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RESEARCH PAPER

Site-Specific Tryptophan Oxidation Induced by Autocatalytic Reaction of Polysorbate 20 in Protein Formulation

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ABSTRACT

Purpose Tryptophan (Trp) oxidation leading to atypical degradation of a protein (Fab) formulated with polysorbate 20 (PS20) was investigated. Such atypical Trp oxidation was discussed in relation to a kinetic model that involves initiation of oxidizing free radical through an autocatalytic reaction.

Methods Ion-exchange chromatography and peptide mapping were used to determine Trp oxidation. Peroxides in PS20 and free radicals in Fab samples were detected by fluorometric assay and electron paramagnetic resonance (EPR), respectively.

Results PS20 with increased peroxides level led to degradation of Fab stored at 30°C. Degradation was characterized as Trp50 oxidation, which was not observed in a Fab variant where His31 was replaced. EPR peaks related to known spin adducts of 5,5 dimethylpyrroline N-oxide were detected in Fab exhibiting Trp oxidation, indicating free radicals were present. Trp oxidation of Fab observed in several drug product lots with different degradation rates fits an autocatalytic reaction model that involves free radicals. EDTA, catalase, and free tryptophan prevented oxidation.

Conclusions A metal-binding amino acid, His31, was responsible for Trp50 oxidation of Fab induced by peroxides in PS20 present in the protein formulation. Oxidation was induced by autocatalytic degradation of PS20 and could be inhibited by antioxidants.

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INTRODUCTION

Oxidation of methionine (Met) in pharmaceutical proteins such as relaxin, interleukin-2, parathyroid hormone, trastuzumab, Orthoclone OKT3, nerve growth factor, insulin-like growth factor I, and human growth hormone has been well characterized and studied (1–9). In contrast, oxidation of Trp in pharmaceutical proteins is less common due to its slow reaction at low temperature (10) and low reactivity of the amino acid residue with peroxides. Therefore, the overall mechanism and kinetics of Trp oxidation on protein pharmaceuticals is not as well understood, although methods for detecting Trp oxidation in monoclonal antibodies have been recently reported (10,11). Oxidation products of free tryptophan, including kynurenine, hydroxytryptohan, N-formylkynurenine, and 3-hydroxykynurenine, have well been identified by characteristic absorbance and fluorescence measurements (10,12,13). The molecular weight and structure of these products are shown in Fig. 1. The molecular weight differences of 4, 16, 32 relative to Trp for kynurenine, hydroxytryptohan, and N-formylkynurenine, respectively, allow for the identification of Trp oxidation in peptides and proteins by mass spectrometry (14). A reversed-phase HPLC method has also been recently developed for routine monitoring of Trp oxidation in the production of a monoclonal antibody (11). Although metal ions are common catalysts involved in many oxidative degradation reactions, it is

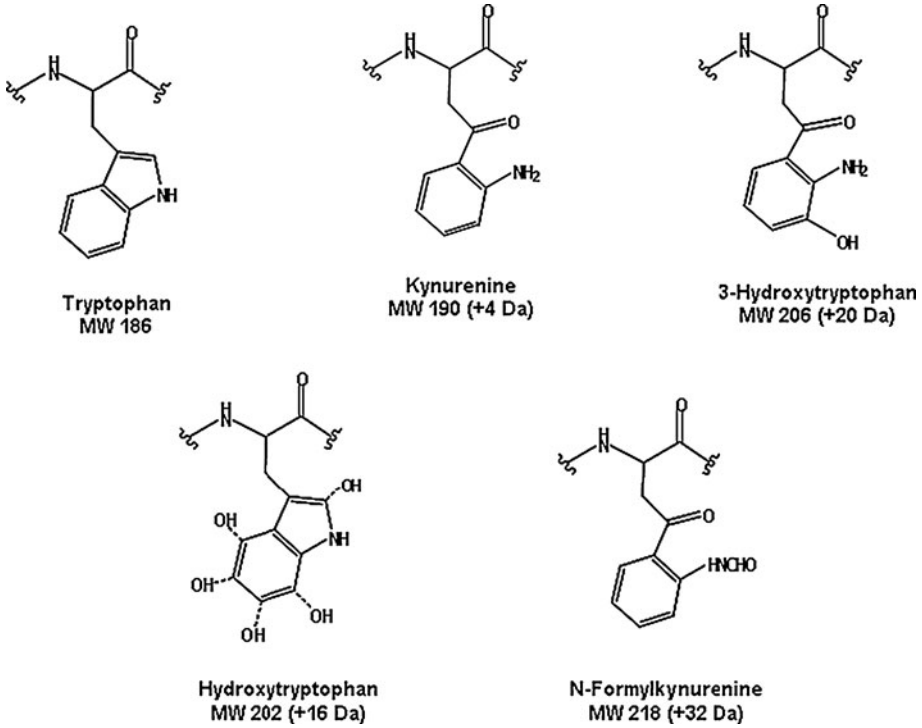

Fig. 1 Structure and corresponding molecular weight of tryptophan, hydroxytryprophan, kynurenine, N-formylkynurenine and 3-hydroxykynurenine.

believed that Trp residues in proteins are not oxidized via a metal-catalyzed reaction because Trp is not a site for binding metal ions (15).

Protein formulations are always developed using various excipients to optimize the stability of drug products during process manufacturing and shelf-life storage. Degradation of some excipients as well as the impurities present may lead to oxidation of proteins over time. For example, nonionic surfactants such as polysorbates with alkyl polyoxyethylene chains that are widely used in pharmaceutical formulations to prevent protein aggregation and surface adsorption can undergo autoxidation to generate peroxides as impurities, which can affect the stability of proteins that are sensitive to oxidation. Peroxide formation in polysorbate 80 under a variety of storage conditions has been examined for its potential to oxidize IL-2 mutein (16). The process of degradation of polyosrbates by autoxidation that involves free radical formation has also been studied (17,18). Free radicals can be initiated by ionizing radiation, light, or free-radical transfer from other molecules (8, 17). It has been shown that Trp residues can be oxidized by ionizing radiation (13,19). Quantitative kinetic studies on peroxide formation in polysorbates alone (17) and protein stability kinetics under the influence of peroxide (16,18) have been previously reported. Nevertheless, to our best knowledge there have been no published kinetic studies that could successfully link the free-radical chain reactions and the induced protein oxidation, especially for oxidation of the Trp residue.

Peroxide formation in polysorbates can be controlled by directly adding antioxidants to the surfactant during manufacture or by removal of oxygen in the headspace of storage container. Some manufacturers package surfactants under nitrogen to prevent the formation of peroxides during storage and shipment. Although antioxidants can also be added to polysorbate-containing pharmaceutical products to protect the drugs from oxidative degradation, the unknown toxicity of many antioxidants and their incompatibility with proteins has hampered their use in protein formulations. Nevertheless, several amino acid antioxidants, including Cys, Met, and Trp, have been shown effective in preventing oxidation of recombinant proteins such as human nerve growth factor, human ciliary neutrophic factor (rhCNTF), and humanized monoclonal antibody HER2 (8,10,20). In addition to oxidation, it has been reported that alkyl hydroperoxides in polysorbate 80 can induce dimerization and subsequent aggregation of rhCNTF (20).

