Microheterogeneity of Recombinant Antibodies: Analytics and Functional Impact

Beate Beyer, Manfred Schuster, Alois Jungbauer,* and Nico Lingg

Antibodies are typical examples of biopharmaceuticals which are composed of numerous, almost infinite numbers of potential molecular entities called variants or isoforms, which constitute the microheterogeneity of these molecules. These variants are generated during biosynthesis by so-called posttranslational modification, during purification or upon storage. The variants differ in biological properties such as pharmacodynamic properties, for example, Antibody Dependent Cellular Cytotoxicity, complement activation, and pharmacokinetic properties, for example, serum half-life and safety. Recent progress in analytical technologies such as various modes of liquid chromatography and mass spectrometry has helped to elucidate the structure of a lot of these variants and their biological properties. In this review the most important modifications (glycosylation, terminal modifications, amino acid side chain modifications, glycation, disulfide bond variants and aggregation) are reviewed and an attempt is made to give an overview on the biological properties, for which the reports are often contradictory. Even though there is a deep understanding of cellular and molecular mechanism of antibody modification and their consequences, the clinical proof of the effects observed in vitro and in vivo is still not fully rendered. For some modifications such as core-fucosylation of the N-glycan and aggregation the effects are clear and should be monitored, but with others such as C-terminal lysine clipping the reports are contradictory. As a consequence it seems too early to tell if any modification can be safely ignored.

1. Microheterogeneity

Since antibodies have emerged as the uncontested number one of biopharmaceuticals,[1] we have seen a multitude of attempts at further improving this type of biotherapeutics by introducing changes into the structure of these molecules in order to make them safer, even more specific or efficient and to further reduce any undesirable additional interactions or potential side-effects.[2,3] Various smaller formats, including single chain variable fragments (scFv), antigen binding fragments (Fab), minibodies etc.[4] as well as antibody structures from alternative sources like camelid antibodies or immunoglobulin new antigen receptors[5] were investigated for their potential use as therapeutics. Additional efforts at engineering were made to create bispecific types of structures that can target two different antigens,[6] or even more recently, to produce antibodies that contain multiple crystallizable fragment (Fc)-domains, showing enhanced effector functions.[7] This additional variety of structures created in the course of the hunt for so-called biobetters, has shifted the focus away from the naturally occurring structural variety and heterogeneity found in unmodified Immunoglobulins (IgGs) (Figure 1). However, the sheer size and complexity of these molecules by itself offers ample possibilities for modifications and changes that result in a rather large number of variants, which can differ in their biophysical properties and even in their biological activity.[8] Without a doubt, an in-depth understanding of this intrinsic structural variety associated with IgG and their engineered derivatives and its consequences in terms of biological function is an important prerequisite for adequately assessing the potential of novel antibody structures and formats.

The protein modifications responsible for the presence of these variants include typical post-translational protein modifications such as glycosylation, enzymatic reactions leading to the removal of C-terminal lysine, as well as non-enzymatic chemical reactions such as deamidation or oxidation. As a result, variants can originate at all stages of the antibody manufacturing process from the expression in cell culture to storage of the purified product.[9–11] A detailed depiction of the most important modifications that can occur in antibodies, can be found in Figure 1.

In order to ensure a consistent safety and efficacy profile of antibody therapeutic products despite this variability,
there is a considerable pressure from the regulatory authorities to closely monitor and compare the microheterogeneity profile associated with a given antibody from batch to batch throughout the entire product development and manufacturing process (Figure 2).\[12,13\]

Nevertheless, the analysis of antibody microheterogeneity and the separation of antibody variants still poses a considerable challenge from a methodological point of view. The sheer number of possible modifications results in a multitude of slightly different variants, some of which are present only in very low amounts. It has been estimated that the total number of potential variants that can be present in a typical antibody sample amounts to $10^8$ different kinds of molecules.\[14\] Even though, it is impossible to dissect an antibody into all these individual variants, this still illustrates the need for particularly sensitive, high-resolution analytical tools to achieve any kind of meaningful product characterization.

This aspect has gained even further importance since the advent of biosimilars in the biopharmaceutical industry. A detailed characterization of the originator molecule is essential for the development of a biosimilar. Additionally, regulatory authorities demand that an appropriate set of methods is put into place so that biosimilarity to and interchangeability with a reference product can clearly be demonstrated.\[15,16\]

Most of the data illustrating the biological consequences of microheterogeneity is derived from in vitro experiments including various kinds of protein-protein interaction measurement methods as well as biological effector function assays. The changes in biological properties of different antibody variants found in these experiments do not necessarily translate directly into an altered potency in the much more complex environment in vivo. Any potential findings about the biological activity of IgG variants should therefore be evaluated critically with a special focus on the available clinical data.

2. Methods for Microheterogeneity Analysis

Due to the small differences between antibody variants, separation and analysis of these molecules is still a challenge from a methodological point of view. Suitable analytical methods have to be sensitive and have high resolution capabilities. The most versatile methods for this application include various types of chromatography, mass spectrometry, and combinations thereof. Modifications resulting in a shift of the antibody’s pI, can be detected by charge based methods such as ion exchange chromatography (IEC), isoelectric focusing (IEF), and capillary electrophoresis (CE).

