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 often result in a mix of proteins containing a distribution of products containing different numbers of active drugs bound in 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. These include increased ionization efficiency and a reduction in ion suppression. CESI-MS separates analytes by their charge and size and is, therefore, a complementary separation mechanism to more traditional techniques, such as reverse phase LC.

This document summarizes the work recently published by the research group at LSMIS at the University of Strasbourg[3]. In this application note we will show how CESI-MS can be used to characterize a gold standard ADC, brentuximab vedotin which is a monoclonal antibody (mAb) which has a cysteine-linked mono-methyl auristatin E (MMAE) which was attached without disrupting the heavy and light chain linkages of the mAb[3]. We will show how CESI-MS will first be used as a nanospray 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, e.g. by analyzing the light chain, Fab and F(ab')2 subunits. Finally a top down analysis of a tryptic digest will be performed by CESI-MS to fully sequence the ADC.

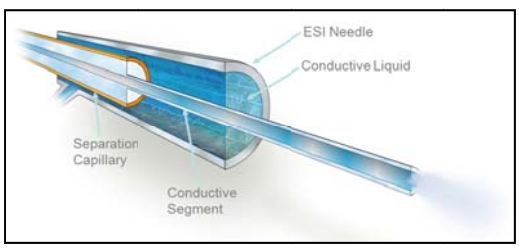

Figure 1: OptiMS- Ultra low flow ESI Interface.

Materials and Methods

Chemicals: All chemicals were Reagent Grade and were purchased from Sigma Aldrich or alternative suppliers[3]. Brentuximab vedotin (BV) was produced by Millenium Pharmaceuticals/Takeda and was buffer exchanged with 200 mM ammonium acetate buffer (pH 7.0) using Amicon filters before intact analysis[3]

Sample Preparation: For middle-up analysis BV was cleaved at the hinge region by proteolysis using IdeS (FabriCATOR, Genovis) to obtain Fc/2 fragments and one 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 was diluted with RapiGest 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 a second reduction step was performed, 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 CESI cartridge (30 µm ID x 91 cm) which was thermostatted using recirculating liquid coolant regulated at 20°C was used. For the analysis of tryptic digests a sample was injected hydrodynamically (10 psi, 60s) and peptides were separated using conditions shown in Table 1 using a background electrolyte of 10% acetic acid. For MS analysis of tryptic digests, a SCIEX TripleTOF® 6600 mass spectrometer was fitted with the NanoSpray® III source. Gas 1 and 2 were not used and the temperature of the interface was set at 150 ºC because ionization at very low flow rates occurs by simply applying the ionspray voltage (1450 V). The curtain gas was set very low at 4 psi (set automatically). MS Data was acquired using a TOF survey scan (m/z 100-2000 amu) which triggered MS/MS acquisition (m/z 100-2000 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 (5min, 50 psi) then with sample (5min, 50psi) and MS data was acquired at 5psi. After each analysis the capillary was flushed with 10% acetic acid (10min, 50psi) to prevent carry over. 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 values ranged from 300 – 400 Vpp, the electrospray voltage ranged from 1200 – 1800 V, drying gas was set at 1.5 L/min and the source temperature was set at 150 °C.

Results

When CESI is used as a NanoSpray 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 in order 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.

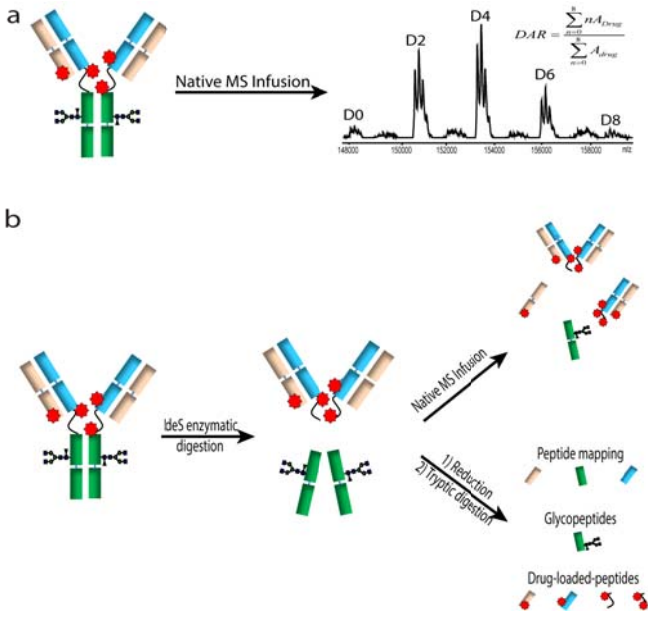

Figure 2: Overview of brentuximab vedotin structural characterization using sheathless CE-MS. (a) Native MS infusion for average DAR determination and drug loaded distribution assessment, (b) middle-up and bottom-up analysis.

Based on charge state deconvoluted mass spectrum of the intact and deglycosylated 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 whose masses corresponding to the intact mAb linked with zero to eight payloads of drug . The mass accuracies of intact BV were in total agreement with results reported in the literature[3].

