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The Use of a 2,2'-Azobis (2-Amidinopropane) Dihydrochloride Stress Model as an Indicator of Oxidation Susceptibility for Monoclonal Antibodies

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ABSTRACT

Protein oxidation is a major pathway for degradation of biologic drug products. Past literature reports have suggested that 2,2-azobis (2-amidinopropane) dihydrochloride (AAPH), a free radical generator that produces alkoxyl and alkyl peroxyl radicals, is a useful model reagent stress for assessing the oxidative susceptibility of proteins. Here, we expand the applications of the AAPH model by pairing it with a rapid peptide map method to enable site-specific studies of oxidative susceptibility of monoclonal antibodies and their derivatives for comparison between formats, the evaluation of formulation components, and comparisons across the stress models. Comparing the free radical–induced oxidation model by AAPH with a light-induced oxidation model suggests that light-sensitive residues represent a subset of AAPH-sensitive residues and therefore AAPH can be used as a preliminary screen to highlight molecules that need further assessment by light models. In sum, these studies demonstrate that AAPH stress can be used in multiple ways to evaluate labile residues and oxidation sensitivity as it pertains to developability and manufacturability.

Introduction

Monoclonal antibodies (mAbs) and their derivatives represent the majority of therapeutic biologics currently under development.[1,2] MAbs are desirable therapeutics because the complementary determining regions (CDRs) can be matured to bind the desired target and the crystallizable fragment (Fc) can be engineered to moderate pharmacokinetics and Fc-mediated effector functions. Traditionally, antibodies have been selected for development largely based on their ability to meet the required therapeutic profile. However, recent advances in protein engineering have allowed the generation of multiple candidates with the requisite functional profile—and candidate selection is now increasingly being influenced by other attributes such as developability/manufacturability.[3-6]

Developability assessments are typically a series of tests designed to identify or predict biochemical and biophysical liabilities that may hinder the successful manufacture or long-term stability of the drug. To accurately identify developability limitations, the stress tests used during these assessments should be robust, reproducible, and related to the stresses that may be experienced by mAbs during manufacturing, shipping, and storage. The assessments are ideally performed sufficiently early in development so that the candidate molecules can be reengineered or manufacturing processes suitably adjusted. Identification of degradation hotspots early in development also allows development scientists to monitor these sensitive sites, allowing for improvements to shelf life stability and reducing the risk of unexpected degradation later in development.[3-6]

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One chemical degradation pathway of particular interest for mAbs is the oxidation of methionine and tryptophan residues. Oxidation of tryptophan or methionine residues in complimentary-determining regions can impact binding,[7] whereas oxidation of Fc methionine residues, particularly M252 (Eu numbering), reduces affinity for FcRn,[8] which consequently decreases plasma half-life.[9] Methionine and tryptophan oxidation have been observed in proteins when exposed to light,[10-12] metal ions from steel tanks or cell culture media,[13,14] hydrogen peroxide,[13,15] reactive oxygen species generated by degraded excipients such as polysorbates,[16-19] or a combination of these species.[15,20]

To assess the susceptibility of mAbs to oxidation by different mechanisms, multiple stress models have been developed, for example, tert-butyl hydroperoxide (t-BHP),[7,21-24] hydrogen peroxide (H_2O_2),[9,21,25] Fenton stress (H_2O_2 in the presence of metal ions),[25] and UV exposure.[7,26] Recently, exposure to radical-generating azo compounds like 2,2-azobis(2-amidinopropane) dihydrochloride (AAPH) has emerged as an important oxidation model for proteins.[27,28] Azo compounds are particularly useful for oxidation studies because they are safe, easy to use and store, readily available, and thermally decompose to generate free radicals at a known and constant rate.[29] AAPH generates peroxyl and alkoxyl radicals on degradation, which preferentially oxidize methionine and tryptophan residues, respectively.[13,27] Ji et al.[13] used AAPH to oxidize tryptophan and methionine residues in parathyroid hormone, a 9.5 kDa protein, and made a side-by-side comparison of AAPH with other chemical oxidation models. AAPH stresses have since been reported for studying the oxidative susceptibility of mAbs, its correlation to solvent accessibility, and opportunities for suppression of, or selective oxidation of, Trp residues.[3,13,25,30-32]

Here, we perform a methodical study of AAPH stress across multiple mAbs and extend the applicability of the AAPH stress model by pairing it with a rapid peptide map method for the study of standard mAbs versus novel format antibodies, comparisons of chemical versus UV-light oxidative sensitivity of mAbs, and evaluation of mAb formulation components. This work demonstrates that the combination of peptide mapping and the AAPH stress allows for a small-scale, fast, predictive test of chemical oxidative susceptibility of mAbs.

Experimental Materials and Methods

Materials

MAb1, mAb2, mAb3, and mAb4 are monoclonal antibodies. BsAb5 is an aglycosylated knob-into-hole bispecific antibody.[33] A version of mAb4 containing the half-life extending Fc YTE mutation[34] was also studied to assess the impact of format on antibody oxidation by AAPH. MAbs and bispecific antibodies were purified by a series of chromatography steps including protein A affinity chromatography and ion-exchange chromatography. Antibodies were formulated in a low ionic strength sodium acetate buffer at pH 5.5 without surfactants or other excipients, unless otherwise specified.

Polysorbate 20 (PS20) and Poloxamer 188 (P188) were obtained from Croda (Edison, NJ) and BASF (Florham Park, NJ) respectively. AAPH was purchased from Calbiochem (La Jolla, CA). Trypsin (mass spectrometry grade) was purchased from Promega (Madison, WI). HPLC-grade acetonitrile and water were purchased from Fisher Scientific (Fairlawn, NJ). Water used for buffer-preparation was obtained from a Milli-Q purification system (Millipore, Bedford, MA).

