Rapid and Simple Determination of PTMs in Monoclonal Antibodies using an Enzymatic HPLC Approach

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Introduction

Post-translational modifications (PTMs) of therapeutic antibodies affect their function and half-life. Rapid characterization of PTMs is thus of utmost importance. Here we present the development of a rapid and simple reversed-phase HPLC based method with high enough resolution for quantification of PTMs such as Lysine truncation, Fc Methionine oxidation and high mannose glycosylation. To improve the resolution in a standard HPLC-setup IdeS (FabRICATOR®) was used to cleave the antibody into subunit domains, F(ab’)2 and scFv. For quantifying the content high mannose a sequential enzymatic digestion approach using three enzymes, Endoglycosidase S (IgGZERO®), Endoglycosidase S2 (GycINATOR®) (1) and IdeS (FabRICATOR®) were used.

Methods

Methionine-Oxidation: Reaction procedure: 9mg/ml mAb, 0.3% H2O2 in sodium acetate buffer pH 5.0. After 0h, 0.5h, 1h, 3h and 6h samples were taken from the reaction vial to a 10kDa minispin filter and ice-cold 10mM sodium acetate buffer pH 5.0 was added. Centrifugation at 10,000g for 10minutes. The remaining volume was diluted using 10mM sodium acetate buffer pH 5.0. After buffer exchange to PBS, pH 7.4, IdeS (FabRICATOR®) was added and allowed to digest for 30 minutes at 37°C.

Lysine-truncation: Cetuximab was obtained from the Swedish pharmacy (Apoteket AB) and used after removal of preservatives by desalting.
The mAb and IdeS (FabRICATOR®) were incubated for 30 min at 37°C in 10 mM PBS, 150 mM NaCl, pH 7.4.

High Mannose: The mAb and the endoglycosidases were incubated for 30 min at 37°C in 10 mM PBS, 150 mM NaCl, pH 7.4. IdeS (FabRICATOR®) was then added and co-incubated for additional 10 min.

HPLC: The reversed phase chromatography was performed on an Agilent 1290 UHPLC system using an ACQUITY BEH 300 C4 column (1.7 um, 2.1x100mm) from Waters. The column was conditioned in 0.1%TFA in MQ water at 65°C, 0.4ml/min, and the antibody fragments were eluted in a slow gradient of 0.1% TFA in 60% acetonitrile/40% isopropanol and detected using UV absorbance at 280nm.

Results

Fig 1. Oxidation of Bevacizumab/Avastin, zoomed on Fc fragment

The Unoxidized Fc peak (Blue) is reduced in size as the oxidation proceeds. A pre-peak appears already after 0.5h of oxidation (data not shown) and is further increased after 1h (Red). This peak conatins Fc with Met 252 and Met 428 oxidized. After 6h oxidation (Green) there is nothing left of the unoxidized peak and the first appearing pre-peak is significantly smaller, i.e. main part of Fc is oxidized in both positions.

Unoxidized Fc

Met 252 & Met 428 ox

Met 252 ox.

Fig 2. Lysine clipping Cetuximab/Erbitux analyzed as intact IgG by RP-UHPLC. It is not possible to detect any Lysine-truncation. Only one major peak can be seen and if both clipped and non-clipped is present this is not resolved using intact IgG… (B)Cetuximab/ Erbitux analyzed by RP-UHPLC after IdeS treatment. The Fc peak is now divided into two peaks, the first contains the c-terminal Lysine and the second is lacking the same.

Unoxidized Fc

1 hour oxidation

6 hour oxidation

Table 1. High-Mannose analysis summary.

<table>
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<th>Sample</th>
<th>EndoS</th>
<th>EndoS+IdeS</th>
<th>EndoS+IdeS+EndoS2</th>
<th>Area % EndoS</th>
<th>Area % EndoS+IdeS</th>
<th>Area % EndoS+IdeS+EndoS2</th>
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<tr>
<td>Cetuximab</td>
<td>-</td>
<td>-</td>
<td>Cetuximab + IdeS</td>
<td>58.7</td>
<td>56.8</td>
<td>19.2</td>
</tr>
<tr>
<td>Cetuximab + IdeS + EndoS</td>
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<td>43.0</td>
<td>44.4</td>
<td>15.7</td>
<td>19.2</td>
<td>14.0</td>
</tr>
</tbody>
</table>

Fig 3. High Mannose determination. Cetuximab/Erbitux analyzed by RP-UHPLC after removal of N-linked Fc-glycans using either EndoS or EndoS2. After deglycosylation the mAbs were further digested using IdeS enzyme generating scFc and F(ab’)2 fragments. Fragments were well resolved (inset) and the scFc peak was studied in more detail. A clear shift in retention time was observed when Fc-glycans were removed using either EndoS and EndoS2 compared to non-deglycosylated mAbs. The chromatographic profile were highly similar when comparing EndoS2 treated mAbs with glycosylated (non-treated) Fc apart from a shift in retention time. However, the EndoS chromatogram reveals differences in the shape and number of peaks.

Fig 4. MALDI-TOF of released glycans. To further scrutinize the difference observed from HPLC analysis the released glycans from Cetuximab was further analyzed by MALDI-TOF. The released glycan profile from the EndoS2 treatment revealed the release of high-mannose structures that were not detected when treated with EndoS. Additionally, the data suggest that EndoS has significantly lower activity on hybrid glycan structures compared to EndoS2.

Conclusions

We have utilized IdeS and its natural ability to efficiently cleave human IgGs into subunits. By reducing their size we could increase the resolution in a RP-HPLC system and thus generate a simple and rapid approach to detect and quantify PTMs such as Lysine truncation and Methionine oxidation. Since IdeS works under physiological conditions there is also a low risk for sample preparation induced modifications. The subunit domain technique was also used when determining the amount high mannose in Cetuximab. In addition to IdeS two other highly efficient enzymes with different specificities, EndoS and EndoS2, can be used simultaneously as IdeS, generating a rapid overview of the high mannose content in Cetuximab. Previously published high mannose content of Cetuximab also correlates well with our HPLC data (2). Increased incubation time or higher enzyme to mAb ratio for EndoS digestion could further improve the accuracy of this method.

References