Mass spectrometry as a tool in ADC development

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Introduction

Antibody drug conjugates (ADCs) combine a monoclonal antibody (mAb) with a cytotoxic drug via a linker. This allows the mAb to target a specific cell type or antigen and deliver a potent molecule without affecting surrounding cells. There are several currently approved ADCs with a variety of linkage chemistries of varying specificities. As this field grows robust characterisation methods will be needed to ensure conjugations are performed in a repeatable and robust manner.

Mass spectrometry (MS) is an appealing technique for protein characterisation as it provides additional information than purely optical techniques with many existing chromatography methods being hyphenated with the appropriate buffers and columns. In addition, MS does not need chromatographic separation of conjugated species to provide drug to antibody ratio (DAR) which can assist on difficult payloads. MS can go further in localising specific residues of conjugation when combined with enzymatic digestions.

In this work we outline a series of MS approaches for characterising various conjugation lots to ensure comparability.

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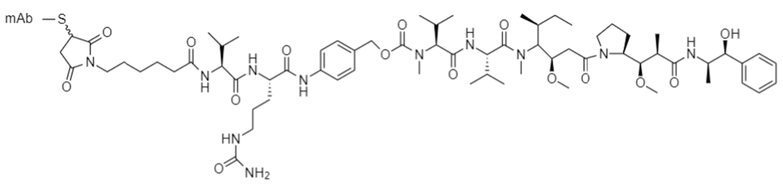

Fig. 1 *Structure of val-cit PABC MMAE payload. The val-cit domain is designed to be cleaved by cathepsin leading to the immolation of the spacer group leaving the active warhead free to block tubulin polymerisation.*

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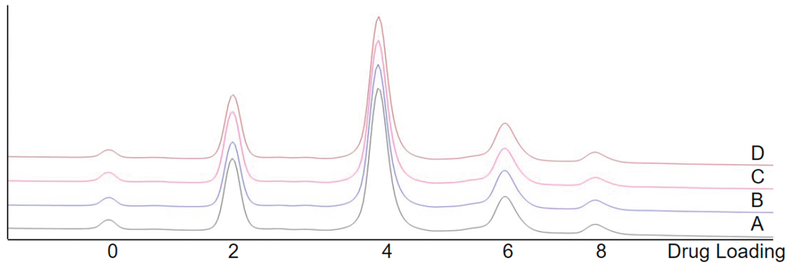

Fig. 2 *A) Hydrophobic Interaction chromatography UV spectrum at 256nm for all four lots. DAR was calculated by HIC as 4 and shown in Table 1.*

	A	B	C	D	
Reduced MS	4.2	3.9	4.1	4.1	4.1 ± 0.1
Reduced Deglycosylated MS	3.9	4.1	3.9	4.0	4.0 ± 0.1
IdeS Reduced Deglycosylated MS	3.9	4.0	4.0	4.0	4.0 ± 0.1
Native MS	3.9	3.9	3.9	3.9	3.9 ± 0.1
Hydrophobic Interaction Chromatography	4.0	4.1	4.0	4.0	4.0 ± 0.1

Table 1 *Assigned DAR across all techniques. Mass spectrometry shows strong alignment across methods and with LC-UV data.*

Methods

Here we outline various analytical strategies to determine the drug-antibody ratio and stochiometry of a several manufacturing lots of a val-cit PABC monomethyl auristatin E (MMAE) conjugate (DAR 4). All data was collected on a SCIEX X500B Mass Spectrometer with an EXION HPLC with buffers of water and acetonitrile each with 0.1% formic acid.

The reduced DAR method was conducted on a bioZen Intact XB-C8 Column (3.6 µm, 50 x 2.1 mm). The peptide digest was performed with a Trypsin/Lys-C mix following denaturation, reduction and alkylation. The column was a bioZen XB-C18 1.8 µm 150 x 2.1mm. The instrument was operated in an information dependent analysis method for MSMS.

The native MS was performed with a bioZen dSEC-2 column (1.8 µm, 50 x 2.1 mm) and 400 mM ammonium acetate (Sigma) as the mobile phase.

References

1. Firth, D.; Bell, L.; Squires, M.; Estdale, S.; McKee, C. A rapid approach for characterization of thiol-conjugated antibody–drug conjugates and calculation of drug–antibody ratio by liquid chromatography mass spectrometry. *Analytical Biochemistry* ***2015****, 485,* 34. https://doi.org/10.1016/j.ab.2015.06.001

REDUCED DAR ANALYSIS

Reduced DAR analysis utilises the same sample prep as a reverse phase PLRP-UV measurement with a fraction of the sample required, loading as little as 100ng on column per measurement. Mass spectrometry allows us to identify peaks directly rather than inferring from shifts and standards, this can greatly aid development when conjugations or proteins behave in unexpected ways. By applying enzymes such as partial digestion and deglycosylation we can assign conjugation locations to protein domains.

