COMPREHENSIVE PTM CHARACTERIZATION OF THE NIST MAB REFERENCE STANDARD USING A HRMS MASS SPECTROMETRY

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INTRODUCTION

The NIST mAb RM 8671 reference standard material can function as a common standard for the biopharmaceutical industry, analytical instrument/software companies, and regulators. RM 8671 can be used to evaluate and improve current analytical technologies and capabilities for determining the physicochemical and biophysical attributes of monoclonal antibodies.

An exhaustive set of characterization data has been collected from QTOF and Orbitrap mass spectrometers and published (ACS Book series: "State-of-the-Art and Emerging Technologies for Therapeutic Monoclonal Antibody Characterization").[1]

In this study, we demonstrate the use of a new bench top IMS QTOF MS controlled by a workflow driven software for common PTM characterization of the new NIST mAb reference standard (RM 8671).

UPLC/Optical/Vion IMS QTOF MS System
- controlled by a complaint ready software

Workflow driven System

T-wave IMS Resolution: ~ 40
Mass Resolution > 50 K
Detection Dynamic Range: 5 order
Mass Accuracy: < sub ppm
CCS Accuracy: < 1% RSD

METHODS

Instrumentation:

ACQUITY UPLC H-Class Bio
BEH C4, 2.1x 50 mm (intact protein/subunit)
BEH 300 C18, 2.1x100 mm (peptide mapping)
BEH amide column 2.1x 150 mm (released glycan with FLR label)
ACQUITY UPLC TUV
ACQUITY UPLC FLR
Vion IMS QTOF MS
Data acquisition modes: MS, DDA, DIA (MS^E, $HDMS^E$)
Instrument Control Software and data processing: UNIFI
Workflows in UNIFI used : Intact Protein, Glycan and Peptide Mapping

Sample Preparations:

Released Glycan:
Glycoworks RapiFluor-MS Glycan Kit was used for glycan preparation.[2] The final products are RapiFluor-MS labeled N-Glycans from NIST mAb.

Intact mAb Analysis:
NIST mAb was diluted in 25 mM ammonium acetate
Injection amount: 0.1 µg for intact protein analysis

Subunit Analysis:
Genovis FabRICATOR (Ides) enzyme used for digestion

Peptide Mapping:
NIST mAb was reduced and alkylated (IAM) under guandine-HCl denaturing conditions. Desalting of the alkylated protein was performed using an NAP-5 cartridge, followed by a 30-minute trypsin digestion.

1. Released Glycan Profiling

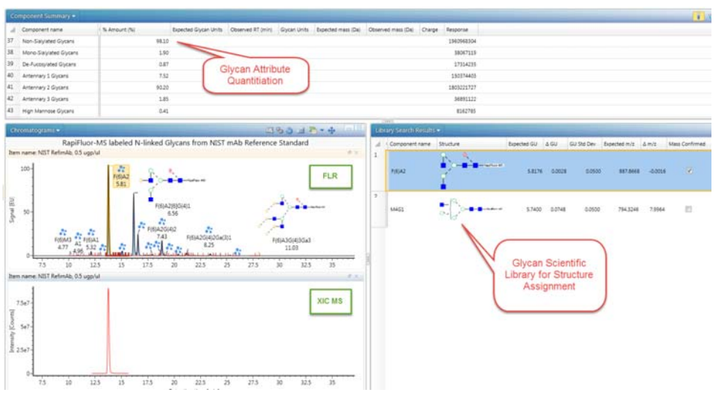
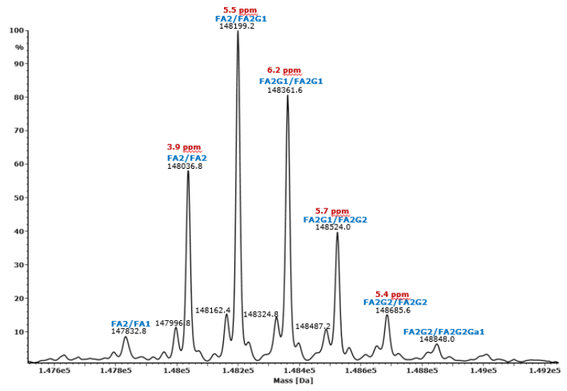


Figure 1. N-linked glycans from NIST RM 8671 were released and labeled with *Rapi*Fluor-MS tag. These glycans were analyzed using a HILIC-UPLC/FLR/MS system for quantification (using FLR integrated peak areas) and identification using an RFMS Glycan GU Scientific library.

