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REPORT

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Assessing in vivo dynamics of multiple quality attributes from a therapeutic IgG4 monoclonal antibody circulating in cynomolgus monkey

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ABSTRACT
Characterization of biopharmaceutical proteins and assessment and understanding of the critical quality attributes (CQAs) is a significant part of biopharmaceutical product development and is routinely performed in vitro. In contrast, systematic analysis of the quality attributes in vivo is not as widespread, although metabolism and clearance of multiple variants of therapeutic proteins administered to non-human primates and human subjects may have a different impact on safety, efficacy and exposure. The major hurdles of such studies are usually sample availability and technical capability. In this study, we used affinity purification coupled with liquid chromatography and mass spectrometric analysis of the digested protein for consistent and simultaneous detection of the full amino acid sequence of a therapeutic IgG4 monoclonal antibody, MAB1. This methodology allowed us to assess in vivo changes of all sequence-related modifications and quality attributes of MAB1 over the duration of a preclinical pharmacokinetic study in cynomolgus monkeys.

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Affinity purification; in vivo; mass spectrometry; monkey; quality attributes

Introduction

Therapeutic biotechnology products, such as monoclonal antibodies and recombinant proteins, are heterogeneous molecules commonly produced in mammalian cells via recombinant DNA technology. Multiple product variants are formed during cell culture processing, purification, and upon storage. Maintaining consistent product quality, and thus controlling multiple product attributes within predefined limits, is imperative for biopharmaceutical manufacturing. Due to the rapid emergence of new upstream and downstream technologies,[1] it is becoming more and more common to have major cell line or process upgrades during late-stage development. In such cases, it is essential to demonstrate the comparability of biologics manufactured by the new processes. [2] One key question that must be addressed is which quality attributes are critical and thus merit rigorous control. [3] In addition, due to increased process yields, fewer batches are used for clinical trials, which limits the clinical experience with the material produced prior to the product approval and commercialization. Such a limitation also generates a pressing need for better understanding of the quality attribute criticality in vivo, which allows a risk-based and scientifically sound control strategy for biopharmaceutical manufacturing to be designed.

Product risk assessment or understanding of quality attribute criticality of biopharmaceuticals is essential for their development and production.[3] Often, the quality attribute evaluation is built upon prior knowledge of similar molecules, and the results are derived from dedicated in vitro studies, although the relevance of those may be incomplete. More recently, there is a growing interest to investigate the criticality of quality attributes through understanding the metabolism and elimination of biopharmaceutical proteins in vivo, i.e., as measured in preclinical and clinical samples.[4] The behavior of multiple quality attributes, including glycosylation, disulfides, glycation, deamidation, and oxidation, and their formation and elimination in animals and humans have been studied, providing a better understanding of in vivo exposure to a particular attribute. [5-18] This information yields useful insight into an attribute's effect on the drug safety and efficacy and greatly contributes to the attribute criticality assessment.

In this work, we investigated the metabolism and clearance of various attributes of a therapeutic humanized IgG4 monoclonal antibody, MAB1, using cynomolgus monkey serum samples from a preclinical pharmacokinetic (PK) study. With this approach, we obtain detailed dynamic attribute information of MAB1 in vivo. Based upon such information, we will be able to provide a more relevant understanding of the product quality attribute criticality, which will contribute to establishing an appropriate process control strategy and help optimize quality and productivity of the biopharmaceutical manufacturing process.

Results

To understand MAB1 quality attributes and their change over time in vivo, we examined serial serum samples from a single ascending dose (SAD) cynomolgus monkey PK study. MAB1 was affinity purified from monkey serum and subjected to

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peptide mapping with mass spectrometric detection (LC-MS). In order to provide sufficient material for affinity purification, serum samples collected at the same time point from 5 individual cynomolgus monkeys (30 mg/kg dosage, 12 time points) were pooled. Quantitative and specific extraction of MAB1 from monkey serum was necessary to minimize serum protein interference and enable accurate LC-MS quantitation. After screening several affinity reagents, we found that a commercially available anti-human IgG4 llama VHH coupled to agarose (CaptureSelect IgG4) had acceptable performance for the affinity purification of MAB1 (Fig. 1A and 1B). Llama VHH is a ~15 kDa single domain antibody fragment that is used as an affinity reagent due to its small size, specificity, affinity and stability.[19,20] Parallel reaction monitoring (PRM) was selected as the mass spectrometric quantitation method, where the peak areas of fragment ions from the peptides of interest were used for quantitation. Following the affinity purification procedure, PRM provides further reduction of the background interference.

