Glycobiology

https://doi.org/10.1038/s41589-024-01755-6

Cracking the diversity of sweet drugs

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Characterization of the heterogeneity in the glycosylation of biotherapeutics is crucial for drug development but challenging. Now, an approach allows the rapid analysis of the glycosylation of intact glycoproteins, without prior processing or separation, enabling the study of glycan composition and quality assessment of any glycoprotein drug.

Most biotherapeutics are glycoproteins, including erythropoietin, antibodies, cytokines, hormones, growth factors and vaccines[1,2]. These drugs carry heterogeneous glycosylation patterns that affect their functionality[3]. Hence, detailed glycosylation analysis is critical for drug approval[1] but current techniques are laborious and do not always cover the full heterogeneous drug landscape[4].

Glycosylation is a metabolic non-template-driven process that relies on a collection of enzymes, operating sequentially and at equilibrium to conjugate and build up carbohydrate chain (glycan) moieties onto proteins as they are synthesized. Within the same cell, proteins will not always populate all of their glycosylation sites; additionally, each glycosylation site could present different glycans, resulting in a broad diversity of glycoprotein variants[3,5]. Consequently, glycoprotein drugs are intrinsically heterogeneous owing to the number and position of glycosylation sites in each of their domains or subunits (macro-heterogeneity) and due to glycan diversity within each of these sites (micro-heterogeneity)[3,5]. Accordingly, glycosylation is an important feature of biotherapeutics that substantially affects drug bioavailability, solubility, activity, stability, pharmacokinetics, immunogenicity, efficacy and safety[3,6]. For example, capping glycans with sialic acids generally improves stability and solubility of biotherapeutics, while under-sialylation leads to rapid clearance by galactose receptors in hepatocytes and liver macrophages, and deglycosylation can dramatically reduce activity with rapid filtration in the kidney[3,6]. Thus, glycan profiling is crucial to ensure drug quality and consistency during manufacturing and is required for market approval[1,2].

Specialized multistep, time-consuming analytical methods are currently used and often require release of glycans from proteins with subsequent complex annotation process, further challenged by heavily glycosylated biotherapeutics[4,7,8]. Thus, simplified glyco-analytical approaches are much coveted[9,10]. In a recent publication in *Nature Communications*, Schachner et al.[11] describe an approach to analyze intact glycoproteins, without any denaturation, digestion or separation, that could facilitate rapid analysis of biotherapeutics.

The team describe an intact mass spectrometry characterization approach, to enable the analysis of glycosylated biotherapeutics that present a highly heterogeneous mixture of variants[11]. This method is applied on the whole glycoprotein–drug mixture, without preprocessing, to rapidly measure molecular weight of its assorted molecular variants. Consequently, it can easily decipher the heterogeneity of glycosylation, as well as subunit mis-assembly or truncated variants, something that could not have been achieved using traditional techniques that use fragmented drug moieties. The approach relies on glycoform fingerprinting using proton transfer charge reduction (PTCR) with gas-phase fractionation in a form of data-independent (DIA) tandem mass spectrometry. Using bioinformatics and correlation with glycoproteomics and glycomics data, glycan barcodes are generated and allow site-resolved glycan composition, including their predicted relative abundances (Fig. 1). The power of the method and its reproducibility are demonstrated on several glycoproteins, each

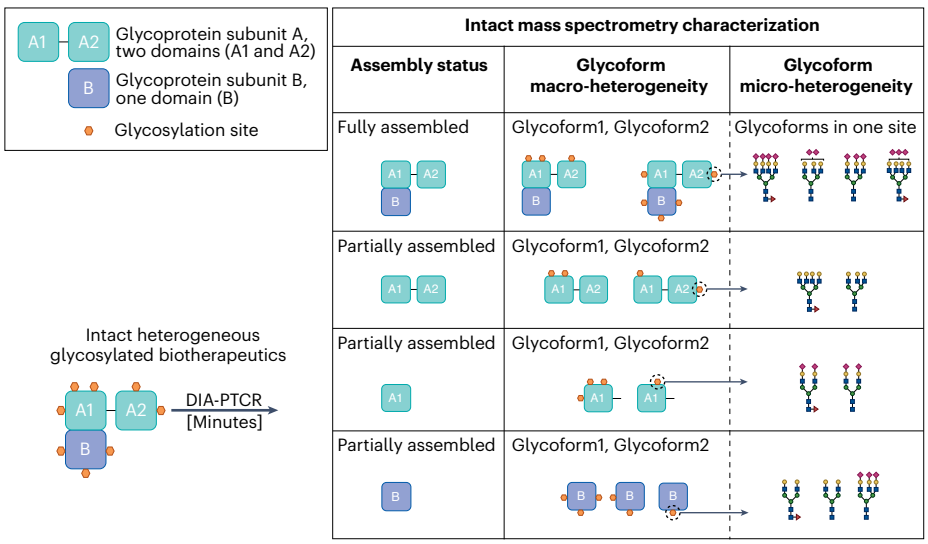

Fig. 1 | Intact mass spectrometry characterization approach for rapid profiling of biotherapeutics. Schematic diagram of the information that can be achieved by the rapid analysis of intact heterogeneous glycosylated biotherapeutics that is done by DIA-PTCR combined with extensive glyco-bioinformatics. This method generates glycan barcodes that allow to rapidly decipher the glycoprotein subunits assembly status and their glycoforms' macro- and micro-heterogeneity through site-resolved glycan composition and abundance, including information regarding subunit mis-assembly variants[11]. Blue square, *N*-acetylglucosamine; green circle, mannose; magenta diamond, sialic acid; red triangle, fucose; yellow circle, galactose.

with different intrinsic complexity and heterogeneity in terms of the number of domains and subunits, the number of glycosylation sites and the diversity of glycan composition. These included ovalbumin, peptide-bound major histocompatibility complex class II complex containing four glycosylation sites, and multidomain-Fc fusion proteins with six to eight glycosylation sites. This efficient and rapid analysis further allowed the correlation of certain glycoforms with optimal functionality. A demonstration with IL-22–Fc fusion variants showed reduced binding to the IL-22 cytokine receptor in variants that are highly sialylated at a key N-linked glycosylation site, while elimination of this glycosylation site led to an almost 4-fold increase in binding to the receptor. Hence, this methodology could expedite quality assurance of biotherapeutics, facilitating linking functionality to specific subpopulations of the glycosylated drug.

Overall, this method provides important innovation to the complex task of analyzing the heterogeneity of biotherapeutics within minutes. In particular, the ability to examine intact glycoproteins adds a new level of information to this type of analysis, including the degree of assembly of the drug's domains or subunits and their glycoforms' macro- and micro-heterogeneity. This could not have been achieved with current techniques that require indirect methodologies to infer such information. Furthermore, the sensitivity of the method to detect minor glycosylation differences, such as the sialic acid composition within each glycoform, highlights the potential to use this approach for batch-to-batch reproducibility of biotherapeutics, monitor glycosylation consistency during production, compare potential biosimilars to original products, and perhaps also to investigate the role of glycosylation in the fine-tuning of biotherapeutic efficacy in vitro and in vivo. While this rapid approach is clearly of potential significance to the field of biotherapeutic design and quality assurance, it requires specialized costly equipment and analytical power, and so it may take time until it is fully validated and integrated into quality assessment workflows.

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Published online: 24 October 2024

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Competing interests

The authors declare no competing interests.