Boosting mass spectrometry-based analytics for biopharma

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Chapter 1

Introduction

Biopharmaceutical antibodies and new antibody formats

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The word biopharmaceuticals is very generic and comprises every pharmaceutical produced by living organisms. That can range from nucleic acids over proteins to complete cells or even blood products, which themselves contain a mixture of several different bioactive compounds. One large class of protein biopharmaceuticals are antibodies, which naturally are produced by immune cells (B cells) and are present in high amounts in the blood. These antibodies - also called immunoglobulins or in short Ig - can be further divided into five isotypes in humans, namely IgG, IgM, IgA, IgE and IgD. IgG is by far the most abundant isotype in blood plasma (more than 70%) and binds with very high specificity and affinity to its antigen on a pathogen (opsonization), activating an immune response and thereby allowing to fight and survive infections. Already in 1981 scientists successfully tested an intravenous immunoglobulin G (IVIg) therapy, based on captured IgGs from healthy donors, to treat children with idiopathic thrombocytopenic purpura[1].

To fight the wide variety of exogenous substances and pathogens, our body produces a plethora of different IgG antibodies - i.e. different clones, each specific for a particular antigen. To increase antigen specificity and treatment efficacy, scientists pursued therapies based on one single clone (monoclonal) antibody (mAb) instead of polyclonal IgG preparations. The development of hybridoma technology (where specific B cell clones are fused with immortalized myeloma tumor cells) revolutionized biopharmaceutical industry due to its capacity to recombinantly produce large amounts of mAbs and is currently the base for many modern mAbs[2]. One of the first marketed mAbs was used to treat diffuse large cell non-Hodgkin's lymphoma. It was applied in combination with chemotherapeutics and showed a significantly elevated cure rate of cancer patients. Since then, around 50,000 patients with this disease are cured each year[3].

Until now, biopharmaceutical companies have developed and marketed 100 mAbs[4] for various types of cancer, autoimmune diseases, cardiovascular or neurologic diseases. Conventional mAbs are large proteins consisting of four subunits (two identical light chains (Lc) and two identical heavy chains (Hc)) with a molecular weight of around 150 kDa. However, pharma industry is continuously improving antibodies (*e.g.* higher stability, binding avidity or improved pharmacokinetics) and conventional mAbs are being slowly displaced by new antibody formats with new or enhanced properties. These recently emerging new antibody formats range from small nanobodies[5] over Fc fusion proteins[6] to bispecific antibodies (BsAbs) and elongated versions thereof[7], allowing to bind two different antigens.

Bispecificity, in particular, opened a myriad of new possibilities of therapeutic applications. An example is the ability to block two different receptors and thereby prevent their activation, allowing to block cell proliferation or inflammatory processes[8]. This mechanism can also be used for cancer therapy to bring cancer cells and immune cells in close proximity[8]. Treatment

with the BsAb Emicizumab was shown to be effective in patients with haemophilia A by replacing the function of the missing factor VIII and mitigating the interaction between factor XI and X[9]. However, next to the benefit of targeting two molecules, bispecificity comes also with major biotechnological and analytical challenges due to the higher structural complexity of these molecules.

Functional BsAbs consist, instead of two, of four different chains: two different Lcs and Hcs (**Figure 1**). Proper assembly of these chains is not trivial and different antibody engineering strategies have been developed over the years. A well-known one is the *"knob-into-hole"* technology which guides the correct assembly of the Hcs[10, 11] (**Figure 1**). To this end, a small amino acid in the CH3 domain of one Hc is exchanged by a large one and vice versa in the second Hc. This way, a knob and a hole are generated, allowing only one possibility to assemble the heavy chains. To further enhance the heterodimer formation of both heavy chains, additional amino acid exchanges, allowing ionic interactions and hydrogen bond formation, can be introduced[12]. Regarding the correct assembly of the different Lcs, the so called *"CrossMab"* technology has been implemented[13-16]. The clue behind this approach is to swap either the complete Fab part on one Hc and its Lc or exchange only the variable part between one Lc and one Hc (**Figure 1**). Another simplistic and broadly established approach is the controlled Fab-arm exchange where BsAbs are generated by reassembly of antigen-binding arms of individually expressed monospecific parental antibodies containing two point mutations[17]. As bispecificity is generally enough to induce the desired therapeutic response, BsAbs often contain additional modifications in the CH2 domain of the Hc to abrogate the binding to Fc receptors and thereby abolish the immune response. Typical modifications are the exchange of the amino acids L234A, L235A, P329G, known in short as LALA-PG mutation[18].

