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RESEARCH PAPER

Characterization of Therapeutic Monoclonal Antibodies Reveals Differences Between *In Vitro* and *In Vivo* Time-Course Studies

Sheng Yin • Cinthia V. Pastuskovas • Leslie A. Khawli • John T. Stults

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ABSTRACT

Purpose To examine and determine the sites and the kinetics of IgG1 mAb modifications from both *in vitro* (rat plasma and PBS) and *in vivo* (rat model) time-course studies.

Methods A comprehensive set of protein characterization methods, including RPLC/MS, LC-MS/MS, iCIEF, capSEC, and CE-SDS were performed in this report.

Results We demonstrate that plasma incubation and *in vivo* circulation increase the rate of C-terminal lysine removal, and the levels of deamidation, pyroglutamic acid (pyroE), and thioether-linked (lanthionine) heavy chain and light chain (HC-S-LC). In contrast, incubation in PBS shows no C-terminal lysine removal, and slower rates of deamidation, pyroE, and HC-S-LC formation. Other potential modifications such as oxidation, aggregation, and peptide bonds hydrolysis are not enhanced.

Conclusion This study demonstrates that *in vivo* mAb modifications are not fully represented by *in vitro* PBS or plasma incubation. The differences in modifications and their rates reflect the dissimilarities of matrices and the impact of enzymes. These observations provide valuable evidence and knowledge in evaluating the criticality of modifications that occur naturally *in vivo* that might impact formulation design, therapeutic outcome, and critical quality attribute assessments for therapeutic mAb manufacturing and quality control.

KEY WORDS critical quality attributes · deamidation · *in vivo* circulated monoclonal antibody modifications · pyroglutamic acid · thioether/lanthionine

ABBREVIATIONS

capSEC	capillary size exclusion chromatography
CE-SDS	capillary electrophoresis-sodium dodecyl sulfate non-gel sieving
iCIEF	imaged capillary iso-electric focusing
LC-MS/MS	liquid chromatography couple with tandem mass spectrometry
RPLC/MS	reverse phase liquid chromatography coupled with mass spectrometry

INTRODUCTION

Therapeutic monoclonal antibodies (mAbs) are now a well-established and common therapeutic platform for the treatment of cancer, autoimmune disease, and other medical conditions. According to the Pharmaceutical Research and Manufacturers of American (PhRMA), in 2008 there were 633 biotechnology medicines in development, and among them there were 192 mAbs (1). Of the four types

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S. Yin • J. T. Stults
Protein Analytical Chemistry, Genentech, Inc.
South San Francisco, California, USA

C. V. Pastuskovas • L. A. Khawli
Department of Pharmacokinetic and Pharmacodynamic Sciences
Genentech, Inc.
South San Francisco, California, USA

J. T. Stults (✉)
1 DNA way, MS 62
South San Francisco, California 94080, USA
e-mail: stults.john@gene.com

of immunoglobulin (IgG), the majority of therapeutic mAbs are IgG1, but examples of each of the other isotypes are also in development. Different hinge-region structures distinguish the four subclasses of IgG's; IgG1 and IgG2 are the two most abundant classes. Although the relative lengths of the hinge and the number of disulfide bonds in the hinge are different among subclasses, all IgG's share more than 80% overall sequence homology, and 95% sequence homology in their constant regions (2). Three IgG1 mAbs representing the main therapeutic mAb subclass are selected for this study.

During development, detailed molecular characterization of the protein is performed to demonstrate that the mAb has the correct primary structure. The manufacturing process seeks to maximize production of the desired product in high purity with minimal and consistent levels of variant forms (e.g. asparagine (Asn) deamidation, aspartic acid (Asp) isomerization, methionine (Met) and tryptophan (Trp) oxidation). The formulation selection likewise seeks to minimize additional formation of the variants, and to maintain the product in a stable form for storage and delivery to the patient (3–5). To the extent that variant forms have differences in activity, immunogenicity, safety, or clearance, they need to be controlled within defined limits. It is remarkable that current manufacturing processes are capable of producing many kilograms of proteins with high proportions of the desired protein.

The modifications that occur during manufacturing and storage can be reliably monitored and controlled. However, additional modifications are likely to occur after the protein is administered to the patient. Many mAbs are administered intravenously, or quickly move into circulation if delivered subcutaneously. The circulating half-life of a typical mAb is 2–3 weeks in humans. The blood is a considerably different environment than the typical drug product formulation. Blood pH is tightly regulated between 7.35 and 7.45 at a temperature of 37°C, containing thousands of proteins including many protein-modifying enzymes (6). Thus, an understanding of mAb modifications during blood circulation is necessary in order to determine the relative importance of modifications that occur prior to administration.

A Critical Quality Attribute (CQA) for a pharmaceutical is defined as a physical, chemical, biological or microbiological property or characteristic that should be within an appropriate limit, range, or distribution to ensure the desired product quality, safety, and efficacy. The understanding of CQA's for protein therapeutics helps to guide the development of the production process and of the formulation. For example, an oxidized methionine variant in the Fc region of a monoclonal antibody that displays a modest change (20% loss) in FcRn binding would preliminarily be categorized as a CQA. As a CQA, the production process would be optimized to try to reduce the amount of that variant, and the formulation would be chosen to limit that oxidation. However, if that oxidized variant was found to form rapidly in the blood stream following administration, that knowledge may justify its classification as a non-CQA. As a non-CQA, the limits for its formation during production and during storage would likely be much less stringent. A goal of our research is to gain a greater understanding of the formation of IgG variants following administration, to inform the assessments for Critical Quality Attributes.

Although well over 100 protein post-translational modifications have been described, only a small number are found to be responsible for the majority of degraded forms in therapeutic proteins including Asn deamidation (4,6–10), Asp isomerization (11,12), Met and Trp oxidation (6,13–18), N-terminal pyroglutamic acid (pyroE) formation (16), C-terminal lysine removal (19), aggregation (20–22), and fragmentation (23–25). Among them, only deamidation (26,27), N-terminal pyroE formation (28), lysine glycation (29), and C-terminal lysine removal (30) have been previously investigated *in vivo*.

In this report, we present the comprehensive characterization at four time points for three therapeutic IgG1 mAbs (mAb-1, -2, -3) after plasma and PBS incubation, and for mAb-1 after *in vivo* administration. The study utilizes cap-SEC, CE-SDS, iCIEF, RPLC/MS and LC-MS/MS to monitor aggregation, fragmentation, deamidation, isomerization, oxidation, C-terminal lysine removal, and N-terminal pyroE formation.

MATERIALS AND METHODS

Materials

Three humanized IgG1 mAbs (mAb-1, mAb-2, and mAb-3) representing the main therapeutic mAb types are selected for this study. They were expressed in Chinese hamster ovary (CHO) cells, and purified by Genentech, Inc (South San Francisco, CA). All three mAbs have N-terminal glutamic acid in their sequence; mAb-1 has λ light chain and mAb-2, -3 have κ light chain. Among the three mAbs, only mAb-2 has substantial C-terminal lysine (8%) in the heavy chain sequence. Formulated reference mAbs were stored in −70°C until their use. Rat plasma with the anti-coagulant, lithium heparin, was purchased from Bioreclamation, Inc (Westbury, NY). The IgSelect anti-human Fc affinity column was purchased from GE Healthcare (Piscataway, NJ). All reagents were of analytical graded and were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise indicated.

Methods

After PBS or rat plasma incubation, three humanized IgG1 proteins, mAb-1, mAb-2, and mAb-3, were purified and

assayed by capSEC, reduced CE-SDS, iCIEF, RPLC/MS, and peptide map LC-MS/MS. In the subsequent *in vivo* experiments, mAb-1 was administered to rats, and blood samples were collected at the same time points as the *in vitro* experiments, and tested with the same assays.

