Application Note

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Identification of NISTmAb Charge Variants by Fully Automated Capillary Isoelectric Focusing and Mass Spectrometry

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Introduction

The NIST monoclonal antibody (NISTmAb) reference material is a therapeutic-grade monoclonal antibody standard developed by the National Institute of Standards and Technology (NIST). Using the NISTmAb facilitates industry-wide technology comparison and analytical method development. One of the critical quality attributes of the NISTmAb, the charge heterogeneity profile, has been comprehensively studied at the NIST, using optimized capillary zone electrophoresis (CZE) and capillary isoelectric focusing (CIEF) assays with ultraviolet (UV) detection. This Application Note demonstrates the direct, high-resolution, mass spectrometry (MS) identification of the NISTmAb charge variants using an online CIEF/MS workflow.

Experimental

Materials

The carrier ampholytes, Pharmalyte having pH ranges 3.0 to 10.0, and 8.0 to 10.5 were purchased from Sigma-Aldrich (St. Louis, MO, USA). The NISTmAb reference material, RM 8671, was purchased from the NIST. LC/MS grade reagents, including water, formic acid, ammonium hydroxide, acetonitrile, and methanol were purchased from Millipore-Sigma (Burlington, MA, USA). The CIEF/MS reagent kits (part numbers CR 3520, CR5520, and CR7720) were provided by CMP Scientific Corp. (Brooklyn, NY, USA).

Online capillary isoelectric focusing/mass spectrometry (CIEF/MS)

Online CIEF/MS was performed on an Agilent 7100 capillary electrophoresis (CE) system (Agilent Technologies, Inc., Santa Clara, CA, USA) coupled to an Agilent 6230B time-of-flight (TOF) LC/MS system using an EMASS-II CE/MS ion source (CMP Scientific Corp., Brooklyn, NY, USA). Electrospray emitters for online CIEF/MS analysis (1.5 mm od, 1.1 mm id, 20 µm tip size) and neutral coating PS1 capillaries (360 µm od and 50 µm id) for separation were from CMP Scientific. A positive electrospray ionization voltage (2.4 kV) was generated by an external benchtop high-voltage power supply that is supplied with the EMASS-II ion source. The distance from the emitter tip to the mass spectrometer was adjusted to 4 mm, as measured by the microscope camera. Samples (0.05 to 0.1 mg/mL) were prepared in various combinations of Pharmalyte with a total concentration of 1.5% (v/v) in the sample solution.

Sample injection and separation

For the 75 cm capillary, the sample solution injection time was set at 40 seconds, following a 40 second injection of the catholyte solution at 940 mbar. The catholyte and sample injection durations were 35 and 30 seconds for the 60 cm capillary. A positive CE separation voltage was then applied at the capillary inlet end to initiate electric focusing. For various lengths of separation capillaries, a suitable CE separation voltage was chosen to generate a field strength of 250 V/cm (that is, 18.8 kV for the 75 cm capillary and 15 kV for the 60 cm capillary). A constant pressure of 5 mbar was applied at the capillary inlet to generate hydrodynamic flow and shorten analysis time.

MS parameters

The capillary voltage (Vcap) on the 6230B TOF LC/MS was set at 0 V. Drying gas was 6 L/min at 365 °C. Fragmentor voltage was 380 V. Skimmer voltage was 300 V. To minimize interference with the nanospray out of the EMASS-II ion source, a single-bore inline nanospray shield was used to divert the drying gas out of the mass spectrometer. The CE/MS method setup, data acquisition, and analysis were performed using Agilent MassHunter workstation software (revision B.09).

Table 1 summarizes this CIEF/MS method.

Table 1. Critical experimental parameters for the CIEF/MS analysis.

