Establishment of A Feasible Method to Quantify Glycoforms of Human IgG1 mAb Drugs in the Production Media as a Component of Process Analytical Technology

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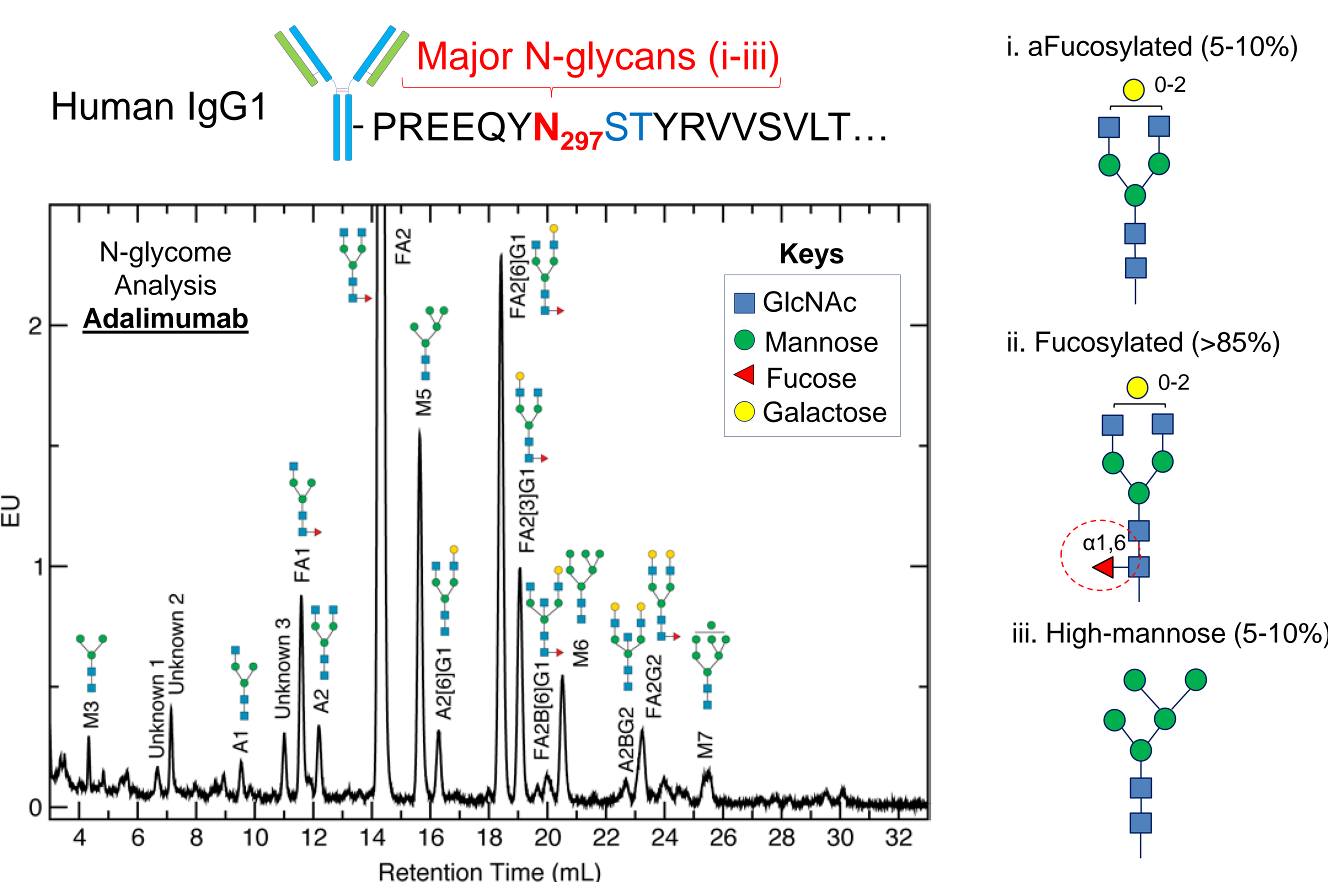
Abstract

