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Demonstrating Comparability of Antibody Glycosylation during Biomanufacturing

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Dr Daryl Fernandes is Founder and CEO of Ludger Ltd. Daryl gained his doctorate from the Glycobiology Institute at the University of Oxford. He was a consultant on biopharmaceutical glycoprofiling to Monsanto and G.D. Searle and helped spin out Oxford GlycoSciences (OGS) from the University. He joined OGS as Process Development Manager and then became Head of Analytical Services. In 1999 Daryl left OGS to set up Ludger, which develops methods to measure and control biopharmaceutical glycosylation and provides glycoprofiling technology to therapeutics companies worldwide.

Glycosylation can significantly affect the *in vivo* safety and efficacy profiles of therapeutic recombinant monoclonal antibodies (rMAbs). In particular, glycans can have a marked influence on IgG Fc effector functions and changes in antibody glycosylation are a major cause of batch-to-batch variability during production. For these reasons, it is essential to measure and control antibody glycosylation accurately and reliably. The FDA and EMEA require biomanufacturers to demonstrate comparability of glycosylation for antibody production batches. These requirements are becoming harmonised under the ICH programme. This article outlines practical steps, compliant with the ICH Q6B and Q5E guidelines, for monitoring glycosylation during antibody production and for demonstrating comparability of rMab glycosylation both during biomanufacturing and after manufacturing process changes.

ANTIBODY GLYCOSYLATION IS COMPLEX AND CAN AFFECT BOTH Fab AND Fc FUNCTIONS

Therapeutic antibodies of the IgG class produced in mammalian expression systems bear two N-glycans in the C_H2 domain of the Fc region. This glycosylation is highly heterogeneous and rMAbs produced in CHO, NSO and other popular cell systems typically contain up to 30 or so different types of glycans at each of the two Fc N-glycosylation sites. This 'microheterogeneity', together with the combinatorial pairing of glycans on the IgG heavy chains, leads to the presence of large numbers of different glycoforms in each product batch.

The Fc glycosylation can significantly modify Fc effector functions such as Fc receptor binding and complement activation. This occurs because the conformation of the immunoglobulin C_H2 region is inherently flexible, but is stabilised by the presence of the N-linked oligosaccharides. Different glycoforms stabilise different Fc conformers. The complex geometries of the immunoglobulin domains and the Fc glycans mean that even relatively small changes in carbohydrate structure can lead to functionally significant changes in protein structure. Furthermore, the effector function activities of different glycoforms can vary significantly and it is possible for a glycoform present in a relatively low abundance to make an inordinately large contribution to specific effector functions.

In addition to Fc glycosylation, some rMAbs exhibit Fab glycosylation – for example, Cetuximab (Erbitux) contains an N-glycan at Asn 99 of the V_H region. Such glycosylation can profoundly influence antigen binding affinity – as one would expect given the relatively large size of such oligosaccharides and their proximity to the antigen binding site.

As a consequence of these phenomena, diversity in Fc glycosylation within an antibody product batch will correspond to diversity in Fc effector functions, and effector function profiles of batches with different Fc glycosylation patterns can vary significantly. Also, antigen-binding affinities will relate to the Fab glycosylation profiles.

ABERRANT GLYCOSYLATION INFLUENCES rMAb SAFETY AND EFFICACY

The structural diversity found in IgG glycosylation must be preserved faithfully during biopharmaceutical production in order to maintain the normal functional diversity of the therapeutic. The consequences of producing an rMAb product batch with non-standard glycosylation can be serious. Safety issues include the production of potentially immunogenic glycoforms. For example, under certain conditions, CHO and murine cells can produce antibodies with oligosaccharides bearing N-glycolylneuraminic acid residues or Galα1-3Gal disaccharide units. These non-human glycans can produce immunogenic responses in patients. Furthermore, batches with aberrant glycosylation can exhibit non-standard efficacy profiles. For example, Genentech found that fucosylated glycoforms of Herceptin showed a 40- to 50-fold decrease in the efficacy of FcγRIII-mediated antibody dependent cellular cytotoxicity (ADCC) compared to the non-fucosylated product.

The potentially serious problems with aberrantly glycosylated product mean that it is essential to characterise and control the glycosylation of antibody therapeutics that rely on Fc effector functions. This is reflected in the regulatory directives and both the FDA and EMEA require biomanufacturers to demonstrate consistent human-type glycosylation for their therapeutic antibodies using validated glycoprofiling systems.

