Analysis of Glycosylation Critical Quality Attributes (GCQAs) of monoclonal antibody (mAb) therapeutics

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Introduction

Glycosylation can significantly decrease the clinical performance of therapeutic monoclonal antibodies (mAbs)[1,2]. For instance, the presence of terminal α1-3-linked galactose[3] or *N*-glycolylneuraminic acid can affect the safety profile and lead to a potential adverse reactions and neutralisation of the drug, thus reducing its therapeutic efficacy. Consequently, regulatory authorities are now tightening the requirements for biopharmaceutical companies to characterise, control and monitor the glycosylation of their therapeutics throughout the product's life cycle.

We use a systematic approach following a quality by design (QbD) framework to characterise glycosylation of glycoprotein therapeutics. Our strategy aligns with current and emerging regulatory guidelines from FDA, EMA and ICH[4] and has three broad steps:

1. Identification and prioritisation of GCQAs by detailed characterisation of the drug glycosylation patterns using a comprehensive range of orthogonal methods.

2. Implementation of appropriate glycan analysis modules to measure the GCQAs throughout the drug's life cycle.

3. Interpretation of the glycan analysis data followed by appropriate actions, if the product falls out of specification (OOS) or trends towards OOS.

Ludger's strategy for identification of GCQAs

Figure 1 outlines the workflow we use for measurement and identification of GCQAs such as **sialylation**, **core fucosylation**, **antennary composition**, **alpha-galactose** and ***N*-glycolyl-sialic acid** in IgG-1 mAb. *N*-glycans are released from the glycoprotein using PNGAse F endoglycosidase then derivatised with procainamide[5] (PROC). Procainamide labelled glycans are run on two orthogonal analysis platforms - HILIC (Hydrophilic Interaction Liquid Chromatography) UHPLC and ESI-MS/MS - generally in hyphenated configuration.

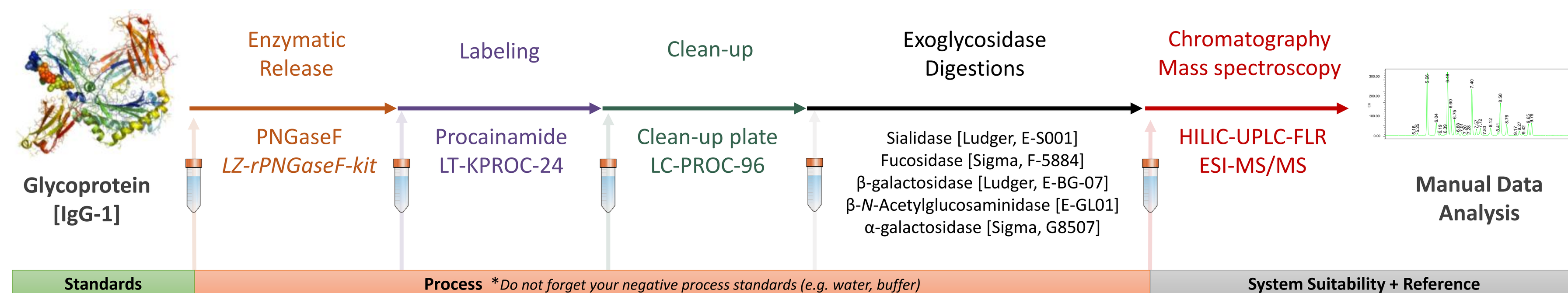
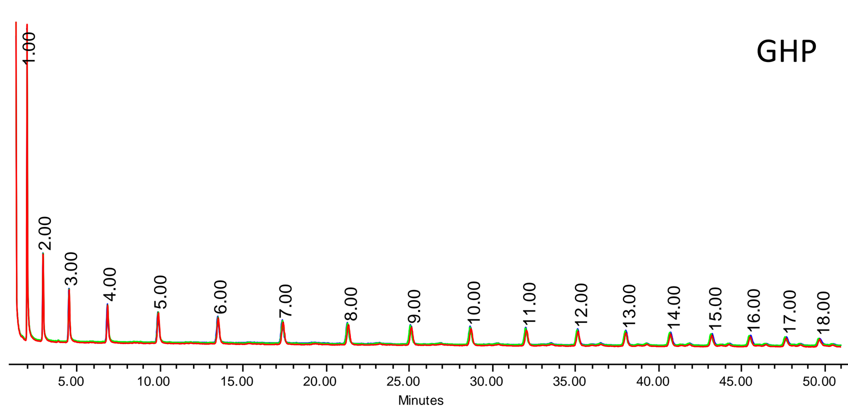


Figure 1. General glycoprofiling scheme for analysis of GCQAs.

