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Chromatographic analysis of the acidic and basic species of recombinant monoclonal antibodies

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The existence of multiple variants with differences in either charge, molecular weight or other properties is a common feature of monoclonal antibodies. These charge variants are generally referred to as acidic or basic compared with the main species. The chemical nature of the main species is usually well-understood, but understanding the chemical nature of acidic and basic species, and the differences between all three species, is critical for process development and formulation design. Complete understanding of acidic and basic species, however, is challenging because both species are known to contain multiple modifications, and it is likely that more modifications may be discovered. This review focuses on the current understanding of the modifications that can result in the generation of acidic and basic species and their effect on antibody structure, stability and biological functions. Chromatography elution profiles and several critical aspects regarding fraction collection and sample preparations necessary for detailed characterization are also discussed.

Introduction

Monoclonal antibodies (mAbs) are heterogeneous in their biochemical and biophysical properties due to multiple post-translational modification and degradation events. Variants are commonly observed when mAbs are analyzed by charged based-separation techniques such as isoelectric focusing (IEF) gel electrophoresis, capillary isoelectric focusing (cIEF) gel electrophoresis, cation exchange chromatography (CEX) and anion exchange chromatography (AEX). These variants are generally referred to as acidic or basic species as compared with the main species. Acidic species are variants with lower apparent pI and basic species are variants with higher apparent pI when antibodies are analyzed using IEF based methods. When analyzed by chromatography-based methods, acidic species and basic species are defined based on their retention times relative to the main peak. Acidic species are the variants that elute earlier than the main peak from CEX or later then than the main peak from AEX, while basic species are the variants that elute later than the main peak from CEX or earlier than the main peak from AEX.

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Although there is general agreement about the profile and amounts of acidic and basic species observed using IEF-based and chromatography-based methods, subtle differences may exist because of differences in the mechanisms of separation. IEF separates antibody variants based on overall charge difference (apparent pI), but, in addition to the overall charge, distribution of charge plays a critical role in the separation of antibody variants by chromatography because it may affect the interaction of antibodies with column resins. The difference between IEF and IEX has been repeatedly demonstrated and reported in literature.[1-3] For example, fractions of a murine mAb with different retention times separated by a weak cation exchange (WCX) column showed identical pI when subsequently analyzed by IEF.[1] A recombinant mAb with either aspartate (Asp) on both heavy chains or one Asp on one heavy chain and one isoaspartate (isoAsp) on the other heavy chain at the same positions had identical pI when analyzed by IEF, and yet these variants could be resolved by CEX.[2] Fab fragments of a recombinant mouse/human chimeric antibody with a pyroglutamate (pyroGlu) on either the light chain or the heavy chain, and therefore with no expected pI difference, could be resolved by a strong cation exchange (SCX) column.[3] These examples indicate that chromatography-based separation is not solely dependent on the overall charges because separation can be achieved even in the absence of charge differences. Therefore, an apparent pI based on theoretical calculation or determined experimentally by IEF-based methods may not be adequate to predict the elution order from chromatography separation methods, although the primary driving force for separation is charge difference.

Charge variants may substantially affect the in vitro and in vivo properties of antibodies. It has been demonstrated using chemically-modified antibodies that charge variation can alter binding to proteins or cell membrane targets, thus affecting the tissue penetration, tissue distribution and pharmacokinetics (PK) of the antibodies.[4-13] It is important to note, however, that these antibodies were deliberately modified and thus were highly enriched with one particular modification. Low abundance acidic and basic species of recombinant mAbs that were formed due to multiple modifications did not show differences in properties such as potency, binding to FcRn and PK compared with the main species or the unfractionated material.[14] As will be discussed, the effects of variants are highly dependent on the nature, location and degree of posttranslational modifications that cause the formation of acidic and basic species.

