ZipChip Charge Variant Analysis Coupled to a SCIEX TripleTOF® 6600+ System Mass Spectrometer

INTRODUCTION

Charge variant analysis is routinely performed during the development and production of mAb based biotherapeutics to assess quality attributes related to product efficacy and safety. Identification of charge variants, particularly new, unexpected variants, is difficult with traditional techniques that are interfaced with optical detection, such as capillary isoelectric focusing and charge exchange chromatography. ZipChip integrates high efficiency charge variant separations of mAbs with high resolution mass spectrometry to enhance the detection and identification of product quality attributes critical to drug development and production.

In this work ZipChip is interfaced with a TripleTOF 6600+ MS. By keeping the molecules in their native form during the ZipChip separation, excellent resolution can be achieved between the charge variant peaks. This separation combined with the high resolution and accurate mass delivered by the mass spectrometer yields unambiguous identifications of many mass and charge variants. In this experiment, the NISTmAb was stressed at elevated pH and temperature to induce deamidation. The mass shift of a deamidation is less than 1 Da. Due to the large number of isotope peaks on intact mAb mass spectra it's not possible to resolve a deamidation without separating the deamidated species from its unmodified pair. The ZipChip separation fully resolves the deamidated species, enabling identification of this and many other species via the SCIEX TripleTOF® 6600+ System.

MATERIALS AND METHODS

Sample Preparation. For initial charge variant analysis NISTmAb was diluted to 1 mg/mL with sample diluent from the Charge Variant - TOF BGE Kit. For forced degradation, NISTmAb was incubated in a phosphate buffer at pH 8 and 40 °C. Time points were taken at 0, 24, 48, 72, 96, and 144 hrs. Prior to analysis the time point samples were diluted and buffer exchanged into sample diluent for a final concentration of 0.85 mg/mL.

Instrumentation. HRN type chips and the Charge Variant - TOF BGE Kit were used for all analyses. The default method conditions for running intact charge variants were used with a 10 second replicate delay as shown in Figure 1.

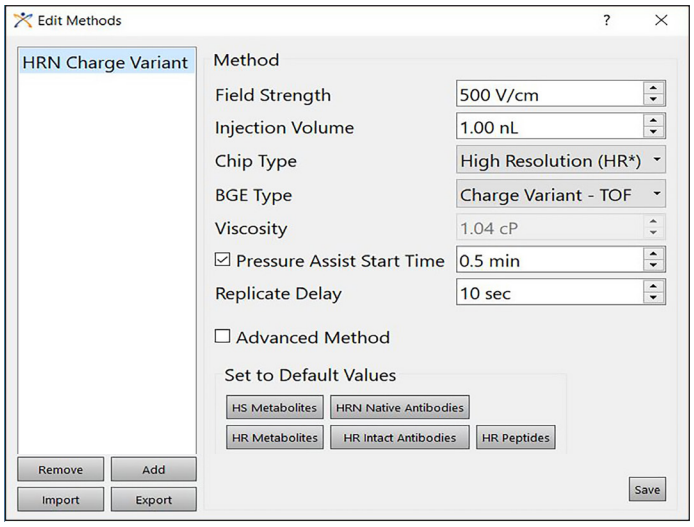

Figure 1: ZipChip method settings used for intact charge variant analysis.

MS analysis was performed using a SCIEX TripleTOF 6600+ mass spectrometer. The curtain gas was set to 10, GS1 at 3 psi, and interface heater at 50°C.

ZipChip mounted to a SCIEX TripleTOF 6600+ mass spectrometer.

Data was acquired from 2,000-7,000 m/z with a declustering potential of 180 and collision energy of 70 with an accumulation time of 1s and time bins to sum set to 100.

Data Processing. Data were viewed and peak areas were generated using SCIEX OS Explorer. Raw mass spectra were deconvoluted using BioPharmaView Flex. For automated mass matching, Intact Protein Target Masses were used as illustrated in Figure 2. The non-glycosylated mass of the NIST mAb with a fixed N-terminal pyroglutamic acid modification (145402.82) was used as the Target Mass. A post-run calibration was applied to processed masses to correct for a systematic mass error.