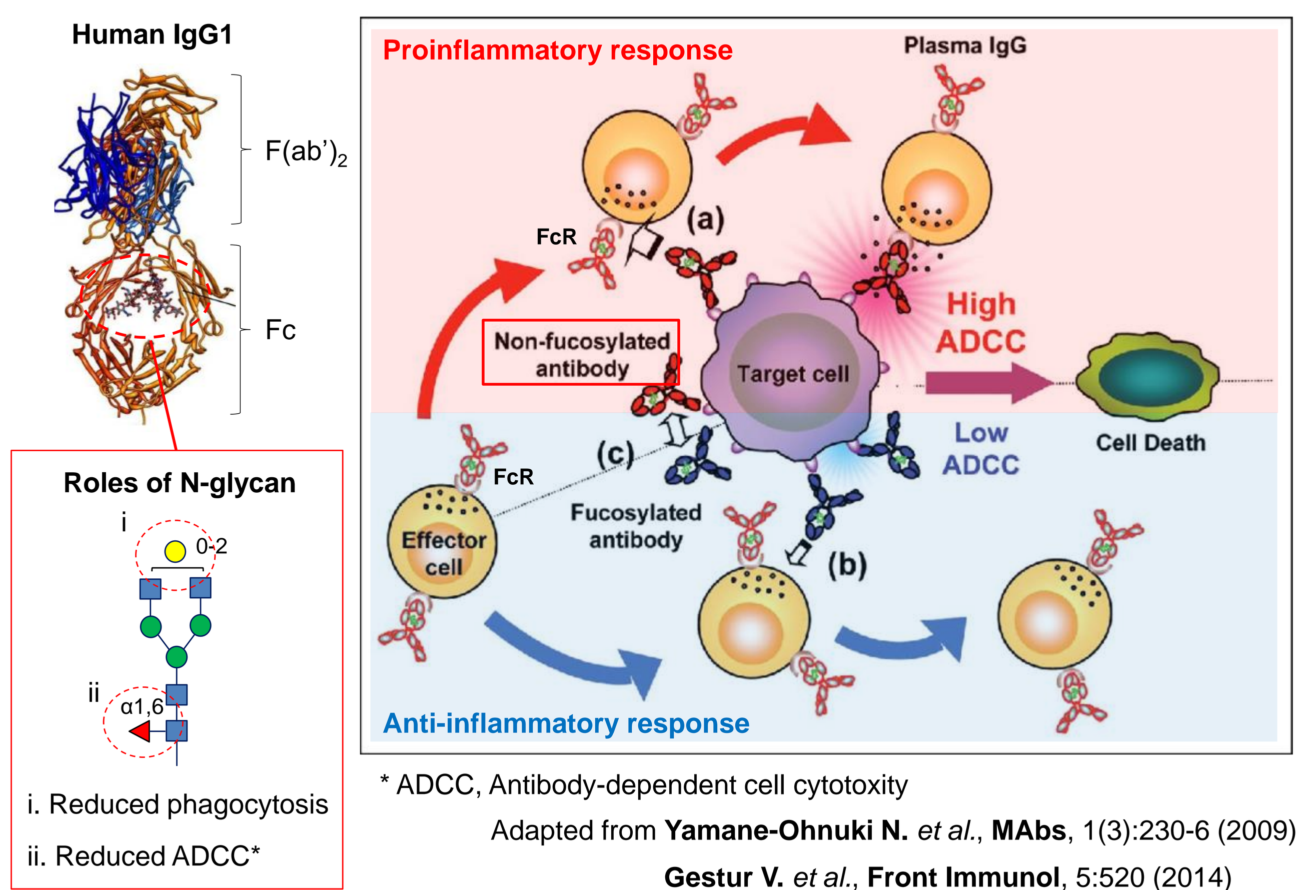
Monoclonal antibody (mAb) drugs constitute a significant portion of all approved therapeutic proteins. IgG1 mAb drugs produced from CHO cells often contain three major glycoforms due to different N-glycosylation at Asn297 residue in their Fc domain: i, aFucosylated; ii, Fucosylated; and iii, High-mannose (***See Introduction 1***). N-glycosylation is a critical quality attribute (CQA) for many IgG1 mAb drugs as it can impact their effector function-related activities, such as antibody-dependent cell-mediated cytotoxicity (ADCC) or complement dependent cytotoxicity (CDC), and pharmacokinetics (PK)/pharmacodynamics (PD), and thus needs to be controlled and monitored (***See Introduction 2***). A small fraction of IgG1 mAb is not N-glycosylated (non-glycosylated) and consequently lacks effector activity. Mass spectrometry and (U)HPLC are the current tools for analysis of these glycoforms of the purified mAbs, which is not practical for an in-process analytical characterization such as PAT (process analytical technology) during advanced manufacturing. To address the unmet analytical need, we generated two sets of mouse mAbs by immunizing mice with chemically synthesized human IgG1 Fc glycopeptides with different glycoforms-conjugated on KLH as immunogens (***See more details in Materials and Methods***). Selected mouse mAb-a hybridoma supernatants specifically recognized non-glycosylated human IgG1, and mouse mAb-c specifically bound to glycosylated human IgG1 and differentiated aFucosylated from Fucosylated human IgG1 upon endoglycosidase treatments in an ELISA assay. The mAb-c requires both sugar-bound Asn297 (Fuc-GlcNAc-Asn or GlcNAc-Asn) and surrounding peptide around the Asn297 for antigenic recognition and binding. Currently, we are further characterizing the purified mouse mAbs (mAb-a and mAb-c) using differently glycosylated human IgG1 mAb drugs, and establishing a Biolayer Interferometry (BLI) method to quantify the major glycoforms of human IgG1 mAb drugs in the production media or unprocessed bulk after treatments with different endoglycosidases: Endo-S, Endo-M, Endo-H, and Endo-F3 that show distinct specificities for their glycoforms. This technology can be potentially developed into a component of PAT. This OPQ-mission relevant project will facilitate development of advanced/continuous manufacturing of IgG1 mAb drugs through identification of glycan-related critical material attributes (CMA) and critical process parameters (CPP).