In the below figures we compare the reverse phase PLRP data with an IdeS digested, deglycosylated set of samples. From the PLRP data we can see comparability between the heavy and light chains on the four samples. The MS goes further and allows us to see where these are present on each protein chain. We can also see in Table 2 that all the masses observed are as expected allowing us to confirm the shifts observed by optical methods are assigned correctly. On this kind of analysis, we can monitor limited PTM's such as oxidation and any linker breakdown over time.

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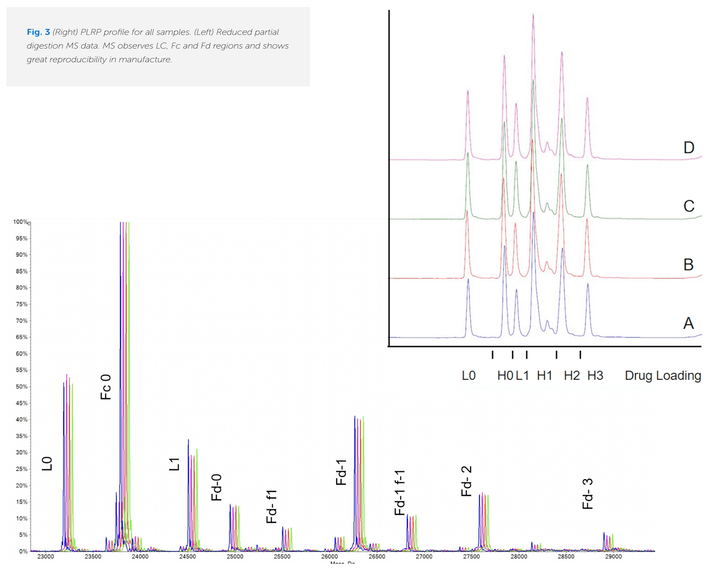
Fig. 3 *(Right) PLRP profile for all samples. (Left) Reduced partial digestion MS data. MS observes LC, Fc and Fd regions and shows great reproducibility in manufacture.*



Chains	SDE-100 Species	Theoretical Average MW (Da)	GMP 1	GMP 2	GMP 3	TOX 3
LC		23189.5	23189.6	23189.6	23189.7	23189.8
	MMAE + Linker - 1	24506.2	24506.3	24506.2	24506.3	24506.4
Fc	Protein Terminal Lys-Loss-1	23787.7	23787.7	23787.7	23787.8	23787.8
Fd* (Deglycosylated)	MMAE + Linker - 1	26264.6	26264.7	26264.8	26264.8	26264.9
	MMAE + Linker - 2	27581.3	27581.6	27581.6	27581.6	27581.7
	MMAE + Linker - 3	28897.9	28898.2	28898.2	28898.2	28898.2
Gln>pyro-Glu - 1*						

Table 2 *Observed protein conjugate masses. High resolution mass spectrometry can confirm that observed peaks are the expected species with >5ppm expected mass accuracy.*

NATIVE SEC-MS

Native DAR analysis requires the least amount of sample handling with the sample able to be injected from a storage buffer into the LC-MS. The data can be simplified with the removal of glycans through Endo-S (Genovis) however this step is not required. It is also the least quantitive and sensitive requiring tens of µg's of conjugate per injection and only providing a DAR. However, the advantages of the intact measurement is any breakdown in stoichiometry between heavy and light chains can be observed unlike other methods where this information is lost.

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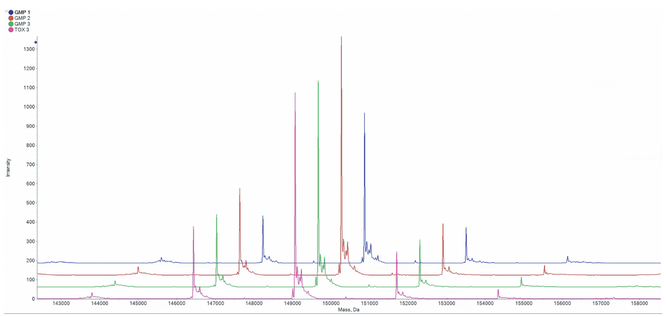