In Vitro Incubation and Purification of mAbs From Rat Plasma or PBS

One milligram of each mAb was spiked into 1 mL of rat plasma or phosphate buffered saline (PBS), pH7.4, to give a final concentration of 1 mg/mL of antibody. Samples were incubated at 37°C in the presence of 0.1% azide for 0, 2, 6, and 10 days. Plasma pH was monitored during the incubation. Incubation time was limited to 10 days due to observed precipitation of plasma proteins after incubation longer than 10 days. Following incubation, plasma samples were stored at −70°C until purification of the mAb with IgSelect anti-human IgG Fc affinity column. During purification, the flow rate was 1 mL/min, and the wash buffer was 20 mM potassium phosphate and 150 mM sodium chloride, pH7.0. Samples were eluted with 0.1 M glycine, pH 3.0. Immediately after elution, the solution pH was adjusted to pH6.0 with 0.825 M Tris buffer. The protein concentration of the eluate was determined with a NanoDrop (Nanodrop, Wilmington, DE). The total volume of the eluate was roughly 5 mL, from which a 0.8 mL aliquot was buffer exchanged and concentrated into water using a 10 kDa MWCO Amicon filter (Millipore, Billerica, MA) to give a final protein concentration of at least 1 mg/mL. Of this concentrated sample, around 60 µL was used for iCIEF and 10 µL was used for capSEC. The remaining 4.2 mL affinity eluate was concentrated with a 10 kDa Amicon filter to at least 1 mg/mL for CE-SDS (approximately 120 µL concentrated samples), reduced intact RPLC/MS (approximately 20 µL concentrated samples), tryptic digestion and LC-MS/MS (approximately 400 µL concentrated samples). The method was optimized to purify humanized therapeutic mAbs, giving a recovery of 85% or higher. The stability of the protein during sample preparation was evaluated using a Bioanalyzer instrument (Agilent, Santa Clara, CA) following the vendor's recommended protocol, and CE-SDS. Incubation in heat-stressed plasma (50°C for 15 min) was used to distinguish the enzymatic reactions in plasma. Reference material (mAb-1) stored 33 months at 5°C was found to be oxidized and was used as a control to test the recovery of modified IgG's. All *in vitro* experiments are performed in triplicate, and a separate set of experiments are performed to ensure the reproducibility and robustness of the experimental design.

In Vivo Administration, Sample Collection and Preparation

To keep the same time-course as the *in vitro* studies, a total of 12 rats were injected intravenously (IV) with a 45 mg/kg dose of mAb-1. Based on separate pharmacokinetic studies, the half-life of mAb-1 in rat was determined as 5.4 days, and the concentrations were estimated to be 400, 280, 161, 100 µg/mL at t=0, 2, 6, and 10 day time points, respectively. At each time point, approximately 4 mL of blood were collected via cardiac puncture from each of three rats. Plasma from each sample was immediately prepared with lithium heparin tubes, and stored at −70°C prior to purification. Purification of *in vivo*-circulated mAb-1 followed the same process as described in the previous section. Due to the clearance of mAb-1 in circulation, to achieve sufficient amount of mAbs for all characterization methods, the day 2 samples were pooled from two rats, day 6 and day 10 samples were pooled from three rats.

All *in vivo* protocols, housing, and anesthesia were approved by the Institutional Animal Care and Use Committees of Genentech Laboratory Animal Resources, in compliance with the Association for Assessment and Accreditation of Laboratory Animal Care regulations.

iCIEF of Incubated mAbs

Charge variant analysis by iCIEF was performed on an imaged cIEF analyzer (iCE280) from Convergent Bioscience (Toronto, ON, Canada). Methylcellulose, p*I* markers, and the testing kit were also purchased from Convergent Bioscience. The ampholyte solution contains 60.9% water (or 2 M urea), 34.5% of 1.0% methylcellulose, 3.2% of pharmalyte, and 0.4% p*I* marker. A 60 µL aliquot of desalted 1 mg/mL mAb in water was mixed with 150 µL of ampholyte solution.

capSEC

A 10 µL aliquot of 1 mg/mL mAb in water was prepared for capSEC experiment. Separations by capSEC were based on a previously described method (31). Briefly, the mobile phase was 200 mM potassium phosphate and 250 mM potassium chloride at pH6.2. The column was a TSK gel super SW3000 stationary phase (Tosoh Biosciences, Montgomery, PA), 300 µm×30 cm, 4 µm particle size, custom-packed from Dionex (Amsterdam, The Netherlands). The temperature was maintained at room temperature, flow rate was 1 µL/min. The chromatography system was an UltiMate 3000 (Dionex, Sunnyvale, CA). A total sample of 20 ng in a volume of 20 nL was injected.

CE-SDS

The CE-SDS separations were performed on a PA800*plus* pharmaceutical analysis system from Beckman Coulter (Brea, CA). Sample vials, caps, reagents and SDS-MW gel buffer were also purchased from Beckman Coulter. Purified mAbs were concentrated with a 10 kDa Amicon filter to at least 1 mg/mL and analyzed with a previously described CE-SDS method (32). In summary, concentrated mAbs were first desalted and then labeled with 3-(2-furoyl)-quinoline-2-carboxaldehyde (FQ dye) (Invitrogen, Carlsbad, CA) in the presence of potassium cyanide (KCN). The labeled mAbs were reduced and denatured with 50 mM dithiothreitol (DTT) and 2% sodium dodecyl sulfate (SDS). CE-SDS gel reagents were loaded from the capillary inlet by pressure at 70 psi. Samples were injected with −10 kV for 10 s, and separations were performed at 15 kV with reverse polarity.

Reverse Phase Reduced Intact RPLC/MS

For reduced RPLC/MS, the mAb was Amicon-desalted and concentrated, added to 100 mM DTT with a 1:1 ratio, and reacted at 60°C for 10 mins. The sample was then diluted to a 6 ng/μL concentration with 0.1% formic acid (FA), centrifuged at 10,000 rpm for 3 min, and 20 μL of supernatant was collected for analysis. The separation gradient was delivered by an Agilent 1200 Infinity HPLC, and samples were separated by an Agilent nanospray chip with a built-in C8 column, equivalent to 75 μm×40 mm column. The detector was an Agilent 6210 TOF mass spectrometer (Agilent, Santa Clara, CA). Mobile phase A was 0.1% FA in water and mobile phase B was 0.1% FA in acetonitrile. A 13 min gradient (20–70% B in 9 min, then re-equilibrate the column with 100% A for 4 min) with a flow rate of 0.4 μL/min was utilized for reduced mAb separation.

Tryptic Digestion and LC-MS/MS

Tryptic digestion was carefully controlled to minimize the deamidation and isomerization as described in the literature (11). The concentrated mAbs were denatured in 6 M guanidine and 10 mM *tris*(2-carboxyethyl)phosphine (TCEP) at 60°C for 10 min. The reduced, denatured mAbs were desalted and buffer exchanged with a NAP-5 column (GE Healthcare, Pittsburgh, PA) into the digestion buffer which contained 20 mM 3-(N-morpholino)propanesulfonic acid (MOPS), 0.5 mM TCEP at pH 7.0. Trypsin digestion was carried out by adding trypsin (Promega, Sunnyvale, CA) to the mAb sample with a 1:40 enzyme:substrate ratio and incubated at 37°C for 90 mins. Digestion was stopped by addition of 3 μL of neat trifluoroacetic acid (TFA) (Thermo Fisher Scientific, San Jose, CA). An Agilent 1200 Infinity Series HPLC (Agilent, Santa Clara, CA) and Thermo Fisher Scientific LTQ XL linear Ion Trap were utilized for LC-MS/MS peptide mapping. A total of 95 μL digested mAbs was loaded and separated on a Phenomenex (Torrance, CA) Jupiter, 150×2 mm, C18 column with column temperature of 55°C. The mobile phase A was 0.1% TFA in water and mobile phase B was 0.1% TFA in acetonitrile. A 173 min gradient (hold at 0% B for 5 min, 0–32% B in 106 min, 32–52% B in 20 min, 52–95% B in 11 min, hold at 95% B for 5 min, drop to 0% B in 1 min, and then re-equilibrate the column with 100% A for another 15 min) with a flow rate of 0.25 mL/min was utilized for peptide separation. The chromatogram was monitored by UV absorbance at 214 nm and by mass spectrometry (MS). The MS acquisition consisted of a full scan, followed with the data-dependent Ultra Zoom MS scan and tandem MS/MS scan of the top seven most intense ions of each full scan. The identification of peptides was determined by Mascot Distiller version 2.1.1.0 (Matrix Science, Boston, MA) search of the mAb sequence. No fixed modifications were specified for the search. The potential modification sites (Met and Trp oxidation, deamidation, glycation, N-terminal pyroE, and C-terminal lysine removal) were verified manually. The quantitative results were determined from the integration of the extracted ion current (XIC) of the ions of interest, using a window of −0.25 to +0.25 m/z.