Introduction 1 - Glycoforms on IgG1



Introduction 2 - MoA of aFuc-IgG1

Core fucosylation negatively regulates immune responses; ADCC*



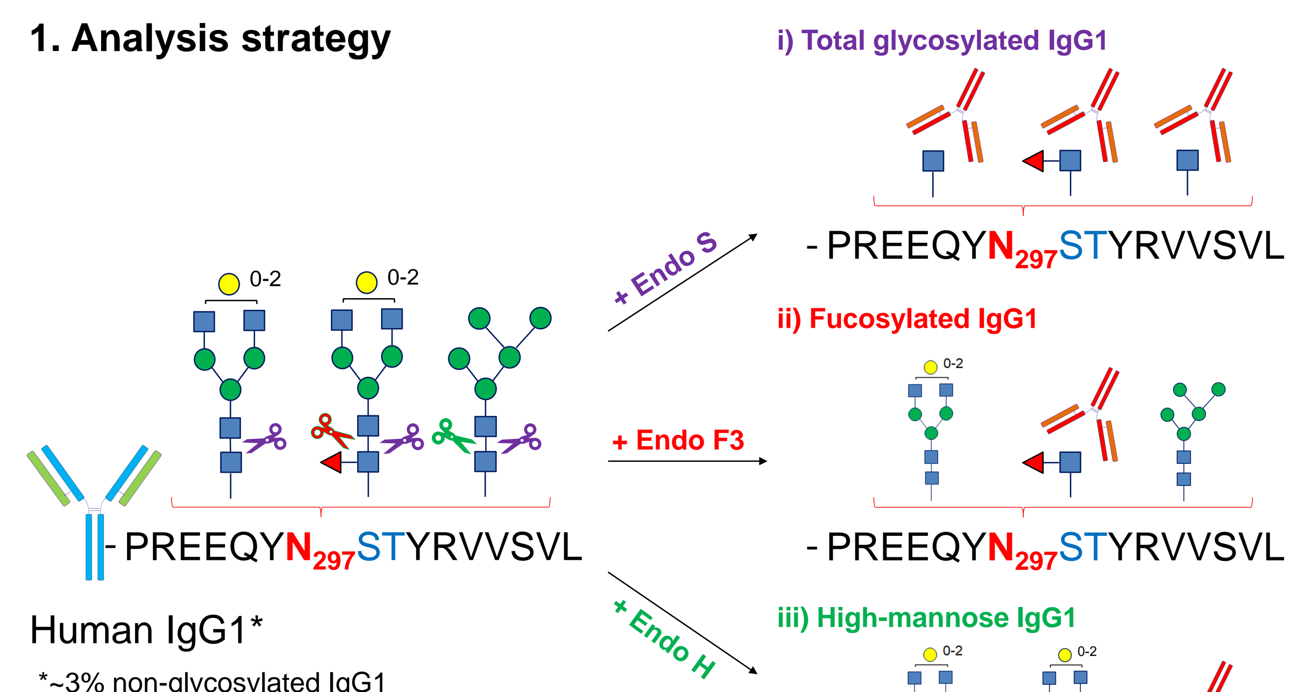
* ADCC, Antibody-dependent cell cytotoxity