Table 1. Modifications that form acidic species

Acidic species	References
Sialic acid	14,17,20,25,26
Deamidation	1,2,14,17,27–39
Non-classical disulfide linkage	40,41
Trisulfide bonds	42
High mannose	23
Thiosulfide modification	43
Glycation	14,44–46
Modification by maleuric acid	21
Cysteinylation	48
Reduced disulfide bonds	14
Non-reduced species	14
Fragments	23,25,36

Table 2. Major deamidation sites of recombinant monoclonal antibodies

Locations	Products	References
Light chain CDR1, Asn30	Asp	2
Light chain CDR2, Asn55	IsoAsp	2
Light chain CDR1, Asn33	Asp and isoAsp	27
Heavy chain CDR2, Asn55	Asp and isoAsp	28
Heavy chain CDR2, Asn55	Not determined	17
Heavy chain CDR2, Asn55	Not determined	29
Heavy chain CDR2, Asn55	Asp and isoAsp	30
Heavy chain, CH3 domain (First and second N in S**N**GPE**N**NYK)		14,31–36
Heavy chain, CH2 domain (QDWL**N**GK)	Asp and isoAsp	32,34,37,38
Heavy chain, CH3 domain (LH**N**HYTQK)	Not determined	14
Light chain, CL domain (First N in LL**N**NFYPR)	Asp and isoAsp	32

This article focuses on characterization of acidic and basic species separated by chromatography techniques and includes information on charge heterogeneity reported since the publication of two reviews in 2008.[15,16] Although acidic and basic species are commonly observed by IEF and cIEF, collecting enough materials from IEF and cIEF for detailed characterization is challenging. Preparative scale IEF-based separation techniques that are suitable for fraction collection exist, but the current knowledge of acidic and basic species is mainly based on characterization of variants collected from chromatography-based methods, which will be the focus of this review.

Main Species

The main species is the antibody that elutes as the major peak on chromatograms. The main species does not necessarily correspond to the unmodified or non-degraded antibody. In fact, the main peak typically consists of species of antibodies with three types of typical posttranslational modifications: (1) cyclization of the N-terminal glutamine (Gln) to pyroGlu; (2) removal of the heavy chain C-terminal lysine (Lys); and (3) glycosylation of the conserved asparagine (Asn) residue in the CH2 domain with neutral oligosaccharides. N-terminal Gln is encoded in the genes of either or both of the light and heavy chains, which can spontaneously cyclize to form pyroGlu after synthesis. Most of the antibodies at the time of analysis contain N-terminal pyroGlu instead of the original Gln and thus elute as the main peak.[3,17,18] Similarly, the heavy chain C-terminal Lys is also encoded in the gene of heavy chain. Incomplete removal of the C-terminal Lys by carboxypeptidase results in antibodies with zero, one or two C-terminal Lys residues.[19] Antibodies without any C-terminal Lys are usually found in the main species,[1,3,17,18,20-23] although an antibody with two C-terminal Lys as the main peak has been reported.[24] Lastly, the conserved Asn residue in the CH2 domain is glycosylated with N-linked oligosaccharides. The main glycoforms of recombinant mAbs from mammalian cell cultures are core-fucosylated complex biantennary structures with zero, one or two terminal galactose residues. The main species serve as an important control for detailed characterization of acidic and basic species.

Acidic Species

Acidic species are defined as the antibody variants that elute earlier than the main peak during CEX or later than the main peak during AEX analysis. The main causes for the formation of acidic species are summarized in **Table 1**.

Sialic acid has been commonly reported to contribute to the formation of acidic species. Sialic acid has been detected in the acidic fractions of a recombinant IgG1 antibody expressed in NS0 cells.[17] Acidic fractions of recombinant IgG1 antibodies expressed in CHO cell lines also contain higher levels of sialic acid.[14,25] The percentage of acidic species was found to be substantially lower when a recombinant mAb expressed in goats was analyzed by CEX after treatment with sialidase, suggesting that the presence of sialic acid is the major cause of these acidic species.[20] Sialic acid has also been detected in the acidic regions of an IgG4 antibody when it was analyzed using CEX.[26]

Deamidation of Asn residues has also been widely reported as a major cause of acidic species. Deamidation occurs both in the variable domains, especially in the exposed and flexible complementarity-determining regions (CDR), as well as in the constant domains (**Table 2**). Deamidation of Asn residues in the CDR region is almost guaranteed to result in the generation of acidic species.[2,17,27-30] Although there is a minimal difference in the pK_a between the side chain of isoAsp and Asp, an antibody and its Fab fragments with isoAsp33 in the light chain CDR1 elute later than the antibody with Asp33 at the same position, and both of them elute earlier than the antibody with the original Asn residues.[27] The fact that an antibody with either isoAsp or Asp at the same position elutes at different retention times suggests a conformational difference that affects the charge

distribution, and thus is evidence of antibody interactions with column resin.

