Lectin-based screening assays for the fast assessment of CQA-related glycosylation of Fc fusion proteins

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Abstract

Fc fusion proteins are biotherapeutics that combine the therapeutic potential of proteins with the additional benefits of the IgG Fc domain. Glycosylation, especially sialylation, is a critical quality attribute (CQA) of Fc fusion proteins and an important parameter to monitor during the discovery and development of these molecules.

In this note we describe a fast lectin-based high-throughput glycan screening assay (Art.-No. PA-201) which is an ideal tool to obtain semiquantitative data on critical CQAs for many samples and show data for three well known Fc fusion proteins: etanercept (Enbrel), aflibercept (Zaltrap, Eylea) and efmoroctocog alfa (Eloctate, Elocta).

In addition, we generated different glycan variants of these molecules by treatment with three different glycosidases to prove the binding specificity of the used lectins. This allowed us to collect evidence for the presence of *O*-glycans on efmoroctocog, which to your knowledge has not yet been reported.

Introduction

Fc fusion proteins are a unique class of diverse molecules in terms of structure and function. Duivelshof et al. provide a comprehensive overview of the currently marketed Fc fusion proteins and discuss the challenges associated with their characterization and CQA analysis including glycosylation[1].

In addition to the *N*-glycans present at the Fc domain, Fc fusion proteins may contain multiple *N*- and *O*-glycosylation sites on the non-Fc protein part. These glycans can be very heterogenously glycosylated. Compared to the usually non-sialylated bi-antennary glycans which are typical for the Fc domain of antibodies, the *N*-glycans of the protein part of Fc fusion proteins are significantly more complex.

Sialylation is a key glycan feature as high levels of sialylation lead to longer serum half-life. It is assumed that sialylation on the protein domain has a larger impact than sialylation on the Fc domain[2].

In addition, the serum half-life of proteins is not only impacted by *N*-sialylation[3], but also *O*-sialylation[4].

The large amount of different glycans leads to huge challenges for HPLC or MS-based glycan analysis methods, in which peaks with high similarity need to be assigned to the correct glycan structures. Lengthy sample preparation procedures render these methods unsuitable for the screening of many samples.

O-glycans pose a special challenge because there are no enzymes available for selectively digesting them from the proteins for further analysis.

Accordingly, there is a need for rapid screening assays such as the PA-201 glycan screening assay, which allows for fast profiling of Fc fusion proteins to speed up the selection of cell lines and cell culture conditions that produce the desired glycosylation of the target protein.

Fc fusion protein characteristics

Aflibercept is a dimeric glycoprotein with a molecular weight of 115 kDa, approximately 15% of which is attributed to glycans. The two extracellular domains of the human VEGF receptors are fused to an immunoglobulin G (IgG)1 Fc domain. Shen et al. [6] reported that each VEGF domain contains two *N*-glycosylation sites, which results in a total of ten *N*-linked glycosylation sites on one molecule (Fig. 1 A). The site occupancy of all but one glycosylation site is close to 100% and glycan profiles are highly heterogenous. The overall degree of sialylation is high, but some glycosylation sites can reach up to 15% of Man5.

Etanercept is a Fc fusion protein comprising two soluble TNFR (sTNFR) molecules, each linked to an Fc part of IgG1. Each sTNFR domain has two *N*-linked and 13 *O*-linked glycosylation sites, whereby the glycans represent about 30% of the whole molecular weight of approximately 150 kDa[5]. The Fc part contains the conserved *N*-glycosylation site within the CH2 domain (Fig. 1 B).

Efmoroctocog alfa is a Fc fusion protein with one molecule of a domain B deleted factor VIII. The molecular mass is approx. 220 kDa. The factor VIII A domain contains 4 *N*-glycosylation sites with highly heterogeneous glycan compositions (Fig. 1 C). High mannose and sialylated glycans are the most abundant glycans on efmoroctocog[7]. In contrast to the other Fc fusion proteins, efmoroctocog is produced in a human HEK 293 cell line. Therefore, the glycan capping sialic acids in efmoroctocog show the human-like 2,6-linkage to the neighboring galactose.

Factor VIII is a heavily *O*-glycosylated protein, mainly in the B domain which is deleted in efmoroctocog, except for a small 14 a.a. fusion region. Qu at al.[8] found one *O*-glycopeptide in the A2 domain of plasma-derived factor VIII, and two were identified on the C domains of recombinant factor VIII (Kogenate, Bayer). Until present, there are no reports on whether there is any *O*-glycosylation present on efmoroctocog.

