Smart ways of modulating and tracking core fucosylation of antibodies in cell-free supernatant samples

Stella Damoser and Anna Johann, PAIA Biotech GmbH, Köln/Germany
Andrea Franke, Daniel Rehm and Susanne Seitz, ProBioGen AG, Berlin/Germany

Abstract

Fucose is a monosaccharide and naturally present in glycoproteins like antibodies. In therapeutic monoclonal antibodies (mAbs) fucosylation is exclusively found as core fucosylation in *N*-glycans on the Fc domain. The absence of core fucosylation has shown significant potential for increasing the antibody-dependent cellular cytotoxicity (ADCC) and may therefore increase the clinical efficacy of antibodies.

Here, we describe how a GlymaxX® producer cell line (ProBioGen AG) generates IgG1 with varying degrees of core fucosylation and how these levels can be easily assessed with a high throughput lectin-based glycan screening assay (PA-201, PAIA Biotech GmbH). This assay can analyze cell-free supernatants directly and measures up to 96 samples in less than two hours total handling time.

We also demonstrate that the results of the supernatant samples correlate very well with standard hydrophobic interaction liquid chromatography (HILIC) after Protein A purification. The lectin-based glycan screening assay represents a much faster and less labor-intensive alternative for the determination of fucosylation levels in monoclonal antibodies.

Introduction

Antibodies lacking core fucosylation show a significantly enhanced FcγRIIIa receptor binding[1], which can lead to up to 100x higher ADCC activity against tumor cells or virus-infected cells. If the clinical efficacy of an antibody is mediated by its ADCC effector function, afucosylated *N*-glycans could help to reduce dose requirement and save manufacturing costs.

ProBioGen´s GlymaxX® technology is an elegant way of glycoengineering. It allows not only the production of afucosylated antibodies, but, by addition of fucose to the cell culture medium, also the precise control of fucosylation levels. This can be useful to fine-tune the effects on receptor binding and ADCC effector function.

The GlymaxX® technology is based on the stable introduction of a gene encoding an enzyme which deflects the cellular pathway of fucose biosynthesis[2].

PAIA Biotech has developed a variety of high-throughput assay products using its patented method for streamlining bead-based assay in microplates[3]. The PA-201 assay is using fluorescent lectins to detect glycosylation on intact antibodies that have been captured by specific beads. A broad portfolio of lectins is available for detecting the different types of *N*- and *O*-glycans.

The assay is intended for the rapid screening of critical glycan features on many samples in cell line and upstream development. Currently available analysis methods require sample purification and preparation (e.g. enzymatic glycan cleavage) and cannot cope with high sample numbers in these early development steps.

The lectin in the PA-201 assay used in this study is capable of detecting fucosylation in cell-free supernatant samples and is not disturbed by the presence of free fucose in the supernatant.

Results

A Trastuzumab expressing GlymaxX® cell line was used to generate samples with different core fucose contents. This was achieved by adding fucose (to final concentrations of 0-1.2 mM) into the cell culture media. After 14 days, cell-free supernatant samples from these cultures were taken and analyzed with the PAIA glycan assay PA-201.

The PAIA assay was performed twice in independent experiments. Each Trastuzumab sample was measured in technical triplicates and lectin binding was calculated. Means and standard deviations (sd) of the two experiments are depicted in **Figure 1.** Increasing amounts of fucose in the culture yielded higher lectin binding rates, which indicate an increased presence of core fucose in the product. Addition of more than 0.7 mM fucose did not lead to a further increase in lectin binding.

Comparison of the lectin binding rates obtained by the two independent experiments show little variability. This demonstrates the repeatability and robustness of the assay despite the presence of cell culture matrix, including fucose.

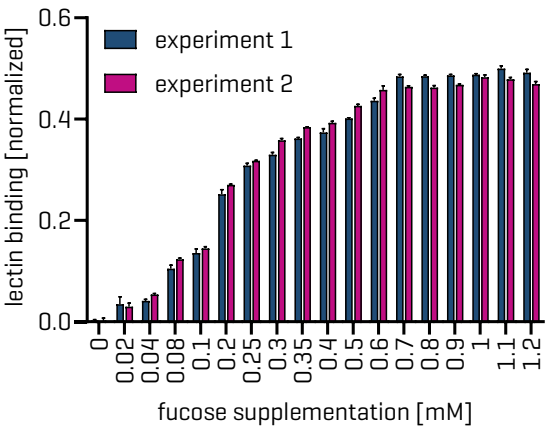

Fig. 1.: Increased fucose concentrations in the cell culture lead to an increase of core fucosylation of Trastuzumab.
Lectin binding of supernatant samples, supplemented with increasing amounts of fucose. Two independent experiments with the PA-201 glycan assay show high reproducibility of the assay with typical CVs of the triplicates of 2%.