Parameter	Value
Capillary Electrophoresis	Agilent 7100A CE
Mass Spectrometer	Agilent 6230B TOF LC/MS
CE/MS Coupling	CMP Scientific EMASS-II CE/MS Ion Source
Separation Capillaries	60 and 75 cm PS1 capillaries (CMP Scientific Corp.)
Anolyte	CIEF/MS reagent kit (CMP Scientific Corp.)
Catholyte	CIEF/MS reagent kit (CMP Scientific Corp.)
Sheath Liquid	CIEF/MS reagent kit (CMP Scientific Corp.)
Sample Buffer	CIEF/MS reagent kit (CMP Scientific Corp.)
Injection	Flush, 35 to 40 seconds (catholyte)
	Flush, 30 to 40 seconds (sample solution)
Separation	15.0 to 18.8 kV, 5 mbar
Electrospray Emitter	20 µm tip size (CMP Scientific Corp.)
ESI Voltage	2.4 kV
Distance from Emitter to MS	4 mm
Distance from Capillary End to Emitter Tip	1.1 mm
Fragmentor Voltage	380 V
Skimmer	300 V
Vcap	0 V
Drying Gas Flow Rate	6 L/min
Drying Gas Temperature	365 °C

Results and discussion

CIFE/MS method development for NISTmAb

The pH value of the main peak of NISTmAb is 9.1. It had initially been challenging to separate the basic and acidic variants of NISTmAb. Figure 1 shows the effect of various combinations of Pharmalyte on the CIEF resolution. Four ratios of the regular range (pH 3.0 to 10.0) and the basic range (pH 8 to 10.5) Pharmalyte were tested: 1:0, 2:1, 1:2, and 1:4. The ratio of 1:4 was chosen for the rest of the study, because the other ratios failed to reveal all the charge variants of NISTmAb. This observation is consistent with previous results from a CIEF/UV study.

Repeat CIEF-MS injections of NISTmAb

Figure 2 shows the results of five overnight CIEF/MS injections of NISTmAb sample. Due to the concern that an extended period with ammonium hydroxide inside the capillary would compromise the capillary neutral coating, we used high pressure (940 mbar) for catholyte and sample injection in the method to quickly move the ammonium hydroxide plug from the capillary inlet to the outlet. This high-velocity catholyte and sample injection may generate laminar flow, resulting in diffusion of the ammonium hydroxide front end, and mixing of the sample plug with the ammonium hydroxide fluid. As a result, the length of effective ammonium hydroxide and sample zones may vary slightly between injections. This may lead to the variation of peak migration time. The RSD of the migration time of the NISTmAb main peak was calculated to be 3.7%.

