

BioPharmaView™ Software as a Robust Tool for Automated Quantitation of Oxidation Sites in Monoclonal Antibody Characterization



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INTRODUCTION

Oxidation of methionine is one of the common post-translational modifications known to occur in recombinant monoclonal antibodies during manufacturing, formulation and the storage processes. Monitoring oxidation is of major concern because it can limit the product's clinical efficacy and/or stability. Mass spectrometry (MS) based methods are commonly used for the determination of oxidation levels, however, availability of suitable software tools for the automated quantitation of sites susceptible to oxidation in antibody characterization is of prime importance. Here we demonstrated BioPharmaView™ software as a robust tool for the detection of oxidation at different levels and for the automated quantitation of oxidation sites.

MATERIALS AND METHODS

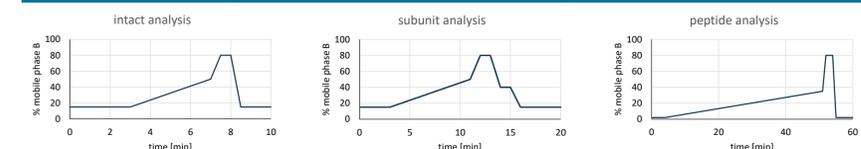
Sample Preparation:

Humanized IgG monoclonal antibodies (mAb) were obtained from the National Institute of Standards (#RM8671). Samples were incubated at 37° C for four hours (or as indicated) using different concentrations of hydrogen peroxide (H₂O₂). Methionine oxidation was subsequently quenched by adding L-methionine. Samples were split into three parts. One part was analysed directly at the intact level. The second part was digested into subunit fragments with IdeS enzyme (Genovis AB) producing a F(ab')₂ and two Fc fragments per mAb molecule prior to LC-MS measurement. The samples of the remaining part were denatured, reduced and alkylated using DL-dithiothreitol and 2-iodoacetamide (Sigma Aldrich). Trypsin (Promega) was added in a ratio of 1:30 (w:w; Trypsin:mAb) followed by an incubation at 37° C overnight. Digestion was stopped by adding formic acid and supernatant was subsequently measured using LC-MS.

HPLC Conditions:

Table 1. HPLC conditions for intact, subunit and peptide analysis.

	Intact and subunit analysis	Peptide analysis
System	SCIEX ExionLC™	SCIEX ExionLC™
Column	C4 (50x2.1mm; 1.7 µm; 30 nm)	C18 (100x2.1mm; 1.7 µm; 13 nm)
Column temp.	75 °C	40 °C
Mobile phase A	0.2 % formic acid in water	0.1 % formic acid in water
Mobile phase B	0.2 % formic acid in acetonitrile	0.1 % formic acid in acetonitrile
Flow rate	0.3 ml/min	0.3 ml/min
Injection volume	2 µl	6 µl (or as indicated)



MS/MS Conditions:

All measurements were carried out in replicates on a X500B instrument – a high resolution quadrupole-time-of-flight (Q-TOF) instrument for routine analysis (Sciex) (Fig. 1).

Intact and subunit samples were measured on TOF-MS level with optimized source parameters.

Detection of peptides was done using an information-dependent acquisition (IDA) method for MS/MS of ten candidate ions per cycle with a total cycle time of 1 s.



Figure 1. Sciex X500B Q-TOF instrument.

Software and Processing

Subsequent data analysis of all levels (intact, subunit and peptide level) was performed using BioPharmaView™ software 2.0.1. including the reconstruction of intact and subunit data. Peptide matching was done using a maximum error of 5 ppm.

RESULTS

Intact Analysis

With increasing concentration of H₂O₂, intact mAb samples showed an increasing mass shift over all glycoforms in combination with broadening of mass peaks (Fig. 2).

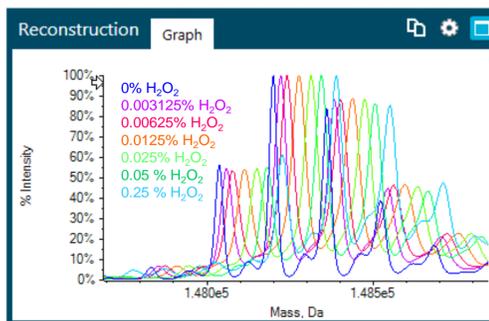


Figure 2. Reconstructed masses of intact mAb samples incubated with different concentrations of H₂O₂ at 37° C for four hours.

