Characterization of NIST monoclonal antibody on Intact, Subunit, and Peptide level with Monitoring of CQA's on the X500B

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ABSTRACT

Monoclonal antibodies (mAbs) can be incredibly complicated with hundreds of amino acids, varying degrees of glycosylation and post-translational modifications which may be critical to product quality. Although analysis via mass spectrometry can uncover a wealth of information of these proteins a synergistic workflow should be used to maximise the data quality and fidelity. Here BioPharmaView™ software and the Sciex X500B QToF have been utilised to provide a complete characterization of the NIST monoclonal antibody standard on an intact, subunit, and peptide level. Additionally the Multi Attribute Monitoring (MAM) workflow in BioPharmaView™ has been used to control Critical Quality Attributes (CQA) including glycosylation. The X500B brings these routine workflows to the hands of more scientists with the user friendly SCIEX OS for confidence in complex characterization.

INTRODUCTION

Biologics and biotheraputics are an area of a great deal of research interest due to their potential to target new and novel systems. A consequence of this is the need for new technology to assist in the development and monitoring of these new molecules, along with adequate standards to ensure comparability between systems and production sites. Biologic compounds contain a great deal of heterogeneity with several modifications which may be critical to efficacy and safety. As the production of these biotherapeutics moves forward there is a need to monitor multiple attributes simultaneously to save time in production and development.

The NISTmAb, humanized IgG1κ Monoclonal Antibody has been extensively characterized and is available as a standard to normalize biotheraputic production and analysis against. The protein can be used as an instrument or process benchmark, or as an internal standard to compare against a developed biotheraputic.

Presented here are the results of a three part study on the SCIEX X500B onto the NISTmAb protein. The protein was characterized via MS as an accompaniment to the paper 'Determination of the NISTmAb Primary Structure'. Intact, subunit and peptide methods were performed to determine the level of CQA's as data was processed in BioPharmaView™ software 3.0.

MATERIALS AND METHODS

Sample Preparation:

Intact:
1µL of NISTmAb, humanized IgG1κ Monoclonal Antibody (NIST, Gaithersburg, MD, USA) at 10 µg/µL was diluted in pure water to 0.01 µg/µL before injection onto the LC/MS system.
Subunit:
10 units of FabRICATOR IdeS enzyme (Genovis) was added to 1µL of NISTmAb, humanized IgG1κ Monoclonal Antibody at 10 µg/µL for digestion at 37℃ with shaking at 400 rpm. Post digestion the sample was diluted in pure water to 0.01 µg/µL before injection onto the LC/MS system.
Digestion:
10µL of NISTmAb, humanized IgG1κ Monoclonal Antibody at 10 µg/µL was diluted in denaturing buffer (7M Guanidine HCl, in 25mM Tris-HCl. pH 6.8) to 100 µL before reduction with 2 µL 500mM DTT for 30 minutes at room temperate with shaking at 400 rpm. Alkylation was performed with addition of 5 µL of 500mM iodoacetamide solution followed by shaking at 400 rpm in the dark for 20 minutes. The sample was buffer exchanged into 50 mM Tris HCl pH 7.9 using a BioSpin 6 column (BioRad).
The sample was digested with the addition 5 µg of trypsin to 50 µg of protein for 30 minutes at room temperate with shaking at 400 rpm before quenching with 1:10 (volume) ratio with 10% TFA. The final sample was diluted in pure water to 0.1 µg/µL before injection onto the LC/MS system.

HPLC Conditions:
A Sciex ExionLC™ System was used for all three applications with a gradient of eluent A water with 0.1 % formic acid and eluent B acetonitrile with 0.1 % formic acid.