Correct interpretation of the influence of certain modifications can be very difficult when no pure preparation of a variant is available. Preparative methods that can generate relatively pure or at least highly enriched pools of variants, such as cation exchange chromatography (CEX) with salt or pH gradients, sometimes after enzymatic treatment, can be instrumental in the elucidation of the effects of microheterogeneity. Continuous advances in such preparative approaches could be observed over the last years.\[17–21\]

For analytical purposes, powerful two-dimensional liquid chromatography mass spectrometry (2D-LC-MS) methods are emerging combining various LC modes, such as CEX, size exclusion chromatography (SEC), hydrophilic and hydrophobic interaction chromatography (HILIC and HIC), or reversed phase (RP), and RP-LC as second dimension, which can be coupled to MS for detection.\[22\] The antibody to be analyzed can either be the intact antibody (top down), or be enzymatically cleaved into larger parts (middle down), that is, Fc and Fab’ fragments with specific proteases such as IdeS, or be cleaved into peptides (bottom up). The most powerful multi-dimensional method for antibody microheterogeneity combines CEX as the first dimension and RP as the second chromatographic dimension.\[23\]

Electrophoretic techniques, including flat gel IEF or CE, again combined with mass spectrometry for identification, can be useful in some cases as an alternative to chromatographic separation.\[24,25\] A comprehensive list of methods for heterogeneity analysis is given in Table 1.

While these techniques can easily provide the necessary resolution and sensitivity for the analysis of antibody heterogeneity, artifacts created during sample preparation can sometimes be an issue.\[26\]
The possible effects of certain kinds of variants on the biological properties and key attributes of antibodies are mostly studied using in vitro methods, see Table 2. The most powerful tools for following binding interactions between proteins and other molecules include label free real-time monitoring techniques such as surface plasmon resonance.[27] Cell based effector function assays are often used to assess the significance that any previously identified differences in antigen or receptor binding may have for antibody-dependent cell-mediated cytotoxicity (ADCC) and complement dependent cytotoxicity (CDC).[24,28]

3. Sources and Effects of Microheterogeneity

3.1. Glycosylation

3.1.1. N-Glycosylation

The conserved, canonical N-glycosylation site in the Fc region of IgG strongly influences the conformation of the antibody. The most commonly observed bi-antennary complex type N-glycan, shown in the inset of Figure 1, stabilizes the conformation of the Fc region to an open structure, whereas the presence of smaller N-glycans lead to a more closed conformation[29] through stabilization of part of the Fc region.[30] Through the destabilization of the Fc region (particularly the C’E loop) and change in the quaternary structure, aglycosylated antibodies show reduced receptor binding.[31]

This glycoform dependent change in Fc conformation has dramatic effects on the interaction of the antibody with various receptors, such as Fc receptors on immune effector cells, parts of the complement system, and neonatal Fc receptors on endothelial cells and monocytes. This has strong implications for effector functions and serum half-life of therapeutic antibodies.[32,33]

N-glycosylation is commonly analyzed by release of the N-glycan using PNGase F, labeling with a fluorescent tag, separation with either HILIC or CE, and detection by fluorescence. This work can usually be coupled to MS for glycan identification via mass and advances in automatization of the process have led to high throughput-screening (HTS).[33]

The branching and terminal residue of the N-glycan is influenced by cell culture conditions such as glucose limitation, dissolved oxygen and pH, with the exact effect being dependent on cell line, and glycoprotein.[34,35] The osmotic conditions and mechanical stress in cell culture do not seem to affect the N-glycan structure.[35] For a detailed review on approaches on how to control antibody glycosylation, the reader is encouraged to read the recent review by Sha et al.[36]

While N-glycosylation is by far the predominant form of glycosylation present in IgG, O-linked glycans can occasionally be found in the hinge region of IgG.[37]

Core Fucose

The presence of core fucose reduces the binding affinity between the IgG Fc region and the FcyRIIIa (CD16) receptor.[33,38] This receptor is crucial in the ADCC, which is a major mode of action for many antibodies. Cell based assays showed a lower required dose in glycoengineered antibodies without core fucose.[39]

Genotypic variation of CD16 exists in the human population, which show vastly different response rates to fucosylated...
antibodies, an effect which might be mitigated by the use of afucosylated antibodies.\[40\]

Absence of core fucose leads to increased binding to FcyRIII, which increases the ADCC response in vitro\[41\] and increases activity in vivo, which leads to a lower required dose in the clinic.\[42\]

Terminal Galactose
Galactose depleted IgG exhibits a significantly reduced binding affinity to C1q\[43–45\] but increased binding to the mannose-binding lectin, which can also activate the complement pathway.\[44\] New findings by Subedi and Barb\[41\] showed that increased galactosylation leads to increased binding to FcγRIII

Figure 2. Examples of microheterogeneity of therapeutic antibodies analyzed by ion-exchange chromatography and linear pH-gradient elution. Antibodies analyzed are commercially available preparations, except for rituximab and palivizumab, which were in-house produced biosimilar candidates, and ch14.18 which was used in a previous research project.\[28\] Data are from Ref.\[106\] and from unpublished work from our lab. The chromatograms have been normalized in respect to the absorbance maximum of the main peak and isoelectric points given are for the main peak.
receptors. This effect is smaller than the change in affinity for afucosylated IgG.

