Journal of Pharmaceutical Sciences xxx (2017) 1-9

Contents lists available at ScienceDirect

Journal of Pharmaceutical Sciences

journal homepage: www.jpharmsci.org

Pharmaceutical Biotechnology

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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ARTICLE INFO

Article history:
Received 31 July 2017
Revised 6 September 2017
Accepted 19 September 2017

Keywords:
AAPH
monoclonal antibodies
oxidation
free radicals
forced degradation
UV light exposure
photo-oxidation
developability

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]

This article contains supplementary material available from the authors by request or via the Internet at https://doi.org/10.1016/j.xphs.2017.09.022.

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https://doi.org/10.1016/j.xphs.2017.09.022
0022-3549/

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 (LC-MS) Tryptic Peptide Mapping

Oxidation of protein peptides were monitored using a tryptic peptide digest followed by 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.