Immunoaffinity nSEC-MS for intact analysis of antibody drug conjugates to evaluate differential clearance of individual drug-load species *in vivo*

Jing Zhang, David Nguyen, Lauren Farr, Abbie Wong, Clark Henderson and Shawna Hengel
Pfizer, USA

This technical note describes a state-of-the-art workflow using immunoaffinity capture followed by native size-exclusion chromatography mass spectrometry (nSEC-MS) to evaluate drug-to-antibody ratio (DAR) and drug load distribution of antibody drug conjugates (ADCs) from *in vivo* plasma samples (Figure 1). This workflow uses anti-idiotype monoclonal antibodies (mAbs) to selectively capture and enrich ADCs, improving method sensitivity and specificity, and allowing characterization of individual ADC drug load species in plasma samples with a concentration as low as ~80 µg/mL. The optimized method was used to determine the longitudinal DAR and percent drug load distribution in a cynomolgus monkey toxicokinetic (TK) study up to 28 days post-dose. Additionally, *in vivo* modifications were identified as free thiol cysteine adducts following deconjugation at later TK timepoints.

Introduction

ADCs have become a significant class of targeted therapies in cancer treatment over the past several decades.[1] ADCs contain 3 main components: a mAb backbone, drug payload and linker that connects the two. The design of ADCs allows targeted delivery of drug payloads, through binding to antigens on cancer cells, internalization into the cells followed by payload release. ADC potency and toxicity are significantly influenced by the average number of payloads conjugated to the mAb (DAR) and how the drug load distribution changes through clearance and deconjugation overtime in circulation.[2]

The complex design of ADCs places a challenge on *in vivo* characterization and bioanalysis. Differences in linker types, payloads and antibody targets are the primary sources of ADC diversity. One major conjugation strategy used in ADC technology is interchain cysteine conjugation which partially reduces the interchain disulfide bonds on the antibody heavy and light chains for covalent conjugation of the drug linker. Due to the nature of the conjugation process, cysteine-linked ADCs can carry 0 to 8 copies of the payload and are dosed as a

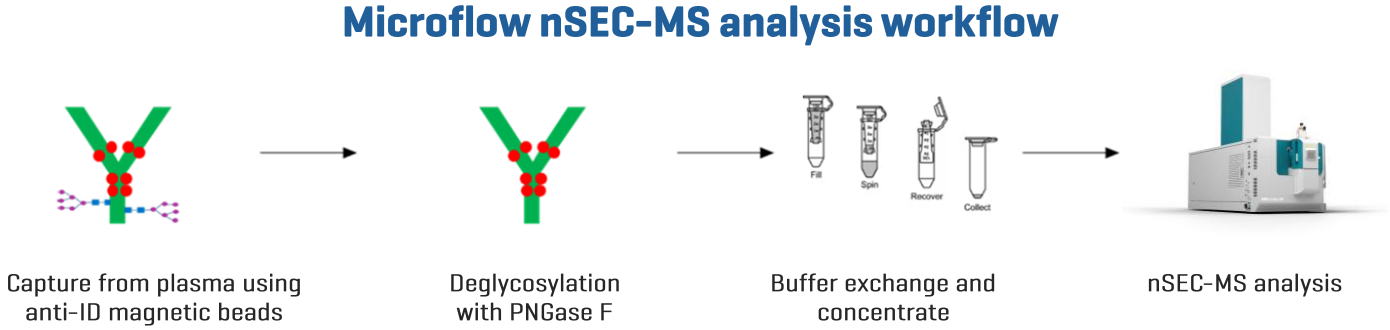

Figure 1. General nSEC-MS sample preparation and analysis workflow. This workflow involves immunoaffinity capture of ADCs from plasma, followed by deglycosylation using PNGase F and nSEC-MS analysis using the ZenoTOF 7600 system.