IMPROVING ANTIBODY THERAPEUTIC PROFILE BY GLYCOENGINEERING

Glycosylation should not be seen merely as a source of headaches for biomanufacturers. Drug developers can take advantage of the relationship between Fc and Fab glycosylation and mAb efficacy by improving therapeutic potency through skilful control of the product's glycoform profile. For example, with Biogen-Idecs Rituximab, an rMAb licensed for treatment of non-Hodgkin's lymphoma, glycoforms bearing bisecting GlcNAc (+Bi structures) and those without core fucose (-F structures) show significantly enhanced efficacy for ADCC-induced killing of malignant B cells *in vitro* compared to the equivalent -Bi and +F forms. In such cases, glycoengineering can be achieved through: manipulation of the protein structure to alter the oligosaccharide profile at each glycosylation site; the use of a cell expression system with improved glycosylation machinery (for example after transfection with the appropriate glycosyltransferases); or isolation of sub-fractions enriched in the more potent glycoforms (such as with lectin affinity chromatography).

DEFINING SPECIFICATIONS FOR ANTIBODY GLYCOSYLATION

Specifications for antibody glycosylation during biomanufacturing are comprised of:

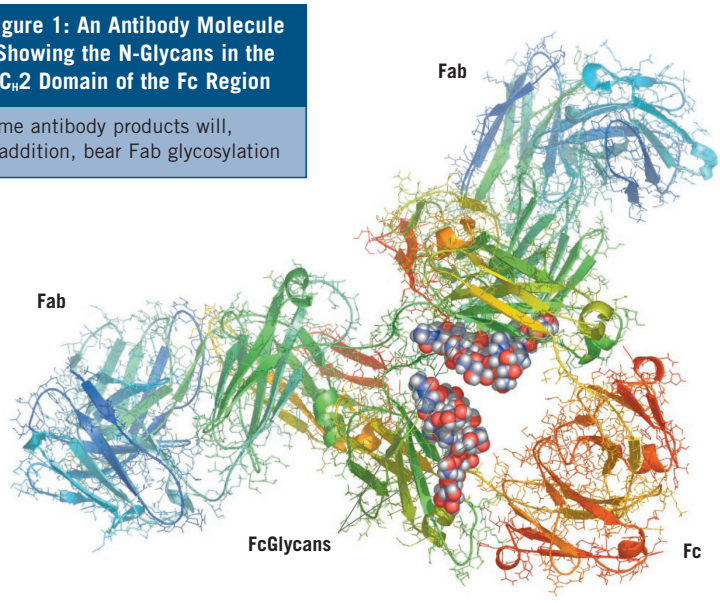
- Molecular specifications that define the limits for the glycoform patterns proven to have the desired product safety and efficacy profiles
- Measurement specifications that relate to the glycosylation parameters measured during product characterisation

The specifications must cover both the desired pattern of human-type oligosaccharides and the upper limits for undesirable (for example non-human or potentially immunogenic) glycans. For antibodies with Fab glycosylation, you should define, wherever possible, molecular specs for the Fc and Fab glycosylation sites separately. Defining the specifications is not trivial – antibody glycoform patterns can be complex. The current licensed therapeutic antibodies typically display a set of up to 32 different biantennary human-type N-glycans (see Figure 1). These oligosaccharides can be classified as follows:

- The number and type of acidic charged sialic acid residues (neutral (N), monosialylated (A1) or disialylated (A2) classes)
- The number of galactose residues (agalacto (G0), monogalacto (G1) or digalacto (G2) classes)
- The presence or absence of a bisecting N-acetyl-glucosamine residue between the two antennae of the glycan (± Bi classes)
- The presence or absence of core fucose (± F classes)

Figure 1: An Antibody Molecule Showing the N-Glycans in the C_H2 Domain of the Fc Region