Deamidation of the Asn residues in the constant region of the CH3 domain with the amino acid sequence SNGQPENNYK has also been widely reported.[31-36] Although the specific products from each site are still debated, it is generally agreed that deamidation occurs only at the first and the second Asn residues in this sequence. Deamidation of other Asn residues in the constant regions including the Asn residue in the CH2 domain with the amino acid sequence LNGK,[32,34,37,38] the Asn in the CH3 domain with the amino acid sequence LHNHYTQK and the first Asn in the sequence LLNNFYPR of light chain constant region[32] has also been reported. Deamidation of Asn residues in the constant regions also most likely results in the formation of acidic species.[14,17,33,36] Various levels of isoAsp were also detected in several acidic fractions of an IgG4 molecule as measured by IsoQuant, which specifically detected isoAsp.[39] It is worth noting that various combinations of deamidation of Asn141 on either one or both of the heavy chains of a murine mAb, and Asn161 on either one or both of the light chains, formed a repeated peak pattern of acidic species when analyzed by CEX.[1]

Although not as widely observed as sialic acid and deamidation, other modifications have also been shown to result in the generation of acidic species. For example, IgG2 with the non-classical disulfide linkage (IgG2B and IgG2A/B) elutes earlier than the molecule with the classical linkage (IgG2A) when analyzed by CEX.[40,41] When analyzed using AEX, antibodies containing a trisulfide bond, which was first identified in recombinant human IgG2, elute later than the main peak, and therefore correspond to acidic species.[42] Interestingly, an antibody variant with high mannose content was also slightly enriched in the acidic fraction of a recombinant mAb, although there is no charge difference between the high mannose oligosaccharides and complex biantennary oligosaccharides with core fucose.[23] This again highlights the fact that chromatography-based separation is not based only on the charge differences, but also on subtle conformational differences. Thiosulfate, which has been used in formulation to prevent oxidation caused by light exposure, can react with the antibody to form acidic species.[43] Glycation, a reaction between reducing sugars and either the side chain of a Lys residue or the N-terminal primary amine, results in the formation of acidic species, as expected, due to neutralization of the positive charges.[14,44-46] Acidic species of a fully human mAb expressed in transgenic goats was identified due to a modification of the light chain and heavy chain N-terminal amine by maleuric acid.[21] Light exposure resulted in earlier elution of an antibody Fab fragment from a CEX column, which was attributed to the modification of a histidine residue in the heavy chain CDR2 region.[47] A recombinant mAb with cysteinylated Cys104 in the heavy chain CDR3 elutes earlier than the antibody after de-cysteinylation.[48] Antibodies with non-reducible species or reduced disulfide bonds[14] and various fragments[23,25,36] are also enriched in the acidic fractions.

Impact on structure, stability and function. The potential effect on structure, stability and function due to various modifications that cause the generation of acidic species is highly dependent on their locations. Modifications that are located in the Fc region may not have a significant effect on Fab fragments because of the flexible hinge region. For example, the presence of sialic acid does not affect antibody potency.[14,17] On the other hand, modifications that are located in the Fab region, especially in the CDR region, are the most likely to have a substantial effect on antigen binding and potency. The impacts of deamidation are summarized in **Table 3**. Deamidation in the CDR regions results in various degrees of reduction in antigen binding affinity and potency of recombinant mAbs.[2,23,27] The effect of deamidation has also been studied using site-directed mutagenesis, where replacement of Asn 55 in the CDR2 of heavy chains by Asp resulted in a 14-fold reduction in the antigen binding affinity of an antibody.[28] Slight differences in the secondary structure are observed between Fab with either isoAsp30 or Asn30 in the light chain CDR1.[27] Interestingly, a Fab with Asp30 has a higher melting temperature (Tm) than the Fab with the original Asn and the Fab with isoAsp has the lowest Tm when studied by differential scanning calorimetry (DSC). The effect of non-classical disulfide bond linkage varies from antibody to antibody. An IgG2 with the non-classical disulfide bond linkage (IgG2B) shares a similar secondary structure as an IgG2A with the classical disulfide bond linkage, but with a smaller hydrodynamic size and different higher order structures and thermal stability.[49] In potency assays, IgG2B has either the same or lower potency than IgG2A.[41,49] The presence of trisulfide bonds in the hinge region has been shown not to affect the thermal stability of an IgG2 antibody.[42] Glycation increases the formation of aggregates without altering antibody conformation,[44] suggesting decreased positive surface charges caused by the glycation of Lys likely result in lower repulsion between antibody molecules. Ten percent glycation of a Lys residue (K65) in the heavy chain CDR2