General Protocol for Liquid Chromatography-Mass Spectrometry Tryptic Peptide Mapping

Oxidation of protein peptides were monitored using a tryptic peptide digest followed by liquid chromatography-mass spectrometry (LC-MS) analysis.[35] MAbs were prepared for LC-MS analysis as follows. Proteins were denatured by diluting 250 μg of each sample with a carboxymethylation reduction buffer to final concentrations of 5 M guanidine HCl, 320-mM Tris, and 2-mM EDTA, pH 8.6. Following denaturation, DTT was added (4-20 μM) and incubated at 37°C for 1 h to reduce the proteins. The samples were then carboxymethylated by the addition of iodoacetic acid in 1N NaOH to concentrations of 40-mM iodoacetic acid, then stored in the dark at room temperature for 15 min. The alkylation reaction was quenched by the addition of DTT to a final concentration of 7.5 μM. The reduced and alkylated samples were buffer exchanged (PD-10 columns or PD-MultiTrap G25 plate; GE Healthcare) into trypsin digestion buffer (25-mM Tris, 2-mM $CaCl_2$, pH 8.2). Sequencing grade trypsin was added at an enzyme to protein ratio of 1:40 by weight to digest the samples. The digestion reaction was incubated at 37°C for 4 h and then quenched by adding neat formic acid (FA) to the sample to a final FA concentration was 3.0%.

Peptide mapping was performed on a Waters Acquity H-Class UHPLC coupled to a Thermo Q Exactive Plus mass spectrometer. Separation of a 10-μg protein injection was performed on an Acquity UPLC Peptide CSH C18 column (130 A, 1.7 μm particle size, 2.1 × 150 mm) with the column temperature controlled at 77°C. Solvent A consisted of 0.1% formic acid (FA) in water and solvent B consisted of 0.1% FA in acetonitrile.

Data were processed using both Thermo Scientific PepFinder™ and Xcalibur™ software. Data were analyzed by integrating extracted ion chromatograms of the monoisotopic *m/z* using the most abundant charge state(s) for the native tryptic peptide and the oxidized tryptic peptide(s). The relative percentage of oxidation was calculated by dividing the peak area of the oxidized peptide species by the sum of the peak area of the native and oxidized peptides. The major tryptophan degradation products (typically +16 and +32, along with +4, +20, and +48 for highly oxidized sites) were summed and used to calculate tryptophan oxidation. Only methionine sulfoxide (M_{+16}) was used to calculate methionine oxidation, as methionine sulfone (M_{+32}) was not observed under these conditions. PepFinder™ analysis was performed by searching the data with a 5 ppm error tolerance. Assignments with "poor" confidence were not included in the further analysis. Where the 2 software packages provided different answers, Xcalibur™ data were reported after manual inspection of the data.

Identification and Assessment of CDR Hotspots by Liquid Chromatography-Mass Spectrometry Tryptic Peptide Mapping

Protein samples were monitored using a tryptic peptide digest followed by a 22 min liquid chromatography-mass spectrometry (LC-MS) analysis. The gradient is shown in Table S1. Full scan accurate mass data were collected at a resolution of 17,500 in positive ion mode over a scan range of 200-2000 *m/z*.

To adequately characterize each peak, a second LC-MS-MS analysis was performed on the 16-h AAPH-stressed sample and corresponding control for each molecule of interest. The gradient is shown in Table S2. Full MS-DDMS2 data were collected using a top 8 method with resolution set to 35,000 for MS scans and 17,500 for MS2 scans. Dynamic exclusion was turned off, and the precursor scan range was a 200-2000 m/z.

Selection of AAPH Stress Conditions

MAbs 1 and 2 (1 mg/mL) were subjected to a 1-mM AAPH stress for up to 48 h at pH 5.5. To assess reproducibility of the stress model, a minimum of 2 samples were produced from individually prepared 11-mM AAPH stock solutions at each time point. AAPH was diluted into mAb solutions to a final concentration of 1 mM. Samples were incubated at 40°C for the following times: 4, 8, 16, 24, and 48 h. An AAPH-free control sample was incubated at 40°C for 48 h. The free radical generating reaction was then quenched with L-methionine in a ratio of 20:1 methionine to AAPH. Samples were buffer exchanged into a sucrose-containing sodium acetate buffer by a PD-10 column (GE Healthcare) and then concentrated to a final concentration of 10 mg/mL using Amicon Ultra Centrifugal Filters (EMD Millipore). Samples were stored at −70°C until oxidation levels were assessed by an LC-MS tryptic peptide map method.

Antibody Developability Assessment Using AAPH

Comparison of AAPH Susceptibility

Following analysis of the AAPH stress time course, 16 h at 40°C was selected as the desired incubation time for comparing molecules and formulation conditions. A 16-h AAPH stress was applied to mAb1, mAb2, mAb3, mAb4 (WT and YTE), and BsAb5 to rank order oxidative susceptibility of CDR tryptophan residues and report oxidation levels in Fv and Fc methionine residues. Samples were prepared, buffer exchanged, and stored as described in the previous section. Oxidation levels were compared with a no-AAPH control incubated at 40°C for 16 h.

Comparison of Light Sensitivity

Photo-stability studies were carried out by exposing samples of mAb1, mAb2, mAb3, and BsAb5 at 10 mg/mL in glass vials to light in an Atlas SunTest CPS+ Xenon Light box (Chicago, IL) with an irradiance level of 143 Wh/m^2 UV and an illuminance level of 3.4 × 10^5 lux h visible light over a 6-h period. Control samples were wrapped in aluminum foil and placed alongside experimental vials. Samples were stored at −70°C until oxidation levels were assessed.

Formulation Assessment Using AAPH

To determine the impact of formulation conditions on antibody oxidation observed via this model, the 16-h AAPH stress was applied to mAbs 1 and 2 in the presence of different formulation excipients. The impact of buffer species was assessed by buffer exchanging mAbs into a low ionic strength histidine buffer or sodium acetate buffer, both at pH 5.5. In addition, the impact of surfactants was assessed. Stocks of 10% w/v PS20 and P188 were prepared and the surfactants were added to protein samples in the sodium acetate buffer to a final concentration of 0.02% w/v. Samples were subjected to the AAPH stress and compared with control as described previously.