In vivo, no significant contribution of mannose-binding lectin induced complement activation to the overall activity could be found. This suggests that the in vivo activity of antibodies is mainly governed by the interaction with cellular FcRs and terminal galactose should lead to a small increase in ADCC and CDC.[41,44]

Bisecting N-acetyl-D-glucosamine (GlcNAc)

Bisecting GlcNAc is only found in humans and not in any of the mammalian cell lines used for antibody production. It seems to have a positive effect on ADCC, but it is hard to distinguish the contribution of bisecting GlcNAc and the accompanying reduction of core-fucose which also leads to higher ADCC.[33]

Sialylation

Glycoforms rich in α2,6 linked sialic acid, inhibit ADCC by upregulation of inhibitory FcγRIIB, an effect which was not observed for α2,3 linked Neu5Ac.[32,33,38] The α2,3 linkage exists in CHO and mouse cell lines, while α2,6 linkage exists in all mammals, including humans.

Antibodies enriched in sialic acid showed decreased in vivo activity in mice, whereas antibodies depleted in these glycoforms showed slightly higher activity in a platelet depletion test.[46] This effect was reported to be associated with reduced binding to FcγRs. Regarding the in vitro binding affinity towards the corresponding antigen and towards FcγRIII receptors no significant change was found for sialylated glycoforms.[41,46]

In terms of therapeutic applications, the presence of sialic acid is beneficial for the anti-inflammatory effect of polyclonal

Table 1. List of analytical methods for the detection of different kinds of modifications on IgG leading to the presence of antibody variants; suitable analytical methods have to be sensitive and have high resolution capabilities.

| Modification                  | Detection method                                           | Comment                                                   | References |
|-------------------------------|------------------------------------------------------------|-----------------------------------------------------------|------------|
| N-glycosylation               | N-glycan release by PNGase F followed by labeling and     | Gives accurate information about overall glycan composition, | [35,104,105]|
|                               | HILIC or CE separation and fluorescence or MS detection  | but not site specific                                     |            |
|                               | Bottom up, middle down and top-down LC-MS                 |                                                           | [21,58]    |
| Glycation                     | Intact LC-MS                                               | Gives information about overall glycation. Can be influenced | [13]       |
|                               | Peptide level LC-MS                                        | by other modifications. No site specific information      |            |
|                               | Boronate affinity chromatography                           | Orthogonal to MS based methods. Needs to be optimized for  | [14]       |
| Terminal modifications        | Charge based: IEX, IEF, CE, can be combined with MS        | High resolution separation of charge variants, IEX         | [24,25]    |
|                               | techniques for identification                              | chromatography as orthogonal method to electrophoretic    |            |
|                               | Bottom-up LC-MS                                            | techniques                                                 | [24,25]    |
| Incomplete signal peptide     | Bottom-up LC-MS                                            |                                                           | [69]       |
| cleavage                      | Bottom-up LC-MS                                            |                                                           | [77,82–84]|
| Amino acid side chain         | Bottom-up LC-MS                                            |                                                           | [57]       |
| modifications                 | Bottom-up LC-MS, at reducing and non-reducing conditions   |                                                           | [86–88]    |
| Disulfide bond variants       | Bottom-up LC-MS                                            |                                                           | [86–88]    |
| Aggregates and fragments      | SEC with light scattering detection, nanoparticle tracking | Large size range needs to be covered. Multiple methods are | [102]      |
|                               | analysis, light obscuration, micro-flow imaging            |                                                           |            |

Table 2. List of analytical methods for assessing changes in biological properties of IgG associated with the presence of protein variants; parameters that have to be taken into account for characterization include antigen binding, binding to various cellular receptors, biological activity, and conformational stability.

| Detection of                          | Method                                      | Comment                                           | |
|---------------------------------------|---------------------------------------------|---------------------------------------------------|---|
| Antigen binding, receptor binding     | Surface plasmon resonance (SPR), (Biacore)  | Label free, real-time monitoring of protein-protein interactions | |
|                                       | Bio-layer interferometry (Octet)            | Label free, real-time monitoring of protein-protein interactions | |
|                                       | Enzyme linked immunosorbent assay (ELISA)   | Plate based technique for quantifying protein-protein interactions | |
|                                       | Isothermal titration calorimetry (ITC)      | Thermodynamic analysis of protein-protein interaction | |
| Biological activity                   | Antibody dependent cellular cytotoxicity (ADCC) assay | Cell based in vitro assay | |
|                                       | Complement dependent cytotoxicity (CDC) assay | Cell based in vitro assay | |
| Conformational changes                | Deuterium exchange MS                       | Measurement of conformational changes/solvent accessibility | |
|                                       | Differential scanning calorimetry (DSC)     | Measurement of thermal stability                   | |