Table 3. Impact of deamidation

Locations	Effects	Reference
Light chain, CDR1	Asp30/Asn30 only 70% as potent as Asn/Asn30	2
Light chain CDR1	Asp30/Asn30 has 39% and isoAsp30/Asn30 has 60% of binding affinity compared with Asn30/Asn30 of 96% binding affinity. Slight difference in the secondary structure of Fab with isoAsp compared with Fab with Asn30. Higher Tm for Fab with Asp30 and lower Tm for Fab with isoAsp30 compared with Fab with Asn30	27
Heavy chain CDR2	14 fold reduction in binding affinity compared with the antibody with Asn	28
Heavy chain CDR2	IsoAsp55 or Asp55 in one heavy chain only 20% potent as antibody with two Asn. Fab with either isoAsp55 or Asp55 has only 50% binding affinity compared with Fab with Asn.	23
Heavy chain CDR2	No impact on potency for fraction with lower level of Asn55 deamidation	17

Table 4. Modifications that form basic species

Basic species	References
C-terminal Lys	1,3,14,17–20,22,24,47,50
N-terminal Glu	3,17,26,51
Isomerization of Asp	2
Succinimide	2,23,56,57
Met oxidation	25,58,59
Amidation	60,61
Incomplete disulfide bonds	51
Incomplete removal of leader sequence	14,17,61
Mutation from Ser to Arg	26,62
Aglycosylation	63
Fragments	25
Aggregates	14,36

of an antibody did not affect its binding affinity.[46] The effects of cysteinylation and decysteinylation have been extensively investigated.[48] The cysteinylation of Cys104 in the CDR3 of the heavy chain can be removed by mild reduction with free cysteine; however, Cys104 after decysteinylation cannot be modified by the alkylation reagent iodoacetimide, suggesting the modification site is less accessible after decysteinylation. While no difference in the hydrodynamic size is measured by ultracentrifugation, the antibody after decysteinylation is retained longer on SEC column, suggesting a stronger interaction with the column matrix. In addition, the antibody with cysteinylation showed decreased thermal stability, was more prone to form aggregates and was only 39% as potent as the decysteinylated antibody.

Structural and functional effects have also been assessed using total acidic regions without separating into fractions with one particular modification enriched. The acidic fractions of a recombinant IgG1 mAb containing 29% sialic acid,17% glycation, 7% non-reducible species, 29% reduced disulfide bonds and 18% deamidation in the Fc region have a slightly lower binding response to FcRn, but have similar in vivo PK in rats as the main peak materials lacking these modifications.[14] On one hand, these results demonstrate that various multiple modifications to such levels may not have substantial effects on potency and in vivo PK and therefore may not be a concern for the development. On the other hand, these results also demonstrate the need to collect fractions that are enriched with one particular modification in order to clearly detect the potential difference between antibodies with or without such a modification.

Basic Species

Basic species are defined as the materials that elute later than the main peak during CEX and earlier than the main peak during AEX analyses. Modifications that result in the generation of basic species are summarized in **Table 4**.

One major reason for the formation of basic species is incomplete removal of C-terminal Lys. MAbs with heavy chain C-terminal Lys are more basic than the main species due to the additional positive charges.[1,3,14,17-20,22,24,47,50] Because carboxypeptidase B (CPB) can specifically remove C-terminal basic amino acid residues, comparison of the chromatograms of antibodies before and after CPB digestion can clearly demonstrate the contribution of C-terminal Lys to the formation of basic species.

