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Analysis of ADCs by Native Mass Spectrometry

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Running head

Analysis of ADCs by native electrospray MS

Summary/Abstract

Mass spectrometry performed in non-denaturing conditions (native MS) has proven its utility for the quantitative and qualitative analysis of antibody-drug conjugates (ADCs), especially when ADCs' subunits involve non-covalent interactions (i.e. cysteine-conjugated ADCs). Its hyphenation to ion mobility spectroscopy (IM-MS) allows differentiation of gas-phase ions based on their rotationally averaged collision cross section providing an additional dimension of conformational characterization of ADCs. More recently, size exclusion chromatography (SEC) appeared as an interesting technique to perform online buffer exchange in an automated way prior to native MS/IM-MS analysis. Online SEC-native MS/IM-MS allows the global structural characterization of ADCs and the assessment of some critical quality attributes (CQAs) required for ADC release on the market, such as drug load distribution (DLD), drug-to-antibody ratio (DAR), the average DAR (DAR_{av}), and the relative amount of unconjugated mAb.

Keywords

Native mass spectrometry, Antibody drug conjugate, Ion mobility mass spectrometry, Size exclusion chromatography-native mass spectrometry

1. Introduction

ADCs are tripartite molecules designed to improve the therapeutic efficacy of monoclonal antibodies (mAbs) through the covalent addition of a cytotoxic molecule via a linker[1]. The heterogeneity associated to ADCs can vary, depending on the bioconjugation chemistry used to attach the cytotoxic molecules to the mAb structure[2]. Several studies have shown the suitability of native mass spectrometry (MS) for ADC analysis[3-13]. The reduced charge state of the ions under non-denaturing conditions facilitates the identification and quantification of all the different DAR populations that can be potentially formed during the conjugation process. Furthermore, the rotationally averaged collision cross section (CCS) of each individual DAR population can be determined by using ion mobility coupled to mass spectrometry (IM-MS) to assess the impact of the conjugation process on the overall conformation of the original mAb[6; 7]. However, sample preparation (desalting) is still mostly manual, labor intensive and time-consuming prior to native IM-MS analysis. SEC-native MS not only allows on-line buffer exchange within few minutes but also provides separation, identification and quantification of high/low molecular weight species[9]. Online SEC-native MS can be easily implemented in high throughput automated environments thus pushing native MS approaches to the forefront of ADC characterization in R&D laboratories.

2. Materials

All solutions are prepared with ultrapure water.

2.1. Mobile phase for SEC-native MS

100 mM AcONH₄, pH 6.8: weigh 3.08 g of AcONH₄ in a 500 mL volumetric flask. Add 400 mL of milli-Q water with stirring. No pH adjustment is needed.

2.2. Deglycosylated ADCs

ADCs were obtained as European Union pharmaceutical-grade drug product from their respective manufacturers. The deglycosylation was performed by incubating for 30 min at 37°C one unit of IgGZERO (Genovis) per microgram of ADC.

2.3. Manual buffer exchange

1. 150 mM AcONH₄, pH 6.8: weigh 578 mg of AcONH₄ in a 50 mL falcon. Add 50 mL of milli-Q water with stirring.

2.4. Cesium Iodide solution for Mass Spectrometer calibration

Prepare a 2 g/L solution of cesium iodide in 50/50 isopropanol/water (v/v).

a. Synapt G2

1. The automated chip-based nanoelectrospray device (Triversa Nanomate, Advion, Ithaca, USA) is operated in the positive ion mode. Set the capillary voltage and the nitrogen nanoflow at 1.75 kV and 0.60 psi respectively.

2. In MassLynx MS Tune windows, set the following parameters:

Operate the instrument in positive and sensitivity modes with a m/z range from 1 000 to 10 000.

Nanoflow+ panel: Sampling Cone: 120 V. Extraction con: 5 V. Source Temperature: 90 °C. The Capillary Voltage and the gas flows are not used in the Nanomate interface setup.

Instrument: Trap and Transfer Collision Energy are turned off.

3. Start a 2 min TOF-MS acquisition with a m/z range from 1 000 to 10 000.
4. Average the signal over the 2 min in the Chromatogram panel. In the spectrum panel, a Spectrum Smooth with ± 10 Smooth window and a number of smooths of 2 with a “Mean” Smoothing method were realized. On the smooth spectrum, the TOF Spectrum Center with a min peak width at half height of 2 and a Centroid top at 80.00 % was selected.

