TOSOH

APPLICATION NOTE

CORRELATION OF FcR AFFINITY CHROMATOGRAPHY WITH GLYCAN PATTERN AND ADCC ACTIVITY OF A THERAPEUTIC ANTIBODY

Antibody-dependent cell-mediated cytotoxicity (ADCC) is an important mechanism of action (MoA) of monoclonal antibodies used in cancer treatment. Selecting suitable cell lines and optimizing culture conditions towards expression of antibody candidates with desired ADCC activity is an essential part of the R&D process. A fast and straight forward approach to easily access ADCC activity would facilitate screening of a large number of clones or monitoring the effect of upstream process variations. Other stages of R&D and production could benefit from fast ADCC assessment as well: comparing biosimilar and originator, detecting lot-to-lot variations, monitoring product stability, to name but a few.

Fc RECEPTOR AND ADCC ACTIVITY

ADCC starts with the binding of the Fab region of an antibody to a target cell, e.g. a cancer cell. Binding of the Fc domain of that antibody to Fcγ receptors on the outer membrane of natural killer (NK) cells triggers degranulation into a lytic synapse and finally the apoptosis of the cancer cell (Figure 1). The glycan micro-heterogeneity of the Fc domain, in particular on the galactose and core-fucose levels[1], influences binding of the Fc domain to Fcγ receptors.

Current ADCC activity tests are either cell based bioassays or surface plasmon resonance (SPR) measurements using immobilized Fcγ receptor. A new approach combines the specificity of the FcγIIIa receptor (FcγRIIIa) with the easy handling of an HPLC method.

For Fc receptor affinity chromatography, a recombinant FcγIIIa receptor ligand is immobilized on a stationary phase.

ANTIBODY-DEPENDENT CELL-MEDIATED CYTOTOXICITY

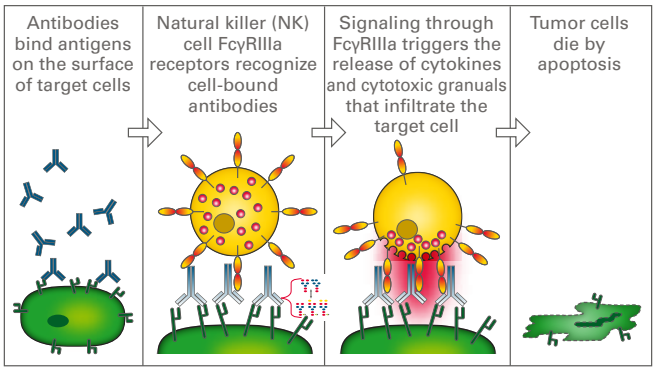

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Figure 1

Glycoforms of an antibody sample can be partly separated based on the strength of their binding to the FcR ligand. Resulting peaks can be assigned to low, medium, and high ADCC activity (Figure 2).

FCγR -AFFINITY CHROMATOGRAPHY

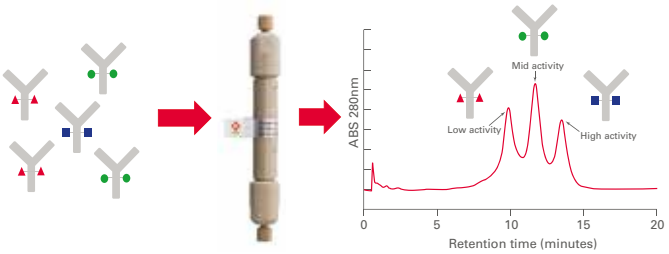

Figure 2

Taking the well-known therapeutic antibody rituximab as an example, this application note demonstrates that the pattern of FcγRIIIa affinity chromatography shows a good correlation with the results obtained by ADCC reporter assay. Fractions collected from HPLC peaks with different receptor affinity also show different glycosylation patterns at the Fc domain.

Rituximab (Figure 3) is a recombinant chimeric human/mouse monoclonal IgG1 antibody, approved in 1997 and used to treat certain autoimmune diseases and types of cancer. Besides other effects of rituximab, its Fc portion mediates antibody-dependent cellular cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC)[2]. N-glycans bound to the Fc domain of Rituximab contain mainly G0F and G1F structures.