Another common modification that contributes to basic species is incomplete cyclization of the N-terminal glutamine (Gln) to pyroGlu of the light chain or heavy chain or both. The Gln is originally encoded in the genes, but it can undergo a spontaneous reaction to result in pyroGlu. Antibodies with the original Gln have a higher apparent pI than the main species and are therefore referred to as basic species.[3,17,26,51]

Isomerization of Asp to isoAsp has been reported widely.[2,33,52-55] Similar to deamidation, isomerization occurs frequently in the CDR regions[2,52-55] and rarely in the constant regions.[33] When analyzed by CEX, a recombinant mAb with isoAsp102 in the CDR3 of heavy chain elutes later than the antibody with the original Asp.[2] This later elution of antibody containing isoAsp compared with antibody containing Asp has also been observed for antibodies containing either isoAsp or Asp as Asn deamidation products.[27] Isomerization of the Asp in the constant region has also been reported,[33] but its relationship to basic species is unclear.

Succinimide, which is the common intermediate for Asn deamidation and Asp isomerization, can also contribute to the generation of basic species.[23,53-56] Recombinant mAbs with succinimide in the CDR regions as the Asp isomerization product elute later than antibodies with Asp at the same positions from CEX.[2,56] An antibody with succinimide 55 as Asn deamidation product in the CDR2 of heavy chain elutes in the basic region on CEX.[23] The presence of succinimide 74 as an Asp isomerization product, which is outside of the CDR regions, also results in the formation of basic species.[57] Succinimide as an deamidation intermediate has also been detected at position 384 in amino acid sequence SN(384)GOPEN(389)N(390)YK and in the peptide LNGK of the constant domains;[32] however, the elution position relative to the original antibody is unclear.

Although not widely observed, several other modifications have also been reported to generate basic species. Antibodies with oxidation of the two conserved methionine (Met) residues in the Fc region elute in the basic region when analyzed by CEX.[25,58] Oxidation of a Met residue in the CDR2 regions of a recombinant mAb also results in the generation of basic species as evidenced by an earlier elution from an AEX column.[59] Amidation of the proline residue after removal of the heavy chain C-terminal Lys and the preceding glycine residue results in the formation of basic species.[60,61] The last three amino acids of antibody heavy chain are PGK, which fits the enzymatic amidation consensus sequence of XGB where X is the target amidated residue and B is a basic amino acid. Therefore, amidation may be a much more common modification for recombinant mAbs, although maybe to a very limited extent in many cases. A recombinant mAb with unformed disulfide bond in the heavy chain variable domain elutes later than the antibody with complete disulfide bond from a CEX column and thus represents a basic species.[51] Antibody variants with incomplete removal of the signal peptides elute in

the basic region.[14,17,61] However, this observation may not be generalized because the elution of antibodies with or without signal peptides may highly depend on the nature of the amino acid in the signal peptide. Recombinant mAbs with a single amino acid mutation from serine to arginine in the light chains[26] or in the Fc region of the heavy chain elute as basic species from CEX columns.[62] An antibody with one aglycosylated heavy chain and one heavy chain with short oligosaccharides elutes as basic species from CEX as well.[63] A non-covalent Fab-Fab dimer elutes later than Fab monomer from a WCX-10 column.[47] Antibody aggregates[14,36] and fragments[25] have been detected as basic species. Because fragments are also enriched as acidic species, the nature of charges of the fragments could play a critical role in affecting their elution order by chromatography.

Impact on structure, stability and functions. Similar to the discussion regarding the acidic species, the locations of modifications that form basic species are critical to whether or not the modifications have any effect on the structure, stability and biological functions. Modifications of either N-termini or C-termini of antibodies are not expected to have substantial effects on antibody structure, stability and functions because these regions are highly exposed and not part of any ligand binding sites. There is no difference in the potency between antibodies with two heavy chain N-terminal pyroGlu compared with the same antibody with one pyroGlu and one Gln.[17] The presence of N-terminal leader sequence of various lengths also does not affect potency.[17] C-terminal Lys did not affect thermal stability of a recombinant IgG1 antibody[64] nor the potency of another antibody mediated by complement-dependent cytotoxicity (CDC).[22] Amidation of the C-terminal proline residue after removal of Lys and Gly has no effect on antigen binding or Fc effector functions.[60] Basic species of an IgG1 antibody composed of 85% C-terminal Lys and 15% leader sequence in the light chain demonstrate similar potency, FcRn binding affinity and PK in rats.[14]

