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Application News

Peptide Mapping Analysis of Monoclonal Antibody / LCMS-9030

A Fast and Simple Workflow for Monoclonal Antibody (mAb) Post-Translational Modifications (PTM) Study Using Shimadzu LCMS-9030 Q-TOF

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User Benefits

- Simple and straightforward workflow for study on post-translational modifications (PTMs) of mAbs.
- High-resolution Q-TOF mass spectrometry LCMS-9030 coupled with Protein Metrics software for data processing can give accurate quantification of amino acid site relative abundance.

■ Introduction

Antibody therapeutics hold significant growth potential in the biopharmaceutical industry. Currently, there is a growing interest in using liquid chromatography mass spectrometry (LC/MS) to characterize monoclonal antibodies (mAbs) to support early to late-stage drug development. mAbs are macro-proteins that consist of two identical heavy chains and two identical light chains, linked together by disulfide bonds. Each mAb contains a fragment antigen-binding (Fab) region that binds to the antigen and a fragment crystallizable (Fc) region that is at its tail. mAbs can be identified by the unique Complementarity Determining Regions (CDRs) found on the heavy and light chains. (Fig. 1a)

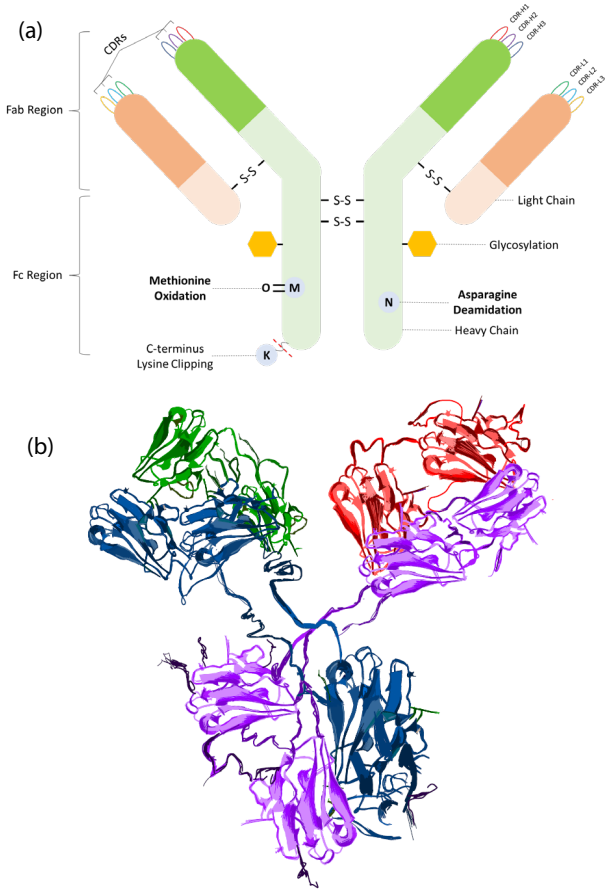

Fig.1 (a) Structure of monoclonal antibody with the common modifications such as Methionine Oxidation and Asparagine Deamination. (b) 3D schematic representation of mAb structure.

In this report, peptide mapping analysis was conducted using NIST monoclonal antibody (NISTmAb) reference material, RM 8671. During the sample preparation, artificial modifications can be introduced to the mAbs such as prolonged exposure to high temperature and incubation conditions. These artificial modifications can increase the oxidation and deamidation relative abundance values. Therefore, it is important to store mAbs correctly and strictly adhere to sample preparation incubation conditions.

Trypsin enzyme was used to digest the mAbs into smaller peptide fragments. The digested peptides were then separated using liquid chromatography and their masses were detected using Shimadzu LCMS-9030 QTOF Data-Dependent Acquisition (DDA) mode (Fig. 2). Data processing was performed using Protein Metrics software to quantify the amino acid site relative abundance.

Fig.2 Q-TOF mass spectrometer LCMS-9030

■ Experimental

Reagents and Chemicals

NISTmAbs Humanized IgG1k monoclonal antibody reference material 8671 was obtained from Merck Pte Ltd. Sequencing Grade Modified Trypsin (V511) was obtained Promega. S-Trap™ mini spin columns was obtained from Protifi. TORAST-H Bio Vial (370-04350-00) was obtained from Shimadzu.

Acetonitrile (LCMS grade) was obtained from commercial suppliers. Formic acid (>99%) of LCMS grade was used as additive in the mobile phase prepared from Milli-Q water.

