Time Course Study of Oxidation Stress Using SCIEX MAM Solution with the X500B QTOF System

Zoe Zhang[2] Fan Zhang[2], Ji Jiang[2], Sean McCarthy[1]
[1]SCIEX Framingham, MA (USA), [2]SCIEX Redwood City, CA USA

During the development of a biopharmaceutical it is critical to closely monitor critical quality attributes, which may be related to safety and efficacy. Traditionally, arrays of assays were employed to track different attributes. In recent years, the concept of a multiple attribute methodology (MAM) has been introduced which has the potential to expand the use of mass spectrometry for tracking and quantification of attributes. The use of mass spectrometry plays a pivotal role in a MAM assay. It provides unparalleled insight into many aspects of a biotherapeutics which can be difficult to determine using other assays. In short, the concept of MAM consists of characterization, attribute definition and monitoring, quantification, and purity assessment via new component detection. Presented here is the use of SCIEX X500B QTOF System to monitor each potential oxidation site on an antibody. Presented here is the application of the MAM workflow for liability assessment using BioPharmaView™ 3.0 software, including the definition of attributes, defining custom calculations and setting Pass/Fail criteria for each attribute.

SCIEX X500B QTOF System

Key Feature of X500B QTOF and BioPharmaView™ software 3.0

- High resolution mass spectrometer for a wide range of biopharmaceutical applications
- Compact benchtop footprint reduces laboratory space requirements
- Easy to use hardware and software accessible for a wide range of users
- Complete MAM solution including both characterization and attribute monitoring

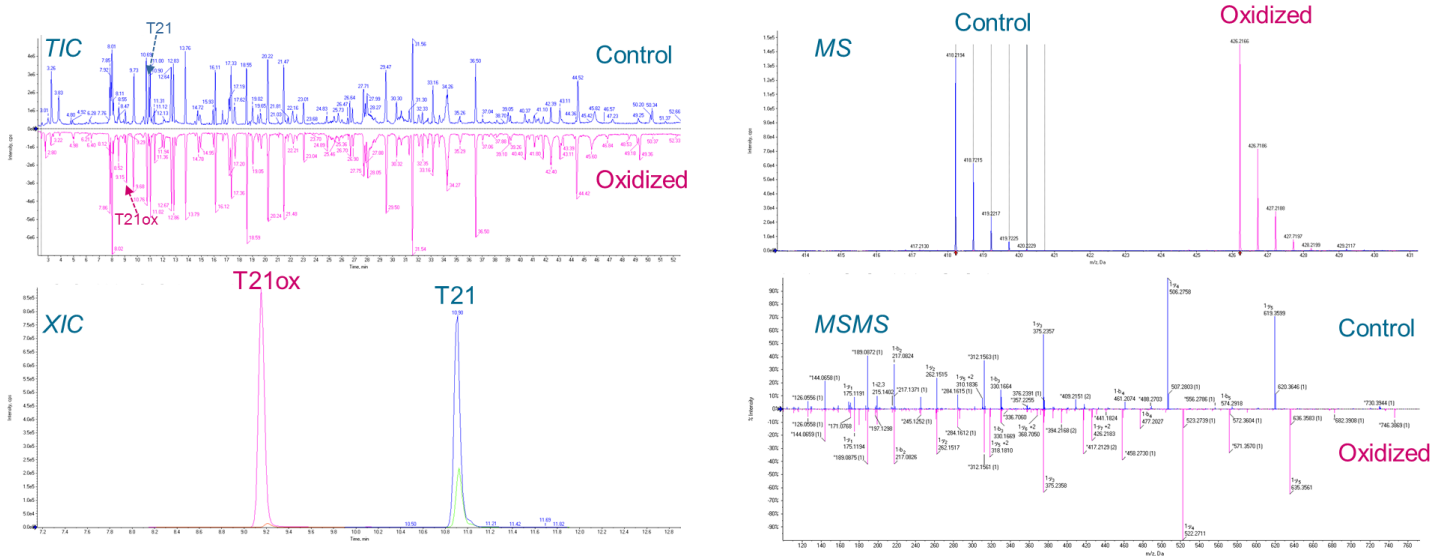

Figure 1. Comparison of oxidation profile for Met-255 between control and 24h stressed NISTmAb digest by TIC, XIC, MS and MSMS

Table 1. Chromatographic Conditions

Parameter	Value
Stationary phase	*Agilent ZORBAX 300 SB-C18 column1.8 µm, 2.1mm X 150 mm*
Mobile phase A	*0.1% formic acid in water*
Mobile phase B	*0.1% formic acid in acetonitrile*
Flow rate	*0.3 mL/min*
Column temperature	*50 ºC*
Injection volume	*3 µL*

Table 2. Chromatographic Gradient

Time (min)	Flow Rate (ml/min)	%A	%B
Initial	*0.3*	*99*	*1*
5.0	*0.3*	*99*	*1*
6.0	*0.3*	*90*	*10*
50.0	*0.3*	*65*	*35*
55.0	*0.3*	*40*	*60*
56.0	*0.3*	*10*	*90*
60.0	*0.3*	*10*	*90*
62.0	*0.3*	*99*	*1*
64.0	*0.3*	*99*	*1*
66.0	*0.3*	*10*	*90*
70.0	*0.3*	*10*	*90*
72.0	*0.3*	*99*	*1*
74.0	*0.3*	*99*	*1*
76.0	*0.3*	*10*	*90*
80.0	*0.3*	*10*	*90*
82.0	*0.3*	*99*	*1*
95.0	*0.3*	*99*	*1*

Table 3. Mass Spectrometry Conditions

Parameter	Value	Parameter	Value
Curtain gas:	*45*	*Time bins to sum:*	*4*
Ion source gas 1:	*35*	*TOF start mass (Da):*	*300*
Ion source gas 2:	*35*	*TOF stop mass (Da):*	*1800*
Temperature(°C):	*250*	*Accumulation time:*	*0.25 sec*
Ionspray voltage:	*5200*	*CAD gas:*	*7*
Scan type:	*IDA MS*	*Declustering potential (V):*	*20*
Polarity:	*Positive*	*Collision energy (V):*	*4*
IDA setting			
Maximum candidates ion	*10*	*Intensity threshold exceeds (counts/s)*	*100*
TOF start mass (Da):	*100*	*TOF stop mass (Da):*	*1000*
Accumulation time (s)	*0.05*	*Declustering potential (V)*	*50*
Declustering potential spread (V):	*0*	*Collision energy spread(V):*	*5*

Methods

Sample Preparation:

A total amount of 990 µg NIST sample was incubated with 0.03% H_2O_2 at room temperature for 24h. An aliquot of 150 µg sample was taken out at different time point (0.5h, 2h, 4h, 8h, 21h, 24h). The samples were quenched with an equal volume of 250 mM Methionine, followed by a buffer exchange with 12.5 mM L-histidine (pH 6.0), using Amicon centrifugal filter (Millipore, 10K, R8EA69651). Samples are subsequently stored at 4 °C before reduction or digestion.

The NIST samples were diluted to 1 mg/mL with the denaturing buffer (7.0 M Guanidine HCL, 100 mM Tris, pH 8.3). The denatured protein was subjected to reduction with DTT at 10 mM DTT at room temperature for 30 minutes, followed by alkylation with iodoacetomide at 20 mM for 20 minutes in the dark at room temperature. The alkylation was quenched with 4 uL of 50 mM DTT, and desalted using BioSpin-6 column followed by the instruction. The sample was then digested with Trypsin/LysC at a ratio of 1:10 (Roche, sequence grade) for overnight at 37 °C. The digestion was subsequently quenched by adding 10% TFA to a final concentration of 1%. Samples were stored at -20 °C before injected into LC-MS analysis.

The sample was analyzed by SCIEX X500B system fitted with a IonDrive™ Turbo V source with TwinSpray coupled with ExionLC™ system. Table 1 and Table 2 describe the liquid chromatography conditions and gradient used. Table 3 describes the mass spectrometry parameters used. The data was processed using BioPharmaView™ software 3.0..

