TOSOH

APPLICATION NOTE

ANALYSIS OF ANTIBODY DEPENDENT CELLULAR CYTOTOXICITY (ADCC) ACTIVITY AND UNDERLYING GLYCAN PATTERN

Antibody dependent cellular cytotoxicity (ADCC) refers to the ability of an IgG antibody to induce death of infected or tumor cells (target cells). The Fab fragments of an antibody bind target cells, while the Fc-region binds to an Fc-receptor (FcR) on natural killer (NK) cells. One of the receptors is particularly implicated in target cell death: FcγRIIIa on NK cells binds the antibody leading to the release of enzymes and proteins responsible for target cell lysis. The N-glycosylation of the Fc-region influences the interaction with FcγRIIIa and, thus, ADCC activity. For instance, terminal galactoses increase the affinity to FcγRIIIa. Hence, the ability of an Fc-region to associate with the receptor as well its glycan structure responsible for affinity are critical quality attributes (CQAs) as they correlate with drug efficacy[1]. Rapid analyzing methods facilitate the screening of cell lines regarding the ADCC activity of antibody products and batch analyses.

A fast solution for analyzing the affinity of antibodies to FcγRIIIa is the affinity chromatography column TSKgel® FcR-IIIA-NPR which uses the receptor as ligand. The glyXera high performance glycoprofiling technology: glyXboxCE™ (glyXera GmbH, Germany), based on multiplexed capillary gel electrophoresis with laser induced fluorescence detection (xCGE-LIF), analyzes N-glycans in more detail[2]. Combining both methods allows for correlating glycan pattern to FcR affinity and ADCC activity. The methods were applied to the analysis of two therapeutic antibodies: rituximab used to treat autoimmune diseases and leukemia as well as adalimumab, a treatment against autoimmune diseases such as rheumatoid arthritis and Crohn's disease.

FcR AFFINITY CHROMATOGRAPHY

The ligand of the TSKgel FcR-IIIA-NPR column is a recombinant version of the FcγRIIIa, the receptor on natural killer cells implicated in ADCC. The column separates antibodies regarding their affinity to the receptor. Typically, three affinities are separated: low affinity, mid affinity and high affinity. Recently, ADCC activity of the fractions was tested in cell-based assays and revealed that ADCC increases along with the affinity of Fc-region to FcγRIIIa[3].

Both monoclonal antibodies, rituximab and adalimumab display low, mid and high-affinity fractions (*Figure 1*).

FcR AFFINITY CHROMATOGRAPHY ANALYSIS OF RITUXIMAB AND ADALIMUMAB

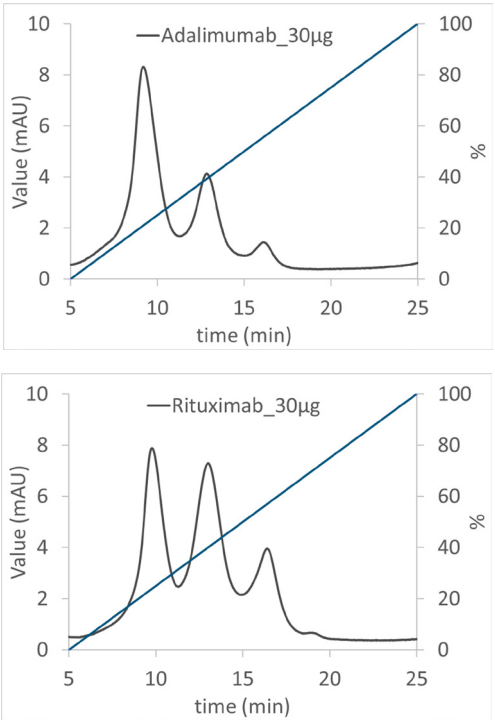


Figure 1

Column: TSKgel FcR-IIIA-NPR (P/N 002351)
Sample: Rituximab (6 g/l), Adalimumab (5 g/l)
Buffer A: 50 mM Na citrate, 150mM NaCl pH 6.5
Buffer B: 50 mM Na citrate, 150mM NaCl pH 4.5
Gradient: 0-5 min: 0%, 5-25min: 0-100%
Flow rate: 0.75 ml/min

GLYCAN ANALYSIS WITH GLYXERA TECHNOLOGY

For both antibodies, fractions of low, mid and high affinity were collected and their N-glycans analyzed using a method developed by glyXera. The fast method allows for linking the glycan pattern to FcγRIIIa affinity.