The linearity of the procedure, combining affinity purification with PRM quantitation, was tested before employing this methodology on the PK serum samples. A series of standards were created by spiking MAB1 into naive cynomolgus monkey serum. The spiked serum standards covered a wide range of MAB1 concentration (from 0 to 800 μg/mL), which was expected to cover the concentration range of the preclinical samples tested based on earlier obtained ELISA data. To extract MAB1, the spiked serum standards were incubated with CaptureSelect IgG4 agarose, as described in the Methods section. After the incubation, the beads were washed to remove the unbound serum proteins and reduce non-specific binding to the resin. The affinity-bound MAB1 was then eluted from the beads and enzymatically digested prior to LC-MS analysis. Three different enzymatic digestion approaches were used: a combination of Endoproteinase Lys C and Trypsin (LysC/Trypsin), a sequential addition of LysC/Trypsin followed by Endoproteinase Asp-N (LysC/Trypsin/AspN), and pepsin alone. Peptides generated by the LysC/Trypsin approach were used for mapping attributes such as oxidation, glycosylation; the LysC/Trypsin/AspN approach was used for mapping disulfide and trisulfide-bonded peptides as it reduced the size of linked peptides for MS detection. The pepsin approach was used for mapping attributes such as deamidation, glycation, pyroglutamate and hydroxyl-lysine, for such attributes are more stable at the low pH digestion condition. Generally, pepsin digestion is rapid (~30 min) and is carried out in an acidic solution (pH~2), which avoids generation of higher pH-related analytical artifacts. However, because pepsin digestion produces certain peptides that are either too small or too large for LC-MS detection, the LysC/Trypsin approach was used for mapping other attributes and with overlapping sequences to complement the pepsin approach (and vice versa) for full sequence and modification analysis. When coupled with PRM analysis, both pepsin and LysC/Trypsin digestion approaches demonstrated good linearity within the tested concentration range (0–800 μg/mL) (Fig. 2A and 2B).

To enable accurate absolute quantitation, a heavy-labeled version of MAB1 was used as an internal standard. The heavy-labeled internal standard was produced by a manufacturing process similar to that used for MAB1, except that lysine and arginine in the cell culture medium were replaced by their heavy-labeled counterparts, ^{13}C and ^{15}N (purity = 99 %). The heavy-labeled MAB1 internal standard was utilized in the LysC/Trypsin approaches since the peptides generated by these enzymes contain either a lysine or arginine residue at the C-terminus. A constant amount of the heavy-labeled MAB1 standard was spiked into the serum samples prior to affinity purification to allow us to normalize for sample preparation-related variability. In this case, the area ratios of ion signals, corresponding to unlabeled and heavy-labeled peptides were plotted against serum MAB1 concentration to generate a standard curve (Fig. 2B). The standard curve demonstrated good linearity. In addition, by comparing MAB1 standards prepared in buffer with MAB1 samples recovered from sera, we

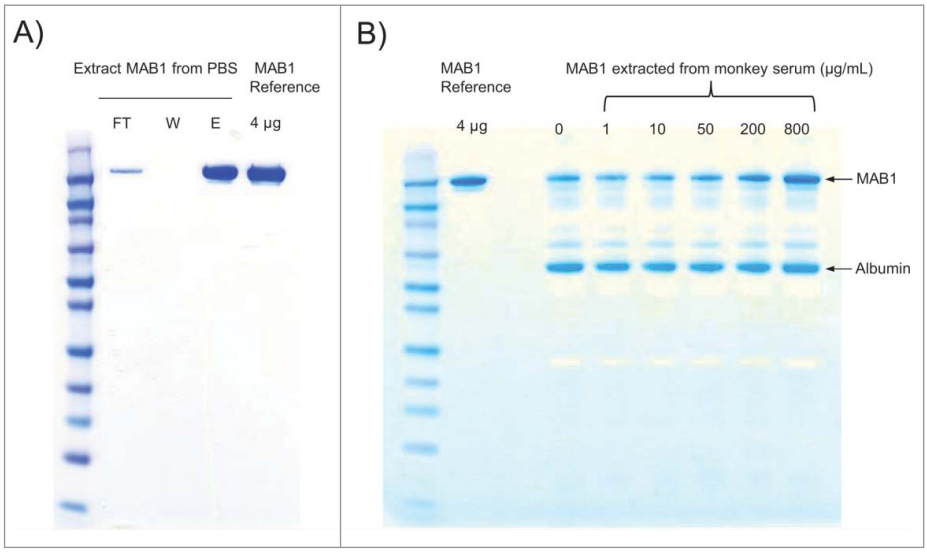

Figure 1. Development of affinity purification method. (A) MAB1 was quantitatively recovered from PBS by affinity purification; Lane 1: molecular weight marker; Lane 2: flow through (FT); Lane 3: wash (W); Lane 4: Eluate (E); Lane 5: 4 μg purified MAB1 as reference. (B) MAB1 can be extracted from monkey serum with acceptable specificity. Lane 1: molecular weight marker; Lane 2: 4 μg pure MAB1 reference; Lane 4–9: Eluates recovered from spike-in serum samples with increasing MAB1 concentration.