© 2017 The Authors. Biotechnology Journal Published by Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim
intravenous gamma globulin (IVIG) therapy. Through which receptors this effect is mediated is still an open question at the moment.\textsuperscript{[47]} This direct connection between the amount of sialylated glycoforms present for a given antibody sample and the anti-inflammatory effect of IgG is also supported by the fact that steady-state serum IgG shows sialylation to a higher extent than serum IgG produced in response to a challenge with a specific antigen.\textsuperscript{[46]}

**High Mannose**

As shown in Figure 1, the high mannose glycan can comprise between five and nine mannose residues. High mannose type glycans display favorable pharmacodynamics and unfavorable pharmacokinetics, that is, high mannose rich glycoforms suffer from faster serum clearance\textsuperscript{[48]} but show an ADCC response almost as high as non-fucosylated complex type glycans.\textsuperscript{[49]} A pathway that degrades high mannose glycans with a higher number of mannose subunits down to the core structure with five mannose via glycosidases, exists in humans.\textsuperscript{[50]}

**Non-human**

Some mammalian glycosylation patterns do not exist in humans, which is why they can cause adverse reactions of the immune system, if present on therapeutic antibodies.\textsuperscript{[33]} Among these non-human epitopes, gal α,1,3 gal and Neu5Gc sialylation, are of particular importance when producing antibodies in mouse cells, as those xenoglycans can lead to hypersensitivity reactions.\textsuperscript{[32]}

**Non-canonical N-glycosylation Sites (Fab Glycosylation)**

Apart from the evolutionarily conserved N-glycosylation in the Fc region, IgG can bear additional N-glycosylation sites in the Fab region, with 15–25% of serum IgG being modified in such a way.\textsuperscript{[51]} These glycans are mostly of the bi-antennary type with a higher degree of sialylation than found in the Fc glycan, due to the better accessibility for glycosyltransferases in the golgi apparatus. Depending on the location, the presence of the glycan can affect the antigen binding negatively or positively. Due to the increased accessibility of the Fab glycan, the serum half-life can be increased through interaction with various glycoprotein receptors.\textsuperscript{[34,51]} Most importantly Fab glycosylation can have anti-inflammatory effects in vitro and in vivo.\textsuperscript{[52,53]}

### 3.2. Terminal Modifications

**3.2.1. C-terminal Lysine Clipping**

Human IgG is normally produced with a sequence of Pro-Gly-Lys residues at the C-terminus of each heavy chain. The C-terminal Lys is then commonly cleaved off by intracellular carboxypeptidases, resulting in the loss of one positive charge unit causing a shift of the molecule’s isoelectric point toward the more acidic side.\textsuperscript{[59]} This modification has been shown to also occur naturally on serum IgGs, which is why it is not considered a critical quality attribute (CQA).\textsuperscript{[60]}

Due to the associated change in the charge state of the antibody, any charge based analytical methods like IEF, cIEF, CEX are very well suited for the detection of variants created by incomplete lysine clipping. Additionally, peptide mass analysis by RP-LC and MS can be used for the detection of variants containing zero, one, or two C-terminal Lys residues.\textsuperscript{[24]}

As the target point for this modification is located far away from any sites that play an essential role for the functionality of the antibody like the antigen binding region or the Fc-region involved in receptor binding, it was commonly believed for a long time that C-terminal lysine clipping does not affect the in vitro or in vivo activity of the molecule, especially the CDC activity.\textsuperscript{[24,61]} This claim was supported by the fact that the presence or absence of the C-terminal Lys residue does not cause antibodies can undergo further transformation into advanced glycation end products (AGEs), which are associated with diabetes, arthritis, aging, and other conditions.\textsuperscript{[56]}

The propensity of a lysine residue for glycation is mainly governed by its proximal amino acid composition, whereas solvent exposure seems to have little effect.\textsuperscript{[57]} The vicinity of carboxylic acids can act as a catalyst for the formation of a stable glycated amino acid. Glycation is generally evenly spread among all susceptible residues, unless a highly reactive lysine residue is present in an antibody.\textsuperscript{[56]}

In general, overall glycation levels of antibodies are low and have no biological effect. In some cases, especially when glycation occurs in the complementarity-determining region (CDR), the antibody can completely lose its ability to bind the antigen.\textsuperscript{[56]} The propensity for glycation of a lysine residue in the CDR can be tested in forced glycation studies, to ensure that glycation during production, storage, or in the patient does not eliminate efficacy of the antibody. Effector functions related to Fc binding seem to be unaffected by glycation. No studies on the biological effect of AGEs on antibodies have been published to date, but since the formation of AGEs can lead to aggregation,\textsuperscript{[56]} the reader is referred to the relevant section in this review.

