Advances in Microchip CE-MS: From Protein Characterization to Nucleic Acids Analysis

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Introduction

Microchip capillary electrophoresis (CE) coupled with mass spectrometry (MS) has revolutionized the characterization of therapeutic modalities in biopharmaceuticals. Here, we present the applications of an integrated workflow that couples the microfluidic CE system (ZipChip) with MS in characterization of biotherapeutics. The generic workflow is rapid, simple, with minimum method development and sample prep. We will showcase 3 distinct application examples using the ZipChip CE-MS workflow:

- **Multi-level characterization of monoclonal antibodies (mAbs)** including charge heterogeneity, glycosylation profiles, and critical quality attributes. The combination of microchip CE and MS provides efficient analysis, enhancing understanding of mAb structure.
- **Characterization of adeno-associated viruses (AAVs)** including intact denatured Viral Protein (VP) characterization as well as peptide mapping to further characterize PTMs of the VP. Examples of the serotype AAV6, AAV8, and AAV9 are shown in this poster
- **Analysis of nucleic acids** covering FLP determination of Oligos as well as Oligo mapping of biological RNAs. Apart from benefits stated above, the workflow requires NO ION PAIRING Reagents, eliminating system contamination.

ZipChip – A closer look

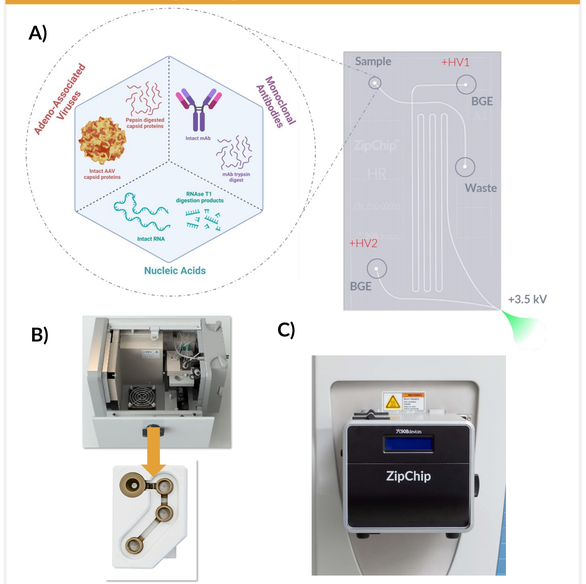


Figure 1. Schematic of the microfluidic chip showing sample and background electrolyte wells and separation channel (A), ZipChip interface showing chip manifold (B) ZipChip mounted on a mass spectrometer (C).

Multi-level Characterization of mAbs

Charge Variant Analysis of mAbs[1]

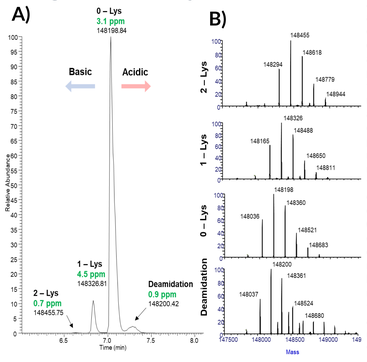


Figure 2 Electropherogram showing acidic, basic and main charge variants of the NSIT mAb (A) Deconvoluted mass spectra of the variants showing glycoforms (B).

Charge variant analysis (CVA) Assessing the charge variant profile is an essential step in characterization of biotherapeutics during development and production, as well as lot release testing. ZipChip provides ready-to-use consumables for a simplified CVA protocol, requiring only dilution and injection and provides in-depth characterization of the different mAb proteoforms. Figure 2A shows CVA results for NIST mAb along with the mass spectra generated by averaging the peaks observed in the electropherogram (2B). When coupled with High Resolution MS (HRMS), this generic approach enables rapid and sensitive detection of charge variants and glycoforms of mAbs for a variety of mAbs and bispecific antibodies without further method development.[2] Furthermore, the peak profiles align with iCIEF, allowing direct MS identification of unknown species.[2] The ZipChip/MS workflow tolerates matrices like detergents and salts, eliminating the need for species removal through cleanup procedures.