RESULTS AND DISCUSSION

ZipChip performs capillary zone electrophoresis separations so molecules separate based on the ratio of their net charge and their hydrodynamic radius in solution. Assuming different forms of the same mAb have similar solution phase conformations, the charge variants separate largely based on charge with the more basic variants migrating through the chip first and the more acidic variants last. Figure 3 shows the separation of unstressed NIST mAb. The mAb standard separates into two basic variants, a main variant, and an acidic variant with migration times in the range of 5.5 – 7.0 minutes.

The ZipChip separation is seamlessly integrated with electrospray ionization and mass spec detection. Therefore, every data point in the separation is actually a unique mass spectrum. For data processing, the data points in each charge variant peak were combined into

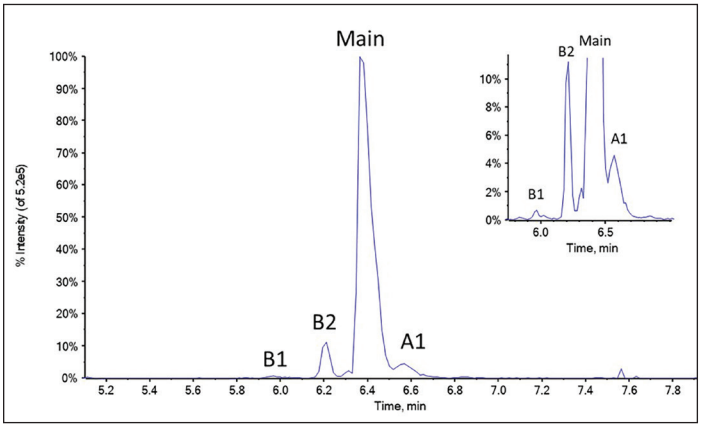

Figure 3: ZipChip charge variant separation of the NIST mAb. Four main variants are separated: Basic 1 (B1), Basic 2 (B2), the main species (Main), and an acidic variant (A1). The inset shows a zoom of the lower abundance basic and acidic species.

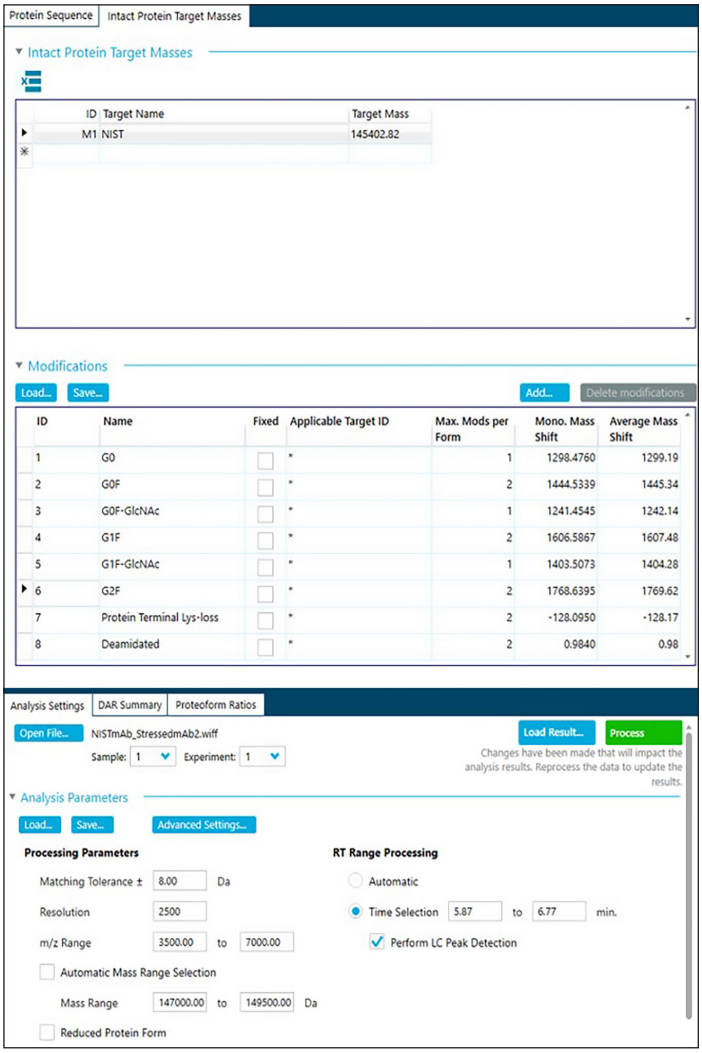

Figure 2: BioPharmaView Flex settings used for processing mass spectra.

