Native CZE-MS of proteins and protein complexes using a neutral OptiMS surface cartridge

Using the CESI 8000 Plus ESI-MS System

Zhichang Yang[1], Zuzana Demianova[1], Fang Wang[1], Sahana Mollah[1], Kevin Jooss[2], Neil L Kelleher[2]

BioPharma

[1]SCIEX-Brea, USA; [2] Northwestern University, Evanston, IL, USA

Native mass spectrometry (nMS) enables the characterization of protein compositions, structure and interactions with ligands, metals and drugs as it preserves the non-covalent interactions during analysis.[1] Traditionally, nMS is performed using direct infusion setup. However, front-end separation (such as, size exclusion chromatography, ion-exchange chromatography) of native proteins/protein complexes are implemented offline. In this technical note, we coupled the CESI 8000 Plus ESI-MS System to a TripleTOF 6600+ system and demonstrated the on-line separation and characterization of a standard protein mixture covering a wide molecular weight (MW) range from 30 kDa to 230 kDa in the native state. The two-period mode capability of the TripleTOF 6600+ system enabled fast parameter optimization in a single run for a balanced and optimized signal of proteins mixtures.

Characterization of proteins in native mode is very important. A detailed description of protein functions requires the characterization of protein in the native state to understand the protein ensemble conformation, protein/protein interaction, and protein-ligand, metal and drug binding.[2]

Key features

- Capillary zone electrophoresis using Capillary electrospray ionization (CESI) technology offers the capability of separating standard proteins with their native state
- Characterization of proteins and protein complexes ranging from 30 kDa to 230 kDa in native mode with identification of different proteoforms such as glycol- and truncated forms
- High scanning speed of TripleTOF 6600+ system enables multi-period mode for fast MS parameters optimization

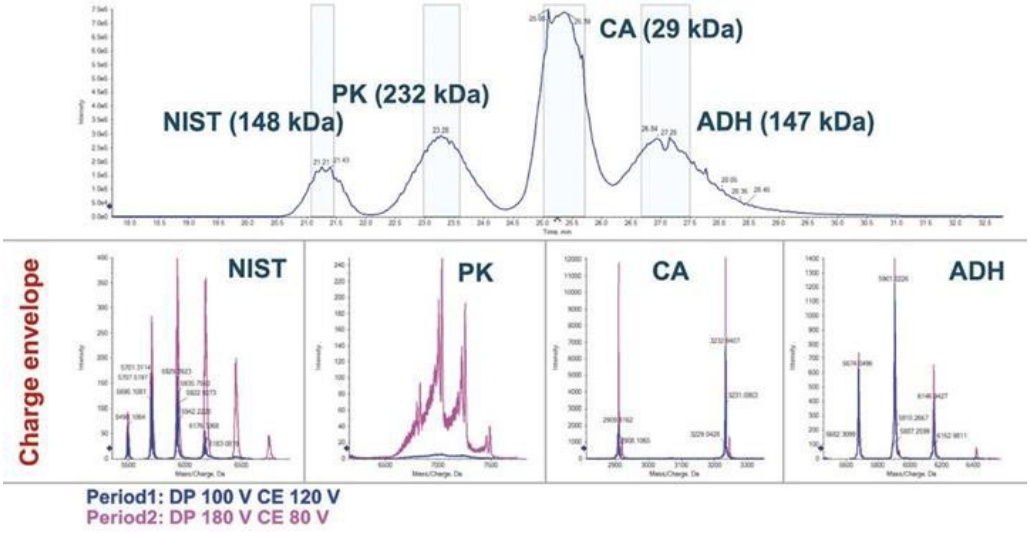

Figure 1. Analysis of protein mixture with 2-period mode. Top panel shows the separation of the 4 proteins by CESI-nMS. The bottom panels show the MS[1] spectra of the four proteins. The 2-period mode had different DP and CE settings applied.