RESULTS

Size Variants

Changes in protein aggregation during the incubation time-course were monitored by capSEC analysis. For PBS-incubated mAbs, no aggregation was observed. For plasma-incubated mAbs, the data showed a slight increase in the high molecular weight species (HMWS) from 0 days to 10 days (see Supplementary Material Figure S1 and Supplementary Material Table SI). The rates of HMWS formation in plasma were below 0.1% per day for all of the mAbs tested. For *in vivo*-circulated mAb-1, the HMWS of mAb-1 did not show an increase; it maintained at its initial relative percentage around 0.3%. No low molecular weight species (LMWS) were observed to change under the conditions tested. To understand whether the IgSelect purification step may lead to loss of aggregated material, a sample of mAb-2 that was enriched in aggregate was evaluated. Recovery of aggregate was 90% from a PBS sample, and 83% from a rat plasma sample, similar to the 85% recovery of monomer, demonstrating that there is no bias in the isolation procedure.

Reduced CE-SDS was performed to monitor fragmentation of the mAbs. No new fragments were observed during PBS incubation. However, for all three plasma-incubated mAbs and *in vivo*-circulated mAb-1 we investigated, the data

showed an increase in an incompletely reduced peak (Fig. 1a). Determined by reduced RPLC/MS, the mass of the incompletely reduced component of mAb-1 is 33.4 Da smaller than theoretical mass of the reduced HC and LC pair (Fig. 2a, b). The LC-MS/MS data (Fig. 2c) further identified the incompletely reduced peak as a thioether-linked tryptic LC peptide (TVAPTECS) and HC peptide (SCDK) through two cysteine residues (HC-S-LC) that normally form the interchain disulfide bond (HC-S-S-LC). During plasma incubation, the rate of HC-S-LC formation was 0.7% per day for mAb-1, 0.2% per day for both mAb-2 and mAb-3 (Supplementary Material Figure S2). For mAb-1, the rate of the HC-S-LC formation was determined as 0.1%, 0.7%, and 0.3% per day for PBS, plasma incubation, and *in vivo* circulation, respectively (Fig. 1b). The reduced CE-SDS experiment (Fig. 3) also demonstrates an increasing HC-S-LC peak during *in vivo* circulation.

Examination of the *in vivo*-circulated mAb-1 also revealed a time-dependent increase in peaks labeled as A and A* (Fig. 3). Based on previous reduced CE-SDS experiments, peak A migrated at the same position as the non-glycosylated heavy chain (NGHC) (33–35). Similarly, peak A* migrated at the position expected for the NGHC of the incompletely reduced HC-S-LC peak. At this time there is no direct evidence to confirm the identity of peaks A and A*, and it is possible that these peaks may also correspond to a HC fragment. Comparing the relative percentage of peaks A and A* with the normalized HC peak, these peaks increased 2-fold after 10 days *in vivo* circulation (Table I). Other peaks that appeared to increase were not listed due to strong interference from co-migrating blood proteins. Peak A did not change in abundance during PBS or plasma incubation.

Charge Variants

The charge distributions of the mAbs were monitored by iCIEF. With incubation in PBS or plasma, the acidic variant peaks of all three investigated mAbs increased in abundance (Supplementary Material Figure S3). The mAb-1 charge distribution after *in vivo* circulation also presented an increase in the acidic peaks. Figure 4 shows results from mAb-1 incubation iCIEF experiments in which the acidic variants increased 0.9% per day during PBS incubation, 2.9% per day during plasma incubation, and 2.1% per day during *in vivo* circulation. Similarly, the acidic variants in the plasma-incubated mAb-2 and mAb-3 increased 2.1% per day and 3.2% per day, respectively (Supplementary Material

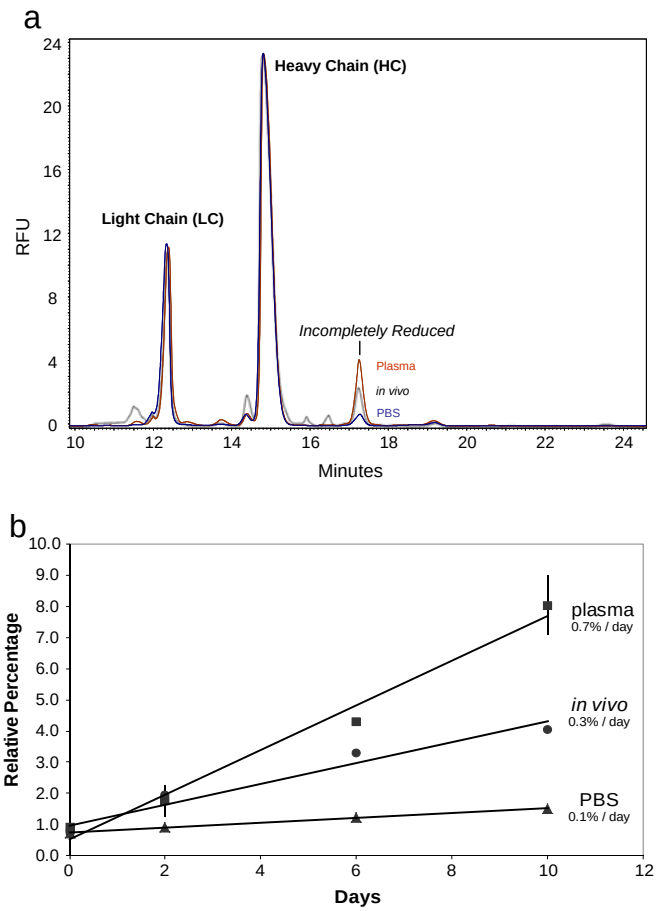

Fig. 1 (**a**) Reduced CE-SDS chromatograms of PBS-, plasma-incubated, and *in vivo*-circulated mAb-1 at 10 days. An increase in an incompletely reduced peak during plasma incubation and *in vivo* circulation is identified asHC-S-LC. (**b**) Rate of change in the HC-S-LC. Each time point in PBS-incubated (▲) and plasma-incubated (■) mAb-1 represents a measurement of n=3 separate incubation experiments with error bars representing the standard deviations. The HC-S-LC increases with 0.1%, 0.7%, and 0.3% per day in PBS (▲), plasma (■) and *in vivo* (●), respectively.