Results

Selection of AAPH Stress Conditions

A time course study (4-48 h) for the AAPH stress was performed to enable selection of a stress condition that would yield good dynamic range of CDR tryptophan oxidation levels using 2 representative oxidatively sensitive mAbs. Results (Fig. 1 and Table 1) are shown here and throughout for Fv residues that demonstrated an increase in oxidation of at least 5% in at least 1 stressed sample compared with the control, as well as for the pharmacokinetically

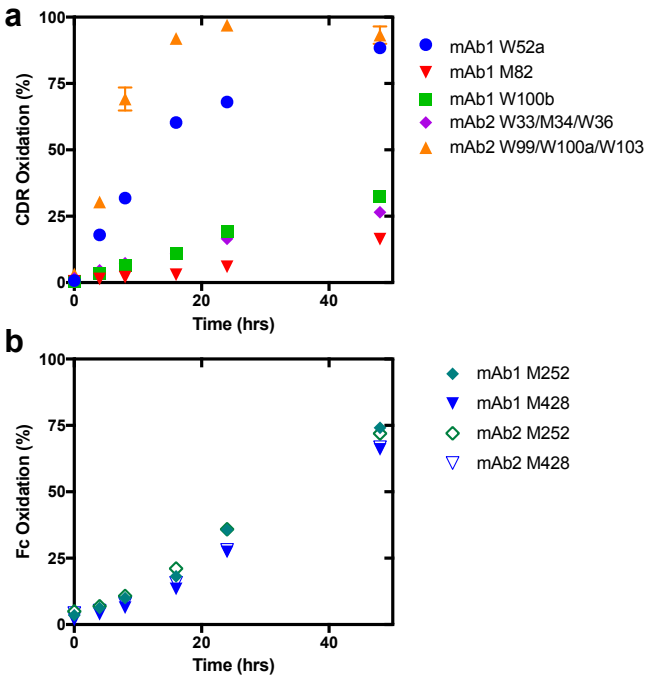

Figure 1. MAbs were subjected to 1-mM AAPH stress at 40°C for up to 48 h. Oxidation as a function of time for CDR residues is shown in (a). The rate of oxidation was rapid for W99/W100a/W103 of mAb2 (orange triangles), intermediate for W52a of mAb1 (circles), and slow for the other 3 peptides studied. The oxidation levels of Fc methionines M252 (triangles) and M428 (diamonds) for mAb1 (solid), and mAb2 (open) are shown in (b). Error bars represent the standard deviation of 2 independent samples at 0, 4, 8, and 16 h, or 4 independent samples at 24 and 48 h.

relevant Fc methionine residues M252 and M428. The oxidation levels produced by the AAPH model were quite reproducible for both Fv residues and Fc methionine residues. The average difference between replicates at each time point was less than 2%. In a subset of cases, peptides could not be adequately monitored by this method due to incomplete recovery on the column (e.g., M358) or poor ionization of large peptides. These peptides were omitted from further analysis (see Fig. S1).

Peptide mapping data indicated that 2 CDR tryptophan residues on mAb1 were sensitive to AAPH stress: W52a and W100b (Kabat numbering used for variable fragment (Fv) residues here and throughout text, see Table 1). Mab1 also had a peptide containing HC M82, a sensitive Fv methionine. For mAb2, 2 peptides, each containing multiple sensitive residues, were identified (CDR H1 W33/M34/W36 and CDR H3 W99/W100a and Fv W103). For these 2 peptides with multiple sensitive residues, the summed oxidation values for each peptide are shown throughout the manuscript. Fc Met residues 252 and 428 in both molecules were found to be sensitive to oxidative stress.

Oxidation as a function of time for W52a, W100b, and M82 in mAb1 and W33/M34/W36 and W99/W100a/W103 in mAb2 is shown in Figure 1a. Oxidation levels start low (≤4%) and increase nonlinearly with time, with final oxidation levels ranging from 16% to 93% after 48 h of AAPH exposure. For the peptides containing a single sensitive residue, the range of observed oxidation was 16% (for M82 of mAb1) to 88% (for W52a of mAb1). For the peptides with multiple potentially sensitive residues, the range in observed oxidation was similar: 26% (for W33/M34/W36 of mAb2) to 93% (for W99/W100a/W103 in mAb2). Oxidation levels of these sites after 48-h incubation at 40°C with no AAPH remained ≤4%, indicating minimal change in oxidation due to thermal incubation alone.

Table 1
Oxidation Sensitive Residues in Model Proteins

Molecule	Residue	Location	Δ Oxidation	
			AAPH (%)	Light (%)
mAb1	HC W52a	HC2	60	3
	HC M82	Fv (non-CDR)	3	<1
	HC W100b	HC3	11	<1
	M252	Fc	15	8
	M428	Fc	12	8
mAb2	HC W33/M34/W36	HC1	10	1
	HC W99/W100a/W103	HC3	87	6
	M252	Fc	16	15
	M428	Fc	11	15
mAb3	LC W94	LC3	68	4
	M252	Fc	11	11
	M428	Fc	8	9
mAb4	HC M34	HC1	5	n.t.
	HC M82	Fv (non-CDR)	5	n.t.
	HC M100	HC3	23	n.t.
	M252	Fc	23	n.t.
	M428	Fc	20	n.t.
BsAb5	HC W52	HC2	86	3
	HC M99	HC3	15	<1
	HC W103	HC3	10	1
	M252	Fc	19	7
	M428	Fc	16	7

Sites were considered sensitive if an oxidation increase of ≥5% was observed under one of the tested stress conditions in the study. The change in oxidation after exposure to 16-h AAPH or 6-h light stress for susceptible residues is shown. MAb4 was not tested (n.t.) by light.

For comparison, progression of Fc methionine oxidation is shown in Figure 1b. Oxidation of the same methionine residues (M252 vs. M428, Eu numbering used for Fc domain here and throughout text) in the 2 mAbs progressed similarly between both antibodies. For all time points in both mAbs, M252 achieved slightly higher oxidation levels compared with M428 (at 48 h, M252 was oxidized to 72%-74% in the 2 molecules vs. 66%-67% for M428).

