Multi-attribute Monitoring of Monoclonal Antibodies by icIEF-MS

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ABSTRACT

Presented are results of comprehensive icIEF-MS analysis of NISTmAb 8671 charge and mass variants. During a 15-minute icIEF-MS separation the percent composition of charge variants were quantitated by UV absorbance at 280 nm, and 130 intact NISTmAb proteoforms were putatively identified through deconvoluted mass spectra. High pI variants were comprised of incompletely processed c-terminal lysines, with detectable levels of glycation. Correspondingly, the main NISTmAb charge profile peak contained completely processed c-terminal lysines. Acidic variants of the NISTmAb charge profile contained glycated, deamidated and terminal N-glycolylneuraminic acid species.

icIEF-MS analysis of deglycosylated NISTmAb was performed to confirm the presence of glycation. Charge variant peaks shifted to lower pI contained a stepwise addition of 162 Da within basic, main, and acidic charge isoforms, providing strong evidence for glycation at lysine residues. Additionally, a continuous shift in the mass amongst the acidic region's nonglycated peaks were observed indicating the presence of deamidated species. Together, the pI and molecular weight attribute measurements of intact and deglycosylated NISTmAb isoforms provided holistic understanding of modifications to the NISTmAb molecule at the intact protein level.

INTRODUCTION

Posttranslational and chemical modifications that occur during the production process of a monoclonal antibodies (mAbs) give rise to a diverse population of proteoforms with potentially differing safety, efficacy, and potency profiles. Charge heterogeneity assays have proven to be highly useful in assessing Critical to Quality Attributes (CQAs) of therapeutic products as many of the alterations a protein undergoes also produces charge isoforms. Imaged capillary isoelectric focusing (icIEF) has been widely adopted for characterizing changes in the charge profile of a protein. Combining icIEF with electrospray ionization (ESI) in a microfluidic chip has resulted in an analytical technique with the ability to identify and measure dozens of molecular attributes on an intact mAb in a single run.

MATERIALS AND METHODS

Sample Preparation:
Buffer components of the NISTmAb 8671 formulation were removed by a Zeba™ Desalting Column 7kDa MWCO (ThermoFisher Scientific Cat. 89883). Desalted NISTmAb was deglycosylated overnight under native conditions at 37ºC with PNGaseF (NEB Cat. P0704S) . After deglycosylation, the enzyme buffer was removed by desalting column. All NISTmAb samples were stored at -80 ºC before use.
To prepare the 200 µL icIEF-MS samples, 80µg of desalted NISTmAb or 40µg of deglycosylated NISTmAb was combined with 3% Pharmalyte 8 to 10.5 pH (Cytiva Cat.17045301), 1% Pharmalyte 3 to 10 pH (Cytiva Cat.17045601), 7.5 mM Arginine (Fisher Bioreagents Cat. BP2505-500) and 6.25 µg pI 8.40 and 9.99 markers. The sample was vortexed, loaded into sample vial and placed into the IntaBio icIEF-MS system.

iCIEF-MS Separation Conditions
The sample was focused using 1% formic acid (anolyte) and 1% diethylamine (catholyte) for 1 min at 1500 V then 1 min at 3000 V followed by 4.5 min at 4500 V. Mobilization and ESI were performed with 25% acetic acid 25% ACN flowing at 2.5 µL/min for 6.0 minutes while 3000 V was applied between the anolyte and mobilizer and 5500 V was applied at the tip.

MS Conditions and Analysis:
A SCIEX TripleTOF® 6600 quadrupole time-of-flight (QTOF) mass analyzer equipped with an OptiFlow™ Interface was used to analyze the mobilized peaks. During the mobilization step the MS was set to scan 2000-6000 *m/z* at 2 Hz. The interface was set at 100 ºC with a curtain gas setting of 15 psi. Deculstering potential was 210 eV and collison energy was at 50 eV.

