Relationship between structure and function: Influence of galactosylation on Fc-mediated binding and functional properties of adalimumab

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Introduction

The N-glycan profile of an antibody plays a critical role in product stability, immunogenicity or pharmacokinetics and can also significantly influence the Fc-region mediated effector functions of antibodies, such as antibody dependent cellular cytotoxicity (ADCC) and complement dependent cytotoxicity (CDC).

β-galactosylation is known to be crucial for CDC activity, since terminal galactose enhances binding of antibody Fc regions to the C1q complement protein, which is required for CDC. β-galactosylated glycans can also increase antibody Fc-region binding to the FcγIIIa receptor, which can give rise to enhanced ADCC activity.

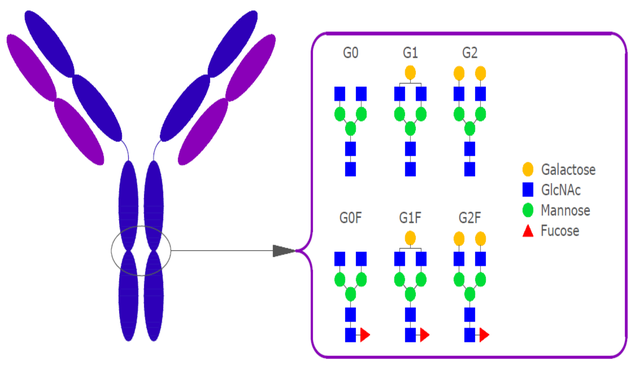

Figure 1. Examples of N-linked glycans in therapeutic mAbs

Product characterization is key to successful biological drug development. Comprehensive characterization of new therapeutic monoclonal antibodies requires a deep understanding of their structural and functional critical quality attributes (CQAs), which greatly impact product potency, stability and safety.

We evaluated the criticality of glycan structure on antibody Fc-region mediated effector functions through the application of a highly resolving N-glycan assay, in combination with binding and cell-based assays. In order to demonstrate the influence of terminal galactose on the effector functions of adalimumab, we removed β-galactose from N-glycans using β-1,4 galactosidase (Fig 2.) and evaluated the impact of the changes in glycosylation profile on CDC and ADCC activity.

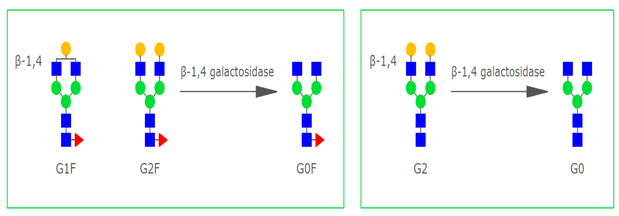

Figure 2. β-1,4 galactosidase digestion

Methods

Enzymatic glycan remodeling

In order to remove terminal galactose moieties, adalimumab was added to β -1,4 galactosidase (Sigma) diluted with 50 mM PBS, pH 6 and digested overnight at 37ºC (16 hours). The antibody samples were purified after enzymatic digestion using Nab™ Protein A spin column and the buffer was exchanged into PBS, pH 7.2. Mock-digested adalimumab (mock control), was prepared along with enzyme treated adalimumab (enzyme treated) in the absence of β -1,4 galactosidase.

Stock adalimumab (untreated), adalimumab mock control and adalimumab enzyme treated samples were run in parallel in each of the following assays.

N-glycan analysis by CE-LIF

N-linked oligosaccharide analysis was performed using the SCIEX Fast Glycan Labelling and Analysis Kit (Sciex). N-glycans released from samples following PNGase enzyme digestion were labelled with charged fluorophore. The fluorophore-labelled glycans were separated and analyzed by capillary electrophoresis (CE) equipped with a laser-induced fluorescence (LIF) detector. Glycans were identified using the GU Value Software (Sciex).

C1q binding assay

Samples from adalimumab dilution series were added to the ELISA plate. After overnight incubation at 4ºC, 2µg/mL of human C1q (Sigma) was added to the wells and bound C1q protein was detected using an anti-C1q-HRP conjugated antibody (Abcam) followed by addition of TMB substrate. The reaction was stopped with 1M HCl, and the absorbance was read at 450nm. Data generated was reported as relative binding potency (RP) values.