mixture of the different drug load species. nSEC coupled with high resolution MS (HRMS) is a powerful tool to measure intact drug load species of cysteine-linked ADC under non-denaturing conditions. Valliere-Douglas *et al.* reported the first nSEC-HRMS method for direct measurement of the intact mass of ADC drug load species.[3,4] Hengel *et al.* successfully measured ADC drug load distribution and distribution changes following an ADC dose in preclinical and clinical samples using affinity capture with nSEC-HRMS.[5,6]

The method described here employed the affinity capture nSEC-MS approach for intact analysis of ADCs with novel chemotypes using the ZenoTOF 7600 system (Figure 1), which provides high resolution and sensitivity. The assay was used to analyze non-clinical TK study samples to characterize the TK profile of *in vivo* DAR and the percent distribution of individual drug load species in cynomolgus monkeys.

Methods

Equipment: KingFisher Apex (Thermo Fisher), I-Class HPLC system (Waters) and ZenoTOF 7600 system equipped with an OptiFlow source (SCIEX).

Capture reagents preparation: Streptavidin-coupled magnetic beads (Promega, Z5482) were washed and resuspended in PBS. Biotinylated anti-idiotype mAbs were added to the beads and rotated at room temperature for 1 hour with a final mAb concentration of 0.3 mg/mL. After incubation, the supernatant was removed, and the beads were washed with PBS and resuspended in PBST.

Sample preparation: ADC quality control (QC) samples were prepared at 80, 120, 200 and 900 µg/mL in cynomolgus monkey plasma. QC and TK study samples (19.2-108 µg per sample with a plasma volume range of 110-240 µL) were incubated with 0.8 mL anti-idiotype mAbs-coupled magnetic beads at 4°C for 1 hour for ADC capture and enrichment. Samples were transferred to a 96-well plate and washed with 900 µL PBST and 900 µL PBS x 2 using KingFisher Apex. ADCs were eluted from beads into 100 µL IgG elution buffer (Fisher Scientific, PI21004). Following elution, 10 µL of 1 M Tris buffer (Sigma-Aldrich, T3038) was added to each well and vortexed at room temperature for 5 minutes. 3 µL of PNGase F (New England Biolabs, P0705L) was added to each well, and the samples were incubated at 37°C and 700 RPM for 2 hours. Sample extracts were transferred to Amicon ultra centrifugal filters with a 30K MW cutoff (Millipore, UFC503008) for buffer exchange with 200 mM ammonium acetate. The sample extract was concentrated to a final volume of ~25-50 µL.

nSEC-MS analysis: ADC sample extracts (15 µL, ranging from 13 to 33 µg) were injected onto a 1.0 mm x 150 mm (5 µm, 300 Å) polyhydroxyethyl-A (PHEA) SEC column (PolyLC, 151HY0503) with an isocratic flow of 200 mM ammonium acetate at 25 µL/min. Salt concentrations were diluted with 0.1% formic acid in water at 125 µL/min after the column before going to the mass spectrometer. All MS data were acquired using the ZenoTOF 7600 system (SCIEX). An intact protein acquisition workflow was used. The MS parameters are set at 5,500 V, 250 V, 1 V and 300°C for spray voltage, declustering potential, collision energy and source temperature, respectively. The gas parameters were 55 psi, 65 psi, 45 psi and 7 for ion source gas 1, ion source gas 2, curtain gas and CAD gas, respectively.

Data processing: Data analysis was performed in totality by the Pfizer team authoring this technical note. Mass spectra were deconvoluted using the Byos Protein Characterization Software (Protein Metrics). An ADC workflow incorporated with an intact mass algorithm was used to deconvolute the mass spectra, generate the peak intensity of each individual drug load species and calculate DAR.

***In vivo* study**: Cynomolgus monkeys were administered 2 intravenous doses of ADCs at 30 mg/kg on Day 1 and Day 22. Whole blood was collected at specified timepoints following each dose period. Samples were kept on ice and processed to plasma within 1 hour of collection. All procedures performed on animals were in accordance with regulations and established guidelines and were reviewed and approved by an Institutional Animal Care and Use Committee or through an ethical review process. Absolute antibody concentrations of the ADCs were determined in-house using a ligand binding platform.