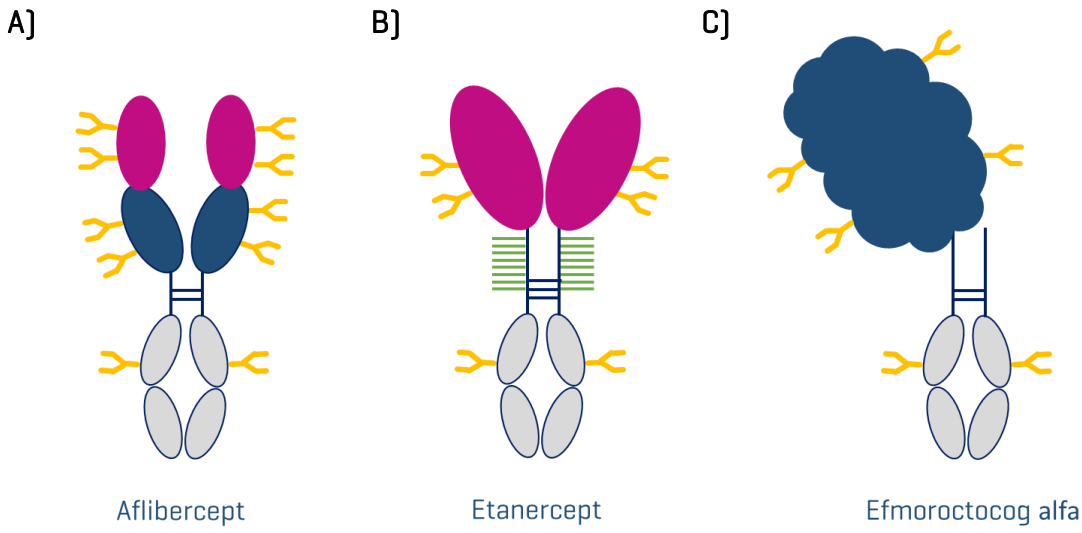

Fig. 1: Schematical representation of the three Fc fusion proteins. *N*-glycans are indicated in yellow, *O*-glycans in green.

PA-201 assay description

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 Fc fusion proteins. Lectins are proteins mainly derived from plants or fungi that exhibit specific binding to certain types of glycans. The result of the assays are lectin bindings for each of the individual lectins used in the assay (table 1).

Lectin name	Lectin specificity
FUC	Core fucose (*N*-glycans)
HM	High mannose glycans (*N*-glycans)
N-tGAL	Terminal galactose (*N*-glycans)
2,6-SA	2,6-linked sialic acids (*N*-glycans)
2,3-SA	2,3-linked sialic acids (*N*-glycans)
O-SA	Sialic acids (*O*-glycans)
O-tGAL	Terminal galactose (*O*-glycans)

Tab. 1: Overview of lectins used in this study

Materials and methods

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

In brief, 50 µL of lectin reagent were added to each well of the 384-well PAIA*plate* and 10 µL of sample (Fig. 2, step 2), previously mixed with PAIA sample preparation solution in a 1:1 ratio, was added.

The Fc fusion proteins were used in concentrations lower than 100 µg/mL which is typical for Fc fusion proteins in the PA-201 assay.

The PAIA*plate* was shaken for 45 minutes on a BioShake HP orbital shaker (Qinstruments, Germany) at 1800 rpm (Fig. 2, step 3). After sedimentation of the beads the plates were measured at 635/660nm on a fluorescence plate reader in the bottom reading mode (Fig. 2, step 5).

PNGase F (New England Biolabs, USA) was used to remove all *N*-glycans overnight at room temperature and immobilized SialEXO and GalactEXO (Genovis, Sweden) were used to specifically cleave off sialic acids and terminal galactose from *N*- and *O*-glycans according to the manufacturer´s protocol (30 minutes at RT).

The concentrations of the digested Fc fusion proteins were determined using the PA-104 Fc titer assay (PAIA Biotech). All Fc fusion samples were diluted to the same concentration prior to the PA-201 glycan assay so that the obtained lectin binding rates are directly comparable for all samples.

