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Biopharmaceutical Sialylation

Dr Daryl L Fernandes at Ludger Ltd elucidates the significance of sialic acids in glycoprotein therapeutics and outlines a scheme for effective profiling and regulatory compliance

Dr Daryl Fernandes gained his doctorate at the Glycobiology Institute, University of Oxford. He was a consultant on biopharmaceutical glycoprofiling to Monsanto and GD Searle and helped spinout Oxford GlycoSciences (OGS) from the University. He joined OGS as Process Development Manager, before becoming the Head of Analytical Services. In 1999, Daryl left OGS to set up Ludger, a company that develops methods to measure and control biopharmaceutical glycosylation and provides a range of glycoprofiling systems and services to pharmaceutical and biotech companies worldwide.

Biopharmaceutical sialylation refers to the type and distribution of sialic acids in the glycans of therapeutic glycoproteins. Sialylation can significantly influence the safety and efficacy profiles of these drugs. In particular, the *in vivo* half-life of some biopharmaceuticals correlates with the degree of oligosaccharide sialylation. Furthermore, the sialylation pattern can be a very useful measure of product consistency during manufacturing (1). Advances in our understanding of these issues have led the FDA, EMEA and other regulators to tighten their rules on glycoprofiling throughout the drug life cycle. These rules are being harmonised under the ICH programme and the ICH Q6B and Q5E guidelines cover requirements for characterisation of biopharmaceutical sialylation during normal biomanufacturing and after biomanufacturing changes. This article is a guide for biomanufacturers on how to assemble a practical, ICH-compliant glycoprofiling scheme for characterisation of biopharmaceutical sialylation.

HUMAN-TYPE AND ABERRANT SIALYLATED GLYCAN STRUCTURES

Sialic acids are a family of 9-carbon monosaccharides with heterocyclic ring structures. They bear a negative charge via a carboxylic acid group attached to the ring as well as other chemical decorations including N-acetyl and N-glycolyl groups. The two main types of sialyl residues found in biopharmaceuticals produced in mammalian expression systems are N-acetyl-neuraminic acid (NeuAc) and N-glycolyl-neuraminic acid (NeuGc). These usually occur as terminal structures attached to galactose (Gal) residues at the non-reducing terminii of both N- and O-linked glycans. The way that NeuAc and NeuGc attach to other sugar residues is different from that of the hexoses found in therapeutic glycoproteins – the glycosidic linkage configurations for these sialyl groups are usually either α2,3 or α2,6 compared to α1,x or β1,x for the hexoses (where x indicates the carbon atom of the adjacent monosaccharide).

Controlling the ratio of NeuAc to NeuGc is critical for biomanufacturers. The reason is that NeuAc is the desired, normal human-type sialylation, while NeuGc is found in non-human glycoproteins and is considered an undesired, aberrant form of sialylation for therapeutic glycoproteins. We should note that our understanding of the safety of NeuAc vs NeuGc is still unfolding, with pioneering work being done in the labs of Professor Ajit Varki and colleagues (2). However, the story for biomanufacturers is simple – you should aim to maximise the ratio of NeuAc to NeuGc. A major influence on this ratio is the composition of the cell culture medium. Varki's work has shown that cells can scavenge sialic acids from glycoproteins in nutrients and incorporate these sialyl groups into their own glycoconjugates. Non-human sera used in media during drug production can contain NeuGc bearing glycoproteins that can end up in the therapeutic. This can result in the final product going out of specification. Measuring the NeuAc to NeuGc ratio with appropriate process analytical technology (PAT) throughout the cell culture, and on media before they are added to the cells, allows you to control culture conditions to keep NeuGc within acceptable levels.

As well as the ratio of the human and non-human type sialyl residues, biomanufacturers should also measure the distribution

of the sialic acids amongst the individual oligosaccharides found on their therapeutic. The relevant glycoprofiles for this are:

- **The glycan charge profile** – this shows the distribution of anionic glycan species – that is the neutral (non-charged), mono-, di-, tri- and tetra-charged glycans. To prove that the anionic charges are due to sialyl residues (rather than, say, phosphorylated or sulfated monosaccharide residues) the charge profile can be determined before and after treatment with a sialidase. Alternatively, sialylated species can be identified by mass spectrometry.