Peptide mapping of mAbs using microfluidic CE-MS reveals important information such as their primary sequence and post translational modifications (PTMs) many of which are considered CQAs. Traditional LC-MS based-peptide mapping methods require extensive sample preparation and long analysis times. ZipChip provides a rapid method for peptide mapping of mAbs and related molecules. The Peptide mapping sample preparation protocol is shown in Figure 3. Ammonium acetate is used to replace Tris buffer for the trypsin digestion, making it directly compatible with ZipChip/MS analysis with no further sample prep steps. When compared with LCMS protocol using TRIS buffer, this workflow demonstrated similar performance for CQAs identified with only 8nL of 1mg/mL of material needed per injection. Figure 4A shows the electropherogram for a NIST mAb digest and as shown in 4B the data returned ~98% sequence coverage. The method was 20 minutes long and identified several PTMs (Table 1) The workflow was applied to different biopharmaceutical formats (IgG4, IgG1, Fc fusion proteins) with great results.[4]

Peptide Mapping Analysis of mAbs[3]

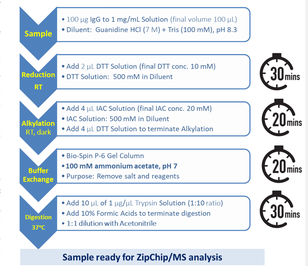


Figure 3. Sample preparation protocol for digestion of mAbs for subsequent ZipChip-MS analysis

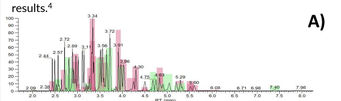


Table 1

CHAIN	RESIDUE MODIFICATION	RELATIVE ABUNDANCE (%)	RSD (%)
HC	M255+Oxidation	1.99	7.1
HC	N300+A1G0	0.52	14.3
HC	N300+A1G0F	3.09	4.6
HC	N300+A2G0F	44.45	0.2
HC	N300+A2G1F	41.39	0.5
HC	N300+A2G2F	9.68	2.0
HC	N300+A1G1F	2.70	1.9
HC	N300+Unglycosylated	1.69	7.1
HC	M361+Oxidation	0.79	5.8
HC	N387+Deamidation	0.94	4.3
HC	N387+Succinimide1	0.71	1.0
HC	M431+Oxidation	1.27	7.4
LC	M4+Oxidation	2.05	18.7

Figure 4. Electropherogram for peptide mapping of NIST mAb (A); Sequence coverage map obtained after data processing of the CE-MS analysis of NIST mAb tryptic digest (B). Table 1 shows PTMs identified in the same peptide mapping analysis.

Characterization of AAV Viral Capsid Proteins

Intact Denatured Analysis of AAV Capsid Proteins[5]

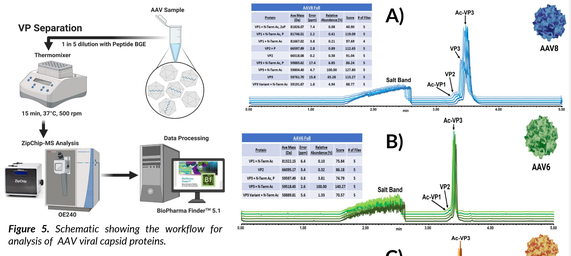


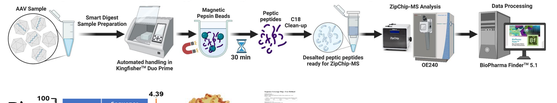
Figure 5. Schematic showing the workflow for analysis of AAV viral capsid proteins.

Intact Denatured Analysis is performed using a ZipChip™ (908 Devices) coupled to an Orbitrap Exploris™ 240 MS (Thermo Scientific). The analysis time was 5 minute using a standard High Resolution (HR) chip (908 Devices) along with a Peptide Background Electrolyte (BGE) containing 4% DMSO. The workflow is shown in Figure 5.