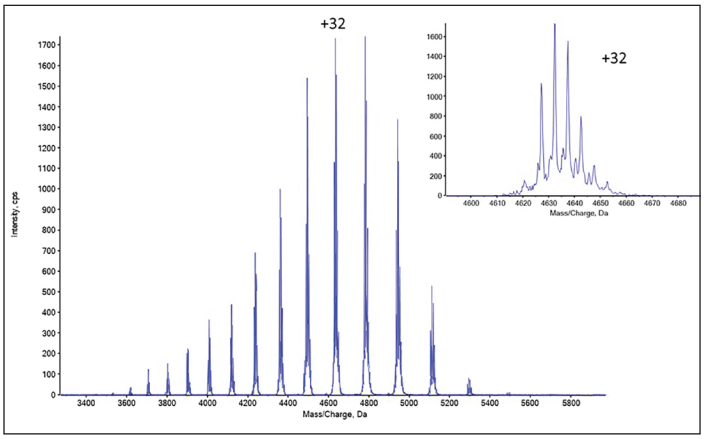

Figure 4: Raw mass spectrum of the main variant of the NIST mAb. The envelope spans 3,500 to 6,000 m/z with +32 as the most abundant charge state. High resolution mass measu-re-ments also allow the glycoforms of the mAb to be measured. This is visible as additional detail within each charge state as shown in the inset.

a unique mass spectrum. The raw mass spectrum for the main variant of NIST mAb is shown in Figure 4. Under the conditions used for this analysis, a charge state envelope is generated from 3500-6000 m/z with the most abundant charge state at +32. The TripleTOF 6600+ provides high mass resolution of the intact mAbs, which can be seen in the finer detail within each charge state of the envelope as shown in the inset of Figure 4. These separate peaks within each charge state are the different glycoforms of the mAb. After processing in BPV Flex, the glycoforms of NIST mAb can be easily identified by their accurate mass. Table 1 lists the identified glycoforms of the main variant of NIST mAb. All of the glycoforms were identified with less than 20 ppm mass error.

The NIST charge variants are baseline resolved in the ZipChip separation making it easy to characterize the charge variants of the mAb. This is particularly important when performing peak identification because a clean, single species mass spectrum can be generated for each charge variant. This significantly reduces complications and ambiguity in identifications due to spectral overlap of species with similar masses, such as with deamidated variants. To identify the charge variants of NIST mAb both their migration relative to the main variant and their mass were considered. The basic and main variants differ in mass by ~128 Da. This

Glycoform	Theoretical Average MW	Observed Average MW	Error (ppm)
G0F - 1; G0F-GlcNAc - 1	147833.95	147833.35	-4.06
G0F - 2	148037.15	148035.31	-12.40
G0F - 1; G1F - 1	148199.29	148198.17	-7.52
G1F - 2*	148361.43	148360.66	-5.17
G1F - 1; G2F - 1	148523.57	148523.64	0.51
G2F - 2	148685.71	148686.94	8.29

Table 1: Glycoforms of the main NIST mAb variant

mass shift is indicative of C-terminal lysine truncation and the observed change in migration time supports that. The first basic variant (B1) has the fastest migration time indicating it is the most highly charged variant. Its mass also agrees with the expected mass for a variant with two C-terminal lysines. B2 is lower in mass by ~128 Da and has a slower migration time indicating a reduced net charge. Finally, the main variant's mass agrees with truncation of both C-terminal lysine residues which is accompanied by a slower migration time.