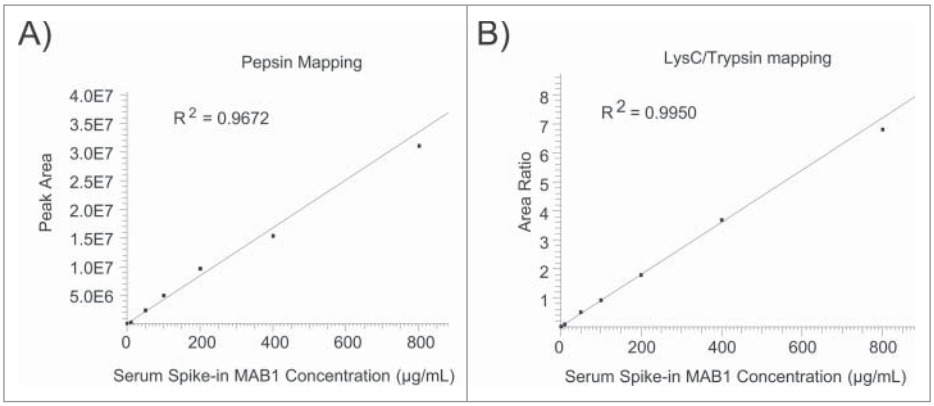

Figure 2. Linearity of parallel reaction monitoring (PRM) quantitation coupled to affinity purification. (A) a representative CDR peptide from pepsin mapping analysis; Y-axis: LC-MS peak area of the CDR peptide; X-axis: MAB1 concentration in spiked-in serum. (B) a representative peptide from LysC/Trypsin mapping; Y-axis: area ratio of the peptide over its heavy-labeled counterpart. X-axis: MAB1 concentration in spiked-in serum;

confirmed that there was no process-related bias for quantifying the levels of quality attributes across a wide range of concentrations (100–800 μg/mL) (Fig. 3A and 3B). As MAB1 serum concentration decreases beyond 100 μg/mL after Day 21, quantitation results beyond this time point are more variable. The time period from Day 1 to Day 21 is deemed sufficient to assess the PK behavior of individual quality attributes. In these method-qualification experiments, MAB1 was immediately extracted from serum after being spiked in, so no change of quality attributes was expected in such a short time frame, and our results confirmed this (Fig. 3A and 3B).

After the affinity purification-PRM procedure was developed, the pooled monkey serum samples from the MAB1 preclinical PK study were analyzed. Twelve time points were included in our analyses starting with one serum sample taken prior to MAB1 administration, and continued with serum taken at 5 minutes, 12 hours, 2 days, 3 days, 7 days, 10 days, 14 days, 21 days, 28 days, 35 days and 49 days post administration of MAB1. The serum concentrations of MAB1, obtained previously by ELISA, were compared with the serum concentration data obtained by the affinity purification-PRM method. Good correlation was observed between the 2 data sets as demonstrated by the strikingly similar PK clearance profiles shown in Fig. 4. While the ELISA uses antigen as the capturing reagent, the affinity purification uses anti-human IgG4 VHH; therefore, the match of the 2 data sets suggests that there is likely no bias of these 2 capturing reagents and quantitation methods toward a particular MAB1 variant (Fig. 4C). The lower concentration detected by ELISA at Day 49 could be due to anti-drug antibody formation, which interferes with antigen binding but not anti-human VHH binding.

Degradation of MAB1 in vivo was investigated by comparing the PK profiles of N- and C-terminal peptides with the total MAB1 concentration, determined as the three3-peptide average (Fig. 5C). If clipping or degradation occurred relatively rapidly, a different PK profile for the terminal peptides compared to the internal-peptide average could be expected due to a more rapid concentration decrease in vivo over time. The results showed no obvious differences, and hence suggested no notable clipping of MAB1 in vivo over the course of the PK study (Fig. 5).

The in vivo behavior of the major N-linked Fc glycoforms was also investigated due to their importance to therapeutic IgG function. Glycoform levels were calculated by dividing the peak area of peptide ions of the corresponding glycopeptide by the peak area sum of all glycosylated variants of the same peptide. The results showed that the relative percentages of the major glycoforms (G0F, G0F-GlcNAc, G1F) were largely unchanged, suggesting that they were cleared at the same rate

