Boosting mass spectrometry-based analytics for biopharma

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Chapter 10

Discussion and future perspectives

Production of biopharmaceuticals requires several levels of characterization to guarantee an effective and safe drug. Similar to other sectors, new products with novel properties are regularly being introduced by the biopharmaceutical industry. This includes optimized conventional antibodies (*e.g.* improved pharmacodynamics or pharmacokinetics) and new antibody formats with new or enhanced properties. However, analytical technologies are not evolving with the same speed, resulting in a higher load/pressure on the analytical laboratories, increasing development cost and eventually product prices. One example are suitable sized platforms to tackle integrated sample preparation and analysis. Another, major analytical hurdle is the investigation of Fc functions of specific mAb proteoforms, enabling to improve conventional antibody therapeutics (efficacy or pharmacokinetics), (re)-define CQAs or produce new antibody formats. Boosting biopharma analytics is therefore crucial and benefits from close collaboration between industry and academia instead of independent development. The mentioned points will be discussed in the following sections of this thesis.

10.1 Automated sample preparation and MS analysis

MS characterization of antibodies, necessary in several stages of mAb development, stability and formulation assessment, is traditionally performed by bottom-up approaches. Recently, the possibility of performing multiple attribute monitoring (MAM)[1] for CQA analysis is gaining more interest. MAM approaches are based on protein digestion (bottom-up) and analysis by LC-MS/MS. In MAM, information on several CQAs can be obtained from one measurement instead of the traditional single CQA assessment using different platforms. Whereas the concept of getting maximum information out of one measurement and remove several analytical platforms increases efficiency, still it is based on a tedious sample preparation (reduction, alkylation, digestion) prior LC-MS/MS analysis. In biopharma laboratories, this sample preparation is either performed in a manual manner[2] or following an off-line automated sample preparation by a pipetting robot[3, 4]. Not only the manual approach feels a bit outdated for a highly innovative sector as the biopharmaceutical industry, but it also has the risk to induce unintended modifications[5] which might stay unnoticed especially when MAM approaches are employed. On the other hand, also robotic platforms for off-line automated sample preparation come with hurdles. A robotic platform needs to be purchased and maintained, trained personal is required and a large amount of samples is needed to make it cost-efficient. Still, following robotic sample preparation, the analysis of the samples by LC-MS needs to be performed, which leads to extended storage times and thereby also increases the risk of unintended modifications. Because in the biopharmaceutical industry samples are often not in the range of hundreds at one time point but rather an almost continuous flow of samples with often only a handful of samples at any given time, robotic sample preparation platforms often feel like using a sledge-hammer to crack a nut.

Chapter 3 presents an elegant solution to perform bottom-up sample preparation and analysis in the same platform, closing the gap for continuous sample analysis and reducing the risk of unintended modifications. The presented concept is based on an commercial 2D-LC system, which is nowadays very common in analytical laboratories. These systems can be easily extended with additional commercial modules and perfectly integrated into the existing software (**Chapter 3**). Also the need for special trained personal is not necessary as most laboratory workers are able to operate an LC system. The samples can be injected directly from formulation without the need for any desalting. All relevant sample preparation and analytical steps from reduction, tryptic digestion over separation of peptides and MS analysis are performed automatically. Whereas in the platform presented in **Chapter 2** some hydrophilic peptides were lost, **Chapter 3** shows improved conditions which allow to obtain very similar results to the standard manual procedure but with lower hands-on time. Also, there is no storage time after sample preparation as in robotic platforms and samples are directly processed online, which avoids the risk of inducing unintended modifications. Next to automation, the use of immobilized enzyme reactors (IMERs) results in more efficient and, therefore, fast digestion. The reduced digestion time is consequence of a higher local trypsin concentration due to immobilization and due to the faster mass transfer between antibody and trypsin[6]. Similarly, the reduction takes only a few minutes instead of 30 min commonly used in the off-line procedure. The method is also applicable to new formats such as BsAbs providing high sequence coverage, comparable to the standard approach.

