Protein Therapeutics

David Neville, Technical Specialist

Reading Scientific Services, Reading Science Centre, Whiteknights Campus,
Pepper Lane, Reading, Berkshire, RG6 6LA
Tel: +44 (0)118 918 4000 Email: enquiries@rssl.com Web: www.rssl.com

Introduction

The use of protein-based biological materials to treat patients suffering from a wide range of diseases is one of the success stories of modern medicine. The diseases treated include diabetes (insulin), growth hormone deficiencies (recombinant human growth hormone), inborn errors of metabolism (glucosylceramidase, alpha-galactosidase), and red blood cell deficiency (erythropoietin). As the market develops, the number of biopharmaceutical products both being submitted for regulatory approval and reaching the end of their patent life is increasing. In the latter case, this has led to the development of replacement/substitute products that have been termed 'biosimilars' or 'biobetters', often deriving their improved performed via post translational modifications such as protein N-linked glycosylation. Hence, establishing the position and extent of protein N-linked glycosylation is crucial to meeting the strict guidelines laid down by the relevant regulatory authorities that must be completed prior to marketing approval being given and there are a variety of analytical approaches that might be taken to provide the necessary information.

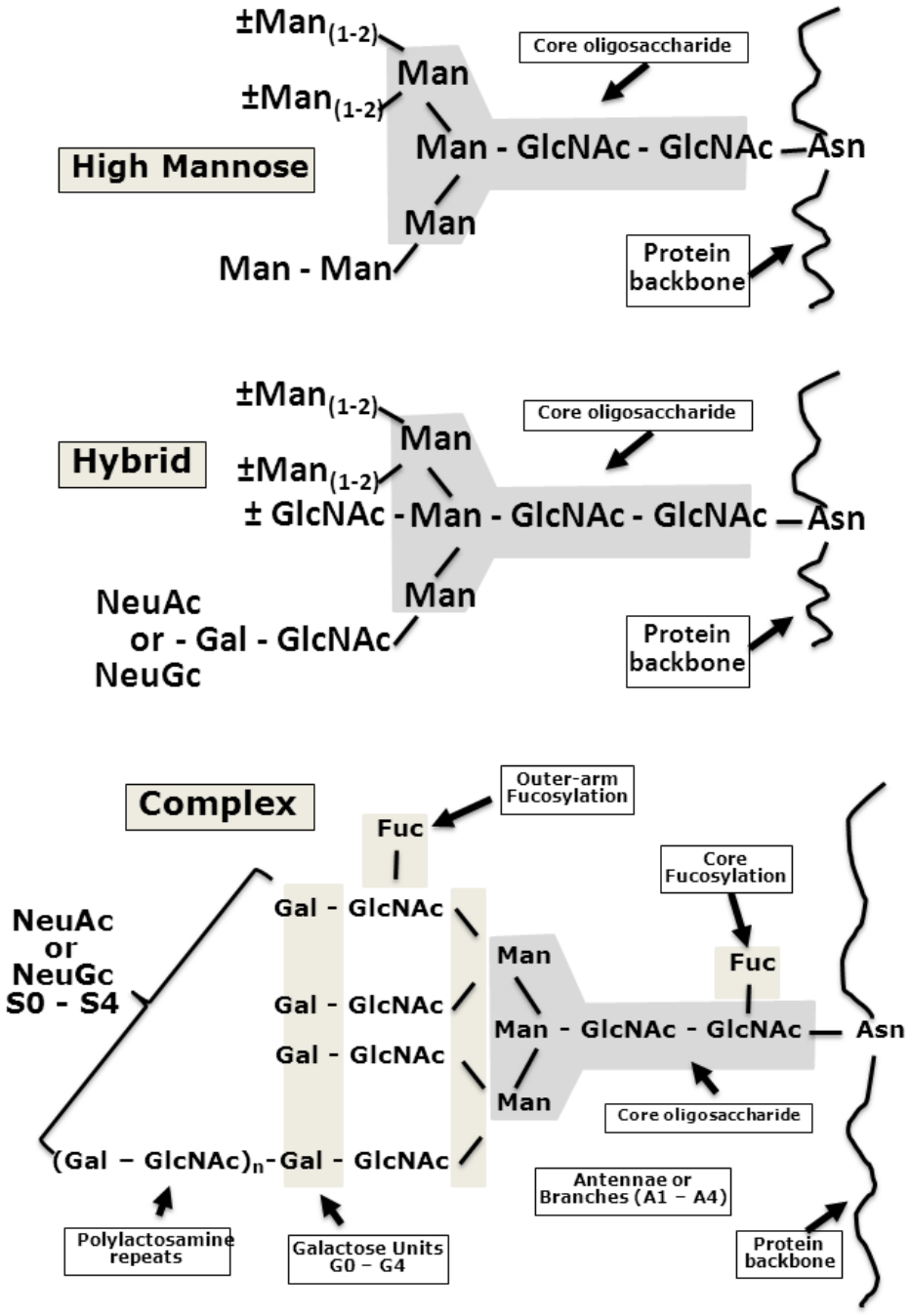
The complexity of post-translational modification of proteins by glycosylation