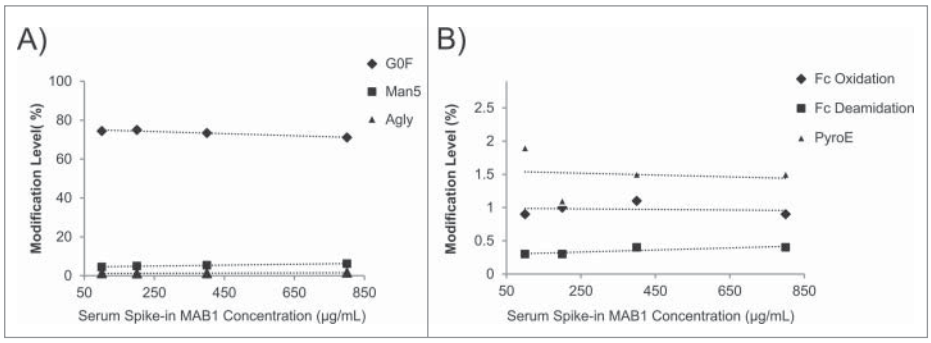

Figure 3. Quantitation of quality attributes across a range of spiked-in samples. The level of an attribute is calculated by the following equation: $\text{Modification level} = \frac{\sum \text{Areas of peptide containing the attribue of interest}}{\sum \text{Areas of all versions of the same peptide}} \times 100\%$. (A) Levels of major glycoforms. The detailed structures of the glycans are listed in **Table 1.** G0F: a glycopeptide containing G0F glycan; Man5: the same peptide containing Man5 glycan; Agly: the same peptide without any glycan attached. (B) Levels of representative oxidation, deamidation and pyroglutamate modifications. Fc Oxidation: a heavy chain peptide containing an oxidized methionine; Fc Deamidation: a heavy chain peptide containing a deamidated asparagine; PyroE: an N-terminal peptide containing cyclized glutamate at the N terminus.

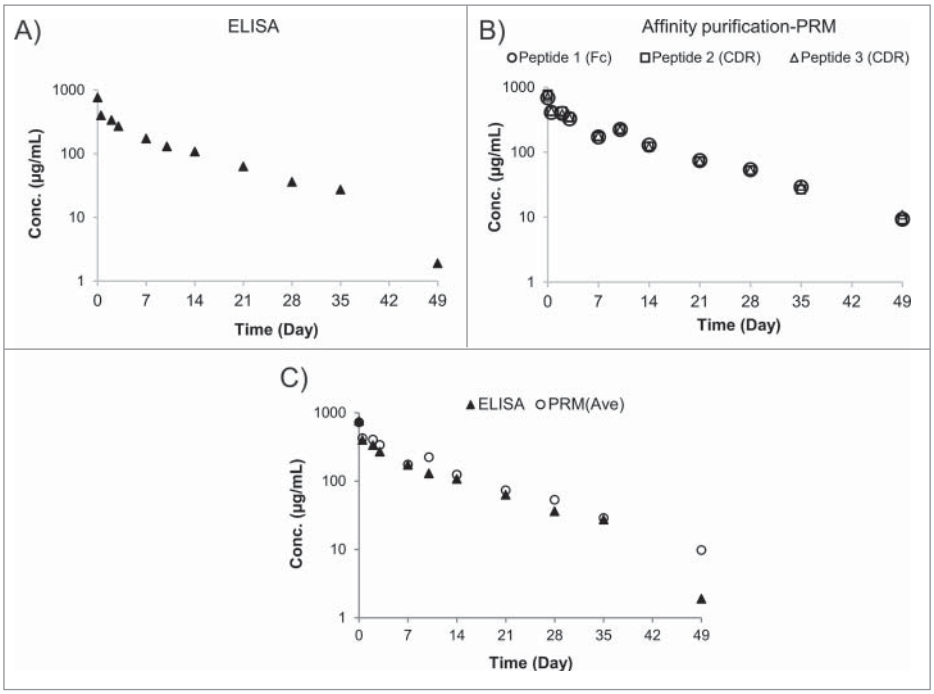

Figure 4. Comparison of serum MAB1 concentration measured by ELISA and Affinity purification-PRM. MAB1 concentrations in serum are plotted against different collection time points along the course of the preclinical SAD study. (A) ELISA data. (B) Affinity purification-PRM data. (C) The overlay of ELISA data and averaged PRM data. The average PRM data were calculated from 3 peptides, which were shown in Fig. 4B.

and that the major glycoform composition of MAB1 remained the same over the 49 day course of in vivo antibody clearance (Fig. 6A). Since preferential clearance of high mannose-containing proteins from circulation had been previously reported,[8] we monitored the clearance of Man5 glycoform in our study because this is the most abundant high mannose species. Indeed, the Man5 glycoform-containing peptides demonstrated significantly faster clearance than the major complex glycoform species (Fig. 6B). Overall, rapid clearance of high mannose species results in decreased drug exposure if the

