Turning to Colour

Daryl Fernandes at Ludger discusses fluorescent labelling methods for glycoprofiling of therapeutic glycoproteins

Biopharmaceutical glycosylation can significantly influence the safety and efficacy profiles of glycoprotein therapeutics, so detailed and accurate monitoring of the glycans is an essential task throughout a drug's life cycle. This typically involves use of an integrated set of glycoprofiling modules designed to measure an array of glycosylation critical quality attributes (GCQAs) specified for that particular drug. A number of these glycoprofiling methods require labelling of glycans or glycan fragments with a fluorescent tag. The labelling aids subsequent separation and quantitation on high performance liquid chromatography (HPLC) or capillary electrophoresis (CE), and can improve analysis by mass spectrometry. This article provides an overview of a quality by design (QbD) inspired approach to the process of selecting glycan fluorescent labels for reliable biopharmaceutical glycoprofiling.

Glycosylation can play an important role in the safety and efficacy profiles of biopharmaceuticals (1). The safety concerns include possible adverse clinical events caused by non-human glycans which are either attached to the drug or associated with non-drug contaminants. For example, IgE-mediated anaphylaxis has been found in certain patients treated with cetuximab (a therapeutic antibody marketed under the name of Erbitux® and used for treatment of metastatic colorectal cancers and cancers of the head and neck). This adverse event has now been attributed to immunogenic non-human type oligosaccharides attached to the drug's Fab region. The immunogenic epitope is a galactose-α-1,3-galactose disaccharide unit found on the non-reducing termini of certain cetuximab glycans (2). This epitope has also been discovered in other biologics produced in mammalian expression systems.

A number of biologics contain the non-human sialic acid N-glycolylneuraminic acid (NeuGc). The implications of this on both the safety and efficacy of the drugs are still being unravelled. The sialic acid naturally synthesised by human cells is N-acetyl-neuraminic acid (NeuAc), and is considered by most drug designers and manufacturers as the desired type of sialylation for biopharmaceuticals. NeuGc is found in non-human glycoproteins and may be introduced into some biologics by the cells of the expression system, either via biosynthesis or by scavenging NeuGc from non-human sera in the culture media. The risks are the possibility of adverse clinical reactions involving the xenobiotic NeuGc epitopes, and reduction in drug efficacy via neutralisation by circulatory anti-NeuGc antibodies. The possibility of such effects occurring is indicated by various studies including that of Nguyen *et al*, which showed that activated human T-cells could incorporate NeuGc, and that anti-NeuGc IgG in human sera bound to these T-cells and initiated complement-mediated killing (3).

Turning to desired glycans, many types of biologics require specific glycosylation patterns for optimal efficacy (1). For example, for some antibodies, the percentage of core-fucosylation of the Fc glycans is inversely correlated with antibody-dependent cellular cytotoxicity (ADCC) activity levels as measured in both *in vitro* and *in vivo* assays. Also, levels of terminal galactose residues can significantly affect antibody CDC activity. With glycoprotein hormones and cytokines such as erythropoietin (EPO), the types of oligosaccharides (for example, N-linked versus O-linked), their antennary structures and degree of sialylation can profoundly affect *in vivo* bioactivity.

A consequence of these phenomena is that, for therapeutic glycoproteins, accurate, detailed and quantitative structural characterisation of the glycosylation is required for comparability studies throughout the drug life cycle. This is accomplished with the use of a number of glycoprofiling modules to measure different glycosylation parameters. Fluorescent labelling of glycans or glycan fragments is a key component of such glycoprofiling (4). There are now many glycan labelling methods to choose from and selection of the ones to use for a particular biologic should be done with care. Use of appropriate labelling methods and analytical platforms allows good separation of the individual species in complex glycan mixtures and reliable quantitation of those species. A poor choice of label for a particular application can lead to inaccurate quantitation of critical glycan species, increasing the risk of failing drug batches with good glycosylation and, more seriously, passing batches with out-of-specification glycosylation patterns.

The rest of this article focuses on the use of fluorescent labels for effective monitoring of the glycosylation of biopharmaceuticals produced in mammalian expression systems. The glycoprofiling modules covered will be for analysis of oligosaccharides, sialic acids and neutral monosaccharides. Their service in a QbD framework for product control will also be explained.

GLYCAN LABELLING IN A QBD FRAMEWORK

Monitoring of drug glycosylation typically requires comparisons of different drug batches, each containing dozens of different glycoforms. Given the complexity of glycosylation and the structural heterogeneity it confers to the therapeutic, the questions arise of what to compare and how to compare it. The short answer to this is 'compare biologically relevant glycosylation features in a quantitative manner'. This can be done using a QbD approach modified to cope with the complexities of biopharmaceutical glycans (1). In essence, this involves comparisons of glycoprofiles where each glycan component is weighted by its relative molar abundance and its influence on the *in vivo* safety and efficacy profiles of the drug. The glycoprofiles are selected to measure glycosylation critical quality attributes (GCQAs) for the particular drug accurately.