N-linked glycosylation is defined by the attachment of an oligosaccharide via an amide bond to an asparagine (Asn) residue of a defined tripeptide sequence (Asn-X-Ser/Thr) where X can be any amino acid except proline. Proteins may contain single or multiple occurrences of this sequence but there is no guarantee that this site will be glycosylated and, even where the site is glycosylated, it is not certain that the site will be one hundred per cent glycosylated or that unique oligosaccharide structures exist at each site. Oligosaccharides play an important function as they are known to be determinants of serum half-life, cellular uptake, intracellular targeting, efficacy and immunogenicity. Due to the importance of oligosaccharides, both biologically and as part of product quality control, Table 1 outlines the types of information that must be discovered as part of the product development process, and Figure 1 shows the different forms of N-linked oligosaccharide commonly found attached to biopharmaceuticals.

Table 1: Information required as part of the drug discovery/submittal process.

	Information Required	Information Obtained
1	Monosaccharide Analysis	Relative composition of monosaccharides (sialic acid, galactose (Gal), N-acetyl glucosamine (GlcNAc), N-acetyl galactosamine (GalNAc), fucose (Fuc) and mannose (Man)
2	Antennary Structures	Relative amounts of antennae or branches present (A1-A4)
3	Fucosylation	Outer-arm or core fucosylation
4	Galactosylation	Number, linkage isomers and presence/absence of α-galactose
5	Sialylation	Number of sialic acid residues per complex structure (S0-S4), linkage, and type of sialic acid - N-glycolyl (NeuGc) or N-neuraminyl (NeuAc)
6	Lactosamine Extensions	Presence of polylactosamine repeat sequences on branches
7	High Mannose Structures	Identify presence of any non-hybrid or complex oligosaccharides

Figure 1: Generic High Mannose, Hybrid and Complex N-linked oligosaccharides. The Complex oligosaccharide demonstrates the relative position of a typical modifications (Items 2 - 6 in Table 1). The shorthand terms for the monosaccharides are defined in Table 1.



It is noticeable that oligosaccharide structures are not just single moieties and this multiplicity of structures is termed 'microheterogeneity'. Indeed, the oligosaccharides attached to proteins at each individual glycosylation site may differ in sialic acid and galactose content, the numbers of antennae or branches, the degree and type of fucosylation, and whether the oligosaccharide is high-mannose, hybrid or complex . All of these facts conspire to add to the complexity of information that must be obtained, and the skill required of the analyst(s).

Analysis of N-linked glycosylation by chromatographic methods

The methods that may be used to analyse oligosaccharides are varied and complex (high performance liquid chromatography, mass spectrometry, lectin-based analysis, enzyme digestions, chemical methods etc.). For ease of analysis, oligosaccharides must be released from the protein while preserving the biological information. Two methods have traditionally been used; chemical and enzymatic release. Chemical release using hydrazine is not favoured as some of the biologically relevant data may be destroyed during the oligosaccharide release and purification. Enzyme release using peptide N: glycosidase F (PNGase F) removes all N-linked oligosaccharides from mammalian proteins. However, if there is a core-fucose residue attached via an α1-3 linkage, as found in plant and some insect oligosaccharides, PNGase F will not work and Peptide: N-Glycosidase A (PNGase A) must be used. As most, if not all, biopharmaceuticals are currently produced using mammalian cell lines there should not be a problem in using PNGase F when performing oligosaccharide structure analysis.