Intact
The intact analysis was performed with a Phenomenex Jupiter® 5 µm C4 300 Å 50 x 2 mm LC column. The sample was desalted on column for three minutes at 15% B with a flow rate of 0.5 mL/min, the gradient was increased to 50% B over four minutes with a flow rate of 0.2 mL/min, before washing at 80% B for one minute with a flow rate of 0.5 mL/min finally regenerating for a further two minutes.
Subunit
The subunit analysis was performed with a Phenomenex Jupiter® 5 µm C4 300 Å 50 x 2 mm LC column. The sample was desalted on column for two minutes at 15% B with a flow rate of 0.5 mL/min, the gradient was increased to 50% B over four minutes with a flow rate of 0.2 mL/min, before washing at 95% B for one minute with a flow rate of 0.5 mL/min finally regenerating for a further two minutes.
Peptide Mapping
The peptide analysis was performed with a Phenomenex Luna® Omega 1.6 µm C18 100 Å 150 x 2.1 mm LC column. The sample was desalted on column for three minutes at 1% B with a flow rate of 0.25 mL/min, the gradient was increased to 35% B over fifty one minutes with a flow rate of 0.25 mL/min, before washing at 80% B for two minutes with a flow rate of 0.25 mL/min finally regenerating for a further three minutes.

Parameter	Value Intact/Subunit/Peptide	Parameter	Value Intact/Subunit/Peptide
Curtain Gas	35 / 35 / 45	Time bins to Sum	120 / 120 / 12
Ion Source Gas 1 (psi)	50 / 50 / 30	TOF start mass (Da)	900 / 900 / 250
Ion Source Gas 2 (psi)	50 / 50 / 40	TOF stop mass (Da)	4000 / 3000 / 1800
Temperature (℃)	450 / 450 / 250	Accumulation Time (s)	1 / 1 / 0.04
Scan Type	TOF MS / TOF MS / SWATH	Declustering Potential	250 / 125-225 / 50
Polarity	Positive	Collision energy (V)	15 /15 / Dynamic

Table 1. Source settings for characterization of NIST mAb on various structural levels.

RESULTS

The X500B was calibrated before the NISTmAb was injected in triplicate. The data was processed in BioPharmaView™ with reconstruction and matching of expected modifications with <10ppm mass error. During reconstruction settings were optimized to best match raw data (Figure 1). Major reconstructed peaks were attributed to G0F/G0F-GlcNAc, G0F/G0F, G0F/G1F, G1F/G1F or G0F/G2F, G1F/G2F, G2F/G2F all assigned with two lysine clips and two N terminal pyroglutamate formations. Minor peaks were also assigned to glycosylated forms with a single or no lysine losses.

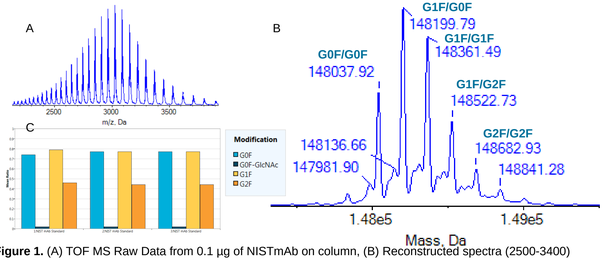

Figure 1. (A) TOF MS Raw Data from 0.1 µg of NISTmAb on column, (B) Reconstructed spectra (2500-3400) (C) Ratio of glycoforms across three technical replicates

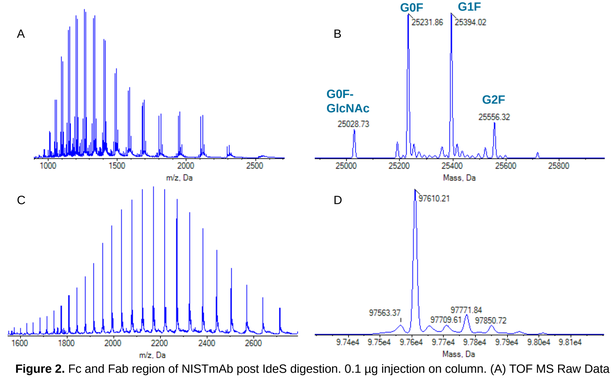

Figure 2. Fc and Fab region of NISTmAb post IdeS digestion. 0.1 µg injection on column. (A) TOF MS Raw Data of Fc region, (B) Reconstructed Fc region, (C) TOF MS Raw Data of Fab region, (D) Reconstructed Fab region.

