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Reducing Risk in Biopharmaceutical Production by Controlling Glycosylation

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GLYCOSYLATION AND RISK IN BIOPHARMACEUTICAL PRODUCTION

The most commercially successful biopharmaceutical to date is recombinant erythropoieitin (EPO). In 2003, the combined sales of EPO variants reached $9.4 billion. However, its commercial success has been tempered by serious manufacturing problems, including QC failure of product batches, concerns about drug safety, intense competition to produce more effective EPO variants and much litigation between competing companies. Many of the problems with EPO relate to the characterisation and control of its glycosylation. These difficulties apply not only to EPO, but to many other glycoprotein therapeutics, and arise because glycosylation can introduce great complexity and heterogeneity into biopharmaceuticals, significantly influence product safety and efficacy, and vary considerably between batches.

In some cases, even small changes in cell culture conditions during production can cause aberrant glycosylation, which may or may not be recognised during final QC. This leads to a number of potential risks:

- True batch failure – the batch fails final QC and is discarded because the glycosylation pattern is out of specification
- False batch failure – the product is within specification, but final QC indicates aberrant glycosylation (for example, because of degradation during sample work-up) and the batch is failed erroneously
- Safety compromised – aberrant glycosylation is not detected during final QC, the product is released and safety is compromised. For example, antigenic components are produced because the protein conformation is altered, new peptide regions are unmasked or there is an increased tendency for aggregation
- Non-standard efficacy – the batch is released with undetected aberrant glycosylation and the *in vivo* efficacy is significantly altered; for example, there is a non-standard serum half-life due to modified sialylation or product solubility is changed

Another major risk with products that do not have well-characterised and controlled glycosylation is the low barrier for competition from manufacturers of follow-on biologics who can more easily demonstrate comparability of their products to yours. The many difficulties with glycosylation have lead the FDA, EMEA and other regulatory bodies to increase pressure on biomanufacturers to demonstrate that they have satisfactory programmes to understand, measure and control glycans.

This article examines the lessons we can learn from EPO and other cases, and how this knowledge can be used to implement an effective glycan measurement and control system designed to minimise the technical, commercial and legal risks associated with glycosylation.

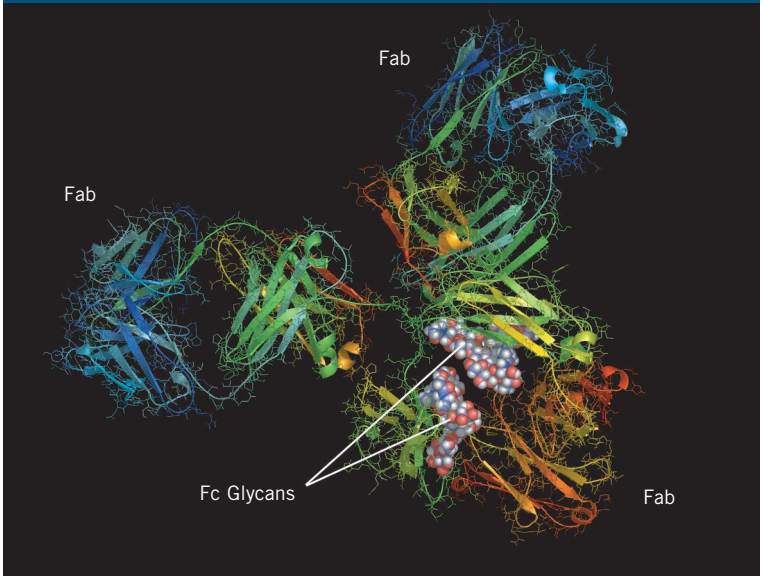
UNDERSTANDING GLYCOSYLATION IN THE NATURAL MOLECULE

The first step is to understand the structure-function relationships for glycosylation in the natural molecule. This must achieve a balance between *in vitro* assays and *in vivo* studies, since changes in glycosylation can significantly affect *in vivo* behaviour of therapeutic glycoproteins, even when *in vitro* activity is unaltered. In the case of EPO, the functional importance of the glycans is indicated by the high degree of sequence homology among mammals where N-glycosylation sites are conserved. Natural human EPO has three N-glycosylation sites and one O-glycosylation site. The glycosylation affects the bioactivity and *in vivo* behaviour of the molecule. It influences serum half-life, protects against proteolysis and aggregation induced by heat and low pH and masks antigenic sites on the protein. Removal of any of the N-

glycosylation sites alters proper biosynthesis and secretion of the molecule, and reduces *in vivo* but not *in vitro* biological activity. Furthermore, correct sialylation is essential for optimal *in vivo* activity, in part by enhancing serum half-life.