Using the time course data, a 16-h stress was selected to enable facile comparisons between molecules and between excipient conditions. This stress time was selected to maximize the range of oxidation observed, ideally yielding quantifiable oxidation of less sensitive residues but without completely saturating more sensitive sites. Although the oxidation level of W99/W100a/W103 in mAb2 was near saturation after 16 h, the tryptophan oxidation levels observed in the rest of this study demonstrated an acceptable dynamic range.

Antibody Developability Assessment Using AAPH

Comparison of AAPH and Light Sensitivity

The 16-h AAPH exposure was used to compare the chemical and light oxidative susceptibility of Fv and Fc residues on 4 mAbs and 1 BsAb. Of the total 64 tryptophan residues in these molecules, 10 were found be sensitive by the AAPH model (Fig. S1). Similarly, of the total 28 methionine residues in these molecules, 16 were found be sensitive by the AAPH model (Fig. S1, note that the peptide containing the potentially sensitive Fc methionine M358 site was incompletely recovered and results were not determined for this site). The oxidation levels of the sensitive Fv residues (i.e., those that exhibited greater than ~5% increase in oxidation on AAPH exposure) were largely located in CDRs (Table 1).[3,30] As intended, a wide range of sensitivities was seen among the sensitive Fv residues using the optimal AAPH stress duration of 16 h (3%-87% increase in oxidation), allowing ranking of oxidative susceptibility. The range of Fc methionine oxidation was more limited: 11%-23% for M252 and 8%-20% for M428 (Table 1).

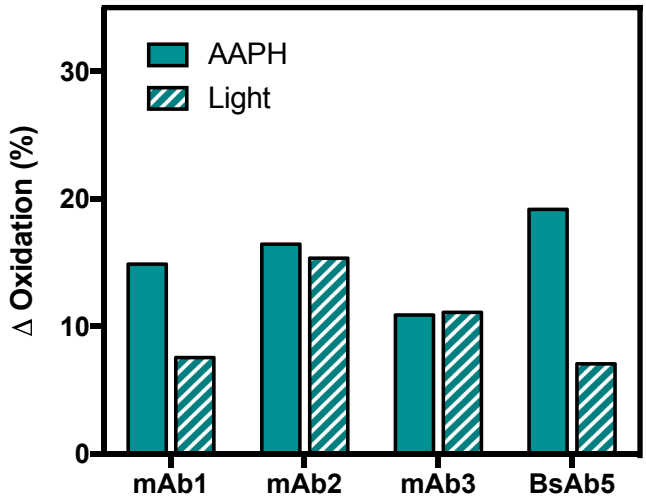

Figure 2. Changes in Fc methionine (M252) oxidation levels after 16 h of AAPH stress or 6 h of light stress for 4 molecules. Note that mAb4 was not tested for light stress due to limited material availability and is therefore not shown.

The selected light stress induced similar levels of Fc methionine oxidation as the AAPH stress model (Fig. 2, note mAb 4 not tested for light stress due to limited sample availability). M252 oxidation was 11%-23% for the different mAbs under AAPH stress and 7%-15% for light stress. Similarly, M428 oxidation levels were 8%-20% by AAPH and 7%-15% by the light stress model. In contrast to the Fc methionine oxidation, the Fv residue oxidation in the light stress model was much more modest (≤6% change) than the AAPH oxidation range (3%-87%), despite the relatively harsh light stress selected (Table 1).

While only residues sensitive to AAPH showed significant oxidative sensitivity under light stress, the converse was not true—not all AAPH-sensitive sites were light sensitive. For example, one of the most sensitive Fv methionines observed by AAPH stress, HC3 M99 of BsAb5 (15% oxidation by AAPH) showed negligible oxidation under light stress. Furthermore, the rank order of the sites by light and AAPH sensitivities were different. For example, for mAb 1, AAPH sensitivity decreased in the order W52a (60%) > M252 (15%) ≥ M428 (12%) ~ W100b (11%) > M82 (3%) whereas light sensitivity decreased in the order M252 and M428 (both 8%) > W52a (3%) > W100b and M82 (both <1%). Looking at a single site across mAbs also showed that rank ordering of AAPH and light susceptibility did not correlate. For example, M252 light oxidation was lowest for BsAb5 of the tested molecules but had the highest AAPH susceptibility (Fig. 2).

These results demonstrate that AAPH is a valuable model to identify individual tryptophan and methionine residues by chemical oxidation risk. However, chemical oxidation risk does not necessarily imply light oxidation risk: residues may be sensitive to chemical oxidation but relatively insensitive to light-induced oxidation. Once the AAPH model identifies chemically sensitive molecules, additional studies are required to understand the light risk of each molecule.

AAPH Assessment of Antibody YTE Mutation Modulated Effector Function

A comparison of wild-type (WT) mAb4 with a variant containing the Fc YTE mutation (M252Y/S254T/T256E) was performed to measure the impact of removing an Fc methionine on oxidation levels of other potentially susceptible residues. Figure 3 shows a comparison of Fv and Fc residues in the WT version of mAb4

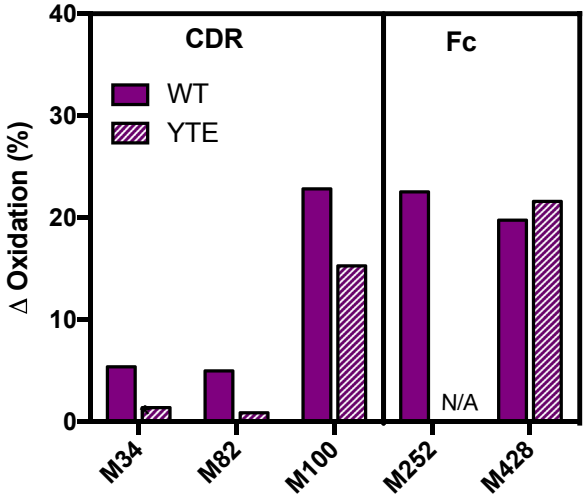

Figure 3. CDR and Fc oxidation of the wild-type (WT) version of mAb4 compared with the YTE mutant after 16 h of AAPH stress at 40°C. Note: in the YTE mutation, M252 is replaced with Tyr (Y252).

compared with the YTE mutant. For Fv methionine residues CDR H1 M34, HC M82, and CDR H3 M100, the oxidation level decreased by 4%-8% in the YTE mutant compared with the WT. Differences in oxidation between the YTE mutant and WT in the common Fc methionine M428 were small (2%). A comparison of the peptide map profiles is shown in Figure S2.

