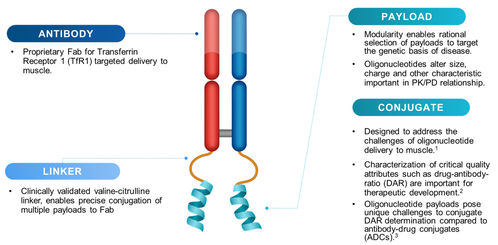
Investigation of drug-to-antibody ratio for FORCE oligonucleotide conjugates using Microchip CE-MS

Benjamin F. Vieira[1], Aditya Kulkarni[2], Kate Yu[2], Peiyi Shen[1], Pei-Ni Tsai[1], Timothy Weeden[1]

[1]Dyne Therapeutics Inc., Waltham, MA, USA, [2]908 Devices, Boston, MA.

BACKGROUND

The FORCE Platform



- The ZipChip is a capillary electrophoresis-based (CE) front-end separation device that can be seamlessly coupled with commercial mass spectrometers, enabling CE-MS.
- This method is sensitive to the surface charge on analytes and is therefore well-suited for characterization of FORCE conjugates where payload influences conjugate isoelectric point (pI).
- In this study, FORCE conjugates containing different oligonucleotide payloads with various chemistries and DAR distributions were subjected to separation using the ZipChip coupled to high-resolution mass spectrometry.
- This method enabled the separation and precise identification of DARs 1-6 and enabled accurate measurement relative to orthogonal methods, such as gel-electrophoresis and reversed-phase liquid chromatography mass spectrometry (RP-LCMS).

METHODS

Conjugate Prep:

Both neutral phosphorodiamidate morpholino (PMO) and charged antisense oligonucleotides (ASO) were chemically activated and subsequently conjugated to the Fab to target a specific DAR range. FORCE conjugates then were purified and fractionated to a specific DAR range, and to remove free Fab and payload. SDS-PAGE and bicinchoninic acid assay (BCA) analysis were performed to understand final DAR, concentration, and yield. Prior to analysis, samples were diluted into prototype background electrolyte (BGE) before loading on ZipChip.

ZipChip:

	PMO	ASO
Chip Type	ZipChip HR	
BGE	Prototype (pH 4.5)	Peptides
Injection Volume	1nL	
Pressure Assist	ON at 0.5min	
Analysis Time	15 min	4 min

Mass Spec: Thermo Q Exactive HF | Spray Voltage: 0 V | Capillary Temp: 250 | Sheath Gas: 2 | Scan Range: 1000-4000 | isCID: 50 | S-Lens RF: 60 | Resolution: 15000 | AGC Target: 1e6 | Max IT: 50 ms | Microscans: 10 |

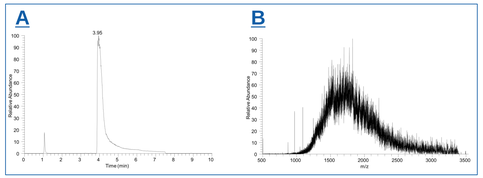
RESULTS AND DISCUSSION

Conjugate Information

	A	B	C	D	E
Payload Type	PMO				ASO
Conc. (mg/mL)	4	11.8	4.67	1.59	15.6
Average MW (Da)	67401.8	64123.8	82702.2	108515.0	54359.9
Average pI	~7.8	~8	~7.45	~6.6	~7.1
Payload Theoretical Mass (Da)	~9841	~9781	~9781	~9781	~6716
Average DAR (SDS-PAGE)	2.2	1.6	3.5	6.1	1.1

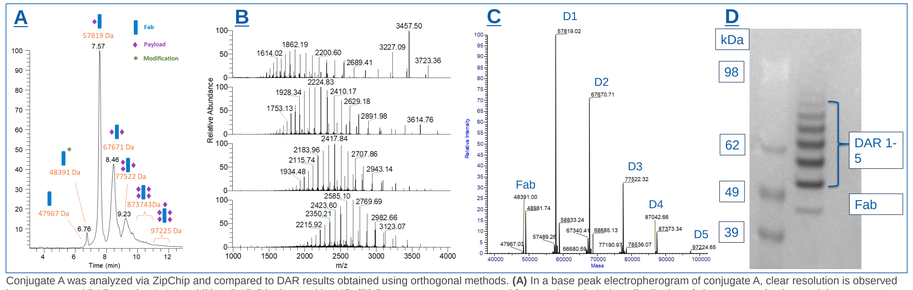
Key data for all test samples used in this study. Payload Theoretical Mass refers to the total mass of the payload and linker as attached to the Fab. Average DAR refers to the average moles of payload per mole of Fab.