Monitoring of overall glycation of an antibody can be achieved rather easily by using boronate affinity chromatography of the intact antibody or fragments or top-down mass spectrometry.\textsuperscript{[34,55,58]} Glycation is also detectable through charge based methods, due to the blocking of a lysine residue, but other modifications can interfere making a clear identification difficult. If quantitative site specific information is required, a bottom up mass spectrometry method after derivatization of the sugar can be used.\textsuperscript{[57]}

**3.1.2. Glycation**

Reducing sugars can react with primary amines on the N-terminus and lysine side chains creating reversible Amadori glycation products. Glycation can occur during cell culture, due to the unavoidable presence of glucose (and sometimes galactose).\textsuperscript{[54]} It can also occur during storage when glucose or maltose is used as an excipient in the formulation of the antibody, and even when sucrose is used as it can undergo hydrolysis into reducing sugars.\textsuperscript{[55]} This glycation during storage has even been observed in a powdered (lyophilized) state.\textsuperscript{[56]} Antibodies can get glycated during administration, as infusions often contain glucose and the process can take several hours at room temperature.\textsuperscript{[54]} Finally, glycation can occur in the patient’s serum, especially in those suffering from diabetes.\textsuperscript{[55]} Glycated
any conformational changes to the antibody’s structure.\cite{62}

However, a recent study by van den Bremer et al. contradicted these findings by reporting that antibody fractions that had undergone complete C-terminal lysine clipping showed a clearly increased CDC activity compared to their counterparts, which still contained lysine residues on both C-termini of the heavy chains. It was then proposed that previous studies may not have been able to identify this link due to a lack of samples containing separated variant fractions rather than mixtures.\cite{61}

### 3.2.2. N-terminal Pyroglutamate Formation

The most common amino acid residues present at the N-termini of both the heavy and the light chains of antibodies are glutamine (Gln) and glutamic acid (Glu). Both of these residues can be target points for modifications resulting in the formation of pyroglutamate (PyroGlu). While the non-enzymatical cyclization reaction of Gln to PyroGlu seems to occur predominantly during protein production in cell culture,\cite{63} the chemical conversion of Gln to PyroGlu happens later during processing and storage of the product and seems to be heavily dependent on buffer conditions.\cite{64,65} Additionally, both reactions can also occur in an enzymatically driven fashion, catalyzed by the glutaminyl cyclase.\cite{66}

Non-enzymatic conversion of glutamate to pyroglutamate has also been observed on antibodies in vivo, without affecting the pharmacological properties of the molecules, such as its safety, pharmacodynamics, and pharmacokinetics.\cite{25,60}

The formation of PyroGlu from Gln is associated with the loss of the positively charged primary amine, which creates variants that have a more acidic isoelectric point.\cite{59} Thus, all charge based methods, including IEF, cIEF, CEX, possibly coupled with mass spectrometry are again well suited for the detection and identification of this type of modification. The conversion of Glu to PyroGlu on the other hand does not result in a change of the net charge of the molecule and can therefore only be analyzed by peptide mapping approaches.\cite{25}

Overall, N-terminal pyroglutamate formation is believed to not have a major impact on the biological activity of IgG simply due to lack of proximity to the more functionally relevant regions in the molecule.\cite{59,67}

### 3.2.3. Incomplete Signal Peptide Cleavage

Like most proteins intended for secretion from the cell, antibodies are initially produced with an N-terminal signal peptide, that initiates translocation of the molecule and is then enzymatically removed to obtain the mature form of the protein. While this cleavage by signal peptidases happens in a very site specific manner in most cases, there is a certain probability of unspecific cleavage that creates variants of the molecule with parts of the signal sequence still attached at the N-terminus. Given the close proximity of this position to the antigen binding region in the three dimensional structure of a folded IgG, it is reasonable to assume that any sequence change at the N-terminus might affect the target affinity of the molecule, even though this has seldom been observed experimentally.\cite{68}

The most straightforward approach to screen for antibody heterogeneity resulting from alternate signal peptide cleavage, would be LC-MS based peptide mapping. An in-depth approach for the functional characterization of these variants would involve the creation of various mutant antibodies containing different versions of the signal peptide by amino acid substitutions followed by subsequent expression, purification, and analysis.\cite{69}

### 3.3. Amino Acid Side Chain Modifications

#### 3.3.1. Asparagine and Glutamine Deamidation

Deamidation of asparagine and glutamine residues is a very common non-enzymatic protein modification that is often reported to be among the leading causes for the charge heterogeneity observed in IgG.\cite{67} For both residues, the reaction adds an additional negative charge to the molecule, which results in the presence of acidic charge variants in the sample with a molecular weight increase of 1 Dalton.\cite{8}

Even though deamidation can happen at virtually every stage from the production of an antibody in the cell to storage of the final product, the rate at which this modification tends to occur is highly dependent on the external conditions including buffer composition, pH, and temperature.\cite{70} For example, it has been reported that incubation of IgG samples at basic pH and elevated temperatures drastically increases the likelihood of deamidation reactions occurring in the sample.\cite{8} As these conditions are often part of the typical sample preparation procedure for LC-MS analysis it can be difficult to identify variants created by this type of modification due to the presence of artifacts resulting from sample preparation.