In this paper, we investigated the root cause for the unexpected Trp oxidation of a recombinant humanized monoclonal antibody fragment (Fab) formulated in the presence of polysorabte 20 to stabilize the protein during formulation and storage. During development of a robust manufacturing process, several Fab lots exhibited different degradation kinetics, subsequently characterized as Trp oxidation. We demonstrated that the increased peroxide level of polysorbate 20 in the Fab formulation during the formulation step of the manufacturing process

accounted for the Trp oxidation of the protein, and the oxidation mechanism involved free radical formation. The atypical oxidation kinetic data and mechanism of degradation fit an autocatalytic reaction model proposed for the PS20. In addition, we studied how the neighboring histidine amino acid residue can play a role in site-specific oxidation of Trp (i.e. Trp 50). Site-specific Trp oxidation resulting in atypical degradation kinetics has not been reported in literature. Finally, the methods of preventing of Trp oxidation were identified for the protein therapeutic.

MATERIALS AND METHODS

Recombinant Humanized Monoclonal Antibody Fragment

Recombinant humanized monoclonal antibody fragment (Fab) was expressed in *E. coli* cells and purified by the Late Stage Purification Department of Genentech, Inc.

Identification and Characterization of Trp Oxidized Species

A Hewlett-Packard 1100 HPLC instrument equipped with a diode-array detector and a solvent delivery system was used to assess the stability of Fab lots 1–6 by cation-exchange chromatography (IEC) method. Samples were injected onto a Dionex Pro-Pac® WCX-10 column (4×250 mm). Separation was achieved with a mobile phase consisting of solvent A (20 mM MES, pH 5.7) and solvent B (200 mM NaCl in 20 mM MES). Peak detection was performed at 280 nm.

The IEC main peak and two main basic peak fractions were collected and analyzed using Lys-C peptide map. Samples were digested for 5 h at 37°C with endoproteinase Lys-C before they were separated with an Agilent 1200 series HPLC system, and the masses of each peptide fragment were determined with an on-line-coupled LTQ linear ion-trap mass spectrometer (Thermo Fisher Scientific, San Jose, CA). A Zorbax 300SB-C8 column (4.6×150 mm) was controlled at 30°C and the column effluent was monitored at 214 nm. Elution was achieved at 1 mL/min with a mobile phase consisting of solvent A (0.1% TFA in water) and solvent B (0.1% TFA in acetonitrile). A linear gradient from 7 to 95% buffer B was performed over 90 min. The effluent from the HPLC was infused directly into the LTQ electrospray ionization source. Electrospray ionization in positive-ion mode was achieved by using a needle spray voltage of 4.5 kV and a capillary voltage of 44 V. Mass spectrometry (MS) spectra interpretation and peptide assignments were accomplished with an automatic database search with a SEQUEST algorithm using BioWorks Browser version 3.2 (Thermo Fisher Scientific) and manual investigation of each matched product ion spectrum.

Polysorbate Study

To investigate if Trp oxidation observed in several of the Fab lots was induced by peroxides in PS20, a simulated handling study was conducted. Increased peroxide levels in PS20 were generated by storing the surfactant under room temperature at 20–25°C without nitrogen overlay for up to 4 weeks. The peroxide was measured to determine the level of increase during storage of the surfactant before it was used to formulate Fab for accelerated stability testing by IEC and analysis of peptide map for Trp oxidation confirmation, if there was any. The IEC results and degradation profile were compared to the Fab formulated with PS20 stored with nitrogen overlay and protected from light. In addition, metal analysis was also performed on the Fab drug product lots (Lots 1, 3, and 6) to determine if Trp50 oxidation was metal-catalyzed.

Determination of Peroxide Level in PS20

Peroxide levels in PS20 were measured using the fluorometric Amplex® Red Peroxidase Assay (Invitrogen, Carlsbad, CA). In this assay, the Amplex® Red reagent, in combination with horseradish peroxidase (HRP), is used to detect peroxides in solutions. The reagent (in the presence of HRP) reacts with peroxides stoichiometrically in 1:1 molar ratio to produce a red-fluorescent oxidation product, resorflurin. This oxidized product has an excitation and emission maxima at approximately 571 and 585 nm, respectively. All samples from the polysorbate study and standard curves were prepared at a peroxide range between 1–200 ppm.

Elemental Analysis of Fab Drug Product Lots

A Perkin Elmer inductively coupled plasma atomic emission spectroscopy (ICP-AES) model 3300DV was used to determine the trace levels of chromium (Cr), iron (Fe), nickel (Ni), molybdenum (Mo), and manganese (Mn) in Fab drug product Lots 1, 3, and 6. The respective levels for each metal in each lot were calculated based on a standard curve containing 0–1 ppm for each metal. Plasma operating parameters were optimized for elemental analysis of drug product lots. Gas flows of 15 L min^{-1} (cooling), 0.5 L min^{-1} (auxillary), and 0.8 L min^{-1} (injector) were used. The power level of 1300 W was employed, and the sampling position was set at 15 mm from the load coil. The Limit of Detection (LOD) for each of the metal analyzed using ICP-AES is 0.001 ppm.

Preparation of Modified Fab to Remove Histidine Residue

To study the role of neighboring His residues in the oxidation of Trp in Fab, the original amino acid sequence of Fab was engineered to replace His at amino acid residue #31 of the heavy chain with asparagine (Asn). His31 is in proximity to the primary site of oxidation (i.e. Trp50). The resulting Fab variant was purified using the same process for the native Fab, prepared in the final drug product formulation using formulation buffer that contained 44 ppm peroxides in the PS20 and stored for up to 3 months at 30°C. The single amino acid modification of the His to Asn in the primary sequence changed the overall surface charge of the protein such that IEC could not be used to collect the two IEC basic peaks for Lys-C peptide map characterization. Therefore, Lys-C peptide map was performed on the full intact Fab molecule instead.

Detection of Free Radicals Using Electron Paramagnetic Resonance (EPR)

5,5-dimethylpyrroline N-oxide (DMPO) prepared to a final concentration of 100 mM was used as a spin trap to detect free radicals present in Fab samples. All EPR spectra were recorded at room temperature and at a resonance frequency of 9.8 GHz using a Bruker ER 041XG spectrometer equipped with an ER 4119HS cavity. Sample analysis was performed on 50 uL by loading into micro-capillaries, sealed with Critoseal (Fisher Scientific), and placed inside standard quartz EPR tubes (Wilmad, 707-SQ-250 M). The spectrometer settings used for the experiments were as follows: microwave power, 25 mW; modulation frequency, 100 kHz; modulation amplitude, 1 G; sweep width, 100 G; time constant, 163.84 ms; conversion time, 81.92 ms; receiver gain, 1×10^6; number of data points, 1024. The EPR spectra obtained were simulated using EPR simulation software SimFonia v.1.2 (Bruker), which is based on the perturbation theory.

Trp Oxidation Inhibition Study

The inhibitory effect of antioxidants on Trp oxidation of Fab was evaluated by adding EDTA (Sigma Chemical), catalase (Sigma Chemical), and L-tryptophan (VWR International) individually at final concentrations of 1 mM, 1 μM, and 10 mM, respectively, to the Fab drug product Lot 6 that had not been exposed to accelerated temperatures. The effect of molecular oxygen on Trp oxidation of Fab was also evaluated by purging the drug solution from product Lot 6 with nitrogen. All samples were stored at 30°C and analyzed by IEC for detection of basic peaks. IEC results were compared to untreated Fab material as control.

