Multi-Attribute Monitoring (MAM) of oxidized NIST mAb using BioPharmaView™ Workflow

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INTRODUCTION

Monitoring and tracking multiple critical quality attributes (CQAs) of biopharmaceuticals by mass spectrometry on a peptide level is a rapidly emerging trend. Using such approaches enables the interrogation of specific sites that are susceptible to desired and non-desired post-translational modifications of amino acids that can occur during manufacturing and storage. Implementing these approaches has been challenging due to the complexity of mass spectrometry data and the limited informatic solutions available to address these needs.

Here we show the multi-attribute monitoring workflow in SCIEX BioPharmaView™ software for monitoring and quantifying post-translation modification while maintaining ease of data acquisition and data processing. NIST monoclonal antibody (mAb) reference standard was subjected to oxidative stress conditions to investigate the impact on protein post-translational modifications and verify the ability of BioPharmaView™ software to monitor these attributes with ease. Additionally, the limit of detection and ability track impurities within a sample was assessed by spiking in known peptides into the NIST mAb digest.

MATERIALS AND METHODS

Materials:
NIST reference standard was purchased from National Institute of Standards (#RM8671). Tris-HCl, iodoacetamide, DTT, methionine, hydrogen peroxide and formic acid were purchased from Sigma (St. Louis, MI, USA). ProteaseMax™ and trypsin were purchased from Promega (Madison, WI, USA). Premixed mobile phases were purchased from VWR (Radnor, PA, USA). PepCalMix was purchased from SCIEX (Framingham, MA, USA).

Sample Preparation:
NIST reference standard was incubated with hydrogen peroxide (0%, 0.003125%, 0.00625%, 0.0125%, 0.025% and 0.05%) at 37° C for four hours to induce oxidative stress. The reaction was quenched with the addition of methionine (50 mM). NIST reference standard was denatured with 1% ProteaseMax followed by reduction with DTT and alkylation by 2-iodoacetamide before digestion with trypsin at a ratio of 1:30 overnight at 37° C. Control sample was divided and a portion was spiked with concentrations of heavy labeled PepCalMix at 0.01, 0.025, 0.05, 0.1 and 0.2 % final molar concentration of NIST.

HPLC Conditions:
An ExionLC™ system with a Waters Acquity UPLC® CSH C18, 1.7 µm 2.1 x 100 mm column at 40° C with a gradient of mobile phase A: water + 0.1 % formic acid and mobile phase B: acetonitrile + 0.1 % formic acid was used at a flow rate of 300 µl/min. A 60 minute gradient was run with an injection volume of 6 µl for the samples incubated with H_2O_2 and 4 µl for the NIST samples spiked with PepCalMix.

MS/MS Conditions:
SCIEX X500B QTOF system with IonDrive™ source and Electrospray Ionization (ESI) probe was used. SWATH® acquisition was acquired using variable windows across a mass range of 350 – 2000 and TOFMS accumulation of 200 ms MSMS data was acquired over 100 to 2000 with an accumulation time of 50 ms.

Data Processing:
Data was processed using BioPharmaView™ software. NIST standard was set as the reference standard and the samples were processed in a batch for oxidation and deamidation attributes. Spiked in samples were processed in another set with NIST set as the reference standard. The peptides and their XIC areas were extracted and plotted from BioPharmaView ™ software. Limit of quantitation (LOQ) and limit of detection (LOD) were calculated.

RESULTS

OXIDATION RESULTS

NIST mAb standard was incubated with varying concentrations of H_2O_2 to determine the susceptibility of oxidation of methionine containing peptides to oxidation. All stressed samples were processed with BioPharmaView™ software and compared across the concentrations of H_2O_2 used.