Figure 6. BPEs of the 5 replicates analysis (overlaid) performed for AAV8 (A), AAV6 (B) and AAV9 (C).

Data in Figure 6 show separation of the three VPs (viz. Vp1, Vp2 and VP3) during the analysis with capability to detect multiple proteoforms for each VP, including phosphorylation and acetylation. LOD testing showed that all Identified proteoforms were detected at concentrations 8.80E6 viral particles, with at least one proteoform of each VP detected at concentrations as low as 2.64E6 viral particles. A list of modified proteoforms detected in the ZIpChip analysis for each of the three AAV serotypes as shown in the inserts.

A) Peptide Mapping Analysis of AAV Capsid Proteins[5]



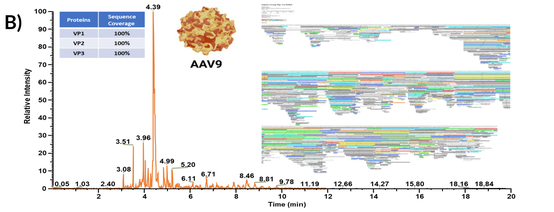


Figure 7. Schematic showing the sample preparation strategy along with analysis followed by data processing for digested AAV capsid proteins (A) Microchip CE-MS separation for peptide mapping and identification of PTMs. CE-MS base peak electropherograms of AAV9 serotype illustrating high sequence coverage (B).

Table 2

Protein	Modification	Peptide Sequence	Average % Abundance	STDEV N=2
AAV9_VP1	Q119=HNO loss_Glutaminide	GAAXXRLLEPLGL	57.21	3.52
AAV9_VP1	A257+Acetylation	ASGGGAPVADNNEGADGVGSSSGNWHCDSGWLGDRVVTTSTRTWVLPTYNNHL ASGGGAPVADNNEGADGVGSSSGNWHCDSGWLGDRVITTSTRT	99.20	0.08
AAV9_VP1	M372+Oxidation	FMPQYGYLTLNDGSQAVGRSSF	1.31	0.12
AAV9_VP1	D383+Succinimide1	LTLADGSQAVGRSSF MPQYGYLTLNDGSQAVGRSSF FMPQYGYLTLNDGSQAVGRSSF	1.03	0.04
AAV9_VP1	M451+Oxidation	EYFPSQMLRTGNNFQF	1.09	0.04
AAV9_VP1	M431+Oxidation	DRLMNPLIDQYL FEWYFNLSSTAMSGDLDRLMNPLIDQ	1.48	0.13
AAV9_VP1	N451+Deamidation	YFLSKTMGSGSNGQDTLNFSNAGPSNM	1.23	0.25
AAV9_VP1	+1431=Phosphorylation	SYAGPSNMAVQGRNYIPGPSYRQQRVSTTVTQNNNSE AYGGRKYKPGPSTRQQRVSTTVTQNNNSE	2.57	0.08
AAV9_VP1	M531+Oxidation	ALGKRNSLNMNPGPAMASHKEGEDRFFPLSGSL FAKPGASKWALAGRNSLNMNPGPAMASHKEGEDRFFPLSGSL	6.55	0.77
AAV9_VP1	-Q657=HNO loss_Glutaminide	QDRDVTLGGPMV	19.68	11.22
AAV9_VP1	Q614=HNO loss_Glutaminide	QGPGWAKPKTDGNPHPSLMQGFGMKHPPPQIL QGPRWAKPKTDGNPHPSLMQGF	34.76	4.88
AAV9_VP1	D625=Succinimide D	AKPKTDGNPHPSLMQGFGMKHPPPQIL	1.88	0.25

Table 2. Identification of PTMs from peptide mapping analysis of AAV9 capsid proteins

Peptide Mapping Analysis for AAVs is performed using the ZipChip/Exploris™ 240 workflow demonstrated on Figure 7A. 10 µg of AAV (determined using NanoOrange protein assay) was **digested** with SMART Digest pepsin magnetic beds on a Kingfisher™ DuoPrime. Desalting is performed using a C18 spin column and then reduced to dryness in a speed-vac. The **Analysis** was performed by reconstitute digested samples in 10uL of the Peptide BGE and loaded directly into an HR chip primed with Peptide BGE. Each run was 20 minutes, and 2 injections were performed for each sample. Results of the peptide mapping analysis for AAV is shown in Figure 7B.