The introduction of high performance anion exchange chromatography (HPAEC) with pulsed amperometric detection (PAD) greatly eased the analysis of oligosaccharides. This technology allowed for the facile separation of neutral from charged, sialic acid-containing oligosaccharides. Additionally, the charged oligosaccharides were separated on the basis of the number of charged residues per oligosaccharide and the underlying branched structure. The technique has some drawbacks in that; (a) it is difficult to interface the column eluate to a mass spectrometer due to the high pH, non-volatile salt content, (b) it may be accompanied by chemical peeling of the core oligosaccharide and (c) it has limited sensitivity of detection for small amounts of oligosaccharide.

Thankfully, these problems have been overcome by the adoption of techniques to incorporate UV-absorbing or, more commonly, fluorescent molecules to the core-GlcNAc residue (formerly attached to the protein backbone Asn residue) of the oligosaccharide.

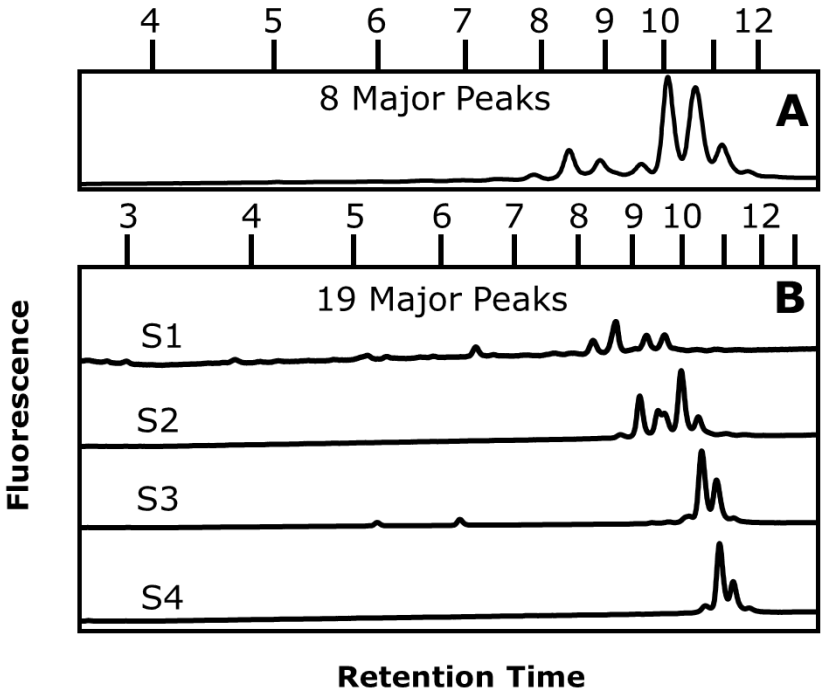
The sensitivity of detection of the oligosaccharide species is dramatically increased by attachment of a fluorescent moiety and a number of different fluorescent reporter groups are reported in the literature (1, 2, 3). These reporter groups influence sensitivity, choice of column, efficiency of separation and mass spectrometric analysis. Hydrophilic interaction chromatography (HILIC) (4, 5, 6) is a form of HPLC commonly used to separate carbohydrates or oligosaccharides. HILIC analysis is routinely performed using either a TSKgel Amide 80 column or a BEH Amide column on HPLC systems with fluorescence detection. Where possible, the retention times of the oligosaccharides are compared to that of an external standard, usually consisting of glucose oligomers, and elution is expressed in terms of glucose unit (GU) values. With the recent advent of ultra-performance liquid chromatography (UPLC), or ultra-high-pressure liquid chromatography (UHPLC), with sub two-micron bead sizes, both the speed of analysis and peak resolution have been dramatically increased As the buffers used in the analysis are volatile it is facile to interface the column eluate to a mass spectrometry system to obtain both mass and sequence information. Where the data generated contains a GU value, which can be referenced to a database, and a mass value, initial assignment of structure is relatively easy. Confirmation may be obtained from the mass spectrometric sequencing experiment in combination with sequence-specific enzyme digestions and further LC analyses.

HILIC does not effectively separate carbohydrates based on charge distribution and therefore, oligosaccharides of similar hydrodynamic volume and polarity may co-elute. This may lead to difficulties in interpretation due to the overlap of data and the masking of small populations of oligosaccharides. Analysis

using HILIC may also be performed in combination with, or following, weak anion-exchange (WAX) or strong anion-exchange (SAX), porous graphitized carbon (PGC) and reverse-phase (RP) separations. The advantage of combining SAX with HILIC, using TSKgel Amide 80, is shown in Figure 2. This shows how it is possible to separate the oligosaccharides into pools according to the number of sialic acids (S1-S4). Thereafter, submitting those pools to HILIC greatly increases the ease of analysis and the number of different structures observed following the analyses.