5. In the Acquity UPLC Console, select “Create Calibration”.
6. In the calibration profile editor, select the right Mass Calibration Profile (CsI in the 1 000 – 10 000 m/z range in positive and sensitivity modes).
7. The Reference Compound (Csi_1000-10000 positive) with reference masses from 1 172.1450 to 7 927.2031 Da) is used for the calibration.

b. Exactive Plus EMR

1. Using HESI source and the 500 µL Hamilton™ syringe, deliver cesium iodide solution with a flow rate set to 10 µL/min.
2. In the MS Tune page manually set the following parameters: Operate the instrument in positive mode with a m/z range from 1,000 to 20,000. Apply 25 eV and 100 eV for the CID and CE parameters, respectively. The source temperature is set at 250°C, the capillary voltage is set to 4 kV and the gas flows is set to 10 u.a. The trapping gas pressure is set to 7 u.a. The ion optics are set to 4 V for injection, inter and bent flatapoles. Set the nominal resolution at 17,500 and activate the EMR mode.
3. To perform the calibration, wait for a stable TIC (<12 %) then select “Calibrate” and check “EMR MS Mass Calibration (pos)”.

2.5. Ion mobility calibrants

1. 10 mM AcONH₄: weight 38 µg of AcONH₄ in 50 mL falcon. Add 50 mL of mili-Q water with stirring.
2. Weigh between 400 µg and 900 µg of concanavalin A from *canavalia ensiformis*, alcohol dehydrogenase from *saccharomyces cerevisiae*, and pyruvate kinase from rabbit muscle. Dissolve each individual calibrant in 100 µL of 10 mM ammonium acetate to reach a final tetrameric concentration between 30-40 µM (**Note 1**).
3. Perform manual buffer exchange using a 50 kD microconcentrator (see 2.3. section).

4. Use the nanodrop spectrophotometer to determine the concentration of the calibrant solutions after manual desalting.
5. Add the required volume of 10 mM AcONH₄ solution to get a final concentration of 10 μ M for each individual calibrant solution.

3. Methods

3.1 ADC sample preparation

1. A microconcentrator with an appropriate MWCO (such as Vivaspın 50-kD cutoff for classical ADCs, Sartorius, Göttingen, Germany) is used for manual buffer exchange.
2. Equilibrate the microconcentrator by adding 500 μ L of 150 mM AcONH₄ at 14 000 g over 2 min and remove the eluate.
3. Repeat step 2 twice.
4. Load between 25-50 μ L of sample. Add AcONH₄ buffer to get a final volume of 500 μ L.
5. Centrifuge for 10 min at 14 000 g to concentrate down to 50 μ L and remove the eluate. Add 450 μ L of AcONH₄ buffer. Repeat the dilution concentration cycle five times.
6. After the last cycle, transfer the 50 μ L of sample to an Eppendorf tube. Protein concentration is determined by UV absorbance using a NanoDrop spectrophotometer (Thermo Fisher Scientific, France).

3.2 Mass spectrometer calibration

a. Synapt G2

1. The automated chip-based nanoelectrospray device (Triversa Nanomate, Advion, Ithaca, USA) is operated in the positive ion mode. Set the capillary voltage and the nitrogen nanoflow at 1.75 kV and 0.60 psi respectively.

2. In MassLynx MS Tune windows, set the following parameters:

Operate the instrument in positive and sensitivity modes with a m/z range from 1 000 to 10 000.

Nanoflow+ panel: Sampling Cone: 120 V. Extraction con: 5 V. Source Temperature: 90 °C. The Capillary Voltage and the gas flows are not used in the Nanomate interface setup.

Instrument: Trap and Transfer Collision Energy are turned off.