A key component of our strategy is the inclusion of: (i) **system suitability standards**; (ii) **process standards; and** (iii) **reference standards**[5].

System Suitability Standard and Reference Standard for GU allocation

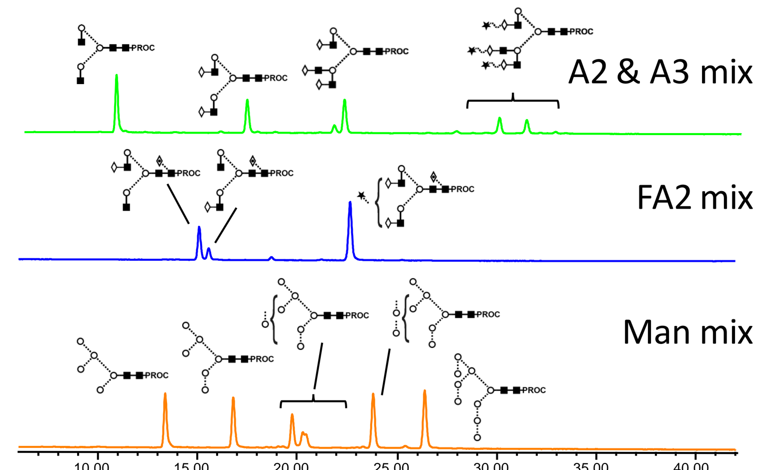


This standard enables an analyst to test the holistic functionality of an analytical system and evaluate whether it is adequate for its intended use.

Procainamide Labelled Glucose Homopolymer (GHP)
Provides GU values that can be used as a primary identification for glycans based on reported values in the literature and databases.

Acceptance Criteria: The peak width at half height for GU10 is less than 0.22 min. The profile should be similar to the profile shown in the Certificate of Analysis. The peaks should be symmetrical and well resolved. At least two runs of GHP should overlay without any drift in retention time.

Process Control



These are used to verify that part of or an entire process has worked correctly. There are four main categories for processes standards in glycoanalysis: release, labelling, release followed by labelling and exoglycosidase sequencing.

Ludger human IgG (hIgG) [Ludger product: GCP-IGG-100U]
Well Characterised glycoprotein run in parallel with samples to assess the release, labelling, clean-up and analyses.

Acceptance Criteria: The profile of the procainamide labelled released *N*-glycans should be similar to the profile shown in the product certificate of analysis.

Reference Standards

A2 & A3 mix

FA2 mix

Man mix

Figure 2. HILIC-UPLC-FLR profiles for the process, reference and system suitability standards

Allow for characterisation by comparison. This can be accomplished by the direct comparison of the chromatographic or electrophoretic retention time of an unknown to that of a standard whose structures have been fully characterised.

Mixtures of *N*-glycans Common to mAb Samples

A2 & A3 mix [mixture of Ludger products: CPROC-NA3-01; CPROC-A3-01C; PROC-NGA2-01; and CPROC-NA2-01]

FA2 mix [mixture of Ludger products: CPROC-FA2G1-01 and CPROC-A1F-01]

Man mix [mixture of Ludger products: CPROC-Man5-01; CPROC-Man6-01; CPROC-Man7-01; CPROCMan8- 01 and CPROC-Man9-01]

Acceptance Criteria: The A2&A3 mix, FA2 mix and Man mix show peaks at expected GU values

In order to fully characterise IgG-1 mAb *N*-glycans and identify GCQAs we:

1. Compared IgG-1 mAb HILIC profiles with reference standards for a primary glycan identification.
2. Performed exoglycosidase sequencing (Figure 3) followed by HILIC-UPLC analysis to determine glycan structures and confirm monosaccharide building blocks, linkage and sequence.
3. Performed HILIC-UPLC-ESI-MS/MS analysis (Figure 4) to confirm/determine glycan structures.