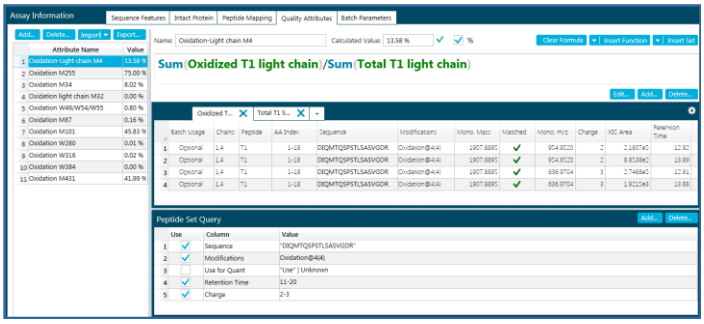

Figure 2 Definition of oxidation attribute on NISTmAb

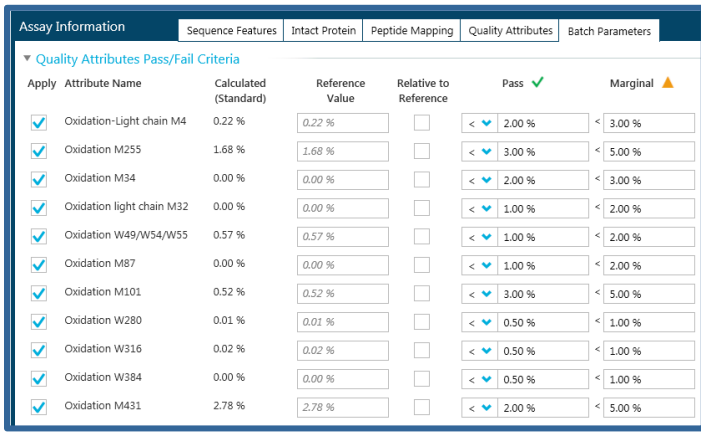

Figure 3 Definition of assay Pass/Fail criteria for each oxidation attribute on NISTmAb

Results and Discussion

Before oxidation liability assessment, the robustness and reliability of MAM assay was validated. A total 7 replicates of NISTmAb tryptic digest were injected and acquired using TOF-MS, IDA and SWATH® acquisition. For data processing, BioPharmaView3™ software 3.0 was used to identify and quantify each oxidation. For each site, a calculation to determine the oxidation level was defined as shown in Figure 2. and Pass/Fail criteria were specified as shown in Figure 3. After data processing, BiopharmaView™ software 3.0 generates a result table (Figure 4) showing the oxidation level on each defined oxidation site. The result were found to be consistent across all seven injections, demonstrating robustness and reproducibility of the assay.

NISTmAb was stressed by 0.03% H_2O_2 at room temperature for 24 hours. 150ug of sample was taken out at different time (0, 0.5h, 2h, 4h, 8h, 21h and 24h) using the protocol defined. Defined oxidation levels were tracked using BioPharmaView™ software 3.0. The results of time course study are presented in Figure 5. As shown, the oxidation level increases for nearly all methionine residues in NISTmAb, however tryptophan residues were largely unchanged under the conditions tested. Met-255 and Met 431 which are located in the Fc region, and Met 101 located in complementarity determining region (CDR) 3 were found to be the most susceptible to oxidation under H_2O_2-incubation conditions. This finding is expected as they are known to be solvent exposed and located on the outside of protein folded structure. Met 34 on CDR1 region and methionine residues on the light chain of NISTmAb were found to be more resistant to oxidation. This is attributed to these residues being less accessible to solvent as they are internal to the protein structure. Tryptophan level remains the same across the incubation time which is consistent with previous literature that tryptophan oxidation is mainly induced by photooxidation[1].

The underlying data for each oxidation site was reviewed using, BioPharmaView™ software 3.0. As an example, shown in Figure 1, the unmodified and oxidized methionine located in the heavy chain T21 peptide is shown. From the mirror plot of TIC, the decrease of unmodified peptide at 10.9 min and increase of oxidized peptide at 9.1 min is clearly observed. In addition, selecting peptides of interest in BioPharmaView™ software 3.0,