3. Start a 2 min TOF-MS acquisition with a m/z range from 1 000 to 10 000.
4. Average the signal over the 2 min in the Chromatogram panel. In the spectrum panel, a Spectrum Smooth with ± 10 Smooth window and a number of smooths of 2 with a “Mean” Smoothing method were realized. On the smooth spectrum, the TOF Spectrum Center with a min peak width at half height of 2 and a Centroid top at 80.00 % was selected.
5. In the Acquity UPLC Console, select “Create Calibration”.
6. In the calibration profile editor, select the right Mass Calibration Profile (CsI in the 1 000 – 10 000 m/z range in positive and sensitivity modes).
7. The Reference Compound (Csi_1000-10000 positive) with reference masses from 1 172.1450 to 7 927.2031 Da) is used for the calibration.

b. Exactive Plus EMR

1. Using HESI source and the 500 μ L Hamilton™ syringe, deliver cesium iodide solution with a flow rate set to 10 μ L/min.
2. In the MS Tune page manually set the following parameters: Operate the instrument in positive mode with a m/z range from 1,000 to 20,000. Apply 25 eV and 100 eV for the CID and CE parameters, respectively. The source temperature is set at 250°C, the capillary voltage is set to 4 kV and the gas flows is set to 10 u.a. The trapping gas

pressure is set to 7 u.a. The ion optics are set to 4 V for injection, inter and bent flatapoles. Set the nominal resolution at 17,500 and activate the EMR mode.

3. To perform the calibration, wait for a stable TIC (<12 %) then select “Calibrate” and check “EMR MS Mass Calibration (pos)”.

3.3 Direct infusion on Exactive Plus EMR for native MS experiment

a. Mass spectrometry setting for native MS experiment

1. Desalt between 100 µg and 150 µg of ADC in 150 mM ammonium acetate.
2. Determine the concentration of the ADC solution after desalting by UV absorbance using NanoDrop spectrophotometer (Thermo Fisher Scientific, France).
3. Prior to ADC mass analysis, perform Exactive Plus EMR calibration with 8 µL of cesium iodide solution (see 2.4).
4. Connect the automated chip-based nanoESI infusion source (Triversa Nanomate, Advion, Ithaca, USA) to the Exactive Plus EMR.
5. In the Tune page, set the following parameters: Positive mode with a m/z range from 1 000 to 14 000. Activate the EMR mode and set the trapping gas pressure at 7 u.a., the ion optics at 8 V, 7 V, 6V for inter, injection and bent flatapoles respectively, the S-lens RF voltage at 200 V, the temperature at 250°C, the fragmentation parameters at 75 eV and 10 eV for CID and CE values, respectively. Select a nominal resolution of 17 500.
6. Start a 2 min acquisition with a stable TIC. The mass spectrum is presented in **Figure 1a**.

b. Data interpretation

1. In BioPharma Finder 2.0, select your raw data file and Intact Protein Analysis.
2. In chromatogram & Source Spectra, select a m/z range from 5 500 to 7 500. In ReSpect Deconvolution Algorithm, set a Model Mass Range from 145 000 to 160 000 with a

mass tolerance of 10 ppm. Select a charge state range from 18 to 35. For the Choice of Peak Model, select Raw File Specific for the Resolution at 400 m/z.

3. Process the algorithm in order to have the deconvoluted spectrum presented in **Figure 1b**.
4. For average DAR (DARav), see 3.4. [Figure 1 near here]

3.4 Direct infusion on Synapt G2 for native MS/IM-MS experiment

a. Mass spectrometry settings for native MS experiment

1. Desalt between 100 µg and 150 µg of ADC in 150 mM ammonium acetate.
2. Determine the concentration of the ADC solution after desalting by UV absorbance using NanoDrop spectrophotometer (Thermo Fisher Scientific, France).
3. Connect the automated chip-based nanoESI infusion source (Triversa Nanomate, Advion, Ithaca, USA) to the Synapt G2 HDMS (Waters, Manchester, UK).
4. The analysis on the Synapt G2 HDMS is performed in sensitive mode.
5. Prior to ADC mass analysis, perform Synapt G2 calibration with 8 µL of cesium iodide solution (see 2.4).
6. In the MassLynx Tune windows set the following parameters: Positive ion mode, m/z range from 1000 to 10000, sampling cone: 180 V (**Note 2**), backing pressure: 6 mbar, source offset: 5 V, source temperature: 90°C, trap collision energy: 10 V, transfer collision energy: 5 V, trap gas: 5 mL/min, scan time: 4 s, and acquisition time: 2 min.
7. Deliver among 10-20 µL of ADC solution at 5 µM concentration.
8. In MaxEnt 1, the ranges are set from 145000 to 162000 with a resolution of 1 Da/channel. The width at half height is set to 2 Da for the uniform Gaussian. The deconvoluted spectrum is presented in **Figure 2b**.
9. For the DARav calculation, see 3.4.[Figure 2 near here]