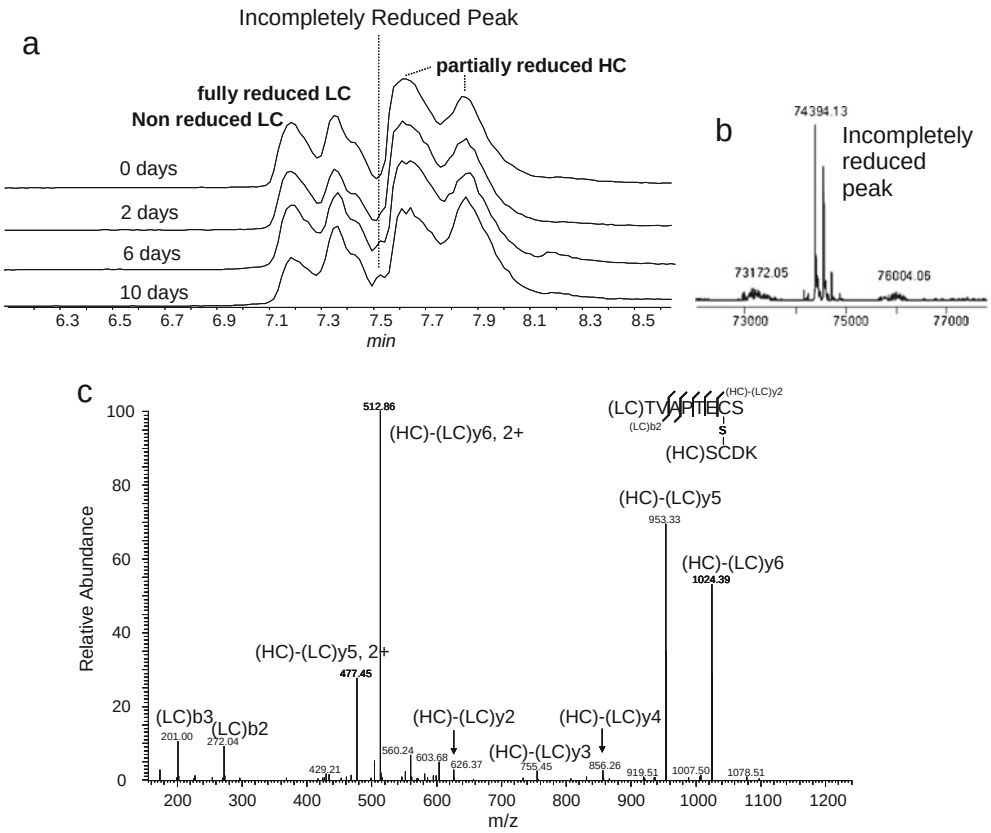

Fig. 2 (**a**) RPLC/MS chromatograms of the whole mixture of reduced plasma-incubated mAb-2, shows an increased incompletely reduced peak which correlates with the CE-SDS observation. (**b**) Deconvoluted RPLC/MS spectrum of the incompletely reduced peak that is 33.4 Da smaller than the summed MW of LC and HC observed from the same chromatogram with the same reducing condition. (**c**) LC-MS/MS spectrum of the thioether-linked tryptic LC peptide (TVAPTECS) and HC peptide (SCDK). It provides the identity of the incompletely reduced peak observed in CE-SDS and RPLC/MS as HC-S-LC.

Figure S3). Further analysis of the acidic variants is based on tryptic peptide map data.

Peptide maps analyzed by LC-MS/MS were used to measure changes in Asn deamidation, N-terminal pyroE formation, Asp isomerization, Met and Trp oxidation, Lys glycation, and C-terminal lysine removal. For deamidation, the data were searched for all potential deamidation sites based on the amino acid sequences, e.g., NG, NS, NA, and NN sites in all three mAbs (single letter amino acid notation is used, for example, NG is Asn-Gly). Of these, a total of four distinctive peptide sequences from three mAbs were found to undergo deamidation during plasma incubation (Supplementary Material Table SIIa). Among the deamidated peptides found, a highly conserved Fc region peptide (PENNY peptide) which has two deamidation (NG and NN) sites is observed in all three studied mAbs. During plasma incubation, the deamidation rate for the PENNY peptide was 1.7% per day for mAb-1, 1.5% per day for mAb-2, and 1.8% per day for mAb-3 (Supplementary Material Figure S4). For mAb-1, PENNY peptide had a deamidation rate of 0.5% per day during PBS incubation, 1.7% per day during plasma incubation, and 1.1% per day during *in vivo* circulation (Fig. 5b).

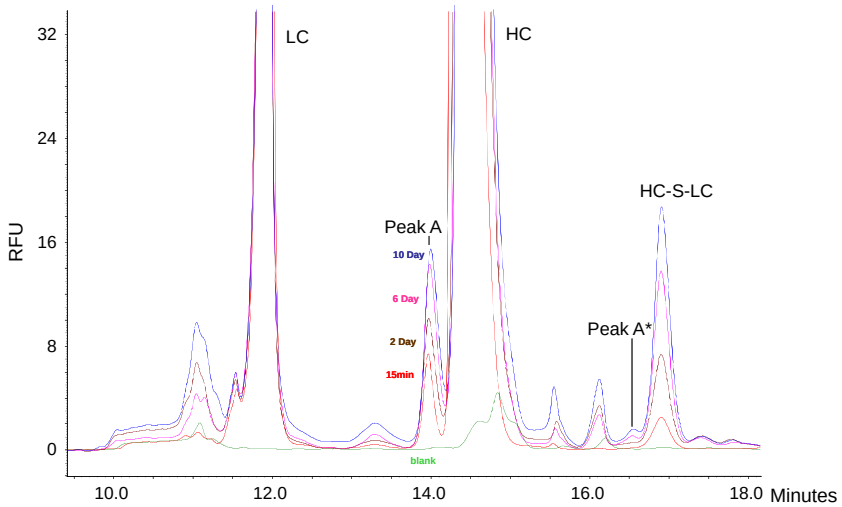

Fig. 3 Reduced CE-SDS electropherograms of *in vivo*-circulated mAb-1 show increased HC-S-LC, Peak A, and Peak A*. The chromatograms at four time points (15 mins, 2 day, 6 day, 10 day) are normalized to HC peak intensity. Data for 0 day Plasma control is labeled as "blank". Unlabeled peaks have not been identified or have an interference from plasma proteins.

Table I Relative Peak Percentage for mAb-1 *In Vivo* CE-SDS

Relative peak %	0 day	2 days	6 days	10 days
HC	100	100	100	100
Peak A	4.2	5.8	8.1	8.5
HC-S-LC	2.0	4.4	7.8	10.4
Peak A*	0.0	0.3	0.7	0.9

For *in vivo*-circulated mAb-1, reduced CE-SDS data of the relative intensity of HC, HC-S-LC, Peak A, and Peak A* are presented in table which shows clear increases during *in vivo* circulation. Total Peak A and Peak A* increased to 2 fold of the original amount

Other Modifications

N-terminal pyroE was formed in all PBS-incubated mAb-1, −2, and −3 with 0.3%, 0.2%, and 0.2% per day, compared with plasma-incubated mAb-1, −2, and −3 with 0.6%, 0.6%, and 0.5% per day, respectively (Supplementary Material Figure S5). *In vivo*-circulated mAb-1 has the same rate of N-terminal pyroE formation as for plasma-incubated mAb-1 (Fig. 5c).

Other modifications such as Met and Trp oxidation, Asp isomerization, or Lys glycation were also investigated but found to show little (<1%) or no change during PBS, plasma incubation and *in vivo* circulation. Unlike mAb-1 and mAb-3 which have <1% C-terminal lysine, mAb-2 has a 8% lysine at the C-terminal of its HC. The C-terminal lysine of mAb-2 was quickly and completely removed at the 2-day time point in plasma, but not in PBS, according to both LC-MS/MS and iCIEF data.

DISCUSSION

Modifications that occur during the manufacture and storage of protein therapeutics are generally well understood and controlled. However, additional modifications may occur *in vivo* after mAb administration, potentially impacting their therapeutic significance. Further investigation into potential mAb modifications in blood is important for understanding the criticality of many product quality attributes, and it is important for production and engineering of therapeutic mAbs. For this reason, we examined mAbs under three conditions of increasing complexity, with samples taken periodically to assess the rates of change.