Influence of Formulation on AAPH Stress Model

MAbs 1 and 2 were selected to study the impact of formulation excipients on AAPH-induced oxidation. Figure 4 shows a comparison of the impact of buffer species on oxidation levels: a histidine-based buffer was compared with sodium acetate. The oxidation levels of Fv tryptophan and methionine residues were consistently higher in sodium acetate compared with the histidine buffer (Fig. 4a). Figure 4b shows the impact of buffer species on Fc methionine residues. The sensitivity of M252 was increased by 3%-4% in histidine compared with sodium acetate, whereas negligible differences in oxidation levels were observed for M428 as a result of buffer species.

A similar study was used to compare the impact of surfactant on oxidation levels (Fig. 5). Addition of 0.02% w/v PS20 or P188 in the buffer modestly increased oxidation of all the sensitive Fv residues by similar levels (2%-10%, Fig. 5a). PS20 and P188 had a greater effect on Fc methionine oxidation, 6%-16% for PS20 and 24%-42% for P188 (Fig. 5b).

Discussion

Selection of AAPH Conditions

A successful model for identifying oxidatively sensitive residues must be able to both differentiate and rank the sites most susceptible to oxidation. A time course study was performed to select the appropriate model conditions. At the selected 16 h time point (under fixed protein, AAPH, and excipient conditions), only the most sensitive residues were oxidized and the range of oxidation showed an appropriate dynamic range for risk ranking. The susceptible Fv residues were located in—or adjacent to—the flexible and solvent-exposed CDR loops (Table 1), a finding consistent with previous reports that free radical–induced oxidation correlates with surface exposure/solvent accessibility.[3,30] The most sensitive tryptophan residues in the studied molecules had increases in oxidation levels of 10%-87%, allowing for clear differentiation of the most sensitive sites. The increases in oxidation level for sensitive CDR methionine residues was lower (3%-23%) but still indicated significant sensitivity differences between residues on each protein (Table 1). Most mAb framework residues were found to be insensitive to AAPH stress under the selected conditions, with the notable exception of the Fc methionines previously reported to be oxidatively sensitive[8,9] (Table 1 and Fig. S1). Collectively, this shows that AAPH indicates sensitive residues, rather than an indiscriminate stress and therefore supports the use of the AAPH model as a developability assessment.

Fc methionine oxidation by AAPH was not dependent on tryptophan oxidation under the selected conditions as evidenced by oxidation of methionine residues in mAb4, which did not contain susceptible tryptophan residues. This observation supports previous mechanistic work that shows AAPH-derived reactive oxygen species can directly and independently oxidize methionine and tryptophan residues (vide infra and Ref. 13). Under the selected conditions, Fc methionine oxidation was similar across molecules (11%-23% for M252, 8%-20% for M428), consistent with the similar structural and chemical environment of these constant domain residues. M252 and M428 oxidative sensitivity trended together with the most susceptible Fc methionine residues being on mAb4 and BsAb5 (an aglycosylated knob-into-hole bispecific antibody). The modest range in Fc oxidative susceptibility between the molecules may be due to subtle surface exposure differences of the Fc methionines due to structural flexibility imparted by Fc-Fv interactions, as well as aglycosylation[36] and knob-into-hole feature for Bsp5. Taken together, this suggests that AAPH can highlight differences in oxidative susceptibility even between candidates with high sequence homology, allowing for oxidative risk ranking performed as part of developability assessments.[3,6,30]

If warranted (e.g., as part of later development), further differentiation of the identified susceptible sites could be performed by tuning AAPH stress conditions (different time/temperature/concentrations). It is worth noting that forcing very high levels of oxidation is not recommended as it may alter the physical stability of the molecule, potentially exposing residues that would otherwise be protected from oxidizing species. Yan et al.[37] have shown that highly oxidized CDR methionine oxidation may slightly perturb the antibody structure. Liu et al.[38] and Koulov et al. have shown that complete Fc methionine oxidation can significantly impact the physical stability of antibodies, resulting in increased aggregation propensity (and, correspondingly, that less aggregation is observed with lower levels of Fc methionine oxidation).[31] Antibody integrity under the conditions used in this study is supported by the lower levels of Fc methionine oxidation and the observation that only surface-exposed tryptophans oxidize.

Comparison of AAPH and Light Sensitivity

In this study, the relationship between chemical oxidative stability and photo-stability of tryptophan and methionine residues in mAbs was investigated. Comparisons between chemical oxidation models and photo-oxidation models in the literature have been limited. Duenas et al.[15] compared chemically induced oxidation by H_2O_2 and t-BHP to light-induced oxidation in rhVEGF. Although this study did show a correlation in oxidative susceptibility between the multiple oxidation models, their findings were limited to methionine oxidation, as H_2O_2 and t-BHP did not induce tryptophan oxidation. Similarly, Liu et al.[24] studied methionine oxidation induced by light and t-BHP in a single mAb finding cooperativity in oxidation of Fc methionines under light conditions but not under