Despite all these biotechnological advances, production of BsAbs often comes with misassembled side products, such as light chain dimers, wrongly assembled heavy or light chains or entire monospecific antibodies (either covalently or non-covalently bound), which have to be carefully monitored, both qualitatively and quantitatively[16, 19, 20]. Especially the latter ones are considered critical because of their increased immunogenicity, due to lower stability and, therefore, higher probability to form aggregates[21].

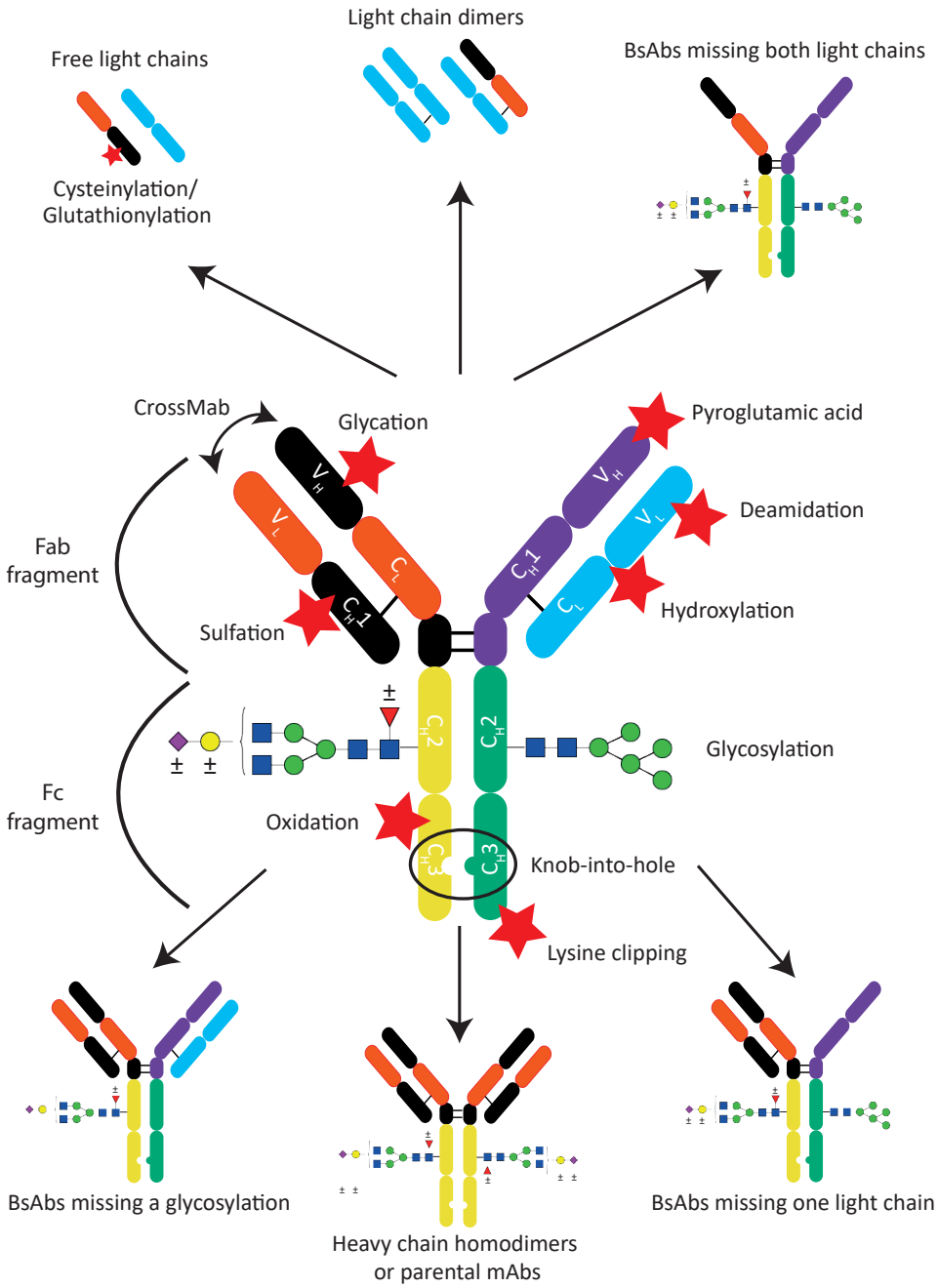

Figure 1. Common heterogeneity and modifications of BsAbs. BsAbs have a IgG-like blueprint containing two disulfide bridges connecting the two heavy chains and two additional connecting the corresponding heavy and light chain. The central BsAb illustrate typical PTMs and modifications from the engineering process. Misassembled products and antibodies missing a glycosylation site are depicted in the side figures.