RESULTS

Atypical Degradation Characterized as Trp Oxidation

Analyses of Fab drug product lots by IEC method revealed that some lots stored at 30°C had different degradation rates and profiles compared to the others as indicated by the loss of IEC main peak over time. Among the six drug product lots of Fab investigated, Lot 6 degraded at a significantly faster rate after 1 month of storage at 30°C, and Lots 3–5 degraded faster after 3 months but at a different rate. Lot 2 also degraded slightly faster than Lot 1 after 2 months at 30°C (Fig. 2). Despite the atypical degradation behavior of Lots 2–6, no difference in stability was observed among all six lots when stored under the recommended conditions at 2–8°C for long-term storage. Analysis of the IEC chromatograms of all six lots revealed the increase in two major peak species in the basic region of the chromatogram that accounted for the loss in IEC main peak (Fig. 3). Characterization of these two IEC basic peaks was accomplished by collecting the IEC main and two basic (Basic-1 and Basic-2) peaks followed by MS analyses of the Lys-C peptide map on the respective peak fractions. IEC Basic-1 fraction was found

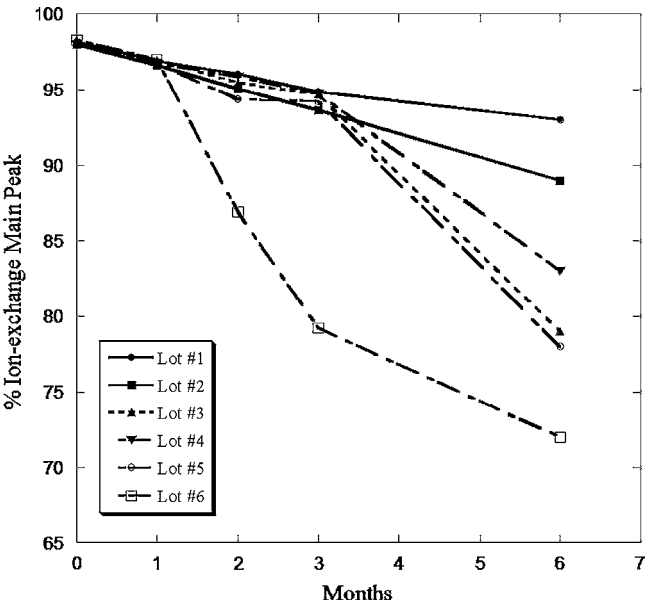

Fig. 2 The degradation of Fab drug product stored for 6 months at 30°C as determined by IEC. Fab typically degrades in a linear fashion as shown by Lot 1. In Lot 6, a significant increase in degradation was observed between 1 and 2 months of storage. In other lots (Lot 2–5), a significant increase in main peak degradation was observed between 3 and 6 months of storage.

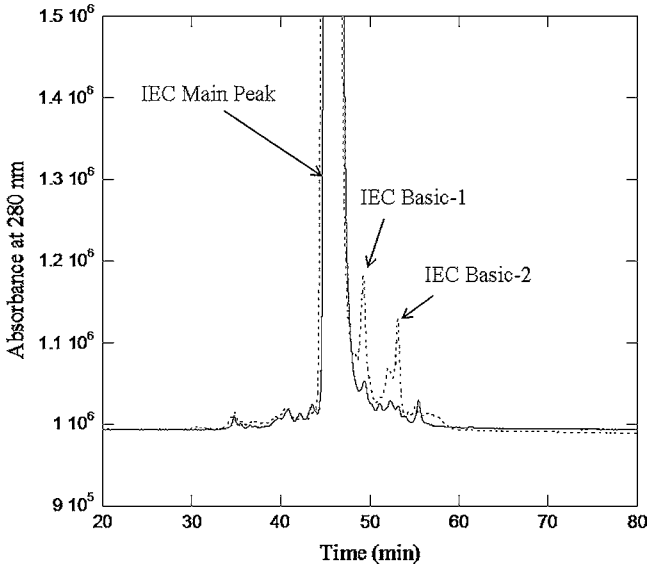

Fig. 3 IEC chromatograms showing the effect of temperature on Trp oxidation. Samples were incubated for 2 months at 30°C. This chromatographic overlay shows the degradation of a typical Fab lot (*solid line*) compared to Lot 6 with increased Trp oxidation (*dotted line*).

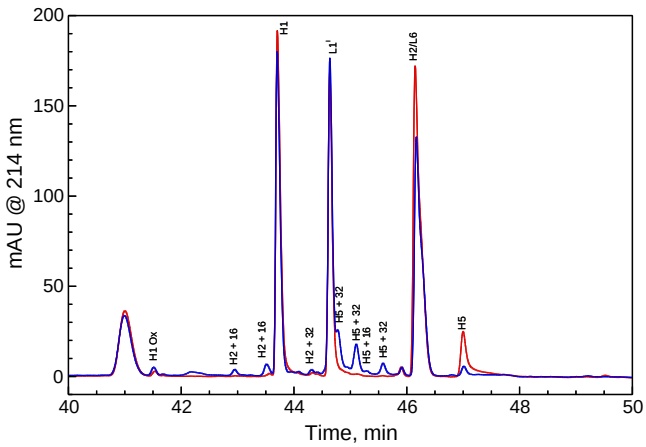

Fig. 5 Lys-C peptide map of Fab IEC main peak and peak 2 fractions. Samples incubated at 30°C were analyzed by IEC. The main peak and Basic-2 peak fractions were collected and analyzed by Lys-C peptide map. Lys-C pepride map of the Basic-2 fraction contains unidentified oxidized species of Trp (blue line) compared to the peptide map of the main peak fraction only (red line). "H" and "L" denote peptides from the Fab heavy chain and light chain, respectively. Each peptide is numbered sequentially from the N-terminus.

to contain +4, +16, and +32 atomic mass unit changes to primarily the Trp50 residue in the H2 peptide (Fig. 4). Kynurenine, hydroxytryptophan, and N-formylkynurenine have each been identified as Trp oxidation products with molecular weight differences of +4, +16, and +32, respectively, relative to unoxidized Trp (Fig. 1). The IEC Basic-2 peak fraction was also found to contain hydroxytryptophan and N-formylkynurenine oxidized species of the Trp50 residue of the H2 peptide as well as +16 and +32 atomic mass unit changes to other unidentified Trp residues in the H5 peptide (Fig. 5).

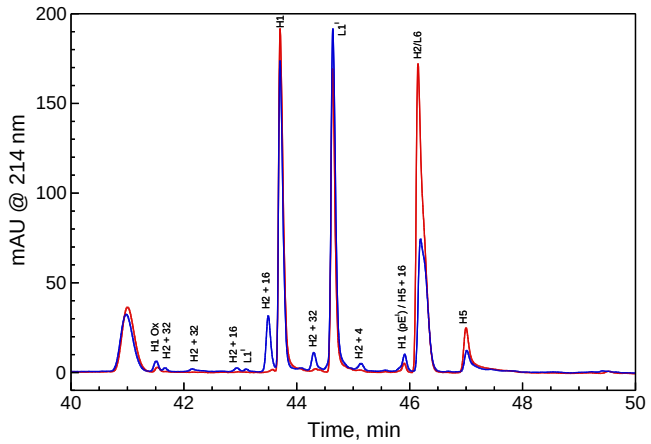

Fig. 4 Lys-C peptide map of Fab IEC main peak and Basic-1 peak fractions. Samples incubated at 30°C were analyzed by IEC. The main peak and Basic-1 peak fractions were collected and analyzed by Lys-C peptide map. Lys-C peptide map of the Basic-1 fraction contains several species identified as Trp oxidation (blue line) compared to the peptide of the main peak fraction only (red line). "H" and "L" denote peptides from the heavy chain and light chain, respectively. Each peptide is numbered sequentially from the N-terminus.