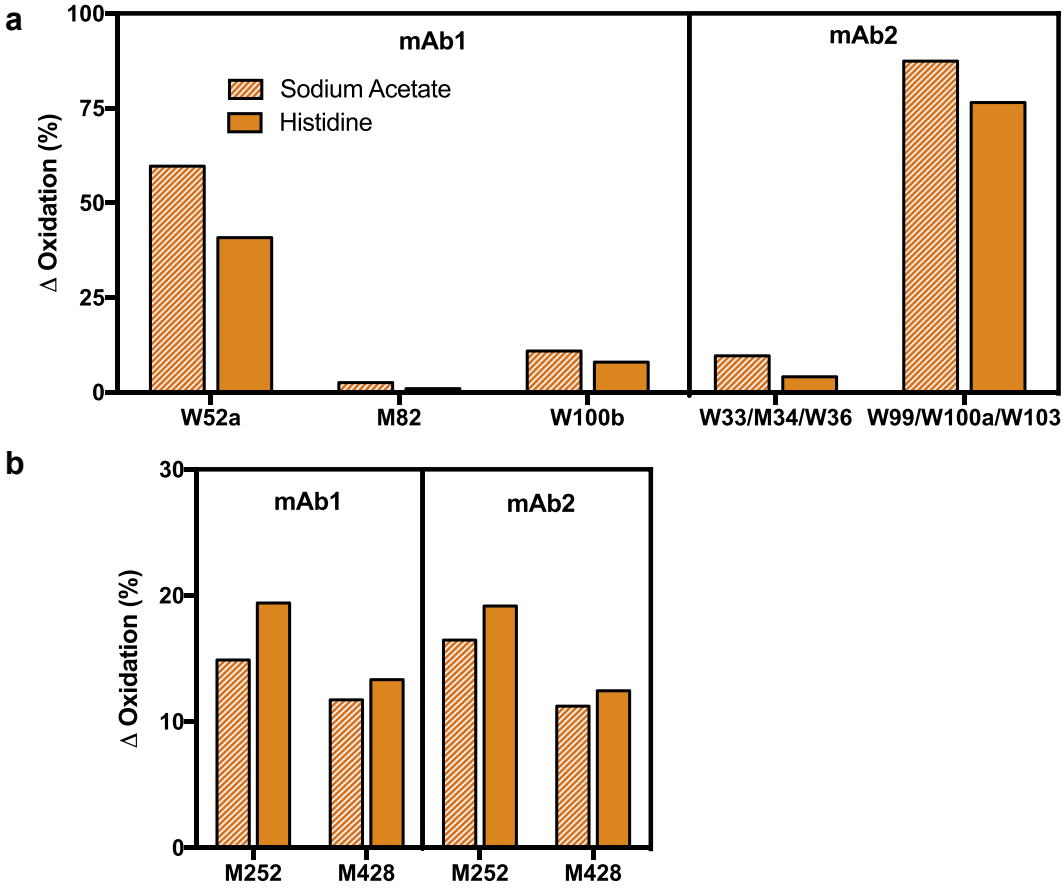

Figure 4. The impact of buffer species on oxidation of CDR residues (a) and Fc methionine (b) in mAbs 1 and 2 after 16 h AAPH stress at 40°C.

t-BHP stress. Grewal et al.[25] applied the Fenton, AAPH, and International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) recommended light oxidation stress models to assess the oxidation protection provided by different indole compounds; however, these studies were limited to a single mAb. To our knowledge, no other studies have been reported that compare the AAPH stress and UV light models to multiple mAbs with the purpose of comparing the oxidation hot spots identified by both mechanisms.

To ensure that the level of oxidation would be sufficiently high to allow for rank ordering of residues, the light stress selected here (143 Wh/m^2 UV and 3.4 $\times$ 10^5 lux-h visible light) was harsher than the conditions reported to represent worse case for typical mAb manufacturing (1.5-3 W h/m^2 UV and 4-26 $\times$ 10^4 lux-h visible light)[25,39] but still considerably less than the ICH light stress ($\geq$200 W h/m^2 of UV and $\geq$1.2 $\times$ 10^6 lux-h of visible light).[40] As expected, the tested antibodies were not highly oxidized under the selected light stress conditions (oxidation ranged from 7% to 15% for Fc methionine residues and <1% to 6% for the CDR residues).

Every light-sensitive residue in this study was observed to be sensitive to AAPH. However, not every AAPH-sensitive residue was found to oxidize under the selected light stress. Furthermore, there was poor correlation between rank order of susceptibility between 2 the stresses for both tryptophan and methionine residues, suggesting that analysis of light stress may be important in further assessing the developability of molecules with AAPH-sensitive sites. One contributing factor to the disparity for rank order of methionine sensitivity between the 2 stress models could be related to the interplay between tryptophan and methionine oxidation under light stress. It has been reported that tryptophan residues generate H_2O_2 under UV light exposure which can subsequently oxidize methionine residues[39]—and therefore tryptophan light sensitivity rank order may influence methionine light sensitivity rank order. The data collected in this study tentatively support a correlation between the rank order of Fc methionine and tryptophan sensitivity to light stress (mAb2> mAb3 > mAb1, BsAb5 for light stress for both, see Table 1 and Fig. 2), but a higher dynamic range of tryptophan light oxidation would be needed to confirm this observation.

Taken together, the study suggests that a molecule with no changes under AAPH conditions may not warrant further light studies. However, AAPH cannot completely replace the need for relevant light studies for molecules with oxidatively sensitive residues. The combination of AAPH and UV light stress studies is necessary for a thorough evaluation of protein oxidation sensitivity to the majority of oxidation stress experienced during manufacturing, administration, and storage.