Analysis of Oligonucleotides and RNAs

Intact Oligo Analysis[6]

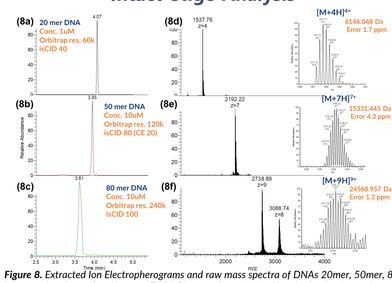


Figure 8. Extracted Ion Electropherograms and raw mass spectra of DNAs 20mer, 50mer, 80mer (8a-8c), mass spectra generated by averaging the peaks of interest ((8d-8f) inserts show M+H adduct in the dominant charge state for each of the Oligo.

The **Intact Oligos Analysis:** The analysis of intact oligos can be easily performed using the ZipChip CE/MS platform. This method is rapid and does not require ion-pairing reagents. For this protocol, the ready-to-use consumables include a High-Resolution Bare Glass (HRB) chip and the ZipChip Oligos BGE. A generic protocol can be used for the analysis of oligos' full-length products (FLP) ranging from 10 to 80 mers. Figure 8 presents example electropherograms and corresponding mass spectra for 20-mer, 50-mer, and 80-mer DNA samples.

Analysis of Partially Digested RNAs[7]

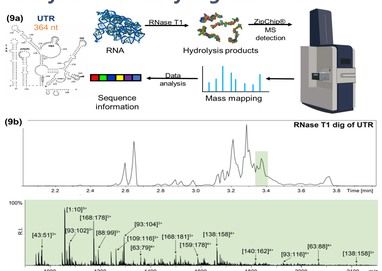


Figure 9. Protocol for digestion and analysis workflow (a); Bottom-up analysis of UTR on ZipChip-timsTOF showing the electropherogram and mass spectrum of peak of interest (b)

Oligo Mapping of a larger biological RNA by a partial digestion using RNAse T1 enzyme was also performed using similar CE-MS protocol. The workflow for this protocol is shown in Figure 9a. The method has an analysis time of 4 minutes. The sample used was HIV-1 5'-UTR (untranslated region) RNA (UTR), with 364 nucleotides. After a digestion time of 15 min an number of digestion products that were generated were analyzed using ZipChip. Figure 9b shows the electropherogram and an example mass spectrum for one of the peaks of interest. 100% sequence coverage of UTR was achieved.

Conclusion

- ZipChip CE/MS is a versatile platform for analysis of a variety of therapeutic and pharmaceutically important molecules such as mAbs, AAVs and nucleic acids and both intact and partially digested levels.
- This microfluidic technology offers a rapid method of analysis for all formats; ready-to-use consumable kits eliminate the need for method development.
- Due to its mass spec friendly BGE solutions and lack of ion-pairing reagents, switching between methods is extremely simple, thereby reducing critical bottlenecks in analytical workflows in a biopharmaceutical mass spectrometry laboratory.

References

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4. Carillo, S.; Kulkarni, A. (2023, April). Direct Microchip CE/MS Peptide Mapping Workflow for the Analysis of mAb CQAs [Webinar] https://908devices.com/resource/zipchip-webinar-ls-zc-wb-0017/
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6. **Application Note 9.5** "Rapid characterization of oligonucleotides using microfluidic capillary electrophoresis - mass spectrometry by ZipChip. 908 Devices
7. **2023** *Continuing the investigation of microchip capillary electrophoresis coupled with mass spectrometry in the bottom-up characterization of progressively larger RNAs* (Poster); ASMS, Houston, TX, USA.