Also, native conditions promote lower charge states, leading to increased m/z spacing, which facilitates the detection of smaller mass differences between proteoforms,[3] — for example, the identification and quantification of different drug-to-antibody ratio (DAR) populations of antibody-drug-conjugates (ADCs), and proteoforms with similar mass.[4]

nMS provides an effective way to characterize protein in native mode. When analyzing complex protein mixtures by nMS, a front-end separation is necessary to reduce ion suppression and signal superposition. Typically, size exclusion chromatography (SEC),[5] hydrophobic interaction chromatography (HIC),[6] ion-exchange chromatography (IEC),[7] and native Gel-Eluted Liquid Fraction Entrapment Electrophoresis (GELFrEE)[8] have been used for offline protein separation and fraction collection. Protein fractions are then cleaned and directly infused for nMS analysis, mainly because the buffer/electrolytes used in these techniques are not compatible with MS. The set up used in this work, a capillary electrophoresis platform, the CESI 8000, was coupled to the mass spectrometer for the online separation of peptides and intact proteins providing high separation efficiency and sensitivity. More importantly, MS-compatible and volatile aqueous reagents are used allowing the analytes to preserve their native state.

Here, we introduce a capillary zone electrophoresis (CZE) for the analysis and characterization of native protein and protein complexes by coupling the CESI 8000 Plus ESI-MS System to a TripleTOF 6600+ system.

Methods

Sample preparation: Protein stock solutions (10 mg/ml) were desalted, and buffer exchanged into 40 mM ammonium acetate (NH4Ac) (pH ≈ 6.8) using centrifugal MWCO filters. First, the filters were equilibrated with 500 µL of 40 mM NH_4Ac and spun at 12,000 *g* for 5 minutes. Each protein standard was then dispensed into the filter device individually and spun at 12,000 *g* until the sample was concentrated to a volume of 100 µL or less. In this work, ten consecutive buffer exchanges were performed by adding 40 mM NH_4Ac up to 500 µL per spin. NIST Monoclonal Antibody Reference Material 867 (NIST-mAb), was from NIST (Gaithersburg, MD). Carbonic anhydrase II (CA), Alcohol dehydrogenase (ADH); Pyruvate kinase (PK), were from Sigma (St. Louis, MO).

Individual protein samples and a protein mixture were diluted with 40 mM NH4Ac to final concentrations listed in Table 1.

Instrument configuration: CESI 8000 Plus ESI-MS System with the neutral OptiMS surface cartridge (SCIEX, P/N B07368) was coupled with the TripleTOF 6600+ system through a NanoSpray III source and a CESI adapter (SCIEX, P/N B07363). 32 Karat and Analyst TF software 1.8.1 were used to operate the CESI 8000 Plus ESI-MS System.

Native capillary electrophoresis and mass spectrometry: The separation conditions are listed in Table 2. Direct infusion using 15 kV voltage (positive polarity) with 5 psi supplemental pressure at both cartridge ends were applied to optimize the CESI sprayer position and spray stability.

The final parameters are listed in Table 3. A 2 periods MS method was developed to obtain a high-resolution MS spectrum for each protein complex in the prepared mixture (Table 1).

Important: For cartridge longevity and clogging prevention, it is crucial to use a capillary clean-up method and to keep the CESI spray tip emerged in water. The cleaning method consists of rinsing the separation line of the cartridge at 100 psi with 0.1 M HCI for 10 minutes, followed by a water rinse of the separation and conductive lines for 10 minutes each.

Data Processing: SCIEX OS software was used to visualize and perform data analysis.

Table 1. Experimental details of the protein mixture used in this study.

Protein	MW (kDa)	MWCO filter(kDa)	Direct infusion(µM)	Concentration in the mixture(µM)
Carbonic anhydrase II (CA)	*29*	*10*	*1*	*3.75*
Alcohol dehydro-genase (ADH)	*147*	*50*	*1*	*2.5*
NISTmAb (NIST)	*148*	*50*	*5*	*0.5*
Pyruvate kinase (PK)	*231*	*50*	*3.3*	*1*

Table 2. CESI 8000 Plus ESI-MS System separation conditions were used to separate the protein mixture.

