# Multi-level expeditious characterization of adeno-associated virus (AAV) capsid proteins using PATsmart™ ZipChip® microfluidic CE coupled with HRAM MS

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## Highlights

- Analyze AAV9 capsid proteins by Microchip CE/MS peptide mapping
- Achieve sequence coverage up to 100% in 10 minutes
- Identify and characterize post translational modifications (PTM) by HRAM MS/MS
- No method development for CE separation required

## Introduction

Recombinant adeno-associated viruses (rAAVs) emerged as a promising transfer vectors for gene therapy. The icosahedral capsid of AAV vectors is comprised of 60 copies of three capsid proteins viz. VP1, VP2 and VP3 (1:1:10 ratio). Complete characterization of the capsid viral proteins (Intact and peptide mapping) is essential for the safety, quality, and efficacy of AAV products. Compared to proteins such as mAb, AAV analysis is more demanding for sensitivities and resolution as they are more complex in structure, and often limited in sample volumes.

We present a novel approach using microchip CE/MS for the characterization of AAVs. The analysis of intact capsid proteins with the microchip CE/MS has been published elsewhere.[1] Here, we focus on the peptide mapping of the capsid proteins with the microchip CE/MS using AAV9 as an example. Peptide mapping is commonly used in the biopharma industry for protein characterization to confirm the amino acid sequence, product purity and post-translational modifications (PTMs).[2,3] The Microchip CE/MS method enables fast analysis time while obtains sequence confirmation and characterization of post-translational modifications (PTMs). The entire CE/MS workflow (Figure 1) enables separation and identification of low level PTMs with high sequence coverage for AAV capsid proteins in 10 minutes, a 10-15 fold decrease in analysis time with results comparable to the conventional LC/MS approach.[2,3]

## Materials & Methods

### Samples Preparation

An internal AAV9 sample expressed via transient transfection in HEK293 cells using the Gibco AAV-MAX Helper-Free AAV Production was used. The estimated protein concentration was 0.2 µg/µL, which was based on the AAV titer information from the internal collaborator (Thermo Fisher Scientific). The AAV9 sample was first buffer exchanged into water containing 5 mM tris(2-carboxyethyl) phosphine (TCEP) and 5% formic acid using the BioSpin-6 column (Bio-Rad), then enzymatically digested in-solution by pepsin (1 mg/mL in the buffer adjusted to pH 2.0) at 75°C over 2.5 h. The peptide mixture was desalted and further diluted (5x) with the sample diluent [part of the PATsmart™ ZipChip® Peptides kit (Repligen)] before loading to the microchip sample well.

### Instrumentation

The analysis was performed using a PATsmart™ ZipChip® Device (Repligen) coupled to an Orbitrap Exploris 480 mass spectrometer (Thermo Fisher Scientific). A standard High Resolution (HR) chip (Repligen) was used. The chip was primed with the background electrolyte (BGE) from the ZipChip Peptides kit (Repligen). The injection volume was 6 nL, and CE field strength was 500 V/cm. The data dependent MS/MS setup was used for MS/MS.

### Data Processing

Data was visualized using the Qual Browser data analysis software (Thermo Fisher Scientific). Raw data file was parsed and processed with the BioPharma Finder 4.1 software (Thermo Fisher Scientific) and searched against protein sequences of VP1, VP2 and VP3 of AAV9.

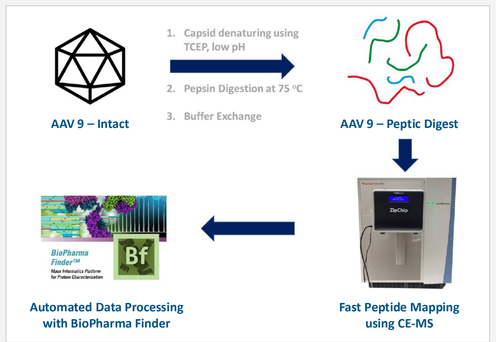


**Figure 1.** Microchip CE/MS workflow for peptide mapping of AAV capsid proteins

## Fast Separation of Peptic Digests

The overall goal of this work was to develop a fast & efficient workflow for peptide mapping of AAV capsid proteins (**Figure 1**). Pepsin was chosen for the AAV capsid protein digestion because it can cleave primarily after bulky hydrophobic amino acid residues.[2,3] In addition, Pepsin is active at low pH which is important to denature the AAV capsid. **Figure 2** shows the base peak electropherogram of the CE/MS AAV9 peptide map, obtained in less than 10 minutes. This microchip CE/MS method is much faster compared to conventional LC methods that may range from 30 – 120 minute depends on whether it is analytical LC or nano LC. For the analysis of larger cohorts of such samples (eg: forced degradation studies), the Microchip CE/MS enables time saving, allows faster turn around time, increases operation efficiencies.