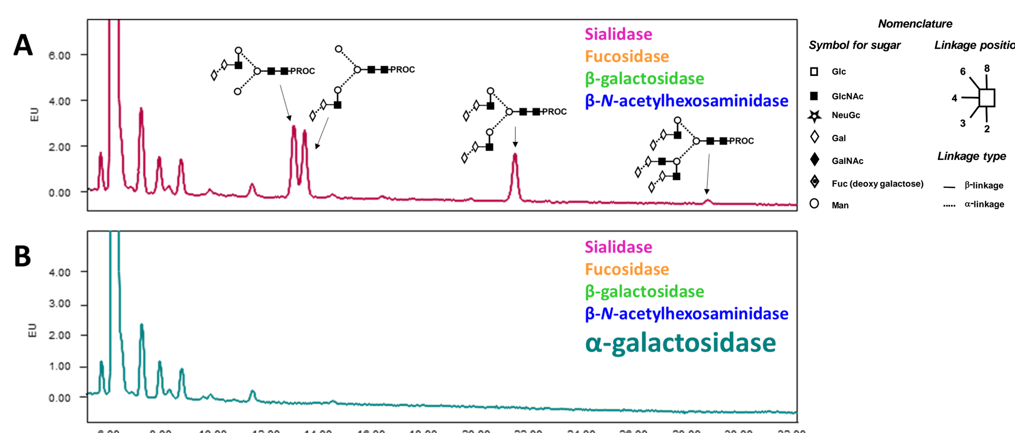


Figure 3. HILIC-UPLC profiles of the procainamide labelled N-glycans from IgG-1 mAb. (A) Profile after removal of sialic acids, fucoses, beta-galactoses, beta-N-acetylhexosamines - leaving glycans with Galα1-3Gal epitope. (B) Identities of Galα1-3Gal bearing glycans were confirmed after treatment with alpha-galactosidase.

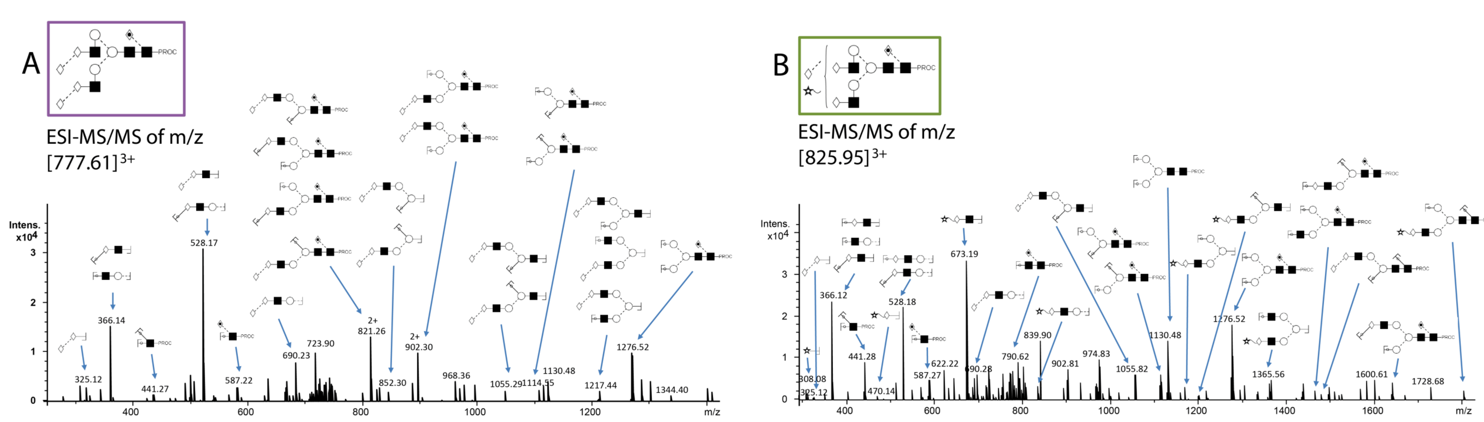


Figure 4. Example of MS/MS fragmentation patterns of procainamide labelled N-glycans released from IgG-1 mAb.