	Event	Time (min)	Value	Direction	Note
1	Rinse	5	100 psi	Forward	0.1 M HCl
2	Rinse	3	100 psi	Reverse	BGE
3	Rinse	5	100 psi	Forward	BGE
4	Injection	30 s	2.5 psi	Forward	Sample
5	Wait	0			Water dipping
6	Injection	10 s	2.5 psi	Forward	BGE
7	Separation	0	3 psi/15 kV	1 min ramp, normal polarity, both	BGE
8	Relay On	1			MS Starts
9	Separation	35	5 psi/1 kV	1 min ramp, normal polarity, both	Ramp down
10	End	40			

Table 3. TripleTOF 6600+ system method conditions.

	Parameter	Period 1	Period 2
1	MS mass range	2,000 – 9,000 m/z	2,000 – 9,000 m/z
2	MS accumulation time	1 s	1 s
3	Curtain gas	5 psi	5 psi
4	Polarity	positive	positive
5	Source temperature	175°C	175°C
6	Declustering potential (DP)	100 V	180 V
7	Collision energy (CE)	120 V	80 V
8	Collision activated dissociation (CAD)	7 V	7 V
9	Ion spray voltage	2050 V*	2050 V*
10	Sum to bin	100	100

** Value is tuned during direct infusion.*

Results and discussion

CZE under native conditions and charge envelope analysis

It has been demonstrated that increasing the ammonium acetate (NH_4Ac) concentration improved the separation of the four proteins.[3] However, the increased NH_4Ac concentration also resulted in the separation current above the safety limit of 5 µA.[3] Thus, 40 mM NH_4Ac was selected as both background electrolyte (BGE) and leading electrolyte (LE) to preserve the native structure of a standard protein mixture containing four proteins with MW ranging from 30 kDa to 230 kDa (NIST, Carbonic anhydrase, Alcohol dehydrogenase and Pyruvate kinase. The concentrations of the four proteins were adjusted to ensure sufficient signal for each analyte.

Figure 1 shows a typical separation profile. Under native conditions, fewer protonation events occur, leading to lower charge state envelopes and higher m/z range in MS spectrum. This phenomenon can be explained by (i) less protons available at higher pH and by (ii) preserving the tertiary and quaternary structure, where moieties of amino acid sequence is not accessible for protonation. Figure 2 illustrates this behavior. The upper panel shows the typical charge envelope of a denatured state recognized by the high charge state. In contrast, the bottom panel shows the native form of NIST-mAb featured by the lower charge state.

The highest abundance charge states for all four proteins are closer to the results of reference 3 in which the same standard protein mixture was analyzed by an Orbitrap MS with extended mass range (EMR) under native condition, as shown in Table 4, indicating the transferability of the developed native CZE method to different MS instruments.

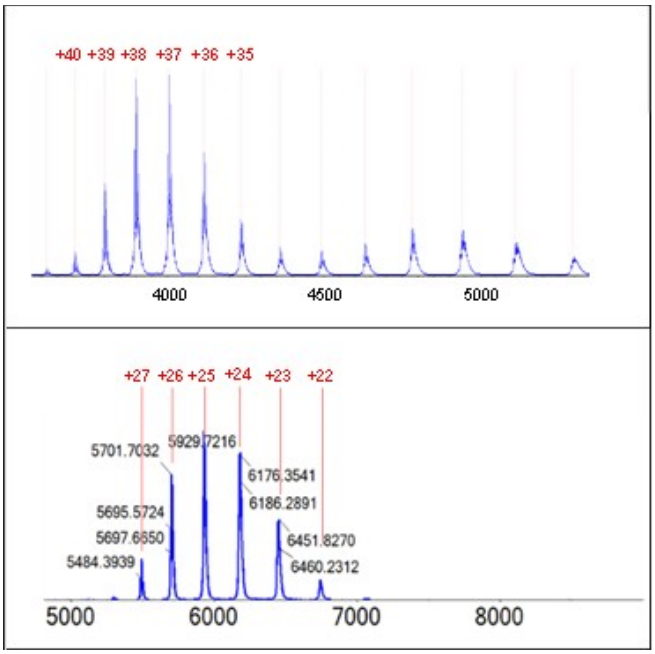

Figure 2. Two charge envelops of NIST- mAb in denatured (upper) and native (bottom) mode. The analysis of NIST- mAb in denatured mode was processed under acidic reverse phase conditions. The analysis of NIST- mAb in native mode was processed under neutral CESI condition.