b. Data interpretation Synapt G2

1. Open the chromatogram option in MassLynx menu.
2. Combine the mass signal over the two minutes acquisition time.
3. Smooth the signal using the following parameters: Smooth window: 10, number of smooths: 2, and smoothing method: Mean (**Figure 2a**).
4. Perform MaxEnt 1 deconvolution with the following parameters: Ranges 145000-165000, Resolution: 1.00 Da/channel, Uniform Gaussian width at half height: 5 Da, and minimum intensity ratios: 33% left and 33% right (**Note 3**).
5. Take the intensities of each individual DAR population to assess the DLD (**Figure 2b**) and calculate the DARav (see section 3.4).

c. Ion mobility mass spectrometry settings for native IM-MS

1. For IM-MS, in the MassLynx Tune windows set the following parameters: Positive ion mode, m/z range from 1000 to 10000, sampling cone: 80 V (**Note 4**), backing pressure: 6 mbar, source offset: 5 V, source temperature: 90°C, trap collision energy: 4 V, transfer collision energy: 5 V, trap gas: 5 mL/min, scan time: 4 s, and acquisition time: 2 min.
2. In the Ion mobility cell set the following parameters: N₂ flow rate: 45 mL/min, He flow rate: 120 mL/min, IM wave height: 40 V, and IM wave velocity 923 m/s (**Note 5**).
3. In the masslynx tune page, activate the Mobility TOF mode in the acquisition mode menu.
4. Deliver among 10-20 µL of ADC solution at 10 µM concentration (**Note 6**).
5. Record the mass spectrum. Do not forget to activate the option “add drift time function” to record the arrival time distribution of each ion (**Note 7**).
6. Deliver among 10-20 µL of each individual solution of IM calibrants previously prepared.

- Record the IM chromatogram of each individual calibrant in triplicate and under identical instrumental conditions as those used for the ADC IM-MS analysis. [Figure 3 near here]

d. Data interpretation Ion Mobility Synapt G2

- In the chromatogram menu of MassLynx, open the IM function.
- Combine the whole mobilogram signal to extract the mass spectrum (**Figure 3a**).
- In your mass spectrum, combine the peak of interest at the half-maximum. The chromatogram menu will automatically open, showing the arrival time distribution associated with the ion selected previously (**Figure 3b**).
- Take the drift time associated with the center of the ATD Gaussian.

e. Calibration curve to convert drift time on collision cross section (CCS)

- Proceed as described in section 3.2.d. to obtain the drift time associated with each peak of the calibrant solutions: For concanavalin A take the drift time of the 19+, 20+, and 21+ charge states, for ADH take the 24+, 25+, and 26+ charge states, and finally for PK take the 32+, 33+, and 34+ charge states.
- Correct the drift times of all these charge states using the following equation (**Note 8**):

$$t_d' = t_d - (c * \sqrt{\frac{m}{z}})/1000$$

- Calculate the reduced cross section (Ω') of the reference charge states of the calibrant systems reported in the literature with the following equation:

$$\Omega' = (\Omega_{literature} \sqrt{\mu})/z$$

- Make a graph $\ln(t_d')$ vs $\ln(\Omega')$, fit the data with a linear equation

$$\ln(\Omega') = a + b \ln(t_d')$$

- Make the final correction of the drift times from the equation:

$$t_d'' = t_d'^X(z|\sqrt{\mu})$$

6. Build a second graph t_d'' vs $\Omega_{literature}$ and fit the data with a linear regression

$$\Omega_{literature} = b t_d''$$

7. Use the b and X parameters to convert the drift time of the different DAR populations of your ADC sample on CCS values (**Figure 3c**) with the following equation:

$$CCS (nm^2) = (t_d - (1.57 \sqrt{\frac{m}{z}} / 1000)^x z (1/\sqrt{\mu}) b$$

3.5 Online SEC-native MS

a. LC setup for SEC-native MS

2. The column used is a BEH SEC column 200 Å, 1.7 µm, 4.6 x 150 mm (**Note 9**). the chromatographic system is an Acquity UPLC H-Class system (Waters, Manchester, UK) comprising a quaternary solvent manager, a sample manager set at 10°C, a column oven at room temperature and a TUV detector operating at 280 and 214 nm.
3. For the analyses of ADC, the elution is performed in isocratic mode with the following flow rate gradient: 0.25 mL/min over 4 min, then 0.1 mL/min over 5.5 min and finally 0.25 mL/min over 2.5 min. (**Note 10**), (**Note 11**).
4. Load the LC method and equilibrate the LC system with ten column volumes of mobile phase (see 2.1).