Adapted from **Yamane-Ohnuki N.** *et al.*, **MAbs**, 1(3):230-6 (2009)

Gestur V. *et al.*, **Front Immunol**, 5:520 (2014)

Materials and Methods

1. Analysis strategy



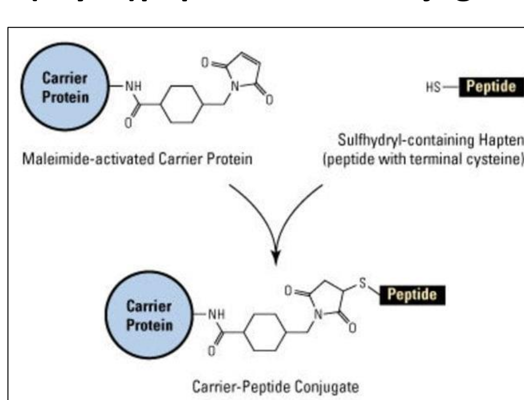
2. Generation of mouse mAbs

(1) Immunogen-a
(Non-glycosylated)

(2) Immunogen-b
(aFucosylated)

(3) Immunogen-c
(Fucosylated)

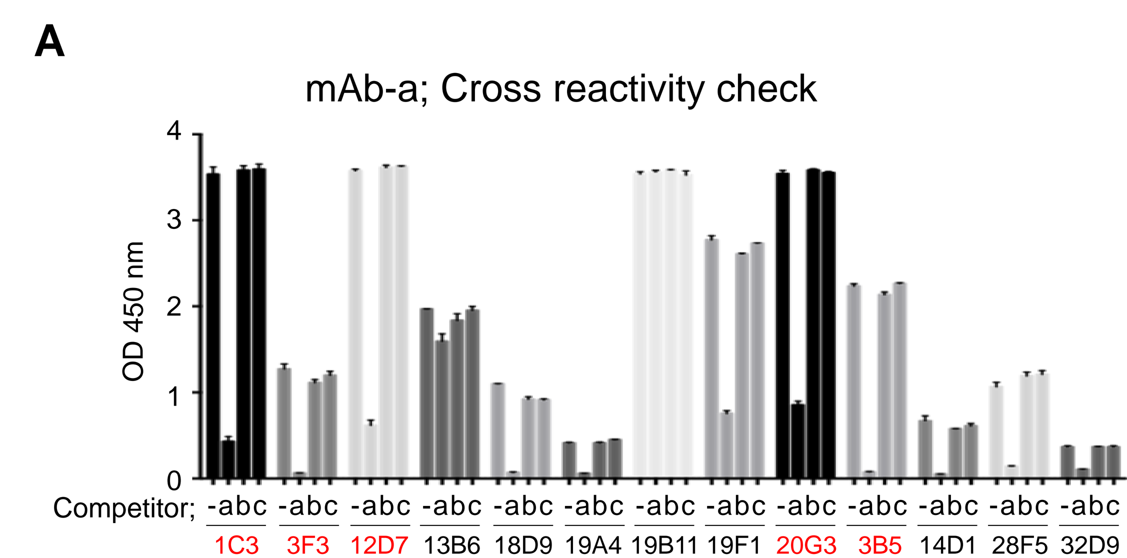
(Glyco)peptides-KLH Conjugates



- **3x Immunization into mice**
- **Screening hybridomas**

Results and Discussion

- **mAb-a (Non-glycosylated);** Initial immunization with Immunogen-a-KLH (Keyhole limpet haemocyanin) conjugates did not induce immune responses. We synthesized more soluble immunogen-a-BCP (Blue carrier protein) conjugates for mouse immunization. Ten hybridoma clones were identified to recognize non-glycosylated Fc peptide (Immunogen-a), and 5 (in red) of them were selected for further characterization (**Fig. 1A**).



- **mAb-b (aFucosylated);** After immunizing with Immunogen-b-KLH, no hybridomas that specifically recognized Immunogen-b were obtained (Data not shown, all hybridomas showed broad cross reactivity across Immunogen-a to c.)
- **mAb-c (Fucosylated);** After immunizing with Immunogen-c-KLH, seven hybridoma clones recognized both aFucosylated and Fucosylated Fc peptides. Five clones (in red) were selected (**Fig. 1B**), and affinity-purified for further characterization (**Fig. 1C**).

