 % with respect to the LC-MS analysis. The new established LBLI method obtained a mean value of R_T 1.11 $\pm$ 0.09 nm. A regression analysis in order to compare both methods was not feasible due to the constant output.

Determination of galactosylation of immunoglobulin G in different mammalian cell culture processes

Significant variations were observed for the galactosylation of the mAb. They ranged from approximately 16.5-33.1% calculated by LC-MS and 0.24-0.70 nm RT for the LBLI analysis (Table 2 & Figure 3b). A linear relationship between the LC-MS and the LBLI responses was observed. Specifically, a function of $r = a + b \cdot p$ with $a = -0.277$ nm (confidence limits: -0.428--0.126) and a significantly positive parameter $b = 0.031$ nm (confidence limits: 0.023-0.038) was obtained. Hence, higher LBLI-responses corresponded to higher proportions of galactosylation (Figure 3b). It follows that with the LBLI tool the galactosylation levels can be successfully measured and data obtained from LC-MS analysis were equal compared to the LBLI results.

The product is continuously secreted into the supernatant. The finally determined glycosylation pattern, represents the accumulated profile from the entire production process. Connecting the overall process performance with the glycosylation can already provide an appropriate picture about certain impacts. However, for a comprehensive understanding of the impact of process and cellular dynamics on glycosylation, straightforward techniques that enable the analysis of samples throughout the process, will be beneficial

Since fucosylation levels stayed constant in all samples of the test case we merely focused on the evaluation of the galactosylation. According to recent studies^{10,28–30} glucose and ammonium can be environmental key factors in context of mAb galactosylation.

Since the IgG is accumulated during the process in the supernatant, the average production (NH_4^+) and consumption (glucose) rate for the entire process should, presupposed that there is an impact, may reflect the overall variance of the glycosylation pattern. The rates $\bar{q}_n$ were calculated according to Eq. 1. However, in the experimental runs different product titres were achieved, which means that different amounts of protein were available and susceptible to posttranslational modification. Accordingly, the rates $\bar{q}_{\text{NH}_4^+}$ and $\bar{q}_{\text{gluc}}$ were set into a relationship with the specific protein production rate $\bar{q}_p$, which was also calculated according to Eq. 1

$$\bar{q}_n = \frac{\sum_{t_0}^{t_{\text{harvest}}} n_{t+1} - n_t + n_{\text{feed}}}{\sum_{t_0}^{t_{\text{harvest}}} \frac{X_{v,t+1} - X_{v,t}}{\mu_t}} \quad (1)$$

n represents the glucose, ammonium or mAb, respectively, X_v the amount of viable cells and μ the growth rate to a given time point. The variable n_{feed} represents the amount of substrate feeded into the system. Hence, for mAb and ammonium this term becomes zero.

The results obtained from different experimental setups indicate that the average specific glucose uptake rate does not correlate with the galactosylation content (Figure 4a). This finding may be attributed to the fact that glucose concentrations were never limiting throughout the processes. Reduced glucose level can negatively influence the galactosylation index due to a reduced availability of uridindiphosphate (UDP) sugars and decrease the galactosylation index^{8,9}. This observation points to the fact that other additional factors might influence the galactosylation.

In this respect, the ratio of mean q_p to q_{NH4+} was calculated (Figure 4b). A linear relationship with the galactosylation levels of the mAb could be determined. The less ammonium per mAb produced, the more galactosylation occurred. A regression analysis performed with all runs resulted in the equation of $y = 0.502x + 0.100$ with a regression coefficient of 0.660. In conclusion our results indicate that the ammonium production impacts the galactosylation profile. This finding is in agreement with the already known fact that high ammonium concentrations can increase the internal pH of the Golgi bodies and subsequently promote the inhibition of the enzymes required for the oligosaccharide processing. Galactosylation and sialylation of mAbs are mainly influenced by this regulation^{3,29}. This result suggests that the production of ammonium per product is a crucial parameter.

In this study we assumed that the rates were constant for an entire process run. Due to this simplification certain dynamics and correlations might be undetectable. For instance, the availability of glucose, due to glucose depletion or a low glucose uptake rate, might have had an influence on the galactosylation level (Figure 4c). It also remains unclear if the ammonium production rate or the ammonium concentration in the supernatant was the real cause for the determined glycosylation pattern (Figure 4d). A clear understanding of the dynamics can only be achieved if the whole process progression is taken into consideration. In principle the presented LBLI method is capable of high throughput analysis. In this respect, the protein

Accepted Article

purification step still remains a bottleneck for this analysis. Therefore, future research will focus on different high throughput (HT) protein purification procedures to reduce the work load and to be capable to identify such process dynamics.