In contrast to modifications on the N-terminal or C-terminal regions, modifications in other regions are more likely to have a substantial affect on structure, stability and biological functions. A Fab with unpaired disulfide bonds is only ~28% as potent as the Fab containing the fully formed disulfide bond.[65] An isolated fraction containing antibody with isoAsp102 as an isomerization product was found to be only 9–21% as potent as the main peak fraction containing the original Asp102 residue.[2] The effect of formation of isoAsp has also been studied using fractions collected from hydrophobic interaction chromatography (HIC) separation of a recombinant mAb. Fab fragments with isoAsp32 in the CDR1 of the light chain had 13% relative binding affinity[53] and were ~23% as potent[65] as the Fab with the original Asp32 residue (136% for the peak with the original Asp and 30% for the peak with isoAsp32), while Fab'(2) with isoAsp32 on one light chain had 42% binding activity compared with Fab'(2) with Asp32.[53] Several studies have demonstrated the effect of succinimide formation on antigen binding affinity and potency. A Fab fragment containing succinimide 55 in the heavy chain CDR2 showed a 50% decrease in the binding affinity compared with a Fab with the original Asn residue, and the antibody with succinimide showed a 70% decrease in potency compared with the antibody with the original Asn.[23] A Fab with succinimide 32 in the light chain CDR1 had 16% binding affinity[53] and ~42% potency (136% for the peak with Asp and 56% for the peak with succinimide).[65] Fab'(2) with succinimide 32 in the CDR1 of one or both light chains had 51% and 22% binding affinity compared with Fab'(2) with the original Asp.[53] In contrast, an IgG2 antibody with succinimide 30 in the light chain CDR1 only showed a 10–20% decrease compared with the antibody with Asp30, a difference that was within the assay variability.[56]

Oxidation of Met residues in the Fc region did not affect antigen binding and potency for a recombinant mAb;[43] however, it did cause conformational changes mainly in the CH2 domain and resulted in decreased thermal stability and increased propensity to aggregate.[66] Since the two Met oxidations are located close to the binding sites of multiple ligands, oxidation of these residues decreases binding to protein A, protein G and FcRn, but has only a minimal affect on Fcγ receptor binding.[67-69] In addition, oxidation of these Met residues resulted in a significant reduction in in vivo half-life.[70] Oxidation of Met residues in the heavy chain CDR2 regions resulted in decreased thermal stability of the antibody as measured by differential scanning calorimetry (DSC).[59]

Strategy for Characterization of Acidic Species and Basic Species

Although fraction collection is almost always the first step toward a thorough characterization of acidic and basic species, it may be beneficial to determine the contribution of sialic acid to the acidic species and C-terminal Lys to the basic species by enzymatic digestion prior to fraction collection. Both sialic acid and C-terminal Lys can be specifically removed by enzymatic digestion under native conditions that will not substantially affect antibody structures. Sialic acid alone can be readily removed using sialidase or with the entire N-linked oligosaccharides using PNGaseF. C-terminal Lys can be readily removed by CPB digestion. Comparison of the percentage of acidic or basic species after these enzymatic treatments will determine the contribution of these modifications to the total of acidic or basic species. If an antibody has a substantial amount of C-terminal Lys, acidic species and basic species associated with the antibody with various numbers of C-terminal Lys will overlap substantially. Thus, treatment with CPB will be necessary to collect fractions that will be enriched with specific type of modifications.

Fractions are typically collected based on the identifiable peaks. There are cases where several low abundant peaks are grouped as one fraction to obtain enough material for further characterization, but it is highly desirable to collect finer fractions because a single peak on a chromatogram may be a result of multiple modifications. Further fractionation using different separation methods is also useful to obtain materials with modifications that are enriched to the level that can be characterized. Further analysis of Fab and Fc fragments obtained from enzymatic digestion can help to localize the acidic or basic species that are either associated with Fab or Fc.