1.1 Antibody proteoforms and their functional variability

Recombinantly (and naturally) produced antibodies often comprise several post-translational modifications (PTMs), resulting in a mixture of different "proteoforms" instead of a single molecular product. Most antibodies contain a conserved N-glycosylation site at Asn297 of the Hc. Therapeutic mAbs mainly exhibit complex type glycans (left glycan in **Figure 1**) and to a minor extent high mannose glycans (right glycan in **Figure 1**). Depending on the production system, the heterogeneity and abundance of certain glycan types can be different[22]. Additionally to glycosylation, other modifications can appear during the production or storage of the antibody. Often encountered antibody PTMs are C-terminal lysine clipping, sulfation, hydroxylation, N-terminal and lysine glycation, deamidation of asparagine residues, oxidation of methionine residues, N-terminal pyroglutamic acid formation and cysteinylation or glutathionylation of cysteine residues[23-25] (**Figure 1**). These modifications can influence the physicochemical properties of the antibody but also its functional characteristics and, therefore, should be carefully assessed.

Antibodies consist of a variable region specific for antigen binding and a constant region which determines the antibody half-life and activates effector cells (via binding to Fc receptors (FcRs)). Therefore, not only the nature but also the position of the modification can determine the functionality, stability and pharmacokinetic behavior of an antibody proteoform. An example is deamidation of asparagine residues, which was shown to occur with high probability in the complementary-determining region (CDR) of antibodies[26]. This can result in reduced antigen-binding affinity lowering drug potency[27-30]. Another PTM which frequently occurs during antibody production or long term storage in formulation buffer, is glycation[31, 32]. Similar to deamidation, it was shown that depending on the position glycation can impact antigen binding[33, 34].

PTMs occurring in the Fc region of antibodies do not directly influence the binding of the antigen but rather influence their pharmacokinetic behavior or effector functions. Oxidation of methionine residues mainly occurs on two conserved residues in the CH2 and CH3 domains, namely Met252 and Met428[35, 36]. Oxidation of these residues alters the structure of the Fc region[37], resulting in a detrimental impact on the binding affinity to the neonatal Fc receptor (FcRn)[38-40] and to a minor extent to other Fc gamma receptors (FcγR)[38, 41]. As the FcRn is responsible for recycling antibodies, a lower FcRn binding affinity shortens the half-life of oxidized proteoforms[39]. Also, glycosylation can influence the pharmacokinetics of antibodies[42]. Whereas aglycosylated antibodies do not show a significant difference in their half-life compared to the glycosylated antibody[43-45], certain glycoforms can have a drastic impact. Some examples are the high mannose glycoforms[46, 47], which are removed by the mannose receptor[46-48] or hypersialylated antibodies, which are cleared from circulation more slowly due to lower binding to asialoglycoprotein receptor[49]. Controversially, other

studies show no difference between sialylated or non-sialylated antibodies[50, 51].

Glycosylation also plays a critical role in the pharmacodynamics of antibodies. These pharmacodynamic effects are depending on the interaction of antibodies with Fcγ receptors and C1q, activating thereby immune cells. This results in complement-dependent cytotoxicity (CDC), antibody-dependent cellular phagocytosis (ADCP) or antibody-dependent cell-mediated cytotoxicity (ADCC)[52]. For example, aglycosylated or hemi-glycosylated antibodies show reduced binding to Fcγ receptors[53-55] or C1q[56], abrogating (or reducing in case of hemi-glycosylated antibodies) CDC and ADCC[53, 55] activity. Within glycoforms afucosylation shows a 50 fold increase in binding affinity to the FcγRIIIa receptor[57, 58] and subsequently an increase in the ADCC activity[59-61]. Complex type glycans normally contain a core fucose, however, some antibodies are engineered to lack this fucose which increases their potency[62]. A similar but less pronounced effect was seen for high mannose glycoforms[63]. Additionally, high mannose glycoforms decrease CDC[63] response and have a lower binding affinity to FcγRIIa and FcγRIIb[46]. These examples show that next to the structural characterization, the determination of binding affinities for each specific proteoform is of utmost importance.

1.2 Mass spectrometry for antibody characterization

The large diversity of antibody proteoforms, side products and engineering processes makes their analysis and characterization not trivial. Mass spectrometry (MS) is a powerful tool for the detailed characterization of antibody proteoforms. In biopharmaceutical industries MS is particularly used in early and late-stage development or for characterization of new occurring peaks during product release. Recently, first reports about validation and implementation of MS based QC methods are also being published[64, 65]. Yet, currently, there is no universal method to assess all the heterogeneity and different approaches are available depending on the antibody and the question to answer. **Figure 2** illustrates the current approaches for MS antibody characterization. *Bottom-up* approaches rely on protein digestion using endoproteases and analysis of the obtained peptides by MS, usually combined with tandem MS (MS/MS) (**Figure 2A**). *Middle-up* approaches often consist of a limited cleavage of the mAb in the hinge region and analysis of the resulting antigen-binding fragment ($(Fab)_2$) and crystallizable fragment (Fc) (**Figure 2B**). To obtain information on the N- and C- termini, these subunits can be further fragmented by different fragmentation techniques (*middle-down*). mAbs can also be analyzed at the *intact level* without or with fragmentation (*top-down*) (**Figure 2C**). Next to structural, the *functional characterization* of mAbs can be performed using MS-based approaches, which can be considered the 4th level of antibody characterization (**Figure 2D**). The utility of these approaches will be discussed in the following sections.