While MS approaches are mainly used in product characterization as well as formulation and stability testing, release testing in quality control (QC) is largely based on separation methods with UV detection. Still, when an unexpected peak appears, an in-depth MS characterization of these signals is required. Direct hyphenation of QC methods to MS is in most cases not doable due to the use of high amounts of non-volatile salts in the mobiles phases. The standard procedure in the pharmaceutical industry is collection of the peaks by several off-line fractionation steps to obtain enough material and characterization by bottom-up LC-MS. In the cases where the new species are not baseline separated, other proteoforms can be cofractionated complicating a confident assignment of the unexpected species. Even if the peak of interest is well resolved from other mAb proteoforms, this process consumes extensive hands-on time for the fractionation and reanalysis. **Chapter 2** describes an automated system where QC separations can be directly implemented, the peak of interest can be fractionated and individually characterized in an on-line bottom up approach. In contrast to the approximately 52 hours necessary in a regular off-line fractionation, reanalysis and characterization by a bottom-up approach of 5 peaks, this platform can perform this process in around 9 hours in an automated way (**Chapter 2**). The developed platforms (**Chapter 2 and 3**) have been very well accepted and are currently established and used on a daily basis in various pharmaceutical laboratories. Furthermore, there has been a high potential and broad interest by academic and different pharmaceutical laboratories represented by the number of articles based on the method presented in

Chapter 2[7-10]. Despite the full automation at the hardware level, in **Chapter 2** two controlling software tools are currently still necessary, which is not a problem itself, but would benefit from a complete software integration by the vendor in future, allowing even further to enhance the implementation of these approaches in biopharma.

The developed platforms focus on trypsin digestion (which is the standard protocol) for generating the peptides. However, some biopharmaceuticals require alternative endoproteases to provide efficient cleavage (*e.g.* LysC or GluC). Until now, next to trypsin, only papain, IdeS or pepsin columns are commercially available, which either cleave only in the hinge region of antibodies (papain, IdeS) or provides non-specific digestion of the Fc subunit (pepsin), limiting the applicability of the approach. To overcome this bottleneck on-line in-solution digestion – *i.e.* without immobilization of the endoprotease in a column – would be beneficial. This would open the possibility to use other endoproteases commonly used in industry, such as LysC or GluC or endoprotease mixtures, to ensure high sequence coverage and detection of all relevant CQAs. However, as mentioned before the digestion using an IMER has the benefit of a relatively high local endoprotease concentration and a very fast mass transfer between endoprotease and antibody. Mixing both enzyme and antibody on-line might result in a lower local endoprotease concentration and autodigestion therefore, decreasing digestion efficiency. A solution could be the use of proper mixers or to non-covalently trap the endoprotease on a column, which could allow higher concentrations of the enzyme and prevent autodigestion partially.

Besides biopharmaceutical characterization, this platform would be very useful to study the pharmacokinetic behaviour of drugs in serum or disease-related antibodies where often a large number of samples have to be analyzed to draw reliable conclusions. However, sample amounts needed for this analytical scale platform are in the range of µg and therefore, miniaturization would be necessary to reduce sample consumption and further boost the MS sensitivity (*i.e.* nano-ESI). Unfortunately, commercial multidimensional systems in a nano or capillary scale are not available yet, and only in-house developed systems have been reported which lack robustness and high throughput applicability. Therefore, technological developments towards miniaturized multidimensional LC systems by LC vendors are necessary to further push their applicability in pharma and clinical laboratories.

10.2 Intact, middle-up/down and native approaches in biopharma

Bottom-up approaches are very useful for the localization of modifications on the protein backbone and determination of the mAb sequence. However, after endoprotease digestion, some information is lost, leaving only pieces of the puzzle instead of the complete picture. Even when the complete puzzle can be constructed from the pieces, the obtained picture can misrepresent the reality. A clear example of this situation is illustrated in **Chapter 9**.