In the case of oligosaccharide profiles, measurement of the relative molar

abundances of individual oligosaccharides is typically achieved by releasing them from the protein backbone, tagging them with stable fluorescent labels at their reducing termini, resolving the labelled glycans on HPLC or CE, then measuring the peak areas of the fluorescence signal. The labelling reaction is a reductive amination (see Figure 1). Neutral monosaccharides can be profiled in a similar way, except that they are released from either the native glycoprotein or released oligosaccharides by breaking the glycosidic bonds using strong acid hydrolysis. Sialic acids are released by mild acid hydrolysis but are labelled in a different way as they do not possess a reducing end (5). Table 1 summarises the core set of fluorescent labels used in our glycoprofiling laboratories.

There are two main reasons for fluorescent tagging of glycans during glycoprofiling.

Labelling Allows Quantitation of Glycans on HPLC and CE

Glycans do not possess strong chromophores, so labelling allows their detection during analytical HPLC and CE by fluorescence or UV/VIS monitoring. In most cases, fluorimetry gives better sensitivity and selectivity than UV/VIS detection and allows use of separation methods involving complex buffer systems that would otherwise interfere with reliable analysis. Unlabelled glycans can be monitored on HPLC by pulsed amperometric detection (PAD), but that requires use of high pH buffers, either in the mobile phase or added post-column to effect de-protonation of the sugar hydroxyl groups. In addition, different glycans exhibit different relative PAD responses, so reliable quantitation is difficult with PAD detection. Mass spectrometry can also be used for detection of unlabelled glycans, but there are even greater problems with variable signal response for different glycans and different MS instruments. Fluorescent labelling is not perfect, but often enables much more reliable quantitation than PAD or MS. When executed correctly, fluorescent labelling gives excellent stoichiometric tagging of glycans with widely varying structures in complex mixtures. For most applications of oligosaccharide profiling, one can assume that the relative peak areas in the fluorescence trace correspond to the relative molar abundances of the glycan structures. With neutral monosaccharides and sialic acids, different species do have different fluorescent yields so calibration curves are required for accurate quantitation.

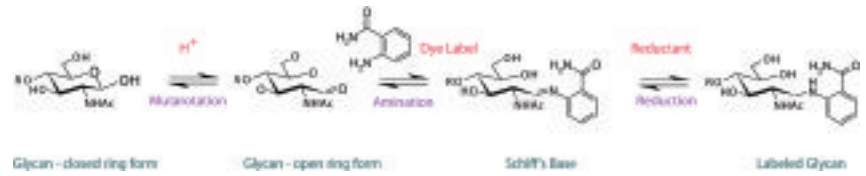


Figure 1: The reductive amination reaction scheme for fluorescent labelling of reducing glycans

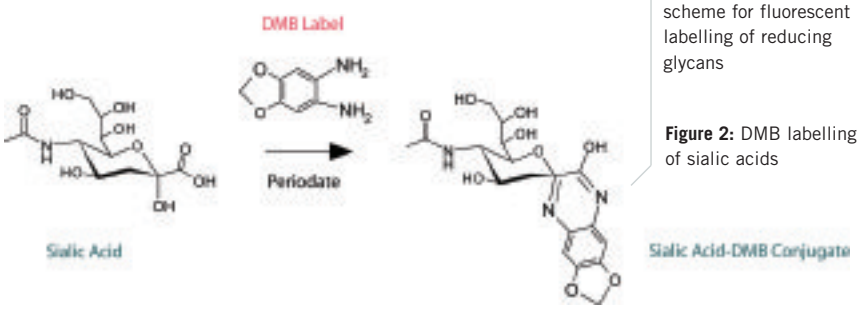


Figure 2: DMB labelling of sialic acids

Labelling Aids Separation of Glycans on HPLC or CE

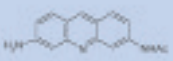
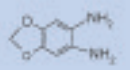
Most fluorescent labels possess aromatic rings that add a degree of hydrophobicity to otherwise completely hydrophilic glycans. The amphipathic nature of the glycan-label conjugates enables separation of complex oligosaccharide mixtures on both hydrophilic and hydrophobic HPLC stationary phases. Such orthogonal separations are essential for resolving oligosaccharides with potentially immunogenic epitopes and other glycans with a critical effect on the biological properties of the drug. Other physicochemical properties of labels can be used to enhance analytical separation. For example, labels with sulphonate groups such as APTS (8-aminopyrene-1,3,6-trisulfonate) can be used to confer strong negative charges on oligosaccharides for rapid separation by CE.