Some antibody products will, in addition, bear Fab glycosylation



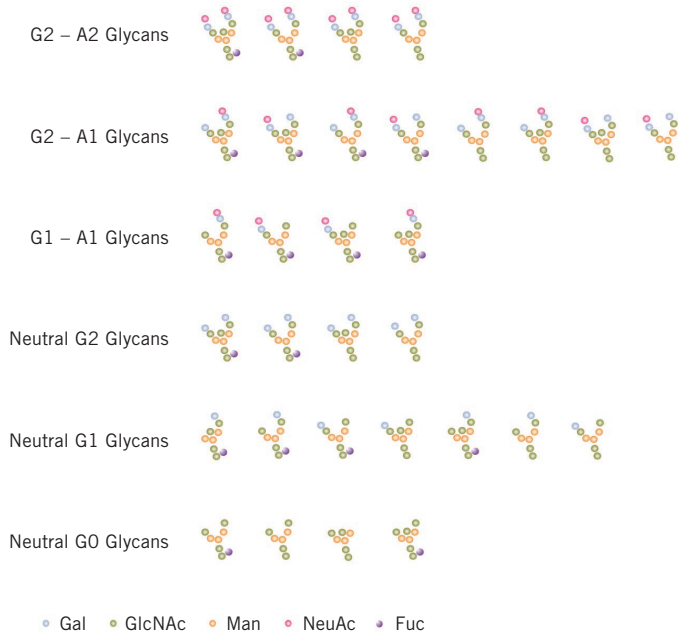
As for aberrant glycans, those that are found in a particular batch will depend on the expression system and the production conditions.

When drawing up the molecular specs, consider:

- The overall glycoform profile obtained during stable production
- Specific Fc glycoforms that contribute most to the desired effector functions
- Specific Fab glycoforms that most affect antigen binding
- Aberrant glycoforms known to appear in your expression system and which either have altered potency or are potentially immunogenic

Figure 2: Human-Type Oligosaccharides Commonly Found in Therapeutic Monoclonal Antibodies

The glycans are classified into G0, G1 and G2 groups according to the number of galactose residues.



MEASURING MAB GLYCOSYLATION PARAMETERS

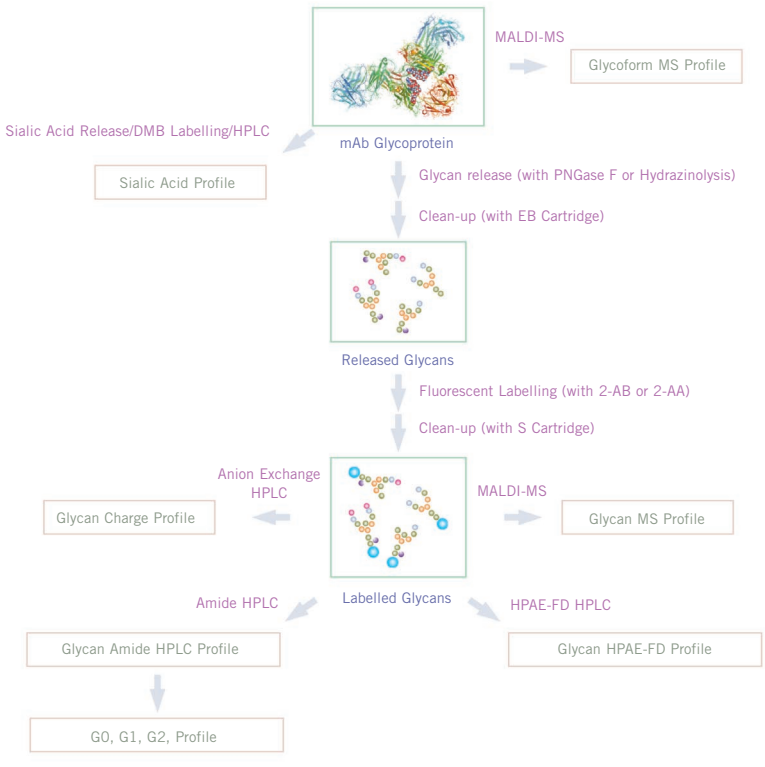
The next stage is to establish a set of structural glycosylation parameters that you can measure to determine if a particular antibody batch conforms to the molecular specs. Candidate parameters include the monosaccharide composition, oligosaccharide profile (for released glycans), glycosylation site profile (for glycopeptides) and the glycoform profile (for the intact antibody). These can be measured in many ways, but biomanufacturers have invariably standardised on antibody glycoprofiling systems with core modules based on HPLC or CE and MS. This approach has many advantages, including regulatory authority approval (standard methods are already used for QC of licensed therapeutic antibodies), good to excellent reproducibility, high accuracy for quantitation, cost effectiveness (you can use analytical instruments already employed for protein characterisation), availability of commercial analysis kits and ease of use. In particular, the system is very flexible – you can mix and match the standard glycoanalysis modules to cope with all stages of drug development from glycoprotein design, to IND submissions and production.

