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Mass spectrometry for glycosylation analysis of biopharmaceuticals

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A B S T R A C T

Biopharmaceuticals are drugs of biotechnological origin used as vaccines or for the treatment of non-communicable diseases, such as cancer or anemia. Due to their high efficacy and specificity, the market for novel and biosimilar biopharmaceuticals is growing immensely. This growth is accompanied by new challenges in quality control and analytical characterization during drug development and production. Glycosylation is one of the structural modifications that occur during production of many protein-based drugs and can have significant effects on their pharmacological properties. Mass spectrometry (MS) is a promising technique for high-quality analytical characterization of glycosylation, starting from early drug development through to final lot release. Here, we review the most recent trends in biopharmaceutical glycosylation analysis by MS with and without coupling to liquid chromatography or capillary electrophoresis, and draw comparisons with established, non-MS methods. We discuss future prospects for the emerging MS approaches for the biotech industry and biopharmaceutical research.

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Abbreviations: AA, Aminobenzoic acid; AB, Aminobenzamide; CE, Capillary electrophoresis; CGE, Capillary gel electrophoresis; CID, Collision-induced dissociation; CIEF, Capillary isoelectric focusing; CZE, Capillary zone electrophoresis; EPO, Erythropoietin; FTICR, Fourier transform-ion cyclotron resonance; HCD, Higher-energy C-trap dissociation; HILIC, Hydrophilic interaction liquid chromatography; HPAEC-PAD, High-performance anion-exchange chromatography with pulsed amperometric detection; HPLC, High-performance liquid chromatography; Ig, Immunoglobulin; IM-MS, Ion mobility-mass spectrometry; IT, Ion trap; mAbs, Monoclonal antibodies; MALDI-TOF, Matrix-assisted laser-desorption/ionization time-of-flight; MS, Mass spectrometry; PGC, Porous graphitized carbon; PNGase, Peptide-N4-(N-acetyl-beta-glucosaminyl)asparagine amidase; PTM, Post-translational modification; QbD, Quality by Design; RP, Reversed phase.

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1. Relevance of glycosylation in biopharmaceuticals

Biopharmaceuticals or biologic drugs have been gaining increasing importance on the drug market, since these therapeutically active biomolecules often have greater efficacy and specificity than classic small-molecule drugs. They have been shown to be particularly promising in the treatment of non-communicable diseases, such as cancer or rheumatoid arthritis, but also as vaccines [1]. Since the prevalence of non-communicable diseases is expected to increase worldwide, the biopharmaceutical market – currently estimated at >US$199 billion – is also expected to grow at annual rate of ~13.5% [2].

Most biologic drugs tend to cause less adverse reactions than small molecules because they are structurally very similar to endogenous proteins, such as immunoglobulin G (IgG)-based monoclonal antibodies (mAbs), cytokines or hormones. Nonetheless, clinical safety and efficacy testing are key pillars in biologic-drug development. Notably, due to the structural variability of biologic drugs as a consequence of the manufacturing process in living cells and their inherent structural complexity, quality assessment and control come at a cost. Post-translational modifications (PTMs) during biopharmaceutical production contribute greatly to the structural variety. Moreover, manipulation and characterization of PTMs is challenging, but very important, since PTMs can significantly affect the physicochemical and pharmacological properties of the therapeutic.

The attachment of sugars (glycosylation) is a common PTM in biologic drugs and it can greatly affect clinical safety and efficacy profiles primarily via its influence on immunogenicity, solubility, protein folding and serum half-life [3,4]. Two main types of glycosylation occur in protein-based drugs: N- and O- linked, where glycan chains are attached to specific asparagine and serine/threonine residues, respectively (Fig. 1). The N-glycan composition of IgG (and mAbs) has been shown to regulate efficacy by affecting its receptor affinity [5]. The impact of O-glycosylation in biopharmaceuticals, such as erythropoietin (EPO) or blood-coagulation factors, is less well investigated due to many obstacles in O-glycan analytics, but O-glycosylation is also expected to play an important role in drug quality [6].

The main obstacles due to glycosylation, which impede biologic-drug manufacture, arise from the following aspects.

