LCMS 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. The 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 instability. Oxidation of either using hydroperoxide 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 LCMS analysis. The commonly used DLT/MS 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-threothritol (DTH), iodoacetamide, tris (hydroxymethyl)-amino methane (Tris Base), t-butyl hydroperoxide (t-BHP) were purchased from Sigma Aldrich. High quality sequence grade trypsin was obtained from Stratagene a division of Agilent Technologies (USA).

Oxidation of mAb using t-butyl hydroperoxide (t-BHP): The monoclonal antibody samples were incubated without any concentration of t-BHP in tris HCl pH-7.5 over night at ambient temperature. The concentration of 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 tryptic digestion.

Tryptic digestion

Tryptic digestion was added to the above force oxidized mAb samples after reduction and alkylation treatment at a ratio of 20:1 (protein to trypsin units). 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.

Instruments

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.

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: G1312A) to the enrichment column and 650 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% A.

Amount of sample injected onto the chip: 50 ng of tryptic digest of protein

Sample analysis: Gradient with an Agilent 1200 Series Nanoflow LC pump as shown below.

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>A (%)</th>
</tr>
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<tbody>
<tr>
<td>initial</td>
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</tr>
<tr>
<td>10</td>
<td>30</td>
</tr>
<tr>
<td>12</td>
<td>50</td>
</tr>
<tr>
<td>14</td>
<td>95</td>
</tr>
<tr>
<td>14.10</td>
<td>95</td>
</tr>
</tbody>
</table>

MS Parameters:

Spectra were recorded in positive ion and centroid mode. 

Vcap: 1900V and drying gas flow of 5 L/min at 32°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 MassHunter Qualitative Analysis, Agilent MassHunter BioConfirm, and Agilent DA reprocessor.

Molecular feature extraction: The raw data (chromatograms) were processed using the Molecular Feature Extractor (MFE) algorithm within Agilent Mass Hunter 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 tryptic 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.

Results and Discussion

Highly accurate peptide mass determination provided by the Agilent 6530 Accurate-Mass Q-TOF LC/MS and easy to use Mass Hunter Bicofirm and Bicofirm analysis software enabled the quick assignment of methionine containing peptide peaks with and without oxidation.

Relative percentage quantification of methionine oxidation in mAbs was demonstrated.

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

References:


Figure 1: Oxidation of mAb containing 1% t-BHP

Figure 2: Tryptic digestion of the MAb sample in the presence of 2 µL of trypsin (1 mg/mL) digested for overnight incubation at 37°C.

Figure 3: The oxidized peptide mass difference spectra showing the oxidation of Met 432.

Figure 4: LC/MS analysis (20 scans) plot of the different oxidation levels of the Met residue using Agilent 6520 Q-TOF system.

Figure 5: The unoxidized peptide mass difference spectra showing the absence of oxidation of Met 432.