Choosing and Using Labels for Oligosaccharide Profiling

The process of choosing a fluorescent label for oligosaccharide profiling should be part of a company's quality risk management programme. If a company wishes to standardise on a single label, then they should select one that allows

Table 1: Fluorescent labels used for biopharmaceutical glycoprofiling

Key: AEX = anion exchange, HPAE-FD = high pH anion exchange with fluorescence detection, PGC = porous graphitic carbon

Fluorescent glycan label	Main applications	Fluorescence wavelengths and common analysis methods
2-AB (2-aminobenzamide) 	Oligosaccharide profiling by HPLC and MS	λ_{ex} = 320-360nm, λ_{em} = 420nm HPLC (amide, C18, AEX, HPAE-FD, PGC), ES-MS, MALDI-MS
2-AA (2-aminobenzoic acid) 	Oligosaccharide profiling by HPLC and MS Monosaccharide profiling by HPLC	λ_{ex} = 320-360nm, λ_{em} = 420nm Oligosaccharides: HPLC (amide, C18, AEX, PGC), ES-MS, MALDI-MS Monosaccharides: HPLC (C18)
APTS (8-aminopyrene-1,3,6-trisulfonate) 	Oligosaccharide profiling by CE	λ_{ex} = 455-488nm, λ_{em} = 510 - 520nm CE
AA-Ac (3-(acetylamino)-6-aminoacridine) 	Oligosaccharide profiling by HPLC	λ_{ex} = 382, 445nm, λ_{em} = 525nm HPLC (amide, C18, AEX), ES-MS, MALDI-MS
DMB (1,2-diamino-4,5-methylenedioxybenzene) 	Profiling of human versus non-human sialylation by HPLC	λ_{ex} = 373nm, λ_{em} = 448nm HPLC (C18)

analysis of the derivatised glycans by a variety of orthogonal separation methods. This flexibility becomes crucial at a number of important stages in the drug life cycle – for example, after an adverse clinical event when you need to search for and identify suspected aberrant glycans that co-migrate with the normal oligosaccharides on your standard separation method, or when you need to transfer production to another site with different analytical platforms in the QC department.

Following best practice, different glycan labels and separation methods should be tried early on in any new campaign, and the combination that gives the most reliable quantitation of the GCQAs determined for that therapeutic glycoprotein should be selected. Analyses should be designed with suitable checks for distortions in quantitation caused by non-glycan contaminants such as buffer salts, or selective loss of glycans during the pre- or post-labelling workup. If resources allow, quantitation of GCQAs should be validated according to the ICH Q2(R1) guidelines, bearing in mind that the list and priority order of the GCQAs may change during product development as knowledge of the structure-activity relationships for the therapeutic substance increases (1).

Companies could also consider using more than one fluorescent label for different types of oligosaccharide analyses on a drug. For example, during clone selection, CE of APTS-labelled glycans would allow rapid screening of oligosaccharides with no or low sialylation. However, if a drug also contains highly sialylated species that are biologically important, then analysis of 2-aminobenzamide-labelled sugars may be better during biomanufacturing, as these are amenable to a wider range of orthogonal HPLC separations that resolve glycans with several sialic acids.

With monosaccharide and sialic acid profiling, there is generally no need for multiple orthogonal separations and labels, as the analytical traces are much simpler than those for oligosaccharides. Nevertheless, analysts should be prepared to expand the range of glycoprofiling methods in their labs if they need to investigate something unusual.

After selecting candidate labelling and separation methods, detailed structure assignments should be made for each type of glycoprofile. Positive identification of labelled glycans in an HPLC or CE trace can be achieved by a combination of the following:

- Comparing sample glycans to well-characterised analytical reference standards (note that co-elution of a standard and an unassigned peak does not necessary mean the structures are the same)
- Using external fluorescently labelled 'ladder' standards (such as a 2-AB labelled glucose homopolymer ladder) and databases of glycan standard retentions to compare retention times of sample peaks
- Conducting detailed structure analysis of the whole sample or isolated fractions by MS, MSn and/or exoglycosidase sequencing (6)

CONCLUSION

The process of selecting oligosaccharide labelling and separation methods and identifying labelled glycan structures is not trivial. However, if it is carried out thoroughly and at an early stage in drug development, companies will be rewarded with an in-depth understanding of a biologically important quality attribute of their therapeutic and can develop a slim, relevant QC method for glycosylation during biomanufacturing, but if required can expand their analyses to investigate anything unusual. In addition, it entails better preparation to adopt the control and design spaces of the QbD paradigm, and the manufacturing flexibility and lower regulatory burden promised for that system.

References

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About the author

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