RITUXIMAB

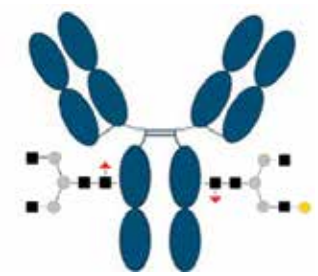

Figure 3

FcR AFFINITY CHROMATOGRAPHY OF RITUXIMAB

In FcγRIIIa affinity chromatography, purified antibody or cell culture supernatant is injected under conditions that promote binding of mAbs to the FcγRIIIa ligand. Elution of bound mAb variants is performed by lowering the pH of the mobile phase in order to disrupt the target/ligand interactions. The higher the affinity of a mAb variant to the receptor, the higher the retention time of the respective peak.

FcR affinity chromatography analysis of rituximab on the new TSKgel FcR-IIIa-NPR column results in three peaks representing variants with low, medium, and high FcγRIIIa affinity (Figure 4a). For subsequent characterization of the three peaks a semi-preparative prototype FcR-IIIA column was used to collect fractions.

Figure 4b shows that the separation pattern on the semi-preparative column is identical to that of the analytical column, although resolution is worse. Fractions out of the three peaks were collected. For each peak, cleaved and 2AB-labeled N-glycans were characterized by HILIC-UHPLC and ADCC activity was analyzed with a standard ADCC reporter bioassay kit (Promega).

FCγR AFFINITY ANALYSIS OF RITUXIMAB ON TSKgel FCR-IIIA-NPR (A) AND A PROTOTYPE SEMI-PREPARATIVE COLUMN (B)[3]

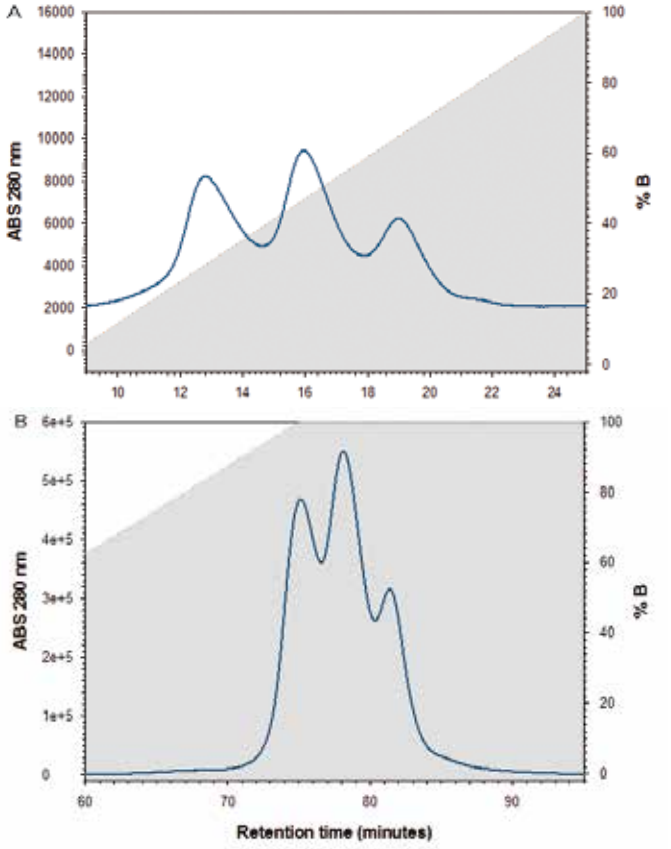

Figure 4

ADCC BIOASSAY OF RITUXIMAB AND FcR AFFINITY FRACTIONS

ADCC activities of the original rituximab sample and the three fractions collected from semi-preparative FcR affinity chromatography were determined with the ADCC reporter bioassays (Promega). The Fc effector reporter bioassay uses the FcγR and NFAT-mediated activation of luciferase activity in effector cells to determine ADCC efficacy and potency of antibodies. Figure 5 shows the ADCC reporter bioassay response to rituximab and to the three fractions collected from FcR affinity chromatography (low, medium, high FcγR affinity).