Subunit Analysis

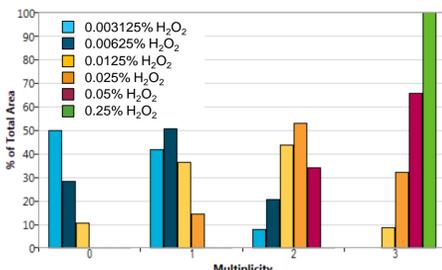


Figure 3. Automated calculation of the percentage of modification of the total reconstructed area for Fc fragment for each sample expressed as multiplicity. Samples were incubated at 37° C for four hours with different concentrations of H₂O₂.

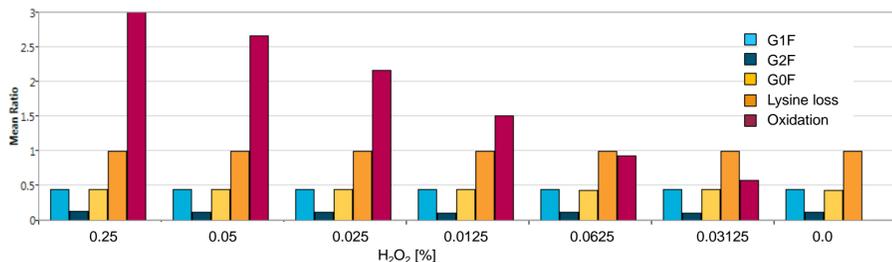


Figure 4. Automated mean ratio calculation of modifications of IdeS digested mAb in BioPharmaView™ software. Ratio calculations are based on reconstructed areas of the Fc fragment. Samples were incubated at 37° C for four hours with different concentrations of H₂O₂ (as indicated).

Reducing complexity by analysing subunits of proteins, is a fast way to have further insight into modifications. For subunit analysis the Fc fragment obtained after IdeS digestion was processed by BioPharmaView™ software. This fragment contains three methionine residues which are prone to oxidation. The control sample did not show any oxidation, whereas all three methionines were oxidised with the highest amount of H₂O₂ addition (green bar, Fig. 3). All other samples showed a mixture of mass peaks indicating 0-3 oxidation events (indicated as multiplicity in Fig. 3) per fragment. Simultaneously the software can also calculate the mean ratio of modification by averaging the results of multiplicity from Fig. 3. As expected, the oxidation (red bar in Fig. 4) is decreasing with decreasing concentration of oxidation agent, while other

modifications (lysine loss, glycosylations G0F, G1F, G2F) are constant over all samples.

Peptide Mapping

Three tryptic peptides (VGYMHWYQQKPGK, DIQMTQSPSTLSASVGDR, DTLMISR) were chosen for further analysis based on ionization and chromatographic behaviour. Oxidized peptides were matched and the percentages of oxidation were calculated automatically and reproducibly by BioPharmaView™ software (Fig. 5). The peptides showed a significant difference in the oxidation level (Fig. 6). However, the lowest detectable percentage of oxidation was determined reproducibly for all peptides (Tab. 3).



Figure 5. Evaluation of peptide DIQMTQSPSTLSASVGDR. mAb sample was incubated with 0.003125 % H₂O₂ at 37° C for four hours prior to digestion. A: Table for automated calculation of percentage of modification B: Extracted ion chromatograms (XIC) on TOF MS level for modified and non-modified peptide. C: MS trace for modified and non-modified peptide. D: MS/MS data of non-modified peptide E: MS/MS data of modified peptide

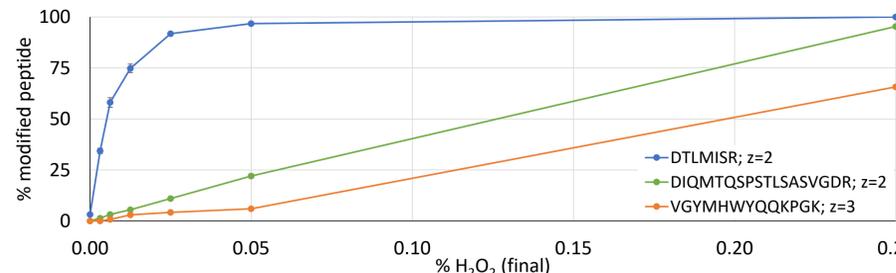


Figure 6. Mean percentages of different modified peptides based on XIC on MS level (n = 3). mAb samples were incubated with H₂O₂ at 37° C for four hours prior to digestion.