To validate the utility of the PAIA glycan assay for measuring core fucose in supernatant samples, the same samples were purified and relative core fucose levels were determined by HILIC/UHPLC.

This data confirmed the results of the PAIA glycan assay: increasing amounts of fucose supplemented to the media lead to an increase of core fucosylation of Trastuzumab (**Figure 2**).

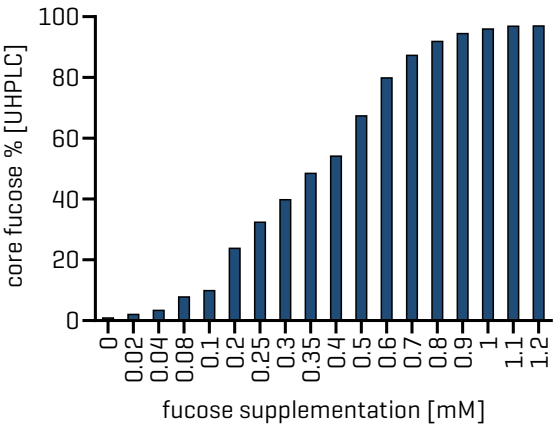

Fig. 2.: Increased supplementation of fucose leads to an increase of core fucosylation of Trastuzumab.
Relative core fucose content of purified samples were determined by HILIC/UHPLC and plotted against the concentration of fucose added to the cultivation media.

The correlation between lectin binding in cell-free supernatant samples and UHPLC data obtained with purified material demonstrates that the PAIA glycan assay can reliably distinguish samples with core fucosylation contents between 0 and 87% (**Figure 3**, dotted line marks sample with 87% core fucose).

For Trastuzumab, the assay shows the highest resolution power for samples with fucosylation levels below 50%. The coefficient of determination (R^2) was calculated and yielded an excellent value of 0.91.

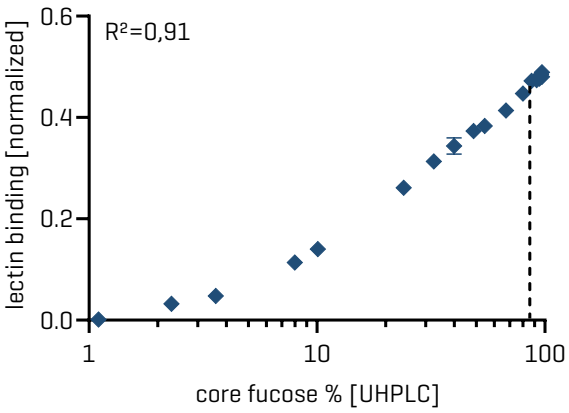

Fig. 3. : Correlation of lectin binding and % of core fucose (UHPLC). Lectin binding from cell-free supernatant samples with PAIA glycan assay correlates very well with UHPLC data from purified samples (R^2=0.91). Lectin binding data: mean and sd from two independent experiments, technical triplicates per experiment.

Conclusion

We used the GlymaxX® technology to produce cell-free supernatant samples containing Trastuzumab that differ in their *N*-glycan core fucose content. These samples were subjected to analysis with the PAIA glycan assay. The resulting lectin bindings were compared to the data obtained by the reference method (HILIC/UHPLC) and an R^2 of 0.91 was calculated.

These results demonstrate that the glycan screening assay (PA-201) represents a fast and accurate method to assess the degree of fucosylation of the IgG1 Trastuzumab.

The data was generated with cell-free supernatant samples and show that the assay generates robust results without the need of sample purification.

The PA-201 assay is therefore an ideal tool to screen fucosylation levels of many supernatant samples and can be a useful tool in the different stages of cell line and upstream development.

Materials and methods

The GlymaxX® technology can be used for glycoengineering of glycoproteins and antibodies. The technology relies on the heterologous, stable, cytosolic expression of the bacterial enzyme GDP-4keto-6-deoxy-D-mannose reductase (RMD) in a cell that is intended to be used for production of a glycoprotein or an antibody by subsequent (or simultaneous) transduction of respective genes of the glycoprotein/antibody.

RMD redirects the de-novo fucose synthesis pathway towards a sugar-nucleotide (GDP-Rhamnose) that cannot be metabolized by the cell and also acts as an inhibitor of the pathway. In a GlymaxX® producer cell, the fucose-content in the glycan of a glycoprotein is stably reduced to a level where 98-99% of the glycans are depleted of fucose if fucose is not provided via the culture medium.