The resulting spectra were analyzed in the IntaBio icIEF-MS workflow in BYOS by Protein Metrics Inc. After deconvolution mass isoforms were identified by a delta mass from a reference that was either 148037.2 Da for the intact NISTmAb or 145148.5 Da for deglycosylated NISTmAb

References

1. Formolo, T., *et al*, Chapter 1 Determination of the NISTmAb Primary Structure, Art and Emerging Technologies for Therapeutic Monoclonal Antibody Characterization Volume 2. Biopharmaceutical Characterization: The NISTmAb Case Study ACS Symposium Series; American Chemical Society: Washington, DC, 2015

2. Hillard, M., *et al,* MABS 2017, Vol. 9, No. 8, 1349–1359

RESULTS

IntaBio icIEF-MS workflow

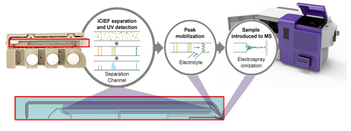

Figure 1 illustrates the streamline process of icIEF-MS analysis. After loading the separation channel with sample an electrical potential is applied between the Anolyte (Anode) and Catholyte (Ground) channels to start the focusing process. During focusing absorbance images at 280 nm are taken of the forming gradient and stacking sample components. At the completion of focusing the ground is switched from the catholyte to the mobilizer channel. The anion in the mobilizer solution disrupts the pH gradient, transport the focused bands by electrophoresis to the incorporated ESI tip for electrospray and downstream MS analysis

NISTmAb Intact Charge Profiles

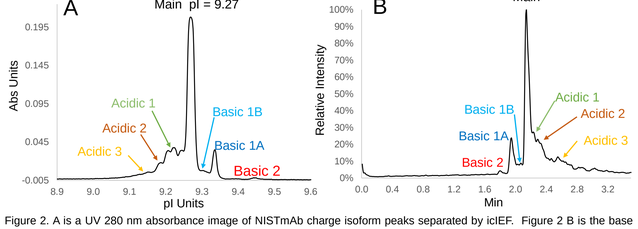

Figure 2. A is a UV 280 nm absorbance image of NISTmAb charge isoform peaks separated by icIEF. Figure 2 B is the base peak electropherogram (BPE) of same corresponding peaks after mobilization, electrospray and analysis by MS. The UV and BPE profiles are mirror images of each other as charge variants with higher pI are the first to be analyzed in the MS.

Deglycosylated NISTmAb Charge Profiles

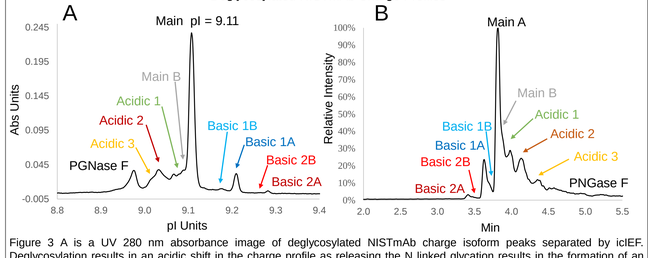

Figure 3 A is a UV 280 nm absorbance image of deglycosylated NISTmAb charge isoform peaks separated by icIEF. Deglycosylation results in an acidic shift in the charge profile as releasing the N linked glycation results in the formation of an aspartic acid residue. Each charge isoform peak in the UV profile has a corresponding peak in the MS BPE. The Main Peak in the MS BPE was split into two sections A and B, as a +1 Da shift was observed in the B section of the main peak.