Conclusion

The glycosylation is a relevant key quality attribute for monoclonal antibodies. It can be affected by many different factors, such as the expression system, process conditions, or media composition and feed protocols and thereof vary from batch to batch³. In this study we showed that the developed LBLI method, verified with data resulted from LC-MS, is feasible to determine the Fc fucosylation and galactosylation of an anti-TNF alpha antibody. Samples containing mAbs with varying glycosylation profiles were used to show the method applicability. By both applied analytical techniques it could be shown that fucosylation remained constant within the experimental design, while galactose varied. Based on the monitored glucose and ammonium levels it could deduce that a low ratio of q_p to q_{NH4+} resulted finally in a reduced galactosylation level in harvest samples. To gain more insight into the very complex process dynamics individual, additional in-process measurements and intra-cellular analysis would be necessary.

The application of HTX-BLI-based instruments in the 32-channel mode and 384 well plates enables 32 individual glycoanalytic measurements to be performed in less than 15 min. Antibodies either captured by Protein A or complementary methods prior reduction under standardized conditions, allows the identification of protected glycan structures within the protein in an efficient manner. This is a huge advantage compared to conventional techniques, where determination of glycosylation patterns are still accompanied with a high workload, expenditures costs and usually requires an advantage expertise³¹. For the future, the LBLI method and the in general the BLI platform offer a simple and inexpensive high throughput (HT) technique for the analysis of several important product related parameters such as the product

titer, the antibody antigen binding kinetics and the glycosylation pattern. Although, in research very often different techniques and equipment are used, in R&D and production the use of only one machinery is definitely advantageous to fulfill all the regulatory needs and the future goals of automatization. The presented technique will accelerate cell line, media and process development but also will be important as a process monitoring tool. To transfer the proposed platform to industrial application, automatization of protein capture need to be established to overcome this bottle neck for future analysis. Currently, different HT purification systems are under examination with the aim to complete the proposed platform.

Acknowledgments

We would like to acknowledge the entire PatPlant research group for their effort in conducting those experiments. We also thank the Austrian Research promotion Agency (FFG) and Bilfinger Industrietechnik Salzburg (Competence Headquarters, 849725) for their support. Furthermore, we thank Assoc. Prof. Kühleitner and Assoc. Prof. Brunner (Institute of Mathematics, University of Natural Resources and Life Sciences) for helpful discussions. The BLI equipment was kindly provided by the EQ-BOKU VIBT GmbH and the BOKU Core Facility for Biomolecular and Cellular Analysis.

References

1. Xu X, Nagarajan H, Lewis NE, et al. The genomic sequence of the Chinese hamster ovary (CHO)-K1 cell line. *Nat Biotechnol.* 2011;29(8):735-741. doi:10.1038/nbt.1932
2. Sommeregger W, Sissolak B, Kandra K, von Stosch M, Mayer M, Striedner G. Quality by control: Towards model predictive control of mammalian cell culture bioprocesses. *Biotechnol J.* 2017;1600546. doi:10.1002/biot.201600546
3. Batra J, Rathore AS. Glycosylation of monoclonal antibody products: Current status and future prospects. *Biotechnol Prog.* 2016;32(5):1091-1102. doi:10.1002/btpr.2366

- Accepted Article
4. Raju TS. Terminal sugars of Fc glycans influence antibody effector functions of IgGs. *Curr Opin Immunol.* 2008;20:471-478. doi:10.1016/j.coi.2008.06.007
 5. Jefferis R. Glycosylation as a strategy to improve antibody-based therapeutics. *Nat Rev Drug Discov.* 2009;8:226. <https://doi.org/10.1038/nrd2804>.
 6. Hossler P, Khattak SF, Li ZJ. Optimal and consistent protein glycosylation in mammalian cell culture. *Glycobiology.* 2009;19(9):936-949. doi:10.1093/glycob/cwp079
 7. Schmelzer AE, Miller WM. Hyperosmotic stress and elevated pCO₂ alter monoclonal antibody charge distribution and monosaccharide content. *Biotechnol Prog.* 2002;18(2):346-353. doi:10.1021/bp010187d
 8. Liu B, Spearman M, Doering J, Lattová E, Perreault H, Butler M. The availability of glucose to CHO cells affects the intracellular lipid-linked oligosaccharide distribution, site occupancy and the N-glycosylation profile of a monoclonal antibody. *J Biotechnol.* 2014;170:17-27. doi:10.1016/j.jbiotec.2013.11.007
 9. Villacrés C, Tayi VS, Lattová E, Perreault H, Butler M. Low glucose depletes glycan precursors, reduces site occupancy and galactosylation of a monoclonal antibody in CHO cell culture. *Biotechnol J.* 2015;10(7):1051-1066. doi:10.1002/biot.201400662
 10. Chen P, Harcum SW. Effects of elevated ammonium on glycosylation gene expression in CHO cells. *Metab Eng.* 2006;8(2):123-132. doi:10.1016/j.ymben.2005.10.002
 11. Brunner M, Fricke J, Kroll P, Herwig C. Investigation of the interactions of critical scale-up parameters (pH, pO₂ and pCO₂) on CHO batch performance and critical quality attributes. *Bioprocess Biosyst Eng.* 2017;40(2):251-263. doi:10.1007/s00449-016-1693-7
 12. Mariño K, Bones J, Kattla JJ, Rudd PM. A systematic approach to protein glycosylation analysis: a path through the maze. *Nat Chem Biol.* 2010;6(10):713-723. doi:10.1038/nchembio.437

