Charge Variant Assessment of Nanobodies at the Intact Level by CESI-MS

Sensitive workflow for the detection of charge variants of nanobodies from small sample volumes

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Introduction

Traditional LC-MS methods struggle to separate proteins that differ in mass by <10 Da. When proteins larger than 10 kDa of nearly identical mass co-elute, their spectra overlap, making it nearly impossible to deconvolute the data and identify the individual species. Therefore, identification of charge variants such as deamidation, which causes a mass charge of just 1 Da, is very difficult using traditional LC-MS approaches. CESI provides a separation mechanism orthogonal to LC-MS-based approaches for separation. The inherent separation efficiency of capillary electrophoresis (CE) provides sharp peaks for better separation and identification. The goal of this work was to separate and detect challenging charge variants, such as deamidation of nanobody proteins at the intact level, without the need for digestion of the sample using CESI-MS.

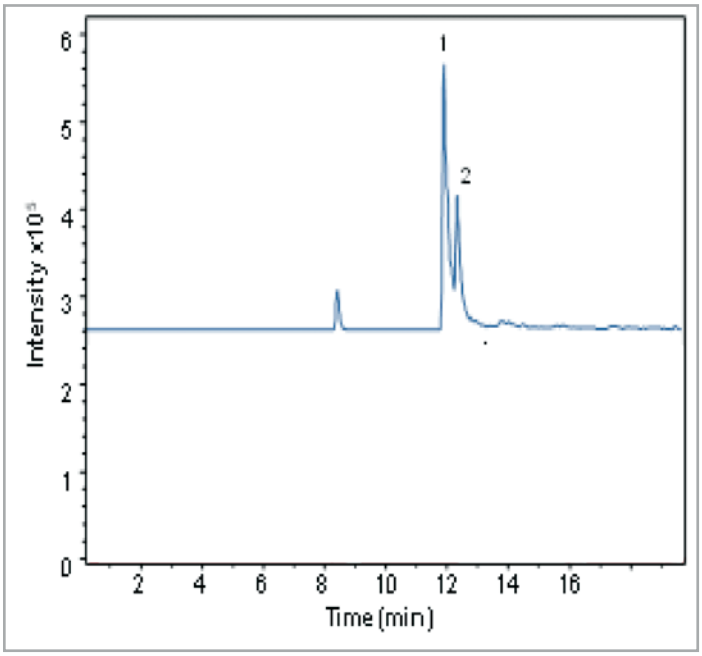

Figure 1. Separation of a nanobody (peak 1) from its deamidation form (peak 2) using CESI-MS.

The CESI 8000 Plus System

Key Features

- Direct combination of capillary electrophoresis with mass spectrometer, enabling the charge-based separation of proteins at very low flow rates, including separation of deamidated forms of the proteins
- Minute (<10 nL) injection volumes from sample volumes of 5 µL possible, which helps in sample limited analysis

Methods

Sample preparation: Nanobodies were C-terminally tagged with Myc-(EQKLISEEDLNG) and 6x-histidine tags, and produced in BL21 E.coli.[1] Periplasmic extracts were prepared by a freeze-thaw cycle of E.coli in phosphate buffered saline (PBS) and the nanobodies isolated by ion-metal affinity chromatography using

Ni-NTA agarose resin. Elution of the nanobodies from the Ni-NTA resin was performed with 250 mM imidazole in PBS. Finally, imidazole was removed via multiple dialysis steps against PBS using a 3.5 kDa MWCO SnakeSkin dialysis membrane.[2] These final nanobody samples were diluted into 10 mM ammonium acetate (pH 3.0) to a final concentration of approximately 1 µM before CESI-MS analysis.

CESI-MS methods. For analysis, the CESI 8000 Plus High Performance Separation-ESI Module was used in conjunction with a neutrally coated separation capillary and coupled to a Bruker maXis HD Ultra-High Resolution QTOF mass spectrometer (Bruker Daltonics, Bremen, Germany) equipped with a nano-electrospray source.1 Prior to each analysis, the capillary was rinsed with water (3 min, 100 psi) and a background electrolyte (BGE) of 50 mM ammonium acetate at pH 3.0 (10 min at 50 psi), and the conductive line was filled with 50 mM acetic acid (3 min, 75 psi). The sample was pressure injected by applying 2.5 psi for 15 s (which was equivalent to 6.3 nL) followed by an injection plug of BGE (0.5 psi for 20 s). The separations were performed at +30 kV (0.5 psi forward pressure, 20 min) with the voltage ramped down at the end of the run. The mass spectrometer was operated in positive ionization mode using a capillary voltage of 1,200 V and drying gas temperature of 180 °C (flow rate 2 L min−1). Mass spectra were collected in profile mode using an m/z range of 500 to 3,000 with molecular mass determinations performed using the “maximum entropy” algorithm of the data analysis software.