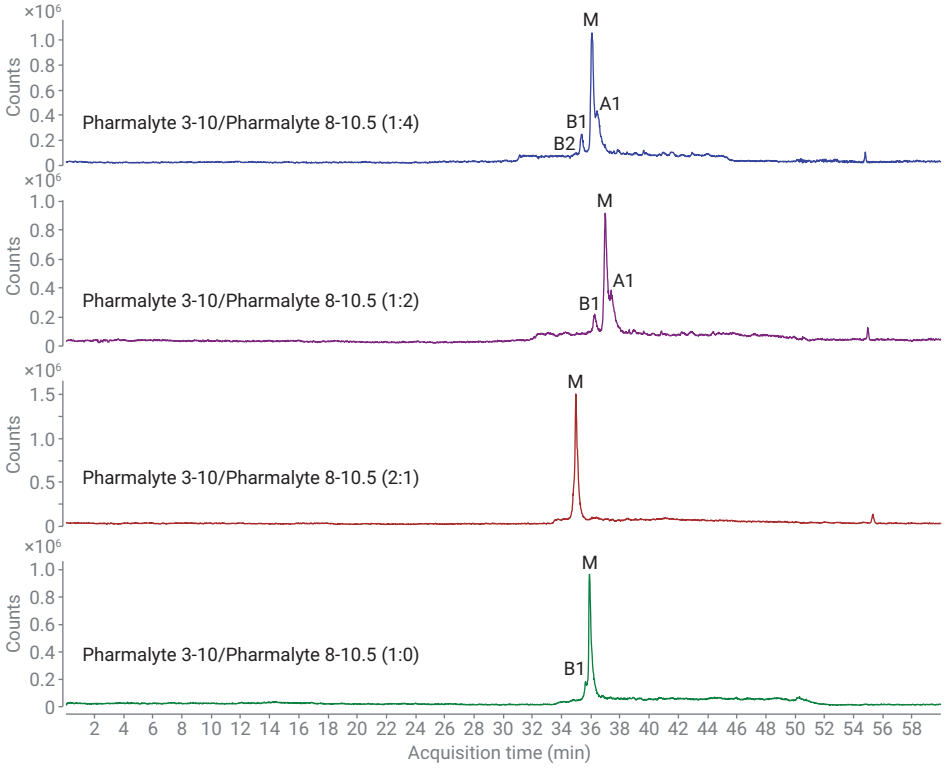

Figure 1. CIEF/MS method development for NISTmAb including the testing of different combinations of ampholytes.

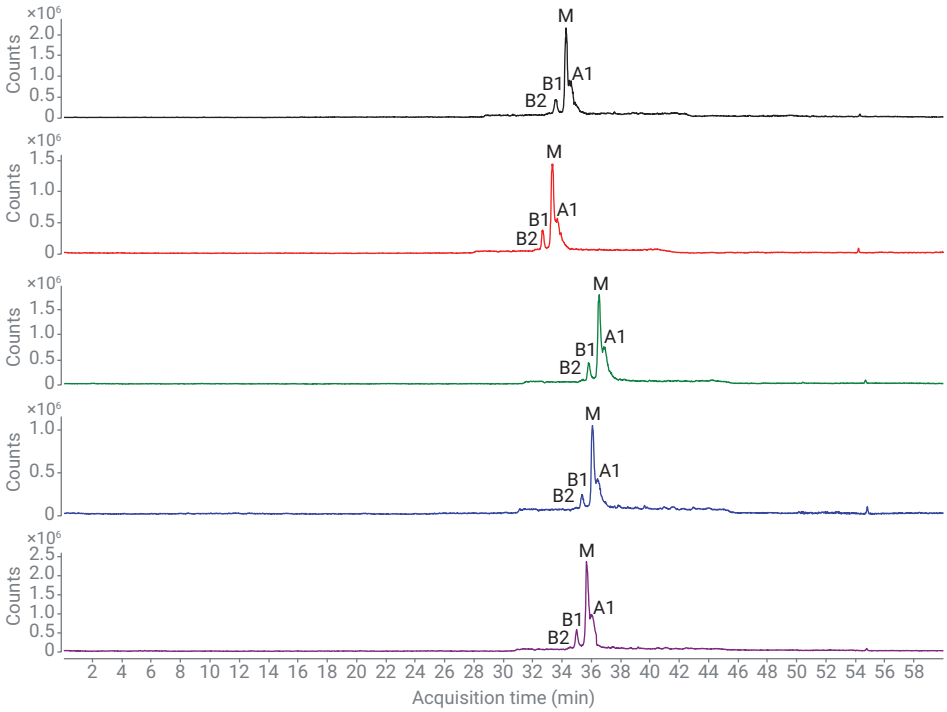

Figure 2. Repeat CIEF/MS analysis of NISTmAb on a 60 cm separation capillary.

Further optimization on the CIFE/MS method for NISTmAb

The CIEF/MS method for NISTmAb was optimized with the intention to obtain further separation between each of the NISTmAb charge variants. Figure 3 shows the five overnight CIEF/MS injections of NISTmAb sample, which were carried out on a 75 cm separation capillary. The resolution shown in Figure 3 is slightly better than that in Figure 2. In particular, the acidic variant (peak labeled as A1) was better resolved on the 75 cm capillary. We found that a longer capillary generates better resolution for CIEF/MS analysis. This may be because a 5 mbar constant pressure was applied during each injection. Under the same pressure, the disturbance on the focusing and subsequent mobilization was less on longer capillaries. The 7100 CE system can reliably deliver 5 mbar for long periods. This special feature of the 7100 CE system is one of the critical factors that made our CIEF/MS method successful.