AAPH Assessment of Antibodies With YTE Mutation Modulated Effector Function

Despite the large success of antibody-based therapeutics for the treatment of diseases, research efforts have been made to continue to improve the clinical efficacy of antibodies through the development of novel formats. Multiple formats have recently been introduced in the clinic including bispecific antibodies, antibody fragments, antibody-drug conjugates, fusion proteins, and modulated effector function antibodies.[41] Development of these novel

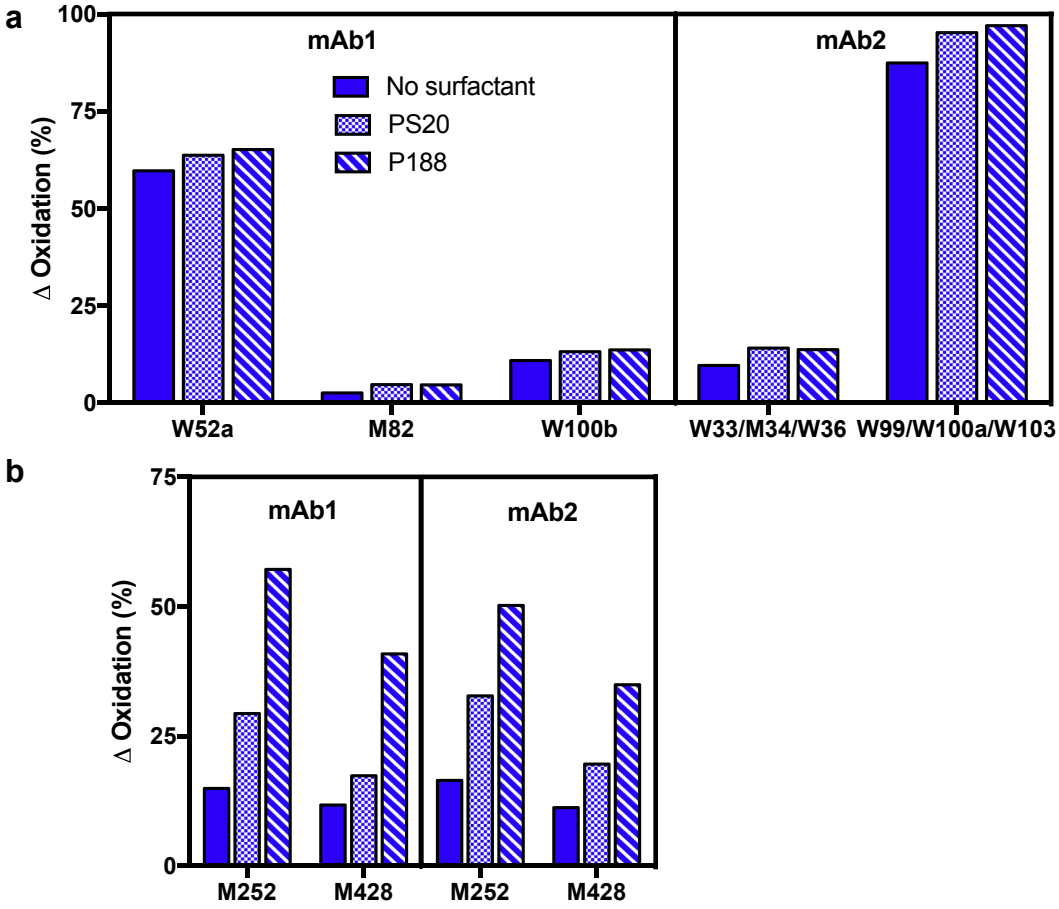

Figure 5. The impact of surfactant on oxidation levels of CDR residues (a) and Fc methionines (b) following AAPH stress (16 h, 40°C) was assessed for mAb1 and mAb2 in the sodium acetate buffer. Addition of PS20 and P188 (both at 0.02% w/v) were compared with a surfactant-free control.

molecules is often strenuous for process engineers and scientists as their complex formats can require challenging and intricate manufacturing processes. Furthermore, these molecules often exhibit biochemical stability properties that differ from a standard mAb, creating a challenge for formulation scientists. As a result, it is necessary to have appropriate and accurate stress models to assess the developability of novel format molecule to facilitate their rapid and safe advance to the clinic. Here, the AAPH stress model was applied to compare the impact of Fc mutations on oxidative susceptibility. The results show that AAPH is an effective stress model to evaluate novel format antibody developability and to determine the impact of structure and mutation on oxidative free radical stability of antibodies.

To show the utility of the AAPH model for assessing the effect of format on antibody oxidative stability, the impact of the YTE mutation on mAb oxidation was studied. The Fc YTE mutations (M252Y/S254T/T256E) increase plasma half-life[34] and result in a replacement of Fc M252 with a tyrosine residue. Levine et al.[42,43] have suggested that methionine residues serve as internal antioxidants to protect residues (20 mg/mL glutamine synthetase under H_2O_2 stress) that are critical for protein activity from oxidation, but the impact on oxidation sensitivity of the YTE mutation has not been studied. Interestingly, oxidation levels of most residues decreased in the YTE mutant compared with the WT, contrary to expectation. Several possible explanations exist for this unexpected result. Previous studies of the conformational dynamics of YTE mutant antibodies have shown that several segments of the mutant have enhanced flexibility compared with WT,[44,45] and this may translate to differences in solvent exposure of the sensitive methionine residues. Alternately, this "antioxidant" effect may only be observed at higher protein concentration. This suggests that M252 may not be critical for protecting mAbs from chemical oxidation damage at low concentrations, but additional studies are warranted, such as comparing a WT and YTE mutant antibody with sensitive tryptophan CDR residues. Furthermore, we can conclude that the AAPH stress is a useful tool for studying novel format antibodies and can potentially be used to help investigate structure-function relationships related to antibody chemical stability.

Influence of Formulation on AAPH Stress Model

The AAPH model has been used to screen antioxidants for formulation development studies in the literature, with the focus being primarily on tryptophan, methionine, and indole derivatives.[13,25] Little has been done to examine the impact of other common formulation components that are known to affect protein oxidation, such as histidine and surfactants.