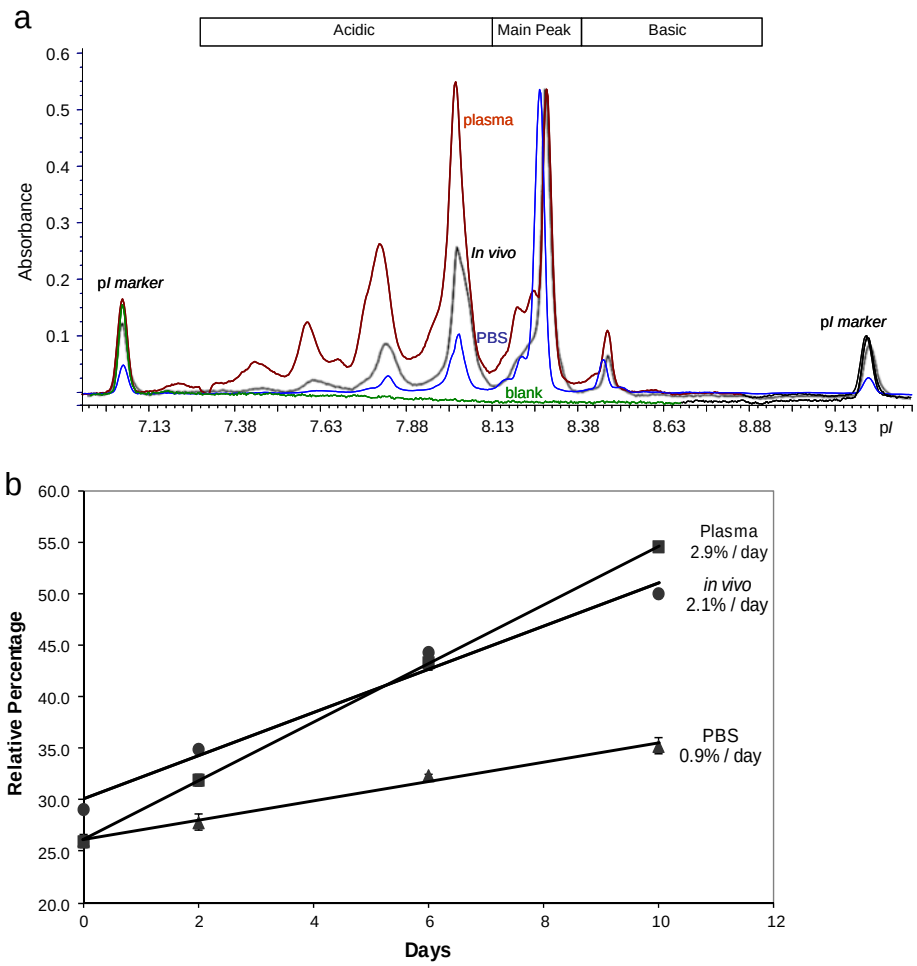

Fig. 4 (**a**) iCIEF electropherograms of mAb-1 after 10 days PBS, plasma incubation, and *in vivo* circulation. Electropherograms are normalized based on the main peak. The relative percentage of the acidic variants is dramatically increased during plasma incubation. Plasma control is labeled as "blank". (**b**) The iCIEF analysis of mAb-1 shows an increase in the acidic peaks during PBS and plasma incubation, and *in vivo* circulation. Each time point for PBS and plasma-incubated samples are in triplicate. Error bars represents standard deviations. Acidic variants increase with rate of 0.9%, 2.9%, and 2.1% per day during PBS (▲), plasma (■) incubation, *in vivo* circulation (●), respectively.

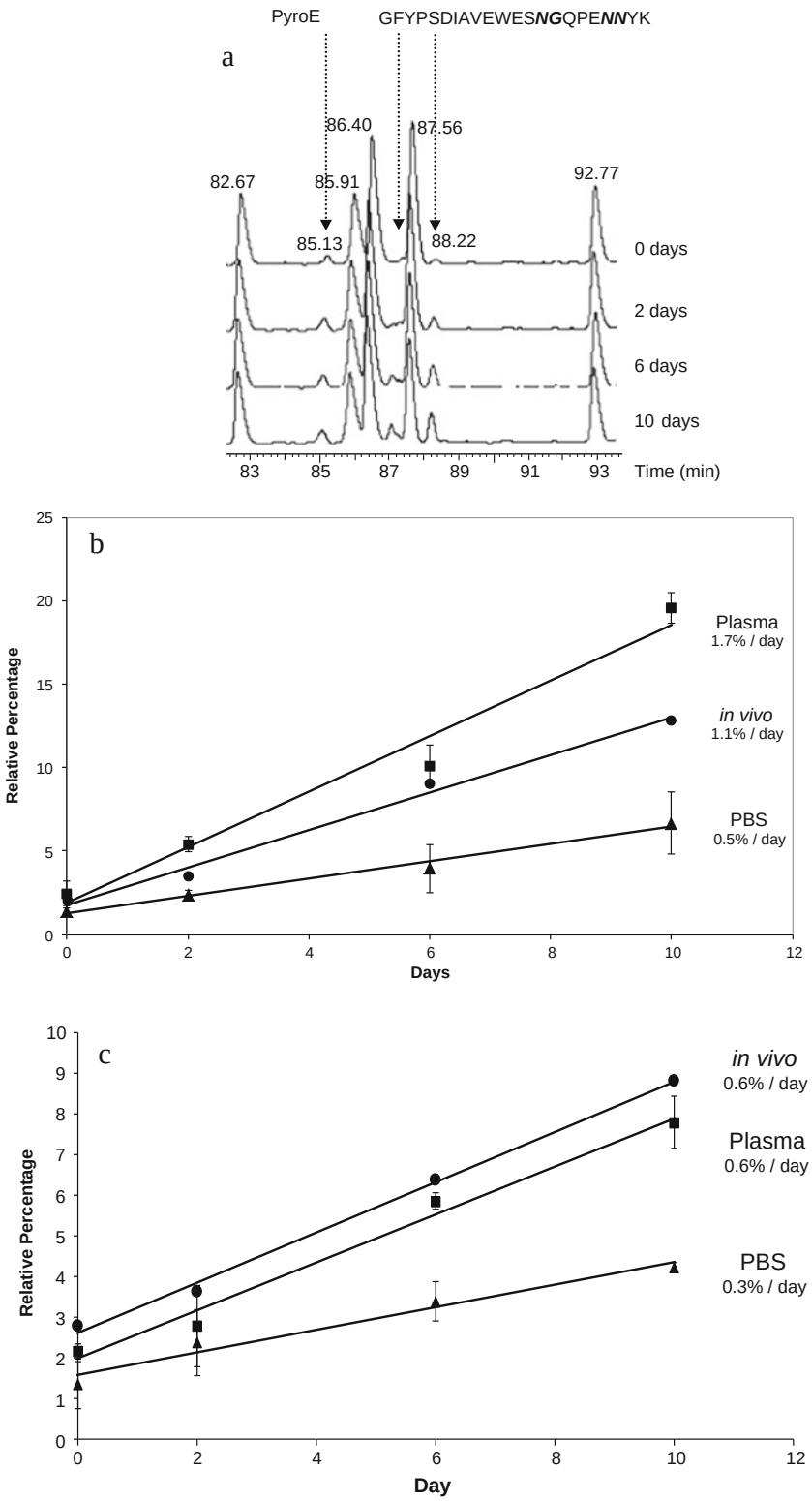

Fig. 5 (**a**) UV chromatograms of peptide maps of 0 day to 10 days plasma-incubated mAb-1. Labeled are the N-terminal pyroE peptide and deamidation of the conserved Fc PENNY peptide (GFYPSDIAVEWESNGQ PENNYK). Increased intensity is observed due to the plasma incubation. The LC-MS/MS determined rate of increase is shown in (**b**) and (**c**). Each data point represents average value of a triplicate incubation experiment, and error bars represent the standard deviation. (**b**) Deamidation rate of PENNY peptide. The overall deamidation rate of NG and NN sites is 0.5% per day in PBS (▲), 1.7% per day in rat plasma (■), and 1.1% per day *in vivo* (●). (**c**) PyroE formation of N-terminal of mAbs. Plasma incubation (■) and *in vivo* circulation (●) have the same effect on the N-terminal pyroE formation (0.6% per day). PBS-incubated (▲) mAb-1 has 2 fold slower rate of pyroE formation (0.3% per day).