- **The detailed oligosaccharide profile** – this involves fine separation and quantitative detection of the various sialylated oligosaccharide species.

Methods for characterising biopharmaceutical sialylation in a production environment by determining the NeuAc:NeuGc ratio, charge profile and detailed oligosaccharide profile are outlined below after some tips on handling sialylated biopharmaceuticals during glycoprofiling QC.

Handling Sialylated Biopharmaceutical Samples

Sialylated biopharmaceutical samples, as well as sialylated glycans derived from biopharmaceuticals, must be handled with care as they can undergo spontaneous desialylation. This can cause tremendous problems and we know of cases where worldwide production of approved biopharmaceuticals has been halted for several days because of desialylation of QC samples during final tests for lot release. Such problems can be avoided by following good sample handling practice.

The three main causes of desialylation of QC samples are acid catalysed degradation, unintended enzymatic treatment and microbial contamination. The first of these can be minimised by, as far as possible, keeping the sample buffered above pH4 and at temperatures lower than 30^{0}C during workup and analysis; storage samples should be frozen at -20^{0}C or lower. Biopharmaceutical glycan samples are particularly vulnerable to acid catalysed desialylation during drying steps. We recommend the use of vacuum drying methods such as centrifugal drying with minimal heating. Make sure your vacuum pump is pulling a good vacuum and that all the seals in the drying system are well-maintained. Avoid the use of warm gas drying methods as these can cause rapid desialylation.

Unintended enzymatic desialylation can occur in a number of ways. The most common are sialidase contamination from pipettors and sialidase contamination of HPLC columns. The latter can occur when sialidase treated samples are loaded onto HPLC columns without adequate pre-chromatography cleanup. The glycosidases can become immobilised onto the column and, being tough enzymes, can remain active and degrade any glycans subsequently loaded onto the column. The rules are:

- Keep things clean

- Use filtered pipette tips

- Clean up enzyme treated samples before injecting them on to your HPLC system

Sample desialylation caused by microbial contamination can be minimised by adding anti-microbial agents such as sodium azide or low concentrations of acetonitrile or toluene to your sample. Take care, however, to check the compatibility of these agents with downstream procedures.

DETERMINING THE NeuAc: NeuGc RATIO

A widely used method for determining the NeuAc:NeuGc ratio is HPLC analysis of sialic acids labelled with fluorescent DMB (1,2-diamino-4,5-methylenoxybenzene). In our experience, when performed with good quality reagents, a robust, optimised protocol and suitable calibration standards, this procedure is capable of giving accurate, reproducible quantitative data. As well as use in final QC for lot release, DMB analysis is fast enough to be used as a PAT method for monitoring levels of non-human sialylation throughout cell culture before product harvesting. This early warning system can save a great deal of time and money, and significantly reduce the risk of product batch failure.

The first step in DMB analysis is release of the sialic acids from the therapeutic glycoprotein. This can be done using mild acid hydrolysis. Typical conditions are incubation of the sample with 2M acetic acid for two hours at 80^{0}C. These can be varied to effect faster release if a rapid analysis is needed. The released sialic acids are then fluorescently labelled using DMB and the tagged conjugates stabilised by reduction with sodium dithionite. Analysis of the DMB labelled sialic acids is done by HPLC with a C18 column and fluorescence detection (λex=295 nm, λem=352nm).

With DMB analysis (as with all glycoprofiling), good calibration is essential for accurate, reliable results. For NeuAc:NeuGc determinations we recommend calibration using three standards:

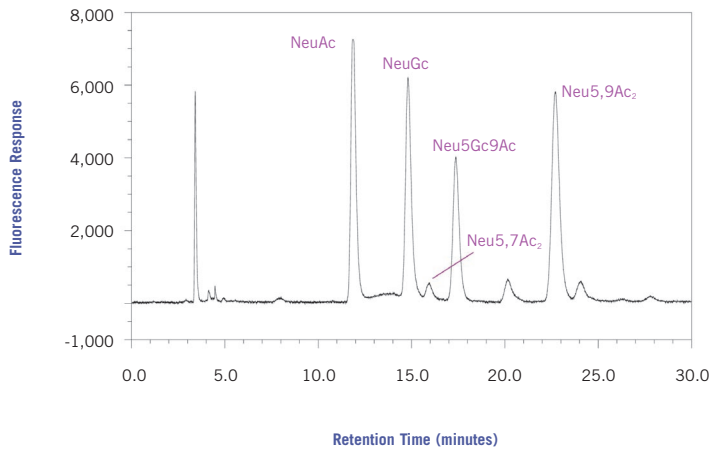
1. A sialic acid reference panel containing a mixture of sialic acids found in human and animal tissue. This is used as a system suitability standard and to help identify any unusual sialic acids in your drugs.

2. A quantitative NeuAc standard. Serial dilutions of this are used to obtain a standard concentration-fluorescence response curve on the HPLC.

3. A qualitative NeuGc standard. This can either be used alone or can be added to aliquots of the NeuAc standard for calibration of retention on HPLC. Figure 1 shows a typical HPLC trace of DMB labelled sialic acid reference panel.

Alternatives to DMB analysis include HPLC of OPD (o-phenylenediamine) labelled sialic acids (3) as well as HPAE-PAD (high pH anion exchange with pulsed amperometric detection) and hybrid variants of these procedures.

Figure 1: Typical Profile for a DMB Labelled Sialic Reference Panel



PREPARATION OF SAMPLES FOR GLYCAN CHARGE AND DETAILED OLIGOSACCHARIDE PROFILES

The glycan charge and detailed oligosaccharide profiles require detachment of the glycans from the protein backbone followed by cleanup and derivatisation. These processes must be done stoichiometrically and with high efficiency.

Glycan detachment can be achieved either enzymatically using a suitable endoglycosidase (such as PNGase F or PNGase A) or chemically (using hydrazinolysis for example). The method you choose depends on several factors, including whether you have N- or O-linked glycans or both on your biopharmaceutical and the facilities in your lab (current hydrazinolysis methods require specialised sample handling facilities). These issues will be the subject of a future article.

Whichever method is used for glycan detachment, the released oligosaccharides should be purified to remove non-carbohydrate material that could interfere with subsequent derivatisation and analysis. This can be done very effectively using solid phase extraction (SPE) cartridges containing electronic interaction resin (EIR). EIR is a remarkable substance which acts like a super hydrophobic phase that binds glycans, allowing efficient removal of contaminating salts, detergents and protein.

The final stage of glycan sample preparation is derivatisation with a fluorescent tag such as 2-aminobenzamide (2-AB) or 2-aminobenzoic acid (2-AA). Labelling with these allows quantitative detection of the glycans on HPLC and CE and improves analysis of sialylated species on MS. These particular tags have many advantages. They are small and hydrophilic, they conjugate to the glycan efficiently and have good spectral properties. Furthermore, they have minimal effect on the specificities of glycosidases, which aids enzymatic glycan sequencing. The labelling reaction for 2-AB and 2-AA is reductive amination and results in attachment of the fluorophore at the reducing terminus of the sugar (that is distal to the end containing the sialyl residues). This allows analysis of sialylated species by enzymatic desialylation with specific sialidases, and is particularly useful when used together with the glycan charge profile.

DETERMINING THE GLYCAN CHARGE PROFILE

The glycan charge profile can be determined by anion exchange chromatography of the fluorescently labelled oligosaccharides. Typical running conditions are HPLC with a sodium acetate gradient using a column containing a polymer based wide pore strong anion exchange (SAX) resin. Weak anion exchange (WAX) columns can be used, but SAX systems seem to be more robust and give more reproducible results. Fluorescence detection of 2-AB or 2-AA labelled glycans on SAX columns can be done using λex = 365nm, λem= 420nm. Figure 2 shows a typical glycan charge profile