Comparing the results of intact protein analysis and a simulation of the intact spectrum from bottom-up results showed quite some discrepancies. Whereas the results were comparable in the case of non-charged glycoforms, sialylated glycans were underestimated in the bottom-up approach resulting in an overall lower mass. Similar observations were made in another report, where erythropoietin was analyzed[11]. The most likely explanation is that the ionization bias on the sialylated species (bearing a negative charge) is higher at the peptide level, due to the lower total number of charges compared to the intact protein which is highly charged. Also the ion transmission energies might not be optimal for each glycopeptide group (*e.g.* sialylated vs non-sialylated glycopeptides), affecting the relative intensity between species. Additional PTMs on the protein backbone would further complicate the situation as co-occurrence of modifications can have an interplay. This example nicely demonstrates the importance to analyze complex glycoproteins on the intact level to get a more real picture of the actual proteoforms. For antibody samples where most PTMs are known, such intact solutions are often enough to provide reliable characterization. Yet, for complex glycoproteins that are first-time characterized, as shown in **Chapter 9** for RBDs, the use of complementary platforms is inevitable. Here using a combination of glycan release, bottom-up and intact MS approaches was necessary to assign all proteoforms of a new protein with very high confidence. After full assignment, however, a comparability analysis of *e.g.* RBD samples could be performed at the intact level to ensure batch-to-batch similarity in a fast and straightforward way with limited sample manipulation.

Next to biases and loss of information on co-occurring modifications – *i.e.* proteoform identity – also some crucial aspects for biopharma characterization cannot be attained by bottom-up approaches. One clear example is the misassembling side products of new antibody formats such as BsAbs. In those cases, the analysis at the intact level is mandatory. In **Chapter 6** we developed a methodology based on CE-MS for the assessment of misassembled side products in BsAb therapeutics. We observed that the engineering process can alter the heterogeneity obtained, highlighting the necessity of these type of approaches. Another benefit of the intact analysis is the possibility to assess the exchange efficiency of bispecific antibodies generated by controlled Fab-arm exchange (**Chapter 7**), which is not possible with stand-alone MS due to the mass similarity of the parental mAbs. Yet, although intact characterization is necessary for macroheterogeneity assessment, it fails on PTM localization. **Chapter 6** shows the benefits of complementary intact and middle-up characterization for BsAbs. Whereas intact analysis allows to get the information on these misassembled side products, the middle-up level allows to localize the proteoforms to a specific subunit and increase certainty in the assignment of proteoforms with small mass differences (higher accuracy). The versatility of CE-MS allowed to assess BsAbs on both levels and determine macro- and microheterogeneity by simply changing the BGE. Some new antibody formats have a modified hinge region (PG LALA mutation), abrogating the use of the classical enzyme IdeS. To overcome this, an alternative enzyme SpeB is reported in **Chapter 6**, able to cleave hinge-region modified antibodies allowing thereby their middle-up

characterization.

Recently top-down and middle-down approaches are gaining more and more attention due to new developments in fragmentation techniques, such as ECD and ETD or UVPD, but also MALDI-ISD, allowing to obtain very good sequence coverage[12]. Whereas middle-down approaches require a hinge-region cleavage and/or reduction of disulfide bridges, top-down approaches require only a desalting step or no prior sample preparation. In both cases localization of the PTMs is possible, however middle-down permits accessibility to the hinge region and thereby boosts sequence coverage. It was shown that middle down sequencing can provide a sequence coverage of around 60-89% depending on the subunit[12], whereas top-down of conventional mAbs reaches only around 30% sequence coverage[13]. Similarly, in **Chapter 4** we reported higher sequence coverage for IdeS cleaved bispecific antibodies compared to top-down fragmentation due to the presence of fragments from the hinge region of the Hc.

Another expanding field, which greatly benefited from the recent technological advances in MS and is clearly gaining momentum is native mass spectrometry. Native MS maintains the higher order structure of the protein during analysis permitting to study protein complexes. The implementation of Orbitrap mass analyzers has revolutionized the field of native MS allowing to analyze native proteins with high resolution. Especially instruments with an extended mass range permit to detect with good resolution antibody and new antibody formats in their native state (~*m/z* 6000-8000) and their complexes (*e.g.* dimers, mAb-FcR complexes ~*m/z* 7000-9000)[14]. Another benefit of native MS is the higher sensitivity due to the lower number of charge states (and therefore lower spread of the signal) compared to denaturing MS. However, many mass spectrometers so far are not able to allow good declustering or desolvation of native proteins to benefit from this increased sensitivity. Orbitrap mass analyzers provide more efficient desolvation compared to FT-ICR or Q-TOF mass analyzers, due to the possibility of storing the ions in an HCD cell with a higher gas pressure[15]. Overall, all these advances significantly boosted native MS in the last few years and first applications of native MS in biopharmaceutical analysis have been demonstrated[16-18] and applied by pharma companies and undoubtedly will be expanded in the next years. In this lines, **Chapter 8** demonstrated the benefits of native MS to study protein-protein interactions and determination of the binding stoichiometry by detection of the formed FcRn-antibody complexes.