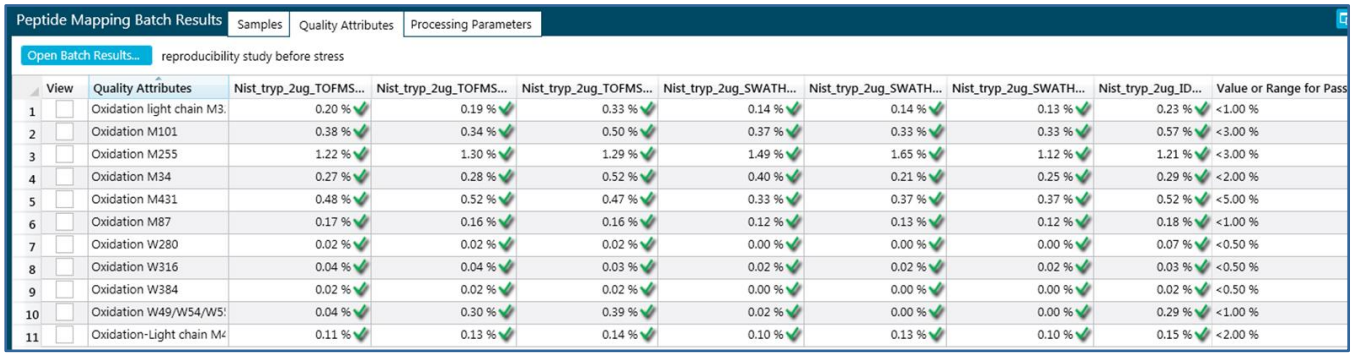

Figure 4. Validation on a MAM assay using seven replicate injections of NISTmAb tryptic digest

Peptide Mapping Batch Results | Samples | Quality Attributes | Processing Parameters

Open Batch Results... MAM time course study final

	View	Quality Attributes	NIST-0h_IDA.wiff2	NIST-0.5h_IDA.wiff2	NIST-2h_IDA.wiff2	NIST-4h_IDA.wiff2	NIST-8h_IDA.wiff2	NIST-21h_IDA.wiff2	NIST-24h_IDA.wiff2	Value or Range for Pass
1		Oxidation-Light chain M4	0.15 %	0.32 %	1.21 %	1.12 %	2.21 %	5.56 %	5.66 %	<2.00 %
2		Oxidation M255	1.91 %	7.18 %	17.22 %	23.41 %	37.21 %	67.20 %	70.63 %	<3.00 %
3		Oxidation M34	0.00 %	0.00 %	0.00 %	0.00 %	0.00 %	0.00 %	2.79 %	<2.00 %
4		Oxidation light chain M3.	0.00 %	0.00 %	0.00 %	0.00 %	0.00 %	0.00 %	2.53 %	<1.00 %
5		Oxidation W49/W54/W5	0.04 %	0.02 %	0.17 %	0.01 %	0.00 %	0.08 %	0.01 %	<1.00 %
6		Oxidation M87	0.00 %	0.00 %	0.00 %	0.00 %	0.00 %	0.00 %	0.00 %	<1.00 %
7		Oxidation M101	0.52 %	1.75 %	5.55 %	9.01 %	20.16 %	47.23 %	66.89 %	<3.00 %
8		Oxidation W280	0.00 %	0.00 %	0.00 %	0.00 %	0.00 %	0.00 %	0.00 %	<0.50 %
9		Oxidation W316	0.00 %	0.00 %	0.00 %	0.00 %	0.00 %	0.00 %	0.00 %	<0.50 %
10		Oxidation W384	0.00 %	0.00 %	0.27 %	0.00 %	0.00 %	0.10 %	0.00 %	<0.50 %
11		Oxidation M431	0.00 %	0.00 %	0.00 %	4.52 %	15.26 %	26.77 %	45.52 %	<5.00 %

Figure 5. Time course study of oxidation stress on NISTmAb at 0, 00.5h, 2h, 4h, 8h, 21h and 24h.

from the list of identified components provides a detailed view of the underlying MS and MS/MS data. By selecting multiple components simultaneously, it is possible to display data using mirror plots. As shown in Figure 1, both the MS and MSMS data enable rapid assessment of mass shifts and profiles for each selected component. In addition, the MS/MS spectra are annotated with identified b and y ions to expedite review of peptide mass spectra.

Conclusions

- BioPharmaView™ software 3.0 is a streamlined software tool for comprehensive attributes monitoring
- The MAM workflow in BioPharmaView™ software 3.0 is generally applicable over a range of applications throughout the biopharmaceutical development process
- The X500B QTOF system coupled to the ExionLC™ system provides highly reproducible data for assessment of protein post translational modifications

References

1. J.P. McCormick, Thomas Thomason Near-ultraviolet photooxidation of tryptophan. Proof of formation of superoxide ion *J. Am. Chem. Soc.*, **1978**, *100* (1), pp 312–313

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Headquarters
500 Old Connecticut Path | Framingham, MA 01701 USA
Phone 508-383-7700
sciex.com

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