In many cases, stressed samples are used to generate higher levels of acidic or basic species for fraction collection. The data are

then extrapolated to determine the nature of the acidic and basic species that are present to an extremely low level in the unstressed drug substance. The observation that the acidic species and basic species in the drug substance and in the stressed sample have the same retention times, however, may not be sufficient to support the conclusion that they are formed due to the same modifications. Further evidence is usually needed to confirm that acidic species and basic species generated at different conditions are the same in nature. Even if the nature of the modifications is the same, the underlined mechanisms may be different. For example, the oxidation of Met256 and Met432 is coupled. Either both residues are oxidized or neither of them is oxidized on one or both heavy chains in drug substance[25] and under accelerated stability conditions.[58] However, commonly used chemical oxidation using tBHP resulted in random oxidation of the two Met residues on the two heavy chains.[58] In another example, only Asp30 of the light chain was observed as the deamidation product in a drug substance, while both Asp and isoAsp was observed in the accelerated stability sample.[2]

Controlling the levels of analytical artifacts is also critical to the characterization of acidic and basic species. Artifacts can be easily introduced during fraction collection and preparation of samples for detailed analysis. First, exposure of the fractions to various pH, agitation, room temperature, and freeze/thaw cycles cannot be completely avoided during fraction collection, concentration, buffer exchange and storage. Modifications such as aggregation, oxidization and deamidation can thus be generated as artifacts. Use of the main peak and the starting material before and after going through similar steps as assay controls is always a good idea. The purity and quality of the collected acidic and basic species also needs to be ensured before carrying out any further studies, including in vitro and in vivo functional assays. Second, artifacts such as deamidation, oxidation and cyclization of N-terminal Gln can occur during sample preparations for analysis. It is always a good practice to include the main peak as a control and carry out side-by-side characterization with the fractions of acidic and basic species. Modifications can also be lost during sample preparation. The formation of succinimide results in a molecular weight decrease of 17 Da if it is an Asn deamidation intermediate or 18 Da if it is an Asp isomerization product. However, succinimide is not stable under typical denaturation, reduction, alkylation and enzymatic digestion conditions used for generating peptides for LC-MS analysis. Similarly, cysteinylation cannot endure the reduction step used for sample preparations for LC-MS analysis. In cases of the loss of modifications during sample preparations, including the main species as a control does not provide much help. Rather, broad knowledge of protein modifications in general and an understanding of the limitations of the sample preparation procedures are very helpful.

Conclusions

Acidic and basic species are commonly observed when recombinant mAbs are produced. Complete elimination of acidic and basic species is unrealistic and unnecessary. There are a few modifications that are probably unique to recombinant mAbs that are either associated with the specific host expression systems (e.g., modification by maleuric acid in goat milk) or occur during the production and formulation (e.g., modification by thiosulfide). Most of the modifications, however, have either been detected or are expected to be present in endogenous human IgG molecules, suggesting a much lower risk as a safety concern. The impact on biological functions is highly dependent on the sites and levels of modifications. For modifications that are localized in the Fc regions, even the presence of higher levels may not have direct effects on the binding affinity. Similarly, modifications that are localized in the Fab regions may not affect Fc-related functions such as receptor binding. The level of modification is also important regarding whether or not there is an effect. For example, the lower level of deamidation of Asn55 in the heavy chain CDR region does not affect the potency of one antibody,[17] but the presence of isoAsp55 or Asp55 in one heavy chain decreases the potency of another antibody to only 20%.[23] Of course, the different effects of deamidation on the two antibodies could be due to their interactions with different antigens, but the percentage of deamidation most likely also plays a role.

Many of the modifications leading to the formation of acidic and basic species have been identified by analyzing fractions collected mainly from chromatography-based methods. There are cases where the acidic and basic species have been fully characterized and can be accounted for by the identified modifications. In many cases, though, fully understanding the nature of acidic species and basic species may still be challenging. Under all circumstances, it is necessary to assess the differences between the acidic or basic species and the main peak with regard to stability and functions. Modifications are likely either critical quality attributes (CQAs) or key process attributes (KPAs). Therefore, information of acidic and basic species is critical to establish comparability and similarity with the ultimate goal to ensure efficacy and safety of therapeutic recombinant mAbs.

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