Intact ADC analysis workflow using nSEC-MS

General sample preparation and analysis protocols were described in the Method section. Figure 1 shows the overall nSEC-MS workflow scheme. This workflow involves immunoaffinity capture of ADCs, followed by deglycosylation with PNGase F, then nSEC-MS analysis using the ZenoTOF 7600 system.

Evaluation of nSEC-MS and immunoaffinity capture methods

MS parameters were optimized using the ADC dosing material, which has a reported DAR of 4.1, and individual drug load percent contribution data from hydrophobic interaction chromatography (HIC) (Table 1). Both neat dosing material and *in vivo* plasma QC samples were used for MS method optimization. Spray voltage, declustering potential and source temperature were evaluated to gain sufficient sensitivity while achieving an accurate DAR measurement and percent contribution of individual drug load species. Using optimized conditions, the average DARs of 4.1 and 4.0 were measured for the neat dosing material (Figure 2) and plasma QC samples (Figure 3), respectively. The percent contributions of individual drug load species were in good agreement with those of the reference dosing material (Table 1).

The affinity capture of ADCs in plasma was optimized. Both the capacity of anti-idiotype mAb biotinylation to streptavidin on beads and the capacity of ADC capture were evaluated. The maximum enrichment amount from plasma was set at ~70% ADC capture capacity to ensure complete capture of heterogeneous cysteine-linked ADCs.

Table 1. Percent contribution of individual drug loads in neat and plasma QC samples in comparison with the dosing material.

Drug load	MS QC	Plasma QC	Dose material
DAR 0	1.11	1.33	2.8
DAR 2	26.3	28.5	21.7
DAR 3	1.89	1.33	1.90
DAR 4	44.2	43.9	38.2
DAR 6	18.8	17.9	22.1
DAR8	7.64	7.04	10.4

Analysis of *in vivo* samples from non-clinical TK study

The optimized method was used to analyze *in vivo* cynomolgus monkey plasma samples from a non-clinical TK study. To monitor the ADC amount enriched for enough sensitivity and complete capture, the antibody concentrations of the ADC from study samples were measured using an in-house ligand binding platform. The measured concentration ranged from 79.2 to 933 µg/mL. At least 19 µg of ADC was enriched with a final sample extract concentration ranging from 850 to 2,200 µg/mL. A 15 µL of the sample extract was injected onto the column. MS data was acquired using the ZenoTOF 7600 system.

