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Analysis of Glycans

The development of biologically derived therapeutic drug products has never been more important, and one of the areas of greatest focus is the generation of drugs based on monoclonal antibodies (mAbs). This class of compound presents many challenges to the separation scientist due to the complexity inherent within the molecule and the number of possible variants that can be produced during and post the manufacturing process. One of the most critical post-translational modifications of glycoproteins, such as mAbs, is the addition of linked glycans. This can result in alteration of the mAb structure, which in turn can affect the efficacy and also the toxicity of the therapy. This article will review the importance of glycan analysis and the approaches that can be employed to successfully determine the type and nature of the glycans that have been formed on a protein after it has been manufactured.

BACKGROUND

The growth of the biopharmaceutical market has seen significant developments in the understanding of the manufacturing process, as well as the process of ensuring the final product quality. From a regulatory aspect this can be seen with the development of the FDA regulations designed specifically for the determination of the purity of a biopharmaceutical drug.[1] This has led to the development of a common workflow that is employed in the routine analysis of the final product. The workflow encompasses a range of different separation techniques, with each one targeting the specific analysis of a genre of covalent or enzymatic modifications to the original protein. One of the most important analyses that is performed within this workflow is the analysis of glycan structure, which can have substantive impact on the shape, efficacy, and toxicity of the mAb.[2]

Monoclonal antibodies are antibodies produced by cloning a unique white blood cell. The mAb has monovalent affinity and will only bind part of the antigen. This can be used either to aid the isolation and detection of a specific antigen as a marker of a disease state or as a therapy to either disrupt signal pathways, or blocking targeted molecule functions resulting in apoptosis of particular cells which are causing a disease state. This level of specificity coupled with the ability to manufacture and refine to a higher level of purity has resulted in a large increase in the development and manufacture of these compounds. As part of the manufacturing process it is feasible for the protein (mAb) to undergo a post translation modification resulting in the addition of glycans to the primary protein structure.

GLYCAN ANALYSIS

The building blocks for glycans are primary sugar groups or monosaccharides, of which a wide range exist, with the primary differentiator being the number of carbons involved in the ring structure 5 (pentose) or 6 (hexose). Subsequent differentiation between the different forms of monosaccharides depends on the relative positions of the hydroxyl groups. Glycans are compounds consisting of one or more monosaccharides that are linked through a glycosidic bond, Figure 1. Figure 2 gives examples of some of the possible structures for the glycans.

The complexity of the resulting glycan structures means that drawing out the full chemical structure is not practical and so instead, a standardised pictorial representation of the different

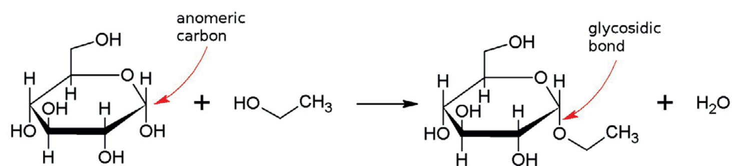


FIGURE 1: Reaction scheme for the generation of a glycosidic bond.

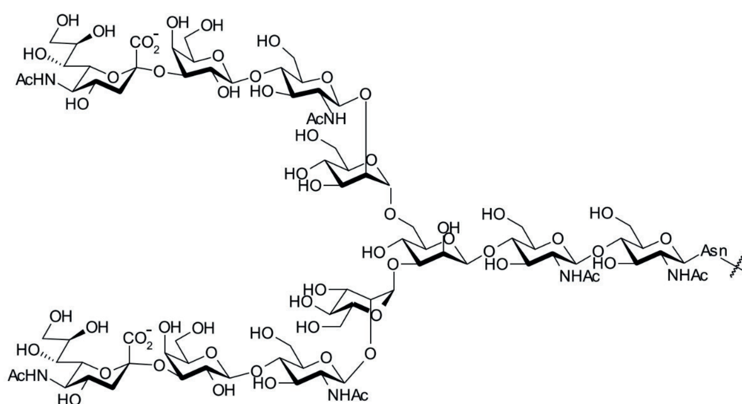


FIGURE 2: Example of N-glycan structure.

glycans was proposed in 1978 by Kornfeld.[3] Table 1 gives a summary of the more common glycan groups along with their pictorial representation. This notation is often referred to as SNFG or Symbol Nomenclature for Glycans.