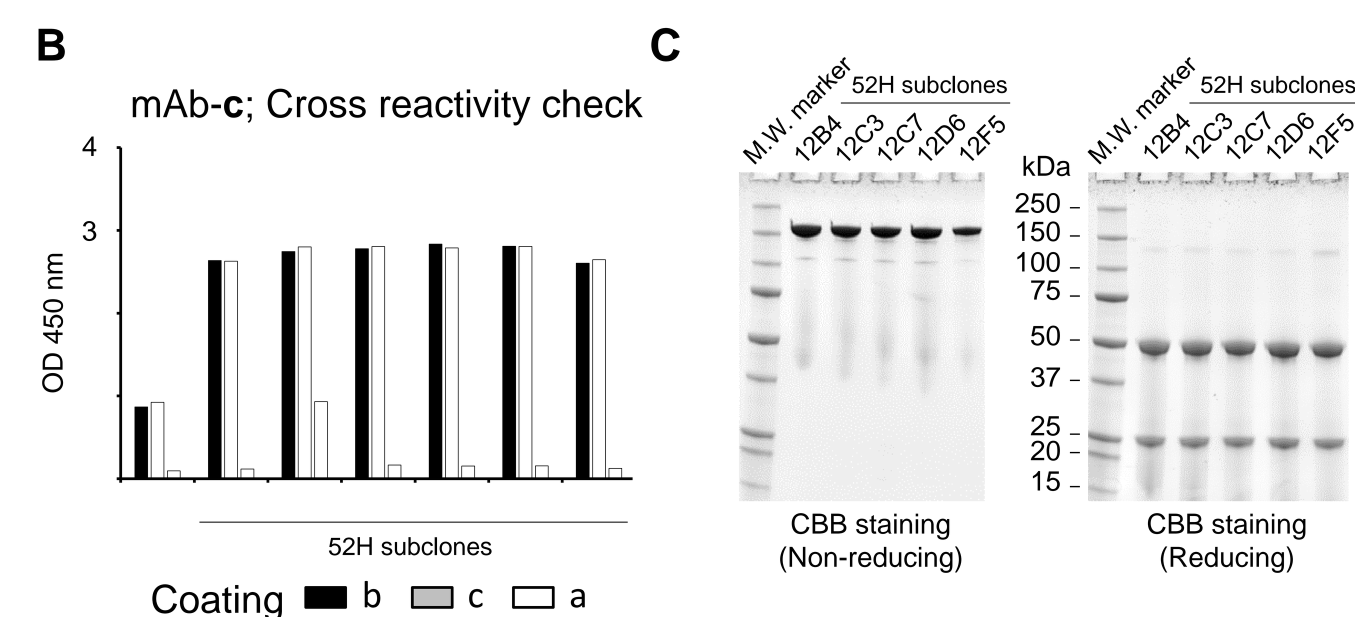


Figure 1. Generation of monoclonal antibodies that recognize non-glycosylated Fc or glycosylated Fc
Several hybridoma clones were screened on ELISA. **(A)** Immunogen-a was immobilized on 96-well plate, and obtained hybridoma sup (1:10 dilution) was added after pretreating sup with immunogen-a, b, or c, as competitors. HRP-labeled with anti-mouse specific IgG was incubated, and signal was measured using TMB substrate (Absorbance at 450 nm). Error bars represent ±1 SD of triplicates. **(B)** Immunogen-a, b, or c, were immobilized on a 96-well plate, and obtained hybridoma sup (1:10 dilution) was added. Five hybridoma clones as in red were selected. **(C)** Antibodies from supernatant of five clones in mAb-c were affinity-purified, and confirmed by SDS-PAGE with CBB staining under reducing (2ME+) or non-reducing condition

Table 1. Relative abundance (%) of major glycoforms of Adalimumab

Adalimumab	Preparation 1			Preparation 2			Six experiments		
N-glycan type	1	2	3	1	2	3	Average	STDEV	CV%
Complex%	87.19	87.42	87.81	87.46	87.69	87.19	**87.46**	0.25	0.29
Total Afuc%	12.97	13.43	13.17	13.03	12.63	13.07	**13.05**	0.26	2.0
Complex Afuc%	3.20	3.22	3.31	3.17	2.89	2.72	**3.09**	0.23	7.4
HM% (M5-M7)	9.39	9.78	9.44	9.48	9.74	9.88	**9.62**	0.21	2.1
Unknown%	2.14	2.38	2.32	2.34	2.56	2.46	**2.37**	0.14	6.0

Mass spectrometry (MS) in Adalimumab

Adalimumab	Preparation 1			Preparation 2			Six experiments		
N-glycan type	1	2	3	1	2	3	Average	STDEV	CV%
Complex%	91.7	91.2	91.5	91.6	91.6	91.8	91.6	0.2	0.22
Total Afuc%	8.9	9.4	9.1	9.1	9.1	9.0	9.1	0.2	1.7
Complex Afuc%	0.98	1.04	1.02	1.00	1.00	1.10	1.02	0.04	3.7
HM (M4-M7)%	7.5	7.9	7.7	7.7	7.7	7.5	7.7	0.1	1.6

0-2 0-2

(U)HPLC; 3.09% 84.37% 9.96%
MS; 1.02% 90.58% 8.27%

- PREEQYN297STYRVVSVL

- **Table 1** showed two analytical measurements of (U)HPLC and mass spectrometry (MS) gave a similar % of glycoforms on Adalimumab, anti-TNF IgG1 mAb drug.