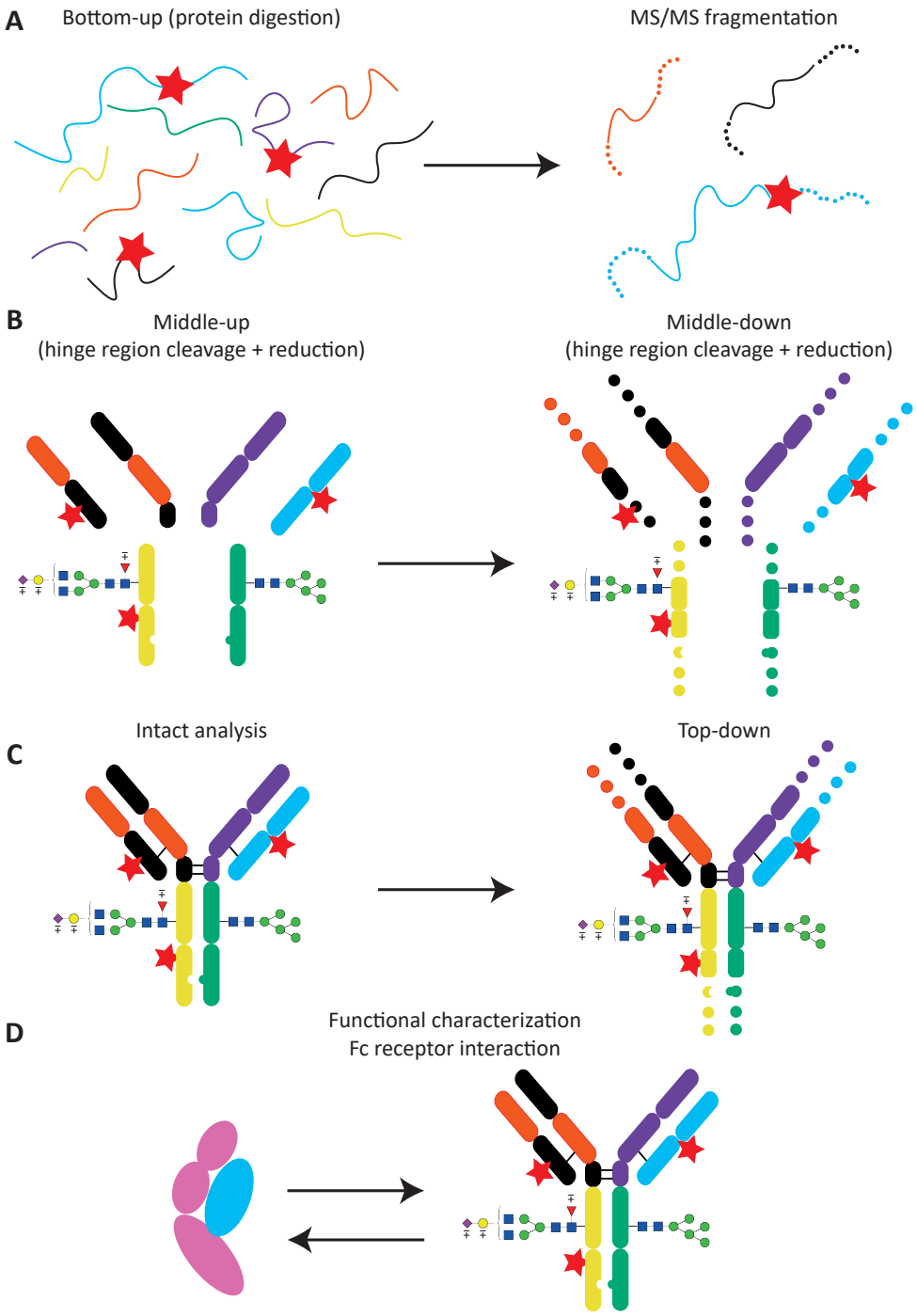

Figure 2: Different levels of mAb analysis. A) Bottom-up and MS/MS fragmentation, B) Middle-up/down, C) intact and Top-down D) functional interaction.

