Structural Characterization of Antibody Drug Conjugates (ADCs) by a Combination of Intact, Middle-up and Bottom-up Approaches using CESI-MS

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Introduction

Antibody drug conjugates (ADCs) represent a rapidly growing class of biopharmaceuticals. ADCs are formed by the conjugation of an active drug species to a monoclonal antibody,[1] and they often result in a distribution of products containing varying numbers of active drugs bound at different locations around the antibody.

Capillary electrospray ionization (CESI) is the integration of capillary electrophoresis (CE) and electrospray ionization (ESI) into a single process in a single device (Figure 1).[2] CESI-MS operates at low nL/min flow rates and offers several advantages, including increased ionization efficiency and a reduction in ion suppression. CESI-MS separates analytes by their charge and size, and it is therefore a separation mechanism that is complementary to more traditional techniques, such as reversed-phase LC.

This document summarizes the work recently published by the research group at LSMIS at the Université de Strasbourg.3 In this application note, we will show how CESI-MS can be used to characterize a gold standard ADC, brentuximab vedotin (BV). BV is a cysteine-linked mono-methyl auristatin E (MMAE) monoclonal antibody (mAb), which was attached without disrupting the heavy and light chain linkages of the mAb.[3] We will show how CESI-MS is used as a nano-spray infusion device to identify the drug antibody ratio of the sample. We will also show how CESI-MS can be used to study the "middle-up" structure for this protein, for example, by analyzing the light chain, Fab and F(ab')2 subunits. Finally, a bottom-up analysis of a tryptic digest will be performed by CESI-MS to fully sequence the ADC.

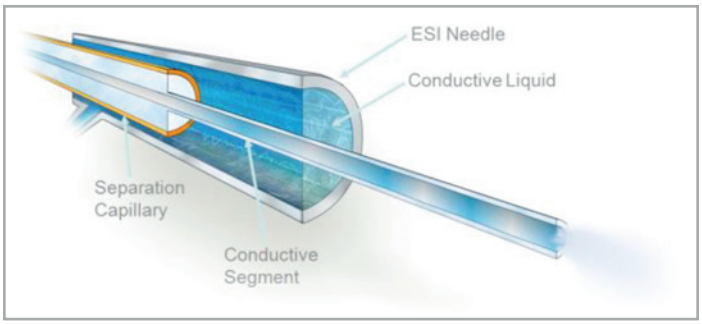

Figure 1. OptiMS - Ultra low flow ESI Interface.

Materials and methods

Chemicals: All chemicals were reagent grade and were purchased from Sigma Aldrich or alternative suppliers.[3] Brentuximab vedotin was produced by Millennium Pharmaceuticals/Takeda.

Sample Preparation: BV was buffer exchanged with 200 mM ammonium acetate buffer (pH 7.0) using Amicon filters before intact analysis.[3]

For middle-up analysis, BV was cleaved at the hinge region by proteolysis using IdeS (FabriCATOR, Genovis) to obtain Fc/2 fragments and an F(ab')2 fragment. After digestion was completed, the sample was again buffer exchanged into 200 mM ammonium acetate buffer (pH 7.0) before CESI-MS analysis.

For bottom-up analysis, a sample of desalted IdeS cleaved ADC, which had undergone buffer exchange and was diluted with RapiGest (Waters) and reduced with dithiothreitol (DTT). Before enzymatic digestion, 10% of acetonitrile was added to the sample. The reduced protein was then digested with trypsin overnight, isopropanol (40%) and formic acid (1% v/v) were added and the final solution diluted using ammonium acetate (50 mM, pH 4.0) to produce a final concentration of 2.2 µM.[3]

CESI-MS method: For the analysis of intact and digested samples, a bare fused silica OptiMS cartridge (30 µm ID x 91 cm) from SCIEX was used and was thermostatted using recirculating liquid coolant regulated at 20°C used. For the analysis of tryptic digests, the sample was injected hydrodynamically (10 psi, 60 s) and peptides were separated using conditions shown in Table 1 with a background electrolyte of 10% acetic acid. For MS analysis of tryptic digests, a SCIEX TripleTOF® 5600+ LC-MS/MS System was fitted with the NanoSpray® III Ion Source. Gas 1 and 2 were not used, the curtain gas was set to 5 psi and the temperature of the interface was set at 150 °C. Ionization at these very low-flow rates occurred by simply applying the ionspray voltage (1,450 V). MS data was acquired using a TOF survey scan (m/z 100–2,000 amu), which triggered MS/MS acquisition (m/z 100–2,000 amu).