Thioether-Linked HC-LC (Incompletely Reduced HC-LC Pair)

Compared with PBS-incubated mAbs that show a less than 1% increase in the incompletely reduced HC-LC peak, plasma-incubated mAbs and *in vivo*-circulated mAb-1 show a much stronger increase in this peak based on the reduced CE-SDS experiment. Non-reduced CE-SDS was tested initially and excluded from further characterization due to strong interference from residual plasma proteins. Reduced

CE-SDS experiments were performed under denaturing conditions with 2% SDS and 50 mM DTT; therefore, the incompletely reduced HC-LC pair is presumed to be a covalent linkage between the two mAb-2 chains. Based on RPLC/MS of the reduced mAb-1, a component that correlates with the time-abundance profile of the incompletely reduced peak has molecular weight matches within 33.4 Da smaller than the theoretical molecular weight of heavy plus light chain. Tryptic peptide LC-MS/MS identifies this species is thioether-linked through the two cysteine amino acids that previously formed the disulfide bond between HC and LC. This modification may be formed by β-elimination of the disulfide bond between LC and HC near the upper hinge region followed by releasing free sulfur and forming a thioether-linked HC-S-LC (23,36–38). It is also important to note that this HC-S-LC exists at a very low level in the reference sample before plasma incubation, and it increases with a much lower rate (0.1% per day) during PBS incubation.

Potential Non-Glycosylated Heavy Chain

The reduced CE-SDS analysis for the *in vivo*-circulated mAb-1 shows increases in peak A and peak A* that were not observed in PBS or plasma. Due to the migration position, these peaks are presumed to correspond to the NGHC. This time-dependent change may be due to slower clearance of the non-glycosylated mAb (or more likely, the hemi-glycosylated form that is the predominant, partially glycosylated mAb species expected for low levels of NGHC). The methodology employed is not able to distinguish the fully-, hemi-, and non-glycosylated forms of the non-reduced mAbs and others have investigated the clearance of several non-glycosylated IgG's. Non-glycosylated IgG's were generated with either site-direct mutagenesis (substitution of the glycosylation site Asn by Gln, Ala or Lys) or biochemical inhibition of N-linked glycosylation (using tunicamycin). The IgG1 with glycosylation site mutation (Asn297Gln) showed the same serum half-life in mice as wild type (39), while mutated IgG2b (Asn297Ala) and IgG3 (Asn297Lys) showed a significantly shorter half-life (39,40). A humanized IgG2 IV injected in human also showed a slight increasing elimination rate comparing to the glycosylated form (41). Comparing FcRn binding properties of genetically and biochemically generated non-glycosylated IgG2b, mutant non-glycosylated IgG2b was indistinguishable from wild type, and yet tunicamycin-treated IgG2 was less stable, showed lower binding to FcRn, and cleared more rapidly (42). These data run counter to our observations. Whether the behavior we observe is antibody-specific, or is a characteristic of a humanized IgG1 in a rat PK model which has not been previously studied, remains to be determined. One additional consideration for differential clearance is the glycan structures present on the mAb. For the *in vivo* experiments there was insufficient sample to determine the glycan structures at each timepoint. However, we know from previous characterization that mAb-1 contains glycan structures that are typical of CHO-derived mAb's, and the high mannose structures, shown by others to influence clearance (41), are present at <1%. Thus, the glycan profile is unlikely to be responsible for the observed changes.

We looked for further evidence that peaks A and A* correspond to NGHC. Currently there is no method to characterize minor peaks from CE-SDS directly. Separately, examination of the LC-MS/MS peptide map data did not reveal a peak for the non-glycosylated form of the normally-glycosylated tryptic peptide (either as Asn or potentially deamidated Asp forms), even for the T=0 sample when the NGHC peak is 4% (The mAb-1 samples used for these experiments have been separately shown to contain 4% NGHC). The small amount of sample available from the *in vivo* rat study meant that low-intensity peaks were not readily observed. The small amount of sample also precluded direct mass spectral measurement of the intact protein from the *in vivo* experiments. Other explanations for the observed relative increase in peak A and A* cannot be ruled out. Peak A was identified by reduced CE-SDS based on the migration time observed as NGHC for other mAbs (33–35). This peak may include, for example, a co-migrating HC fragment with approximately the same molecular weight. Alternately, the increased NGHC species from *in vivo*-circulated mAb-1 may be due to an enzymatic removal of the glycan in circulation. We are not aware of glycosidase activity in normal blood, and the lack of change in this peak during *in vitro* plasma incubation and the lack of mass spectrometry data to confirm the enzymatic removal of glycan argue against an enzymatic deglycosylation mechanism. The nature of the NGHC change remains under investigation.

Deamidation

In mAbs, deamidation has been reported in both highly conserved Fc regions (43,44), and in the complementarity determining regions (CDRs) (12,45,46). The deamidation rates of human proteins have been demonstrated to depend on primary sequence, 3-D structure, pH, and the buffer constituents (7,44,47,48). Based on *in vitro* peptide and protein deamidation studies, it has been argued that deamidation of endogenous proteins is a biologically relevant event that happens in a large percentage of human proteins in plasma and may serve as a "molecular clock" (7,48). Within the Fc conserved region of the mAbs studied here, there are more than twenty potential "hot-spots" based on the sequence. The common deamidation site among all three mAbs is the PENNY peptide from the conserved Fc C_H3 region which has two deamidation sites NG and NN. According to x-ray crystallographic data, this peptide is fully exposed to solvent (49). Protein deamidation also depends

on the pH and the ionic strength of the buffer. Due to a catalytic effect of plasma proteins, the deamidation rate of proteins has been observed to be at least 1.5-fold faster in plasma than in phosphate buffer at the same pH7.4 (7). From our experiments, deamidation of mAb-1 during plasma incubation and *in vivo* circulation occurs approximately 3.0- and 2.1-fold faster, respectively, than the deamidation in PBS buffer (Table II).

Deamidation was confirmed by both LC-MS/MS (peptide level) and iCIEF experiments (protein level). An overall increased deamidation percentage at the peptide level is correlated well with changes in the protein level acidic variants. Comparing the LC-MS/MS peptide map and iCIEF experimental data, LC-MS/MS showed the deamidation of mAb-1 peptides to increase from 3% to 22% after 10 days of plasma incubation. It is most likely that modifications at the mAb molecule level will be a mixture of deamidated and non-deamidated heavy chains. The amount of deamidation found at the peptide level will differ from the amount at the protein level. A 19% (22% minus 3%) increase at the peptide level would correspond, in theory, to a 34% ([1-(1 – 0.19)^2]=0.34) increase in acidic mAb species at the protein level (demidation on one or both HC's). The protein level iCIEF experiment shows that the acidic variants of mAb-1 increase 30% after 10 days of plasma incubation. Therefore, based on LC-MS/MS and iCIEF results, the acidic variants that arise from plasma incubation or from *in vivo* circulation are primarily due to the deamidation of the IgG's conserved Fc region (PENNY peptide).