- Accepted Article
13. Beyer B, Schuster M, Jungbauer A, Lingg N. Microheterogeneity of Recombinant Antibodies: Analytics and Functional Impact. *Biotechnol J*. 2018;13(1):1-26. doi:10.1002/biot.201700476
 14. Zhang L, Luo S, Zhang B. The use of lectin microarray for assessing glycosylation of therapeutic proteins. *MAbs*. 2016;8(3):524-535. doi:10.1080/19420862.2016.1149662
 15. Wallner J, Lhota G, Jeschek D, Mader A, Vorauer-uhl K. Journal of Pharmaceutical and Biomedical Analysis Application of Bio-Layer Interferometry for the analysis of protein / liposome interactions. *J Pharm Biomed Anal*. 2013;72:150-154. doi:10.1016/j.jpba.2012.10.008
 16. Wallner J, Lhota G, Schosserer M, Vorauer-Uhl K. An approach for liposome immobilization using sterically stabilized micelles (SSMs) as a precursor for bio-layer interferometry-based interaction studies. *Colloids Surfaces B Biointerfaces*. 2017;154. doi:10.1016/j.colsurfb.2017.03.015
 17. Jonnalagadda KN, Markely LRA, Guan B, Alves C, Prajapati S. Analytical Methods High-throughput sialylation measurement using lectins on an Octet platform for clone screening. *Anal Methods*. 2016;8(18):7193-7198. doi:10.1039/C6AY01703G
 18. Dalziel M, McFarlane I, Axford JS. Lectin analysis of human immunoglobulin G N-glycan sialylation. *Glycoconj J*. 1999;16(12):801-807.
 19. Liljeblad M, Lundblad A, Pålsson P. Analysis of agalacto-IgG in rheumatoid arthritis using surface plasmon resonance. *Glycoconj J*. 2000;17(5):323-329.
 20. Gruber C, Altmann F. Site-Specific Glycosylation Profiling Using Liquid Chromatography-Tandem Mass Spectrometry (LC-MS). In: Castilho A, ed. *Glyco-Engineering*. Humana Press, New York, NY; 2015:407-415. doi:https://doi.org/10.1007/978-1-4939-2760-9_27
 21. Zboray K, Sommeregger W, Bogner E, et al. Heterologous protein production using

- euchromatin-containing expression vectors in mammalian cells. *Nucleic Acids Res.* 2015;43(16):e102. doi:10.1093/nar/gkv475
22. Reinhart D, Damjanovic L, Kaisermayer C, Kunert R. Benchmarking of commercially available CHO cell culture media for antibody production. *Appl Microbiol Biotechnol.* 2015;99(11):4645-4657. doi:10.1007/s00253-015-6514-4
23. Tan K. Current protocols in immunology. John E. Coligan, Ada M. Kruisbeek David H. Margulies, Ethan M. Shevach and Warren Strober (eds). John Wiley and Sons: New York US\$240 (loose-leaf binder) (1991). *Cell Biochem Funct.* 1991;9(4):295-295. doi:10.1002/cbf.290090416
24. Loos A, Gruber C, Altmann F, Mehofer U, Hensel F, Grandits M. Expression and glycoengineering of functionally active heteromultimeric IgM in plants. 2014;21(7). doi:10.1073/pnas.1320544111
25. Sumar N, Bodman KB, Rademacher TW, et al. Analysis of glycosylation changes in IgG using lectins. *J Immunol Methods.* 1990;131(1):127-136.
26. Aitken A, Learmonth M. Carboxymethylation of Cysteine Using Iodoacetamide/Iodoacetic Acid. In: *Protein Protocols Handbook, The.* New Jersey: Humana Press; 2002:455-456. doi:10.1385/1-59259-169-8:455
27. Armbruster DA, Pry T. Limit of blank, limit of detection and limit of quantitation. *Clin Biochem Rev.* 2008;29 Suppl 1(Suppl 1):S49-52.
28. Hong JK, Cho SM, Yoon SK. Substitution of glutamine by glutamate enhances production and galactosylation of recombinant IgG in Chinese hamster ovary cells. *Appl Microbiol Biotechnol.* 2010;88(4):869-876. doi:10.1007/s00253-010-2790-1
29. Aghamohseni H, Ohadi K, Spearman M, et al. Effects of nutrient levels and average culture pH on the glycosylation pattern of camelid-humanized monoclonal antibody. *J*