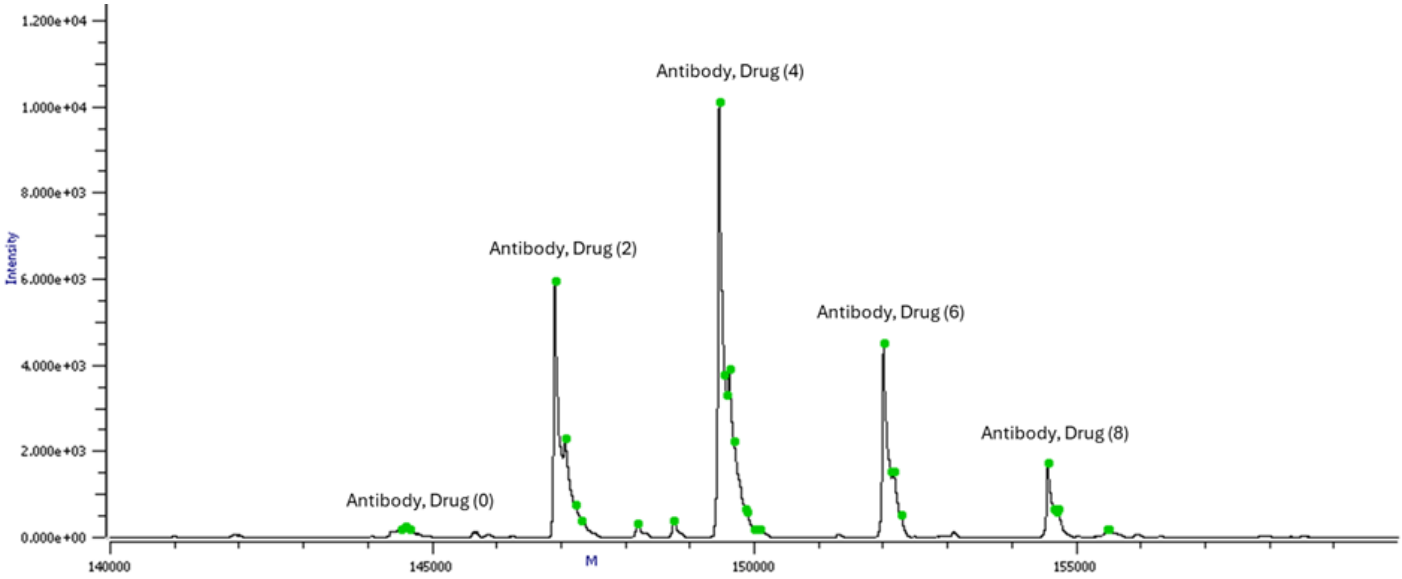

Figure 2. Deconvoluted mass spectrum obtained using nSEC-MS for the neat ADC material with an average DAR of 4.1.

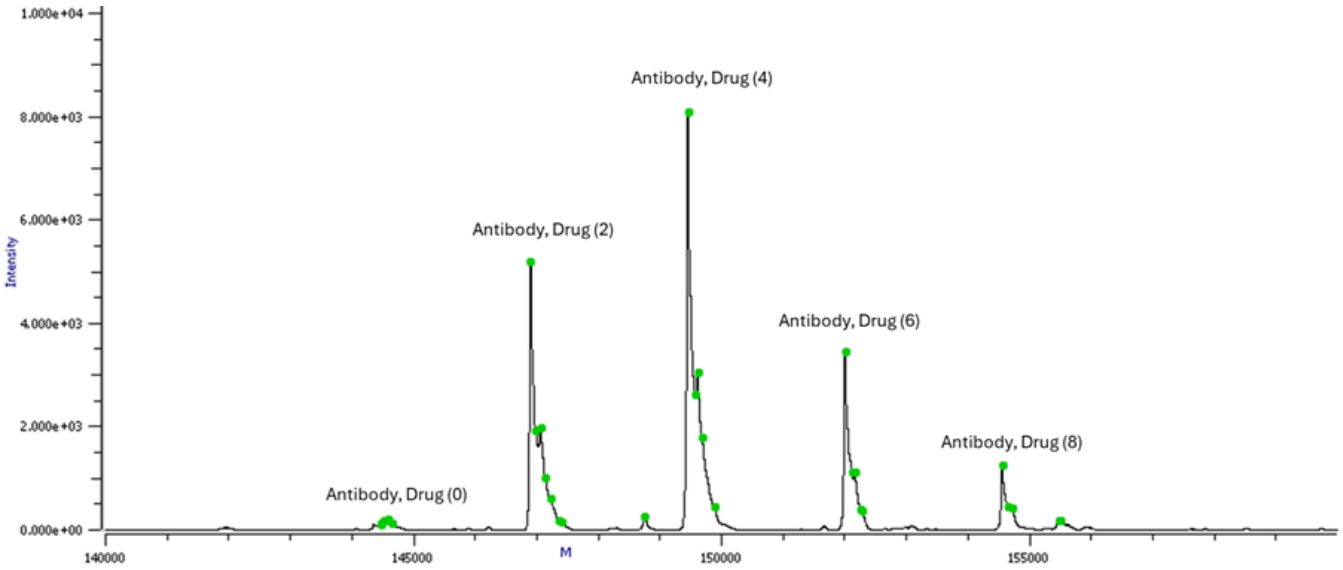

Figure 3. Deconvoluted mass spectrum obtained using nSEC-MS for the ADC plasma QC sample with an average DAR of 4.0.