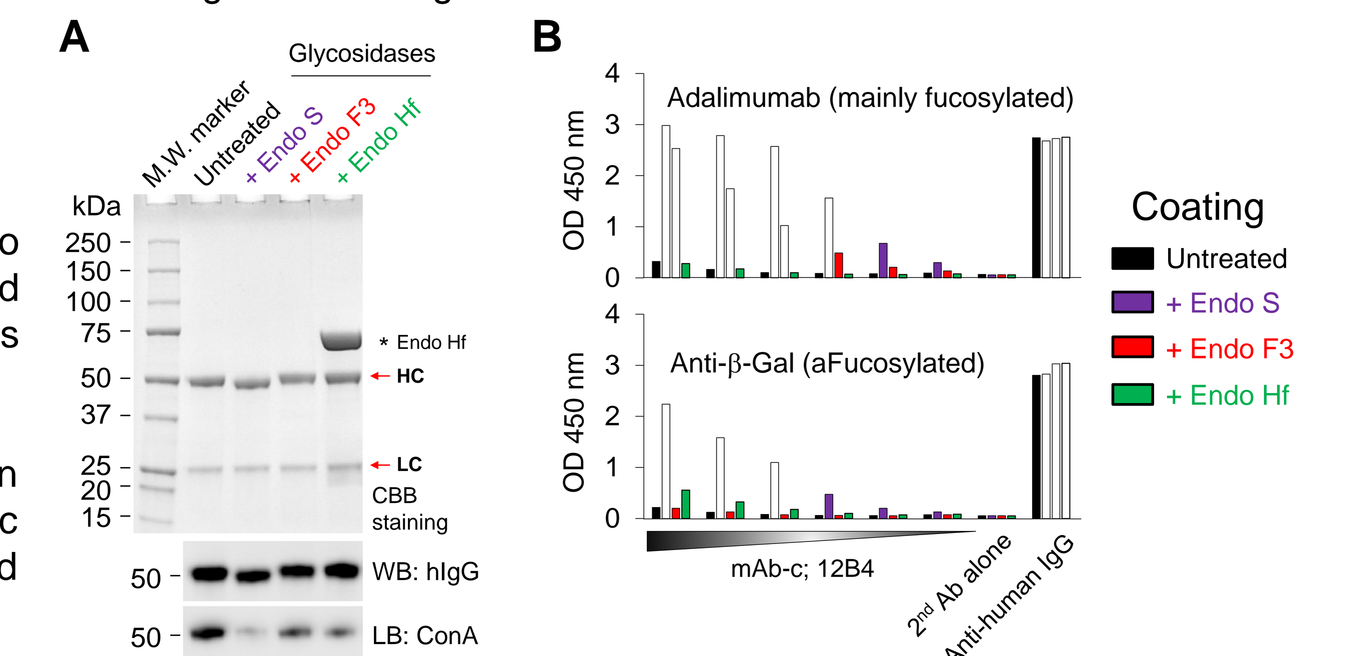


Figure 2. Measurement of glycoforms on Adalimumab using the mAb-c in an ELISA assay
(A) Endo-glycosidase treatments: Adalimumab was treated with endo-glycosidases (Endo S, Endo F3, or Endo Hf) for 1 h at 37°C, and confirmed by SDS-PAGE with CBB staining, Western and lectin blotting. Both heavy chain (HC) and light chain (LC) were indicated as red arrows (50, and 25 kDa, respectively). Asterisk indicates Endo Hf recombinant enzyme (-69 kDa). Lectin Con A stains all types of N-glycans. **(B)** ELISA: Materials from **A** were immobilized on a 96-well ELISA plate, then incubated with purified mAb-c (52H12B4, a 1:3 dilution ratio starting at 1 μg/ml). Anti-human IgG (right column) was used as an internal control for coating, and an aFucosylated human IgG1 (anti-β-Gal, bottom panel) was used as afucosylated hIgG1 control.

- The mAb-c clearly bound to both aFucosylated and Fucosylated IgG with higher OD in samples treated by Endo S than Endo F3 as expected (**Fig. 2B**). The signal from Endo Hf-treated IgG, however, was not detected due to its low efficiency to an intact IgG. Overall, we observed the trends of major glycoforms within Adalimumab compared to **Table 1**. The Anti-β-Gal IgG1 (aFucosylated) had 74.44 % of aFucosylated and 25.56% of high-mannose, as supposed to no fucosylated glycoform confirmed by Endo F3 treatment.

Conclusion

- Mouse mAbs that specifically recognize unglycosylated and glycosylated IgG-Fc (mAb-a, and mAb-c, respectively) have been generated.
- Preliminary ELISA data indicated both mAbs will be useful for an application to feasibly quantify major glycoforms of human IgG drugs.