Methods for the glycosylation analysis of therapeutic antibodies

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Summary

Immunoglobulins (antibodies) are an important part of the humoral immune response in humans. They can be divided into five different isotypes, namely IgA, IgD, IgE, IgG and IgM. The IgG isotype is further subdivided into four subclasses: IgG1, IgG2, IgG3 and IgG4. Each IgG comprises two heavy chains and two light chains which are linked by disulfide bonds forming a Y-shaped molecular structure. Today therapeutic monoclonal antibodies produced in the biopharmaceutical industry are of key importance in the treatment of various diseases and are among the top-selling medicines world-wide. They are applied in many disease areas including oncology (non-small cell lung cancer, melanoma, neuroblastoma, gastric cancer, colorectal cancer, breast cancer and many more), autoimmune diseases (rheumatoid and psoriatic arthritis, ankylosing spondylitis, psoriasis, Crohn's disease, multiple sclerosis and many more) and infectious diseases (anthrax infection). Hundreds of therapeutic antibodies are in development and more than fifty monoclonal antibodies are approved or in review in the European Union or the United States. As such the development and commercialization of therapeutic antibodies is and continues to be of utmost importance for the pharmaceutical industry.

IgGs are glycosylated with a glycosylation in the Fc part at asparagine 297, characterized by the sequon Asn-X-Ser/Thr (where X is not proline but can be any other amino acid). Three different types of biantennary N-glycans are generated: high-mannose type glycans, hybrid glycans and complex glycans. The glycosylation of biotherapeutics, as it can have an impact on bioactivity, pharmacodynamics/pharmacodynamics and immunogenicity, has to be consistent and to a large extent human-like to prevent adverse events. Therefore monitoring of the IgG glycosylation is of great importance in cell line development, clone selection, process characterization and process validation studies, as well as for comparability purposes and release. A wide range of different analytical technologies are employed in the biotech industry. Some are separation based others are mass spectrometry based and can be principally subdivided in three groups: 1. Analysis of the intact molecule and deduction of the overall glycan composition; 2. Enzymatic or chemical release of the glycans and measurement of the glycans; 3. Proteolytic cleavage and measurement of glycopeptides.

For process development, medium development and clone selection there is a great demand for need concerning high-throughput glycoanalysis in the biotech industry.

The biological and clinical role of IgG Fc N-glycosylation, especially their role as critical quality attributes (CQAs) is reviewed in **Chapter 2.** In addition Fc glycan information from published literature as well as internal data is evaluated concerning their impact on patient safety, immunogenicity, bioactivity and pharmacodynamic and pharmacokinetic behavior.

Mass spectrometry is applicable for structural characterization of protein glycosylation and site-specific structural characterization at the level of glycopeptides. In **Chapter 3** the development of a fully automated high-throughput method based on the glycopeptide level is described. A robust system was obtained with samples prepared in 96-well plates. After direct capturing of the IgGs from the fermentation broth with immobilized protein A and subsequent tryptic digestion, the glycopeptides are purified by hydrophilic interaction (HILIC) solid-phase extraction and injected by means of a robot to a electrospray ionization mass spectrometer (ESI-MS) in the positive mode. A recently published method was adapted to robotics, ESI-MS instead of MALDI-MS was used and several steps of the procedure were optimized. The relative abundances of the main glycoforms were in good agreement with several alternative methods. The method was successfully applied to measurement of the glycosylation state in the course of a fermentation run. With this approach it is possible to deduce the appropriate fermentation time for the desired glycosylation and to investigate the impact of alterations in the fermentation process on the consequent glycosylation. It was found that it is crucial to have the right concentration of acetonitrile and trifluoracetic acid in the HILIC purification step. The method was found to be accurate, precise and reproducible and as such can be used to aid in clone selection, for media development, glycomic applications, process development and process characterization.