RESULTS

2. Intact Protein Analysis (Major Glycoforms)



Glycoforms	Theoretical mass (Da)	Experimental Mass (MaxEnt 1)	Mass Accuracy ?ppm
FA2	146591.8	146593.6	12.3
FA2G1	146754	146754.4	2.7
FA2G2	146916.1	146919.2	21.1
FA1/FA1	147630.8	147629.6	-8.1
FA2/FA1	147834	147834.4	2.7
FA2/FA1G1	147996.1	147996.0	-0.7
FA2/FA2	148036.4	148037.4	3.9
FA2/FA2G1	148198.6	148199.2	5.5
FA2/FA2G1+K	148327.5	148324.8	-18.2
FA2G1/FA2G1	148360.7	148361.6	6.2
FA2G1/FA2G1+K	148489.6	148487.2	-16.2
FA2G1/FA2G2	148522.8	148523.2	5.7
FA2G1/FA2G2+K	148651.8	148650.4	-9.4
FA2G2/FA2G2	148685.0	148685.6	5.4
FA2G2/FA2G2Ga1	148847.7	148846.4	-8.7

Figure 2. MaxEnt 1 deconvoluted spectrum of intact NIST RM 8671 is shown here. The intact protein was on-line desalted using an RP C4 column. Mass accuracy for the major glycoforms are listed in the table. High levels of lysine truncation were observed for high abundance glycoforms.

3. Subunit Analysis (Glycoforms, and Global Glycation Measurements)

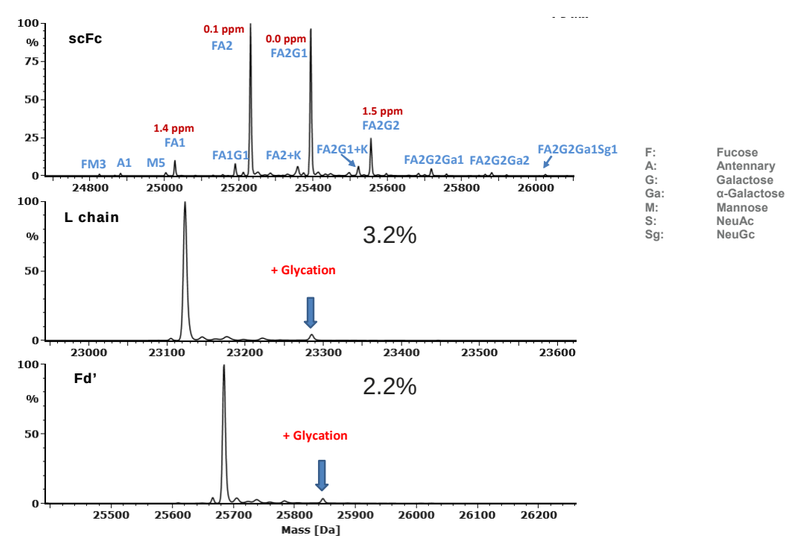


Figure 3. MaxEnt 1 deconvoluted subunit data are show here. The glycan distribution from the ScFc subunit is highly similar to the released glycan profile (see Figure 1.); the global glycation level for the LC and Fd region were measured at 3.2% and 2.2% respectively.

4. Peptide Mapping (PTM Analysis)

C-terminal Lysine Truncation

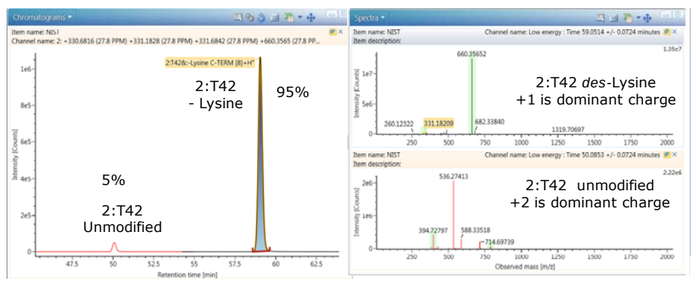


Figure 4. C-terminal lysine truncation was calculated to be 95%. Quantitation in UNIFI can be performed in two ways: 1) combined ion counts from all charge states; or 2) from only one charge state. The C-terminal tryptic peptide with lysine shows a dominant charge state at +2, while the truncated version has the +1 as the dominant ions. Therefore, quantitation using sum of all charge states provides more accuracy for the modification% calculation.

Deamidation

Chain	Position	Mod %
H	N78	0.19
H	N86	0.09
H	N162	0.21
H	N279 or N289	0.22
H	N318	0.33
H	N328	N/A
H	N387	1.98
H	N392	
H	N437	1.27 (new)
L	N136	0.20
L	N151	0.28
L	N157	0.43

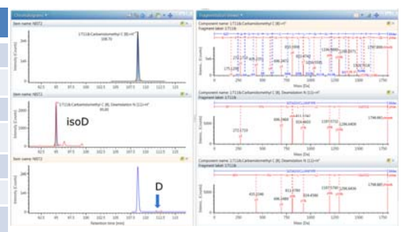


Figure 5. Identified deamidation sites and modification% are summarized in the table above. Deamidation at N437 was not reported previously in the ACS Book Chapter (Ref.1).

The XICs and spectra above are examples of chromatographically separated D and isoD forms of the light chain peptide that were deamidated at position N136.

