**LC/MS Studies on Oxidation of Monoclonal Antibody Using Micro-Fluidic Based HPLC Chip Coupled to an Accurate-Mass Q-TOF**

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**Background**

Protein oxidation is one of the major concerns in biopharma industry. Proteins are sensitive to oxidation during production, formulation, storage etc. The amino acids such as methionine (Met) cysteine (Cys), tryptophan (Trp), and histidine (His) are known to be sensitive towards oxidation. It has also been demonstrated that oxidation can lead to loss of desired activity of the protein biotherapeutics. Oxidation of methionine residue in monoclonal antibody (mAb) is one of the major concerns as it can lead to product with affected binding. Forced oxidation studies either using hydrogen peroxide or t-BHP is performed in order to investigate the possible sites that are susceptible to oxidation and the effect on the activity due to these oxidations. In this study, methionine residues of monoclonal antibody were forced oxidized using t-BHP at various concentrations. Further the Met residues that were susceptible to oxidation were analyzed by generating peptide mapping using trypsin and LC/MS analysis. The commonly used DILMRPS peptide sequence was used for quantifying the degree of oxidation in the monoclonal antibody.

**Experimental**

**Materials and methods**

Immunoglobulin G (IgG) was obtained from ProMab Biotechnologies, Inc. DL-Dithiothreitol (DTT), iodoacetamide, Tris (hydroxymethyl)-ammonomethane (Tris Base), t-butyl hydroperoxide (t-BHP) were purchased from Sigma Aldrich. High quality sequence grade trypsin was obtained from Stratagenla a division of Agilent Technologies (USA).

**Oxidation of mAb using t-butyl hydroperoxide (t-BHP)**

The monoclonal antibody samples were incubated with different concentration of t-BHP in tris HCl pH=7.5 over night at ambient temperature. The concentration used for the studies were 0.1%, 0.3%, 0.6%, 1%, 1.5% and 3%. After overnight reaction the treated samples were vacuum dried and were further subjected to trypsin digestion.

**Oxidation of mAb using hydrogen peroxide**

2 μL of antibody (1 mg/ml) was diluted with 20 mL of Milli-Q deionized water, and 2 μL of H$_2$O$_2$ (50% concentration) was added to this solution for oxidation. The oxidation reaction was kept for 20 min at room temperature before further analysis.

**Trypsin digestion**

Trypsin was added to the above force oxidized mAb samples after reduction and alkylation treatment at a ratio of 20:1 (protein to protease w/w). The reaction was kept for overnight incubation at 37°C. The enzymatic activity was quenched by adding 1 μL of 10% formic acid solution. The samples were either immediately analyzed by LC/MS or stored at -80°C until LC/MS analysis.

**Instrumentation**

The Agilent 1200 Series HPLC-Chip/MS Interface (PN: G4240A) was coupled with the Agilent 6520 Accurate-Mass Q-TOF LC/MS System for LC/MS analyses.

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**Results and Discussion**

**LC Parameters:**

HPLC-Chip: 5 μm, ZORBAX 300SB-C18 (300A), 40 nL enrichment column and a 75 μm x 43 mm analytical column. Flow rate: 3 μL/min from an Agilent 1200 Series Capillary Pump (PN: G1328A) to the enrichment column and 600 nL/min from an Agilent 1200 Series Nanoflow LC pump (PN: G2226A) to the analytical column.

Solvents: 0.1% formic acid in water (A): 90% acetonitrile in water with 0.1% formic acid (B).

**Sample Loading:** With an Agilent 1200 Series Capillary Pump at 3% initial.

**Data analysis:** Spectra were recorded in positive ion and in centroid mode.

**MS Parameters:**

Vcap: 1500V and drying gas flow of 5 L/min at 320°C was used.

**Fragmentor voltage:** 175V

Data were acquired on high resolution (3200 m/z), 4GHz. For MS only mode - range 300-3200m/z.

**Data analysis:** The data obtained from LC/MS were analyzed using features contained in the following software packages: Agilent MassHunter Qualitative Analysis, Agilent MassHunter BioConfirm, and Agilent DA repressor.

**Molecular feature extraction:** The raw data (chromatograms) were processed using the Molecular Feature Extractor (MFE) algorithm within Agilent MassHunter Qualitative Analysis software.

**MS/MS spectrum assignments:** B and Y ions workflow of Bioconfirm was used to assign the MS/MS data obtained for the mAb trypsin digest.

**Define and match sequence:** Both the light and heavy chain sequences were digested using trypsin containing 2 missed cleavages with preferred modification of oxidation to generate a theoretical peptide digest list. The compounds extracted using MFE were matched against this list.

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**Conclusions**

Highly accurate peptide mass determination provided by the Agilent 6520 Accurate-Mass Q-TOF LC/MS and easy to use Mass Hunter Bioconfirm and Differential analysis software enabled the quick assignment of methionine-containing peaks with and without oxidation.

Relative percentage quantification of methionine oxidation in mAb was demonstrated:

*Met526* and *Met342* which are in the CH2-CH3 interface of mAb are more prone to oxidation than the buried methionine is noted as described in previous studies. Hence showing additional benefits in accessing the about-mass resolve Met residues in mAb.

MassProfiler Pro offered a set of statistical analysis tools which allowed easy separation of oxidized samples from un-oxidized samples.

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References:
