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EXPERT OPINION

In vivo characterization of therapeutic monoclonal antibodies

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Introduction

Therapeutic monoclonal antibodies (mAbs) are complex drug molecules with a high degree of heterogeneity, including charge variants, aggregates, fragments, and post-translational modifications (PTMs). PTMs can be introduced by chemical or enzymatic modifications over the lifespan of mAbs during production, storage, and *in vivo* circulation. Common PTMs in mAbs include glycosylation, deamidation, oxidation, glycation, N-terminal glutamine cyclization, and C-terminal lysine processing. These product-related modifications are termed as product quality attributes (PQAs). A given PQA has the potential to impact the safety and/or efficacy of a therapeutic mAb. A PQA may enhance immunogenicity or off-target bindings, resulting in changes of the safety profile of a therapeutic mAb. A PQA may change the interaction of a therapeutic mAb to its target antigen, and therefore alter the efficacy. The PQAs that impact on drug safety and/or efficacy are considered as critical quality attributes (CQAs). Control of PQAs, especially CQAs, within predefined acceptance criteria is required by regulatory agencies to ensure product quality, consistency, and stability. During drug development, manufacturing processes aim to maximize the production of a desired product in high purity with minimal and consistent levels of variant forms. Formulation development seeks to stabilize mAb products and minimize additional formation of variants during storage. The modifications occurred during mAb production and storage can be reliably monitored and controlled. However, additional modifications can occur after a mAb is administered to patients. mAbs are typically administered intravenously or subcutaneously, and quickly get into the circulation of patients. The circulating half-life of a typical mAb in human blood system is 2-3 weeks. The blood environment is remarkably different from formulation buffers and storage temperatures. Typical mAb drug products are formulated at a pH of 5-6 with multiple excipients, such as sugars, salts, amino acids, and surfactants. The formulation buffer, pH, and excipients are optimized to enhance mAb solubility/stability and to reduce viscosity as well as the formation of aggregates/particulates. In contrast, blood is a dynamic environment with thousands of proteins including many protein-modifying enzymes, and is tightly regulated at a pH of 7.4 and a temperature of 37 °C. The clearance and modification of a mAb in blood circulation may result in changes of the PQA profile, leading to increased attribute exposure and decreased effective drug exposure to patients, and therefore causing the concerns of immune responses and loss of efficacy. To this end, a recent FDA guidance for industry recommends sponsors to evaluate susceptibilities of therapeutic protein products to modifications in the *in vivo* milieu as early as in product design and development, in order to facilitate product engineering for enhancing the product stability under *in vivo* conditions [1]. Therefore, *in vitro* and *in vivo* characterization of PQAs can monitor the attribute stability before and after drug administration and

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determine the attribute exposure to patients, which can help to establish attribute criticality and control strategy during the design, engineering, selection and development of therapeutic mAbs.

Mass spectrometry based mAb PTM characterization methods

The most common type of therapeutic mAbs is γ-immunoglobulin (IgG). The IgG is a glycoprotein comprised of two identical heavy chains (HC) and two identical light chains (LC). Each heavy chain contains one variable (VH) and three constant domains (CH1, CH2, and CH3), whereas each light chain contains one variable (VL) and one constant domain (CL). Each heavy chain is connected to a light chain by a disulfide bond, and the two heavy chains are linked in the hinge region by multiple disulfide bonds. Antigen binding regions are located at three loops in both VH and VL domains, which are termed as complementarity determining regions (CDRs). The VH and CH1 domains together with the disulfide linked light chain are called the fragment antigen-binding (Fab). The hinge region disulfide bond linked CH2 and CH3 domains together are called the fragment crystallizable (Fc) region. A single N-linked antennary oligosaccharide is located on a conserved asparagine residue in the CH2 domain. Based on heavy chain sequences and inter-chain disulfide bond patterns, IgGs are classified into four group, IgG1, IgG2, IgG3, and IgG4. IgG1, IgG2, and IgG4 are widely used as therapeutics, whereas IgG3 is rarely used due to the short half-life in serum.