10.3 Affinity CE for the selective functional characterization of antibody proteoforms

Antibody biopharmaceuticals consist of a plethora of proteoforms, each with a potentially different function. Well-known examples are afucosylation and its increased affinity to the FcγRIIIa[19] or the decreased binding of oxidated antibodies to the FcRn[20]. These are extreme

examples that show a severe biological effect. However, many more proteoforms could/ have not been addressed with existing binding techniques (*e.g.* SPR or ELISA) because of their inability to distinguish between different species such as different proteoforms. These techniques determine an overall affinity for the mixture or require the production/isolation of enriched antibody proteoforms, which often do not result in a 100% pure form and therefore do not provide confident results[21].

The challenge of proteoform-specific binding affinity assessment can be clearly illustrated through the example of glycosylation. Let's assume that we have two samples containing a high and low amount of core fucose and they are tested for their FcγRIIIa binding. Obviously, the mentioned techniques will determine a difference between both samples and link the increased binding affinity to antibody afucosylation. However, still the question of the impact of other glycan features such as galactosylation is not solved. To test for this, glycoengineered high and low galactosylated samples have to be analyzed in the context of high and low core fucose. However, alteration of galactosylation will also slightly change fucosylation levels, biasing the results. Furthermore, highly galactosylated antibodies may comprise 2 to 4 galactoses per antibody and low galactosylated ones from 0 to 2 making it hard to point out which galactoses influence the binding[21]. Additionally, galactosylated samples often exhibit sialic acids, which can also reduce binding to the FcγRIIIa receptor in context of low levels of fucose but high amounts of bisection[19]. In addition to the challenge of producing pure glycoforms, additional proteoforms often appear during production and storage, such as deamidation or oxidation, influencing the binding. On top of this, the situation gets even more complex if we consider the heterogeneity of the receptors (*e.g.* different glycoforms), which can also influence binding affinity and should be taken into account[22]. This example shows the urgent need for methods able to determine the binding affinities in a proteoform specific manner.

Affinity LC-MS (AC-MS) has recently demonstrated its ability to study binding of proteoforms with different glycosylation or oxidation status to FcγRIIIa and FcRn[23, 24]. AC-MS offers information in relative binding (dissociation) of different proteoforms but in contrast to SPR or ELISA do not provide quantitative information (*i.e.* affinity constants). Although AC-MS opens possibilities for proteoform-selective binding studies, it comes still with certain limitations and drawbacks. After binding to the receptor, immobilized in the stationary phase, the proteoforms are eluted by a pH stress to disrupt the interaction. For instance, in FcγRIIIa elution is achieved using a pH gradient from pH 5 to 3 which is not a physiological pH. These conditions are far from a biological representation and the results should be carefully interpreted. Furthermore, in AC-MS (and most binding techniques) the receptor is immobilized to the stationary phase. This obviously does not represent the native situation where the receptors are located in the cell membrane and allowed to form in some instances higher-order structures. In addition, the stationary material can cause secondary interactions between the antibody and the stationary phase which can not be distinguished from actual

binding. These secondary interactions are dependent on the protein sequence resulting in different elution times for different antibodies even if they exhibit the same binding affinity. To exclude unspecific binding, negative controls with no binding to FcRs (*e.g* same antibody with a LALA-PG mutation to silence the binding) are necessary for each particular antibody, hampering the possibility of comparing binding affinities of different antibody samples or application to antibody mixtures[25]. Commercial availability of Fc receptor columns in AC-MS is another major challenge as now it is limited to FcRn and FcγRIIIa. Receptor immobilization and self-packing of the columns are possible, but it requires high knowledge, plenty of the receptor and can lead to high lot to lot variations. As mentioned before, the heterogeneity of the Fc receptor (*e.g.* glycosylation) can also influence the binding to the antibody[22]. Therefore, glycosylation changes of the receptor would require new packing of affinity LC columns which is a very tedious process.