The Standard Glycoprofiling analysis module of glyXboxCE was employed for glycan analysis. The Standard Glycoprofiling by glyXera consists of N-glycan release, N-glycan labeling, post-labeling clean-up, xCGE-LIF measurement and data analysis (see *Figure 2*).

WORKFLOW OF GLYCAN ANALYSIS WITH GLYXBOXCE™

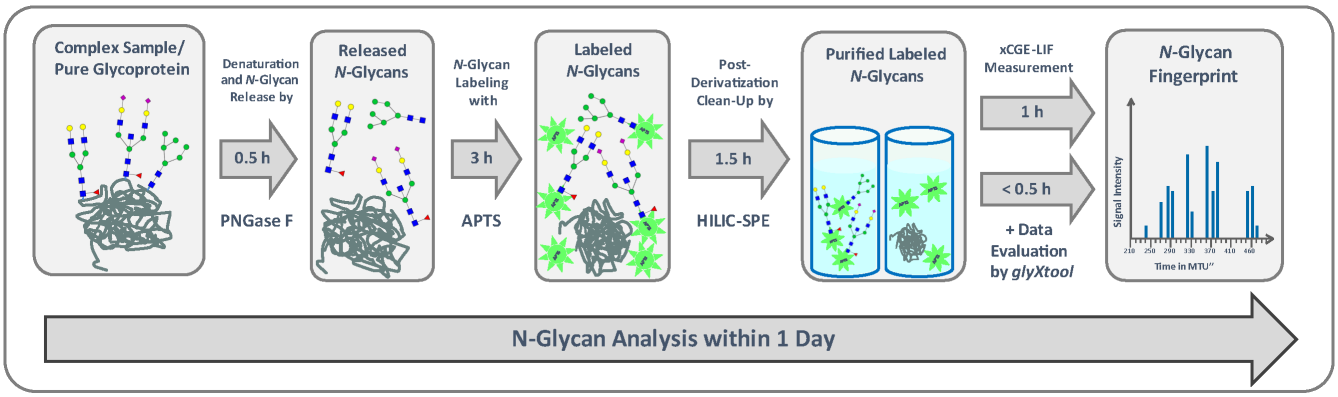


Figure 2

Data processing, and evaluation for Standard Glycoprofiling were performed with glyXtoolCE™ software (glyXera GmbH, Germany). The Limit of Quantification (LOQ) was determined using the signal-to-noise ratio of each N-glycan analysis. All peaks above LOQ were included in the calculation of the Total Peak Height (TPH). By performing migration time alignment (using glyXera's proprietary orthogonal double migration time alignment technology) and normalizing the signal intensity to the TPH, the characteristic glycan composition pattern (Glycofingerprint) of each sample was obtained. These Glycofingerprints were further refined into Glycoprofiles by annotating the peaks with N-glycan structures retrieved from the integrated glycan database of glyXtoolCE via migration time matching.

With glyXtoolCE, hundreds of samples can be processed, evaluated and compared in parallel[4]. By merging and matching individual peak lists into a single, unified Glycoprofile (Unity-Profile Comparison (UPC) function), glyXtoolCE facilitates comparison of samples in a straightforward and intuitive manner.

