Boosting mass spectrometry-based analytics for biopharma

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English Summary

Antibody-based therapeutics are widely used to treat various diseases. Initially, antibodies were enriched from blood specimens before administration to patients but nowadays most are produced recombinantly, either as conventional antibodies or with optimized functions. These recombinantly produced antibody therapeutics commonly carry a plethora of post-translational modifications (PTMs) that potentially alter their function and therefore need to be monitored to ensure high safety and efficacy (**Chapter 1**). Although several analytical platforms have been established for the characterization of conventional monoclonal antibodies (mAbs), aspects such as automation, the appearance of unexpected PTMs in novel antibody formats or proteoform-specific functional characterization still require further analytical developments.

Chapter 2 and **3** address automation of bottom-up approaches for fast antibody characterization. Automation allows analysis at higher sample throughput thereby reducing hands-on time and minimizing the risk of introducing unintended modifications. **Chapter 2** is focused on integrating bottom-up characterization after a charge variant separation in a multidimensional liquid chromatography (LC) platform. The described method allows in-depth characterization of separated variants in an automated way avoiding tedious collection of chromatographic fractions. Furthermore, the approach provides a solution to overcome offline sample preparation, including reduction, overnight tryptic digestion and LC-mass spectrometry (MS)(/MS) analysis. In **Chapter 3** this new online tryptic digestion approach was further developed. Antibody sequence coverages similar to the ones obtained by offline procedures were achieved by optimization of parameters such as column, flow rates and temperature. In addition, this chapter provides a new platform for direct analysis of formulated mAb and bispecific antibody (BsAb) samples without the need of any sample pre-treatment allowing to process several samples in a short timeframe and thereby providing an alternative to robotic platforms.

Although bottom-up approaches allow in-depth characterization of monospecific antibodies, the newer antibody formats often require additional solutions to localize a modification with regard to their quaternary structure. For example, in BsAbs, many heavy chain proteolytic peptides are identical even though the chains are partially different, hampering the possibility to assign PTMs to specific subunits. **Chapter 4** describes middle-up/down and top-down approaches based on matrix assisted laser desorption ionization (MALDI)-MS for localization of PTMs of BsAbs at the subunit or amino acid level. The applicability of the methods was demonstrated by the analysis of glycated BsAb samples. Several glycations were detected at the subunit level, for which the modified site could be determined when close to the N- or C-terminus by applying in source decay (ISD) fragmentation. In **Chapter 5** the MALDI-ISD approach was additionally applied to an antibody presenting a very labile modification, *i.e.* sulfation. Since ISD is a soft fragmentation technique it allowed to assign the sulfation to

a tyrosine residue, which was not possible with other fragmentation techniques. Next to sulfation **Chapter 5** also describes the assignment of a 4-hydroxyproline which occurred on new antibody formats. Finally, an in-house *in silico* tool was developed allowing to predict the appearance of modifications already during mAb or BsAb design and thereby allowing to modify the sequence before expression to avoid them.

Chapter 6 describes an alternative approach to study BsAbs at the middle-up and intact level based on capillary electrophoresis (CE)-MS**.** Two sister BsAbs, with same antigen target but different engineering process were employed. In this chapter, a novel hinge-region endoprotease (SpeB) was explored as alternative to the commonly used IdeS for antibodies with a modified hinge region compared to IgG1. Application of the CE-MS method for the analysis of BsAbs at the intact level allowed to characterize side products, such as BsAbs missing chains, free light chains carrying different PTMs or BsAbs missing one N-glycosylation. This type of macroheterogeneity required the analysis with limited sample treatment to avoid loss of information on their quaternary structure. This intact CE-MS approach was in addition applied to monitor correct assembly of BsAbs. **Chapter 7** therefore demonstrates the applicability of intact CE-MS to monitor the exchange efficiency during bispecific antibody production using a controlled Fab-arm exchange. The method was suitable for antibodies with and without Fab glycosylation and to assess degradation products occurring during elongated storage.

Equally important next to characterization of antibody PTMs is to evaluate the impact of these modifications on antibody functionality. Functional characteristics are often addressed using binding techniques, such as ELISA or SPR. However, these techniques provide an overall response for a mAb sample requiring tedious fractionation or production of single variants. In **Chapter 8** these difficulties are addressed and a technique is described to study antibody interactions in a proteoform specific-manner based on mobility shift affinity CE-MS without the need for any prefractionation. The developed approach focused on studying the interaction of different antibody proteoforms with the FcRn receptor, responsible of antibody half-life. Affinity information was obtained by means of their different mobility shifts upon addition of the receptor while hyphenation to MS provided direct structural information. Furthermore, it is shown that by using different amounts of FcRn in the background electrolyte K_D values can be simultaneously determined for each proteoform.

The last part of this thesis (**Chapter 9)** is focused on the characterization of recombinantly produced SARS-CoV-2 receptor binding domain (RBD). Due to the complexity and limited knowledge on the RBD, a multilevel approach was necessary to obtain an entire picture. To this end, released O-glycan analysis, bottom-up N-glycopeptide analysis, MALDI top-down fragmentation and CE-MS intact analysis were combined with different endo- and exoglycosidase treatments to obtain a complete assignment of glycoforms and PTMs. Two RBD samples produced in different cell types (CHO and HEK293) were characterized

revealing specific heterogeneity. Furthermore, the chapter describes the localization of the O-glycosylation site in the RBD for the first time. This was achieved by a combination of intact and top-down MS analysis. After structural characterization, binding assays with antibodies from SARS-CoV-2 patients and ACE2 were performed to study potential differences in binding between the two RBD samples.

The final chapter (**Chapter 10**) comprises a general discussion of this thesis. The chapter discusses the need of automated sample platforms in the pharmaceutical industry and how they could be further improved. Furthermore, the relevance for intact and middle-up/down native MS is discussed giving special attention to the discrepancies between bottom-up and intact data based on the observations reported in **Chapter 9**. Another discussion point is the functional characterization of mAb PTMs with various techniques, further developments and a view on potential clinical use to study the implications of antibody proteoforms in diseases. Finally, some personal thoughts on the (sometimes) limited relation between industry and academia and some possible strategies to strengthen this relationship are shared.