Multi-level structural and functional characterization of therapeutic glycoproteins by mass spectrometry

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SUMMARY

Therapeutic proteins have been successfully developed for advancing medical treatments. They are usually large molecules produced by host cells and have a high degree of complexity compared to synthetic small molecule-based therapeutics. The complexity is mainly attributed to the heterogenic nature of post-translational modifications (PTMs). Glycosylation is one of the main drivers of protein heterogeneity. Since each modification may potentially impact the safety and efficacy, analytical methods for the structural and functional characterization of protein-based therapeutics are highly demanded.

Chapter 1 introduces an overview of therapeutic proteins. Common PTMs of therapeutic proteins are described with a focus on glycosylation. The functional relevance of glycosylation is exemplified for Immunoglobulins (Igs), a major class of therapeutic proteins. Further, common approaches and challenges for the analytical characterization are introduced. Mass spectrometry (MS) is a key technique for the analysis of therapeutic proteins. Thus, the fundamental ionization techniques for the analysis of biomolecules, electrospray ionization (ESI) and matrix-assisted laser desorption ionization (MALDI) are described. Finally, the scope of the thesis is outlined.

The (glyco)peptide-centered analysis of therapeutic proteins is important for the site-specific characterization of PTMs. Bottom-up approaches are generally very data-rich, particularly for glycoproteins, due to the high structural diversity of glycosylation. **Chapter 2** presents a data analysis pipeline for streamlined processing of glycopeptide information from reversed-phase liquid chromatography MS/MS. A tryptic digest of IgG and IgA, enriched from human plasma, was analyzed in this study. Several recent bioinformatics tools, namely Byonic, GlycopeptideGraphMS and LaCyTools, are integrated. In addition, drawbacks and limitations of current glycoproteomics approaches are described. This chapter provides a guide for the efficient analysis of glycoproteomics data with a focus on maximizing glycopeptide identification and increasing the reliability of relative quantification.

The sample preparation strategy is highly relevant for a successful and efficient analysis of glycoproteins by bottom-up approaches. One of the main challenges is to obtain peptides bearing only a single glycosylation site. The addition of artificial protease cleavage sites via cysteine aminoethylation is exploited for the glycoproteomic analysis of recombinant erythropoietin (EPO) in **Chapter 3**. This approach adds additional tryptic cleavage sites between two glycosylation sites which could previously only be assessed by less specific proteases. It is demonstrated that the developed strategy, relying on trypsin as a sole protease, significantly reduced the data complexity and allowed for a shorter analysis time. Moreover, the advantage of the new method for

multiple attribute monitoring is presented.

Glycoproteomic approaches are important for an in-depth site-specific characterization of glycoproteins with multiple glycosylation sites. However, the information on the combination of glycoforms is lost. Valuable information on the actual proteoform heterogeneity is commonly obtained by intact mass profiling. ESI MS-based methods are usually the first choice for intact mass analysis due to higher proteoform resolution and increased stability of glycosylation features (i.e. sialic acids) compared to MALDI MS. **Chapter 4** demonstrates the feasibility of MALDI Fourier transform cyclotron resonance (FT-ICR) MS for the intact analysis of the complex and highly sialylated glycoprotein EPO. It is shown that the instrumental superiority and an appropriate choice of the MALDI matrix allow characterizing intact proteoforms by MALDI FT-ICR MS with comparable performance to ESI MS-based methods. The developed method is fast and offers a straightforward data analysis of doubly charged ions omitting the need for any charge deconvolution. This makes the approach highly relevant for developing high-throughput applications for the intact mass analysis of glycoproteins.

Monoclonal antibodies (mAbs) show a diversity of glycoforms (i.e., pairings of conserved Fc glycans) which may differentially impact the safety and efficacy. The structure-function characterization of mAb glycoforms is commonly addressed by glycoengineering of specific features and subsequent analysis by binding assays to relevant immune receptors. This approach is time-consuming and limited by the purity and variety of glycoforms that can be achieved. Fc gamma receptor IIIA is an important receptor for assessing antibody-dependent cellular cytotoxicity. **Chapter 5** describes a novel method based on Fc gamma RIIIa affinity chromatography (AC) hyphenated to MS, which allows unpreceded insights into the structure-function relationship of mAb glycoforms. Individual glycoforms can be easily ranked by their affinity without the need for glycoengineering.

The high mAb glycoform resolution achieved by Fc gamma RIIIA AC-MS may be hampered for mAbs with additional heterogeneity (e.g. Fab glycosylation). Therefore, the analysis of Fc moieties by a middle-up approach is desirable to decrease the proteoform complexity for a more confident glycoform assessment. In **Chapter 6**, the Fc gamma RIIIA method is further developed by evaluating different hinge-cleavage proteases for middle-up Fc gamma RIIIA AC-MS. Kgp is found to be a suitable protease for retaining Fc gamma RIIIA affinity of Fc moieties. The advantages for reducing the ambiguities in glycoform assignments are demonstrated for the analysis of Fab glycosylated cetuximab.

Chapter 7 shows the development of a novel Fc gamma RIIIB AC-MS method. Fc gamma RIIIB plays a major role in neutrophil activation, which is an important mechanism of action of therapeutic mAbs. The use of an Fc gamma RIIIb AC column is reported for the first time. The applicability of the method to a panel of different classical and glycoengineered mAbs is demonstrated. Furthermore, this chapter

compares the glycosylation profiles of human and recombinant Fc gamma RIIIB. In addition, the IgG subclass specificity of Fc gamma RIIIb AC-MS is shown.

Finally, the analytical challenges for the characterization of therapeutic glycoproteins are discussed in **Chapter 8**. The first part describes perspectives towards higher data analysis efficiency and reliability of bottom-up and intact mass analysis. In addition, potential biopharmaceutical applications for cysteine aminoethylation and intact mass analysis by MALDI MS are discussed. In the second part, the role of AC-MS for supporting critical quality attribute assessment of mAbs is highlighted. Technological perspectives are described for enhancing the functional understanding of mAb proteoforms. Finally, technical aspects of AC-MS are discussed and compared to other binding assays for applications in the biopharmaceutical industry.