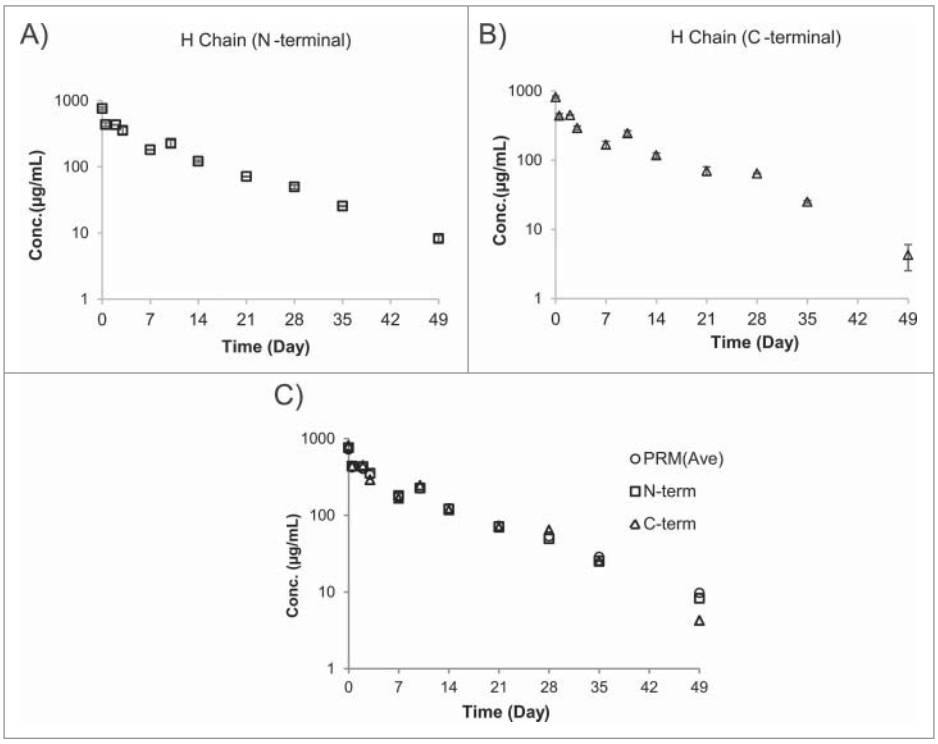

Figure 5. In vivo dynamics of terminal peptides. (A) Heavy chain N-terminal peptide. (B) Heavy chain C-terminal peptide. (C) The overlay of heavy chain N- and C-terminal peptides with the three3-peptide average, which was shown in Fig. 4C.

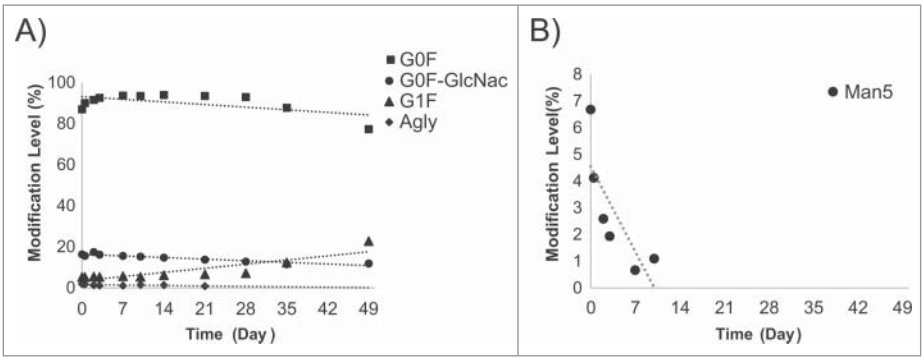

Figure 6. In vivo dynamics of major glycoforms. Modification level $= \frac{\sum \text{Areas of peptide containing the attribue of interest}}{\sum \text{Areas of all versions of the same peptide}} \times 100\%$. (A) Levels of major glycoforms stay relatively stable during the course of the SAD study. The detailed structures of the glycans are listed in Table 1. G0F: a glycopeptide containing G0F glycan. G0F-GlcNAc: the same glycopeptide containing G0F-GlcNac glycan. G1F: the same glycopeptide containing G1F glycan. Agly: the same peptide without glycan. (B) Man5 glycoform was quickly cleared in vivo. Man5: the same glycopeptide as in Fig 6A containing Man5 glycan.

amount of the high mannose containing protein was significant in a biopharmaceutical drug preparation.[8] It calls for close monitoring of high mannose level during production.

In vivo deamidation of several susceptible asparagine residues was examined using pepsin digestion and peptide mapping. It was determined that pepsin digested samples had lower deamidation levels than trypsin digested samples (data not shown). This discrepancy was most likely attributed to the acidic pH employed for pepsin digestion, which suppressed sample processing-induced deamidation that occurs with enzymatic digestion performed in non-acidic conditions with longer incubations. Thus, pepsin digestion was chosen for measurement of in vivo deamidation. As previously determined both in vitro and in vivo, N386 in the heavy chain constant domain is highly susceptible to deamidation.[17] Rapid deamidation of this residue was also observed in our study (Fig. 7A). The rapid increase of asparagine deamidation in vivo suggests that patient exposure to deamidated species is primarily dependent on in vivo modifications that occur after administration of the drug. In other words, deamidation that may occur during production and storage likely plays a less significant role in deamidation-related drug exposure.