Results

Figure 5 and Table 1 summarise the results of Ludger's glycoprofiling scheme applied to quantitative characterisation of the *N*-glycans of an IgG-1 mAb. This drug contains a complex mixture of glycan structures, several of which co-elute on HILIC-LC and/or have the same mass composition. The relative proportion of glycans was determined by a matrix of HILIC-UHPLC analyses on procainamide labelled glycan samples treated with various exoglycosidase mixtures and HILIC-UPLC-ESI-MS/MS analysis.

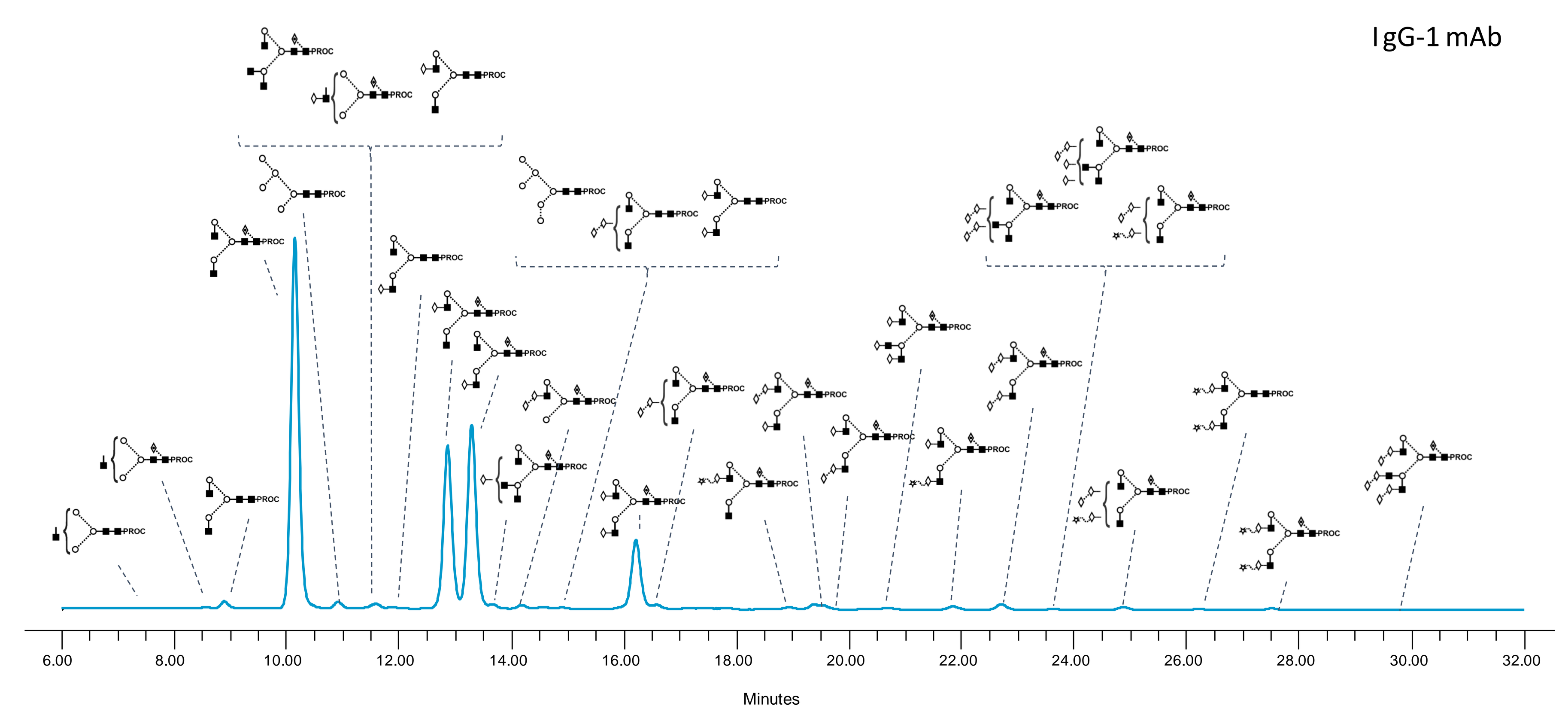


Figure 5. HILIC-UPLC profiles of procainamide labelled N-glycans released from IgG-1 mAb. Structures have been assigned by a ***range of orthogonal*** *methods including exoglycosidase sequencing and mass spectrometry.*

Structure	HILIC-UPLC		ESI-MS/MS						Degree of Certainty			
	GU	% Area	Composition				Mass found	Mass Calculated	GU	Exoglycosidase digestions	m/z at GU	MS/MS at GU
			Hex (H)	HexNAc (N)	Fucose (F)	NeuGc (Sg)						
A1[6]	4.72	0.09	3	3	0	0	$[667.28]^{2+}$	$[667.30]^{2+}$	Y	Y	Y	Y
FA1	5.10	0.21	3	3	1	0	$[740.30]^{2+}$	$[740.33]^{2+}$	Y	Y	Y	Y
A2	5.20	0.82	3	4	0	0	$[768.80]^{2+}$	$[768.84]^{2+}$	Y	Y	Y	Y
FA2	5.59	39.7	3	4	1	0	$[841.81]^{2+}$	$[841.87]^{2+}$	Y	Y	Y	Y
Man5	5.97	0.27	5	2	0	0	$[727.78]^{2+}$	$[727.81]^{2+}$	Y	Y	Y	Y
FA3	6.05	0.56	3	5	1	0	$[943.36]^{2+}$	$[943.41]^{2+}$	Y	Y	Y	Y
A2[6]G1			4	4	0	0	$[849.81]^{2+}$	$[849.86]^{2+}$	Y	Y	Y	Y
FA1G1			4	3	1	0	$[821.35]^{2+}$	$[821.35]^{2+}$	Y	Y	Y	Y
A2[3]G1	6.14	0.29	4	4	0	0	$[849.32]^{2+}$	$[849.86]^{2+}$	Y	Y	Y	Y
FA2[6]G1	6.43	18.8	4	4	1	0	$[922.85]^{2+}$	$[922.89]^{2+}$	Y	Y	Y	Y
FA2[3]G1	6.55	21.7	4	4	1	0	$[922.84]^{2+}$	$[922.84]^{2+}$	Y	Y	Y	Y