Using this notation, the resulting glycan structure is then depicted by a series of shapes and colours representing the different forms of the monosaccharides present. The overall structure of the glycan, including whether it be linear or branched, and at what point branching occurs is also detailed. In glycoproteins, glycans can attach through a variety of different groups on the protein, however the glycans are typically linked through an N (nitrogen) linkage or an O (oxygen) linkage to the protein structure. N-linked glycans are attached to a nitrogen of asparagine or arginine containing side-chains in the endoplasmic reticulum. This modification occurs at a specific sequence of amino acids; Asn-AAX-Ser or Asn-AAX-Thr, where AAX is any amino acid except proline. The glycan may be composed of N-acetylgalactosamine, galactose, neuraminic acid, N-acetylglucosamine, fucose, mannose, and other monosaccharides.

O-linked glycans are attached to the hydroxyl oxygen of serine, threonine, tyrosine, hydroxylysine, or hydroxyproline side-chains. Unlike the N-glycans, a specific sequence of amino acids where the glycan attaches has not been determined. In mAbs, glycan attachment occurs in the crystallisable region (Fc), as shown in Figure 3.

Determination of the glycosylation profile of biopharmaceuticals is essential because glycosylation significantly impacts the stability and function of mAbs, including mediation of antibody-dependent cell-mediated cytotoxicity (ADCC) and complement dependent cytotoxicity (CDC).[5] N-glycosylation, stabilizes the structure of a mAb, making it less prone to folding[6] and less prone to aggregation.[7] Afucosylation of mAb N-glycans can result in increased binding affinity of mAbs to receptors present on the surface of leukocyte effector cells, which can enhance ADCC.[8,9] Several studies have suggested that terminal sialic acid residues on glycans mediate anti-inflammatory responses, reduce ADCC in vivo[10] and inhibit allergic reaction.[11] Galactosylation does not affect ADCC; however, the presence of galactose residues on N-glycans may lead to an increase in CDC[12,13] or anti-inflammatory activity.[14] High-mannose N-glycans have been shown to correlate with accelerated clearance of mAbs from the blood, decreasing circulating half-life of the drugs.[15-17] Therefore, control of the glycosylation pattern and the ability to monitor the correct levels of glycan formation is required to ensure

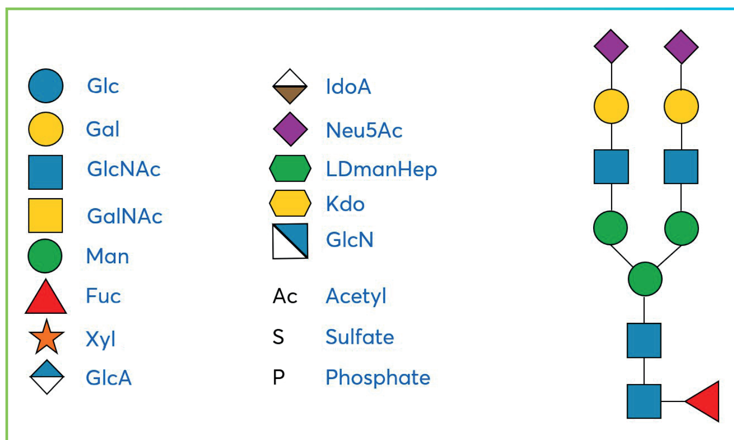


TABLE 1: Pictorial representation of a series of glycans, with an example of the molecular form for one of these. This list is not exhaustive, but a comprehensive list can be found in reference 4.

