Future Drug Discovery

Editorial

Measurement of macro- and micro-heterogeneity of glycosylation in biopharmaceuticals: a pharmacopeia perspective

Jingzhong Guo*,[1], Huiping Tu[1] & Fouad Atouf[1]
[1]Global Biologics, Science Division, United States Pharmacopeia, Rockville, MD 20852, USA
*Author for correspondence: tim.guo@usp.org

"In addition to micro- and macro-heterogeneity, the diversity and lack of harmonization of analytical methods makes it challenging to measure glycosylation consistently. In this regard, public quality standards developed through an agreed-upon process play an essential role to ensure high-quality biopharmaceutical products."

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In recent decades, biopharmaceuticals have emerged as an important new class of drug with the promise to treat diseases that have eluded traditional chemical drugs. Because of their potential to improve clinical outcomes, the number of biopharmaceuticals is expected to grow in the coming decade. According to Medgadget, the biopharmaceutical market is projected to increase at an annual rate of approximately 9% and be worth $395 billion by 2025 [1]. The majority of biopharmaceuticals are recombinant proteins developed by biotechnological methods, 66% of which are glycosylated [2–4]. Glycosylation can significantly affect the efficacy, stability, immunogenicity, clearance, pharmacokinetics and pharmacodynamics of biopharmaceuticals. Therefore, accurately measuring and controlling glycosylation is necessary to assure product safety and quality, as well as to meet regulatory expectations [2–5]. In this article, we provide an overview of the technologies and challenges associated with analyzing glycosylation from a pharmacopeial perspective.

Complexity of glycosylation

Glycosylation is a post-translational modification in which glycans are attached to specific sites on a protein. The pattern of glycosylation varies from cell to cell, tissue to tissue and species to species due to the varying expression of hundreds of glycosyltransferases and glycosidases located throughout the Golgi apparatus and endoplasmic reticulum [5]. There are four types of glycosylation: N-linked, O-linked, C-glycosylation and glycosylphosphatidylinositol anchor. N-linked glycosylation occurs at the consensus sequence of N-X-S/T (where X stands for any amino acid except proline). O-linked glycosylation involves attaching glycans to the hydroxyl groups of serine or threonine. C-glycosylation attaches α-mannopyranosyl to tryptophan and the glycosylphosphatidylinositol anchor is a glycolipid linked to the C-terminus of a protein.

Glycosylation can occur at multiple sites on the protein, leading to a mixture of diverse structures termed macro- and micro-heterogeneity [2,3,6,7]. Macroheterogeneity refers to the site occupancy or completeness of glycosylation, while microheterogeneity concerns the variations of glycan structure at a specific site. Importantly, both forms of heterogeneity can significantly impact the physical and biochemical properties of a protein [2,3,6–8]. Therefore, accurately measuring and controlling the glycosylation macro- and micro-heterogeneity of a product during the manufacturing process is necessary to ensure its quality.

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Measurement of macro- & micro-heterogeneity of glycosylation

Measuring glycosylation is difficult because of multiple levels of heterogeneity of glycoproteins combined with the inherent variations in manufacturing processes. To address this challenge, a variety of analytical approaches are used to monitor the production process and acquire the necessary information. The main analytical technologies used include HPLC, MS, capillary gel electrophoresis (CGE), isoelectric focusing (IEF) and lectin-based approaches [2–4,6]. These methods can be used independently or in combination to measure protein glycosylation by analyzing the entire glycoprotein directly, by fragmenting the protein into glycopeptides or by releasing the attached glycans from the protein surface [2–5].

Direct measurement of glycoproteins

Glycosylation results in changes to the mass and electrostatic properties of the intact protein. These difference can be monitored with a minimal amount of sample manipulation by using analytical techniques that can discriminate between small variations in size and charge, including strong anion exchange chromatography, polyacrylamide gel electrophoresis, IEF, MALDI-TOF MS and ESI–MS [4,5]. Recently, CE and cIEF have become popular because of their improved resolution and throughput.

Analyzing intact glycoproteins is challenging when the target protein is large and is glycosylated at multiple sites. Some of these challenges can be overcome by combining different analytical methods; for example, using high resolution MS coupled with UHPLC, CE or cIEF.HPLC. The recent development of lectin microarray technology provides another option for direct profiling of intact glycoproteins [4]. The integration of lectin affinity purification with native MS has shown the potential to become a generic method for the determination of microheterogeneity of complex glycoproteins [9]. Though technology development helps, these approaches provide only a general profile of the glycosylation heterogeneity present in the sample; a combination with other analytical approaches is needed for detailed analysis of glycosylation heterogeneity [10].

Analysis of glycopeptides

The analysis of glycopeptides provides more comprehensive information than the direct examination of the intact glycoproteins, including glycosylation site, degree of occupancy and identification of glycan structure. Glycopeptides are produced by enzymatically digesting the glycoprotein. The glycopeptides are then separated and/or enriched before analysis. By itself, MALDI-MS is suitable for the rapid profiling of glycopeptides. However, the preferred method is usually a combination of MS with a separation technique (e.g., HPLC or CE).