MS-based Comprehensive Analysis of Intact mAb CQAs

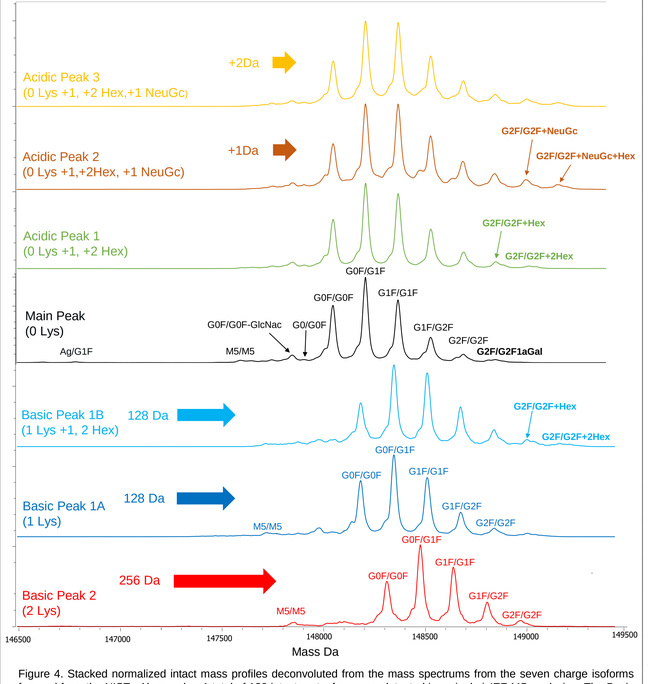

Figure 4. Stacked normalized intact mass profiles deconvoluted from the mass spectrums from the seven charge isoforms focused from the NISTmAb sample. A total of 130 intact proteoforms are detected in a single icIEF-MS analysis. The Basic 2 and 1 A/B charge variants with a delta mass of +256 and +128 Da respectively are identified as c-terminal lysine variants. Additionally, with Basic 1B the relative intensity of the glycoforms changes and masses above G2F/G2F at +162 Da intervals are observed suggesting that this is a glycated fraction of the 1 c terminal lysine variant. Similar glyccoform patterns are detected in Acidic isoforms 1, 2, and 3 indicating that glycated fractions for completely process c terminal NISTmAb are also present in the charge profile. Intact masses that correspond to NeuGc terminated glycans are observed in Acidic Peaks 2 and 3. Both these same isoforms have G0F/G0F masses shifted in +1Da mass intervals from the Main Peak, a pattern consistent with deamidation. Neutral mass isoforms, especially glycosylation are readily measured by icIEF-MS. Amongst the N-linked glycans detected are high mannose, aglycosylated, afucosylated and galactosylated complex structures including GalαGal.

Confirming Glycation and Deamidation with Deglycosylated NISTmAb

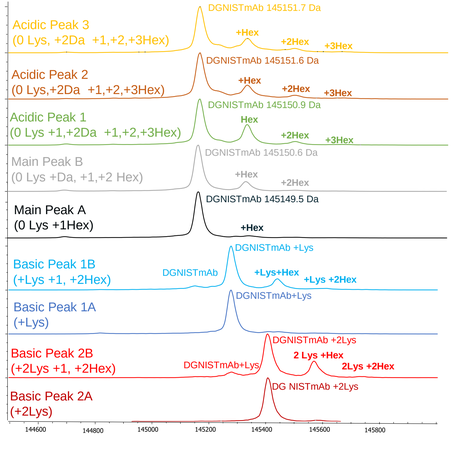

Figure 5 contains stacked normalized deconvoluted mass profiles for the charge isoform peaks of the deglycosylated NISTmAb sample shown in figure 3. Removal of the N linked glycans allows for the detection of glycation events (Hex) without convolution. In addition, the resulting increase in signal intensity, allows for a more precise of mass estimation which is useful when measuring deamidation. Basic Peak 2 A/B, and 1 A/B are shifted +256 and +128 Da from the Main Peak respectively confirming that they are C terminal Lys variants. Up to +2 Hex events per NISTmAb molecule are also confirmed with these Basic isoforms. With the Main and Acidic species up to +3 Hex events are confirmed. The nonglycated isoform (DGNISTmAb) increases in mass moving from Main A to Acidic 3. Mass shifts a total of +2.2 Da confirming that up to 2 deamidation events per molecule are present in the NISTmAb structure

CONCLUSIONS

- Fast, most straightforward way from icIEF to MS with no intermediate sample prep steps.
- Sensitive and comprehensive, requiring only 120 ng of NISTmAb to characterize seven charge variant peaks, and identify 130 molecular features (CQA's)
- Definitive results for intact mAb characterization and identification

TRADEMARKS/LICENSING

PROTEIN METRICS