Glycosylation also plays an important role in the *in vivo* function of antibodies. IgG molecules bear two N-glycans in the Cγ2 domain of the Fc region. This glycosylation is highly heterogeneous – typically, antibodies produced in mammalian expression systems contain over 30 different types of N-glycans. This 'microheterogeneity', together with the combinatorial pairing of glycans on the heavy chains, leads to the presence of very large numbers of different glycoforms in each product batch. Fc glycosylation is known to significantly influence Fc effector functions such as Fc receptor binding and complement activation. This arises because the conformation of the immunoglobulin Cγ2 region is inherently disordered but is stabilised by the presence of aparagine-linked glycans. Different glycoforms stabilise different Fc conformers. The complex geometries of the immunoglobulin domains and oligosaccharides mean that even relatively small changes in glycan structure can lead to functionally significant changes in protein structure. Furthermore, the effector function activities of different glycoforms can vary significantly. This means that a glycoform present in a relatively low abundance can make an inordinately large contribution to specific effector functions. In this way, diversity in Fc glycosylation leads to significant diversity in Fc effector functions. This has consequences for biomanufacturers, and the current thinking is that the natural structural diversity found in IgG glycosylation should be preserved faithfully during biopharmaceutical production in order to maintain functional diversity of the therapeutic. In short, it is felt that it is essential to characterise and control the glycosylation of antibody therapeutics that rely on Fc effector functions.

Figure 1: Structure of an IgG Molecule showing N-glycosylation of the Fc Region



DETERMINING GLYCOIMPORTANCE *IN VIVO*

Knowledge of the glycan structures and functions in the natural molecule makes it possible to assess the probable *in vivo* glycoimportance of the therapeutic molecule to one of the following categories: glycoessential, glycoconcern or glycoirrelevant – depending on whether therapeutic effectiveness is influenced by glycosylation significantly, moderately or not at all. The glycoimportance class offers a guide as to how much effort should be put into controlling glycosylation of your therapeutic.

CONSIDERING GLYCOSYLATION THROUGHOUT THE DRUG LIFE CYCLE

Knowledge of the glycan structures and functions in the natural molecule will prepare you to consider glycosylation throughout the drug life cycle – from design of the polypeptide backbone, engineering of glycosylation sites, selection of the cell expression system, clonal selection and optimisation of the cell culture conditions, through to characterisation for drug approval, product manufacturing and final QC for product lot release. Note that the type and extent of glycoprofiling work to be done will vary considerably depending on the glycoimportance class and the particular stage in the drug life cycle.

SETTING MOLECULAR AND MEASUREMENT SPECIFICATIONS

Setting specifications for biopharmaceutical glycosylation is not straightforward. The main problems are: the products are invariably heterogeneous and exceedingly complex, our understanding of the structure-function relationships for glycans is not complete; and current tools for glycoanalysis are imperfect. The heterogeneity and complexity of glycosylation means that each product batch represents not a single point but rather a multidimensional hypercube in an n-dimensional structural parameter space. This means that glycan specifications for biopharmaceuticals must accommodate variation in several types of glycosylation parameter.

A useful approach to adopt is to draw up two sets of specifications – molecular and measurement:

Molecular Specifications

First, use your understanding of the structure-activity relationships for the glycosylation of the natural molecule to define an initial set of molecular specifications which determine the limits for the glycoform distributions so far proven to be safe. Secondly, modify the initial molecular specs to apply to the therapeutic product. Thirdly, when setting the molecular specs, be aware that even structurally related products (for example, a family of MAbs) could have different glycan structure-function profiles – so treat each product as a new case.