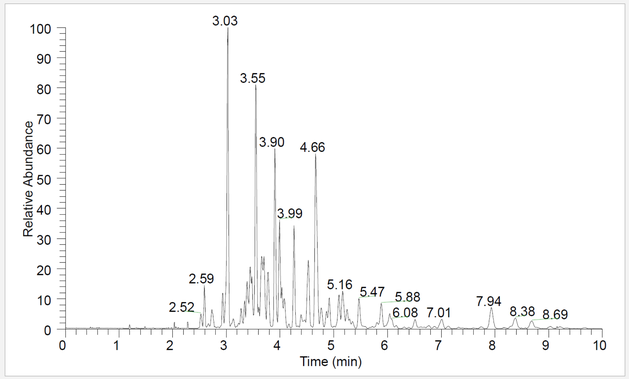


**Figure 2.** CE/MS Base Peak Electropherogram of the peptic digested AAV9 capsid proteins

## High Sequence Coverage by MS and MS/MS

High quality MS and MS/MS spectra were obtained from the CE/MS analysis, resulting the capsid protein sequence coverages of 97.3% (VP1), 100% (VP2) and 100% (VP3) respectively. AAV viral proteins share the same C-terminus sequence, differ only in the N-terminus sequences. Here, the N-terminal sequences for VP2 and VP3 were confirmed from the MS/MS data (Figure 3), and the N-terminal sequence for VP1 was confirmed by high resolution accurate mass MS data.

| Protein | Sequence Coverage |
|---|---|
| VP1 | 97.3% |
| VP2 | 100% |
| VP3 | 100% |

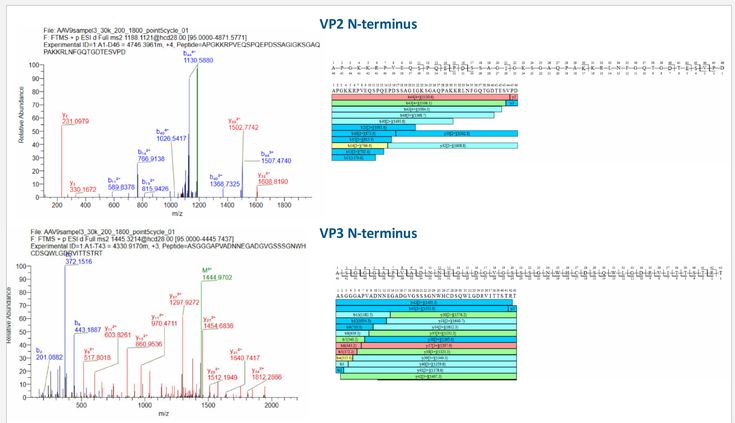


**Figure 3.** N-terminal sequences for VP2 (top) and VP3 (bottom) confidently identified using MS/MS for the peptic digested AAV9 capsid proteins

## Sensitive Identification of PTMs

In addition to fast peptide mapping, the Microchip CE/MS method was able to resolve important PTMs such as phosphorylation, deamidation and oxidation. Figures 4a-4c shows the extracted ion electropherograms of the unmodified (Peak 1) and the corresponding modified peptides (Peak 2). The MS/MS spectra of the modified peptides are shown in Figures 4d-4f. Even low abundance PTMs were identified with high confidence score in the BioPharma Finder software. A total of 30 unique PTMs were identified for VP1 with a confidence score of 90% or higher.

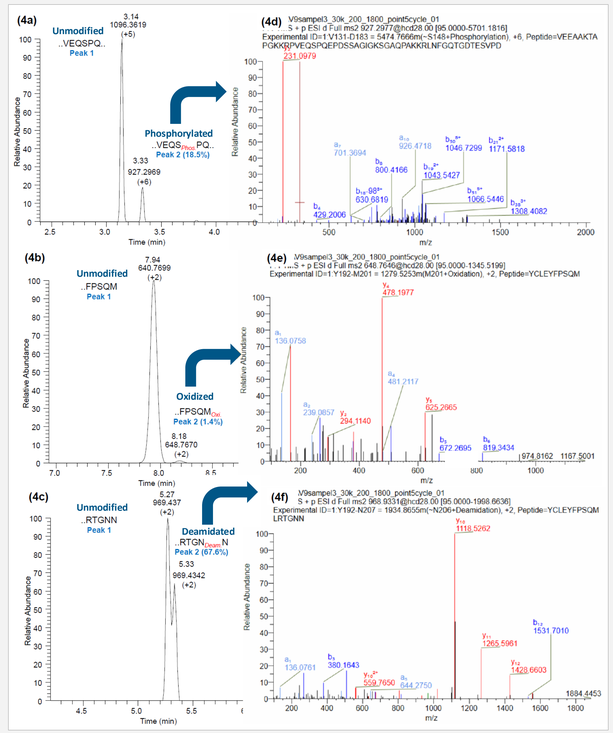


**Figure 4.** Microchip CE/MS separation and identification of selected PTMs using MS/MS data

## Conclusions & Future Work

- Rapid Microchip CE/MS peptide mapping of AAV9 capsid proteins (<10 minutes)
- ZipChip is plug and play with ready to use HR chip & Peptides BGE, no method development
- High sequence coverage for all three viral capsid proteins, as good as LC/MS methods
- Sensitive characterization of PTMs for the capsid proteins with clean MS and MS/MS data
- **Future work:** Repeat the same protocol to other AAV serotypes; Apply the microchip CE/MS protocol for the analysis of intact capsid proteins

## References

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