For adequately evaluating the impact, that this modification can have on the functionality of an IgG molecule, the different sites at which deamidation can happen have to be taken into account. When occurring in the Fab region, deamidation of asparagine has been reported to cause a change in the thermal stability of the IgG molecule.\cite{71} A deamidation site present in the CDR, on the other hand can result in a decreased target affinity and potency.\cite{71–73} However, it has been proposed that this reduction in binding affinity might be caused by a structural change in the antigen binding region induced by the presence of the succinimide intermediate rather than by the endproduct of the deamidation reaction.\cite{74}

It has been confirmed, that Fc deamidation occurs in vivo at rates that are comparable to the ones observed in vitro at physiological conditions.\cite{75}

#### 3.3.2. Aspartic Acid Isomerization

Isomerization of aspartate to isoaspartate often occurs as a follow-up of a deamidation reaction, especially on aspartate residues that are directly followed by a glycine in the sequence. From a biophysical point of view isoaspartate would be slightly more acidic than the aspartate precursor. However, the isomerization reaction also introduces an additional methyl group, which is likely to cause a change in the three dimensional structure of the molecule.\cite{8}
This hypothesis is also supported by the fact that antibody variants containing isoaspartate instead of aspartate have been reported to elute later in cation-exchange chromatography.\textsuperscript{[76]}

The standard method for analysis of this type of modification is again LC and LC-MS based peptide map.\textsuperscript{[77]} However, due to the time-consuming nature of this approach the search for alternative methods is still ongoing.\textsuperscript{[78]}

Similar to the deamidation of asparagine and glutamine residues, the location of potential isomerization sites in the three-dimensional fold of the IgG molecule is of decisive importance for the impact of this modification on the biological activity of the antibody. If occurring in one of the CDRs, the formation of isoaspartate from aspartate can cause a significant loss of antigen binding activity.\textsuperscript{[79]}

3.3.3. Methionine Oxidation

Compared to other amino acids, methionine is the most prone to be modified by oxidation. Thus, it is commonly believed to function as an antioxidant in proteins, protecting other residues, which are more essential for the functionality of the molecule, from oxidative damage.\textsuperscript{[80]} From a biophysical perspective, the oxidation of methionine to methionine sulfoxide increases the size and the polarity of the amino acid side chain resulting in reduced hydrophobicity and increased affinity for formation of hydrogen bonds.\textsuperscript{[81]} The charge state of the molecule is not affected by this modification. Variants created by methionine oxidation can therefore not be resolved by any charge based methods like IEF or CEX which leaves RP chromatography and LC-MS peptide map as the most commonly used analysis methods for the detection of this kind of modification.\textsuperscript{[82]}

With regards to antibody functionality, it has been proposed that an increasing number of oxidized methionine residues is linked to a decrease in affinity toward the neonatal receptor FcRn and might thus result in shorter serum half-life.\textsuperscript{[83]} Especially methionine residues 252 (M252) and 428 (M428) would seem to be relevant target points in this context as they are both located in the Fc part of the molecule close to the binding site for FcRn. Later research, however, suggests that while oxidation of M252 has a considerable impact on binding toward FcRn, oxidation of M428 seems to play a less important role and might even counteract the effect of M252.\textsuperscript{[84]} Additionally, it seems as if the number of oxidized M252 residues on the individual molecule is of decisive importance for the effect on FcRn binding and pharmacokinetics. Only molecules with both heavy chains oxidized at M252 show a significantly faster clearance whereas a single oxidized M252 per antibody exhibits no significant effect.\textsuperscript{[84]}

3.4. Disulfide Bond Variants

The typical disulfide bond arrangements for all four subclasses of IgG has first been described in the 1960s by Milstein et al.\textsuperscript{[85]} The most widely known is probably the disulfide bond structure of IgG\textsubscript{1}, as depicted in Figure 1 with 12 intrachain disulfide bridges, one interchain bond between the heavy and the light chain of the Fab fragment, and two additional interchain linkages located in the hinge region. The classical structures of the other IgG subclasses differ substantially from this one in the location of the two cysteine residues keeping the Fab fragment connected and in the number of disulfide bridges in the hinge region (two for IgG\textsubscript{1} and IgG\textsubscript{4}, four for IgG\textsubscript{2}, and eleven for IgG\textsubscript{3}). For IgG\textsubscript{1} the disulfide bridge in the Fab fragment is formed by the last two cysteines in both the heavy and the light chain, whereas for IgG\textsubscript{2}, IgG\textsubscript{3}, and IgG\textsubscript{4}, the last cysteine residue of the light chain is linked to the fifth cysteine in the heavy chain.\textsuperscript{[85]}

As these disulfide bridges form the only covalent linkage between different parts of the polypeptide chain, it can reasonably be expected that they play an essential role for the correct folding and the structural stability of an IgG molecule. Any variations in these disulfide connections therefore deserve special consideration. The state of the art methods for analyzing these variants are RP-LC and MS-based peptide map both under non-reducing conditions.\textsuperscript{[86]} Using a mass tag or a fluorescent dye for labeling before the tryptic digest of the protein also allows the detection of free SH-groups in non-reduced peptide map.\textsuperscript{[87–89]}

3.4.1. Non-classical Linkage

Variants with disulfide bridge arrangements differing from the classical linkage structure described above have been commonly found only in IgG\textsubscript{2} and IgG\textsubscript{4}.\textsuperscript{[85]} For both subclasses the isoforms observed in MS analysis resulted from variations in the interchain connections between cysteins located in the Fab fragment and in the hinge region. For IgG\textsubscript{2}, three possible variants have been reported, with one being described as a structural intermediate between the other two.\textsuperscript{[90,91]} For IgG\textsubscript{4}, an equilibrium exists between two variants, one of which contains intrachain connections in the hinge region of the heavy chains instead of two interchain bonds.\textsuperscript{[92,93]}