Sample preparation

100 µg of NISTmAb was first reduced using 1 M dithiothreitol (DTT) by heating it at 95 ˚C for 10 mins. Then, it was alkylated using 100 mM iodoacetamide (IAM) by incubating it in the dark at room temperature for 30 mins. The reaction was quenched by adding 12% phosphoric acid. Next, the mAbs were transferred to the S trap mini spin column and washed with 90% methanol in 100 mM triethylammonium bicarbonate (TEAB). The mAbs were then captured on the S-Trap mini spin column. Trypsin digestion was performed using a 1:20 (w/w) enzyme-to-protein ratio and incubating at 47 ˚C for 1 hr. The digested peptides were eluted using 50% acetonitrile: 50% water: 0.2% formic acid. Peptides were dried using refrigerated vacuum concentrate at 4 ˚C and reconstituted in 80 µl of 0.1% formic acid in water in a low protein TORAST-H Bio vial.

Table 1 Analytical conditions on LCMS-9030 QTOF

LC Conditions	
Column	Shim-pack Arata™ Peptide C18 2.0 × 150 mm, 2.2 µm (P/N: 227-32806-03)
Flow Rate	0.2 mL/min
Mobile Phase	A: 0.1% Formic acid in Water B: 0.1% Formic Acid in Acetonitrile
Elution mode	Gradient elution, 65 mins
Gradient Program	%B conc.: 0-2.5 min., 2%; 2.5-5.0 min., 2% to 5%; 5-50 min., 5% to 38%; 50-55 min., 38% to 90%; 55-65 min., 90% to 2%
Oven Temp.	65 ˚C
Injection Vol.	5 µL
Interface Conditions (LCMS-9030)	
Interface	Heated ESI at 4.00 kV
Interface Temp.	300 ˚C
DL Temp.	250 ˚C
Heat Block Temp.	400 ˚C
Nebulizing Gas	3 L/min
Heating Gas Flow	15 L/min
Drying Gas Flow	15 L/min
Data acquisition (Q-TOF)	
MS Mode	DDA positive
DDA event #	2-8
TOF m/z range	200-2000 m/z
DDA m/z range	50-2000 m/z
CE	35.0
CE spread	17.0
Event Time (s)	0.100
Pulser Inj. Time	193

Finally, the peptides were injected and analyzed by a Shimadzu LCMS-9030 Q-TOF. The analytical conditions are shown in Table 1.

Data Processing

Data files were uploaded onto Protein Metrics PTM workflow with the following modifications and workflow parameters as per Table 2.

Quantification of the amino acid site relative abundance modification is done using Protein Metrics software according to the formula:

$$\text{Relative abundance modification \%} = \frac{\text{Extracted–Ion Chromatograms (XICs) of modified peptide}}{\text{XICs sum of all modified peptides + wildtype peptides}} \times 100\%$$

■ Results and Discussion

To conduct peptide mapping analysis, trypsin enzyme was used to cleave the NISTmAb into small peptide fragments, and sample analysis clean-up was done using S-Trap mini spin column. The results showed 100% sequence coverage obtained with the identified CDR peptides (Fig 4). The PTM data collected from the peptide mapping analysis can be trended to monitor the characteristics of mAbs during the different phases of drug development.

Peptides were eluted between 2 to 40 mins with good separation as shown in Fig 3. Peaks were well separated, which is important when performing DDA mode as the 7 most intense precursor ions are chosen for MS/MS fragmentation.

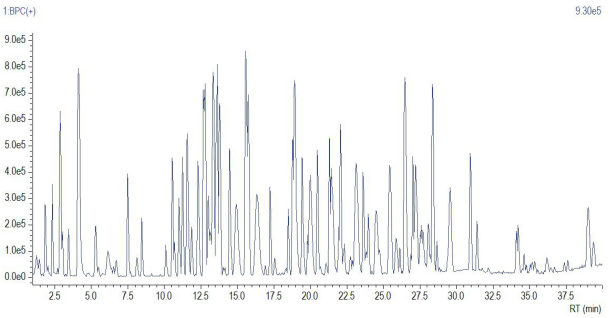

Fig. 3 Peptide mapping analysis Base Peak Chromatogram (BPC) using LabSolutions Insight Explore™.

Table 3 gives the summary of the overall calculated amino acid site relative abundance percentage.

Protein Metrics software automatically calculates the relative percentage of PTM of each individual modified amino acid on the peptides based on the ratio of extracted ion chromatograms (XICs) corresponding to the modified and unmodified wildtype peptides.