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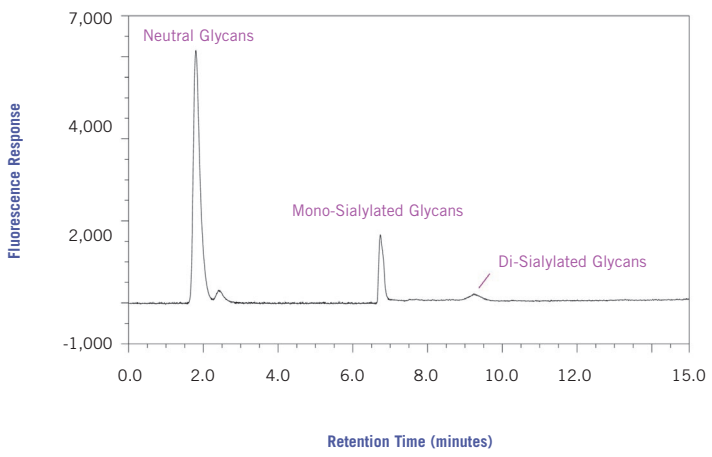
Figure 2: Typical Glycan Charge Profile of 2-AB Labelled Oligosaccharides



of a 2-AB labelled oligosaccharide mix containing asialo (neutral charge), mono-, di- and tri-sialylated species. After treatment with *Arthrobacter ureafaciens* sialidase (a broad specificity sialidase), all of these collapse down to the asialo glycan position.

DETERMINING THE DETAILED SIALYLATED OLIGOSACCHARIDE PROFILE

A detailed sialylated oligosaccharide profile can be obtained by fluorescence amide HPLC of the 2-AB and 2-AA labelled glycans. This glycoprofiling method is widely used for quality control of approved biopharmaceuticals including erythropoietin (EPO) and monoclonal antibodies. The advantages include excellent reproducibility, good quantitation and ease of use. The main disadvantage is the long run-time, but for most applications this is more than compensated for by the quality of the data. Typical running conditions for amide HPLC are a binary gradient from 65 per cent to 50 per cent (v/v) acetonitrile in 50mM ammonium formate pH4.4 over 75 minutes with fluorescence detection as for the charge analysis.

The essential calibration standards for glycoprofiling by amide HPLC are fluorescently labelled glucose homopolymer (GHP) ladder and fluorescently labelled sialylated and asialo glycans. The GHP ladder is composed of a mixture of labelled glucose oligomers (typically from the monosaccharide to the 22-mer and larger). This is run before and after the test samples and allows comparison of data on different days and between different laboratories. The labelled glycan standards are periodically run on the system and their retention positions relative to the GHP oligomers are checked against tables of standard retentions. This calibration is especially important for the sialylated species, the retention positions of which can vary between HPLC systems. Once calibrated, the amide glycans will allow straightforward checks of the consistency of biopharmaceutical sialylation patterns.

There are a number of alternatives to amide HPLC including MS, CE, HPAE-PAD and HPAE-FD (high pH anion exchange chromatography with fluorescence detection). Mass spectrometry is an excellent tool for glycan structure determination, but current MS methods generally give poor quantitation for glycoprofiling – especially for sialylated glycans, which often have attenuated mass spec signals compared to non-anionic oligosaccharides. Capillary electrophoresis of fluorescently labelled glycans is being increasingly used for rapid oligosaccharide profiling. Fluorescent tags suitable for CE glycoprofiling include APTS (8-aminopyrene-1,3,6-trisulfonic acid), AA-Ac (3-(acetylamino)-6-aminoacridine) and 2-AA. HPAE-PAD has been widely used for sialylated oligosaccharide profiling since the 1980s. The method is straightforward and the column resolution is excellent. However, there are concerns over quantitation by PAD and some glycoprofiling labs are moving away from HPAE-PAD towards using HPAE-FD on 2-AA or 2-AB labelled glycans. The latter gives excellent results and is used by some leading biopharmaceutical companies as the principal glycoprofiling QC method for drug lot release.

CONCLUSION

In this article, we have looked at the importance of biopharmaceutical sialylation, what it is, and how to characterise it in a biomanufacturing environment using an ICH-compliant glycoprofiling scheme composed of three glycoprofiling modules for determining the type and distribution of desired and aberrant sialic acids. Use of this or a similar scheme will help you to ensure the production of consistent, safe and effective biopharmaceuticals. ◆

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