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High throughput peptide mapping method for analysis of site specific monoclonal antibody oxidation

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ABSTRACT

Oxidation of therapeutic monoclonal antibodies (mAbs) often occurs on surface exposed methionine and tryptophan residues during their production in cell culture, purification, and storage, and can potentially impact the binding to their targets. Characterization of site specific oxidation is critical for antibody quality control. Antibody oxidation is commonly determined by peptide mapping/LC–MS methods, which normally require a long (up to 24 h) digestion step. The prolonged sample preparation procedure could result in oxidation artifacts of susceptible methionine and tryptophan residues. In this paper, we developed a rapid and simple UV based peptide mapping method that incorporates an 8-min trypsin in-solution digestion protocol for analysis of oxidation. This method is able to determine oxidation levels at specific residues of a mAb based on the peptide UV traces within <1 h, from either TBHP treated or UV light stressed samples. This is the simplest and fastest method reported thus far for site specific oxidation analysis, and can be applied for routine or high throughput analysis of mAb oxidation during various stability and degradation studies. By using the UV trace, the method allows more accurate measurement than mass spectrometry and can be potentially implemented as a release assay. It has been successfully used to monitor antibody oxidation in real time stability studies.

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1. Introduction

Post-translational modifications (PTMs) often occur on therapeutic proteins leading to heterogeneity of molecules [1–3]. The common forms of PTMs include deamidation, isomerization, and oxidation, incomplete disulfide bond formation, glycosylation, glycation, N-terminal glutamine/glutamate cyclization, and C-terminal lysine processing [1,3–6]. PTMs can impact the biological function of the proteins [2]. Monoclonal antibodies (mAbs) are a class of biologics that have a great stability, and have been approved for treating a wide variety of diseases including cancers, and immunological disorders [7–10]. Therapeutic mAbs are highly heterogeneous and contain a variety of PTMs [1,3]. Among observed modifications, oxidation of methionine, tryptophan, and tyrosine residues on the exposed surface is a common occurrence that could occur during any stage of antibody production, purification, formulation and storage [1,3–5,11,12].

The consequence of oxidation greatly depends on the location of the oxidized residues. Oxidation of residues located in the complementarity-determining region (CDR) of an antibody can negatively impact the binding to the target antigens. Oxidation, specifically of methionine and tryptophan side chains, has been shown to result in conformational changes [13,14], to affect antibody binding to Fc receptors [5,11] and antigens [15], and to impact mAb stability and half-life [14,16]. Oxidation of the susceptible Met residues may also result in an increase in immunogenicity [17]. Therefore monitoring oxidation is particularly important for the quality control of mAbs during drug development, production, and storage [2].

Hydrophobic interaction chromatography (HIC) [18], reverse phase-HPLC coupled with Fabricator digestion [19], and Protein A chromatography [20] have been utilized for protein oxidation analysis. Even IEX chromatography has been implied in resolving the oxidized mAb species [1,21]. Recently, mixed mode SEC chromatography has been used for analysis of oxidized methionine and tryptophan variants of mAbs [22,23] and IgG4 half

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molecule exchange [24] using certain types of SEC columns. The HIC and mixed mode methods take advantage of the change in the hydrophobicity of oxidized protein in the separation of oxidized species from native species. Most methods afore-mentioned need method development for each antibody and are limited in analyzing only the overall levels of oxidation and are unable to determine the oxidation sites. Thus far the most common method to characterize site specific oxidation in proteins is peptide mapping via RP-HPLC–MS, which is a very effective method for identifying/locating and quantitating PTMs in protein molecules [12,25–28]. The oxidation determination is performed based on the UV or extracted ion chromatographic peak areas of the oxidized and non-oxidized peptides [29]. In this general method, proteins of interest are first denatured in guanidine HCl or urea, reduced with DTT or other reducing agents, followed by alkylation of the peptides [17,29,30]. The treated proteins are digested with trypsin or other proteases, and generated peptides are separated by RP-HPLC for mass spectrometry analysis. The sample digestion step for the method is a time consuming process that could take up to 24 h at 37 °C, a step that could potentially induce artifacts [31] such as asparagine deamidation [4,32–34], N-terminal glutamine cyclization [35], and methionine oxidation [36,37]. The long preparation time also hampers the high throughput analysis for a large number of samples in the forced degradation studies and process development. A simple and fast peptide mapping method for routine analysis that is independent of the mass spectrometry component is highly desirable to monitor site specific oxidation.