Methionine oxidation, another common quality attribute for therapeutic IgGs, is often monitored during process development, manufacturing, and also in stability studies. Previous literature reported that M249 in the heavy chain is involved in FcRn binding, and that oxidation of this residue impairs this binding, leading to increased clearance.[16] Our data show that the proportion of oxidation on M249 stays relatively constant during the first 10 d (Fig. 7B). However, oxM249 becomes undetectable beyond Day 10. Approximately 1% oxMet249 is detectable at MAB1 concentration of 100 μg/mL (Fig. 3B), while total MAB1 concentration remains >100 μg/mL beyond Day 10 and up to Day 21. Thus, oxM249 levels decrease from ~2.5 % to below ~1%, which is consistent with potentially increased clearance of oxM249 variants via FcRn-related pathways.

While the human IgG4 subclass is known for forming half antibodies and for disulfide shuffling with endogenous counterparts,[21] MAB1 includes a hinge mutation S228P (Kabat numbering) that stabilizes the inter-chain disulfide linkages[22] and prevents the disulfide mediated shuffling. Nonetheless, it was still of interest to investigate the in vivo stability of the disulfide linkages of MAB1 experimentally. To reduce disulfide scrambling during digestion, N-ethylmaleimide was added to block free cysteine. In addition, LysC/Trypsin digestion was combined with AspN digestion to produce shorter disulfide-linked peptides to facilitate MS analysis. It appears that all of the disulfide bonds, including both intra-chain and inter-chain ones, are relatively stable in vivo (Fig. 8A). No obvious scrambling was detected. Interestingly, a trisulfide variant was quickly cleared out and/or converted to the disulfide bonded peptide as it was only detected at the 5 minute time point (Fig. 8B). The conversion of trisulfides to disulfides is consistent with a published in vitro study in reducing buffer.[23] The fast clearance/conversion suggests that the residual level of trisulfide is not of particular concern for drug safety and efficacy.

Discussion

Due to the complexity of preclinical and clinical samples, it is often necessary to isolate therapeutic proteins prior to analysis, which is technically challenging given the high abundance of endogenous protein background. In cases where the biopharmaceutical protein of interest is an antibody, the difficulty of specific isolation may be even more problematic because of the similarity between endogenous and therapeutic antibodies. Affinity purification is often the method of choice for isolating protein and antibody targets from complicated physiological

Table 1. Glycan structures.

Glycan Abbreviation	Composition	Structure
G0F	GlcNAc2Man3(Fuc)GlcNAc2	
G0F-GlcNAc	GlcNAc1Man3(Fuc)GlcNAc2	
G1F	Gal1GlcNAc2Man3(Fuc)GlcNAc2	
Man5	Man5GlcNAc2	

Mannose
Fucose
N-acetyl glucosamine
Galactose

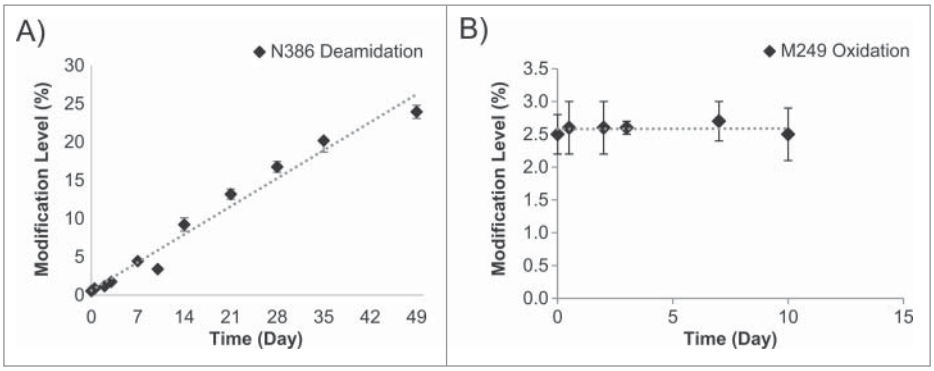

Figure 7. Change of deamidation and oxidation levels *in vivo*. Modification level $= \frac{\sum \text{Areas of peptide containing the attribue of interest}}{\sum \text{Areas of all versions of the same peptide}} \times 100\%$. A) Deamidation on N386 rapidly increases in vivo; B) Oxidation on M249 stays relatively constant up to day 10.

matrices. [24] Depending on the target and matrix, different types of capturing reagent can be used, including anti-human IgG antibodies, recombinant antigens or anti-idiotypic antibodies. One major concern of this affinity approach is a potential bias toward one or several variants. By comparing ELISA and affinity-purification MS data, we've demonstrated that there is no noticeable bias. It is worth noting that antigen, which binds to the complementarity-determining region (CDR) of MAB1, was used in ELISA as a capturing reagent, while affinity purification uses anti-human llama VHH as a capturing reagent, which binds to the Fc region of MAB1. The fact that 2 different capturing reagents provide comparable results supports the claim that there is no detectable bias of these 2 methods. In addition, we compared the composition of MAB1 in buffer and MAB1 extracted from serum by peptide mapping MS, and showed comparable levels of the quality attributes of interest, as described in the Results section.