Mobility-shift ACE approaches showed the ability to determine affinity constants between proteins or peptides and small molecules[26, 27], and recently between small proteins[28]. In **Chapter 8** we demonstrated that ACE-MS can also be exploited to study antibody-FcR binding, clearly overcoming the drawbacks from the previously mentioned techniques. This is due to the fact that the receptor is free in the capillary and that the approach monitors differences in equilibrium (not only dissociation as compared to AC-MS). This allows to study binding affinity under physiological conditions without any pH stress. As the receptor is free in the BGE, it can form higher-order structures in the CE capillary. Furthermore, the receptor can be titrated by simply adding different concentrations to the BGE, permitting to obtain quantitative information on the binding (*i.e.* affinity constants, K_ds). Especially when using MS, the affinity of each proteoform can be determined in a mixture (even if overlapping) without the need of producing highly pure glycoengineered proteoforms. Hyphenation with MS also permits to simultaneously assess receptor heterogeneity (which is detected as a constant signal in the MS) and to monitor the complexes formed between antibodies and the receptors. The latter provides valuable information on the binding stoichiometry as shown in **Chapter 8**, where antibodies in complex with one (1:1 binding) or two FcRn molecules (1:2 binding) were observed. Furthermore, depending on the complexity of the receptor, monitoring the complexes formed can potentially deliver some information on the influence of receptor heterogeneity in the binding.

While the approach can be applied in a straightforward way to monitor relative binding of proteoforms, K_d determinations still suffer from lack of robustness. This is mainly due to the fact that electrophoretic mobilities can be affected by subtle changes in buffer characteristics (*e.g.* receptor concentration, viscosity) or in the capillary wall (*e.g.* adsorption). To correct for these changes we used a marker protein which does not have any interaction with the FcRn receptor. However, as the marker protein migrates before the antibody proteoforms, changes occurring after can not be corrected. Including a second marker protein (one migrating before and the other after the antibody) would allow for better correction on

differences in migration time and more accurate electrophoretic mobility determination. Furthermore, recent software developments permit easy conversion of electropherograms to electrophoretic mobilities using two markers, permitting easy implementation of this strategy[29, 30]. This would permit to monitor very small differences in affinity with high confidence and to determine K_d values more accurately.

Adding the receptor in the BGE comes with high flexibility. A receptor can be easily exchanged for another one permitting multiple applications with the same capillary (*e.g.* various FcRs). Furthermore, ACE-MS can be employed to study the influence of receptor heterogeneity on the binding by simply reversing the approach, avoiding the necessity of receptor engineering. For the reverse approach, instead of the receptor, the antibody can be added to the BGE and the receptor injected, allowing to separate receptor proteoforms based on their different affinity to the antibody. In contrast to AC, where milligrams of receptor are necessary, in ACE only a few micrograms are required for a study as the capillary is filled with nanograms of the receptor. This permits applications to receptors that are difficult to produce and where limited amounts are available. Next to FcRs, the high mannose receptor is of high interest in the biopharma industry due to its involvement in the clearance of high mannose antibody proteoforms. The high mannose receptor (and many other C-type lectin family receptors) bring however additional challenges as they often show calcium dependent binding. The addition of calcium to the BGE would result in a drastic decrease of the MS signal due to adduct formation between the antibody and calcium ions. To overcome this drawback alternative strategies, such as a partial filling of the capillary with the receptor, would be necessary to avoid MS signal suppression, yet such approaches still have to be explored.

In summary, mobility-shift ACE-MS is a highly flexible tool, able to determine affinity constants in a proteoform-specific manner, is closer to native conditions than AC-MS approaches, provides very rich structural information and requires very low amounts of antibody and receptor. Overall for future, this line has the possibility to help optimizing current antibody drugs and can also find clinical relevance to study different binding behavior of antibodies in disease.