For the analysis of antibodies mainly two ionization techniques are used, electrospray ionization (ESI) and matrix assisted laser desorption/ionization (MALDI). In MALDI the sample is spotted together with a matrix on a MALDI target plate and allowed to crystallize. The crystallized samples are then transferred to the vacuum and targeted with a laser which leads to desorption and thereby ionization of the sample. Depending on the matrix used, MALDI is considered a soft ionization technique, which allows to detect labile proteoforms[66]. Another characteristic of MALDI is, that it mainly generates singly charged ions, which simplify data analysis[67]. However, to enhance the mass accuracy and dynamic range of the MS - opening the possibility to analyze larger proteins - the generation of multiply charged ions is sometimes favorable[68-70]. Fragmentation in MALDI can either be performed post-source or by in-source decay (MALDI-ISD). Whereas post-source fragmentation is not efficient for large proteins as mainly singly charged ions are generated in MALDI, MALDI-ISD results in efficient fragmentation and good sequence coverage[71]. Similar to the intact MALDI analysis the resulting fragment ions are mainly singly charged, simplifying data analysis. This fragmentation technique has shown to be very powerful for conventional antibody top-down or middle-down characterization[72-74]. A drawback of this technique is that no precursor ions can be selected, but rather many molecules present in the sample are fragmented simultaneously, challenging data analysis of complex mixtures.

In ESI, the molecule of interest (dissolved in a liquid) is dispersed into the ionization chamber via a sprayer needle onto which a high voltage is applied (often assisted by a coaxial gas flow and heat), resulting in charged droplets and via desolvation in ionized proteins in the gas phase[75, 76]. In contrast to MALDI, where mainly singly charged ions are formed, ESI delivers multiply charged ions. Fragmentation of these ions can either be performed in the source, although these examples are rather rare for proteins[77], or more commonly after the ionization process. This often involves selection of precursor ions and, thereby, targeted fragmentation. Most fragmentation techniques for bottom-up are high-energy collisional dissociation (HCD) or collision-induced dissociation (CID), while for top/middle-down mainly ultraviolet photodissociation (UVPD), electron-capture dissociation or electron-transfer dissociation (ETD) are used[78]. For detection of fragment ions and intact proteins, commonly time of flight (TOF)[79], Fourier transform ion cyclotron resonance (FTICR)[80] or Orbitrap[81] mass analyzers are used.

Upfront separation of proteoforms with small mass differences is beneficial for confident structure assignments. Whereas MALDI-MS is mainly used as stand-alone technique, ESI-MS is commonly combined with an upfront separation technique[82, 83]. Separation techniques that can be directly coupled with MS and that have shown to be helpful for antibody characterization - and to a lesser extent new antibody formats – are reversed phase liquid chromatography (RPLC), hydrophilic interaction chromatography (HILIC), ion exchange chromatography (IEC) and size exclusion chromatography (SEC)[20, 21, 84-87]. Another technique that is gaining attention (especially for middle-up and intact antibody characterization) is

Capillary Electrophoresis (CE)-MS. Hyphenation of CE with MS has been traditionally done by using a sheath-liquid to close the electric circuit. However, this sheath-liquid interface leads to dilution of the sample providing limited sensitivity. Different solutions have been developed to overcome this problem, such as nanoflow sheath-liquid or sheathless interfacing, which have demonstrated similar performance but boosted sensitivity compared to conventional sheath-liquid CE-MS[88, 89].

1.3 Current analytical challenges in biopharmaceutical industry

While several MS-based analytical platforms have been developed and implemented for antibody characterization in the biopharmaceutical industry, the continuous development of novel antibody therapeutics opens a demand for new and improved analytical methods. Some of the current analytical challenges addressed in this thesis are described below.

1.3.1 Automation of bottom-up approaches

With the rising number of antibody applications and additional possibilities arising from new antibody formats, the pipelines of pharmaceutical companies are heavily filled. This also implies an increase in molecules that need to be characterized in various stages from early research until approval by the authorities, formulation development or stability studies using stress conditions (*e.g.* oxidative stress, high temperature or high levels of glucose)[29].

Bottom-up approaches combined with LC-MS are the gold standard for antibody PTM assessment[29, 90, 91]. They are commonly employed in pharmaceutical companies for the initial characterization of mAbs to define CQAs (arising during elongated storage or forced degradation) and during QC release testing of marketed antibodies to characterize unknown peaks. Product variants and PTMs need to be confidentially assigned and a risk assessment performed to ensure patient safety and drug efficacy.

Typical sample preparation in bottom-up approaches require multiple steps (denaturation, disulfide bridge reduction, alkylation and enzymatic digestion) resulting in extensive hands-on and incubation times (~18 hours for trypsin) and increasing the risk of inducing artificial modifications[92-94]. For chromatographic peak characterization the process is even more complex as the peak needs to be tediously fractionated prior bottom-up characterization. Such workflows can easily take several days and often lack a confidential peak assignment due to overlapping with other proteoforms.