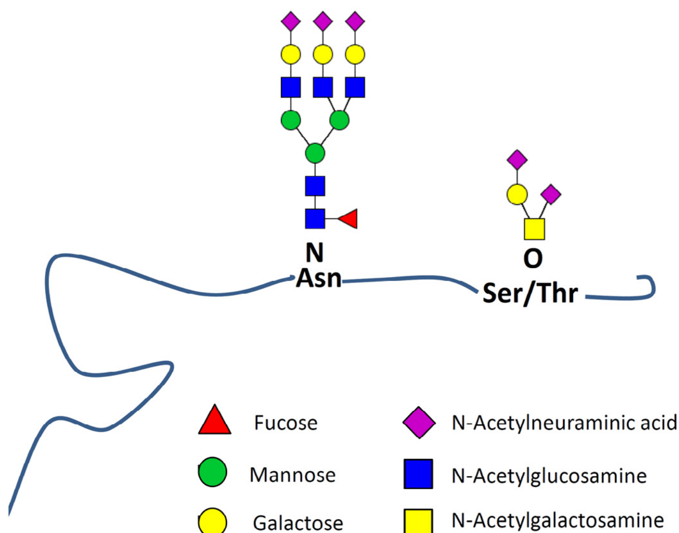

Fig. 1. Model glycoprotein with exemplary glycan structures. Commonly found in biopharmaceuticals are N- and O- type glycosylation, in which oligosaccharides are attached to an asparagine residue and to a serine or threonine, respectively.

- **Complexity.** Glycans are oligomers themselves, and proteins may carry many different glycans, adding to the structural complexity of the overall glycoprotein.
- **Microheterogeneity.** Different glycosylation sites on a protein may carry different glycans. Site-specific glycosylation may contribute to functional variation and influence clinical safety and efficacy.
- **Variability**. Cell-culture conditions partly define the glycosylation phenotype. Changes in glycosylation patterns due to inconsistency of manufacturing conditions are the major source of batch-to-batch variability.

To address these challenges, the biopharmaceutical industry is employing new analytical technologies to improve the characterization of glycosylation during drug design and manufacture. Accordingly, guidelines from health agencies including US Food and Drug Administration, European Medicines Evaluation Agency, and the International Conference on Harmonization contain specifications for drug glycosylation [4].

The variability of drug glycosylation embodies potential risks to patients and exposes companies to litigation for producing unreliable therapeutics. In this context, the Quality by Design (QbD) concept offers solutions. In practice, this entails identifying, characterizing and optimizing Glycosylation Critical Quality Attributes (GCQAs) starting at the early stages of drug development through to post-approval batch release (Fig. 2) [7]. So far, the best GCQAs evaluated include:

- the number of sialic acids attached to the glycan/glycoprotein, known to affect serum half-life of various biopharmaceuticals;
- the distribution of the main mAb glycoforms, which indicates process consistency; and,
- the presence of non-human, potentially immunogenic glycan structures, such as N-glycolylneuraminic acid and alpha1,3-galactosylation.

QbD in glycoprotein-drug development is supported by a range of orthogonal methods for structural and quantitative glycosylation analysis. In practice, these are predominantly drawn from three complementary analytical platforms:

- high-performance liquid chromatography (HPLC);
- capillary electrophoresis (CE); and,
- mass spectrometry (MS).

This review points out the most recent trends and future directions in MS methods used in development and production of biologic drugs. We discuss the advantages of MS for rapid, high-throughput and high-automation glyco-analysis and the challenges of using MS for drug-glycosylation characterization.

2. Current methods in glycosylation analysis of biopharmaceuticals

In principle, the whole range of methods available for glycan analysis, as extensively reviewed in [8], can also be applied to glycosylation analysis in biopharmaceuticals. However, glycosylation analysis in a biotech laboratory is limited to only a small choice of approaches with, preferably, high degrees of robustness, repeatability, accuracy, speed and automation providing qualitative and/or quantitative information sufficient to meet the requirements of regulatory agencies (Table 1).

Previously, the main focus was on batch-to-batch consistency of glycosylation, which is implied in the quality control at a later stage of drug development, as depicted in Fig. 2. However, a more thorough evaluation of biopharmaceutical glycosylation at all stages of

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