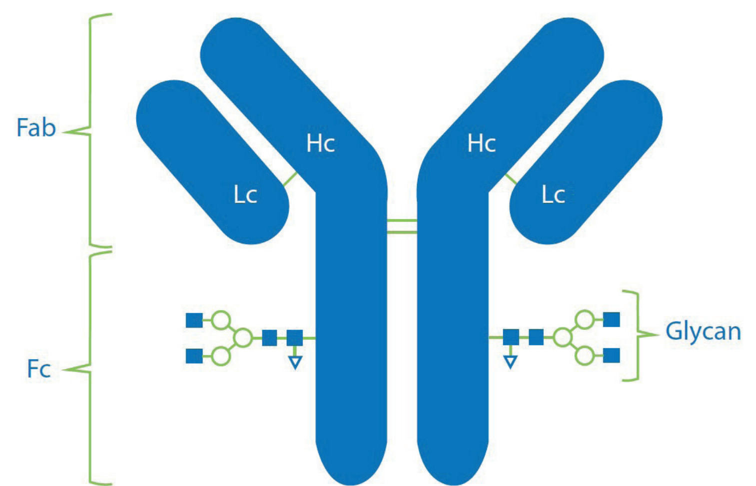


FIGURE 3: Schematic showing glycosylation regions of a monoclonal antibody Fc = Crystallisable fragment, Fab = Antigen-binding fragment, Hc = Heavy chain, Lc = Light chain.

adherence to lot release specifications, safeguarding efficacy and reducing toxicity.

As can be seen from above, the N-glycans have a profound impact on the potential efficacy and toxicity of the mAb and it is necessary to ensure that the levels and composition of N-glycans is monitored carefully, as there is potential for these to be generated via post translational modification (PTM) processes during manufacture. ICH (Q6B),[18] part of the global regulations relating to the production of biological products, states that the carbohydrate content and structure should be determined as part of the characterisation and confirmation of the biological product. The glycan structure of a mAb is a critical quality attribute (CQA) and is something that should be routinely monitored. There are a variety of analytical approaches that can be employed to analyse the levels of N-glycans;

- Analysis of the intact protein
- Analysis of the glycopeptide formed after a tryptic digest
- Analysis of released N-glycans formed by the enzymatic action of PNGase F

The latter of these approaches is the most favoured within the biopharmaceutical industry with the resulting N-glycans typically being tagged with a fluorescent marker (commonly 2-AA – 2 Aminobenzoic acid or 2-AB – 2 Aminobenzamide), Figure 4, since polysaccharides do not absorb strongly in the UV or visible range, and do not ionise particularly well within the mass spectrometer. 2AA and 2AB can be also used as mass spectrometric tags, however an array of alternative derivatising reagents have been utilised for the determination of glycans using mass spectrometry detection that provide a higher level of sensitivity.[19]

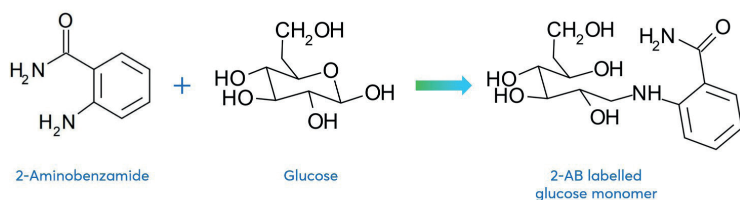


FIGURE 4: Derivatisation of glucose with a 2-AB fluorescent tag commonly used in glycan analysis.

The preferred mode of separation for a series of glycans is HILIC (hydrophilic interaction liquid chromatography). The addition of the fluorescent or mass spectrometric tag does not impact on the overall polarity of the glycan, and since the partition coefficient is low, HILIC is an ideal approach to separate these very polar molecules.[20,21] Indeed, the first reported application of HILIC was for the analysis of sugars, and it is an approach that is commonly used within the food industry, typically using a neutral polar stationary phase. The use of a charged HILIC stationary phase would add no benefit to the separation since the glycans in this form would not be charged.

In a HILIC mode of separation, the stationary phase interacts not only with the analyte molecule but also with the mobile phase, Figure 5. The stationary phase is a polar or charged species that will preferentially attract the more polar aqueous component of the mobile phase resulting in a water-enriched layer around the stationary phase substrate material. This is very similar to liquid-liquid partitioning and so the first stage of the separation can be considered as a liquid-liquid separation, which is then followed by an adsorption process with the surface of the stationary phase defining how the subsequent retention of the analyte molecule occurs.[22]