While the non-classical linkage variants of IgG\textsubscript{2} showed a significant difference in their biological activity in in vitro assays,\textsuperscript{[90]} it is commonly believed that the IgG\textsubscript{4} disulfide isoforms impact the stability of the molecule but not the biological activity as indicated by mutagenesis studies.\textsuperscript{[93,94]}

3.4.2. Free Sulphydryls

Incomplete formation of disulfide bonds is a common phenomenon in both recombinant as well as endogenous antibodies and results in the presence of molecules containing free SH-groups.\textsuperscript{[85]} The extent to which this incomplete disulfide bridge formation occurs seems to be largely dependent on external conditions. For example, it was possible to reduce the level of free SH groups present in a given antibody by adding copper sulfate to the cell culture.\textsuperscript{[95]} Additionally, it seems as if lacking disulfide bridges can be repaired to a certain extent in vivo, which becomes apparent when analyzing antibodies that had been incubated in serum as well as with samples recovered from rat serum after previous administration.\textsuperscript{[74]}

Based on the highly reactive chemical nature of free thiol groups, it has been proposed that free sulphydryls in antibodies
might promote the formation of dimers, which could impact the safety of a therapeutic antibody.

Any impact on biological activity that the presence of incomplete disulfide bonds might have, seems to strongly depend on the individual antibody samples as well as on the location of the resulting free SH-groups in the 3D structure of the molecule. In some cases other non-covalent interactions might be enough to compensate for a missing disulfide bridge leaving the overall structure as well as the functional characteristics of the molecule unchanged. For other therapeutic antibodies, however, it has been claimed that the presence of unpaired cysteines can result in a loss of potency. Specific proteolytic cleavage of a disulfide bridge in the hinge region, on the other hand, has been shown to cause a loss of effector functions. Based on these reports it seems to be necessary to evaluate the impact of incomplete disulfide bonds on the biological activity of an antibody on a case-by-case basis.

### 3.4.3. Beta Elimination and Trisulfide Bond Formation

Beta elimination is the decomposition process of disulfide bridges back to cysteine residues via a dehydroalaneine and persulfide intermediate stage. Subsequent crosslinking of dehydroalaneine and cysteine results in the formation of a non-reducible thioether bond.

Trisulfide bond formation on the other hand, has been proposed to occur during fermentation, resulting from the interaction of an already formed disulfide bond with hydrogen sulfide dissolved in the surrounding media.

While it has clearly been reported for trisulfide bonds that no associated change in antigen binding and biological activity of antibodies could be observed, for thioether linkage it has been proposed that the change in bond length might in certain cases lead to a change of the orientation of the Fab fragment, which could potentially impact antigen binding properties.

### 3.5. Other Variants

#### 3.5.1. Aggregates and Fragments

While aggregates can be considered part of antibody macroheterogeneity rather than microheterogeneity, their substantial effect on pharmacological properties justifies a brief discussion of these important CQAs. Aggregation can be reversible or irreversible, can create small oligomers, nanoparticles, subvisible particles, or visible particles and can occur at any time between manufacture and administration. Different types of stresses typically produce characteristic size populations. Prolonged stress can lead to the formation of larger aggregates and particles from smaller populations. Aggregation can lead to changes in the molecule which reveal new epitopes, leading to an immune reaction to the aggregates. Effects even worse can be observed with aggregates that present native epitopes in such a fashion, that anti-drug antibodies (ADAs) are produced in the patient, leading to neutralization of not only the aggregates, but also the antibody monomer. The ability of aggregates to induce ADAs depends on size, solubility, conformation, and other modifications.

Analytical methods for aggregates must cover a wide analytical size range, which is why no one method is adequate for the full characterization. Methods include size-exclusion chromatography, often coupled to light scattering, nano particle tracking analysis, light obscuration, and micro-flow imaging.

### 4. Conclusion

For the development of better antibodies, antibody fragments, and derivatives, the molecular and cellular action must be fully understood. Furthermore the gap between understanding in vitro results and data from in vivo studies in animals and clinical observations must be closed. The state-of-the-art analytical methods are highly advanced, but we have to keep in mind that the number of possible variants in an antibody preparation is almost infinite. Therefore a more pragmatic approach is necessary to assess different modifications in isolated form. On the other hand this complicates bridging the gap between in vitro, in vivo, and clinical outcome. When ADCC is important for the action the in vivo activity is mainly governed by the interaction with cellular Fc receptors. Serum half-life is influenced by glycosylation and interaction which neonatal Fc receptors, and a new generation of antibodies is in development taking this effect into account. Also an increased in vivo activity of defucosylated antibodies has been observed. C-terminal lysine clipping has recently been reconsidered as an important modification. It was proposed that previous studies may have overlooked its influence due to a lack of samples containing separated variants.