Polysorbate Study

The peroxide level in the PS20 increased from a starting level of 11 ppm to 44 ppm as measured by fluorometric Amplex® Red Peroxidase Assay after storage at room temperature without nitrogen overlaid in the storage container. When the surfactant containing 44 ppm peroxide was used to formulate Fab for accelerated stability study at 30°C, the protein degraded at a faster rate than the Fab prepared using PS20 containing 11 ppm peroxide as determined by the loss of IEC main peak over time (Fig. 6). Upon analysis of the IEC chromatograms for the samples stored at 30°C for 6 months, increase in peak areas of IEC Basic-1 and IEC Basic-2 that resulted in an increased loss of IEC main peak area was observed for the Fab formulated with the PS20 containing 44 ppm peroxide. The IEC basic peaks were further characterized and confirmed by Lys-C peptide map as the oxidation of Trp residue of Fab, primarily Trp50. The IEC chromatogram was also comparable to Fab Lot 6, which showed increase in peak areas of IEC basic peaks (Basic-1 and Basic-2) at 30°C for 2 months as shown in Fig. 3.

Trace Amounts of Metals Detected in Fab Drug Product Lots

The results from the ICP-AES analysis showed that only trace amounts of Cr, Fe, Mo, Mn, and Ni were present in Fab

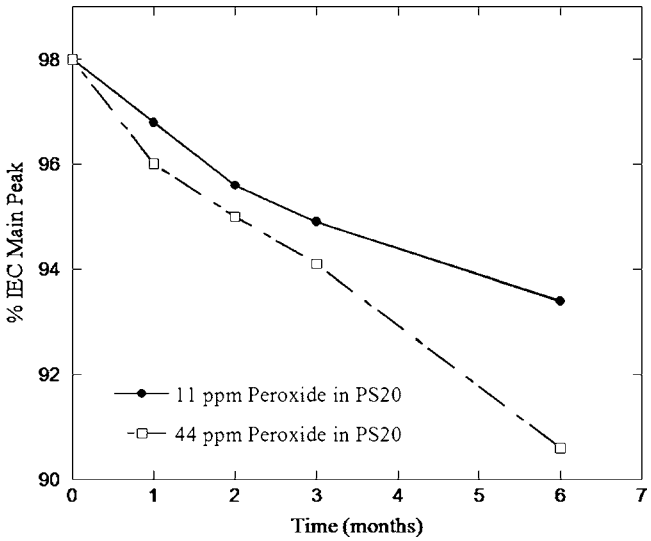

Fig. 6 Fab samples prepared using PS20 containing 11 ppm peroxide were compared to samples prepared using the PS20 (44 ppm peroxide) after 4 weeks of exposure to room temperature without nitrogen overlaid. All samples were stored up to 6 months at 30°C and analyzed by IEC.

Lots 1, 3, and 6 (Table I). The total amount of metals ranged between 0.03 ppm (Lot 6) to 0.08 ppm (Lot 1). Interestingly, Lot 6, which displayed the greatest amount of Trp oxidation, had the lowest amount of total metals measured by ICP-AES. Conversely, Lot 1, which displayed a typical degradation profile for Fab, contained the largest amount of total metal content of the three lots tested.

Site-Specific Trp Oxidation of Fab

As shown in Fig. 7a, the heavy chain of a native Fab contains a His at position 31 in the amino acid sequence. A variant of Fab was made to replace the His with an Asn residue at this position (Fig. 7b). The Lys-C peptide maps of the native Fab and Fab variant after they were formulated with PS20 containing 44 ppm peroxides and stored at 30°C for 3 months are shown in Fig. 8. A slight shift in retention times for the H1 and the oxidized H1 peptides were observed for the Fab variant relative to the native Fab due to the change in hydrophobicity as a result of the single amino acid change from His to Asn. For the native Fab sample, a peak eluted at a retention time of approximately 44 min between the L1 and H2/L6 peptides was characterized in this region to contain two known oxidation products of Trp50. These two products were kynurenine and hydroxytryptophan. For the chromatogram of the Fab variant, no Trp50 oxidized peak eluted in the same region of the Lys-C peptide map as a result of replacing the His31 residue with Asn31 in the heavy chain Fab variant.

Table I Metals Analysis for Fab Lots by ICP

Sample	Increase Trp50 oxidation	Metal content (ppm)					
		Cr	Fe	Mn	Mo	Ni	Total
Fab Lot 1	No	0.030	0.009	0.001	0.001	0.041	0.082
Fab Lot 3	Yes	0.003	0.018	0.003	0.003	0.012	0.039
Fab Lot 6	Yes	0.001	0.005	0.002	0.001	0.024	0.033

Presence of Free Radicals in Fab Solutions

Free radicals formed in the Fab samples prepared in the polysorbate study were detected by using the spin trap DMPO. Solutions of Fab formulated with PS20 containing 44 ppm of peroxide with DMPO were analyzed by EPR and found to contain seven distinct EPR peaks (EPR spectrum A in Fig. 9). Four of these EPR peaks (Peaks 1, 3, 5, and 7) matched up well with the simulated EPR spectrum of a known DMPO-OR spin adduct which was the oxidized species of DMPO (EPR spectrum B in Fig. 9). The other three EPR peaks (Peaks 2, 4, and 6) matched up with another simulated EPR spectrum of a known DMPO spin adduct which had undergone either disproportion into hydroxylamine and C-2 substituted 3*H*-pyrrole *N*-oxide or oxidation to an alkoxyl alkyl aminoxyl species (EPR spectrum C in Fig. 9) (21). Figure 10 shows the experimental EPR sprectra of DMPO-spin adducts of Fab solutions prepared with PS20 containing 44 ppm peroxide, PS20 containing 11 ppm peroxide and PS20 containing 44 ppm peroxide in the presence of 1 mM EDTA and stored for 2 weeks at 30°C. The decrease in EPR intensity of the EDTA-containing sample indicated that the presence of metal ions could initiate the formation of free radicals.

Inhibition of Trp Oxidation

The percents of total IEC Basic Peaks (Basic-1 and Basic-2) of Fab drug product Lot 6 spiked with oxidation inhibitory agents prior to storage at 30°C for 3 months are shown in Table II. After the addition of EDTA to Lot 6 at a final concentration of 1 mM, there was only a 1.9% increase from the initial time point in the amount of IEC basic peaks compared to more than a 14.1% increase for Lot 6 without EDTA. Oxidation of Trp in Fab increased only from 1.4% to 3.1% after 3 months in the presence of 1 μM catalase. Purging the Fab drug product vial with nitrogen lowered the amount of Trp oxidation to 2.8% after 3 months at 30°C. The addition of 10 mM L-tryptophan (free Trp) to Fab Lot 6 before placing it at 30°C also effectively reduced the amount of oxidation to 4.4% after 3 months of storage. In

Fig. 7 (**a**) Amino acid sequence of Native Fab heavy chain. The Trp50 site of oxidation is highlighted in orange, while the His31 is highlighted in yellow. (**b**) Modified amino acid sequence of Fab variant heavy chain. The Trp50 is highlighted in orange, while the Asn31 is highlighted in yellow. The Asn residue at the 31 position of this amino acid sequence replaced His.

a

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          10        20        30        40        50
EVQLVESGGGLVQPGGSLRLSCAASGYDFTHYGMNWVRQAPGKGLEWVGWIN
      60        70        80        90        100
TYTGEPTYAADFKRRFTFSLDTSKSTAYLQMNSLRAEDTAVYYCAKYP
           110       120       130       140       150
YYYGTSHWYFDVWGQGTLVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKD
     160       170       180       190       200
YFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLG
           210       220       230
TQTYICNVNHKPSNTKVDKKVEPKSCDKTHL
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b

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          10        20        30        40        50
EVQLVESGGGLVQPGGSLRLSCAASGYDFTNYGMNWVRQAPGKGLEWVGWIN
      60        70        80        90        100
TYTGEPTYAADFKRRFTFSLDTSKSTAYLQMNSLRAEDTAVYYCAKYP
           110       120       130       140       150
YYYGTSHWYFDVWGQGTLVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKD
     160       170       180       190       200
YFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLG
           210       220       230
TQTYICNVNHKPSNTKVDKKVEPKSCDKTHL
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the absence of these measures, the amount of IEC basic species increased from approximately 2–10% between the 1-month and 2-month time points at 30°C and up to 16% after 3 months under the same storage condition (Table II).