Mass spectra of NISTmAb charge variants

Figure 4 shows the *m/z* mass spectra of the NISTmAb charge variants (B2, B1, M, and A1), generated by averaging each of the peaks in the first injection, shown in the top panel of Figure 3. These mass spectra are of high quality, with essentially no interference from the ampholytes.

Charge variants of NISTmAb identified by CIEF-MS analysis

Figure 5 shows the deconvoluted masses for the spectral data shown in Figure 4. Agilent BioConfirm software (revision B.09.00) was used to deconvolute the *m/z* spectra. The two basic variants of NISTmAb were identified to be C-terminal lysine variants. The acidic variant shows a +162 Da mass shift from the main species of NISTmAb. This indicates that the acidic variant is a result of lysine glycation on NISTmAb.

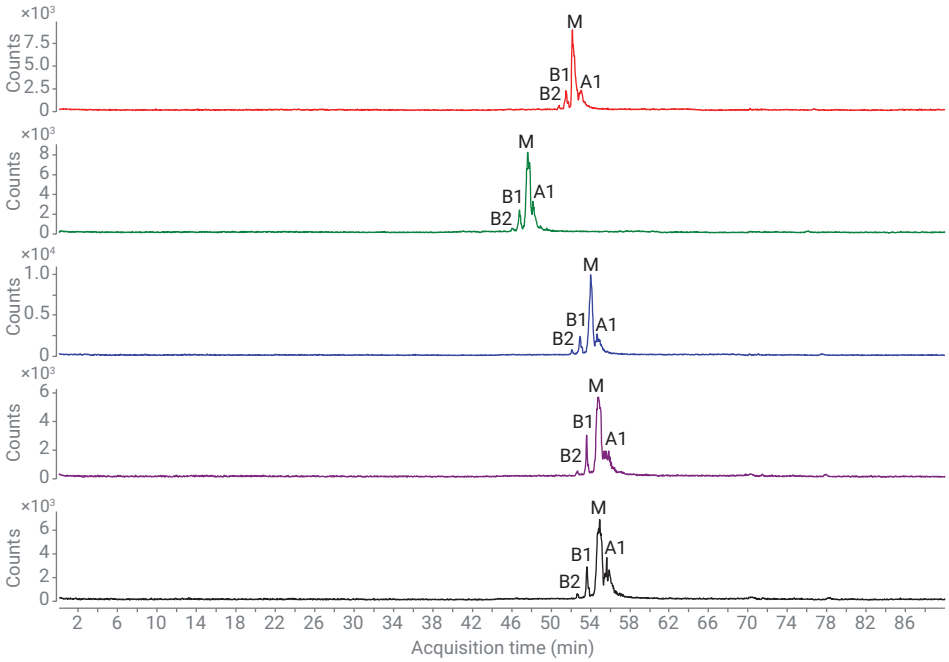

Figure 3. CIEF/MS analysis of NISTmAb on a 75 cm separation capillary.