10.4 Industry and academy: A symbiosis?

Symbiosis happens very often in nature. One concrete example are honeybees and flowers. Honeybees get nectar and pollen from multiple flowers and thereby transport pollen from flower to flower fertilizing them. Everyone has a benefit. The honeybees have food and the population can grow. The plants will produce seeds allowing also to increase their population, which is again positively influencing the honeybees. Industry and academia could also make a perfect symbiosis due to their different interest and complementarity. When both work closely together, the industry which does not have time to test and exploit new technologies or develop new methods can give these questions to academia, that does have time but

struggles to get access to interesting problems and/or the molecules. The industrial partner does get access to people with a lot of know-how and the time to develop new methods that can be transferred, whereas the academia gets funding from the industrial partner and can promote and exploit their research.

Another principle in some bees is nectar robbing. Here bees bite a hole in the flower to get the nectar bypassing therefore, the pollination. This way the bees can get their nectar, but flowers can not reproduce and the population can not grow, which is not beneficial for both of them in the long term. To minimize the threats, plants often develop protective mechanisms to hinder them from the robber bees. Although this situation is rarely seen in nature due to obvious unsustainability reasons, this relationship can be seen often between academia and industry. The industry is constantly worried that academia could "steal" something from them and therefore developed mechanisms, which make collaborations very difficult. In many cases, instead of collaborating, pharmaceutical companies try to do everything on their own. In others, the collaborations are not very successful due to too many hurdles and agreements that need to be signed *(e.g*. IP rights, MDAs, MTAs), publication approvals and other confidentiality aspects because pharmaceutical industries want to protect their property or distrust the academic groups.

However, this is company dependent and there are companies that have successful collaborations for years, whereas others never or barely collaborate. This PhD thesis has been achieved in the frame of a very successful and fruitful academic-industry collaboration. A key aspect for this success was a previous internship performed at the industrial partner and the two additional short secondments performed during the project. This allowed the establishment of a relation of trust and widened the vision on the real interest and challenges faced by industry. Unfortunately, not all researchers have these opportunities and experiences, as either the secondment in industry requires a lot of paperwork and initial training for the student (often secondments below three months are not even considered by industry as the student need a 2-3 weeks training before entering to the lab) or the industry does not have the technology that is being exploited in the project. Big consortia such as the Marie Curie Horizon 2020 consortium Analytics for Biologics (A4B) help to establish initial contacts but still suffer from these problematics. As a matter of fact, from 15 PhD students in the A4B consortium only few spent some time at one of the industrial partners. To encourage these internships industrial partners should facilitate secondments to students and grant some exceptions regarding training regulations (as in most of the cases the student will not work on business-critical projects) and the academic supervisors should understand that even if there is no rocket science happening during the secondment period, this would be highly beneficial for the student. Internships not only expand the capabilities and experience of the students but also allow to strengthen the industry-academia relationship. In fact, the exchanges should not be limited to students but rather be open to other personal to tear this wall between industry and academia down. This would also benefit researchers for

possible late changes in their career paths. Shared positions could also have a tremendously positive influence but yet, are rarely supported from both sides.

Funding by governmental institutions should be more supportive and should invest more in these collaborations. It is often thought that pharmaceutical industries should sponsor most of the collaboration costs instead of being supported by public funds. In many cases, this can cause the industrial partners to be hesitant regarding collaborations as they feel academy is only interested in their money instead of seeing the real benefit of the collaboration. However, the COVID-19 pandemic showed that also the societal benefit is dependent on a strong pharmaceutical industry. Without tests, a vaccination or a treatment of the COVID-19, lockdowns would have been longer and the economy would have suffered even more. Many companies based their vaccines on academic developments and academic groups saw how their discoveries could be applied and save lives. Besides all the negative sides, maybe the corona pandemic has a positive influence on the global vision of industry-academia relations. An example is the development of a pandemic institute financed by the Fraunhofer society and the Bavarian government in collaboration with the Roche diagnostic division aimed to get a better understanding on infectious diseases and built directly next to Roche (Penzberg) to promote the collaboration[31].

10.5 References

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