Table 2 PTM Workflow Parameters

Processing Nodes		
Precursor Mass Tolerance	15.00 ppm	
Digestion Missed Cleavages	2	
Glycans	N-glycan 59 common biantennary.txt	
Modifications	Targets	Fine Control
Carbamidomethyl +57.021464	C	Fixed
Oxidation +15.994915	M, W	Variable-common 1
Deamidated +0.984016	N, Q	Variable-common 1
Gln -> pyro-Glu -17.026549	Peptide N-term Q	Variable-common 1
Gln -> pyro-Glu -18.010565	Peptide N-term E	Variable-common 1
Lys-loss -128.094963	Protein C-term K	Variable-common 1

Fig.4 NIST amino acid sequence coverage

Table 3 Amino acid site relative abundance %

						MS Id ←	1
						MS Alias name	NIST
Protein name	Start AA ↑	Var. Pos. Protein ↑	Sequence (unformatted) ↑	Mod. Names	Mod. AAs ↑		(%)
Heavy Chain	1	1	QVTLR	Gln->pyro-Glu/-17.0265	Q		99
			QVTLRESGPALVKPTQTLTLTCTFSGFSLSTAGMSVGWIR	Gln->pyro-Glu/-17.0265	Q		1.04
	137	162	STSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTY ICNVNHKPSNTK	Deamidated/0.9840	N		2.81
		162,178	STSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTY ICNVNHKPSNTK	Deamidated/0.9840; Deamidated/0.9840	N,Q		12.4
	252	255	DTLMISR	Oxidation/15.9949	M		2.88
	278	289	FNWYVDGVEVHNAK	Deamidated/0.9840	N		2.5
	292	300	TKPREEQYNSTYR	NGlycan/1079.4017	N		5.57
				NGlycan/1241.4545	N		23.2
				NGlycan/1403.5073	N		9.23
				NGlycan/1444.5339	N		33.4
				NGlycan/1606.5867	N		17.2
				NGlycan/1768.6395	N		3.86
				NGlycan/876.3223	N		1.9
				NGlycan/1038.3751	N		5.61
	296	300	EEQYNSTYR	NGlycan/1241.4545	N		100
	305	314	VVSVLTVLHQDWLNGK	Deamidated/0.9840	Q		0.659
			VVSVLTVLHQDWLNGKEYKCK	Deamidated/0.9840	Q		2.1
		318	VVSVLTVLHQDWLNGK	Deamidated/0.9840	N		0.942
			VVSVLTVLHQDWLNGKEYK	Deamidated/0.9840	N		26
			VVSVLTVLHQDWLNGKEYKCK	Deamidated/0.9840	N		2.2
	326	328	VSNKALPAPIEK	Deamidated/0.9840	N		0.767
	348	348	EPQVYTLPPSREEMTK	Glu->pyro-Glu/-18.0106	E		1.79
			EPQVYTLPPSREEMTKNQVSLTCLVK	Glu->pyro-Glu/-18.0106	E		1.59
		364	EPQVYTLPPSREEMTKNQVSLTCLVK	Deamidated/0.9840	N		2.1
	364	364	NQVSLTCLVK	Deamidated/0.9840	N		3.14
		365	NQVSLTCLVK	Deamidated/0.9840	Q		3.14
	374	387	GFYPSDIAVEWESNGQPENNYK	Deamidated/0.9840	N		13.8
			GFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSK	Deamidated/0.9840	N		4.95
		387,389	GFYPSDIAVEWESNGQPENNYK	Deamidated/0.9840; Deamidated/0.9840	N,Q		6.51
			GFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSK	Deamidated/0.9840; Deamidated/0.9840	N,Q		5.57
		389	GFYPSDIAVEWESNGQPENNYK	Deamidated/0.9840	Q		11.7
			GFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSK	Deamidated/0.9840	Q		3.8
		389,392	GFYPSDIAVEWESNGQPENNYK	Deamidated/0.9840; Deamidated/0.9840	Q,N		6.51
		392	GFYPSDIAVEWESNGQPENNYK	Deamidated/0.9840	N		11.7
			GFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSK	Deamidated/0.9840	N		3.8
	413	437	LTVDKSRWQQGNVFSCSVMHEALHNHYTQK	Deamidated/0.9840	N		3.1
	420	437	WQQGNVFSCSVMHEALHNHYTQK	Deamidated/0.9840	N		5.4
	443	450	SLSLSPGK	Lys-loss/-128.0950	K		76.4
Light Chain	108	136	TVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPR	Deamidated/0.9840	N		4.26
	126	136	SGTASVVCLLNNFYPR	Deamidated/0.9840	N		5.16
	145	151,154	VQWKVDNALQSGNSQESVTEQDSKDSTYSLSSTLTLSK	Deamidated/0.9840; Deamidated/0.9840	N,Q		21.3
		157	VQWKVDNALQSGNSQESVTEQDSKDSTYSLSSTLTLSK	Deamidated/0.9840	N		4.03
	207	209	SFNRGEC	Deamidated/0.9840	N		2.33