Although the amount of a therapeutic protein purified from preclinical or clinical samples may be limited and some contaminants from complex biological matrices (serum, plasma) may still be present, high mass accuracy and high resolution mass spectrometry with its selectivity, specificity, sensitivity and dynamic range, is an excellent tool for qualitative and quantitative analysis of such samples. Moreover, peptide mapping with MS detection can provide useful information on multiple attributes from a single analysis,[25] thus maximizing the gain of knowledge while sample amount is limited. We utilized the high resolution and fast cycling time of a Q-Exactive Plus mass spectrometer to quantify the quality attributes of interest by PRM, which uses fragment ions for quantitation, thus further reducing the background interference.[26] Another major challenge is the choice of an appropriate internal standard. The usual heavy peptide standards, although easier to obtain, cannot take into account the variations during affinity purification and digestion. Instead, we produced a heavy lysine and arginine labeled version of MAB1 using a similar manufacturing process. Heavy-labeled MAB1 is added to the serum sample as an internal standard prior to affinity purification, thus normalizing sample processing variability and facilitating absolute quantitation.

In this study, MAB1 does not show any notable clipping at its termini in vivo over the course of the PK study. In addition, the disulfide bonds remain stable. It is consistent with the previously reported studies demonstrating that hinge-mutated IgG4 is stable in vivo.[22] It has been observed that trisulfide-containing isoforms are quickly converted to disulfides or cleared, which indicates that the impact of this modification in vivo is not significant.

The level of major glycoforms remains relatively constant as a function of circulation time. However, high mannose glycoforms, such as Man5 and Man6, exhibit preferential clearance, which is

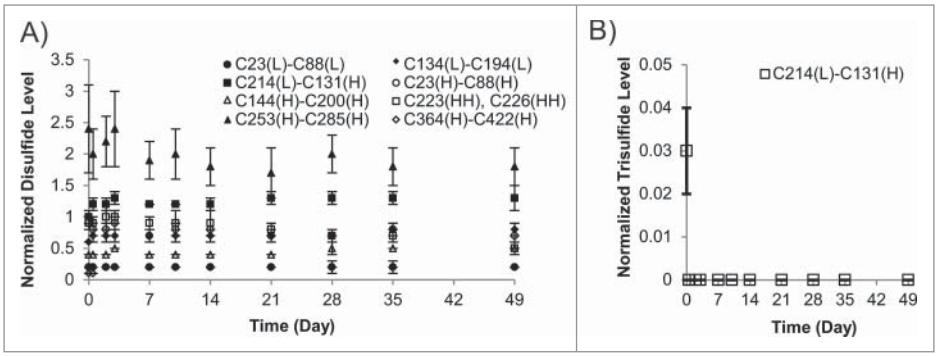

Figure 8. In vivo dynamics of disulfide and trisulfide bonds. Peptides are classified according to the cysteine positions. A peptide in Fc region without disulfide linkages or potential modifications is selected as an internal reference peptide. H: heavy chain. L: light chain. Normalized disulfide level $= \frac{\text{Area of disulfide linked peptide of interest}}{\text{Area of the internal reference peptide}}$. Normalized trisulfide level $= \frac{\text{Area of trisulfide linked peptide of interest}}{\text{Area of the internal reference peptide}}$. (A) Disulfide peptides stay relatively constant; (B) Trisulfide peptides rapidly decrease. It is only detectable at the 5 min time point.

consistent with previously reported results [8] and can be attributed to the mannose receptor mediated clearance. [27] As MAB1 variants containing Fc high mannose glycans are cleared more rapidly than other glycoforms, the quantitative effect of those on PK profile should be considered. Although faster clearance of Man5 glycoform is not significant for this particular study, the level of high mannose glycoforms in antibody-based therapeutics may potentially affect the PK properties. Thus, high mannose glycoforms can potentially be critical product quality attributes with a significant impact on clearance if present at higher levels, which makes it important to control high mannose species during process development and manufacture.

Oxidation is not increased, which suggests that serum is not an oxidative environment. On the other hand, the neutral pH (7.4) is sufficient to induce rapid deamidation on certain solvent-exposed asparagine residues. Due to the rapid increase, most of the deamidation-related drug exposure arises from in vivo deamidated species. Thus, deamidation levels generated upon manufacturing and storage play a less important role on the exposure to this attribute, which is mostly driven by the conversion kinetics in vivo.