b. Native mass spectrometry settings for SEC-native MS experiment

1. Prior to ADC mass analysis, perform Synapt G2 calibration with 8 µL of cesium iodide solution (see 2.4).
2. For the tune page parameters, the Synapt G2 HDMS is operated in sensitive mode with a capillary voltage of 3.0 kV. The sample cone and pressure in the interface region were set to 180 V for lysine conjugate and 120 V for cysteine conjugate (**Note 2**) and 6 mbar, respectively. Source and desolvation temperature were set to 100 and 450°C, respectively. Desolvation and cone gas flows were set at 750 and 60 L/hr, respectively.

3. The MS file is setup with the following parameters: Start time: 0 min; End time: 12 min; Polarity: Positive; Analyser Mode: Sensitivity; Dynamic Range: Normal; Sensitivity: Normal; Low Mass: 1 000 Da; High Mass: 10 000 Da; Scan Time: 1.5 sec (Continuum); No Trap or Transfer CE. Events for flow state are created to direct salts to waste: 0 min Flow State directed to Waste, 4 min Flow State directed to LC, 9 min Flow State directed to Waste.
4. In MassLynx, create a sample list with the MS file, Inlet file, MS tune file. Specify the sample position (Bottle). The injected volume should be comprised between 1 and 10 μ L. No manual desalting is needed prior SEC-native MS experiment due to online desalting provided by SEC.

c. Data Interpretation

1. Chromatogram (**Figure 4a**) and TIC are obtained in the Chromatogram panel.
2. In MaxEnt 1, the ranges are set from 145000 to 162000 with a resolution of 1 Da/channel. The width at half height is set to 2 Da for the uniform Gaussian. The deconvoluted spectrum is presented in **Figure 4b (Note 12)**.
3. For the DARav calculation, see 3.4. [Figure 4 near here]

d. Ion mobility settings for SEC-native IM-MS experiment

1. In the MassLynx Tune window set the following parameters: Positive ion mode, m/z range from 1 000 to 10 000, sampling cone: 80 V, backing pressure: 6 mbar, source offset: 5 V, source temperature: 100°C, Desolvation temperature: 450°C trap collision energy: 4 V, transfer collision energy: 5 V, trap gas: 5 mL/min.
2. In the Ion mobility cell set the following parameters: N₂ flow rate: 45 mL/min, He flow rate: 130 mL/min, IM wave height: 40 V, and IM wave velocity 923 m/s.
3. The MS file is setup with the following parameters: Start time: 0min; End time: 12 min; Polarity: Positive; Analyser Mode: Sensitivity; Dynamic Range: Normal; Sensitivity:

Normal; Low Mass: 1 000 Da; High Mass: 10 000 Da; Scan Time: 1.5 sec (Continuum);

No Trap or Transfer CE. Check Add Drift Time Function and Maintain Mobility Separation. Events for flow state are created to direct salts to waste: 0 min Flow State directed to Waste, 4 min Flow State directed to LC, 9 min Flow State directed to Waste.

4. In MassLynx, create a sample list with the MS file, Inlet file, MS tune file. Specify the sample position (Bottle). The injected volume has to be comprised between 1 and 10 μL . Record each IM experiment in triplicate to calculate a more reliable collisional cross section.
5. Deliver among 1-10 μL of each individual solution of IM calibrants (see 2.5.2).
6. Record the IM chromatogram of each individual calibrant in triplicate and under identical instrumental conditions as those used for the ADC SEC-native MS analysis.
7. For data interpretation, see **3.2.e**

3.6 Average DAR calculation and Drug Load Distribution

The average DAR value represents the sum of relative peak intensities in the mass spectrum for each DAR multiplied by its corresponding number of drugs. It was calculated from the relative peak intensities measured from the raw mass spectra, by using the following equation

$$DAR_{av} = \frac{\sum_0^8 k \cdot I_k}{\sum_0^8 I_k}$$

Where k is the number of drugs and I_k is the relative peak intensity of DAR_k .