	Filename	% Sequence Coverage	LC Met 4	LC Met 32	HC Met 34	HC Met 87	HC Met 101	HC Met 255	HC Met 361	HC Met 431
1	16_NIST_digest_SWATH_60min_0%.wiff2	99.2	0.00 %	0.49 %	0.00 %	0.09 %	0.00 %	1.27 %	0.00 %	0.23 %
2	25_NIST_digest_SWATH_60min_0.003125%.wiff2	95.2	0.67 %	1.15 %	42.28 %	0.24 %	0.00 %	30.88 %	36.07 %	7.97 %
3	34_NIST_digest_SWATH_60min_0.00625%.wiff2	99.2	3.99 %	1.41 %	61.39 %	0.51 %	0.00 %	53.61 %	62.87 %	18.91 %
4	40_NIST_digest_SWATH_60min_0.0125%.wiff2	99.2	6.15 %	3.16 %	82.38 %	1.89 %	0.00 %	77.58 %	75.23 %	22.54 %
5	49_NIST_digest_SWATH_60min_0.025%.wiff2	89.1	13.80 %	4.22 %	92.42 %	2.33 %	0.00 %	91.93 %	82.73 %	28.51 %
6	57_NIST_digest_SWATH_60min_0.05%.wiff2	92.9	22.43 %	7.08 %	96.47 %	3.74 %	0.00 %	97.80 %	98.87 %	42.35 %

Figure 1. 8 Peptides containing Methionine residues were monitored using Quality Attribute feature in BioPharmaView™. % Oxidation calculated for samples 1-6 which were treated with increasing amount of H_2O_2

Peptide DTLMISR was one of the peptides that changed dramatically with increasing H_2O_2 concentration.

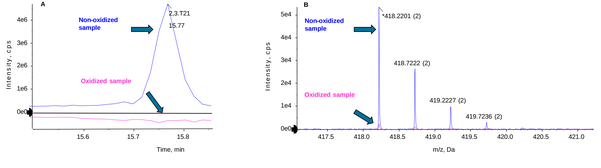

Figure 2: Chromatograms (A) and XIC spectra (B) for the non-oxidized form of peptide DTLMISR from control sample (blue) and 0.05% treated (pink) sample.

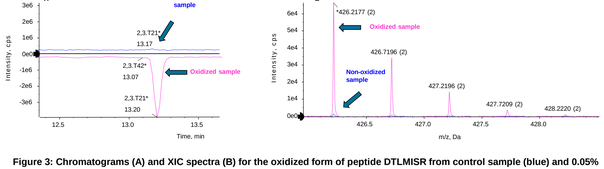

Figure 3: Chromatograms (A) and XIC spectra (B) for the oxidized form of peptide DTLMISR from control sample (blue) and 0.05% treated (pink) sample.

The level of methionine oxidation was dependent on the peptide as seen by the plot of % oxidation vs % H_2O_2 used.

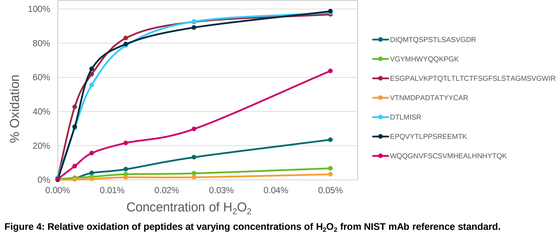

Figure 4: Relative oxidation of peptides at varying concentrations of H_2O_2 from NIST mAb reference standard.

Three of the methionine containing peptides which were all from the heavy chain of the mAb were highly susceptible to oxidation. The remaining methionine containing peptides only showed partial to little susceptibility to oxidation. One peptide showed no susceptibility to methionine oxidation using H_2O_2.

DEAMIDATION

While oxidation was expected to increase with increasing concentration of H_2O_2, other post-translational modifications were interrogated to see if the levels of H_2O_2 would also affect them. Several potential deamidation sites were monitored using BioPharmaview™ software. Of the peptides monitored, only one peptide, GFYPSDIAVEWESNGQPENNYK, showed a significant increase in deamidation .

Table 1: Deamidation was assessed at different concentrations of H_2O_2. Only a subset of tracked deamidation sites shown.

	Filename	% Sequence Coverage	SGTASVVCLLNNFYPR	VYACEVTHQGLSSPVTK	NQVVLK	FNWYVDGVEVHNAK	GFYPSDIAVEWESNGQPENNYK	NQVSLTCLVK
1	18_NIST_digest_SWATH_60min_0%.wiff2	95.2	2.19 %	0.20 %	12.02 %	10.86 %	0.00 %	7.82 %
2	25_NIST_digest_SWATH_60min_0.003125%.wiff2	95.2	2.21 %	0.21 %	10.74 %	9.74 %	5.24 %	7.85 %
3	32_NIST_digest_SWATH_60min_0.00625%.wiff2	99.5	2.38 %	0.22 %	9.88 %	10.35 %	6.87 %	7.70 %
4	41_NIST_digest_SWATH_60min_0.0125%.wiff2	95.5	2.07 %	0.16 %	11.41 %	10.48 %	12.59 %	7.13 %
5	50_NIST_digest_SWATH_60min_0.025%.wiff2	89.4	1.98 %	0.14 %	10.81 %	10.84 %	14.45 %	7.19 %
6	58_NIST_digest_SWATH_60min_0.05%.wiff2	0.0	1.89 %	0.19 %	11.19 %	11.36 %	14.47 %	7.02 %