So, in this case, eight peaks are observed when performing HILIC alone, though other species may become apparent if combined with mass spectrometry and/or linkage-specific enzyme digests followed by further HILIC separation. By contrast, nineteen different oligosaccharides are observed following HILIC analysis of the SAX-separated oligosaccharides.

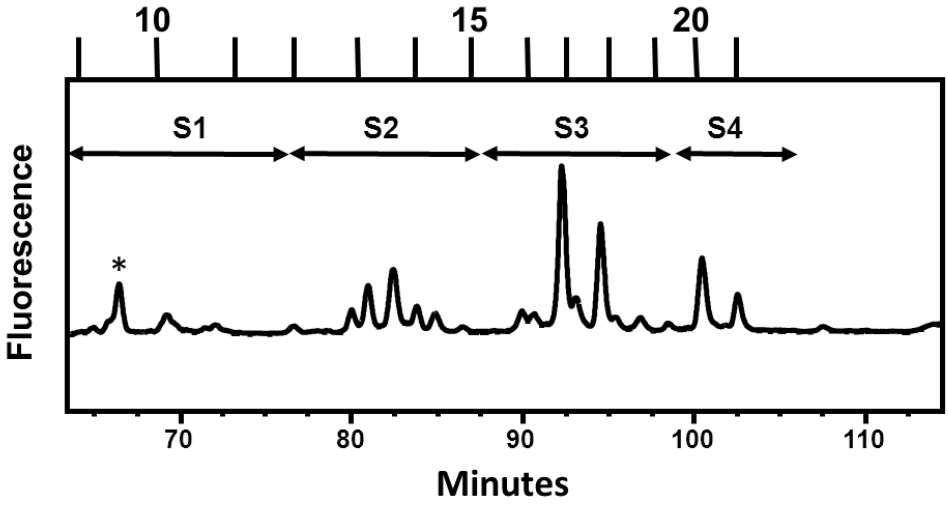
Figure 2: The advantage of combining strong anion-exchange (SAX) chromatography and hydrophilic interaction liquid chromatography (HILIC). N-linked oligosaccharides from Fetuin are analysed following PNGase F release from protein and derivatisation with anthranilic acid by (A) HILIC alone and (B) HILIC of the individual pools of sialylated oligosaccharides obtained following SAX chromatography. The numbers above the chromatograms refer to the retention times of the glucose oligomer external standards.



Recently, it has become possible to perform the same analysis using an internal standard that provides more accurate GU values (7). The GU values obtained with different column matrices may be used to generate oligosaccharide retention chromatography databases (8-11). Additionally, it is possible to combine differing chromatographies, hydrophilic interaction and anion-exchange, using a single column or a tandem column arrangement (12-16). This has been termed mixed-mode chromatography. It is also now possible to obtain GU values following hydrophilic interaction anion-exchange chromatography (HIAX) (15) Figure 3 shows HIAX separation of charged Fetuin oligosaccharides, as also shown in Figure 2, and demonstrates the advantage of one column technology to separate oligosaccharides according to charge and hydrophilic interaction. Twenty one oligosaccharides are observed that contain one to four sialic acid

residues (S1-S4). This is due to the better separation using this column or, some species are lost when performing SAX or WAX followed by HILIC analysis. This method also uses volatile buffers and may also be interfaced with mass spectrometry to enable the acquisition of mass and sequence information for each oligosaccharide.

Figure 3: Hydrophilic interaction anion-exchange (HIAX) chromatography. N-linked oligosaccharides from Fetuin are analysed following PNGase F release from protein and derivatisation with anthranilic acid. The retention times of the sialylated Fetuin oligosaccharides containing one to four sialic acid residues (S1-S4) are shown. The numbers above the chromatogram refer to the retention times of the glucose oligomer external standards. The species marked by * is a non-sialylated species.



In conclusion, the analysis of N-linked oligosaccharides is not a facile undertaking. There are a number of different column technologies available. The most sensitive methods are based on derivatising the free oligosaccharides with a fluorescent reporter molecule. These reporter molecules then influence the sensitivity, separation capabilities and use in mass spectrometric experiments. There is no single technique that provides all the required information and a combination of HPLC, mass spectrometry and enzyme digests will be needed. This requires that the analysts are experienced in a number of analytical practices that are necessary for oligosaccharide structure determination.

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