Robotic platforms are useful to save hands-on time on sample preparation (reduction alkylation and digestion) and contribute to standardization such as reducing sample handling errors. Such approaches can process up to 96 samples simultaneously and reduce digestion time to 4 hours[95]. Still, samples require further LC-MS analysis which leads to additional

storage time. Despite the full pipelines of pharmaceutical industries, 96 samples are barely analyzed at once, but rather 10 to 20 samples, not ideal for a robotic platform which requires experienced users and time to program. In short, an intermediate automated solution for bottom-up sample preparation and analysis (and applicable for QC peak characterization) is still missing.

Using multidimensional LC, few steps have been made to perform an online digestion of protein, either by mixing the trypsin and the sample in a pressurized sample loop[96] or by developing immobilized trypsin reactors (IMERs). Such multidimensional systems with an on-line trypsin digestion step, followed by a RPLC separation and MS detection have been described to characterize apolipoproteins[97], protein mixtures and antibodies[98, 99]. However, these systems are not user friendly and they are not able to fractionate peaks from a pre-separation. Furthermore, none of these systems permits protein reduction, resulting in peptides connected by disulfide bridges, thereby complicating data analysis.

1.3.2 Complexity of new antibody formats and unexpected modifications

Although pharmaceutical labs have established an armament of analytical techniques for characterization of conventional mAbs, new antibody formats comprise higher complexity bringing new analytical challenges.

In the particular case of BsAbs, additional side products are often encountered as consequence of the misassembling of different antibody chains – *i.e. macroheterogeneity*. Especially of interest are the homodimers of Hcs, which can show high immunogenicity[21]. To analyze this type of side products sample preparation should be minimized and the analysis needs to be performed at the intact level. Bottom-up approaches are not suitable for monitoring BsAb misassembling as high-order structures are lost during endoprotease digestion. MS has been extensively applied for the analysis of intact conventional mAbs. However, the macroheterogeneity can be compromised in stand-alone MS as aggregate artifacts or disassembling can happen during the ESI process[20]. Hyphenation with a separation technique allows discrimination of pre-existing complexes from ESI artifacts due to the introduction of an orthogonal dimension. SEC-MS permits to monitor free chains, incomplete assemblies (*e.g.* BsAbs missing a Lc or Hc) and aggregates. However, different antibodies (*i.e.* homodimers or parental monospecific antibodies depending on the production mechanism) can not be resolved by SEC as they are in the same mass range[20]. Although MS can provide mass distinction between antibodies, homo- and heterodimers often exhibit similar masses resulting in signal superposition and difficult assignment. Chromatographic techniques suitable for homodimer and parental antibody separations are hydrophobic interaction chromatography (HIC) and IEC, however, they normally require high amount of non-volatile salts in the mobile phases making hyphenation to MS not straightforward[84, 85]. CE-MS has shown great potential in the characterization of intact and

hinge region cleaved conventional mAbs[100, 101], although the applicability for BsAbs has never been explored so far.

Next to this, new formats can undergo non-conventional or unexpected modifications due to different exposure of amino acids, presence of additional linker elements or amino acid exchanges compared to conventional antibodies[102-104]. For instance, an unexpected modification that can occur during antibody production is hydroxylation of amino acids such as lysine or proline. Commonly these modifications are observed in proteins such as collagen to enhance the water solubility[105]. However, recently they have been found on antibodies and more frequently on new antibody formats[103, 106] and seem to occur during fermentation processes through protein translation errors and misincorporation of hydroxyproline instead proline[107]. Because the mass difference is exactly identical to that of oxidation, characterization of this modification is further complicated if there is an amino acid prone to oxidation nearby (*e.g*. methionine, tryptophan, cysteine or histidine).