This increase of deamidation of the PENNYK peptide only occurred that the highest concentration of H_2O_2 (0.05%) and specifically at GFYPSDIAVEWES**N**GQPE**N**NYK sites.

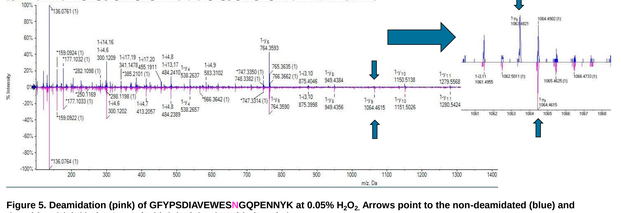

Figure 5. Deamidation (pink) of GFYPSDIAVEWESN**GQPENNYK at 0.05% H_2O_2. Arrows point to the non-deamidated (blue) and deamidated (pink) site. Zoom in (right) of the deamidation site).**

LIMIT OF DETECTION

In order to estimate the limits of detection, heavy labelled peptides were spiked at concentrations of 0.2 – 0.01% relative to the NIST mAb reference concentration. Data was batch processed in BioPharmaView™ software.

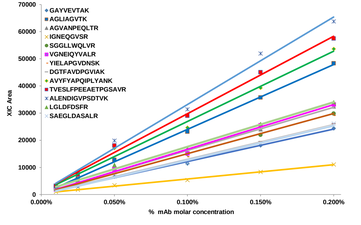

Table 2: LOQ and LOD for spiked peptides expressed as % mAb concentration

Peptide	LOQ	LOD
GAYVEVTAK	0.00116	0.00035
AGLIAGVTK	0.00081	0.00024
AVGANPEQLTR	0.00075	0.00023
SGGLLWQLVR	0.00215	0.00065
VGNEIQYVALR	0.00090	0.00027
YIELAPGVDNSK	0.00036	0.00011
AVYFYAPQIPLYANK	0.00158	0.00047
TVESLPPEAETPGSAVR	0.00083	0.00025
ALENDIGVPSDTVK	0.00053	0.00016
LGLDFDSFR	0.00043	0.00013
SAEGLDASALR	0.00403	0.00121
LDSTSIPVAK	0.00241	0.00072

Figure 6: Plotted concentration vs XIC for 13 heavy labelled peptides.

LOQ and LOD extrapolated from S/N values of lowest concentration analyzed and are shown in Table 2. As per pharmaceutical industry standards, LOQ is defined as the concentration at which S/N = 10:1, and LOD is defined as the concentration where S/N = 3:1. All S/N values calculated on raw, unsmoothed data using 1SD of noise, subtracting influence from blank injections if present. LOQ values for the peptides analyzed range from $3.6x10^{-4}$ to $4.0x10^{-3}$, expressed as a percentage of mAb molar concentration (or 3.6 to 40 ppm), and LOD values for the peptides analyzed range from $1.1x10^{-4}$ to $1.2x10^{-3}$, again expressed as a percentage of mAb molar concentration (or 1.1 to 12 ppm).

CONCLUSIONS

X500B QTOF system was tested for its ability to identify post translational modifications and to determine a limit of detection to ensure that low abundance attributes are identified and quantified. In this poster, we show

- Using oxidative stress, susceptibility of methionine residues to oxidation can be monitored using BioPharmaView™ with ease.
- In addition to the above, tracking of deamidation levels using the software indicated that the peptide GFYPSDIAVEWESNGQPENNYK was affected by increasing concentrations of H_2O_2.
- Limits of quantitation and detection, based on signal-to-noise values calculated from low concentration standards, are shown to be easily in the low ppm (parts per million) range, demonstrating the capability of the software to track low abundant attributes of biologics reliably

TRADEMARKS/LICENSING

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