After IdeS digestion the samples were not reduced leaving the disulfide bonds intact, this results in two Fc fragments (CH3 and Ch2 region) and a single Fab fragment with two light chains and the VH, CH1 and Hinge region of two heavy chains per mAb digested (Figure 2). The Fc region was assigned the glycoforms G0F-GlcNAc, G0F, G1F, and G2F all with a single lysine loss. Minor forms were observed without lysine clipping. The Fab region was mainly observed with a single pyroglutamate formation at the C-terminus with an additional minor form identified with a non specific glycation.

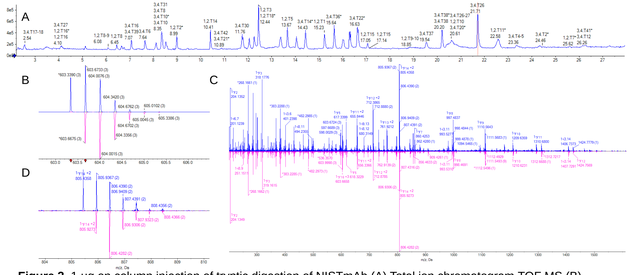

Figure 3. 1 µg on column injection of tryptic digestion of NISTmAb (A) Total ion chromatogram TOF MS (B) TOF MS of peptide VVSVLTVLHQDWLNGK as the amidated (blue) and deamidated (pink) form. (C) MS/MS spectra MS of peptide VVSVLTVLHQDWLNGK as the amidated (blue) and deamidated (pink) form. (D) Zoom in on fragment y-14 on amidated (blue) and deamidated (pink) form of peptide

MS/MS Conditions Peptide Mapping:
The peptide mapping experiments were performed with SWATH® acquisition utilizing a 100ms TOF MS spectra followed by twenty five SWATH windows ranging from 200-1500 *m/z* each with a 40ms acquisition with rolling collision energy.

Upon data processing in BioPharmaView™ 3.0 with three repeat injections 100% sequence coverage was obtained at MS level and 98.6% coverage at MS/MS level. BioPharmaView™ 3.0 automatically assigns peptides based on MS and MS/MS data for both data-dependent and data-independent (SWATH) workflows. Several critical quality attributes (CQA's) were monitored to determine inherent heterogeneity in the sample. Sample impurities were also monitored in the form of auto-tryptic peptides. Figure 4 shows a table with 3 biological replicates compared to a standard. Green ticks indicate the value lies within an acceptable boundary, an orange triangle is a marginal value that requires review, a fail is shown with a red cross. The deamidated peptide containing PENNYK is also shown across two repeats.

	# Unique Peptides	# Impurities	% Sequence Coverage	Pass/Marginal/Fail	N-terminal pyro Glu	C-term des Lys	M255 oxidation	PENNYK-Deamidation	PENNYK-Ammonia loss	Total Deamidation
1	386	5	100.0	Marginal	0.80 %	88.64 %	2.57 %	8.09 %	8.07 %	0.32 %
2	387	7	100.0	Marginal	0.79 %	88.59 %	2.78 %	4.70 %	4.70 %	0.16 %
3	380	4	100.0	Marginal	0.76 %	88.34 %	2.62 %	8.10 %	8.10 %	0.32 %
4	352	5	100.0	-	0.87 %	88.67 %	2.46 %	7.42 %	7.36 %	0.31 %

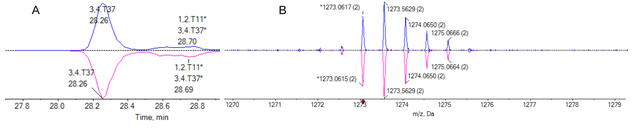

Figure 4. Batch pass/marginal/fail from BioPharmaView™ 3.0. A and B show deamidated peptide GFYPSDIAVEWESNGPENNYK across two biological repeats in XIC (A) and MS (B) .

CONCLUSIONS

The SCIEX X500B provides a fast, robust, and reliable instrument for complete characterization of biotheraputics including accurate intact and subunit mass along with high sequence coverage of peptide digests. BioPharmaView™ 3.0 provides the further ability to automatically assign modifications on the intact along with monitoring of CQAs on the peptide level.

REFERENCES

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TRADEMARKS/LICENSING

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