Localization of PTMs is also an important aspect, as the position of the modification will determine the effect on functionality. Localization can be focused on antibody chains (Fab_2, Fc, Hc, Lc) or amino acid level. For BsAbs, chain localization is especially important as small variations introduced during BsAb engineering can result in different heterogeneity of counter chains (*e.g.* Hc and Hc'). Here, bottom-up workflows can, in some cases, result in equal peptides hampering localization to the specific chain. Middle-up/down approaches - where the BsAb is reduced and/or partially cleaved using hinge region endoproteases - can be a solution for these cases (**Figure 2B**). Techniques which have proven to be suitable for subunit analysis are HILIC- or RP-MS[21, 86, 108], CE-MS[109, 110] and MALDI-MS[73, 74]. To localize PTMs at the amino acid level top-down or middle-down approaches are employed[72-74, 111]. Top-down applications in particular require minimal sample preparation (*i.e.* desalting or buffer exchange), thereby decreasing the risk of introducing unintended PTMs. More challenging is the localization of labile PTMs (*e.g.* sulfation) as they can be lost during standard "harsh" fragmentation, such as CID or HCD[112, 113]. A way to localize sulfation can be to use a soft fragmentation techniques such as ETD or a combination between ETD and CID (ETciD)[112], which allows in some cases to keep the PTM on the protein backbone. However this technique requires enrichment of sulfated proteoforms to enable localization of the PTM, because still the sulfation is lost partially and additionally the fragmentation is in some cases not trivial. Even though there are several intact, top-down or middle-up/down applications for conventional antibodies the ability of these platforms and approaches for the characterization of the more complex BsAbs has barely been explored.

1.3.3 Binding assessment in a proteoform-specific manner

After monitoring different modifications and proteoforms the next question arising is how these modifications influence the characteristics and functionality of the therapeutic product. As discussed at the beginning of this section, these proteoforms can have a severe impact

on pharmacokinetics and pharmacodynamics and the assessment of the affinity for each single proteoform is therefore very important. Unfortunately, approaches for the functional characterization of individual proteoforms in mixtures are largely missing. Techniques used traditionally to determine binding affinities are surface plasmon resonance (SPR) or enzyme-linked immunosorbent assay (ELISA). These techniques, however, are not able to distinguish between different proteoforms and give an overall response for all proteoforms in a sample. To study the binding affinity of specific proteoforms, either isolation or proteoform specific production in an "as pure as possible" form is required[39, 60, 114-116]. However, these processes are very tedious and can still induce confounding by the presence of small amounts of other (glyco)variants[57, 116]. Affinity LC tried to overcome these drawback and columns for the assessment of FcRn and FcγRIIIa have been developed[117-120]. This allowed to determine the relative binding of different mAb proteoforms in mixtures. However, columns for all Fc receptors or other receptors are not available and the determination of quantitative binding affinities is not possible and it requires pH stress for elution. All the above mentioned approaches have the immobilization of the receptor in common. How far this reflects the natural situation, which in some cases requires dimerization of the receptor before binding is questionable. ESI-MS has also been employed for monitoring protein-binding in a proteoforms specific manner[121, 122]. However, gas phase binding often deviates from in solution as many complexes are gas-phase unstable[123]. Therefore, immobilization-free in-solution binding techniques that provide affinity constants in a proteoform-selective manner are desired but still missing.

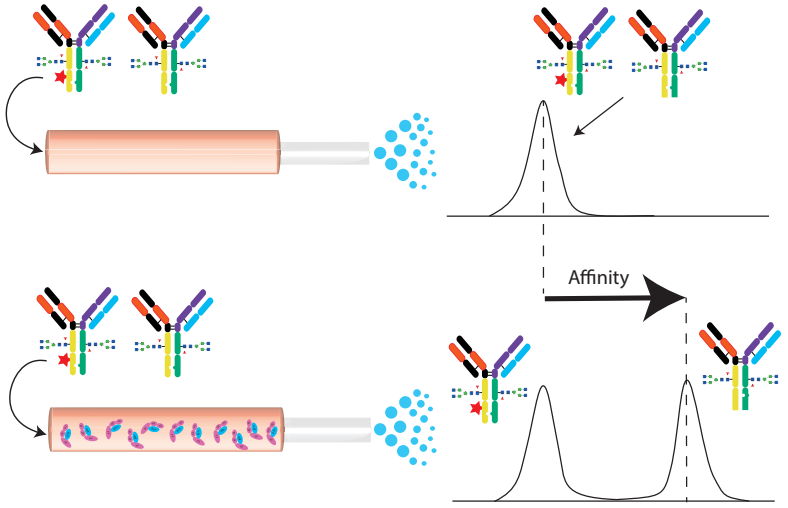

Figure 3: Illustration of the principle of mobility-shift affinity CE. Example of two mAb proteoforms without (upper panel) and with an interaction partner in the BGE (lower panel). The example shows a separation of a antibody proteoform which does not interact with the receptor (with red star), whereas the other antibody proteoform migrates later, due to its affinity to the receptor. This order of migration is only the case if the receptor has a lower electrophoretic mobility compared to the antibody.