30. Allen M, Millett P, Dawes E, Rushton N. Lactate Dehydrogenase Activity as a Rapid and Sensitive Test for the Quantification of Cell Numbers in vitro. *Clin Mater.* 1994;16(4):189-194. doi:10.1016/0267-6605(94)90116-3
31. Huffman JE, Pučić-Baković M, Klarić L, et al. Comparative Performance of Four Methods for High-throughput Glycosylation Analysis of Immunoglobulin G in Genetic and Epidemiological Research. *Mol Cell Proteomics.* 2014;13(6):1598-1610. doi:10.1074/mcp.m113.037465

Figure and table legends

Figure 1: IgG antibody and N-glycan structures. Schematic representation of a glycoprotein IgG (mAbs contains only Fc glycosylation). The disulfide bonds stabilizing the tertiary and quaternary protein structure are also shown.

Figure2: (a) Sensorgram of a typical test performance, including baseline steps (A, C), lectin loading (B) and association of reduced immunoglobulin G (D). (b) Dose-response curve of IgG by serial dilution of one reduced sample (Run 12), diluted to 15, 7.5, 3.75 and 1.875 $\mu\text{g/mL}$ in sample diluent. RCA120 lectin was immobilized on Streptavidin sensor tips prior IgG association. The linear signal curve resulted in an equation of $y = 0.0381x - 0.0084$ and in a correlation coefficient of 0.9986

Figure 3: (a) The proportion of fucosylated glycoforms determined via LC-MS and the responses of the lectin based BLI (LBLI) assay in nm depicted for all runs. (b) Linear regression analysis of all 13 CHO cell culture fed batch runs performed in 15 L pilot scale. The responses in [nm] of LBLI assay were compared with obtained galactosylated glycoforms in [%] of LC-MS measurements. Dots and error bars represent the mean Rt values and standard deviation of LBLI triplicate measurements, and the corresponding results from a single LC-MS run (without standard deviation). The regression line and its single confidence band (dashed line) are plotted. The linear regression line resulted in the equation of $y = 0.0309x - 0.277$ and a correlation coefficient of 0.8925.

Figure 4: The impact of the ratios q_P to q_{gluc} (a) and to q_{NH4^+} (b) on the LBLI response or respectively the galactosylation proportion of the mAb. The regression lines and coefficients are

depicted. (b) Regression line depicted was performed without the outlier. The arrow indicates the outlier. The confidence band (dashed line) are plotted. Example trends of process run 12 for (c) glucose concentration and the respective specific rate and (d) ammonium concentration and the respective specific rate.

Table 1: Experimental design of the fed-batch processes. Experiments are presented by the time point (hours [h]), type of shift, temperature or feed change. F1, F2, and F3 represent the additional glucose concentration in the feed, consisting of 10, 20 and 30 g/L, respectively.

Run number	Shift 1	Shift 2	Shift 3	Shift 4
1	36.3 °C/F3 (72 h)			
2	36.3 °C/F3 (72 h)			
3	37 °C/F3 (72 h)	37 °C/F1 (192 h)		
4	34 °C/F1 (72 h)			
5	34 °C/F2 (72 h)			
6	34 °C/F2 (72 h)			
7	34 °C/F2 (72 h)			
8	34 °C/F2 (72 h)			
9	34 °C/F2 (72 h)	37 °C/F2 (120 h)	34 °C/F1 (192 h)	31 °C/F1 (240 h)
10	31 °C/F2 (72 h)	34 °C/F2 (120 h)	37 °C/F3 (192 h)	34 °C/F3 (240 h)
11	34 °C/F1 (72 h)	31 °C/F1 (120 h)	31 °C/F2 (192 h)	34 °C/F2 (240 h)
12	37 °C/F2 (72 h)	34 °C/F3 (120 h)	31 °C/F2 (192 h)	34 °C/F1 (240 h)
13	34 °C/F3 (72 h)	37 °C/F2 (120 h)	31 °C/F2 (192 h)	37 °C/F3 (240 h)