FcγIIIa receptor binding assay

The binding affinity of all samples for recombinant human FcγIIIa protein was determined using surface plasmon resonance (SPR) technique on a Biacore T200 instrument (GE Healthcare). Briefly, multi cycle kinetics for each sample was performed using multiple different concentrations of adalimumab as analyte flowing over a directly immobilised FcγIIIa surface (CM5 chip) and the resulting K_D value established using Biacore software.

Complement dependent cytotoxicity assay

The CDC assay was performed using a recombinant mTNFα CHO-K1 target cell line (Promega). Briefly, cells were treated with sample dilution series in the presence of pooled human serum complement for 2h at 37ºC. The CDC activity was measured using a luminescence based end point reagent. Data generated was reported as relative potency (RP) values.

Antibody dependent cellular cytotoxicity assay

ADCC was performed using a recombinant mTNFα CHO-K1 target cell line (Promega). Fresh human peripheral blood mononuclear cells (PBMCs) were isolated from healthy blood donors by gradient centrifugation. To detect ADCC activity of Adalimumab, the target cell line was treated with a sample dilution series followed by the addition of PBMCs. After overnight incubation, the ADCC activity was detected using a luminescence based end-point reagent. Data generated was reported as relative potency (RP) values.

Results

N-glycan analysis by CE-LIF

CE-LIF analysis showed that the level of galactosylated species of Adalimumab changed significantly following β-1,4 galactosidase treatment, with modifications detected in fucosylated (G0F, G1F, G2F) and afucosylated species (G0, G1, G2). After β-1,4 galactosidase treatment, glycans G2F and G2 were no longer detected. The level of G1F decreased from 11.8% to 0.6%, while a 13.3% increase was observed for G0F. All other N-glycans in Adalimumab enzyme treated sample remained unchanged (Fig 3; Table 1).

Table 1. N-glycan analysis summary results.

N-Glycan Type	Area (% of total glycan species detected)	
	Adalimumab mock control	Adalimumab enzyme treated
G0F	73.562	86.861
G1F	11.806	0.611
G2	3.74	Not detected
G2F	0.913	Not detected