One particular appealing mode of affinity CE (ACE) for proteoform-specific binding assessment is the mobility shift approach. In this approach, the capillary is filled with one interaction partner followed by an injection of the second one. After applying an electric field, the proteoforms that do not interact will maintain their electrophoretic mobility while proteoforms interacting with the receptor will change their electrophoretic mobility migrating in a different place (**Figure 3**). This technique has been used to study the interaction of proteins with metal ions[124, 125] and demonstrated the ability to obtain quantitative binding information in equilibrium constants (K_d). Yet, these studies were conducted without MS detection, excluding the possibility to determine the affinity of overlapping proteoforms in a mixture simultaneously (*e.g.* oxidation or glycosylation). Protein-protein ACE-MS was for the first time shown in an exploratory study for the interaction between two small test proteins[126]. The system was based on sheath-liquid CE-MS providing limited sensitivity and hampering the applicability to larger or more complex proteins such as antibodies and Fc receptors. Exploiting and developing ACE-MS for proteoform-specific antibody Fc-binding would overcome one of the major current challenges in biopharma characterization.

1.4 Scope of the thesis

The aim of the research described in this thesis is the development of new MS-based analytical platforms that permit to address current limitations and challenges in the characterization of antibody and antibody-derived therapeutics as described in **Chapter 1**.

For detailed structural characterization of therapeutic proteins including PTM assessment commonly bottom-up proteomics strategies are applied. The analytical methodologies are well-established for antibody samples and identification of unknown peaks for quality control (QC) purposes, however sample preparation requires extensive hands-on time - from several hours to days - and increases the risk of inducing unintended modifications. In this thesis this drawback is addressed by integrating all the steps in an automatized multidimensional LC-MS (mD-LC-MS) platform. In **Chapter 2**, a mD-LC-MS method was developed for the on-line bottom-up characterization of peaks separated on ion exchange chromatography. The approach drastically reduced the total analysis time and proved several benefits over conventional offline sample preparation. In **Chapter 3** the novel mD-LC-MS platform was further optimized to increase retention of highly polar peptides by adaptation of the flow rates of the different dimensions. The optimized method provided sequence coverages comparable to the standard offline workflow and allowed detection of hydrophilic PTMs missed prior to optimization. The mD-LC-MS approach was intended to directly analyze formulated mAb samples without pre-separation. The developed platforms are currently in use in various pharma laboratories.

In contrast to bottom-up approaches - requiring endoprotease digestion - top-down or middle-down MALDI-ISD approaches offer the possibility to sequence N- and C-termini of mAbs and subunits (Hc and Lc) with minor sample preparation. **Chapter 4** shows the ability of MALDI-(ISD)-FT-ICR-MS for the analysis of new antibody formats with a higher degree of complexity, such as BsAbs. Next to sequence information, PTMs close to the N- or C-terminus could be localized by MALDI-ISD. Due to the “softer” fragmentation process, very labile modifications, as shown for sulphation in **Chapter 5**, can be retained on the protein backbone and confidently assigned.

Next to classical PTMs, production of BsAbs also yields misassembled side products, which might lead to immunogenic side reactions. To monitor mispairing, the native structure of the antibodies have to be maintained during the analysis (*i.e.* at the intact level). CE-MS has shown to be a powerful technique for the characterization of intact proteins and was employed in **Chapter 6** to analyze two homologous BsAbs. Next to their intact analysis the BsAbs were analyzed at the middle-up level to localize PTMs to specific subunits (Lc1, Lc2, Fd’1, Fd’2, (Fc/2)1 or (Fc/2)2). Similarly, **Chapter 7** provides a sheathless CE-MS approach to monitor the exchange efficiency of in-house produced BsAbs. Here, the presence of highly heterogenic Fab-glycosylated antibodies required a separation prior to MS characterization.

The functional characterization of antibodies in a proteoform-selective way remains one of the major unaddressed challenges of pharmaceutical companies, which still relies on complex engineering and/or isolation strategies. **Chapter 8** presents an innovative approach based affinity CE-MS for the simultaneous assessment of structure and function of antibodies. The method permitted to determine affinities in a proteoform-specific manner and to study binding stoichiometry. The approach was employed to monitor the influence of oxidation on the interaction with the neonatal Fc receptor.

In the course of this PhD thesis, the COVID-19 pandemic appeared, imposing a drastic change of habits and priorities in our lives but also in our research. Recombinant SARS-CoV-2 proteins are essential instruments in the fight against COVID-19. In **Chapter 9**, the receptor binding domains (RBD) of the S protein produced in two different mammalian systems (HEK293 versus CHO) were characterized. Here, the lack of previous structural information instigated the necessity of a multilevel MS approach (intact, glycopeptide and released glycans). Also, the functional differences between both RBD samples, such as binding to anti RBD antibodies and to the ACE2 receptor, were evaluated.

In the last chapter, **Chapter 10**, the results are discussed with special emphasis on the aspects of automation, top/middle-down and native MS, CE and especially affinity CE in an industrial setting. In addition, the potential and benefit of collaborations between industry and academia are discussed.

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