Action	Time (min)	Pressure (psi)	Direction	Voltage (kV)	Solution
Rinse	3.5	100	Forward	0	0.1 M NaOH
Rinse	1.5	100	Forward	0	Water
Rinse	3	100	Forward	0	0.1 M HCl
Rinse	5	100	Forward	0	10% Acetic acid
Rinse	2	75	Reverse	0	10% Acetic acid
Rinse	60s	10	Forward	0	Sample Vial
Injection	10s	5	Forward	0	10% Acetic acid
Separation	35	0.1	Forward	20	10% Acetic acid
Voltage	2	10	Forward	1	10% Acetic acid

Table 1. CESI separation conditions used for the analysis of tryptic digests.

For nano-spray infusion, the CESI capillary was flushed with 10% acetic acid (5 min, 50 psi) and then with sample (5 min, 50 psi), and MS data was acquired at 5 psi. After each analysis, the capillary was flushed with 10% acetic acid (10 min, 50 psi) to prevent carryover. For these nano-spray infusion experiments, MS data was acquired using a maXis 4G system (Bruker). The maXis system was optimized for each analysis using the actual sample and ion funnels with values that ranged from 300–400 Vpp. The electrospray voltage ranged from 1,200–1,800 V, drying gas was set at 1.5 L/min and the source temperature was set at 150 °C.

Important:

- A separation current above 5 µA might cause permanent damage to the separation capillary.
- Generally, please do not apply >2000V to generate electrospray as it may result in capillary damage.

Results

When CESI is used as a nano-spray infusion device, the sample is simply pushed to the MS detector at low nL/min flow rates.[4] Desalted intact BV was analyzed in this mode to confirm the molecular weight of this ADC and measure the drug to antibody ratio (DAR), which is the drug loading on the antibody and typically ranges from 0 to 8. An example of the data achieved is shown in Figure 2, which gives an overview of the analysis of BV by CESI-MS.

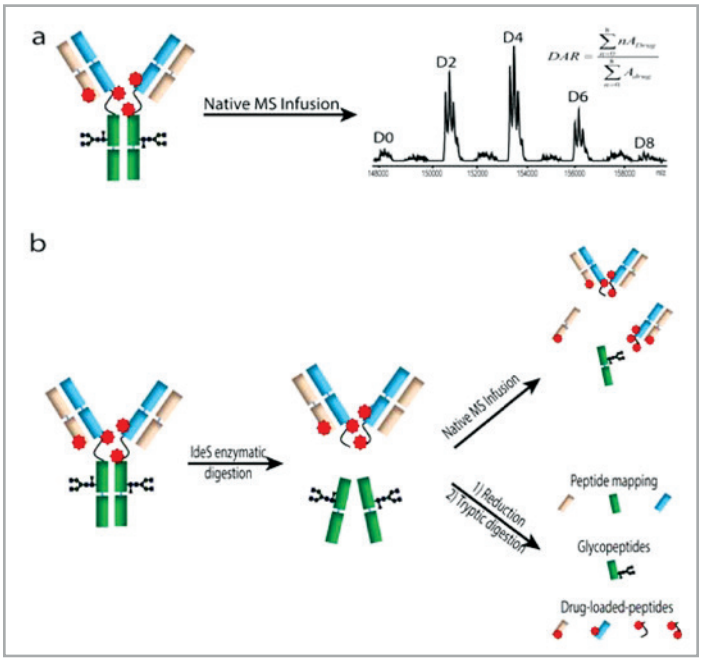

Figure 2. Overview of brentuximab vedotin structural characterization using sheathless CE-MS. (a) Native MS infusion for average DAR determination and drug loading distribution assessment. (b) Middle-up and bottom-up analysis.