FA3G1	6.66	0.56	4	5	1	0	$[1024.37]^{2+}$	$[1024.43]^{2+}$	Y	Y	Y	Y
FA1[6]G1Ga1	6.80	0.51	5	3	1	0	$[902.33]^{2+}$	$[902.38]^{2+}$	Y	Y	Y	Y
Man 6	6.91	0.37	6	2	0	0	$[808.84]^{2+}$	$[808.78]^{2+}$	Y	Y	Y	-
A2G1Ga1			5	4	0	0	$[930.84]^{2+}$	$[930.83]^{2+}$	Y	Y		
A2G2			5	4	0	0	$[930.84]^{2+}$	$[930.89]^{2+}$	Y	Y	Y	Y
FA1[3]G1Ga1	7.00	0.24	5	3	1	0	$[902.33]^{2+}$	$[902.38]^{2+}$	Y	Y	Y	Y
FA2G2	7.34	8.89	5	4	1	0	$[669.61]^{3+}$	$[669.61]^{3+}$	Y	Y	Y	Y
FA2[3]G1Ga1	7.46	0.56	5	4	1	0	$[669.61]^{3+}$	$[669.62]^{3+}$	Y	Y	Y	Y
FA3G2	7.61	0.18	5	5	1	0	$[732.22]^{3+}$	$[731.31]^{3+}$	Y	Y	Y	Y
FA3G2	7.69	0.26	5	5	1	0	$[737.31]^{3+}$	$[737.22]^{3+}$	Y	Y	Y	Y
FA3G1Ga1	7.72	0.21	5	5	1	0	$[737.31]^{3+}$	$[737.22]^{3+}$	Y	Y	Y	Y
FA2[6]G1Sg1	7.97	0.10	4	3	1	0	$[717.94]^{3+}$	$[717.96]^{3+}$	Y	Y	Y	Y
FA2[3]G1Sg1	8.10	0.34	5	4	1	1	$[771.98]^{3+}$	$[771.93]^{3+}$	Y	Y	Y	Y
FA2G2[6]Ga1	8.22	1.09	6	4	1	0	$[723.60]^{3+}$	$[723.63]^{3+}$	Y	Y	Y	Y
FA2G2[3]Ga1	8.45	0.10	6	4	1	0	$[723.60]^{3+}$	$[723.63]^{3+}$	Y	Y	Y	Y
FA3G3	8.57	0.28	6	5	1	0	$[791.28]^{3+}$	$[791.33]^{3+}$	Y	Y	Y	Y
FA2G2Sg1	8.89	0.50	5	4	1	1	$[791.28]^{3+}$	$[791.33]^{3+}$	Y	Y	Y	Y
FA2G2Ga2	9.12	0.78	7	4	1	0	$[771.93]^{3+}$	$[771.98]^{3+}$	Y	Y	Y	Y
FA2G2Ga1Sg1	9.39	0.16	6	4	1	1	$[825.94]^{3+}$	$[826.00]^{3+}$	Y	Y	Y	Y
FA3G2Ga2 and FA3G3Ga1 (&/or)			7	4	1	0	$[845.64]^{3+}$	$[845.33]^{3+}$	Y	Y	Y	Y
FA2G2Ga1Sg1	9.73	0.43	6	4	1	0	$[825.95]^{3+}$	$[826.00]^{3+}$	Y	Y	Y	Y
A2G2Sg2	10.11	0.26	5	4	0	2	$[825.62]^{3+}$	$[826.00]^{3+}$	Y	Y	Y	Y
FA2G2Sg2	10.50	0.32	5	4	0	2	$[874.29]^{3+}$	$[874.68]^{3+}$	Y	Y	Y	Y
FA3G3Ga3	11.17	0.14	9	5	1	0	$[953.38]^{3+}$	$[953.30]^{3+}$	Y	Y	Y	Y