The deamidation rate observed in plasma-incubated mAb-1 is 1.5 fold faster than it was *in vivo*. A PK study of the acidic variants of a different IgG1 mAb has shown no clearance difference relative to the main peak (27). Another study in a different mAb (IgG2) showed a deamidation rate that was the same between human *in vivo*-circulated and PBS-incubated mAb (26). However, the rate of deamidation depends on a number of factors, including the sequence, structure, and matrix. In our study, among the three IgG1 mAbs investigated, they each present similar, faster deamidation rates in plasma or *in vivo* than in PBS.

Table II Observed Rates of mAb-1 Modification

	HC-S-LC	Acidic variants	Deamidation	Pyroglutamic acid	NGHC
PBS	1.0	1.0	1.0	1.0	–
Rat plasma	9.0	3.0	3.2	2.1	–
Rat *in vivo*	4.3	2.2	2.2	2.2	total amount doubled

Comparison between 10-day rat *in vivo* circulated, rat plasma incubated, and PBS incubated mAb-1. The rate of formation of each modification in rat plasma incubation or *in vivo* is normalized to the rate observed for PBS incubation. For example, HC-S-LC is formed 9 fold faster in rat plasma than in PBS, and 4.3 fold faster *in vivo* than in PBS

N-Terminal PyroE Formation and C-Terminal Lysine Removal

Non-enzymatic N-terminal PyroE formation has also been observed. Similar to other modifications observed in this study, plasma-incubated mAbs display a faster pyroE formation rate, 0.6% per day, compared with PBS incubation. Unlike the other changes described above, pyroE forms at the same rate during plasma incubation or *in vivo* administration. For PBS-incubated mAb-1, N-terminal PyroE increases at the rate of 0.3% per day. PyroE formation is not an enzymatic reaction, but it is influenced by pH, temperature and oxygen level (50). Recent studies on IgG2 mAbs show pyroE formation rates of 0.31% and 0.15% per day for two IgG2's in PBS, and 0.32% and 0.18% per day for the same two IgG2's in a human *in vivo* PK study (28). From the same study, the authors also reported a much lower pyroE formation on LC of IgG2, and found that LC pyroE formation rate is strongly influenced by the accessibility of LC N-terminal glutamic acid, yet not on the HC N-terminal glutamic acid (28). The higher and consistent pyroE formation rate that we observe in plasma and *in vivo* suggests the plasma/blood matrix could be a factor in modifying the accessibility of N-terminal glutamic acid for chemical modification.

Removal of C-terminal lysines from mAb heavy chains has been well investigated both *in vitro* and *in vivo* (19,30). In our study, a fraction of mAb-2 (average of 8%) has C-terminal lysine, and we find it is removed within 24 h after plasma incubation. The process is slowed significantly in heat-stressed plasma (50°C, 15 mins) which supports the C-terminal lysine removal is an enzymatic process. The non-enzymatic N-terminal PyroE formation rate is not affected by heat-stress, as expected.

Other Modification Processes

Other modification processes including oxidation, aggregation, Asp isomerization, glycation, and fragmentation were also examined, yet no significant changes were observed. Aggregation showed no appreciable increase in PBS or *in vivo* and only a 1% increase after 10 days incubation in plasma. Lysine glycation was determined to be unchanged and always less than 1% after incubation, which is consistent with recently published work (29). Fragmentation has two mechanisms: peptide hydrolysis and β-elimination (23,25). The HC-S-LC generated due to β-elimination is discussed in the previous section. At pH7.4, peptide hydrolysis frequently occurs at the upper hinge region, generating Fab and desFab fragments.

Due to the anti-human Fc purification column utilized in this study, the Fab fragments were not isolated. The potential desFab fragments, if present, could not be identified unambiguously using capSEC and CE-SDS due to a combination of limited resolution and interference from endogenous rat plasma proteins.

CONCLUSION

Full characterization and comparison of mAbs during PBS incubation, plasma incubation, and *in vivo* circulation were performed for the first time. A number of protein modifications were found to increase in three different therapeutic IgG1's during incubation in PBS and plasma, and *in vivo* circulation. For plasma-incubated and *in vivo*-circulated mAbs, deamidation, pyroE formation, and HC-S-LC were formed at a faster rate than the PBS-incubated mAbs. For *in vivo*-circulated mAb-1, an increase in a peak thought to be NGHC was also observed. C-terminal lysine removal for mAb-2 was observed to occur quickly in plasma incubation *and in vivo* circulation. Other common modifications such as oxidation, Asp isomerization, aggregation, glycation, and peptide hydrolysis fragmentation do not show significant changes after PBS, plasma incubation or *in vivo* circulation. These results clearly demonstrate that even though significant efforts are devoted to developing processes and formulations to produce consistent and stable products, many changes occur quickly following drug administration. Conversely, some common modification processes observed under stressed conditions in formulation buffer (e.g., oxidation, aggregation) were not observed for the antibodies studied during plasma incubation or *in vivo* circulation.

The rates of change and the observations of increased HC-S-LC and potentially NGHC suggest that the *in vivo* system cannot be fully represented by pH-controlled PBS or plasma incubation studies. Characterization after plasma incubation tends to over-estimate the modification rate relative to *in vivo* conditions, and characterization after PBS incubation likely under-estimates those same modifications. Both conditions may miss potentially important modifications; therefore, characterization following *in vivo* circulation is necessary to understand fully the changes that may occur *in vivo*. A number of modifications observed after IV injection of an antibody, e.g., Fc "PENNY" peptide deamidation, C-terminal lysine removal, and pyroE formation, are known to occur endogenously and to occur quickly; therefore, they may be generally considered as non-critical quality attributes for this route of administration. We continue to study therapeutic protein modifications following administration and their potential impact to patients, to aid in the assessment of critical quality attributes.

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REFERENCES

1. PhRMA. Biotechnology research continues to bolster arsenal against disease with 633 medicines in development. Med Dev, Biotechnol. 2008.
2. Wingren C, Alkner U, Hansson U-B. Antibody classes. ELS, John Wiley & Sons, Ltd, 2001.
3. Steinmeyer DE, McCormick EL. The art of antibody process development. Drug Discovery Today. 2008;13:613–8.
4. Jenkins N, Murphy L, Tyther R. Post-translational modifications of recombinant proteins: Significance for biopharmaceuticals. Mol Biotechnol. 2008;39:113–8.
5. Kozlowski S, Swann P. Current and future issues in the manufacturing and development of monoclonal antibodies. Adv Drug Delivery Rev. 2006;58:707–22.
6. Correia IR. Stability of IgG isotypes in serum. mAbs. 2010;2:221–32.
7. Robinson NE, Robinson AB. Deamidation of human proteins. Proc Natl Acad Sci U S A. 2001;98:12409–13.
8. Li N, Kessler K, Bass L, Zeng D. Evaluation of the iCE280 analyzer as a potential high-throughput tool for formulation development. J Pharm Biomed Anal. 2007;43:963–72.
9. Vlasak J, Ionescu R. Heterogeneity of monoclonal antibodies revealed by charge-sensitive methods. Curr Pharm Biotechnol. 2008;9:468–81.
10. Vlasak J, Bussat MC, Wang S, Wagner-Rousset E, Schaefer M, Klinguer-Hamour C, *et al.* Identification and characterization of asparagine deamidation in the light chain cdr1 of a humanized IgG1 antibody. Anal Biochem. 2009;392:145–54.
11. Yu XC, Joe K, Zhang Y, Adriano A, Wang Y, Gazzano-Santoro H, *et al.* Accurate determination of succinimide degradation products using high fidelity trypsin digestion peptide map analysis. Anal Chem. 2011;83:5912–9.
12. Huang L, Lu J, Wroblewski VJ, Beals JM, Riggin RM. *In vivo* deamidation characterization of monoclonal antibody by LC/MS/MS. Anal Chem. 2005;77:1432–9.
13. Pan H, Chen K, Chu L, Kinderman F, Apostol I, Huang G. Methionine oxidation in human IgG2 Fc decreases binding affinities to protein A and FcRn. Protein Sci. 2009;18:424–33.
14. Liu D, Ren D, Huang H, Dankberg J, Rosenfeld R, Cocco MJ, *et al.* Structure and stability changes of human IgG1 Fc as a consequence of methionine oxidation. Biochemistry. 2008;47:5088–100.
15. Lam XM, Yang JY, Cleland JL. Antioxidants for prevention of methionine oxidation in recombinant monoclonal antibody her2. J Pharm Sci. 1997;86:1250–5.
16. Liu H, Gaza-Bulseco G, Faldu D, Chumsae C, Sun J. Heterogeneity of monoclonal antibodies. J Pharm Sci. 2008;97:2426–47.