Two examples of native protein characterization (NIST- mAb and pyruvate kinase) are illustrated in figures 3 and 4, respectively. The MS spectrum of NIST- mAb charge envelope shown on the top panel of Figure 3, revealed the proteoforms with different glycosylation patterns with good resolution and signal. The reconstructed spectrum shows good glycoform distribution of NIST - mAb with relative abundance information in the bottom panel. The largest protein complex, pyruvate kinase (PK)—a 231kDa, tetramer—was well separated from the other three, as shown in Figure 1. The full MS spectrum of PK shows the detection of both the tetramer and the monomer at higher m/z and lower m/z range respectively, (top panel of Figure 4).

The charge envelopes of the monomers and tetramer are shown in the middle panels of Figure 4. After deconvolution, the bottom panels show the reconstructed spectra of both the monomer and the tetramer, possibly from PTMs and truncations. Further MS^2 analysis may be required to confirm the identification of the proteoforms.

Multi-period method for rapid MS method optimization

The NIST- mAb and PK analysis demonstrate the ability of the CESI technology to facilitate the protein characterization in native mode. The stability of native assemblies is sensitive to the MS parameters, especially those controlling the energy imposed on the ions within the instrumentation (collision energy and declustering potential).[2] Optimal MS parameters vary depending on the target analyte, and is essential to obtain the highest quality data. The fast-scanning capabilities of the TripleTOF 6600+ system allows for multi-period analysis mode where different settings of declustering potential (DP) and collision energy (CE) can be tested in a single run. In this work, a two-period mode was applied with different DP and CE settings in each period. Figure 1 shows the impact of the declustering potential (DP) and collision energy (CE) on the signal intensity of PK. Period 1 (data in blue trace) was set with DP of 100V and CE of 120 eV, whereas period 2 (data in pink trace) was set with DP 180V and CE of 80 eV. The comparison between the two setups indicates that a higher DP promotes a more efficient desolvation of the tetramer while displaying a good quality charge envelope.

Instead of adjusting DP and CE per protein through direct infusion, the 2-period mode enables fast parameter optimization within a single run for protein mixture analysis. For complex sample analysis such as proteomics, where pure protein standard is not available, the multi-period capability canbe a powerful tool to improve data quality more efficiently.