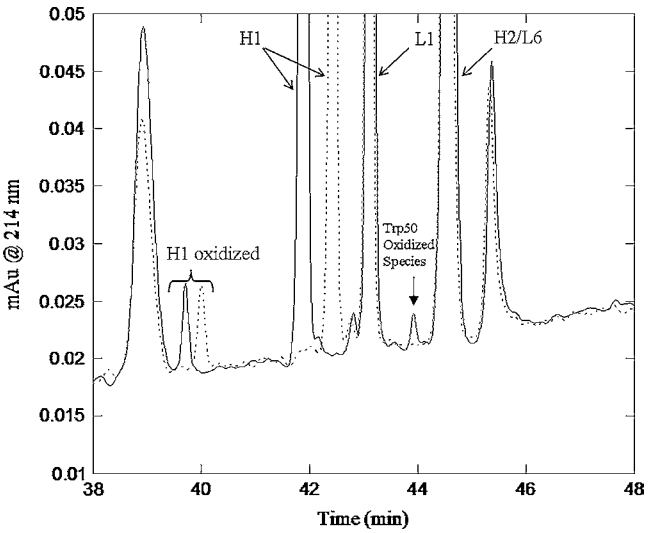


Fig. 8 Lys-C peptide map of Fab variant engineered with Asn31 in the heavy chain instead of His. Fab engineered with the amino acid change (*dotted line*) and native Fab sequence (*solid line*) were prepared using PS20 with 44 ppm peroxide and stored for 3 months at 30°C. Removal of the His resulted in a decrease in the Trp50 oxidized species.

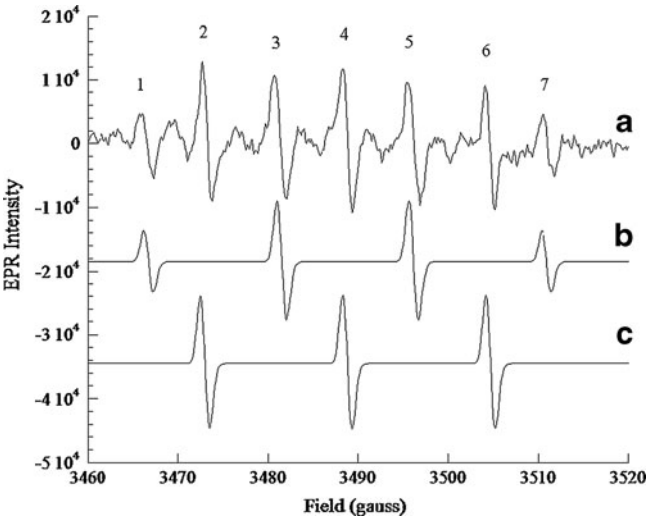


Fig. 9 Experimental EPR spectrum of DMPO-spin adducts from a Fab solution compared to simulated EPR spectra for two potential DMPO-spin adducts. (**a**) Measured EPR spectrum for DMPO-spin adduct from Fab solution; (**b**) Simulated EPR spectrum of the DMPO-OR spin adduct; (**c**) Simulated EPR spectrum of DMPO-spin adduct of hydroxylamine and C-2 substituted 3*H*-pyrrole *N*-oxide or oxidation to an alkoxyl alkyl aminoxyl species.

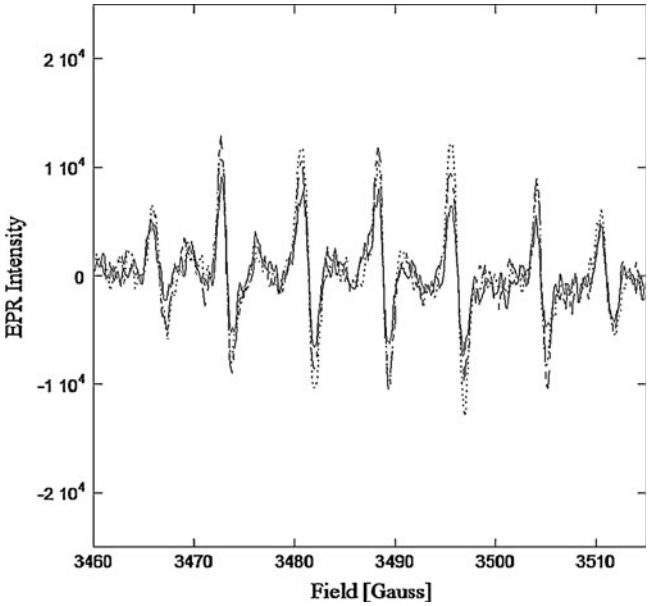


Fig. 10 EPR spectra of DMPO-spin adducts generated from reactions with free radicals in Fab solutions. The EPR spectra for DMPO-spin adducts for Fab samples prepared using PS20 (*dotted line*) with 44 ppm peroxide, PS20 (*dashed line*) with 11 ppm peroxide, as well as a Fab sample prepared using PS20 with 44 ppm peroxide and spiked with 1 mM EDTA (*solid line*) are shown.

DISCUSSION

Peroxides in Polysorbate Induced Oxidation of Trp in Fab

The primary degradation pathway in Fab drug product lots (Lots 3–6) that exhibited atypical degradation was the oxidation of Trp50 residue in the heavy chain of Fab. Kynurenine (+4), hydroxytryptophan (+16), and N-formylkynurenine (+32) were each identified by MS characterization of Lys-C peptide map in both the IEC Basic-1 and Basic-2 peak fractions. Interestingly, significant oxidation was not detected in either Met or His residues and appears to be specific for Trp, particularly for Trp50. Trp oxidation was not observed during formulation development even after Fab solutions were spiked with strong oxidants such as hydrogen peroxide (H_2O_2) or tert-butylhydroperoxide (t-BHP). When H_2O_2 at final concentrations of 1, 100, 300 or 1000 ppm was spiked into Fab solution containing 100 or 500 ppm ferric chloride, Trp oxidation was not detected. However, when the H_2O_2 concentration was increased to 3,000 ppm (only 1–2% of Fab in solution), Trp oxidation was observed in the presence of 500 ppm ferric chloride in the Fab solution. These results suggest that Fab is not susceptible to Trp oxidation by H_2O_2 where hydroxyl radicals are involved. When a protein is susceptible to Trp oxidation by H_2O_2, the presence of such strong oxidant at 100 ppm level or less (not at 3,000 ppm) with trace amount of transition metals (ppb levels) could lead to Trp oxidation within hours of incubation. Therefore, alkyl peroxyl radicals rather than hydroxyl radicals could be involved in the Trp oxidation observed for the Fab drug product lots (Lots 2–6), which required a long period of time (in terms of months) and higher temperatures (30°C and 40°C) for the oxidation to occur.