Measurement Specifications

Choose a set of glycosylation parameters that can be measured and would provide a good indication of product batches lying

outside the molecular specs. Secondly, select tools and standards that allow you to measure the chosen glycosylation parameters accurately and reproducibly. Finally, select a reference batch of your product, but note that it may not lie in the very centre of molecular specification space.

Review and Modify

These specifications must be reviewed and adjusted throughout the lifetime of the drug – especially after significant manufacturing process changes. This strategy increases your chances of developing a reliable, flexible and suitable system for glycan characterisation and gives you an advantage over competitors with less developed systems.

CHOOSING WHICH GLYCOSYLATION PARAMETERS TO MEASURE

It is not possible to measure every type of glycosylation parameter. Consequently, for each stage of the product's life cycle, you must measure a subset of parameters that give a reliable indication of conformance to the molecular specifications. There are four main groups of glycosylation parameter to choose from:

Monosaccharide Profile

This profile indicates the sugar monomer composition. The general consensus is that monosaccharide profiles are of limited use since, in many cases (for example, with MAb Fc glycans) functionally significant variations in glycosylation may not produce noticeable changes in the monosaccharide profile. Secondly, there are serious problems with the accuracy and reproducibility of many monosaccharide analysis technologies. However, monosaccharide profiles can allow you to monitor for gross changes in oligosaccharide class distributions, and help direct sequencing strategy.

Oligosaccharide Profile

This is the most widely used type of glycoanalysis for final QC of biopharmaceuticals. It can provide valuable data on the relative quantities of either particular groups of glycans or individual species. This allows you to monitor certain types of change in the carbohydrate patterns (for example, altered levels of sialylation or increases in branching) and can help identify aberrant glycosylation.

Glycosylation Site Profile

This consists of the set of oligosaccharide profiles for each glycosylation site in the glycoprotein. This information is invaluable when particular glycosylation sites are known to have particular influence on, for example, product expression levels, bioactivity or stability.

Glycoform Profile

This estimates the relative abundances and glycosylation site profiles of each of the glycoforms in a product sample. The quantitative glycoform profile is the highest level of glycan characterisation and, with current technology, is the most challenging to produce.

Each of these profiles can be done at different levels of resolution, structural detail and quantitation.

SELECTING APPROPRIATE GLYCOANALYSIS METHODS

Commonly used methods include:

Monosaccharide Profile

Most monosaccharide analyses start with acid hydrolysis of the glycoprotein to release the monosaccharides, followed by derivatisation then analytical separation. Derivatisations include reductive amination labelling with a wide range of fluorescent or chromophoric tags and chemical modifications of the hydroxyl groups. Analytical separations include HPAE-PAD or HPAE-FD chromatography on underivatised or fluorescently labelled monosaccharides, hydrophobic and hydrophilic phase HPLC and anion exchange chromatography with borate buffers, GC/MS, PAGE and CE.

Oligosaccharide Profile

A typical oligosaccharide profiling scheme for biopharmaceutical QC involves release of glycans either with enzymes (for example PNGase F) or chemical methods (such as hydrazinolysis), fluorescent labelling at the sugar reducing termini and analysis by a combination of HPLC (for quantitation) and mass spectrometry (for structure determination).

Glycosylation Site Profile

A typical glycosylation site analysis involves proteolytic digestion of the glycoprotein, purification of the glycopeptide fraction, isolation of the glycopeptides of each glycosylation site (for example, by hydrophobic phase HPLC) then oligosaccharide profiling of each of the sites.

Glycoform Profile

Current methods for effective glycoform profiling of biopharmaceuticals are limited. However, promising methods in development include those involving capillary electrophoresis, anti-glycan receptors and mass spectrometry on the intact glycoproteins.