In this paper, we present a rapid and simple UV-based peptide mapping method that incorporates an ultrafast trypsin digestion procedure for general quantitative analysis of mAb site specific oxidation. Most ultrafast proteolytic digestions use immobilized enzyme or microwave or ultrasonic-assisted procedures [31]. This is not amenable to routine analytical settings in the pharmaceutical industry due to the difficulty of achieving consistency and reproducibility. We utilized an 8-min reproducible, in-solution digestion procedure to generate peptides of mAbs, and used the optimized RP-HPLC condition to separate oxidized variants from native peptides. With no need to use expensive reagents and mass spectrometry, this method is able to determine oxidation levels at specific residues of a mAb within <1 h. Following the initial characterization by MS, this simple method can be readily implemented in routine or high throughput analysis by using UV absorbance for monitoring mAb oxidation during stability and forced degradation studies. By using the UV trace, the method provides better accuracy measurement of oxidation than the extracted ion counts from MS measurements.

2. Materials and methods

2.1. Chemicals

Ammonium bicarbonate (cat # 09830-500G) was purchased from Fluka; urea (cat # 33247-250G) was from Sigma-Aldrich. Dithiothreitol (DTT) (cat # 20291) was purchased from Pierce. Sequencing grade modified Trypsin (cat # V5113) was from Promega. Water (cat # W6-1) and acetonitrile (cat # A955-1) were from Fisher chemical; Trifluoracetic acid (TFA) (cat # 28904) was obtained from Thermo Scientific.

2.2. Monoclonal antibodies (IgGs)

Antibodies mAb A (IgG1) and mAb B (IgG4) were produced in CHO cells, and purified via a multiple-column purification process including Protein A chromatography. Antibodies were kept in their respective buffers for stability. The protein concentrations were determined by UV absorbance at 280 nm, and mAbs were provided at 25 mg/mL.

2.3. Forced degradation

Stressing of antibodies were performed according to our previous procedure [22].

2.3.1. Light stress

Each IgG under investigation (200 μL per exposure condition) was aliquoted into quartz cuvettes or HPLC glass vials. Light exposure was conducted in a photostability chamber (Caron model 6545-2) at 25 °C. The levels selected ranged from 0X to 2X light exposure, where 1X = light exposure level equivalent to 1.2 million lux hours of white light and 200 W h/square meter of UV light based on ICH guidelines. After each designated exposure level was reached, the samples were covered and placed inside a dark box.

2.3.2. TBHP treatment

mAb A was diluted to 5.0 mg/mL and incubated at 25 °C for 0, 3, and 24 h in an HPLC vial in the presence of 1.0% TBHP. The antibody samples were buffer exchanged into their respective formulation buffer with a 5 mL Zeba spin desalting column (7 kDa MWCO). The column was first equilibrated with formulation buffer three times, and the entire sample (4 mL or 6 mL) was loaded into the column and centrifuged for four minutes at 1000*g*.

2.4. Ultrafast tryptic digestion

Digestion of mAb samples was performed by our previous method [38]. mAb samples were diluted to 6 mg/mL in 20 mM ammonium bicarbonate, pH 7.8. 5 μL of diluted mAb sample (30 μg) was thoroughly mixed with 10 μL of the reduction buffer (8 M urea in 20 mM ammonium bicarbonate and 10 mM DTT), and denatured and reduced at 70 °C for 3 min in a thermo-mixer. Subsequently, the reduced antibodies were digested by adding 100 μL of trypsin (6 ng/μL) in 20 mM ammonium bicarbonate at an enzyme to protein ratio of 1:50. The samples were incubated at 37 °C for 5 min. The digestion was terminated by mixing with 4.8 μL of 20% TFA. The digested samples (~120 μL) were stored at −80 °C until analysis by RP-HPLC or LC–MS.

2.5. LC–MS condition

Digested samples were analyzed by LC on Waters ACQUITY UPLC coupled with Waters Xevo G2 QTOF MS system. For LC analysis, 30 μL (~7.5 μg) of digested sample was injected and separated on a HALO ES-C18, 2.1 × 150 mm, 2.7 μm (Part No.: 92122-702) column at 0.4 mL/min at 60 °C. The peptides were eluted by mobile phase gradients as follows. The initial gradient was 88% mobile phase A (H_2O) and 2% mobile phase B (100% acetonitrile) for 1 min. Mobile phase C (1% TFA) was 10% for the entire LC run. Mobile phase B was increased to 18% in 14 min, then increased from 18% to 40% in 30 min, and then to 45% in 5 min. In final wash step, mobile phase B was increased from 45% to 88% in 3 min and then hold at 88% for 4 min. The column was equilibrated by decreasing mobile phase B to 2% in 1 min and keeping at 2% for 7 min. The autosampler was kept at 5 °C. UV absorbance was set at 214 nm and 280 nm. Mass spectrometry was performed on Xevo G2 QTOF and parameters were set as follows. Tune page settings were: capillary 3.0 kV, sampling cone at 35, extraction cone at 4.0, source temperature at 110 °C, desolvation temperature at 350 °C, cone gas at 20 L/h, desolvation gas at 600 L/h. Data were acquired at positive and resolution modes with calibration by using sodium iodide. GFB (glu-fibrinopeptide B) solution was used as lock mass. Data analysis was analyzed by using MassLynx and BiopharmLynx software.

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