Glycation Site Occupancy

Chain	Residue & Position	Mod %
H	K13	0.08 (new)
H	K58	0.24
H	K66	N/A
H	K77	0.46
H	K136	0.42
H	K255	0.43
H	K249	0.32
H	K251	0.53
H	K291 or K293	0.05
H	K329	2.73
L	K38, K41	0.4
L	K44	0.09
L	K52	4.77
L	K102	0.59
L	K125	0.27 (new)
L	K148	0.59
L	K168	0.54
L	K182	0.91
L	K187	1.97
L	K189	0.12
L	K206	0.18

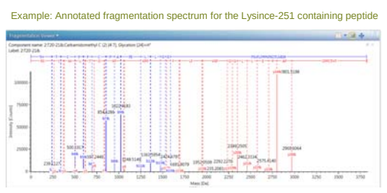
Peptide ID and quantitation criteria:

1. Mass accuracy for precursor and fragment ions (< 5 ppm).
2. Minimum of 3 b/y ions
3. Peptide quantitation was achieved with ion counts from all charges from MS^E acquisitions
4. mod% calculation (see the equation below)

$$\text{mod\%} = \frac{\text{modified peptide ion counts}}{\text{modified peptide + unmodified peptide ion counts}}$$

Figure 6. A summary of all identified glycation sites and relative percent glycation is shown here. All glycated peptides were confirmed by MSMS fragmentation. Two new glycation sites were identified in this study.
The annotated fragmentation spectrum is used to demonstrate the assignment of the Lysine-251 containing peptide. The glycation at this site was not reported previously in the ACS book chapters.

Example: Annotated fragmentation spectrum for the Lysince-251 containing peptide



Oxidation (Met)

Chain	Residue & Position	Mod %
H	M34	2.6
H	M87	0.45
H	M101	1.64
H	M255	2.08
H	M361	4.04
H	M431	0.33
L	M4	0.67
L	M32	0.47

Figure 7. Oxidation sites and oxidation percentages are summarized in the table.

5. Future Work: Utilizing Ion Mobility Separation

Significant reduction in LC/MS run times
Reducing interfering ions for precursor and fragment ions
Automated CCS value generation for all peptide components

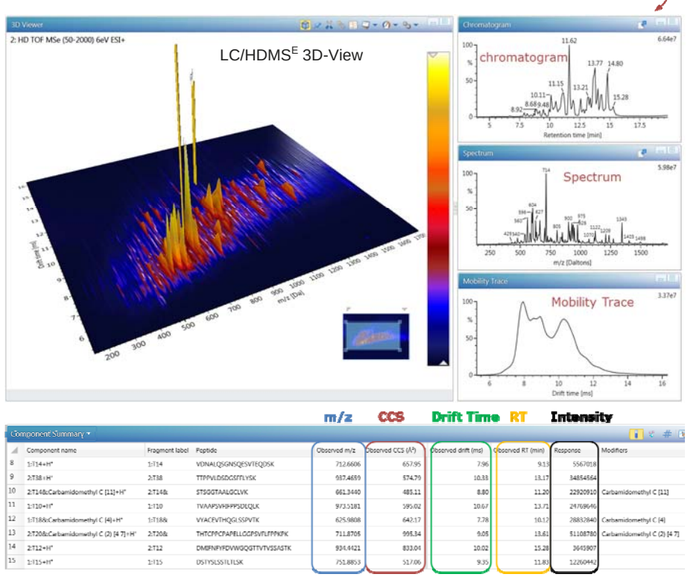


Figure 8. 3D-view of the NIST mAb peptide mapping data collected in $HDMS^E$ mode (data independent acquisition mode with ion mobility separation). One of the advantages of $HDMS^E$ mode of acquisition is to reduce the LC gradient time (to 10 min) without sacrificing of sequence coverage. In addition, CCS and drift time values for each peptide component were automatically generated and saved into a scientific library. CCS values along with other analytical measurements such as LC retention time, m/z, and z are characteristic to each peptide component, therefore, can be used as search parameters for targeted data processing.

CONCLUSIONS

- **NIST RM 8671 was characterized using the latest Vion/IMS QTof MS system controlled by UNIFI Scientific Informatics System.**
- **Established analytical workflows with UNIFI were used for the comprehensive analysis of this reference standard and included:**
  - ⇒ **Accurate MW measurements for major glycoforms achieved at the intact protein level.**
  - ⇒ **Glycation levels in the LC and Fd region by subunit analysis.**
  - ⇒ **A thorough investigation of PTMs, including C-terminal lysine truncation, deamidation, oxidation, and glycation.**
  - ⇒ **N-linked glycan profiling using *Rapi*Fluor-MS tagging chemistry and an RFMS Glycan GU library built into UNIFI.**
- **Attributes from the characterization data can be incorporated in targeted monitoring for mAb development and QC (*see P-131-W*).**
- **Future work will be focused on utilizing CCS values (from IMS) and accurate mass information to monitor critical quality attributes for biologics.**

References

1. Schiel et al.; State-of-the-Art and Emerging Technologies for Therapeutic Monoclonal Antibody Characterization Volume 1-3. ACS Symposium Series; American Chemical Society: Washington, DC, 2014.
2. Lauber et al: Rapid Preparation of Released *N*-Glycans for HILIC Analysis Using a Labeling Reagent that Facilitates Sensitive Fluorescence and ESI-MS Detection, *Anal. Chem.*, **2015**, *87* (10), 5401–9.

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