Important

- A separation current above 5 µA might cause permanent damage to the separation capillary and its coating.
- In general, please do not apply >2,000 V to generate electrospray as it may result in capillary damage.

Results and discussion

There is a growing interest in the potential therapeutic use of nanobodies as possible alternatives to mAbs. Nanobodies are antibody fragments derived from the variable domains of heavy- chain-only antibodies and are relatively small proteins (12-15 kDa) exhibiting a binding capacity and specificity like mAbs while exhibiting higher chemical stability.[1]

Mass spectrometry (MS) has become a common technique for antibody characterization and can provide data on protein structure, purity and heterogeneity. MS has been used for the analysis of intact protein pharmaceuticals as well as peptides obtained after their proteolytic digestion. Intact protein (and top-down) analysis is beneficial for biopharmaceutical analysis as it offers a general picture of the heterogeneity of the protein population while inducing minimal changes caused by sample treatment. Reverse-phase LC separates species based on their hydrophobicity and often struggles to separate proteins of very similar size, such as charge variants of the same protein. CESI-MS, on the other hand, separates proteins based on their charge to hydrodynamic volume ratio.[3] While deamidation corresponds to a gain of 1 Da only, it also adds an excess negative charge on the protein, allowing CESI to separate these variants. Currently available MS systems are accurate enough to distinguish a mass shift of 1 Da on proteins less than 30 kDa, enabling deamidation charge variant identification.

The nanobody preparations Nb1 and Nb2, both with peptide chains of approximately 15 kDa, were tested. The bivalent construct Nb1-35GS-Nb1, generated via a fusion of 2 Nb1 domains attached through a 35-mer peptide linker ((- GGGGS-)7),[1] was also analyzed. During method development, the biggest increase in peak resolution was obtained by minimizing the pressure applied to the inlet capillary during the separation. A pressure of 0.5 psi, providing an overall flow rate of 5 nL/min, produced stable spray and good resolution, and it was therefore selected for protein analysis.

The analysis of the monovalent Nb1 sample produced 2 partially separated peaks migrating at 12.0 and 12.5 min (Figure 2A) of 14590.97 and 14591.90 Da, respectively (Table 1). The mass observed for the first migrating peak agreed with the theoretical mass of Nb1. The second peak, which had an area of about 52% relative to peak 1, was assigned to a deamidated form of Nb1 as this modification caused a reduction in pI and a change of molecular mass (+0.984 Da). The bivalent Nb1 construct (Nb1-35GS-Nb1) yielded a main peak at 13.3 min (peak 4 in Figure 2B), which could be assigned to the intact conjugate (deconvoluted mass, 28835.74 Da; Table 1). The significant peak (5) migrating closely after the main peak had a mass difference of +0.93 Da from the native Nb1-35GS-Nb1 and highlighted the presence of considerable deamidation in the sample. In Figure 2B, 3 minor peaks were also observed (Table 1) and these could be assigned to the monovalent Nb1 and its deamidated form, and possibly to the Nb1 molecule carrying a fragment of the 35GS linker (15527.70 Da). The analysis of the Nb2 nanobody is depicted in Figure 2C and highlighted significant heterogeneity. Up to 22 peaks were observed between 12 and 17 min. Apart