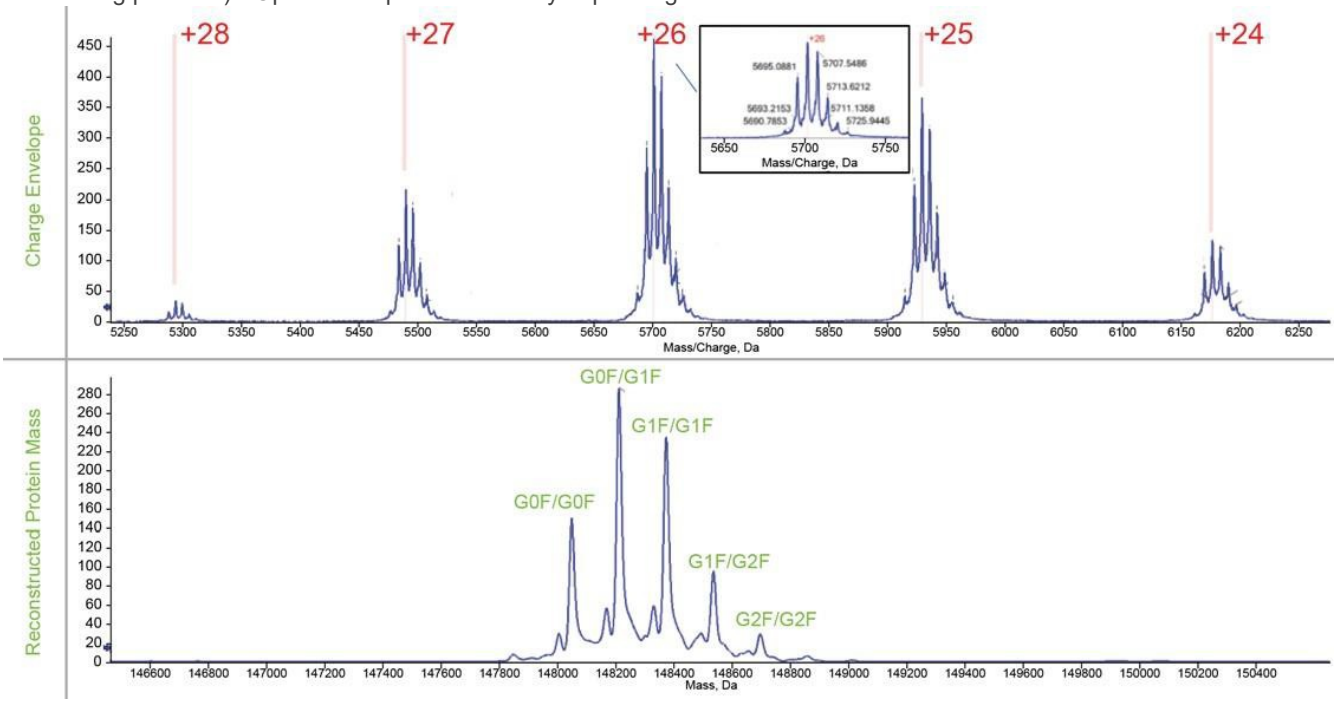

Figure 3. Characterization of NIST - mAb in native mode. The top panel shows the charge envelop of NIST - mAb. The bottom panel shows the reconstructed spectrum of NIST - mAb with different glycoforms.