4. Notes

1. Ion mobility calibrants have to be chosen depending on the mass and the charge state of the analyzed mAb/ADC. Here we chose to use ion mobility calibrants that range from 100

kDa to 230 kDa and exhibit positive charge states from 15+ to 35+. Usually ADCs are proteins around 150 kDa with 20+ to 28+ positive charges under our experimental conditions.

2. The sampling cone (V_c) voltage is a crucial parameter on Waters instrument. A high voltage (180 V) increases transmission of high m/z ions along with more efficient desolvation[14]. However, high V_c values also favor dissociation of noncovalent assemblies, especially for cysteine linked ADCs. An intermediate V_c value of 100-120 V is thus usually more adapted. An optimum value has to be found to provide compromise between good quality MS signal and maintaining ADC integrity.

3. The parameters of the MaxEnt1 deconvolution are protein dependent. In particular, the mass range and the FWHM have to be optimized for each mAb/ADC. The latter has to be chosen as a function of the resolution of the experimental mass spectrum.

4. The sampling cone voltage applied in the source of the mass spectrometer is an important parameter for native IM-MS experiments. Ion heating can occur as a consequence of the effect of this acceleration voltage and eventually can lead to conformational changes (protein unfolding) on protein structures while ions are transmitted into the mass spectrometer. Be sure that the sampling cone voltage you are using allows the transmission of your molecular ions of interest and does not induce any structural changes. From our experience, 80 V provides a good MS signal (intensity and resolution) without modifying ADCs conformation.

5. These parameters are going to drive the separation of the ions in the ion mobility cell. They have to be carefully optimized to ensure ion focusing (He flow) prior to IM separation and provide the best separation (N_2 flow, wave height, and wave velocity[15]). These parameters are protein dependent.

6. When performing native IM-MS experiments, we slightly increase the concentration of the analyte compared to native MS experiments to compensate the loss of sensitivity due to the activation of the IM device.
7. Record each IM experiment in triplicate to calculate a more reliable collision cross section.
8. The c constant has been determined empirically and it is specific of the Synapt G2 mass spectrometer. For ion signals lower than 5000 m/z , $c = 1.41$. For ion signals above 5000 m/z , $c = 1.57$ [16].
9. Other columns are available for SEC-native MS experiment. For a unique fast desalting goal, shorter column dimension (i.e. BEH SEC 4.6 x 30 mm, 200 Å, Waters) could be used. For a separation goal, longer column dimension (i.e. BEH SEC 4.6 x 150/300 mm, 200 Å, Waters) is preferred.
10. The flow rate gradient provides improved sensitivity on the Synapt G2 HDMS mass spectrometer.
11. The flow rate gradient method is a generic method which can be easily adapted. If the LC method is changed, do not forget to change events in MS file for directing salts to waste.
12. SEC-native MS coupling provides higher MS spectral resolution and mass accuracy due to the more efficient desalting afforded by SEC compared to manual desalting.

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6. Figure Captions

Figure 1. Native MS analysis of deglycosylated brentuximab vedotin (Cysteine conjugate) on Exactive Plus EMR. a) Native mass spectrum corresponding to brentuximab vedotin. b) Deconvoluted mass spectrum representing the drug load distribution with the average DAR.

Figure 2. NanoESI MS analysis in non-denaturing conditions of deglycosylated Inotuzumab Ozogamicin (IO), a lysine ADC, after direct infusion. a) Full scan native mass spectrum of IO representing the different DAR populations (from DAR 2 to DAR 8).

Figure 3. NanoESI IM-MS analysis in non-denaturing conditions of deglycosylated site-specific DAR4 ADC[8] after direct infusion. a) Native mass spectrum of deglycosylated site-specific DAR4 ADC. b) Arrival time distributions (ATDs) of different DAR 4 charge states observed in the mass spectrum. c) Experimental collision cross section ($^{TW}CCS_{N2}$) and drift times corresponding to the different charge state populations upon ion mobility separation.

Figure 4. SEC-native MS analysis of deglycosylated inotuzumab ozogamicin (Lysine conjugate). a) SEC-UV chromatogram corresponding to ADC monomer. b) Deconvoluted mass spectrum representing the drug load distribution with the average DAR.

7. Figures

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