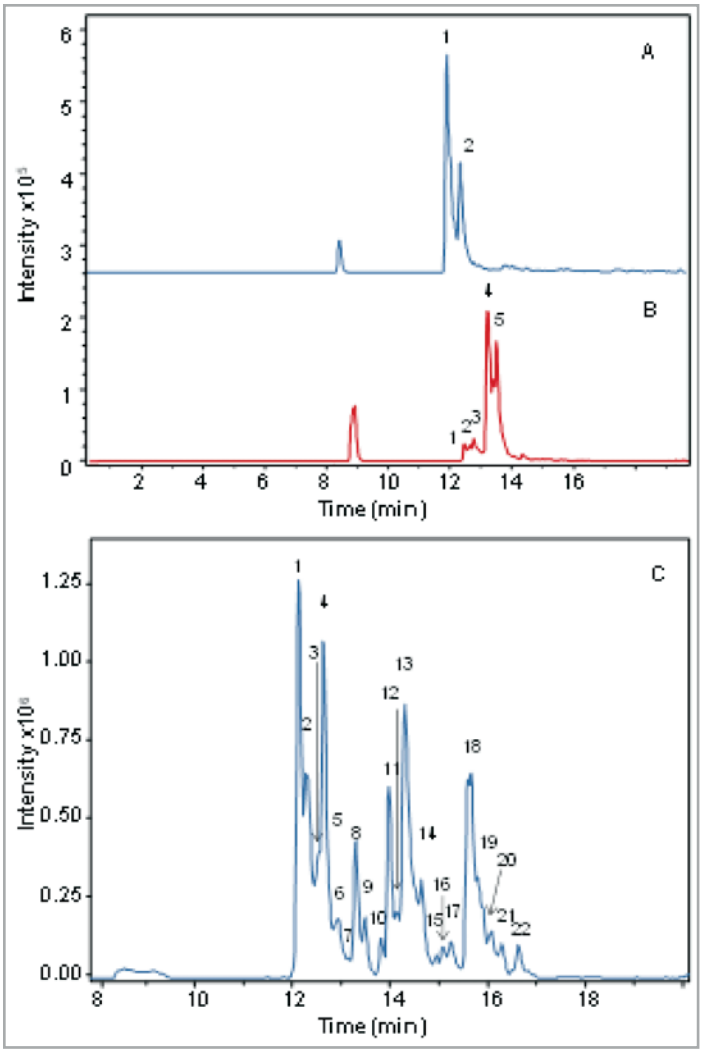

Figure 2. Base peak electropherograms (CESI-MS) for nanobodies Nb1 (panel A), Nb1-35GS-Nb1 fusion (panel B) and Nb2 (panel C).

from the native sample peak 2, other peaks appeared to originate from products of truncation, a result of non-specific digestion of the Myc tag originated by residual proteases in the sample (Table 2). Next to truncated species, several deamidated forms of Nb2 were observed (Table 2). Nb2 has multiple asparagine residues and, in this example, CESI-MS was capable of separating several isomeric species resulting from deamidation at different asparagine sites of Nb2 (e.g., peaks 3, 5 and 6 and peak 7 correspond to singly and doubly deamidated forms of Nb2, respectively).

Peak[a]	Observed mass (Da)	Theoretical mass (Da)	Assignment[b]	Δ Mass (Da)
Nb1				
1	14590.97	14591.18	Nb1	0.21
2	14591.90	14592.17	deam Nb1	0.27
Peak[a]	**Observed mass (Da)**	**Theoretical mass (Da)**	**Assignment[b]**	**Δ Mass (Da)**
Nb1				
1	14590.97	14591.18	Nb1	0.21
2	14591.90	14592.17	deam Nb1	0.27

a Peak numbering according to Figures 2A (Nb1) and 2B (Nb1-35GS-Nb1).
b deam: deamidated.

Table 1. Assignment of species observed during CESI-MS of Nb1 and Nb1-35GS-Nb1 as shown in Figures 2A and Figure 2B (MS spectra can be found in Haselberg et al.[4]

Peak[a]	Observed mass (Da)	Theoretical mass (Da)	Assignment[b]	Δ Mass (Da)
1	1639.79	1640.61	E129-H142	0.82
2	15498.58	15499.16	Nb2	0.58
3	15499.57	15500.15	1-deam Nb2	0.58
4	1953.47	1954.05	L126-H142	0.58
5	15499.55	15500.15	1-deam Nb2	0.60
	1640.79	1641.58	1-deam E129-H142	0.79
6	15500.54	15501.16	2-deam Nb2	0.62
7	15500.54	15501.16	2-deam Nb2	0.62
8	1640.78	1641.61	1-deam E129-H142	0.83
9	1954.53	1955.04	1-deam 126-H142	0.51
10	15087.41	15087.76	Nb2 − (H140-H142)	0.35
11	1953.98	1955.04	1-deam L126-H142	0.82
12	15088.30	15088.75	1-deam Nb2 −(H140-H142)	0.45
13	13562.69	13563.15	Nb2 − (L126-H142)	0.45
14	13875.88	13876.54	Nb2 − (E129-H142)	0.66
15	14004.94	14005.66	Nb2 − (E130-H142)	0.72
16	13305.97	13306.84	Nb2 − (Q124-H142)	0.87
17	13434.50	13434.97	Nb2 − (K125-H142)	0.47
18	14604.39	14605.26	Nb2 − (A136-H142)	0.87
19	14605.38	14606.25	1-deam Nb2 − (A136-H142)	0.87
20	14605.39	14606.25	1-deam Nb2 − (A136-H142)	0.86
21	14606.38	14607.24	2-deam Nb2 − (A136-H142)	0.86
22	14606.38	14607.24	2-deam Nb2 − (A136-H142)	0.86

a Peak numbering according to Figure 2B.
b 1-deam, singly deamidated; 2-deam, doubly deamidated.

Table 2. Assignment of species observed during CESI-MS of Nb2 as shown in Figure 2C.

Conclusions

In this study we have demonstrated that CESI-MS can separate charge variants of nanobodies and nanobody fusion proteins including deamidated forms as well as products of truncation of the antibody which happened during preparation of the protein samples.[4]

Further information

For further information on this topic we would like to refer readers to the full scientific publication on which this application note is based.[4]

References

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