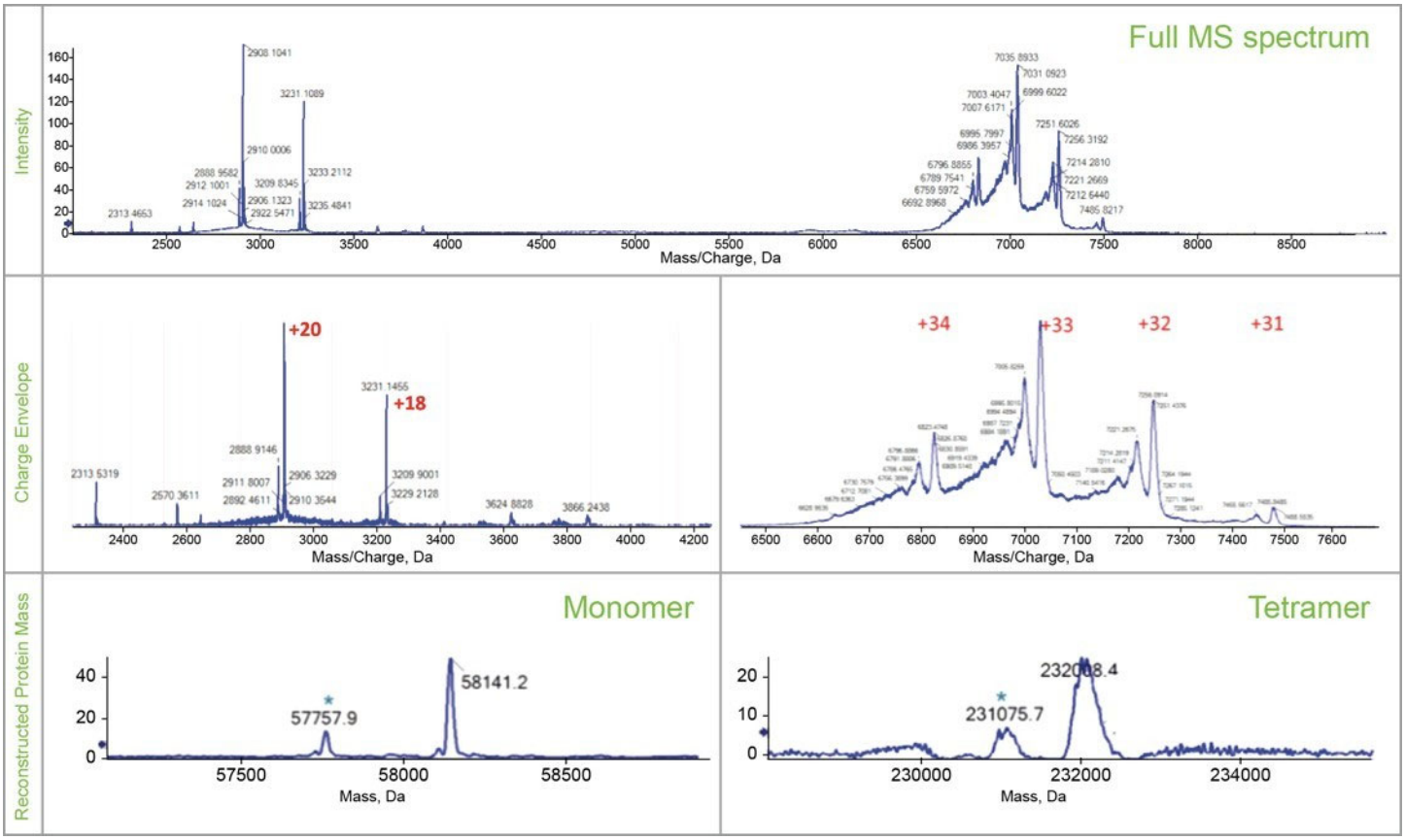

Figure 4. Characterization of the pyruvate kinase in native mode. Top panel shows full MS spectrum of PK tetramer and PK monomer. Middle panels show the charge envelopes of the PK monomer and PK tetramer. Bottom panels show the reconstructed spectra of the PK monomer and PK tetramer.

Table 4. Comparison of highest abundance charge states.

Protein	SCIEX	Reference 2
Carbonic anhydrase II (CA)	*+9*	*+9*
Alcohol dehydro-genase (ADH)	*+25*	*+25*
NISTmAb (NIST)	*+26*	*+24*
Pyruvate kinase (PK)	*+33*	*+32*

Conclusions

- The CESI 8000 Plus ESI-MS System coupled with the TripleTOF 6600+ system enabled the separation of a mixture of four standard proteins in their native where their features were preserved. The MW of the four proteins ranged from 30 kDa to 230 kDa.
- The CZE-CESI-MS separation in native mode showcased different proteoforms such as the glycoforms of NISTmAb.
- A two-period mode was applied during native CZE-CESI-MS analysis with different DP and CE settings. The two-period mode enabled fast parameter optimization to achieve high-quality data.

References

1. Heck AJ et al. Native mass spectrometry: a bridge between interactomics and structural biology. Nat Methods. 2008 Nov;5(11):927-33.
2. Shehu A et al. On the characterization of protein native stateensembles. Biophys J. 2007 Mar 1; 92(5): 1503-11.
3. Jooß K et al. Standard procedures for native CZE-MS ofproteins and protein complexes up to 800 kDa. Electrophoresis. 2021 May;42(9-10): 1050-1059.
4. Hernandez-Alba O et al. Analysis of ADCs by Native MassSpectrometry. Methods Mol Biol. 2020; 2078: 197-211.
5. Kükrer B et al. Mass spectrometric analysis of intact humanmonoclonal antibody aggregates fractionated by size- exclusion chromatography. Pharm Res. 2010 Oct; 27(10): 2197-204.
6. Debaene F et al. Innovative native MS methodologies for antibody drug conjugate characterization: High resolution native MS and IM-MS for average DAR and DAR distributionassessment. Anal Chem. 2014 Nov 4; 86(21): 10674-83.
7. Muneeruddin K et al. Characterization of intact protein conjugates and biopharmaceuticals using ion-exchange chromatography with online detection by native electrospray ionization mass spectrometry and top-down tandem mass spectrometry. Anal Chem. 2015 Oct 6; 87(19): 10138-45.
8. Skinner OS et al. Native GELFrEE: a new separation technique for biomolecular assemblies. Anal Chem. 2015 Mar 3; 87(5): 3032-8

Headquarters
500 Old Connecticut Path | Framingham, MA 01701 USA
Phone 508-383-7700
sciex.com

International Sales
For our office locations please call the division headquarters or refer to our website at
sciex.com/offices