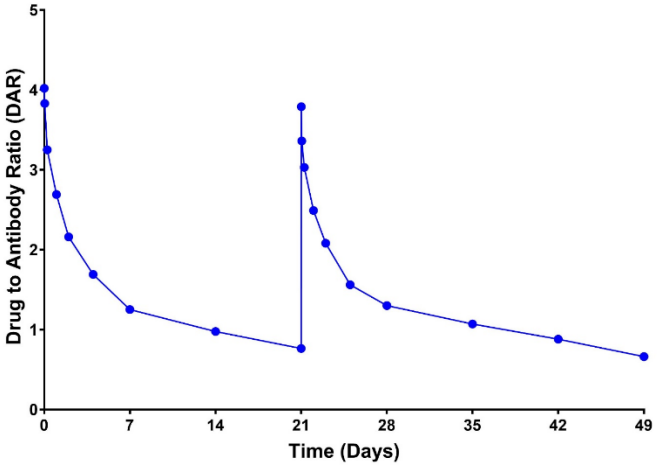

Figure 4. *In vivo* profile of the average DARs measured from the cynomolgus monkey plasma.

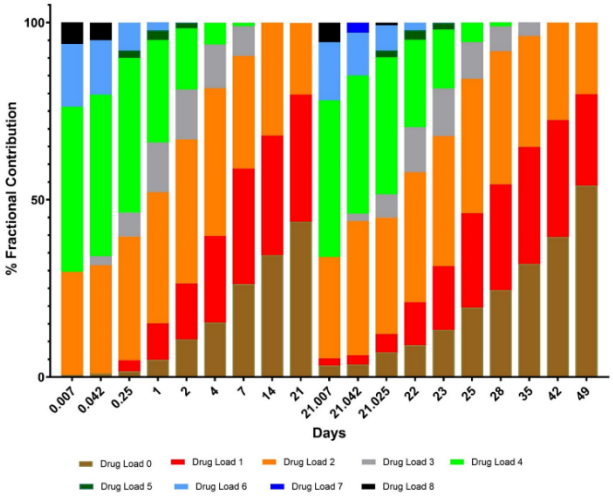

Figure 5. Percent contribution of individual drug load species from *in vivo* cynomolgus monkey plasma.

The ADC drug load profiles were obtained for each timepoint of the *in vivo* samples. The intensities of the drug load species were used to calculate the average DARs (Figure 4) and percent contribution of each species (Figure 5). A full drug load distribution of DAR 0 to DAR 8 was observed at the 10 min and 1 hr timepoints from *in vivo* plasma samples. At the 10 min timepoint, the average DAR and percent contribution profile of the *in vivo* sample (Figure 6A) were comparable to those of the neat and plasma QC samples (Figures 2 and 3). Starting from 1 hr, the species with odd DAR values (e.g. DAR 1 and DAR 3) were observed (Figure 6B) due to the drug linker deconjugation through reverse Michael addition.[7,8] The percent contribution of higher drug load species decreased rapidly in the first few days due to deconjugation and clearance. The DAR 0–2 species were dominant at the late time points (Figure 6C). The DAR profiles obtained after the second dose followed a similar pattern to that of the first dose (Figures 4–6). The reverse Michael addition for maleimide drug linkers generated a free thiol residue, which can react with free cysteine. This led to the addition of ~120 Da to each drug load species. The cysteine-modified DAR 1 and 3 drug load species were observed after Day 2 (Figures 6B and 6C).

Conclusions

- Intact protein analysis of ADCs with novel drug linkers from *in vivo* samples requires ADC immunoenrichment and MS instrumentation with high resolution and sensitivity for accurate DAR calculations and drug load distribution characterization.
- The optimized affinity capture nSEC-MS method was successfully used to characterize the *in vivo* disposition of ADCs in cynomolgus monkeys in a non-clinical TK study.
- The DAR and drug load distribution profiles provide abundant information to understand the new ADC chemotypes, which is critical for early-stage drug development.