ICH COMPLIANT GLYCOPROFILING SCHEMES

Figure 3 outlines an ICH Q6B compliant glycoprofiling scheme suitable for product lot release of a therapeutic antibody. It consists of various profiles that are measured on either the intact glycoprotein or the released, fluorescently labelled glycans. These profiles cover the key glycosylation parameters that relate to the *in vivo* safety and efficacy profiles of a therapeutic mAb that requires

Figure 3: A Glycoprofiling Scheme Suitable for Therapeutic Monoclonal Antibodies

This satisfies the ICH Q6B guidelines on glycan characterisation for product lot release. In practice, QC labs could determine a subset of the glycan profiles depending on the particular drug and the analytical facilities available.



consistent human-type Fc glycosylation. The scheme has many advantages for biomanufacturers. First, it will give reliable, relevant information on the glycosylation pattern of the product. It is built around standardised methods that are currently used for lot release of licensed therapeutic mAbs. It relies on analytical instrumentation commonly used in biopharmaceutical QC labs. It is flexible; you can adapt and optimise it for your product and your laboratories. Furthermore, this flexibility extends beyond QC for product lot release. Consider the scheme as a set of integrated analysis modules that can either be supplemented with other modules or slimmed down to cope with your glycoprofiling requirements throughout the development of your antibody, ranging from design of the glycoprotein, clonal selection, choice of the cell expression system, optimisation of culture conditions and IND submissions, through to bioprocess monitoring and demonstrating comparability after manufacturing changes. The profiles in the core mAb glycoprofiling scheme are as follows:

Sialic Acid Profile

This indicates the relative levels of potentially dangerous non-human N-glycolyl-neuraminic acid (NeuGc) and desired human-type N-acetyl-neuraminic acid (Neu5Ac). The Neu5Ac level is related to the *in vivo* clearance rate of the therapeutic.

Glycan Amide HPLC – G0, G1, G2 Profile

This indicates the overall glycosylation pattern and allows you to monitor: (a) specific glycoforms important for maintaining standard drug potency (for example glycoforms with bisecting GlcNAc or non-fucosylated oligosaccharides that have controlling influence on activation of the immune response) and (b) aberrant glycoforms that could cause adverse reactions (such as those containing the potentially immunogenic non-human Gallili antigen Galα1,3Gal).

Glycoform MS Profile

This gives you a rapid check on the overall glycoform pattern of the intact mAb. Be aware that, with current MS technology, the data will be only semi-quantitative. However, it can be an effective process analytical technology (PAT) tool and, when supplemented with other tests, will allow you to optimise conditions during biomanufacturing runs so you make a consistent product.

Glycan MS Profile

MS analysis on the fluorescently labelled glycans supplements the glycan amide HPLC profile. The structural information can be enhanced with fragmentation of the oligosaccharides either in the instrument (MS^n) or by enzyme treatment (using exoglycosidases such as sequencing grade sialidase, galactosidase, fucosidase and hexosaminidase). This approach helps give greater resolution of the glycan profiling by allowing rapid verification of oligosaccharide primary structures.

Glycan Charge Profile

This allows you to monitor the overall level of sialylation. The resolution is relatively low compared to the amide and HPAE-FD profiles. However, the method is simple and relatively fast, the quantitation is good and the data can be invaluable. Use it like a pulse check for healthy glycosylation; if the charge profile is abnormal, you know something is wrong and should investigate further.

Glycan HPAE-FD (High pH Anion Exchange with Fluorescence Detection) Profile

This can be used as an HPLC fingerprint of mAb glycosylation. It has good resolution for both neutral and sialylated oligosaccharides and better reproducibility and quantitation than the related HPAE-PAD (HPAE with pulsed amperometric detection) method.

CONCLUSION

Biomanufacturers are required to measure and control the glycosylation of therapeutic monoclonal antibodies. However, mAb glycosylation is complex and accurate, reproducible glycoprofiling can be challenging. This article has outlined the principles of an effective glycoprofiling scheme that satisfies the requirements of the ICH Q6B guidelines, is based on validated technology and can be implemented by quality control labs with standard analytical resources. Furthermore, the system is flexible and can be modified for glycan characterisation at all stages in the antibody drug development cycle, from early stage studies through to IND submissions. ◆

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