Fig.2. : Depiction of the workflow of the PA-201 assay in an exemplary well of the 384-well PAIA*plate*

Glycan profiles

Fig. 3 shows the glycan profiles of the three Fc fusion proteins, giving an easy-to-interpret representation of the overall features of the analytes. All molecules were measured at the same concentration allowing a direct comparison of lectin binding signals.

In comparison, Aflibercept is the molecule with the highest overall ability to bind lectins. This is due to the large number of *N*-glycosylation sites and the fact that these show different glycan profiles. Some of them contain up to 15% Man5 and most of them contain some terminal galactosylation and high degrees of fucosylation[6]. In addition, aflibercept is highly *N*-sialylated and binds the 2,3-SA lectin, but it does not bind the 2,6- and the O-SA lectin (Fig. 3 A).

Etanercept, in contrast, has significantly less *N*-glycosylation sites and therefore displays rather little binding of *N*-glycan specific lectins. The O-SA lectin shows high binding due to the presence of glycans on the 13 *O*-glycosylation sites (Fig. 3 B).

Efmoroctocog shows very high binding of the lectin that detects 2,6-linked sialic acids which occurs in human cell lines. Accordingly, efmoroctocog shows no binding of the 2,3-SA binding lectin. Efmoroctocog strongly binds the HM lectin, which is in accordance with literature that reports high mannosylation in the C-domain and lower levels of galactosylation and fucosylation at the A domain[7], resulting in low signals for the N-tGAL and FUC lectins.

We also observed an evidently high signal for O-SA (Fig. 3 C) which was unexpected because no data about *O*-glycans on efmoroctocog have been published.

This led us to conduct enzymatic digestions to verify the specificity of the lectins and confirm the presence of *O*-glycans on efmoroctocog.

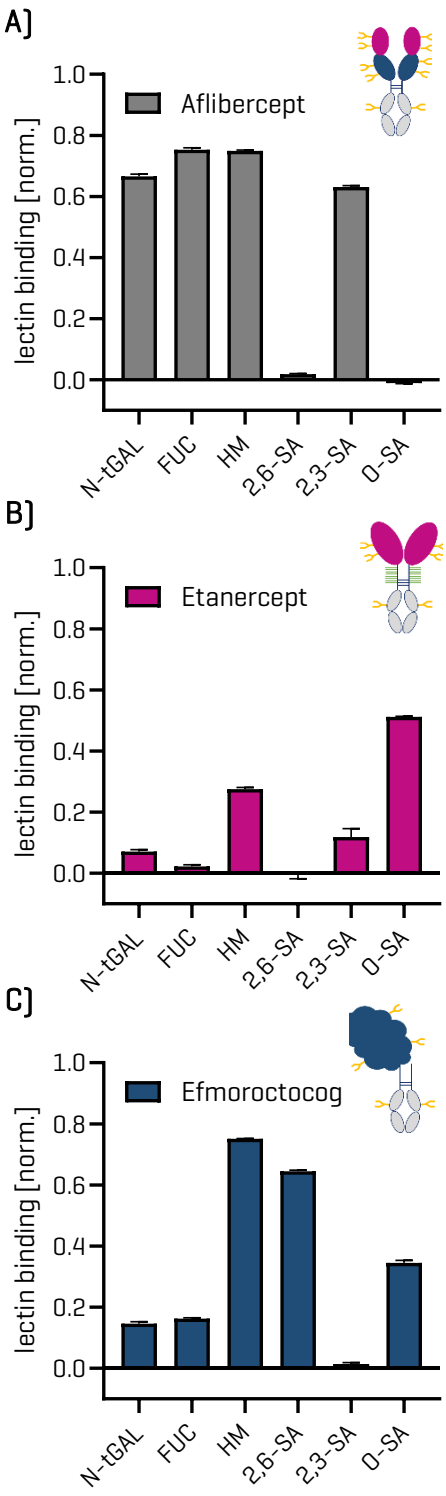

Fig.3. : Glycan profiles of aflibercept, etanercept and efmoroctocog obtained with the PA-201 glycan assay

PNGase and SialEXO digestion

First, we used two enzymes separately: PNGase F to cleave off all *N*-linked glycans and SialEXO to remove all sialic acids from *N*- and *O*-glycans.

We digested two molecules: etanercept (Fig. 4 A) as an example for a Fc fusion protein with both *N*- and *O*-glycosylation and aflibercept (Fig. 4 B) as a molecule without any *O*-glycosylation.