For the development of the latest generation of antibody therapeutics, modifications which improve potency or pharmacokinetics have been taken into account. Although there is already a deep understanding of the cellular and molecular mechanisms associated with antibody variants and their biological consequences, the clinical proof for the effects observed in vitro and in vivo is still missing for many types of modifications. In our opinion the future development of new antibodies and the improvement of currently approved antibodies as biobetters could lead to an increased understanding of the relationship of microheterogeneity and clinical outcomes. This will in turn improve the quality of antibody therapy. However, the current wave of biosimilars might slow down this progress as companies strive to produce exact copies of already approved antibodies rather than new and improved products.

### Acknowledgements

The authors would like to thank Dr. Rainer Hahn (i.m.c.) for his critical review of the manuscript. This work has been supported by the Federal Ministry of Science, Research and Economy (BMWF), the Federal Ministry of Traffic, Innovation and Technology (bmvi), the Syrian Business Promotion Agency SFG, the Standortagentur Tirol, the Government of Lower Austria and Business Agency Vienna through the COMET-Funding Program managed by the Austrian Research Promotion Agency FFG.
Conflict of Interest

The authors declare no commercial or financial conflict of interests.

Keywords

immunoglobulin, IgG, monoclonal, variants, isoforms, pharmacokinetic, pharmacodynamics, safety

Received: July 14, 2017
Revised: August 8, 2017
Published online: September 25, 2017

[1] S. Aggarwal, Nat. Biotech. 2014, 32, 32.
[2] B. C. Kremkow, J. Y. Baik, M. L. MacDonald, K. H. Lee, Biotechnol. J. 2015, 10, 931.
[3] C. A. Castro Jaramillo, S. Belli, A-C. Cascais, S. Dudał, M. R. Edelmann, M. Haak, M-E. Brun, M. B. Otteneder, M. Ullah, C. Funk, F. Schuler, S. Simon, MAbs 2017, 1.
[4] A. F. U. H. Saeed, R. Wang, S. Ling, S. Wang, Front. Microbiol. 2017, 8, 495.
[5] D. König, S. Zielonka, J. Grzeschik, M. Empting, B. Valldorff, S. Krah, C. Schröter, C. Sellmann, B. Hock, H. Kolmar, Curr. Opin. Struct. Biol. 2017, 45, 10.
[6] N. Nuñez-Prado, M. Compte, S. Harwood, A. Álvarez-Méndez, S. Lykkenmark, L. Sanz, L. Álvarez-Vallina, Drug Discov. Today 2015, 20, 588.
[7] Q. Wang, Y. Chen, M. Pelletier, R. Čtvirková, J. Bonnell, C. Y. Chang, A. C. Koksal, E. O’Connor, X. Gao, X. Q. Yu, H. Wu, C. K. Stover, W. F. Dall’Acqua, X. Xiao, MAbs 2017, 1.
[8] H. Liu, G. Gaja-Bulsoco, D. Falduto, C. Chumsae, J. Sun, J. Pharm. Sci. 2008, 97, 2426.
[9] H. Liu, G. Ponniah, H. M. Zhang, C. Nowak, A. Neill, N. Gonzalez-Lopez, R. Patel, G. Cheng, A. Z. Kita, B. Andrien, MAbs 2014, 6, 1145.
[10] J. G. L. Tan, Y. Y. Lee, T. Wang, M. G. S. Yap, T. W. Tan, S. K. Ng, Biotechnol. J. 2015, 10, 790.
[11] C. Villacrés, V. S. Tayi, E. Lattová, H. Perreaud, M. Butler, Biotechnol. J. 2015, 10, 1051.
[12] M. M. van Beers, M. Bardor, Biotechnol. J. 2012, 7, 1473.
[13] A. M. Goetze, M. R. Schenauer, G. C. Flynn, MAbs 2010, 2, 500.
[14] S. Kozłowski, P. Swann, Adv. Drug Deliv. Rev. 2006, 58, 707.
[15] U.S. Department of Health and Human Services, F. a. D. A., Center for Drug Evaluation and Research, Center for Biologics Evaluation and Research, 2015.
[16] U.S. Department of Health and Human Services, F. a. D. A., Center for Drug Evaluation and Research, Center for Biologics Evaluation and Research, 2017.
[17] A. Creasy, G. Barker, G. Carta, Biotechnol. J. 2017, 12, 1600636.
[18] B. Hintersteiner, N. Lingg, E. Janzek, O. Mutschlechner, H. Loibner, A. Jungbauer, Biotechnol. J. 2016, 11, 1617.
[19] B. Hintersteiner, N. Lingg, P. Zhang, S. Woen, K. M. Hoi, S. Stranner, S. Wiederkum, M. Ullah, C. Funk, F. Schuler, S. Simon, MAb 2016, 8, 1548.
[20] E. Wagner-Rousset, S. Fekete, L. Morel-Chevillet, O. Colas, N. Corvia, S. Cianferani, D. Guillarme, A. Beck, J. Chromatogr. A 2017, 1498, 147.
[21] Y. Leblanc, C. Ramon, N. Bièhoreau, G. Chevreux, J. Chromatogr: B. Analit. Technol. Biomed. Life Sci. 2017, 1048, 130.
[22] D. Stoll, J. Danforth, K. Zhang, A. Beck, J. Chromatogr: B Analit. Technol. Biomed. Life Sci. 2016, 1032, 51.