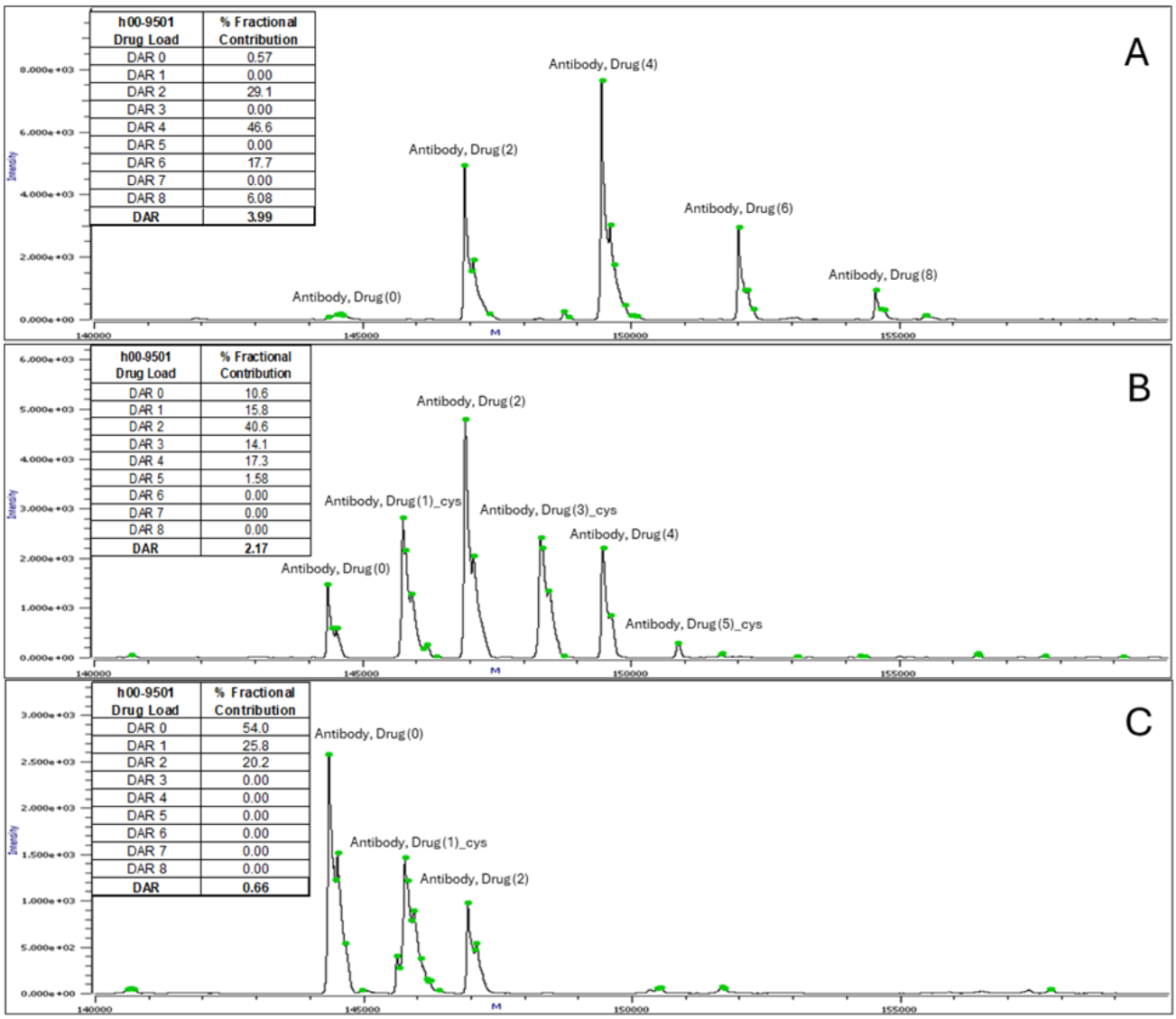

Figure 6. *In vivo* drug load distribution of the ADC at 10 min (A), 2-day (B) and 49 days (C) post first dose in cynomolgus monkey plasma. The insets show the percent distribution of each DAR species at each time point.

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Headquarters
250 Forest Street | Marlborough,
MA 01752 USA
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