PNGase F digestion led to an almost complete loss of binding of the *N*-glycan specific lectins N-tGAL, FUC, HM and 2,3-SA whereas the binding of O-SA to etanercept remained (Fig. 4 A left), clearly indicating that the HM, N-tGAL, FUC and 2,3-SA lectins are *N*-glycan specific.

SialEXO treatment, which removes all sialic acids from *N*- and *O*-glycans resulted in a complete loss of binding of the respective lectins 2,3-SA and O-SA (Fig. 4. right). This result proves that these lectins specifically bind to sialic acids on *N*- and *O*-glycans.

The lectin binding of N-tGAL and O-tGAL increased after SialEXO digestion of both molecules because the galactose sugars became exposed after removal of the capping sialic acids.

This demonstrates that the N-tGAL and O-tGAL lectins do not tolerate capping sialic acids and that both specifically bind terminal galactose.

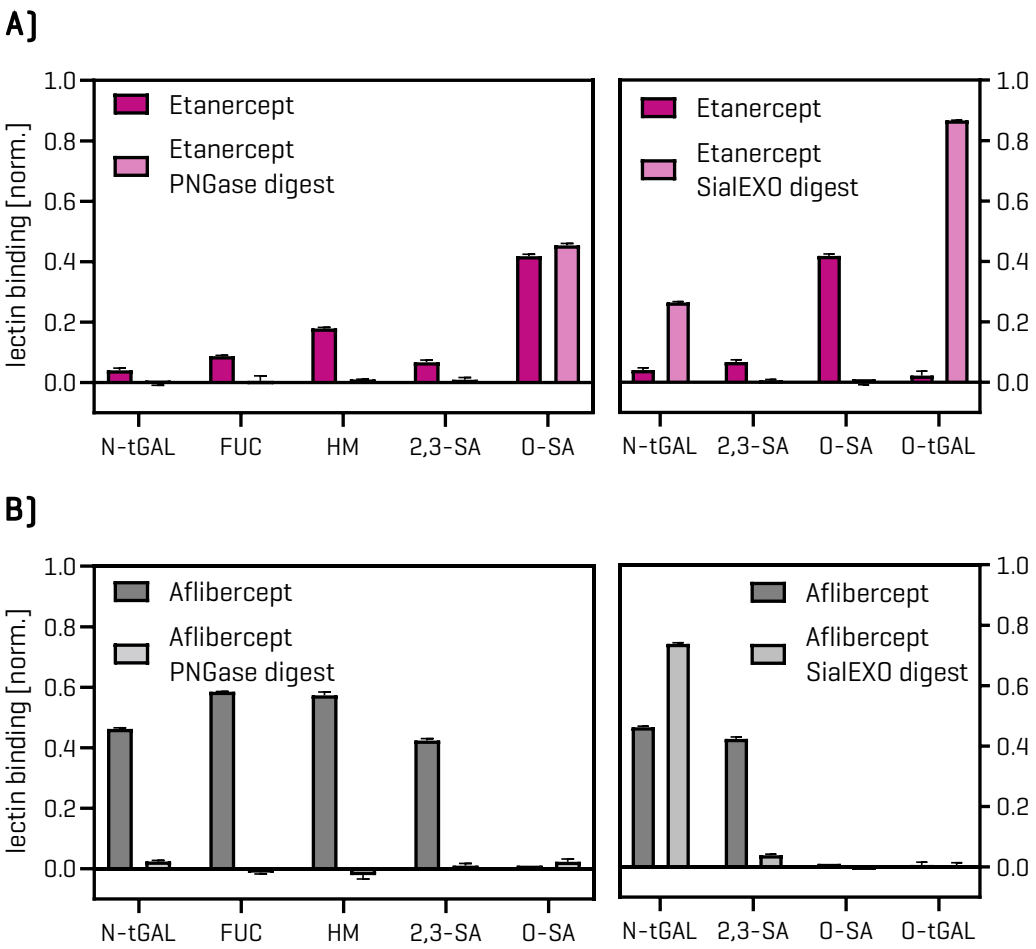

Fig. 4: PA-201 glycan profiles of untreated etanercept (A) and aflibercept (B) compared to PNGase F and SialEXO digests to demonstrate specificity of markers towards indicated glycan features. Efmoroctocog could not be digested because of incompatibility with the reaction buffer.