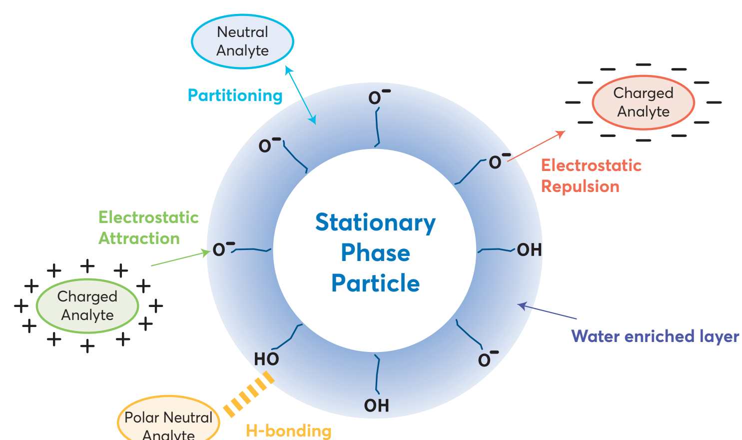


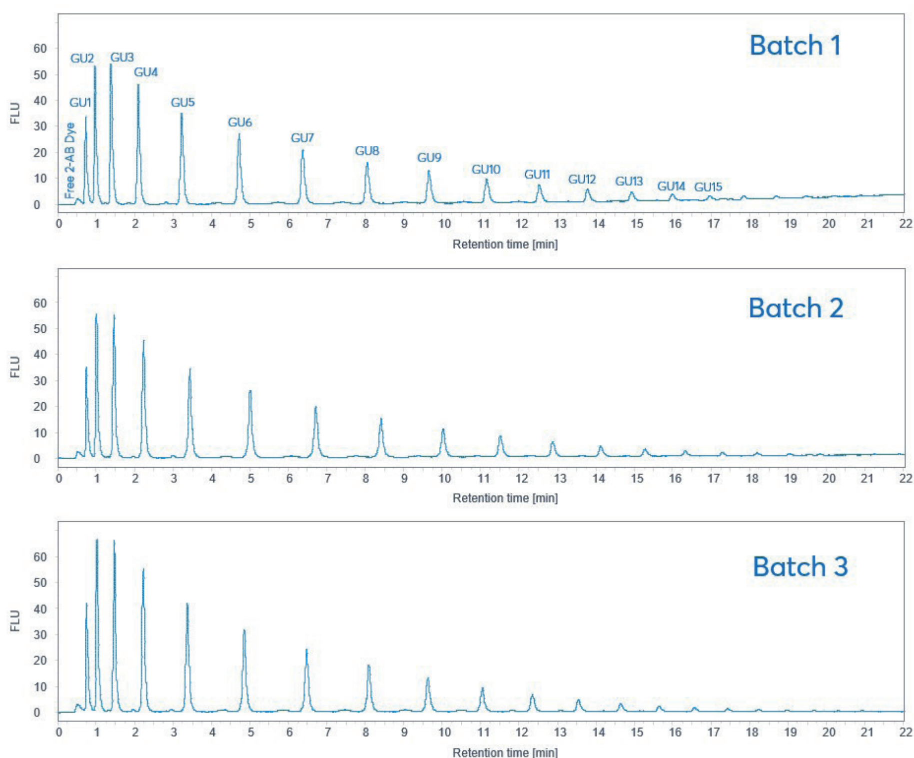
FIGURE 5: Schematic of interactions between different types of polar analytes and the stationary phase in HILIC mode.[23]

UNDERSTANDING THE HILIC MODE OF SEPARATION

McCalley and Neue demonstrated the existence of the water-rich layer on the silica surface under typical HILIC conditions;[24] they were also able to observe that the water-rich layer increased in thickness as the aqueous content in the mobile phase increased up to 30%. The authors provided indirect evidence that hydrophilic partitioning is the main retention mechanism at higher water content, however other interactions (such as hydrogen bonding) might become more relevant as the water content decreases. Subsequently, McCalley demonstrated the existence of a very complex mechanism, consisting of a combination of hydrophilic partitioning interaction, adsorption, ionic interactions and even hydrophobic interactions.[25] Buszewski and Noga have performed an extensive review of different HILIC retention models, which cover mechanisms that depend on the analyte characteristics, the mobile phase composition and the nature of the stationary phase.[26] An understanding of the HILIC retention mechanism allows for better design of the chromatographic conditions to elute a series of glycans. This understanding has been applied to develop a separation of a series of glycans derived from the degraded dextran.