In metal-catalyzed oxidation, histidine has been reported to act as an antioxidant by quenching reactive oxygen species.[46,47] However, in photo-oxidation studies, histidine is known to produce photosensitizers, which can increase mAb oxidation levels.[48] Here, under the AAPH stress conditions, the histidine buffer resulted in decreased CDR residue oxidation levels and modestly increased Fc methionine residue oxidation (Fig. 4). To better understand this observation, a more detailed look at the AAPH

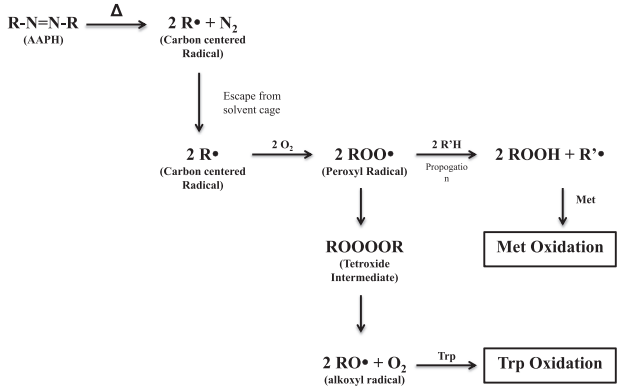

Figure 6. Schematic of the thermal decomposition of AAPH resulting in tryptophan oxidation by alkoxyl radicals and methionine oxidation by alkyl hydroperoxide (figure adapted from Ji et al.[13] and Werber et al.[27]). Histidine, like free tryptophan, likely reacts with alkoxyl radicals, absorbing the free radical stress responsible for oxidizing tryptophan residues in antibodies under some conditions.

mechanism was undertaken. AAPH is thought to generate a peroxyl radical that can undergo 2 distinct propagation reactions: to form an alkyl hydroperoxide- and carbon-centered radical (Fig. 6 upper pathway) or an alkoxyl radical and oxygen (Fig. 6 lower pathway).[27] The alkyl peroxide would be capable of nucleophilic oxidation of methionine residues, whereas that alkoxyl radical could mediate free radical oxidation of tryptophan residues.[13] In this light, the results of the study suggest that under the tested conditions histidine, like free L-tryptophan,[13,25] can alter the balance of tryptophan and methionine oxidation, potentially by quenching the alkoxyl radicals that induce tryptophan oxidation but not the peroxyl radicals. On a molecular level, the electron-deficient alkoxyl radical species could react with the electron-rich aromatic ring of histidine, resulting in the absorption of the oxidative stress. As such, AAPH with the presence of free histidine, may be skewed toward an alkyl hydroperoxide-induced stress—similar to tert-butyl hydroperoxide stress, a known Met-specific oxidant.[7,13] The extent of this effect would be impacted by histidine, AAPH, and protein concentration.

Based on these results, it is recommended that histidine buffers be avoided during mAb developability assessments to assure appropriate detection of oxidatively sensitive residues. This is to assure that inherent oxidation sensitivity of the residues can be detected during the assessments but does not preclude histidine buffers being used in clinical or commercial mAb formulations. If it is desirable to understand the relative risks for mAbs intended for formulation in histidine buffers, it is recommended that the concentrations of histidine and protein in such developability assessments be held constant so that the impact on oxidation sensitive residues is consistent between molecules for ranking purposes.

Like histidine, polysorbates used to provide agitation and processing protection in many mAb formulations have been observed to impact antibody oxidative stability.[16-18] Multiple reports have suggested that polysorbate can auto-oxidize during storage to form alkyl hydroperoxides and other species that can subsequently lead to increases in mAb oxidation.[16,18] Consistent with this literature, the studies here show that addition of AAPH (which mimics and likely propagates polysorbate degradation) to a PS20-containing buffer does indeed increase methionine oxidation (and more minimally, tryptophan oxidation) in the tested mAbs. The observation that methionine residues are more strongly impacted by the presence of PS20 in the AAPH model than tryptophan residues is consistent with published mechanisms of PS20 degradation which contain alkyl hydroperoxides, species which can directly mediate methionine oxidation.[49] When combined with agitation risk assessments, the AAPH model here may provide formulation scientists an assessment of how to balance oxidative risk with the agitation protection provided by PS20 or other surfactants.[50] Although auto-oxidation of poloxamers have not been as thoroughly studied, thermally induced oxidation of neat poloxamer 407 in air has been shown to produce both alkoxyl and peroxyl radicals.[51] The results shown here indicate that P188 may itself be oxidized by AAPH, which in turn, may produce reactive species that further increase protein oxidation. Therefore it may be that the risk of poloxamer-induced oxidation of mAbs is less dependent on the P188 raw material (as auto-oxidation of P188 under typical conditions has not been reported) and more dependent on the risk of exposure to exogenous oxidants (which may initiate P188 degradation resulting in reactive oxygen species that mediate oxidation of the mAb). Additional studies are warranted to better understand the impact of P188 on oxidation susceptibility under additional oxidation models such as H_2O_2, Fenton stress, light, and long-term storage, and under different protein: poloxamer ratios.

Cumulatively, this work underscores that oxidation is dependent not only on inherent mAb properties but also on the formulation conditions and that the AAPH model can be helpful in assessing oxidative sensitivity between different formulation conditions for a given mAb. One note of caution is that the relative rates of AAPH degradation mechanisms are pH sensitive[27] and therefore AAPH should not be used to model stress between formulations of different pHs.

Conclusion

Our studies demonstrate that oxidation achieved by AAPH is quite reproducible and tunable, which enables its use for a number of applications in mAb developability and formulation development. While AAPH is not expected to mimic the exact conditions experienced during mAb manufacture and storage, it provides relative oxidative risk rankings. We show that the AAPH model can be used to evaluate the oxidation susceptibility of novel format antibodies such as bispecific antibodies and modulated-effector function antibodies in addition to standard mAbs. Furthermore, we show that the model, when paired with a rapid peptide map, can provide formulation scientists with a significant understanding of the oxidation sensitivity of a molecule, enabling decisions of whether the molecule is sufficiently stable for manufacturing or requires mAb reengineering, lyophilization, or antioxidant formulations. Finally, we have expanded on previous uses of AAPH for formulation development to show the impact of formulation to free radical stress, reinforcing the importance of evaluating the susceptibility of formulation components to oxidative stress, and the impact that degradation has on antibody stability. Collectively, this work shows how AAPH can be used for developability assessments as well as formulation development.

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