Our study has clearly demonstrated the feasibility of simultaneously monitoring multiple quality attributes of biopharmaceutical antibodies from preclinical samples. The PK profiles of the molecule (i.e., non-modified peptide sequences) as well as the potential attributes (i.e., the modified peptide sequences) were measured simultaneously in the analysis. The information obtained during this study will be integrated into the risk assessment for MAB1 development. It is worth noting that, although therapeutic antibodies share a largely conserved framework, quality attributes may still have different dynamics in vivo due to the differences in multiple factors, such Ig subclass, mechanism of action, affinity to target and nonspecific off-target affinity, delivery method, antibody engineering, as well as clinical indication, dosage and regiment of administration. As a result, it can be useful to carry out specific in vivo studies for individual therapeutic antibodies. In addition to facilitating development efforts, knowledge gained in vivo can also benefit early discovery, process changes and development of biosimilar drugs. We are in the process of extending our study to human subjects, with improved affinity purification assays and increased throughput. It is expected that studies of the behavior and changes of quality attributes of biopharmaceuticals in vivo will be widely implemented, and make substantial contributions at different stages of biopharmaceutical protein discovery and development.

Methods

Reagents

Trypsin (sequencing grade, V5111 and V5117) was purchased from Promega (Madison, WI); Lysyl Endopeptidase (Mass Spectrometry Grade, 125–05061) was purchased from Wako Chemicals (Richmond, VA); Pepsin (Porcine stomach mucosa, 02195367) was purchased from MP Biomedicals (Solon, OH); Endoproteinase AspN was purchased from New England BioLabs Inc. (P8104S) and Roche Diagnostics (11054589001). Dithiothreitol (DTT, D5545), iodoacetamide (IAA, I6125) and N-ethylmaleimide (NEM, E3876–5G) were purchased from Sigma-Aldrich (St. Louis, MO). Phosphate-buffered saline 10X Solution (PBS, BP399-500), Tris buffer (45-000-236), 1.0 N hydrochloric acid solution (HCl, SA48-500), tris(2-carboxyethyl)phosphine hydrochloride (TCEP-HCl, PI-20490), Progenta Anionic Acid Labile Surfactant II (AALS II, 5 mg, 50-121-8307), Cynomolgus monkey serum (027-AS-MP-5ML), LC/MS grade water (W6-4), acetonitrile (ACN, A996-4), formic acid (FA, optima LC/MS grade, A117-50) and Pierce Spin Columns (69705) were obtained from Fisher Scientific (Fairlawn, NJ). CaptureSelect™ IgG4 (Hu) Affinity Matrix (290005 and 290010) was purchased from Life Technology (Woburn, MA).

Preparation of standard solutions in serum

Eight μL MAB1 (60 μg/ μL) was mixed with 592 μL blank cynomolgus monkey serum to obtain an 800 μg/mL stock solution. The stock solution was further serially diluted with cynomolgus serum to make standard solutions at different concentrations (400, 200, 100, 50, 10, and 1 μg/mL), with each standard in 50 μL volume. An equal volume (50 μL) of 100 μg/mL heavy-labeled (Lys8 and Arg10) MAB1 was added to each standard solution.

In vivo PK study

A group (N=5) of cynomolgus monkeys were given a single dose of MAB1 (30 mg/kg) intravenously. Serum samples were collected at 12 time points up to Day 49. For each time point, sera from all 5 monkeys were pooled before analysis.

Affinity purification of MAB1 from monkey serum

Anti-human IgG4 llama VHH-based affinity resin (CaptureSelect™ IgG4 (Hu)) was used as the capture reagent for purifying MAB1 from monkey serum. 50 μL affinity resin was loaded onto a spin column and washed with PBS buffer. 50 μL serum sample was diluted with 450 μL PBS and added to the resin. After several washes with PBS, bound MAB1 was eluted with 100 μL of 10 mM HCl (pH 2). The eluate was split for 3 different enzymatic mappings, i.e., LysC/Trypsin, LysC/Trypsin/AspN and Pepsin mappings. Heavy-labeled MAB1 was added as internal calibrant to serum samples prior to affinity purification.

LC-MS analysis

Digested peptide mixture was analyzed by an LC-MS system consisting of a Thermo Ultimate HPG-3400RS LC and an Agilent 300SB-C18 HPLC column (40°C, 5 μm particle size, 2.1 mm i.d. × 15 cm) coupled to a Thermo Q-Exactive Plus mass spectrometer. Mobile phase A (0.1% formic acid in water) and mobile phase B (0.1% formic acid in acetonitrile) were used for linear gradient elution (from 2 to 28% B in 40 min). The flow rate was maintained at 200 μL/min. Parallel Reaction Monitoring (PRM) was carried out with the following parameters: MS resolution at 35,000, AGC target ion value at 5e5, maximum injection time at 100 ms, isolation window at 2.5 m/z, and HCD energy optimized for each peptide.

Data analysis

Mass chromatograms were extracted with a 10 ppm tolerance window for product ions from each targeted precursor ion. The extracted peaks were integrated by Thermo LCquan 2.9 QF1. The integrated area was further normalized by the area of the corresponding heavy-labeled peptide. The normalized ratios were then plotted against MAB1 concentrations to obtain a standard curve, which was fitted to a linear regression with 1/x2 weighting factor and used for determination of PK sample concentration. In the cases of pepsin mapping, where heavy calibrant is not added, the peak areas from the standard solutions were directly used for determining sample concentration.

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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