Mass spectrometry (MS) based mAb PTM characterization methods can be classified into three approaches: the top-down approach (i.e. intact mass measurement), the middle-up approach (i.e. mAb fragment analysis), and the bottom-up approach (i.e. peptide mapping). The top-down and middle-up approaches require less sample preparation and consumption, and therefore have higher throughput as compared to the bottom-up approach. However, these two approaches only provide modification information at the global structure level. In contrast, the bottom-up approach could locate the modification site to a specific amino acid residue.

Intact mass measurement often uses high end quadrupole-time-of-flight (Q-TOF) mass spectrometers and advanced mass deconvolution algorithms to measure molecular masses of intact mAbs with mass accuracy within a few Daltons. Intact mass measurement reveals the mAb molecular weight, the glycosylation profile, and a few of modifications (e.g. glycation) at the protein level.

To locate the modification into a specific chain or domain, mAb can be cleaved into a few large fragments prior to mass analysis. One mAb fragment method is to reduce disulfide bonds to generate light chains and heavy chains. Another mAb fragment method is the limited enzyme digestion under native condition. For example, papain digestion can generate two Fab and one Fc fragments, whereas Ides can generate one $F(ab')_2$ and two Fc/2 fragments.

Although the middle-up approach can locate a modification to a specific region of a mAb, it could not locate the modification at the residue level. The bottom-up approach not only confirms the mAb sequence but also identifies and quantifies site-specific modifications. In the bottom-up approach, mAb is subjected to denaturation, reduction, alkylation, and protease digestion. The resulted peptides are separated by reversed-phase C18 chromatography coupled to a mass spectrometer for MS and MS/MS analyses. MS/MS can be used to identify and locate modifications. The extracted ion peak areas of the modified peptide and the native peptide were integrated to calculate the relative abundance of the modified peptide. Peptide mapping can also be performed under the non-reduced condition, which preserves disulfide bonds and therefore can be used to confirm the disulfide bond linkages.

To analyze the structures and the relative abundance of N-linked glycans, mAbs are treated with endoglycosidase PNGaseF to release the N-linked glycans, followed by glycan derivatization with fluorescent reagents, such as 2-anthranilic acid (2-AA) or 2-aminobenzamide (2-AB). The labeled glycans are then separated by hydrophilic interaction chromatography and detected by a fluorescence detector and a mass spectrometer (HILIC-FLR-MS). The MS spectra provide the identity of the glycans, and the fluorescent absorption of each HILIC separated glycan peak represents the relative abundance.

For *in vivo* characterization, a therapeutic mAb needs to be purified from the sera of subjects using immunopurification strategies prior to the mass spectrometry based characterization. For animal toxicology and pharmacokinetics studies, anti-human IgG antibodies can be used to pull-down the therapeutic mAb. For human clinical studies, the anti-idiotypic antibody and a target antigen can be used to purify a corresponding therapeutic mAb.

***In vivo* mAb modifications**

Many *in vivo* modifications of mAbs have been reported, including disulfide bond rearrangement, deamidation, formation of N-terminal pyroglutamate, removal of C-terminal lysine, glycation, and oxidation. Some of these modifications behave differently between *in vitro* and *in vivo*, leading to the reassessment of attribute crit-

icality. The IgG antibody subclasses contain a total of 12 intra-chain disulfide bonds within each anti-parallel β barrel domain, an inter-chain disulfide bond between the light chain (LC) and the heavy chain (HC), and multiple inter-chain disulfide bonds between the hinge region of the HCs [2]. Disulfide bonds are static to stabilize the structure of a mAb. However, disulfide bonds can undergo dynamic rearrangements *in vivo* while circulating in human blood [3], which may alter the structure and function of a mAb. Thus, *in vivo* rearrangement of disulfide bonds needs to be monitored.