LC–MS is a powerful strategy for qualitative analysis of glycosylation heterogeneity. It has also proven to be successful for quantitatively monitoring site-specific glycan heterogeneity; however, ionization efficiency of a glycopeptide may be affected by the glycans attached to the peptide [6]. For accurate determination of the site occupancy, calibration of ionization efficiency with reference peptides may be necessary. The most commonly used LC–MS method is RP–HPLC–MS. However, other chromatographic media provide different separation selectivity. For example, hydrophilic interaction liquid chromatography (HILIC) has better performance for separation of hydrophilic glycopeptides, while porous graphitic carbon can be used to separate glycopeptide isomers [4]. Some highly hydrophilic or hydrophobic glycopeptides are hard to recover from LC–MS analysis [4,11]. CE-MS provides an alternative method for analysis of these glycopeptides, including isomers.

For in-depth characterization of glycosylation sites and glycan components in glycoproteins, a tandem MS/MS analysis of fragments resulting from collisional induced dissociation or electron transfer dissociation is frequently used [2–4,7,12]. Collisional induced dissociation is used to get information about the glycan moiety, while electron transfer dissociation provides information about the glycopeptide sequence and location of glycosylation. The main challenge with these advance technologies is hard to implement in quality-controlled environment.

Analysis of released glycans

The glycan moiety attached to a protein can be released enzymatically or chemically. This approach provides information about the diversity of glycans present on the glycoprotein, and it is the most commonly used approach for characterization of N-glycosylation because N-glycans have more clinical relevancy and can be released more quantitatively than O-glycans [5]. Released glycans have no endogenous signal to allow for real-time monitoring. Therefore, chemical derivatization is commonly used to add a fluorescent tag that facilitates glycan quantitation [2–5,11,13]. The most commonly used derivatization reagents include 2-aminobenzoic acid (2-AA), 2-aminobenzamide (2-AB) and procainamide for HPLC with fluorescent detection or 9-aminopyrene-1,3,6-trisulfonic acid (APTS)

for CE with laser-induced fluorescence detection. HILIC provides better glycan separation than other media and is the preferred form of LC. Though MALDI-MS can be used for rapid glycan profiling without separation, HILIC coupled with fluorescent detection plus MS becomes more popular as it provides both qualitative and quantitative results in a single experiment.

However, challenges exist as 2-AA (or 2-AB) labeled glycans have a weak MS response due to their ionization efficiencies [14,15]. Development of new labeling methods such as RapiFluor-MS (Waters Corporation, MA, USA) and Instant PC (Agilent Technologies, CA, USA) has addressed these challenges. They provide high responses in fluorescent detection and MS, also allow for much faster labeling of released glycans. This significantly reduces the time needed for glycan profiling [14,15]. As with glycopeptide analysis, MS/MS is needed for a detailed analysis of the structures of released glycans. However, some glycans have multiple isoforms, and MS alone cannot resolve isomers. The implementation of ion-mobility makes it possible to separate structural isomers using MS [3,4]. In addition, glycan isomers can be identified through the use of specific exoglycosidase digestions, followed by LC–MS/MS analysis [3].

Roles & perspectives of pharmacopeia

In addition to micro- and macro-heterogeneity, the diversity and lack of harmonization of analytical methods makes it challenging to measure glycosylation consistently [2,3,16]. Both experimental and data mining procedures need to be standardized. However, glycosylation analysis technologies advance quickly, creating challenges to harmonization. In this regard, public quality standards developed through an agreed-upon process play an essential role to ensure high-quality biopharmaceutical products.

As a nonprofit, independent standards-setting organization, the United States Pharmacopeia (USP; MD, USA) plays an important role in improving global health through public standards and related programs that help ensure the quality, safety and benefit of medicines and foods. The USP has published several general chapters that provide general guidelines or test procedures for glycosylation analysis. USP Chapter 1084 includes general information on glycoprotein and glycan analysis [5]; chapter 212 presents procedures for oligosaccharide analysis [17]; while chapter 129 provide procedures for analysis of N-glycans and sialic acid in monoclonal antibodies [18].

The USP also offers reference standards for analysis of glycosylation, including a mixture of oligosaccharides for qualitative and quantitative analysis. Reference standards that support glycan analysis procedures for several recombinant glycoprotein products (i.e., including human coagulation factor VIIa, IFN-β-1a, EPO and mAbs) are also available. Finally, other pharmacopeias also provide public standards that support glycosylation analysis of biopharmaceutical products. The European Pharmacopoeia publishes a general chapter (2.2.59) with procedures for glycan analysis of glycoproteins [19], as well as several monographs describing glycan analyses of various glycoproteins.

The precision, accuracy and interlaboratory performance will be significantly improved by using standardized procedures vetted through a multistakeholder participation approach. As a convener, the USP hosts roundtables and workshops to engage with stakeholders and explore opportunities for standardization. The Glycosylation Analysis for Biopharmaceuticals workshop brings together representatives from the pharmaceutical industry, regulatory agencies and academia to discuss the role of protein glycosylation as a critical quality attribute. This type of collaborative approach fosters open dialog and consensus-building, leading to the development of reliable public standards.

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