Table 1. Summary of GU, % Area, ESI-MS/MS and digestion data from IgG1- mAb procainamide labelled N-glycans.

Our current workflow allowed us to produce a high resolution glycan map, detect and quantify serval GCQAs (Figure 6). This information can be used by drug developers for regulatory submission.

Sialylation

The degree of sialylation can impact the function and biological half-life of biopharmaceuticals.

Results:
2.11% of glycans is sialylated

NeuGc is a non-human sialic acid. It's presence can lead to potential adverse reactions and neutralisation of the drug by anti-NeuGc antibodies

Results:
2.11% of glycans contains a non-human sialic acid (NeuGc)

N-glycan branching

N-glycan branching can influence the physicochemical properties and the metabolic turnover by modulating the overall charge, isoelectric point, size, and valence of mAbs.

Results:
Mono-antennary 1.62%
Bi-antennary 95.22%
Tri-antennary 2.17%

α1-3-linked galactose

Galactose α1-3 linked to beta galactose is a non-human glycan epitope. Its presence can lead to potential adverse reactions and neutralisation of the drug by anti-alpha-galactose antibodies.

Results: 4.22% of glycans contains the non-human epitope Galα1-3Gal.

High mannose

High mannose glycans increase serum clearance of IgG.

Results: 0.64% of high mannose type glycans

Differently galactosylated glycans

G0 G1 G2

G0 interacts with mannose binding protein to (i) activate complement and (ii) facilitate serum clearance.

Results: G0:0.82%; G1: 1.22%; G2: 0.37%

Core fucosylation

Absence of core fucose on IgG results in enhanced antibody-dependent cell-mediated cytotoxity activity (ADCC).

Results: 96.49% of glycans contains a core fucose

Figure 6. Identified GCQAs from IgG-1 mAb.

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