ADCC REPORTER BIOASSAY RESPONSE TO RITUXIMAB

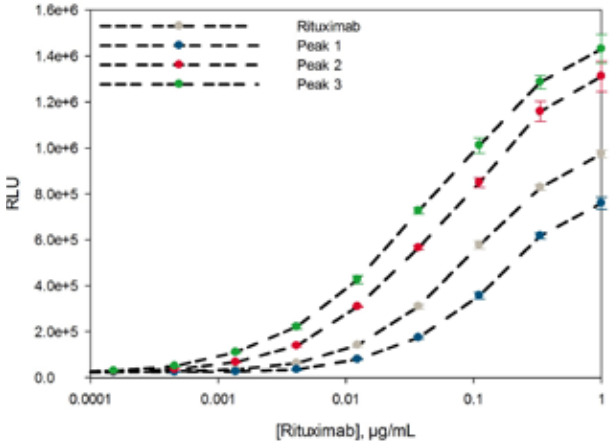

Figure 5

ADCC reporter bioassay response to rituximab (grey), fraction 1 (low FcRγ affinity, blue), fraction 2 (medium FcγR affinity, red) and fraction 3 (high FcγR affinity, green)[3]

EC 50 VALUES OBTAINED BY THE REPORTER BIOASSAY TEST

Antibody	EC_{50} (µg/mL)
Rituximab	0.098
Peak 1	0.153
Peak 2	0.072
Peak 3	0.049

Table 1

The dose-response curve of ADCC data was fitted with a 4-parameter model using Sigma plot.

Table 1 shows the EC50 values obtained by the reporter bioassay test. The lower the EC50 value, the higher the ADCC potency. As expected, peak three (high FcRγ affinity, green) shows highest ADCC potency and efficacy in the bioassay. Peak two shows intermediate, and peak 1 shows lowest ADCC efficacy and potency. ADCC efficacy and potency of the original rituximab lies between the low and medium affinity fractions.

GLYCAN ANALYSIS OF FCR AFFINITY FRACTIONS

The glycan profile of the collected fractions was analyzed by hydrophilic interaction chromatography. Figure 6 shows the glycan pattern of the FcR affinity fractions compared to a glycan library. 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).

HILIC ANALYSIS OF OLIGOSACCHARIDES OF THE THREE FcR AFFINITY FRACTIONS

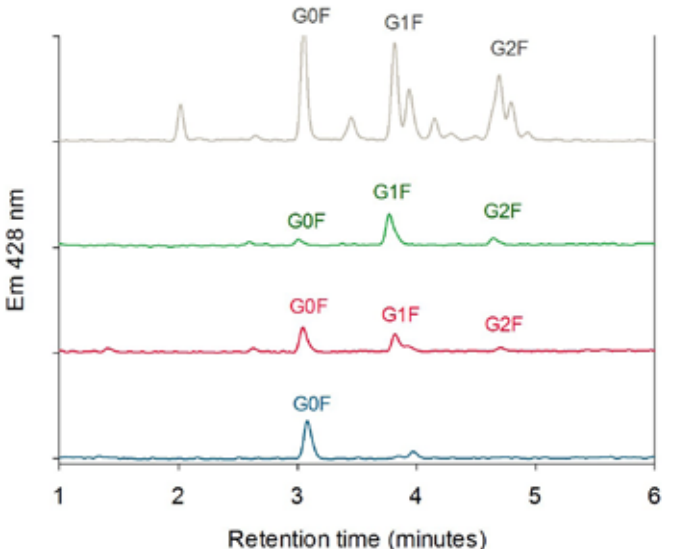

Figure 6

HILIC analysis of oligosaccharides of the three FcR affinity fractions (Peak 1 blue, Peak 2 red, Peak 3 green) compared with a 2-AB labelled biantennary glycan library (grey) [3]

CONCLUSION

The ADCC activity bioassay results show that high retention on TSKgel FcR-IIIA-NPR corresponds to a high ADCC activity. The HILIC-UHPLC glycosylation pattern analysis of the FcR affinity fractions also matches the common understanding that terminal galactose units of Fc-glycans typically enhance affinity to FcγRIIIa and ADCC activity while core fucose units decrease ADCC activity of antibodies. These results confirm that FcγRIIIa affinity chromatography allows fast assessment of biologic activity and glycoform pattern of antibodies.

REFERENCES:

1) D. Reusch and M. L. Tejada, Glycobiology 2015, 25 (12): 1325-1324; doi: 10.1093/glycob/cwv065
2) G.J. Weiner; Semin Hematol. 2010; 47 (2): 115-123; doi: 10.1053/j. seminhematol.2010.01.011
3) Master Thesis Leila Ghaleh, TU Darmstadt

Rituximab kindly provided by Rentschler Biopharma