This two-factor identification of variants based on migration pattern and mass information becomes even more important when considering the acidic variant. One of the most common acidic modifications is deamidation, which causes a mass shift of ~1 Da and is not detectable at the intact mass level without a

	RT	Theoretical MW	Observed MW	Error (ppm)	Modifications
B1	5.96	148293.49	148293.26	-1.53	G0F - 2
	5.96	148455.63	148456.42	5.31	G0F - 1;G1F - 1
	5.96	148617.77	148616.31	-9.83	G1F - 2
	5.96	148779.91	148780.33	2.77	G1F - 1;G2F - 1
B2	6.20	147962.13	147958.73	-22.94	G0F - 1;G0F-GlcNAc - 1;Protein Terminal Lys-loss - 1
	6.20	148165.32	148166.04	4.87	G0F - 2;Protein Terminal Lys-loss - 1
	6.20	148327.46	148327.70	1.62	G0F - 1;G1F - 1;Protein Terminal Lys-loss - 1
	6.20	148489.60	148490.53	6.29	G1F - 2;Protein Terminal Lys-loss - 1
	6.20	148651.74	148652.90	7.83	G1F - 1;G2F - 1;Protein Terminal Lys-loss - 1
	6.20	148813.88	148813.71	-1.18	G2F - 2;Protein Terminal Lys-loss - 1
Main	6.38	147833.95	147833.35	-4.06	G0F - 1;G0F-GlcNAc - 1;Protein Terminal Lys-loss - 2
	6.38	148037.15	148035.31	-12.40	G0F - 2;Protein Terminal Lys-loss - 2
	6.38	148199.29	148198.17	-7.52	G0F - 1;G1F - 1;Protein Terminal Lys-loss - 2
	6.38	148361.43	148360.66	-5.17	G1F - 2;Protein Terminal Lys-loss - 2
	6.38	148523.57	148523.64	0.51	G1F - 1;G2F - 1;Protein Terminal Lys-loss - 2
	6.38	148685.71	148686.94	8.29	G2F - 2;Protein Terminal Lys-loss - 2
A1	6.61	148038.13	148039.11	6.60	Deamidated - 1;G0F - 2;Protein Terminal Lys-loss - 2
	6.61	148200.27	148202.39	14.31	Deamidated - 1;G0F - 1;G1F - 1;Protein Terminal Lys-loss - 2
	6.61	148362.41	148364.72	15.53	Deamidation - 1; G1F - 2; Protein Terminal Lys-loss - 2
	6.61	148524.55	148525.65	7.37	Deamidated - 1;G1F - 1;G2F - 1;Protein Terminal Lys-loss - 2
	6.61	148686.69	148684.20	-16.75	Deamidated - 1;G2F - 2;Protein Terminal Lys-loss - 2