Table II Methods to Inhibit Tryptophan Oxidation in Fab at 30°C

Time point (months)	% IEC basic peaks in lot 6 with oxidation inhibitory agents[1]				
	No oxidation inhibitor	1 mM EDTA	1 μM catalase	Vial purged with N_2	10 mM L-tryptophan
0	1.6	1.1	1.4	1.6	2.0
1	1.9	1.7	2.0	2.0	3.5
2	9.9	3.1	4.1	2.3	4.1
3	15.7	3.0	3.1	2.8	4.4

[1] % IEC basic peaks was used as an indicator for Trp oxidation.

Even though Fab was not observed to be susceptible to H_2O_2-induced oxidation, other organic peroxides such as alkyl peroxides found in polysorbates have long been linked to the kinetics and mechanisms for the oxidation of amino acids (17–21). Such alkyl peroxides may be generated through autoxidation of surfactant upon storage. Organic peroxides in surfactants can easily induce protein oxidation, especially in the presence of metal ion as catalyst. In fact, it was demonstrated in the polysorbate study that increase in Trp oxidation in the Fab could be induced after the peroxide level increased from 11 to 44 ppm in the PS20 used to formulate the protein. The low metal contents ranging from 0.03 to 0.08 ppm detected in Fab drug product (Table I) indicated that trace amount of metal ions was enough to initiate Trp oxidation of Fab when peroxide level in PS20 was as low as 44 ppm. Metal ions (e.g. Fe) are commonly found in pharmaceutical products as impurities when stainless steel components are used during manufacture. The polysorbate study results suggested that the increase in Trp oxidation observed in Fab Lots 2–6 at 30°C over time (Fig. 2) was probably due to the increase of peroxides generated during storage that was used to formulate the respective drug product lot. Therefore, it is very important to implement all necessary measures to keep the alkyl peroxide level of PS20 used within tight specifications for the manufacturing process of Fab. Increase in Trp oxidation has not been observed in the manufacture of Fab drug product since the peroxide level of PS20 raw material was kept below 10 ppm.

Role of Neighboring Histidine on Trp50 Oxidation

The results from the polysorbate study indicated that oxidation specific to the Trp50 residue can increase when

the alkyl peroxide level in PS20 used in the formulation of Fab was as low as 44 ppm. More importantly, storage of PS20 in an uncontrolled manner (i.e. without light protection and nitrogen overlay) resulted in a 4-fold increase in the amount of such peroxides, and its use in the formulation of Fab led to an increase in Trp50 oxidation. Since the oxidation was specific to a single amino acid residue, we believe that the reaction must involve organic peroxides and metal ions present in trace amounts that are in close proximity to the affected amino acid site. Metal ions have been shown to react with peroxides, causing metal-catalyzed oxidation of proteins. Metal-catalyzed oxidation is often triggered by the Fenton reaction, where highly reactive free radicals (e.g. hydroxyl and alkyl radicals) are generated from hydrogen and alkyl peroxides (10,12,13,22). Under specific conditions (e.g. heat) and in the presence of an initiator such as metal ions (e.g. Fe^{2+} or Fe^{3+}), one molecule of oxidant may generate a peroxide free radical (initiation), which in turn reacts with the remaining oxidant molecules through a chain reaction to form more peroxide radicals (propagation) (8,18). Some of the radicals thus generated are extremely reactive and are capable of attacking tryptophan residues in protein, thereby causing the protein oxidized (23).

Amino acids displaying metal-binding capabilities play an important role in complexes that bind metal ions in biological systems. For example, His is the primary amino acid responsible for the binding of Fe^{2+} in the complex of erythrocytes (24). The location of such residues may play a role in site-specific metal-catalyzed oxidation, especially when the conformation of the protein can place them in close proximity to oxidation-susceptible amino acids. Work has been done to show that the metal-catalyzed oxidation is related to the location of metal-binding amino acids (9,22). In the primary amino acid sequence of the Fab heavy chain, five His residues are found at the 31, 107, 174, 210, and 231 positions. His174, His210, and His231 most likely do not play a role in bringing metal ions close to Trp50 due to their locations at the N-terminal end of the heavy chain. The three-dimensional structure of Fab is shown in Fig. 11, which illustrates the position of Trp50 in relation to a proposed complex of three His residues: His31 and His107 from the Fab heavy chain and His56 from the Fab light chain that could trap metal ions in close proximity to the Trp50 site. All other oxidation-susceptible amino acids are either not close to the metal ions and/or are sterically hindered from the metal ions. Therefore, the potential metal binding effect of a His residue (i.e. His31), which is the closest to Trp50 site, was investigated by replacing it with Asn in the heavy chain of Fab (Fig. 7b). The absence of a Trp50 oxidized peak in the Lys-C peptide map chromatogram (Fig. 8) suggests that His plays a role in facilitating the oxidation by forming a complex that can bind metal ions in proximity to this primary site of oxidation.

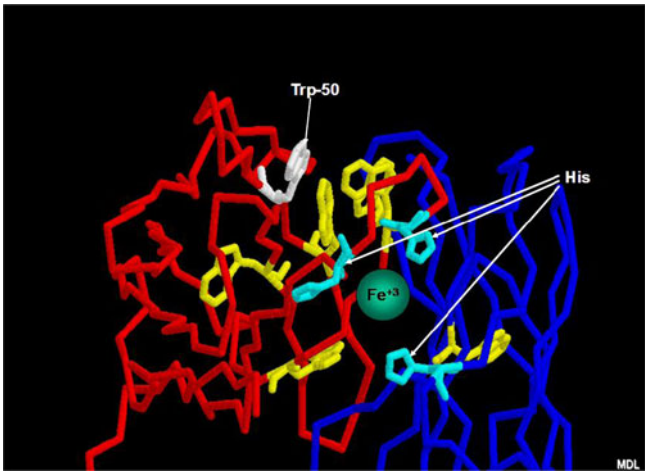


Fig. 11 Three-dimensional structure of Fab. Fab heavy chain is shown in orange, while the light chain is shown in blue. His residues are in light blue, while Trp residues are shown in yellow. Trp50 is highlighted in white.

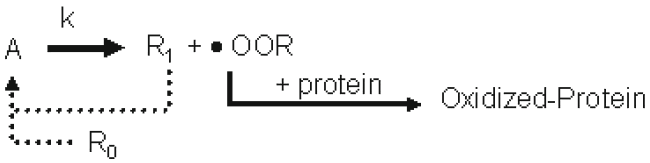
Mechanism and Kinetics of Trp Oxidation in Fab Drug Product Lots

Metal-catalyzed oxidation often involves the generation of free radicals. To confirm that Trp oxidation of the Fab drug product occurred as a result of such a mechanism involving free radicals, EPR was used. Free radicals are difficult to detect because they are highly reactive with a relatively short half-life. The use of spin traps employing an organic nitrone or nitroso compound are often added to the radical generating system to ensure rapid reaction with any radicals to give a stable and detectable nitroxide radical adducts (25–27). In the polysorbate investigation study, EPR using the spin trap DMPO detected free radicals in the Fab samples formulated using peroxide-containing PS20. In the presence of a metal chelating agent (EDTA), EPR intensity decreased in these samples, which suggests that the mechanism for polysorbate-induced Trp oxidation in Fab could involve metal-ion-catalyzed free radical formation.