Asparagine (Asn) deamidation is a common non-enzymatic modification. The side-chain carbonyl group of Asn is vulnerable to the nucleophilic attack by the nitrogen of the n+1 peptide bond, forming an unstable five-member ring intermediate, succinimide (Asu), which rapidly hydrolyzes to form aspartate (Asp) and isoaspartate (isoAsp) under basic pH. Therapeutic mAbs are usually formulated at slightly acidic pH, which preserves the succinimide form. After administration, the accumulated succinimide hydrolyzes to Asp and/or isoAsp under the physiologic pH. In mAbs, deamidation has been reported in the Fc regions [4,5] and the CDRs [6-12]. Deamidation in the CDR regions could affect drug efficacy [10,12]. Deamidation can accumulate more rapidly in *in vitro* and *in vivo* serum samples than that in PBS or formulation buffers[7, 13, 14], which raises concerns about loss of potency and the potential immunogenicity of the therapeutics after administration.

Both glutamine and glutamate at the N-termini of proteins can cyclize to pyroglutamate chemically or enzymatically. The conversion of glutamine occurs much faster than that of glutamate. The N-termini cyclization is resistant to amino peptidases digestion, preventing the degradation. Although glutaminyl cyclase, an enzyme that catalyzes this conversion, is found in human blood [15], the formation of pyroglutamate from glutamate of mAbs *in vivo* is a pH dependent non-enzymatic reaction [14,16], as it replicated the rate constant in PBS *in vitro*.

The C-terminal lysine residue of a mAb heavy chain is susceptible to be removed by unidentified basic carboxypeptidase during protein expression [17]. Partial removal of the C-terminal lysine leads to charge heterogeneity. The remaining lysine residue of mAbs was found to be rapidly removed within an hour through an enzymatic process [18]. As a result, control of the initial lot-to-lot level of C-terminal lysine removal may not be critical.

Glycation is a non-enzymatic modification of primary amines (e.g. lyinse side-chains or N-termini) resulting from incubation with reducing sugars (e.g. glucose, fructose) *in vitro* or during circulation *in vivo*. The degree of glycation of a specific residue depends on protein sequence, structure, and sugar concentration. Glycation of a mAb *in vitro* is reversible in the absence of glucose [19]. In contrast, *in vivo* glycation in human blood increases at a slow rate [20]. The evaluated level of glycation in the Fc region was found to have no impact on binding to Fc receptors [20]. Since glycation of lysine residues will impair the cleavages of lysine residues by trypsin, other enzymes, such as Asp-N or Glu-C, can be used to generate proper peptides for glycation quantitation.

The N-linked Fc glycan is the biantennary complex type composed of a core pentasaccharide with various sugars attached to the core, resulting in mass heterogeneity. The glycan profile of mAbs is an important PQA that needs to be well controlled. The two major N-linked glycan classes are the fucosylated biantennary oligosaccharide and the high-mannose oligosaccharide. The high-mannose species are cleared much faster in human blood than the fucosylated biantennary oligosaccharides [21,22].

Oxidation of methionine is another common post-translational modification. Oxidation of Met252 and Met428 in the Fc region can impact on the binding to Fc receptors [23]. However, no significant accumulation of oxidation *in vivo* was observed [14].

Potential differential clearance of the native and the modified mAb forms in blood circulation needs to be taken into account when conducting *in vivo* characterization of PQAs, because the increased modified form in relation to the native form could be a result of faster clearance of the native form. *In vitro* experiments and knowledge of chemical modification mechanisms can help to distinguish differential clearance from chemical conversion. If the *in vivo* chemical conversion can be reproduced *in vitro* either in physiological buffers or serum incubations, the increase of the modified form is likely due to the chemical conversion. On the other hand, if the *in vivo* chemical conversion cannot be duplicated and the mechanism of the modification is implausible, the increase of modified form may be a result of differential clearance.