Table 2: Identified variants of the NIST mAb

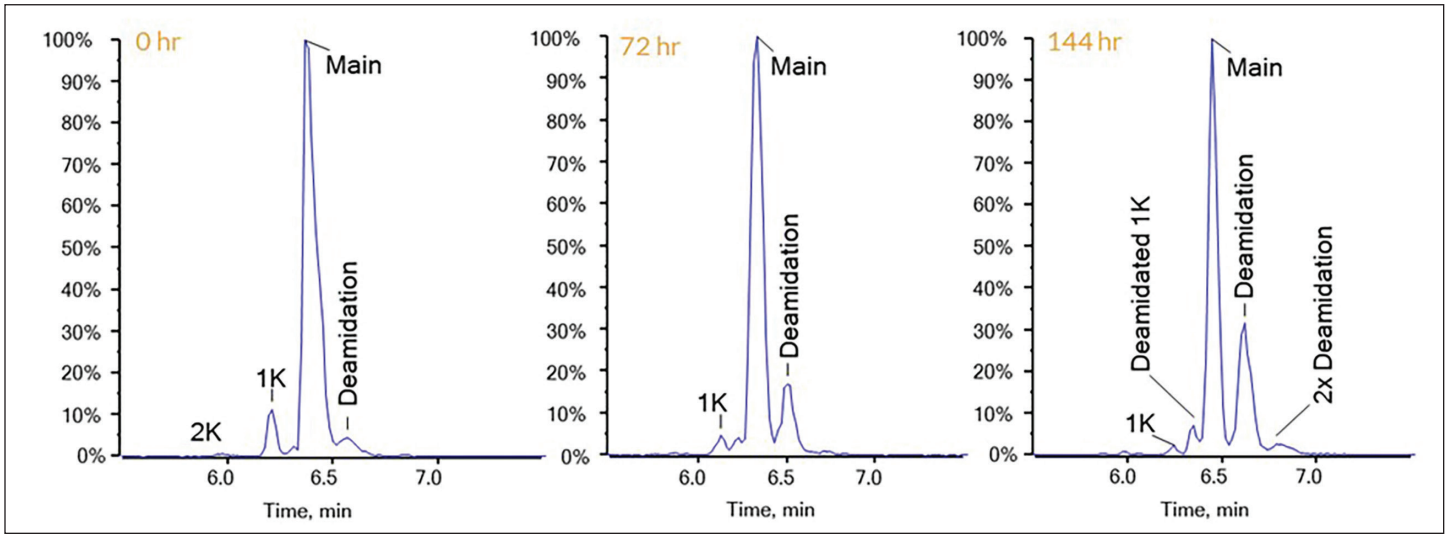

Figure 5. Charge variant separations of the 0, 72, and 144-hour time points. The deamidated peak increases in intensity with incubation time. By the 144 hr time point, deamidated forms of the basic variants are visible as well as a second acidic variant attributed to multiply deamidated mAb.

separation prior to MS detection. Since this modification is so difficult to detect at the intact level observing a shift in migration time becomes mandatory criteria for identifying this modification. The processed masses for the acidic peak are very similar in mass to the main variant. The G0F/G1F glycoform of the main and acidic species differ in mass by 4.2 Da. This can easily be within the measurement error for a molecule of this size. However, the shift in migration time confirms that the acidic variant is a different species. This combined with the small mass shift suggests deamidation. Table 2 lists all the identified variants of the NIST mAb. Including charge variants and variants were identified.

The time points from the forced degradation experiment were analyzed using the same method parameters. The charge variant separations of the 0, 72, and 144 hr time points are provided in Figure 5. The complexity of the charge variant profile is noticeably increased by the 144 hr time point. Lower abundance variants are present between the basic and main variant in addition to a second acidic species at a longer migration time. Processing the raw mass spectra reveals that the new basic peaks are elevated deamidation of B1 and B2, while the second acidic variant is likely due to multiple deamidations on the mAb. Deamidation of the main and basic variants appears to be proportional. For simplicity, the peak areas of the main and acidic variants were generated and plotted as % of the main variant to measure the % deamidation (Figure 6. The abundance of the first acidic variant increases steadily throughout the course of the incubation while the second acidic variant is not detectable until the last timepoint. At the longest time point, ~45% of the mAb was deamidated with ~5% multiply deamidated. The glycoforms of the mAb over the course of the incubation were also compared. Figure 7 compares the relative percent of the 6 major glycoforms from each timepoint. No major variations in glycoform composition were observed due to stressing the mAb.

CONCLUSIONS

ZipChip coupled to the SCIEX TripleTOF 6600+ provides information-rich charge variant analysis of biotherapeutic mAbs. Peak identification is greatly simplified by utilizing the mass information in addition to the charge variant migration time. For all charge variants both the migration time and the mass were used

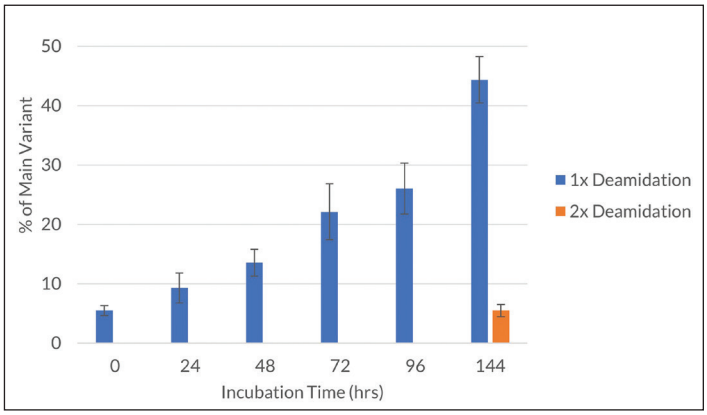

Figure 6: Plot of peak area ratios as % of main with respect to incubation time. By the 144-hour time point approximately 45% of the mAb was deamidated and approximately 5% of the mAb was deamidated multiple times.

to assign post translational modifications. This two-factor identification is necessary to assign challenging post translational modifications like deamidation. Twenty one variants of the NISTmAb were identified including deamidation, C-terminal lysine truncation and glycoforms. For a set of stressed NISTmAb samples relative quantitation showed an increase to ~45% of the main variant for deamidated mAb and ~5% for multiply deamidated mAb while glycoform distribution remained largely unchanged. ZipChip coupled to the SCIEX TripleTOF 6600+ MS is an asset for in-depth characterization of biotherapeutic mAbs.