The range of glycoanalysis techniques can be bewildering. For example, purification of glycans after PNGase F release can be done by solvent precipitation of either glycans or protein, solid phase extraction using hydrophobic or electron interaction resins, size exclusion chromatography or hydrophilic interaction chromatography. These methods vary in their performance, reliability and suitability for work on glycoprotein therapeutics and some can give non-stoichiometric recovery of oligosaccharides, leading to distorted glycoprofiles.

Use of inappropriate technology can lead to serious problems for biomanufacturers. These have included: delays in release of product and suspension of manufacturing after failure at final glycoprofiling QC; non-reproducible QC; and use of QC methods with insufficient resolution to detect aberrant glycosylation.

The difficulties in choosing appropriate glycoanalysis methods have been compounded by problems with the quality and supply of key reagents and instrumentation. In recent years, these have included technologies for automated hydrazinolysis, carbohydrate gel electrophoresis and recombinant PNGase F. In many cases, QC labs have responded to these problems by switching to in-house technology or use of commercial glycoanalysis kits based on non-proprietary technology.

Fortunately, there are initiatives that will provide help in choosing suitable glycoanalysis methods. For example, in Europe, the UK Department of Trade and Industry (DTI) has set up the Measurements for Biotechnology PC2 Biopharmaceuticals Microheterogeneity Programme. The practical outcome of this will be a comprehensive selection guide for glycan characterisation methods for biopharmaceuticals.

OPTIMISING AND VALIDATING YOUR INTEGRATED GLYCOPROFILING SYSTEM

Your chosen glycoanalysis methods need to fit together to form an integrated glycoprofiling system. This will involve a number of multistep processes and you may need to modify or add steps so that the system works smoothly and gives accurate, reproducible results.

During validation, you should demonstrate that your glycoprofiling system gives equivalent results to other validated systems using a panel of well-characterised standards. Depending on the parameters you need to measure, these could include a selection of glycoproteins, glycopeptides, glycan libraries, individual glycans, monosaccharides and derivatised versions of those compounds.

Validation should be done at two levels – qualitative (where you check that your system identifies the structures declared in the standards) and quantitative (where you check your system gives relative abundances for either groups of structures or individual structures equivalent to those declared for the standards). If you get a mismatch, look for sources of structural modification or selective loss during sample processing or misinterpretation of your experimental data; identify problem areas by using standards introduced at different points throughout the sample processing pipeline. This will also allow you to select a set of standards to use routinely as part of operational qualifications for your glycoprofiling system.

You can reduce your development work and increase system reliability by standardising on key glycan analysis modules throughout your organisation. This can be done either by using in-house technology with reagents and protocols controlled by one group or by using commercial glycotechnology kits that are already used in FDA and EMEA approved analysis procedures.

CHARACTERISING AND OPTIMISING YOUR BIOPHARMACEUTICAL PRODUCTION SYSTEM

You can now use your validated glycoprofiling system to characterise the glycosylation machinery of your biopharmaceutical production system. In particular, you need to understand which factors influence glycosylation and how the glycans change for your product. Pay particular attention to cell culture conditions such as pH, temperature, nutritional status, cell age and time of harvesting – all can influence glycosylation significantly. This should allow you to engineer your product and optimise your production system in order to achieve the desired glycosylation pattern for your target bioactivity profile consistently and minimise batch to batch variation.

REVIEWING AND UPDATING YOUR GLYCAN CONTROL SYSTEM

We are still at the junior stages of understanding biopharmaceutical glycosylation, but our knowledge is constantly improving. Take advantage of these improvements by implementing a programme of periodically reviewing and updating your specifications, analysis methods and the knowledge base relating to the glycans of your product. Where appropriate, get advice from a specialist glycoanalysis group – but check their track record first. All this will help you to maintain a glycosylation control system that is efficient and relevant to the production of your therapeutic glycoprotein.

CONCLUSION

This article has outlined a systematic approach for effective measurement and control of biopharmaceutical glycosylation. The benefits of this are manifold. Such a system will help you to produce a more consistent and better characterised product with reduced manufacturing problems and fewer issues regarding product safety and non-standard efficacy. In short, you should be able to reduce the many technological, commercial and legal risks associated with the complexity and variability of biopharmaceutical glycosylation. ◆

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