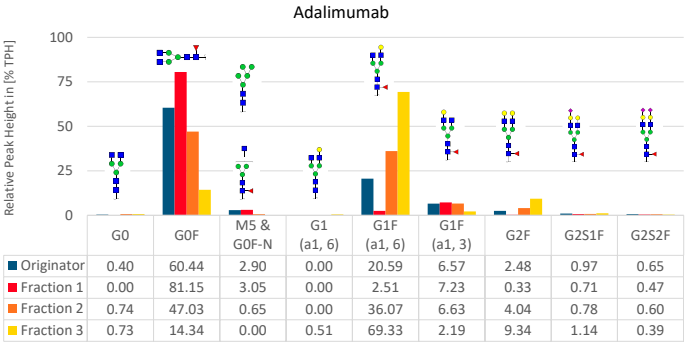
GLYCAN ANALYSIS OF FcR AFFINITY FRACTIONS

The glycan profile of the collected fractions was analyzed by glyXera technology. *Figure 3* shows the glycan pattern of the unfractionated mAbs and each FcR affinity fraction of the individual peaks, elucidated by Standard Glycoprofiling with glyXboxCE. The antibody glycoforms collected in peak 3 (highest affinity) show mainly galactose containing N-glycans (G1F and G2F). Peak 2 glycoforms contain more G0F glycans than Peak 3 and glycoforms collected in Peak 1 (lowest affinity) show predominantly fucosylated glycans without galactose units (G0F). The glyXera glycosylation pattern analysis of the FcR affinity fractions matches the common understanding that terminal galactose units of Fc-glycans typically enhance affinity to FcγRIIIa and ADCC activity.

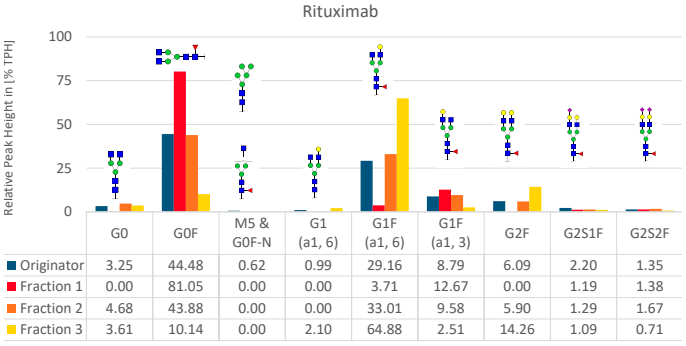
CONCLUSION

Two methods were combined to relate antibody N-glycosylation with its affinity to FcγRIIIa, known to indicate ADCC activity of an antibody. FcR affinity of a sample is analyzed in only 20 minutes on the TSKgel FcR-IIIA-NPR column, a detailed analysis of the underlying glycan

GLYXERA ANALYSIS OF THE GLYCAN STRUCTURES OF THE UNFRACTIONATED RITUXIMAB AND ADALIMUMAB AND THE THREE FcR AFFINITY FRACTIONS



	G0	G0F	M5 & G0F-N	G1 (a1, 6)	G1F (a1, 6)	G1F (a1, 3)	G2F	G2S1F	G2S2F
Originator	0.40	60.44	2.90	0.00	20.59	6.57	2.48	0.97	0.65
Fraction 1	0.00	81.15	3.05	0.00	2.51	7.23	0.33	0.71	0.47
Fraction 2	0.74	47.03	0.65	0.00	36.07	6.63	4.04	0.78	0.60
Fraction 3	0.73	14.34	0.00	0.51	69.33	2.19	9.34	1.14	0.39



	G0	G0F	M5 & G0F-N	G1 (a1, 6)	G1F (a1, 6)	G1F (a1, 3)	G2F	G2S1F	G2S2F
Originator	3.25	44.48	0.62	0.99	29.16	8.79	6.09	2.20	1.35
Fraction 1	0.00	81.05	0.00	0.00	3.71	12.67	0.00	1.19	1.38
Fraction 2	4.68	43.88	0.00	0.00	33.01	9.58	5.90	1.29	1.67
Fraction 3	3.61	10.14	0.00	2.10	64.88	2.51	14.26	1.09	0.71

Figure 3

pattern of a whole series of samples takes effectively only a few minutes per sample, due to parallelization and automation with glyXera technology. Thus, the combination of fast affinity chromatography and high-throughput glycosylation pattern analysis is a rapid and screening-compatible approach to compare antibody batches or production cell lines.

REFERENCES:

1) D. Reusch and M. L. Tejada, Glycobiology 2015, 25 (12): 1325-1324; doi: 10.1093/glycob/cwv065
2) S. Cajic et al. (2021) Capillary (gel) electrophoresis-based methods for immunoglobulin (G) glycosylation analysis. In: Pezer M. (ed) Antibody Glycosylation. Experientia Supplementum, vol 112 in prep. Springer, Cham.
3) Correlation of FcR affinity chromatography with glycan pattern and ADCC activity of a therapeutic antibody
4) Hennig R et al. (2016), Biochimica et Biophysica Acta - General Subjects. 1860, 1728-1738.