Figure 1. Result by Traditional RP LCMS Method



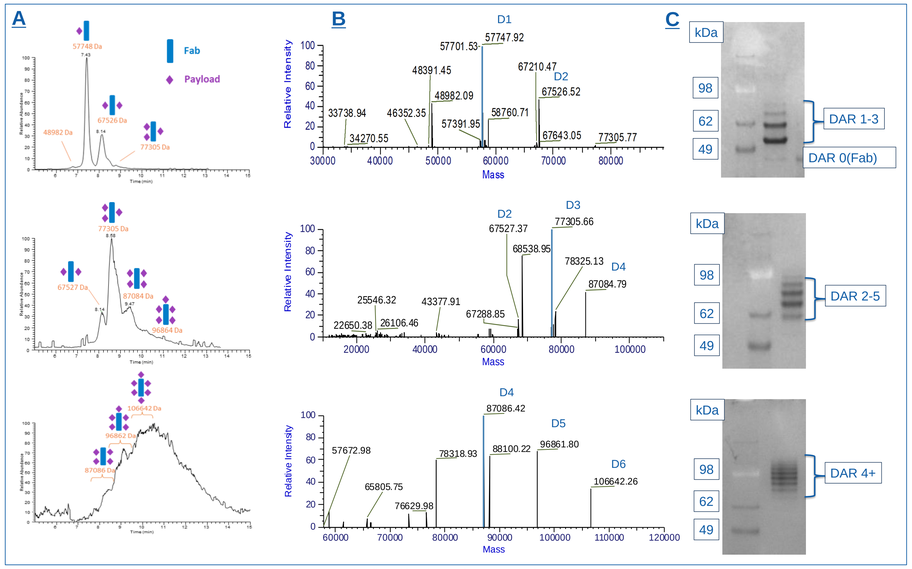
5 µg Conjugate A was injected for routine RP-LCMS analysis. In brief, LCMS analysis was performed by elution from a Thermo Scientific MabPAC RP column using a gradient of 32% to 60% acetonitrile with 0.1% formic acid over 5 minutes. A Thermo Scientific Q-Exactive mass spectrometer was used to collect spectra. **(A)** Total-ion chromatogram (TIC) shows robust ionization of conjugate at RT 3.95 under described source conditions. **(B)** Average spectra collected across the conjugate peak. Simultaneous ionization of all DAR species at once generates an extremely complex spectra that cannot be interpreted or deconvoluted to resolve the mass of each DAR species.

Figure 2. Analysis of Conjugate A by ZipChip



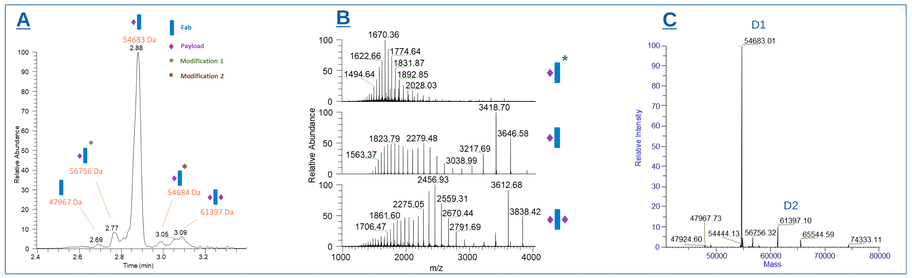
Conjugate A was analyzed via ZipChip and compared to DAR results obtained using orthogonal methods. **(A)** In a base peak electropherogram of conjugate A, clear resolution is observed between several DAR species 1-4. In addition, DAR 5 is detected by MS. **(B)** Raw mass spectra averaged from each peak. A clear distribution of charge states is observed, in contrast to spectra obtained by RP-LCMS. **(C)** Deconvoluted masses generated using the sliding window algorithm in BioharmaFinder v5.0. Peak masses match theoretical masses for DAR0 (Fab) and DARs 1-5. The method allows for better characterization of DAR species compared to SDS-PAGE. **(D)** SDS-PAGE image showing bands of each DAR species. While DAR distribution is clearly observed by SDS-PAGE, MS detection allows for the rapid and precise identification of both DAR species and any related modifications or impurities.

Figure 3. Analysis of Conjugate B-D by ZipChip



Conjugates B-D were fractionated to examine whether CE-MS DAR results align with orthogonal assay methods such as SDS-PAGE. It is challenging to determine whether the bands observed in the SDS-PAGE gel are higher valency DAR species (DAR 4+), aggregates, dimers, or other unrelated molecules. With ZipChip-based CE-MS, we can precisely determine the mass of each DAR species present, ruling out the possibility of HMW aggregates affecting the quality of each fraction prepared for research studies. **(A)** Base peak electropherograms for Conjugates B-D. Separation is observed between DAR species in the sample that are confirmed by MS detection. **(B)** Deconvoluted spectra for each conjugate. Most DAR species present in each sample are resolved, along with any related modifications or impurities. Even in Conjugate D, which contains a distribution of high DAR species, mass was confirmed up to DAR 6. **(C)** SDS-PAGE Analysis of conjugate fractions. ZipChip analysis confirms the mass of DAR species observed by SDS-PAGE. CE-MS also identifies additional conjugation impurities that are not resolved by SDS-PAGE.

Figure 4. ZipChip Analysis of Charged ASO Conjugate E



To determine if ZipChip methods can also be used to analyze conjugates with a negatively-charged payload, conjugate E containing an ASO payload was analyzed. **(A)** Base peak electropherogram for conjugate E as analyzed using ZipChip. Several species are resolved by CE. **(B)** Average spectra for each peak contain clearly resolved charge states for conjugate DAR species. **(C)** Deconvoluted spectra for the sample. Peak 1 was found to contain impurities related to the DAR 1 species and residual DAR 0. Peak 2, the major peak, was found to contain predominantly DAR 1. The last peaks examined contain both DAR 2 and a conjugation-related impurity. These results show that CE-MS can resolve and ionize conjugates with negatively-charged payloads for MS characterization.

CONCLUSIONS

- ZipChip microchip CE-MS resolves DAR species electrophoretically and enables generation of superior MS spectra.
- Analysis of purified conjugate fractions matches well with SDS-PAGE results and allows precise identification of DAR species across multiple payload chemistries.
- CE-MS allows for the rapid and robust characterization of FORCE conjugates.

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DISCLOSURE INFORMATION

B.F.V., P.S., P.T., and T.W. are employees and shareholders of Dyne Therapeutics. A.K. and K.Y. are employees of 908 Devices.

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