Using the same protocol and experimental conditions as the intact analysis a middle-up digested 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 three separate regions across the mass range 2000 - 6000 Da (Figure 3). When deconvoluted these multiply charged protein envelopes depicted in Figure 3 show that free Light Chain (LC) protein subunit (MW = 25040.1 ± 0.1 Da) had one drug molecule linked to it as well as two 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 sub unit (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 as to the location of the drug molecules within BV.

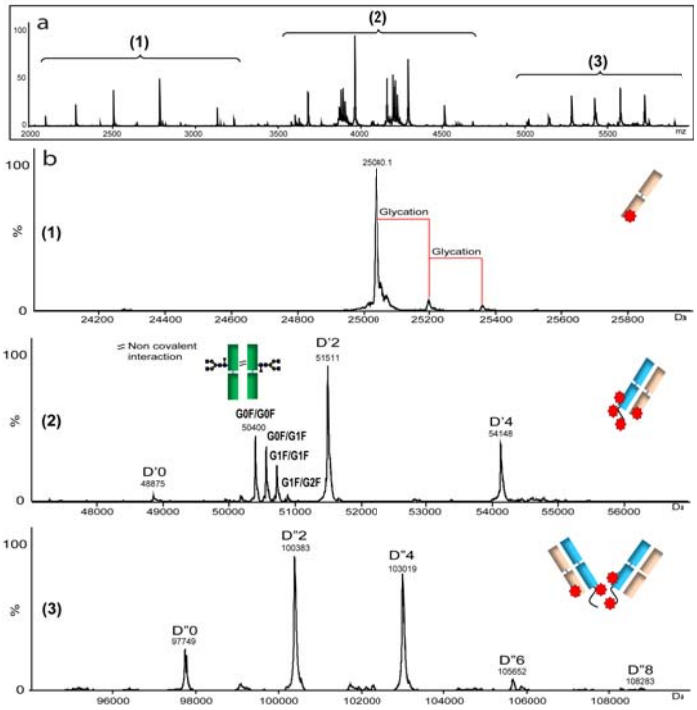

Figure 3: (a) MS spectra corresponding to Native MS NanoESI infusion of middle-up BV. (b) Charge state deconvoluted mass spectra of (1) LC-drug conjugated subunit, (2) Fab subunits with the incorporation of 0 to 4 drug molecules and Fc/2 homodimers and (3) F(ab)'2 subunits with the incorporation of 0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

CESI-MS has previously been used in the analysis of tryptic digests of mAbs[5] so the next set of experiments performed on BV were the bottom up analysis of a tryptic digested sample to determine the location of modification sites (PTMs), the location of the drug linked to the mAb as well as the amino acid sequence of BV. The tryptic 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]. When injected all the peptide peaks in the sample had migrated to the end of the capillary and been detected in less than 35 minutes. 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 effect the separation of the tryptic peptides with the detection of small (3 amino acids) to large peptides (63 amino acid long) possible.

Modified peptides (including N-glycopeptides) were detected in the analysis, e.g. TKYPREEQYN^{297}STYR was observed to have 11 glycoforms. Regarding drug-loaded-peptides, 4 were detected (Figure 4) this was aided by the presence of organic solvent in the sample preparation which prevented loss of these hydrophobic modified peptides.

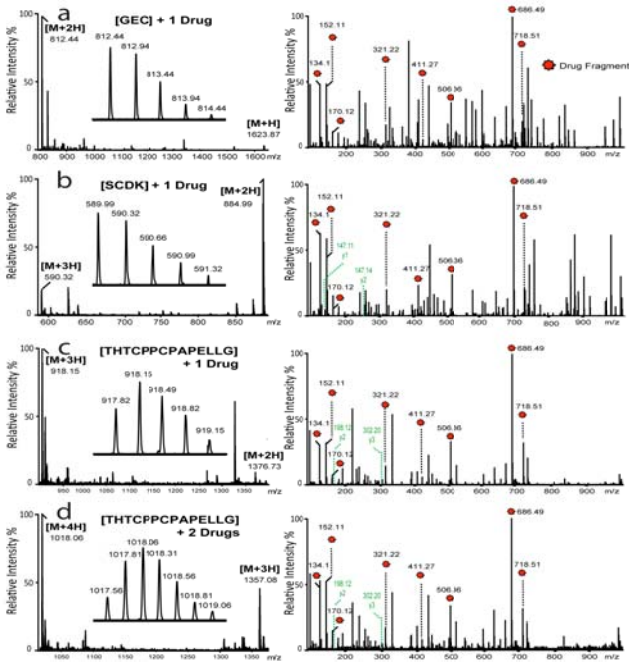

Figure 4: MS and MS/MS spectra of drug-loaded peptides. (a) [GEC] - 1 drug, (b) [SCDK] -1 drug, (c) [THTCPPCPAPELLG] - 1 drug and (d) [THTCPPCPAPELLG] - 2 drug molecules.

Identification of these drug containing peptides was confirmed by MS/MS data analysis which highlighted the presence of several diagnostic drug fragment ions. One 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 two drug molecules and these two different peptides migrated at different times.

Conclusions

A CESI-MS protocol for structural characterization of ADC molecules has been developed. Using CESI-MS in two 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 location of other PTMs of the ADC.
- Characterization of drug loaded peptides from 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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