Fig. 4 *Intact native spectra over incubation time.. Native MS slightly underestimates DAR due to suppression of hydrophobic species ionisation but overall shows alignment of all the samples with minimum sample handling.*

PEPTIDE DIGESTION

Tryptic digestion allows us to break a protein down into reliable and reproducible small peptides of sizes between ~300-4000 Da. The advantage of working with these small peptides is they ionize more efficiently than large proteins, they can be separated based upon they hydrophobicity upon reverse phase chromatography, and within the MS they can be fragmented using collision induced dissociation to provide confirmation of the sequence of each peptide and the location of any PTMs. For ADCs, conjugated peptides are observed to have a noticeable increase in hydrophobicity separating them from un-conjugated species and MS and MSMS can confirm the localisation of each modification. In addition, MS can determine if there are any off-target conjugation events.

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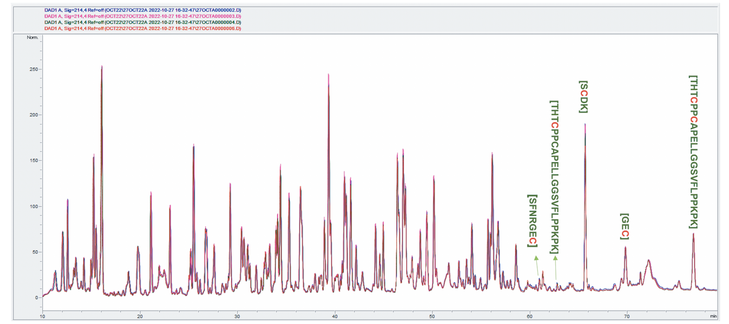

Fig. 5 *UV peptide fingerprint at 254 nm. Repeated digestions of the samples produced highly similar peptides.*

UV fingerprinting (Figure 5) shows highly similar peptides present in all samples and MSMS confirms this. Conjugated peptides appear after 60 minutes and are labelled. Figure 6 shows individual peptides confirmed via MSMS with modified amino acids in red and conjugations in orange.

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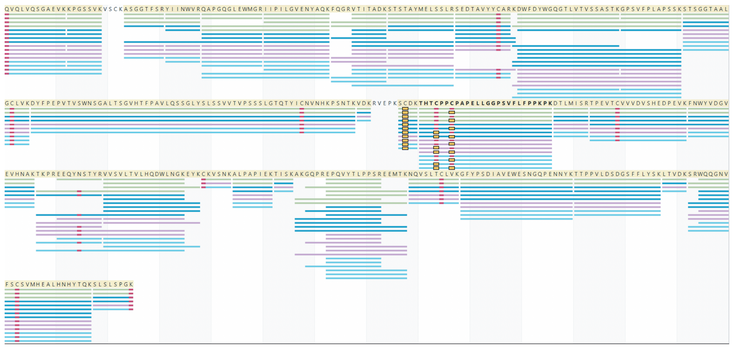
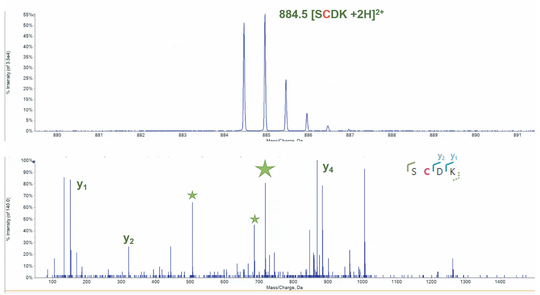


Sample	Auto-Validated Coverage %
GMP 1	96.8
GMP 2	96.4
GMP 3	96.2
TOX 3	97.4

Fig. 6 *(Top) Observed peptides confirmed via MSMS fragmentation.*

(Below) Example conjugated peptide SCDK. Peptides are assigned based upon their MSMS fragments and scored versus a false discovery rate. Indicator fragments (green stars) from the payload are used to aid assignment



Conclusions

Here we present various levels at which mass spectrometry can assist in ADC development. Reducing subunit MS can complement PLRP analysis providing information on unknown peaks and as a DAR assessment if chromatographic separation is not possible. Native MS provides a similar DAR analysis to HIC and can confirm stoichiometry of bispecific or non-covalent additions. Peptide digestion allows any conjugation events to be localised on specific amino acids. The addition of MS allows the elevation of nearly any chromatography method to a more data rich tandem approach. Future work will see the addition of charge variant MS and HIC-MS to our portfolio.

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