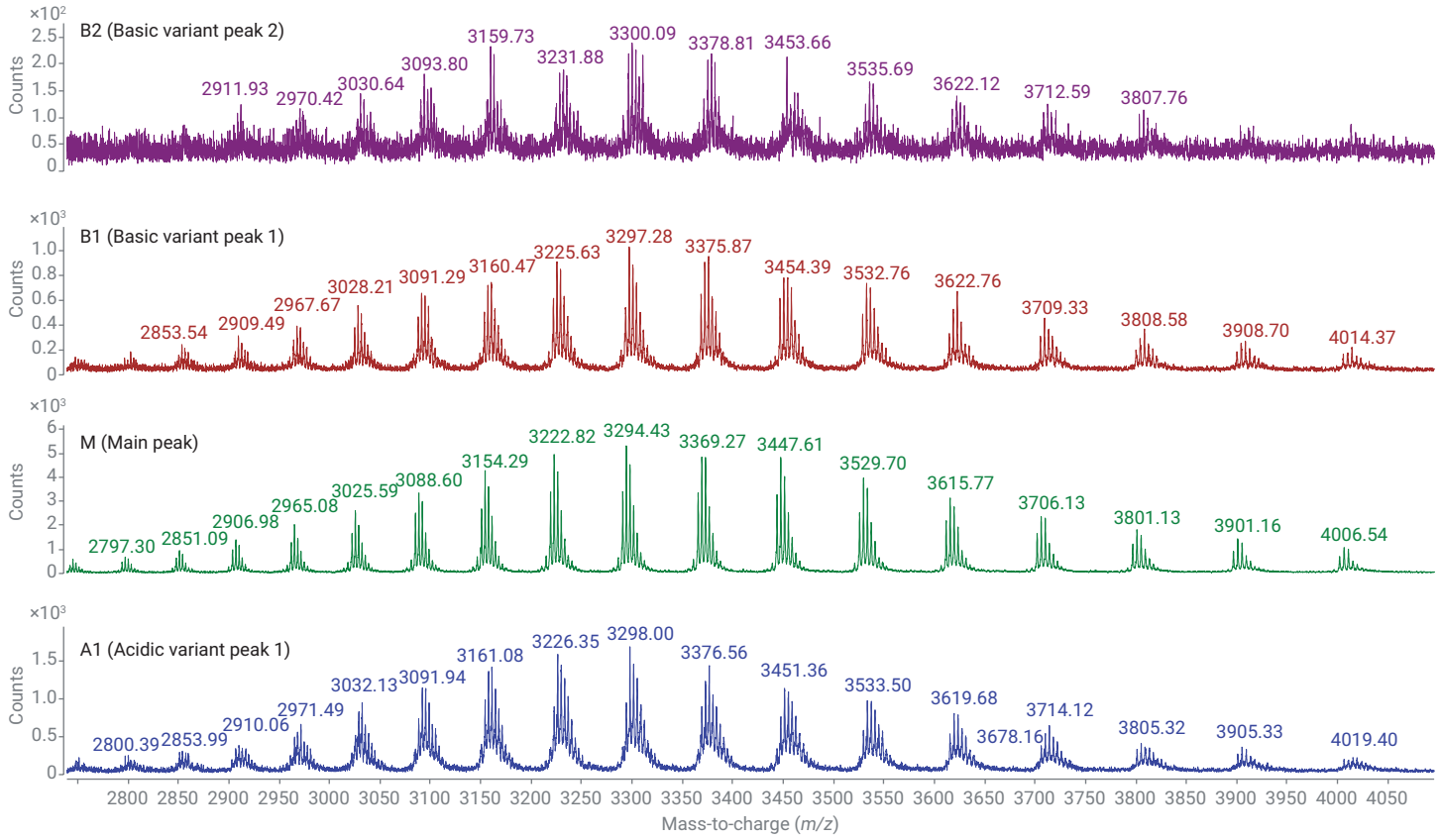

Figure 4. Mass spectra of NISTmAb charge variants.