Based on the charge state of the deconvoluted mass spectrum of the intact BV shown in Figure 2, the average DAR value was between 3.8 and 3.9. A distribution of baseline resolved species of BV was observed with masses that corresponded to the intact mAb linked with 0 to 8 payloads of the drug. The mass accuracies of intact BV were in total agreement with the results reported in the literature.[3]

Using the same experimental conditions as the intact analysis, a middle-up sample was analyzed to elucidate the location of the drug loading on the mAb, which had been cleaved at the hinge region (Figure 2). The raw data highlighted multiply charged protein peaks in 3 separate regions across the mass range of 2,000–6,000 amu (Figure 3). When deconvoluted, the multiply charged protein envelopes depicted in Figure 3 showed that the free light chain (LC) protein subunit (MW = 25040.1 ± 0.1 Da) had 1 drug molecule linked to it as well as 2 glycation modifications. The smaller Fab subunit (approximately 48–55 kDa) had 0–4 molecules of drug linked to the protein and was present as a dimer. The F(ab')2 subunit (approximately 97–108 kDa) had 0–8 drug molecules attached to the protein subunit. As the drug units were spread across multiple regions of the mAb, the IdeS middle-up sample could not be used to calculate the DAR for ADCs, but does give information about the location of the drug molecules within BV.

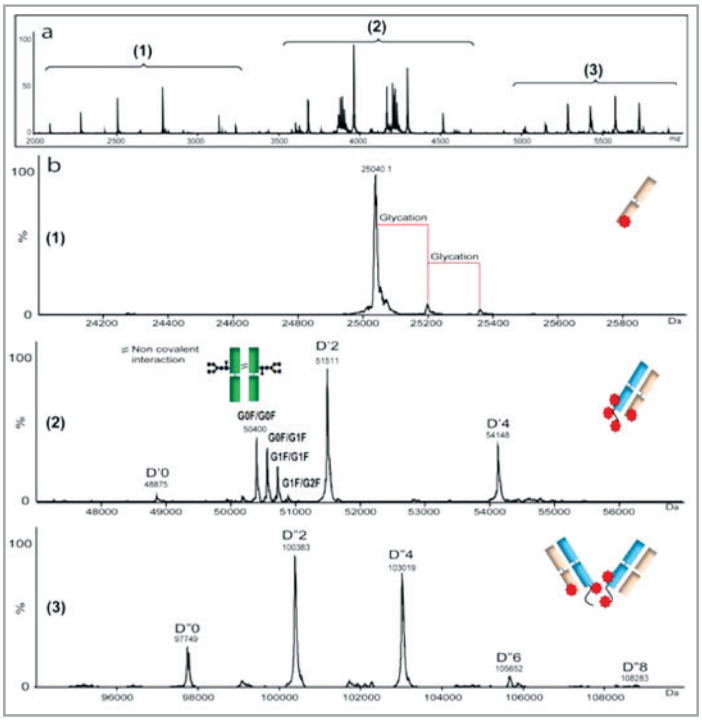

Figure 3. (a) MS spectra corresponding to native MS nano ESI infusion of middle-up BV. (b) Charge state deconvoluted mass spectra of (1) LC- drug conjugated subunit, (2) Fab subunits with the incorporation of 0 to 4 drug molecules and Fc/2 homodimers and (3) F(ab')2 subunits with the incorporation of 0 to 8 drug molecules.