However, the fucose content can be adjusted to a certain level by supplementing the culture medium with fucose. Thus, a single cell line is sufficient to produce both completely fucosylated or afucosylated antibodies and those with a defined intermediate fucosylation level.

Cell culture: ProBioGen's CHO.RiGHT® Cell Line Development Platform was used to generate stable IgG1 expressing GlymaxX® cell lines. Experiments were performed using ProBioGen's chemically defined CD-C6 culture medium with chemically defined feeds, glucose, and fucose ranging from 0-1.2 mM.

Cells were seeded at $0.4x10^6$ viable cells/mL in TubeSpin® Bioreactor 50 (TPP Techno Plastic Products AG) and incubated at 180 rpm, 37°C and 8% CO_2 in a Multitron incubation shaker (Infors HT). The 14-day fed-batch included a temperature shift to 34°C.

Analysis of samples included determinations of cell viability, cell density, selected metabolites, and product titer.

Purification of material and determination of fucosylation by HILIC: Purification of harvest supernatants was performed by using Protein A HP SpinTrap™ columns (Cytiva). In a next step, samples were desalted and further processed using the "Gly-XTM N-Glycan Rapid Release and Labelling with instantPC"-Kit (Prozyme) according to the manufacturer's specifications.

Subsequently, labeled glycans were analyzed by HILIC by applying a Shimadzu Nexera X2 UHPLC. Chromatography was carried out in an AdvanceBio Glycan Mapping column (2.7 µm, 2.1 x 150 mm, Agilent). The column temperature was set to 60°C. Mobile phase A consisted of 100 mM ammonium formate buffered to pH 4.4. Mobile phase B consisted of acetonitrile. Gradient elution was used at a flow rate 0.6 mL/min. Labeled glycans were detected by fluorescent detection (excitation at 285 nm, emission at 345 nm).

PAIA Technology: The PA-201 glycan screening assay format is a sandwich immunoassay, in which capture beads bind the Fc domain of the analyte and fluorescence labeled lectins bind the glycans present on the antibodies. The whole assay is performed in the special 384-well PAIA*plate* which allows to separate the beads by simple sedimentation and measure the plate afterwards. This measurement determines the amount of unbound lectin in a well.

Lectin binding [normalized] is calculated using a negative control without analyte:

$$1 - \left[\frac{\text{fluorescence signal of sample}}{\text{fluorescence signal of negative control}} \right]$$

Thus, high lectin binding represents high levels of fucosylation present in analyte.

PAIA assay Workflow: In this study a core fucose-specific lectin was labeled with a fluorophore and used as a marker molecule. The fluorophore excits and emits in the red wavelength range (640nm/665nm), which is most robust against spectral interference from cell culture components.

The PAIA glycan assay for Fc containing proteins (Art.-No. PA-201) was performed according to the manufacturer´s protocol.

In brief, supernatant samples were diluted in a 1:10 ratio to final concentrations between 0.44 and 0.47 mg/mL using Tris-buffered saline with Tween20. The diluted samples were mixed with PAIA sample preparation buffer in a 1:1 ratio and shaken on a Thermomixer comfort (Eppendorf) for 10 min, 700 rpm at room temperature. 50 µL of lectin reagent were added to each well of the 384-well PAIA*plate* and 10 µL of the treated sample were added, **Figure 4 (A)**. The PAIA*plate* was shaken for 45 minutes on a BioShake HP orbital shaker (Q Instruments) at 1800 rpm **(B)**. After sedimentation of the beads **(C)** the plate was measured on a fluorescence microscope (Synentec) **(D)**.

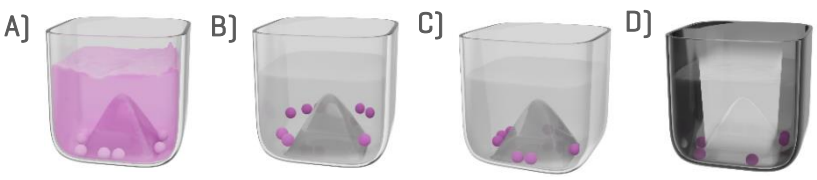

Fig. 4 : Depiction of the workflow of PAIA assays. A) Addition of reagent and sample to the well. B) Mixing of components to allow formation of bead-analyte-marker complex. C) Sedimentation of beads with bound analyte and marker. D) Fluorescent read-out of unbound marker.

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