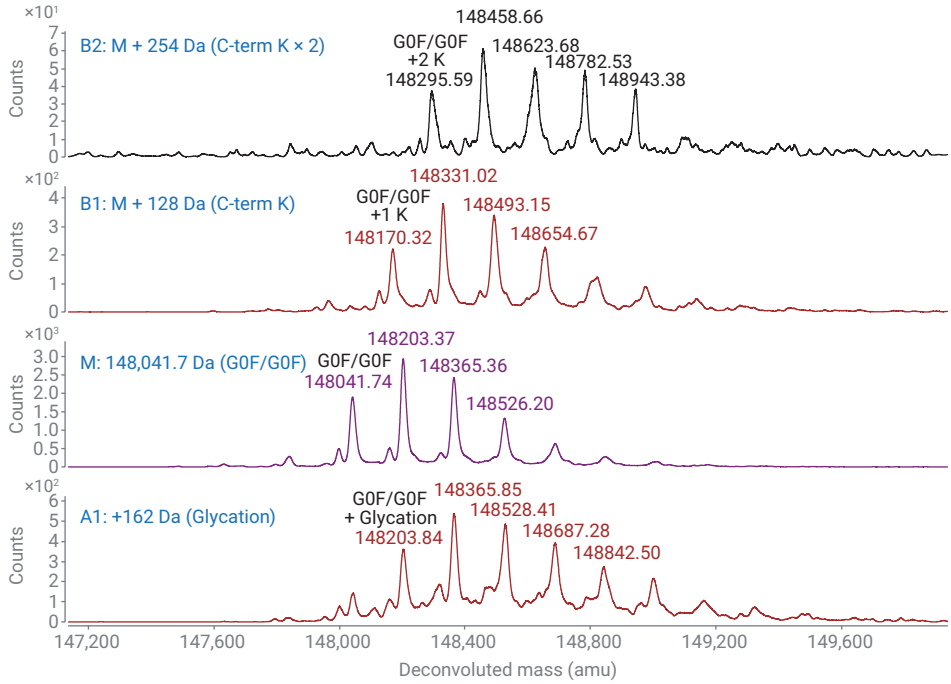

Figure 5. Deconvoluted masses of NISTmAb charge variants.

Reproducibility of NISTmAb charge variants identification by CIEF/MS analysis

Table 2 shows the NISTmAb charge variants identification results from the five overnight CIEF/MS injections of NISTmAb shown in Figure 3. The deconvoluted masses out of the CIEF/MS peaks are consistent. After taking an average of the deconvoluted masses from all five injections, the charge variants peaks of NISTmAb are identified as: two C-terminal lysine variants (B2), one C-terminal lysine variant (B1), and one lysine glycation variant (A1). The standard deviations of the deconvoluted masses are: 1.60 Da for basic variant B2, 1.42 Da for basic variant B1, 0.67 Da for the main peak, and 0.89 Da for the acidic variant A1. These values indicate that the precision in mass measurements is directly related to ion abundance.

Table 2. Reproducibility of five CIEF/MS injections of daratumumab.

Injections	B2	B1	M	A1
1	148,295.59	148,170.32	148,041.74	148,203.84
2	148,298.00	148,170.25	148,041.86	148,204.68
3	148,294.05	148,168.04	148,040.31	148,202.65
4	148,296.97	148,167.80	148,041.05	148,204.12
5	148,297.47	148,170.83	148,041.80	148,204.94
Average (Da)	148,296.42	148,169.45	148,041.35	148,204.05
Std. Dev. (Da)	1.60	1.42	0.67	0.89
ΔM (Da)	+255.07	+128.10		+162.70

Conclusion

This Application Note describes the direct high-resolution mass spectrometry identification of NISTmAb charge variants using a fully automated online CIEF/MS method implemented on Agilent 7100 CE and 6230B TOF LC/MS systems. This CIEF/MS workflow is enabled using an electrokinetically pumped sheath liquid nanospray capillary electrophoresis/mass spectrometry (CE/MS) coupling technology. The excellent performance of a CMP Scientific EMASS-II CE/MS ion source on Agilent systems is demonstrated by the confident identification of NISTmAb charge variants analysis from consistent CIEF/MS results. This study is a continuation of our previous CEIF/MS Application Note, with a primary focus on the NISTmAb molecule. This Agilent-CMP Scientific CIEF/MS workflow enables direct mass spectrometry identification of intact monoclonal antibody charge variants separated by capillary isoelectric focusing. The excellent resolution and mass spectrometry accuracy demonstrated in this Application Note further paves the way for a broad application of our Agilent-CMP Scientific CIEF/MS workflow for the MS identification of mAb charge variants.

References

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