CESI-MS has been previously used for the analysis of tryptic digests of mAbs.[5] The next set of experiments performed on BV were the bottom-up analysis of a tryptic digest to determine the location of post-translational modification (PTM) sites, the location of the drug linked to the mAb and the amino acid sequence. The tryptic digestion protocol had been adapted from the classical approach to improve the overall digestion, which was affected by the presence of the drug molecules bound to the mAb.[3] CESI-MS analysis took less than 35 min. Due to the sensitivity and efficiency of the CESI-MS analysis, 100% sequence coverage could be obtained for BV in a single injection with the identification of the peptides based on their accurate molecular weight as well as sequence data from MS/MS analysis. The presence of the organic solvent in the sample preparation did not seem to have an effect on the separation of the tryptic peptides demonstrated by the detection of small (3 amino acids) to large (63 amino acid) peptides.

Modified peptides (including N-glycopeptides) were detected in the analysis, for example, TKYPREEQYN[297]STYR was observed to have 11 glycoforms. Regarding drug-loaded-peptides, 4 were detected (Figure 4), which was aided by the presence of organic solvent in the sample preparation to prevent the loss of these hydrophobic modified peptides.

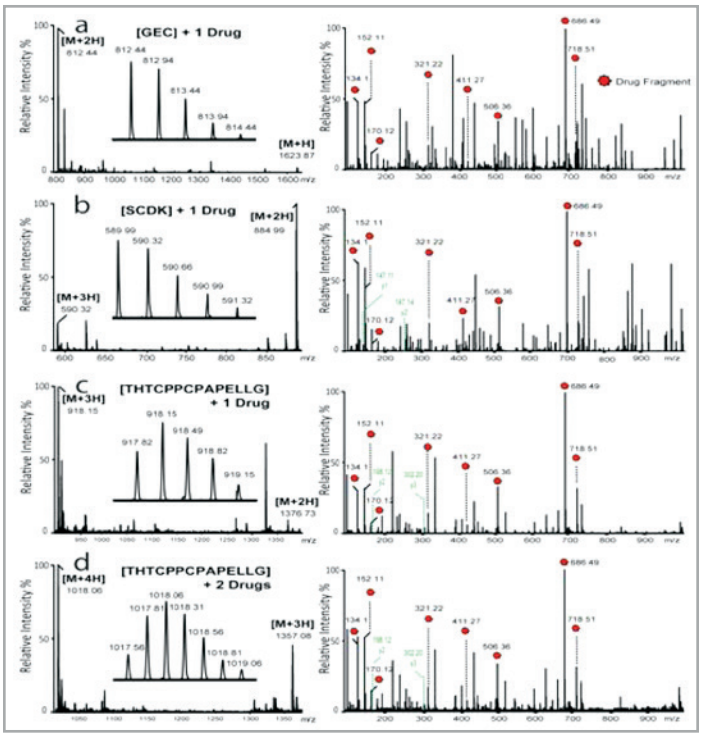

Figure 3. MS and MS/MS spectra of drug-loaded peptides. (a) [GEC] - 1 drug, (b) [SCDK] -1 drug, (c) [THTCPPCPAPELLG] - 1 drug and (d) [THTCPPCPAPELLG] - 2 drug molecules

Identification of these drug containing peptides was confirmed by MS/MS data analysis, which highlighted the presence of several diagnostic drug fragment ions. A single drug loaded peptide was located on the light chain of the mAb (GEC), and the other peptides were present on the heavy chain. The THTCPPCPAPELLG peptide actually had the potential of containing 2 drug molecules, and these 2 different peptides have been separated by CESI-MS.

Conclusions

A CESI-MS protocol for structural characterization of ADC molecules has been developed. By using CESI-MS in 2 different modes, several properties of an ADC could be confirmed, including:

- DAR ratio calculation using native conditions
- Drug distribution on the F(ab')2 and Fc/2 using nano-spray infusion CESI-MS analysis of an IdeS digested middle-up sample using native conditions
- 100% sequence coverage of the ADC and identification of the drug location as well as the location of other PTMs
- Characterization of drug loaded peptides by analysis of MS/MS spectra

For further information on this topic we would like to refer readers to the full scientific publication on which this application note is based.[3]

References

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