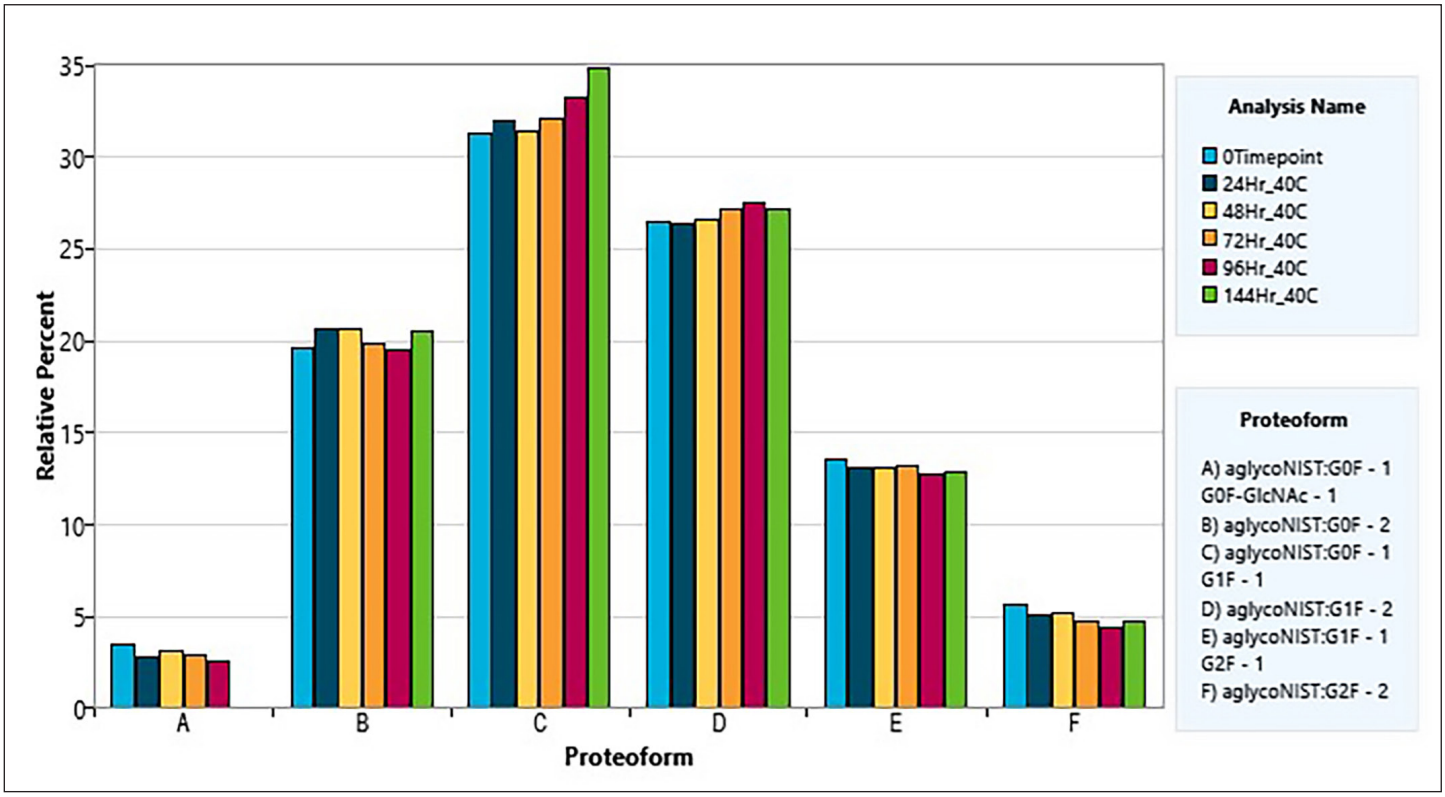

Figure 7: Comparison of the major glycoform composition over the course of the stress experiment. No major changes in glycoform composition were observed.

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