Free radicals have long been linked to the oxidation of protein therapeutics. For example, the formation of alkyl peroxyl free radicals from polysorbates has been documented to cause protein oxidation (16). Previous work has also shown that peroxyl radicals could be produced from dissolved oxygen reacting with alkyl radicals that are generated from chemical "initiators" under the influence of light or temperature (16–19). Other studies demonstrated that the peroxyl free radical could be generated through the catalytic reaction of ferric and/or ferrous ions on organic peroxide (28–31). The overall mechanism for oxidation due to these reactive free radicals has been proposed to involve initiation, propagation, and termination steps of the radicals. We hypothesize

that the characteristic kinetics for Trp oxidation of the Fab in this investigation study are linked to the rate at which these free radicals are formed. Initially, the rate of oxidation proceeds slowly because few free radicals are present. As more free radicals are formed, oxidation increases, then slows and terminates as the oxidant and free radicals are eventually consumed. As a result, the time course of protein oxidation displays a sigmoid profile which is similar to the degradation profile shown in Fig. 2 for the Fab drug product lots. Such a kinetic profile has been reported for autocatalytic reactions, a mechanism in which at least one of the products acts as a catalyst to further promote the reaction (32–35).

In this study, we made an attempt of applying the following catalytic reaction mechanism for peroxide-free radical generation to analyze the oxidation kinetic data from the six Fab lots. Schematic representation of the reaction is shown as follows:



where A is the oxidant with peroxide impurity in PS20; R_0 is the initial catalyst, metal ions; R_1 is the reaction intermediate, assumed to have the same catalytic activity as R_0; • OOR is the alkyl peroxide radical; and k is the reaction rate constant.

First, a conversion of PS20 peroxide oxidant (A) is initiated by a metal ion catalyst (R_0). This initiation yields an alkyl peroxide radical (• OOR) and a reaction intermediate (R_1). R_1 then acts equivalently to the initial catalyst (R_0) to start more peroxide conversions. After each "autocatalytic reaction" cycle more R_1s are generated, thus further accelerating peroxide radical generation. These autocatalytic reaction kinetics are simpler than the complicated series of initiation and propagation reaction proposed in literature, yet still account for the propagation nature of the peroxide radical formation. We also hypothesize that upon formation, the peroxide radical attacks protein imminently (presumably the Trp50 is very close to the catalysts), thus causing the protein to oxidize. As such, protein oxidation continues until all the oxidants are consumed and no more alkyl peroxyl radicals (• OOR) can be generated.

Based on the autocatalytic reaction mechanism proposed above, the rate equation for oxidant A consumption is

$$-r_A = -\frac{dC_A}{dt} = kC_A C_R$$

where C_R is the sum concentration of C_{R0} and C_{R1}. Because one A consumed generates one R_1, and R_1 is the same as R_0 in catalytic activity, the total number of moles of A and R remain unchanged as A is consumed. Accordingly, the following material balance applies at any given time during the reaction

$$C_A + C_R = C_{A0} + C_{R0} = C_0 \text{ (constant)}.$$

Thus, the rate equation becomes

$$-r_A = -\frac{dC_A}{dt} = kC_A(C_o - C_A).$$

Finally, the integrated form for the rate equation can be expressed as follows:

$$1n\left[\frac{M + X_A(t)}{M[1 - X_A(t)]}\right] = C_{A0}(M+1)kt \qquad (1)$$

where

$$M = \frac{C_{R0}}{C_{Ao}} \qquad X_A(t) = \frac{C_{Ao} - C_A(t)}{C_{Ao}}$$

This equation implies that a plot of the kinetic data on the time and concentration coordinates using a known value of C_{A0} and C_{R0} should yield a straight line passing through zero. The reaction rate constant k can be calculated from the slope of the straight line plot. Such procedure has been used as a standard method to analyze the kinetic data of an autocatalytic reaction mechanism described above (35). However, in the present study, the C_{A0} and C_{R0} values may vary lot by lot; thus, they have to be determined using the calculation procedure described below.

Fab drug product Lot 6 had increased Trp oxidation after storage for 1 month at 30°C. After 3 months at 30°C, the loss of IEC main peak in this lot appeared to be much less significant. Therefore, after this time point, it is plausible that all the oxidant content had been completely consumed via the autocatalytic reaction proposed above. Since each oxidant generated a peroxide radical to oxidize a Fab molecule, the initial oxidant content (C_{A0}) in this lot was calculated to be 3.4×10^{-2} mM, based on the fact that 27% of the initial Fab concentration (1.25×10^{-4} M) was oxidized upon storage for 6 months. Accordingly, this C_{A0} value was employed to determine C_{R0} and k for Lot 6 using the following algorithm:

Step 1. Assume a C_{R0} value to generate a value of M (C_{R0}/C_{A0}) correspondently.
Step 2. Apply M to calculate the concentration term (left-side) of Eq. 1 for each stability time point.
Step 3. Plot the concentration and time terms to verify whether the plot yields a straight line passing through zero; if not, repeat step 1 through step 3 until the best regression fit is obtained.

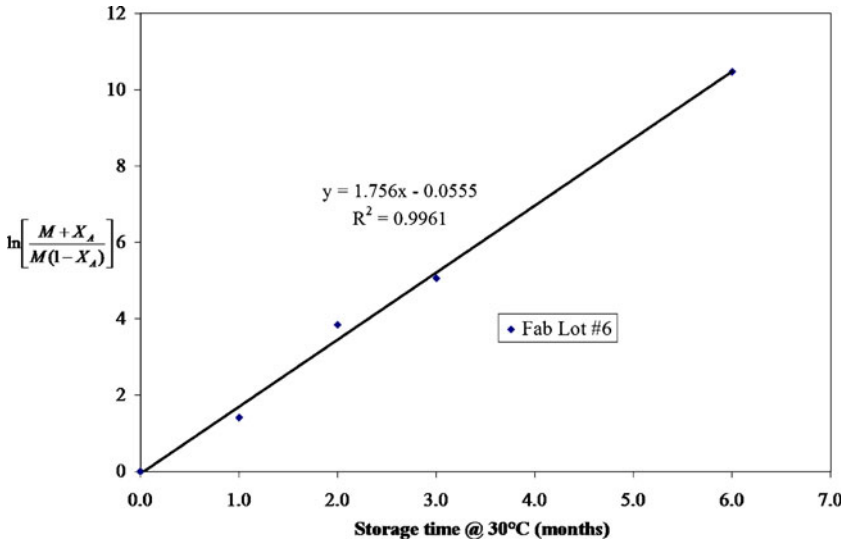


Fig. 12 Fab Lot 6 best-fit at 30°C. The integrated rate equation was tested against the 6-month IEC main peak data of Fab drug product Lot 6, which had increased Trp oxidation after storage. An excellent fit to the proposed reaction model with a regression value ($R^2 = 0.9961$) was obtained.

Step 4. Calculate reaction rate constant k from C_{A0}, the final C_{R0}, and the slope of the straight line plot.

We tested the rate Eq. 1 with the six-month IEC main peak data of Fab drug product Lot 6, which had increased Trp oxidation after storage for 1 month at 30°C, and found an excellent fit with the proposed reaction model, as evidenced by the high linear regression value ($R^2 = 0.9961$) shown in Fig. 12. Through iterated calculation, the initial concentration of the free radical formation initiator was determined to be 5.7×10^{-4} mM. Using C_{R0} and C_{A0} concentrations, we calculated the rate constant for the autocatalytic reaction, k, to be 5.15×10^4 L / mole * month.