Furthermore linearity and reproducibility were tested by injection of different volumes for a sample showing low oxidation levels of around 0.7%. Both peptides - the modified and the non-modified peptide - showed a very good linearity over all injections (Fig. 7). The percentage of modification was very stable over all injections (Fig. 8).

Table 2. Mean percentages and %CV of oxidized peptides at lowest detectable oxidation level. mAb sample were incubated with H₂O₂ for four hours prior to digestion.

peptide	%H ₂ O ₂	% modified	%CV
DTLM[Oxi]ISR	0	3.2	3.1
DIQM[Oxi]TQSPSTLSASVGDR	0.003125	1.4	7.1
VGYM[Oxi]HWYQQKPGK	0.00625	2.2	7.7

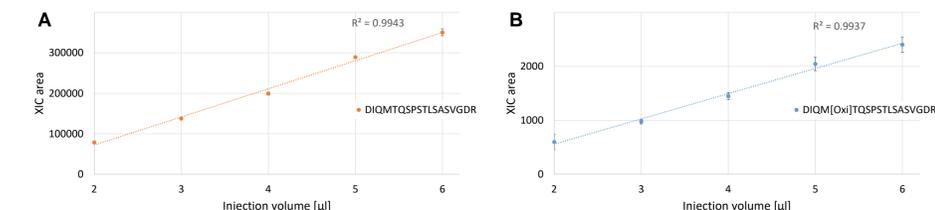


Figure 7. Linearity assessment of peptide DIQMTQSPSTLSASVGDR (z = 2). Mean XIC areas and standard deviations for non-oxidized (A) and oxidized (B) peptide are shown for different injection volumes (n = 3). mAb sample was incubated with 0.003125% H₂O₂ for 120 min prior to digestion.

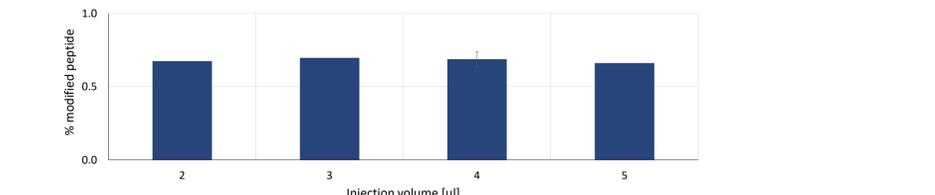


Figure 8. Reproducibility assessment of peptide DIQMTQSPSTLSASVGDR. Mean percentages of oxidized peptide based on XIC areas are shown over different injection volumes (n = 3). mAb sample was incubated with 0.003125% H₂O₂ for 15 min.

CONCLUSIONS

The results from intact, subunit and peptide mapping level were consistent over all samples: Incubation of mAb samples with higher concentrations of H₂O₂ is forcing higher oxidation levels of methionine. Intact levels can already give an indication of oxidation observed by mass shifts and peak broadening. For further insight into oxidation events, subunits can be used. Subunit analysis is offering a fast way of determination of oxidation in terms of sample preparation as well as run time and processing time, being particularly suitable for the analysis of many samples e.g. during biotherapeutic development. In order to determine the exact position of an oxidation event and for quantitation of very low oxidation levels of biotherapeutic proteins, assessment on peptide level is necessary. Data acquisition using the SCIEX X500B Q-TOF instrument combined with data processing using BioPharmaView™ software was demonstrated to be reliable tools for the automated detection of oxidation on all levels, facilitating the monitoring of critical quality attributes by automated calculation of percentages of modifications on peptide level.

TRADEMARKS/LICENSING

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