GLYCAN APPLICATION

HILIC is an ideal mode of separation for released N-glycans, with the ideal column being a neutral but polar stationary phase. The number of possible glycan structures is immense, and it would not be feasible to demonstrate that a specific column could separate a particular glycan structure and so instead, to demonstrate the applicability of column, a series of glycans containing an increasing number of glycan units (GU) is separated. Figure 6 shows the resulting chromatogram for a series of increasing number of conjugated glycan units (GU), often referred to as a glycan ladder or in this case a dextran ladder, as the primary source of the sample was derived from the partial hydrolysis of



CONDITIONS

Column:	**Avantor® ACE® 1.7 Glycan**
Cat No.:	EXL-1716-1002
Dimensions:	100 x 2.1 mm
Mobile Phases:	A: 100 mM ammonium formate (pH 4.5) B: MeCN
Gradient:	

Time (mins)	%B
0	75
24	52.5
24.2	40
24.4	40
24.6	75

Flow Rate:	0.5 mL/min
Injection:	1 µL
Temperature:	55 °C
Detection:	FLD, λ_{ex} 260 nm, λ_{em} 430 nm
Sample:	2-AB labelled Dextran Ladder standard

FIGURE 6: Separation of dextran ladder standard on three Avantor® ACE® Glycan columns packed with three different batches of packing material.

dextran. The separation uses a relatively shallow gradient on an Avantor® ACE® Glycan column, with the strong solvent being 100 mM ammonium formate (aq). It can be seen that the hydrophilicity of the 2-AB labelled glycans increases with increasing number of glycans units. Excellent peak shape is obtained in all cases and the batch-to-batch reproducibility between the columns is also very good.

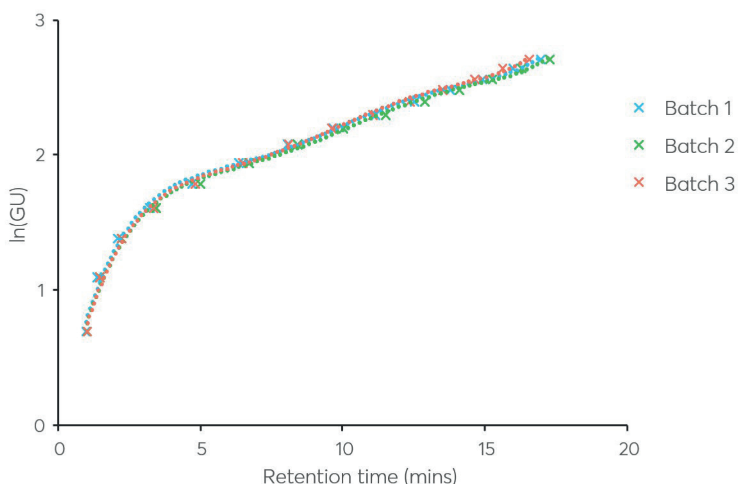
It is possible to calibrate the number of glucose units versus the retention time, with the resulting relationship being of the form;

$$Ln(GU) = at_r^5 + bt_r^4 + ct_r^3 + dt_r^2 + et_r^1 + constant$$

This calibration line can then be used to determine the GU values of unknown samples, allowing putative structures to be assigned based on matching GU values from a database. However, it should be noted that the calibration curve is specific for a series of glycans, in this case a series of linear glucose units, and would not be applicable for another series of glycan units. Figure 7 shows three overlays of the calibration line used for the glycan units. The calibration line can be used in conjunction with MS identification to confirm the structure of unknown glycans being eluted from the HPLC column.

CONCLUSION

This article has reviewed the importance of glycan analysis and presented the common approach that is used within the biopharmaceutical industry to comply with the regulatory demands for critical quality analysis. The approach of tagging enzymatically cleaved glycans and then subsequent separation using HILIC with fluorescent detection has been discussed and an example using the Avantor® ACE® Glycan column has been presented. This column has very high stability and also excellent batch-to-batch reproducibility and is ideal for the routine separation of glycans, as is demonstrated by the high resolution of the glycan ladder.



	Calibration coefficient					
	a	b	c	d	e	r^2
Batch 1	0.00003	-0.00136	0.02622	-0.23596	1.06166	0.996
Batch 2	0.00002	-0.00122	0.02396	-0.22078	1.02167	0.996
Batch 3	0.00003	-0.00143	0.02715	-0.24160	1.08258	0.997

FIGURE 7: Overlay of three calibration lines generated from dextran ladder standard on three batches of Avantor® ACE® Glycan column, showing the relationship between the number of glycan units and the retention time.

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