17. Martin WL, West Jr AP, Gan L, Bjorkman PJ. Crystal structure at 2.8 Å of an FcRn/heterodimeric Fc complex: Mechanism of ph-dependent binding. Mol Cell. 2001;7:867–77.
18. Ji JA, Zhang B, Cheng W, Wang YJ. Methionine, tryptophan, and histidine oxidation in a model protein, PTH: Mechanisms and stabilization. J Pharm Sci. 2009;98:4485–500.
19. Harris RJ. Processing of c-terminal lysine and arginine residues of proteins isolated from mammalian-cell culture. J Chromatogr, A. 1995;705:129–34.
20. Cleland JL, Powell MF, Shire SJ. The development of stable protein formulations: A close look at protein aggregation, deamidation, and oxidation. Crit Rev Ther Drug Carrier Syst. 1993;10:307–77.
21. Cromwell M, Hilario E, Jacobson F. Protein aggregation and bioprocessing. The AAPS J. 2006;8:E572–9.
22. Shire S, Cromwell M, Liu J. Concluding summary: Proceedings of the AAPS biotec open forum on "aggregation of protein therapeutics". The AAPS J. 2006;8:E729–30.
23. Cohen SL, Price C, Vlasak J. B-elimination and peptide bond hydrolysis: Two distinct mechanisms of human IgG1 hinge fragmentation upon storage. J Am Chem Soc. 2007;129:6976–7.
24. Cordoba AJ, Shyong B-J, Breen D, Harris RJ. Non-enzymatic hinge region fragmentation of antibodies in solution. J Chromatogr, A. 2005;818:115–21.
25. Gaza-Bulseco G, Liu H. Fragmentation of a recombinant monoclonal antibody at various ph. Pharm Res. 2008;25:1881–90.
26. Liu YD, van Enk JZ, Flynn GC. Human antibody Fc deamidation *in vivo*. Biologicals. 2009;37:313–22.
27. Khawli LA, Goswami S, Hutchinson R, Kwong ZW, Yang J, Wang X, *et al.* Charge variants in IgG1: Isolation, characterization, *in vitro* binding properties and pharmacokinetics in rats. mAbs. 2010;2:613–24.
28. Liu YD, Goetze AM, Bass RB, Flynn GC. N-terminal glutamate to pyroglutamate conversion *in vivo* for human IgG2 antibodies. J Biol Chem. 2011;286:11211–7.
29. Goetze AM, Liu YD, Arroll T, Chu L, Flynn GC. Rates and impact of human antibody glycation *in vivo*. Glycobiology. 2012;22:221–34.
30. Cai B, Pan H, Flynn GC. C-terminal lysine processing of human immunoglobulin G2 heavy chain *in vivo*. Biotechnol Bioeng. 2011;108:404–12.
31. Rea JC, Moreno GT, Lou Y, Farnan D. Validation of a pH gradient-based ion-exchange chromatography method for high-resolution monoclonal antibody charge variant separations. J Pharm Biomed Anal. 2011;54:317–23.
32. Michels DA, Brady LJ, Guo A, Balland A. Fluorescent derivatization method of proteins for characterization by capillary electrophoresis- sodium dodecyl sulfate with laser-induced fluorescence detection. Anal Chem. 2007;79:5963–71.
33. Gennaro LA, Salas-Solano O, Ma S. Capillary electrophoresis–mass spectrometry as a characterization tool for therapeutic proteins. Anal Biochem. 2006;355:249–58.
34. Liu Y, Salas-Solano O, Gennaro LA. Investigation of sample preparation artifacts formed during the enzymatic release of n-linked glycans prior to analysis by capillary electrophoresis. Anal Chem. 2009;81:6823–9.
35. Ma S, Nashabeh W. Carbohydrate analysis of a chimeric recombinant monoclonal antibody by capillary electrophoresis with laser-induced fluorescence detection. Anal Chem. 1999;71:5185–92.
36. Nashef AS, Osuga DT, Lee HS, Ahmed AI, Whitaker JR, Feeney RE. Effects of alkali on proteins. Disulfides and their products. J Agric Food Chem. 1977;25:245–51.
37. Tous GI, Wei Z, Feng J, Bilbulian S, Bowen S, Smith J, *et al.* Characterization of a novel modification to monoclonal antibodies: Thioether cross-link of heavy and light chains. Anal Chem. 2005;77:2675–82.
38. Linetsky M, Hill JMW, LeGrand RD, Hu F. Dehydroalanine crosslinks in human lens. Exp Eye Res. 2004;79:499–512.
39. Tao MH, Morrison SL. Studies of aglycosylated chimeric mouse-human igg. Role of carbohydrate in the structure and effector functions mediated by the human IgG constant region. J Immunol. 1989;143:2595–601.
40. Wawrzynczak EJ, Cumber AJ, Parnell GD, Jones PT, Winter G. Blood clearance in the rat of a recombinant mouse monoclonal antibody lacking the N-linked oligosaccharide side chains of the CH2 domains. Mol Immunol. 1992;29:213–20.
41. Goetze AM, Liu YD, Zhang Z, Shah B, Lee E, Bondarenko PV, *et al.* High-mannose glycans on the Fc region of therapeutic IgG antibodies increase serum clearance in humans. Glycobiology. 2011;21:949–59.
42. Hobbs SM, Jackson LE, Hoadley J. Interaction of aglycosyl immunoglobulins with the IgG Fc transport receptor from neonatal rat gut: Comparison of deglycosylation by tunicamycin treatment and genetic engineering. Mol Immunol. 1992;29:949–56.
43. Sinha S, Zhang L, Duan S, Williams TD, Vlasak J, Ionescu R, *et al.* Effect of protein structure on deamidation rate in the Fc fragment of an IgG1 monoclonal antibody. Protein Sci. 2009;18:1573–84.
44. Chelius D, Rehder DS, Bondarenko PV. Identification and characterization of deamidation sites in the conserved regions of human immunoglobulin gamma antibodies. Anal Chem. 2005; 77:6004–11.
45. Harris RJ, Kabakoff B, Macchi FD, Shen FJ, Kwong M, Andya JD, *et al.* Identification of multiple sources of charge heterogeneity in a recombinant antibody. J Chromatogr, B: Biomed Sci Appl. 2001;752:233–45.
46. Wakankar AA, Borchardt RT, Eigenbrot C, Shia S, Wang YJ, Shire SJ, *et al.* Aspartate isomerization in the complementarity-determining regions of two closely related monoclonal antibodies. Biochemistry. 2007;46:1534–44.
47. Brennan TV, Clarke S. Spontaneous degradation of polypeptides at aspartyl and asparaginyl residues: Effects of the solvent dielectric. Protein Sci. 1993;2:331–8.
48. Robinson NE, Robinson AB. Molecular clocks. Proc Natl Acad Sci U S A. 2001;98:944–9.
49. DeLano WL, Ultsch MH. de AM, Vos, Wells JA. Convergent solutions to binding at a protein-protein interface. Science. 2000; 287:1279–83.
50. Airaudo CB, Gayte-Sorbier A, Armand P. Stability of glutamine and pyroglutamic acid under model system conditions: Influence of physical and technological factors. J Food Sci. 1987;52:1750–2.