Sequential digestions

To further characterize the *O*-glycan specific lectins *O*-SA and *O*-tGAL, etanercept was sequentially digested with PNGase F, SialEXO and GalactEXO, which removes terminal galactose from *N*- and *O*-glycans (Fig. 5).

O-tGAL shows no binding to etanercept or PNGase F treated etanercept, whereas O-SA binds to both, which indicates that the *O*-glycans in the molecule are highly sialylated.

SialEXO treatment efficiently prevented binding of O-SA to etanercept and caused strong binding of the terminal O-tGAL lectin, demonstrating that the O-SA lectin is specific for sialylated *O*-glycans.

In the final step we applied GalactEXO to remove the terminal galactose from the *O*-glycans. This resulted in a complete loss of O-tGAL binding, proving specificity of this lectin to terminally galactosylated *O*-glycans.

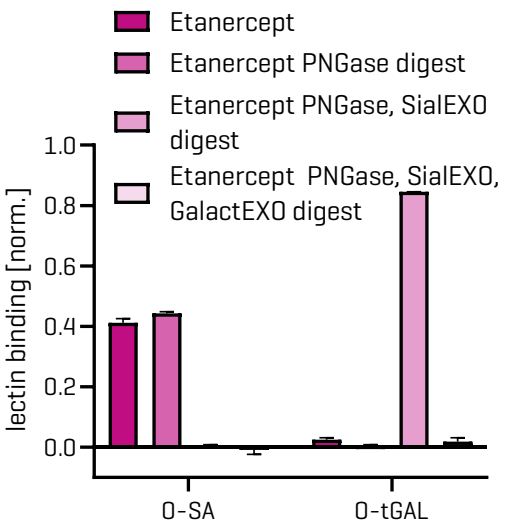

Fig. 5: Lectin binding to etanercept samples treated with PNGase F and further digested with SialEXO and GalactEXO to confirm specificity of O-tGAL lectin towards terminal galactose of *O*-glycans

Finally, we wanted to confirm the initial observation that efmoroctocog binds considerable amounts of the *O*-glycan specific lectin O-SA, suggesting that efmoroctocog contains *O*-glycans.

After digestion with SialEXO efmoroctocog displayed high O-tGAL lectin binding while O-SA lectin binding was suppressed (Fig. 6). Efmoroctocog therefore behaves like the highly *O*-glycosylated etanercept which suggests that efmoroctocog also contains *O*-glycans.

These *O*-glycans can be characterized with the two lectins O-SA and O-tGAL in the PA-201 screening assay.

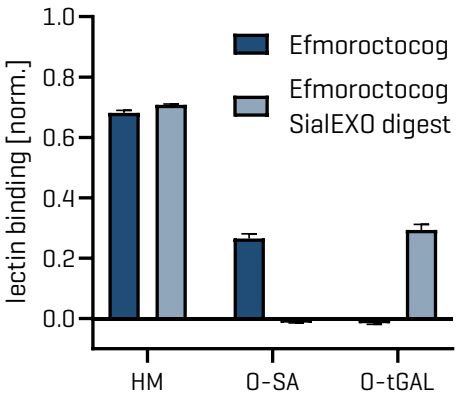

Fig. 6: Lectin binding to efmoroctocog and the efmoroctocog SialEXO digest to confirm presence of sialylated *O*-glycans

Conclusion

In the present study we demonstrate that the lectin-based glycan screening assay PA-201 is a fast and accurate method to assess the vastly diverse and highly heterogenous glycan profiles of Fc fusion proteins.

Enzymatic digestions of the Fc fusion proteins were successfully used to confirm that the presented lectins are indeed specific binders for *N*- or *O*-glycans. The PA-201 can distinguish glycans with terminal galactosylation and sialylation and detects these two types of glycosylation on *N*– and *O*-glycans separately.

In addition, we present evidence for the presence of *O*-glycans on efmoroctocog, although efmoroctocog only contains a small portion of the main *O*-glycan bearing domain of factor VIII.

Due to the easy workflow and simple sample preparation the PA-201 glycan assay is capable of analyzing hundreds of samples within few hours and minimized samples preparation. It is therefore perfectly suitable for screening many samples from the cell line and bioprocess development and generating CQA-related rankings of clones and cell culture conditions.

A wide range of specific lectins is available for initial profiling of Fc fusion proteins and a selection of the most CQA-relevant lectins can then be used to screen large numbers of samples with increased throughput.

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