Since the stability studies of all the six drug product lots were conducted at the same 30°C temperature, the autocatalytic reaction rate constant k derived from Lot 6 should be applicable to the remaining five lots. Thus, this k value was applied to determine C_{A0} and C_{R0} for each of the remaining lot using the following algorithm:

Step 1. Assume a C_{A0} value
Step 2. Assume a C_{R0} value
Step 3. Apply M (C_{R0}/C_{A0}) to calculate the concentration term (left-side) of Eq. 1 for each stability time point.
Step 4. Plot the concentration and time terms to verify whether the plot yields a straight line passing through zero; if not, repeat step 2 through step 4 until the best regression line is obtained.
Step 5. Calculate new C_{A0} from k, the assumed C_{R0}, and the slope of the straight line plot to verify whether the new C_{A0} is identical to the assumed C_{A0} in step 1; if not, repeat step 1 through step 5 until an appropriate C_{A0} value is found.

Figure 13 shows that this kinetic data analysis yielded an excellent fit to the proposed autocatalytic reaction model for all the Fab product lots' stability as shown in Fig. 2. Table III summarizes the calculated oxidant and initiator contents for each lot. The initiator contents calculated as "Fe equivalent" appear to be in the same ppm range as the total analyzed metal contents shown in Table I. The oxidant concentrations shown in Table III were not further converted to peroxide equivalent because the exact chemical structure was not known. Nevertheless, our study indicates that the Trp oxidation of the Fab in all the drug product lots fits a kinetic model that involves initiation of oxidizing free radical through an autocatalytic reaction between a trace amount of oxidant and initiator. The initiation and rate at which oxidation occurs are a function of the oxidant (such as oxygen and peroxide) and the concentration of free radical initiator (such as iron). The initial peroxide contents calculated for Fab Lots 3–6 were relatively higher than Fab Lots 1 and 2 (Table III). This explains why Trp oxidation was observed much earlier (i.e. after 1 or 3 months) and follows a sigmoidal degradation pattern.

Prevention of Trp Oxidation

Our proposed autocatalytic reaction mechanism for Trp oxidation of Fab involves a complex interaction between one or more oxidants and free radical initiators. Furthermore, only trace amounts of oxidants and initiators were needed for the unusual degradation to occur. We evaluated this reaction mechanism by treating Fab drug product with oxidation inhibitory agents. The use of EDTA has long been shown to effectively reduce oxidation observed for protein therapeutics such as HER2 (8). In this study, addition of EDTA reduced Trp oxidation of Fab drug

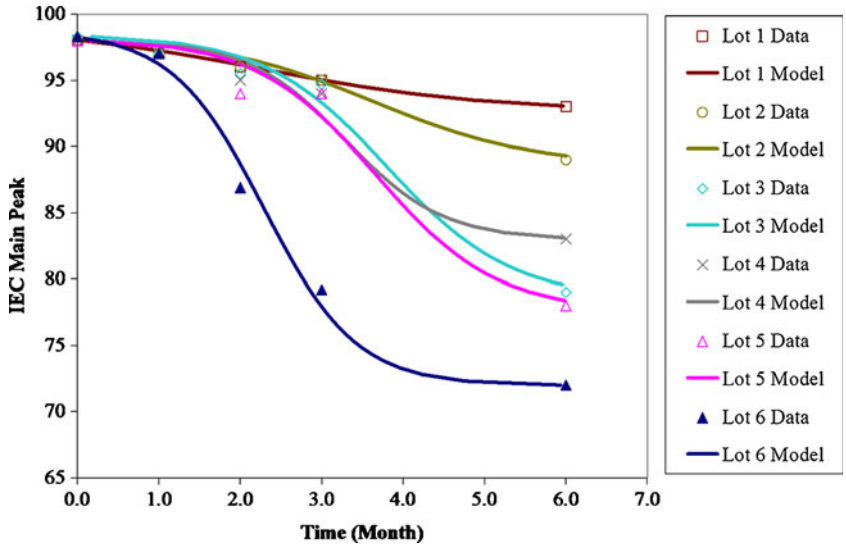


Fig. 13 Fab Lots 1–6 best-fit % IEC main peak *vs.* storage time at 30°C. The autocatalytic rate constant, k, obtained from Lot 6 was applied on other five Fab drug product lots and yielded excellent fits to the actual IEC data.

product Lot 6 stored for three months at 30°C. We propose that the metal-chelating properties of EDTA limited the oxidation by removing the metal ions present in the protein solution. Furthermore, the reduction of EPR intensity in our study also suggests that the mechanism for Trp oxidation is through the generation of free radicals. After the addition of EDTA, there was an insignificant amount of free radical initiator present in order for the oxidation reaction to occur. Likewise, the reduction of Fab Trp oxidation after addition of a peroxide scavenger (catalase) confirmed that removal of this oxidant from the Fab drug solution effectively reduced the autocatalytic reaction from proceeding. Oxygen present in the drug product vial was confirmed to play a role as oxidant in the observed Trp oxidation of Fab. Once the vial was purged with nitrogen, the amount of Fab Trp oxidation reduced significantly. Finally, the reduction of Trp oxidation after the addition of free Trp as antioxidant to the Fab drug solution illustrates that Trp used as a formulation excipient can limit the oxidation by competing with Trp residues on the protein to react with the oxidants and free radical initiators. Free Trp as free radical scavenger in the Fab solution could easily gain access to the complex of the three His residues (His31 and His107 of Fab heavy chain and His56 of Fab light chain) where free radicals were mostly initiated in these metal-binding sites. Although the use of these oxidation inhibitors can be a way to protect pharmaceutical drug products from oxidative degradation, their toxicity and compatibility with proteins and/or excipients in parenteral formulations need to be evaluated carefully before use.

CONCLUSION

Based on the results from these studies, we concluded that the degradation observed in accelerated stability (30°C) of Fab drug product Lots 2–6 was characterized as oxidation of Trp induced by the free radical generated from high peroxide level in PS20. The overall structure of Fab played an important role in degradation as the oxidation was specific to a single Trp50 in close proximity to a metal-binding amino acid, His. Free radical generation and propagation were hypothesized through an autocatalytic reaction from the peroxide in PS20. We applied such reaction model successfully to interpret the atypical degradation profiles observed from

Table III Summary of Peroxide and Initiator Contents for All Six Product Lots

Lot #	Protein Conc. (mg/mL)	Oxidant CAo	Initiator CRo		Linear fit from Eq. 1	
		(mM)	(mM)	Fe equivalent (ppm)	R^2	Slope
1	10	1.1E-02	2.4E-03	0.13	0.9987	0.713
2	10	1.9E-02	5.7E-04	0.03	0.9820	1.021
3	6	2.5E-02	2.2E-04	0.01	0.9638	1.311
4	10	3.2E-02	1.8E-04	0.01	0.9699	1.661
5	6	2.6E-02	2.2E-04	0.01	0.9690	1.356
6	6	3.4E-02	5.7E-04	0.03	0.9961	1.756

the drug product lots. Our studies on polysorbate-induced Trp oxidation of Fab drug product confirmed that a high level of protective measures to keep peroxide level of the surfactant low is critical for formulation of protein therapeutics for long-term storage.

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