In **Chapter 4** of this thesis the development of another largely automated high-throughput glycosylation profiling method for the analysis of immunoglobulin G on the glycan level is described. A DNA Analyzer as multiplexing capillary-gel-electrophoresis (CGE) with laser induced fluorescence (LIF) for detection was used. After purification with protein A beads and PNGaseF digestion, the released glycans were labeled with 8-aminopyrene-1, 3,6-trisulfonic acid (APTS) in 96-well plates, which was followed by the simultaneous analysis of up to 48 samples. The quantitative data evaluation was carried out automatically using in-house developed

software. As the DNA Analyzer cannot be directly coupled to a mass spectrometer, the peak assignment was conducted by HILIC-UPLC-MS/MS of the APTS-labeled glycans combined with peak fractionation and subsequent CGE-LIF analysis of the MS-characterized fractions. Normalization can be conducted with DNA fractions because the characterized peaks can be linked directly to base pair units in the developed software, and hence the peaks can be unambiguously assigned in each run. The CGE-LIF DNA analyzer system had a separation power comparable to HILIC-UPLC after APTS-labeling and 2-AB labeling, respectively.

With the described experiments it was shown that the CGE-LIF method has an excellent separation power, a very good repeatability and intermediate precision, and possesses a high robustness. The method was also successfully applied to measurement of the glycosylation state over the course of a fermentation run. As expected for fermentation of therapeutic antibodies in CHO cells, the relative amount of G0F increases with time and, accordingly G1F glycan decreases.

In Chapter 5 and Chapter 6 a comprehensive comparison of methods for the analysis of therapeutic immunoglobulin G Fc-glycosylation profiles are described. In **Chapter 5** comparison of separation based methods and in **Chapter 6** the comparison of mass spectrometry based methods are compared. A therapeutic antibody reference material was analyzed 6-fold on two different days, and the methods were compared for precision, accuracy, throughput and other(method quality) attributes. Although low in abundance special emphasis was placed on the detection of sialic acid-containing glycans. HILIC-HPLC of 2-aminobenzamide (2-AB)-labeled glycans was used as a reference method in both parts of the study. The seven separation based methods showed excellent performance in terms of accuracy, precision and separation, and are well suited for the purpose of analyzing Fc-glycosylation of IgG1. The relative quantitation for the individual glycan species was comparable. In principle, all methods could be used as release methods, and validation should be straightforward. In our hands, the Reference Method, HILIC (2-AB), is optimally suited for release. The method found to be best suited for high throughput was DSA-FACE (APTS), where 96 samples can be analyzed in parallel. If all glycan structures of a mAb must be quantified, the use of two methods in parallel is advantageous. With respect to the mass spectrometry based methods, they may be compromised by in-source decay. Thus, it is not always possible to distinguish between glycan species present in the sample and species produced by in-source fragmentation. Nevertheless with the exception of ESI-MS Heavy Chain,

the most prominent glycan species could be detected and quantified with high accuracy and precision using the methods evaluated. In addition, the results obtained with all separation-based methods (those without mass spectrometric detection) and the mass spectrometric methods were very similar in regard to the robustness and accurate detection and quantitation of low abundance glycoforms.

In **Chapter 7** the in vitro glycoengineering of IgG1 and its effect on Fc receptor binding and ADCC (antibody-dependent cell-mediated cytotoxicity) activity is described. The influence of IgG1 Fc galactosylation and sialylation on the effector functions were investigated using samples produced by in vitro glycoengineering. A variety of analytical assays including Surface Plasmon Resonance (SPR) and recently developed FcγR affinity chromatography, as well as an optimized cell-based ADCC assay were applied. No impact of sialic acid- containing Fc glycans on ADCC activity, FcγRI, and FcγRIIIa receptors could be shown, however a slightly improved binding to FcγRIIa was observed. In contrast a galactosylation-induced positive impact on the binding activity of the IgG1 to FcγRIIa and FcγRIIIa receptors and ADCC activity could be demonstrated.

As the final chapter **(Chapter 8)** of this thesis, a general discussion, which places the individual chapters into context, is included. The limitation, strengths, areas for application and perspectives of the glycosylation analysis methods are discussed. Furthermore challenges and future trends concerning glycoanalytical methods for biotherapeutics and structure-function relationship investigations with the help of in vitro glycoengineering are discussed.