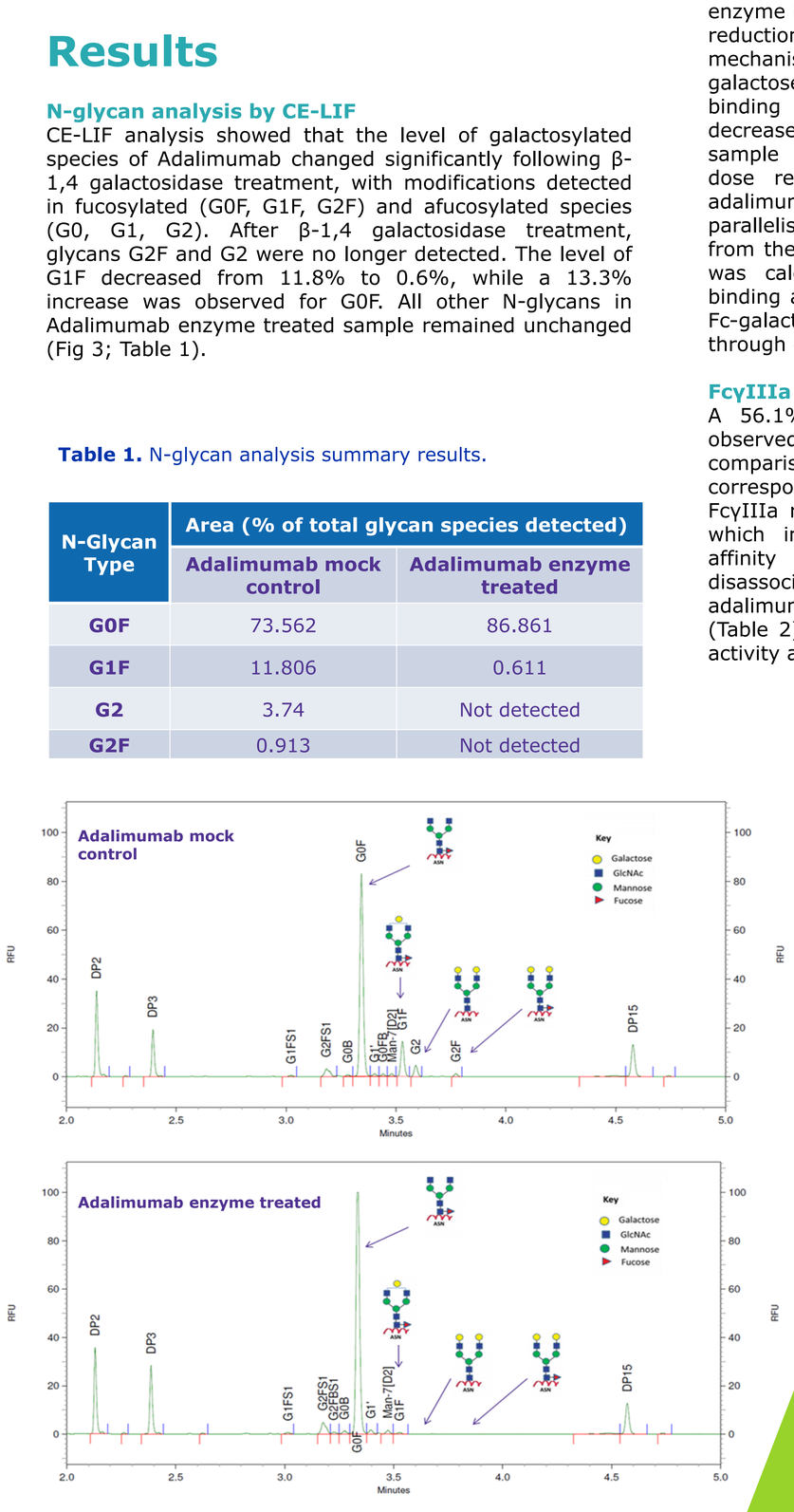

Figure 3. N-glycan analysis of mock control and enzyme treated adalimumab using CE-LIF.

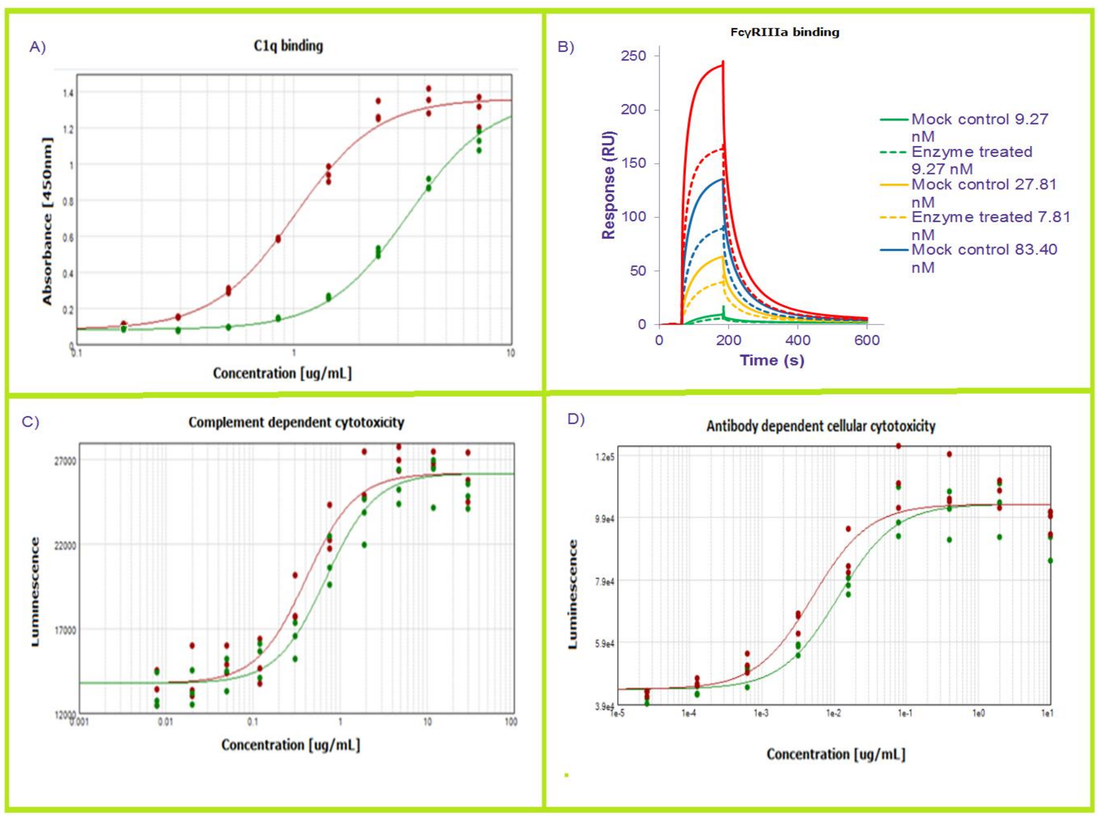

Figure 4. A) C1q binding ELISA, red: mock control, green: enzyme treated sample. **B) FcγIIIa receptor binding by SPR. C) CDC assay** red: mock control, green: enzyme treated sample. **D) ADCC assay** red: mock control, green: enzyme treated sample.