In vivo PQA exposure modeling

The next step, also the most important step, is to use *in vivo* characterization data to access the impacts of PQAs on drug safety and efficacy. The two-compartment pharmacokinetic model is the most common model used to describe the pharmacokinetic behavior of a therapeutic mAb[24]. After intravenous administration, the drug is disturbed rapidly from the central compartment (e.g. plasma) to the peripheral compartment (e.g. tissue) by a first-order process. After the distribution process is completed and the equilibrium is reached, the drug concen-

tration in the plasma declines at a rate dependent on drug elimination. The plasma drug concentration-time profile can be described by the two-compartment model equation as $C_p(t)=Ae^{-\alpha t}+Be^{-\beta t}$, where A and B are the hybrid coefficients when α and β are the hybrid first order constants for the rapid distribution phase and the slow elimination phase, respectively. The equation can be solved by nonlinear-fitting to the drug plasma concentration determined by ELISA or LC-MS/MS based quantification. To quantitatively estimate the impact of PQAs on drug safety, the patient exposure to a PQA over the lifespan of a therapeutic mAb in the patient's circulation, from the point of the drug administration to the point of complete clearance of the drug, needs to be determined. The relative percentage of a PQA, such as deamidation, is often described by the first-order reaction equation as $P(t)=1-(1-P_0)\cdot e^{-kt}$, where P_0 is the initial percentage of modification before drug administration and k is the rate constant. The equation can be solved by nonlinear-fitting to the percentage of the attribute in relation to the total mAb determined by *in vivo* characterization discussed above. The attribute plasma concentration-time profile can be described as the product of the drug plasma concentration-time profile and the attribute percentage-time profile, $C_a(t)= C_p(t)\times P(t)$ [25]. The patient exposure to the drug is represented by the integrated area under the curve of the attribute plasma concentration-time profile. Thus, a higher attribute formation rate *in vivo* will result in a higher patient exposure to the attribute. This model can be used to simulate the quantitative impacts of the initial lot-to-lot attribute difference on the attribute exposure. If an attribute formation is much faster relative to the drug clearance, the exposure to the attribute will be relatively high regardless the initial attribute levels, resulting small differences in the patient exposure. In this case, process and storage controls of the initial attribute level have little influence on the patient exposure. On the other hand, if an attribute formation is slow and the drug clearance is relatively fast, the difference in the initial levels of attributes become more significant due to large difference in the patient exposure. In this case, process and storage controls of the initial attribute level become more critical.

The mAb with a given attribute may have reduced potency as compared to the native antibody. A pure fraction of a mAb with a given attribute can be obtained by chromatography enrichment or mutation, and then the relative potency of the mAb with the given attribute can be determined by binding assay or bioassay. The subject exposure to the effective drug over time can be used to quantitatively estimate the impact of a PQA on drug efficacy. The effective drug concentration-time profile can be described as the product of the drug plasma concentration-time profile and the sum of potency-time profile, $C_e(t)=\sum\text{Potency}(t)\cdot C_p(t)$, where $\sum\text{Potency}(t)$ is the sum of the potency of the native mAb and the modified mAb. The patient exposure to the effective drug is represented by the integrated area under the curve of the effective drug concentration-time profile. This model can be used to simulate the quantitative impacts of the initial lot-to-lot attribute difference on the effective drug exposure, which can help to establish the attribute criticality.

Conclusion and perspective

In vitro characterization of PQAs is important to ensure product quality and consistency at the time of a lot release. However, attributes are not necessarily static after administration, but may change *in vivo* and impact on drug safety and efficacy, resulting in altering attribute criticality. Thus, the biopharmaceutical industry and the regulatory agencies have paid great attentions to the *in vivo* behaviors of PQAs. *In vivo* characterization of PQAs can determine the attribute stability, and help to model the patient exposure to attributes and to the remaining effective drug. Both *in vitro* and *in vivo* characterization help evaluate the impacts of PQAs on drug safety and efficacy and establish the attribute criticality and control strategy, leading to development of therapeutic mAbs with better safety and efficacy.

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