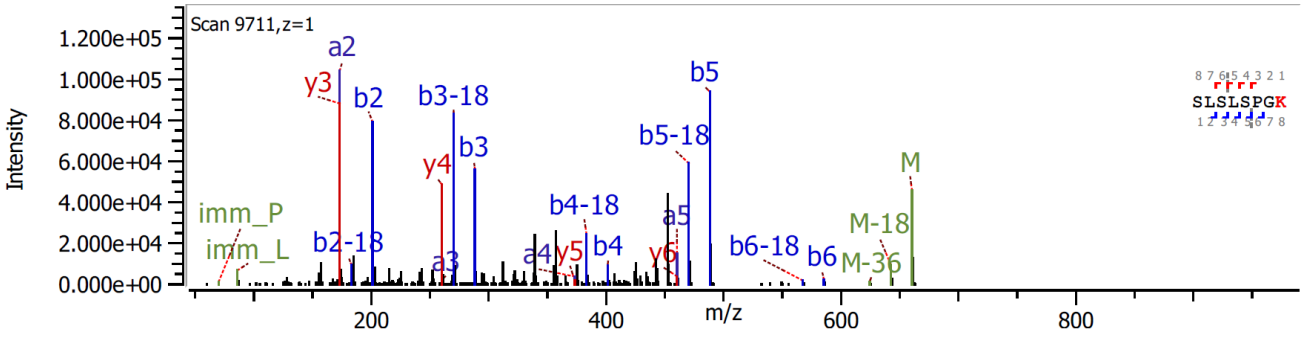

Fig.5 MS Spectra of modified peptide with loss of amino acid Lysine (K) at heavy chain amino acid position 450

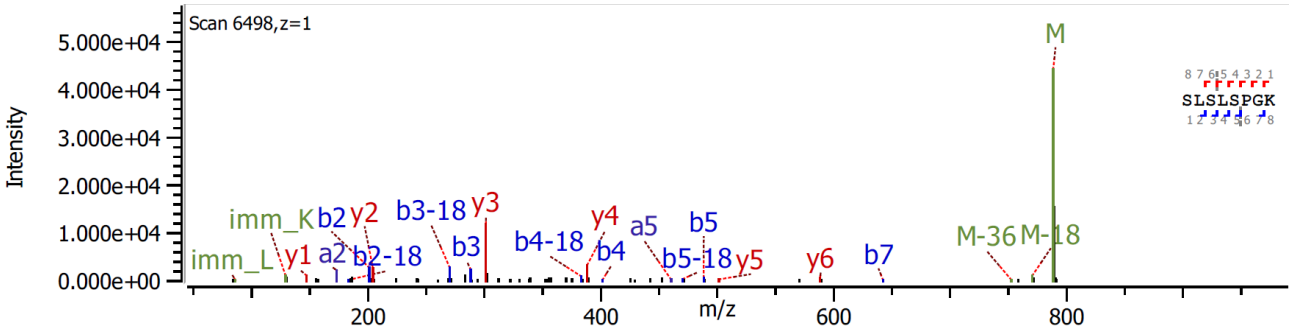

Fig. 6 MS Spectra of unmodified wildtype peptide without the loss of amino acid Lysine (K) at heavy chain amino acid position 450

Common modification hotspots such as C-terminal lysine clipping and N-glycosylation consensus NXS/T ("X" refers to any amino acid residue except proline) at heavy chain position 300 is showing a high relative abundance of more than 75%. This is aligned with the basic characteristics of mAbs. Peptide sequence 'DTLMISR' is the only identified peptide for oxidation and it shows a low relative abundance value of less than 5%. Overall relative abundance values of deamidation are below 30%.

Fig. 5 and Fig. 6 show the differences in the MS spectra between the modified peptide with the loss of lysine at heavy chain amino acid position 450 and unmodified wildtype peptide. Protein Metrics software automatically labels the iminium ions in green with the corresponding amino acid. Peaks in green color labelled with M-X mean there is a neutral loss on X. X could be a loss of 1 water which is M-18 or a loss of 2 water which is M-36.

■ Conclusion

During drug development, it is crucial to closely monitor the changes in the PTM amino acid relative site abundance percentage values, such as methionine (M) oxidation or asparagine (N) deamidation, as it can impact the efficacy, stability and immunogenicity of mAbs.

This study has demonstrated a simple and straightforward workflow to quantify the amino acid site relative abundance using peptide mapping analysis. Shimadzu LCMS-9030 QTOF mass spectrometer coupled with Protein Metrics software provides high mass resolution to accurately identify, separate, and quantify the modified peptides with small mass shift differences.

The use of peptide mapping analysis for PTM study can provide valuable insights into the mAbs characteristics to support early to late-stage drug development.

[Acknowledgments]

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■ References

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