C1q binding and CDC

A significant decrease in CDC activity for enzyme treated sample was observed (40.7% reduction, Fig 5). To investigate the mechanism by which loss of terminal galactose affects CDC, we examined the C1q binding affinity by ELISA. A significant decrease in C1q binding for enzyme treated sample was observed, however, since the dose response curve for enzyme treated adalimumab was not similar enough to pass parallelism criteria, the relative EC_{50} value from the unconstrained dose response curves was calculated to determine relative C1q binding activity (Fig 4A). Results showed that Fc-galactosylation increases CDC activity through enhanced binding affinity to C1q.

FcγIIIa binding and ADCC

A 56.1% reduction in ADCC activity was observed for enzyme treated sample in comparison to mock-digested control. A corresponding decrease was also detected in FcγIIIa receptor binding using an SPR assay which indicated a 20.8% of reduction in affinity (K_D). The association (k_a) and disassociation (k_d) of the enzyme treated adalimumab showed decreased rate constants (Table 2), correlating to the change in ADCC activity assay.

In order to investigate the true influence of galactose loss on binding and activity of Adalimumab, Adalimumab mock control was used as Reference Standard (100%), since it had gone through the same glycan remodelling process as enzyme treated sample. No significant differences in activity were observed between stock adalimumab and mock control. For all assays, results obtained from the analysis of untreated adalimumab indicated the assays had all performed as expected based on trending data for adalimumab using the binding and cell based functional assays established by BioReliance® Services.

Table 2. SPR data summary

Parameter	Adalimumab mock control	Adalimumab enzyme treated
k_a (1/Ms)	2.06 x 10^5	6.79 x 10^4
k_d (1/s)	0.03936	0.01639
K_D (M)	1.913 x 10^{-7}	2.416 x 10^{-7}
Relative K_D (%)	100	79.2

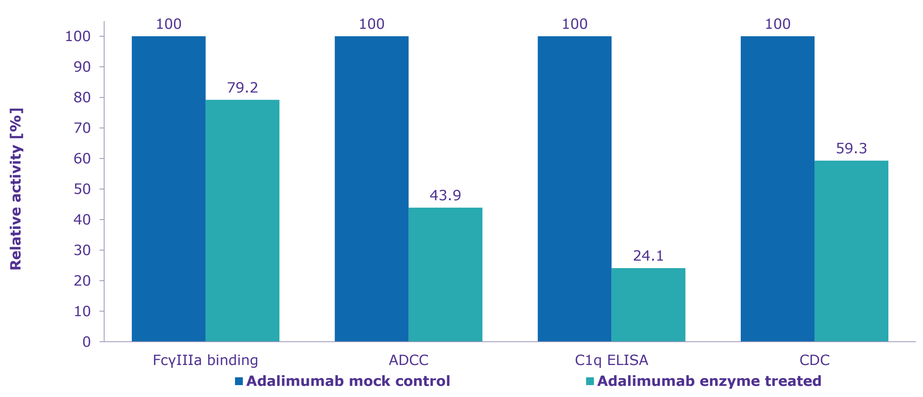

Figure 5. Relative activity summary

Summary

We have developed a highly sensitive package of binding and cell-based methods in order to conduct comprehensive characterization of the functional activity of adalimumab. We combined this with analysis using a commercial kit based analysis of N-Glycan structure to assess the impact of glycosylation patterns on Fc-mediated effector functions. Our data confirmed that glycan structure is a critical quality attribute of adalimumab, and highlights the importance of investigating glycan composition during the development of new therapeutic antibodies. The results obtained were consistent with literature reports showing that a lack of terminal galactose disturbs the effector functions of examined antibody.

In summary (Fig 3, Fig 5):

- Adalimumab mock control contained 16.4% of galactosylated species (G1F, G2, G2F). After enzyme treatment , the content of galactosylated species decreased to 0.6%, which was associated with;
  - An overall reduction in binding affinity to FcγIIIa (20.8%) and C1q protein (75.9%).
  - A 56.1% reduction in ADCC activity (RP)
  - A 40.7% reduction in CDC activity (RP)

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