COMPREHENSIVE AAV CHARACTERIZATIONS USING HRMS: CAPSID PROTEINS, EMPTY/FULL CAPSID RATIO AND SSDNA ANALYSIS

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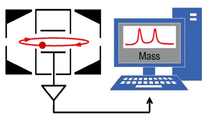
Highlights

- Methods for UPLC-TOF-MS analysis of both trypsin digested AAV5 and intact capsid proteins of several AAV serotypes.
- Demonstrate the utility of charge-detection MS (CDMS) to analyze ssDNA isolated from AAV9 by anion-exchange chromatography (AEC).
- Empty/Full AAV measurements using orthogonal methods (AEC, SEC and CDMS).

Instrumentations and Samples

BioAccord LC-MS System
(Intact Protein and Peptide Mapping)

ACQUITY H-Class Bio
(SEC and AEC for Empty/Full Capsid analysis, ssDNA Fractionation.)



Charge-Detection Mass Spectrometry [1]
MegaDalton Solutions. (Empty/Full Capsid Analysis)

Figure 1. Instrumentations used for the extensive characterization of rAAVs: 1) BioAccord LC-MS system; 2) CDMS (charge detection MS); 3) AEC (Anion Exchange Chromatography), and SEC (Size Exclusion Chromatography) with TUV detector.

Samples:

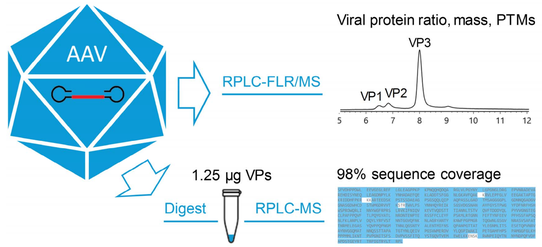
rAAV serotypes (1, 2, 5, 6, 8, 9) were from Vigene Biosciences.
The AAV 8 used in the CDMS analysis was from BioReliance.

Column Chemistries:

- **Intact Protein RPLC-MS:** ***ACUITY BEH C4***
- **Peptide Mapping RPLC-MS:** ***ACQUITY BEH C18***
- **AEC fractionation:** ***Protein-Pak Hi Res Q***
- **SEC:** ***BEH SEC Guard Column, 125Å, 1.7 µm, 4.6 mm X 30 mm***

Informatics software: UNIFI 1.9.4 and Empower 3.0

Capsid Proteins



Serotype	VP1			VP2			VP3		
	Observed mass (Da)	AA sequence	Theoretical mass (Da)	Observed mass (Da)	AA sequence	Theoretical mass (Da)	Observed mass (Da)	AA sequence	Theoretical mass (Da)
AAV1	81,289	2(Ac)-736	81,286	66,096	139-736	66,093	59,517	204(Ac)-736	59,517
AAV2	81,854	2(Ac)-735	81,856	66,486	139-735	66,488	59,974	204(Ac)-735	59,974
AAV5	80,336	2(Ac)-724	80,336	65,283	139-724	65,283	59,463	199(Ac)-724	59,463
AAV6	81,324	2(Ac)-736	81,322	66,094	139-736	66,096	59,518	204(Ac)-736	59,519
AAV8	81,668	2(Ac)-738	81,667	66,519	139-738	66,519	59,805	205(Ac)-738	59,805
AAV9	81,292	2(Ac)-736	81,291	66,210	139-736	66,210	59,732	204(Ac)-736	59,733

Table 1. Observed (average) mass, theoretical amino acid sequence, and theoretical masses of the capsid proteins from six AAV serotypes analyzed using the RPLC-MS methods.[2]

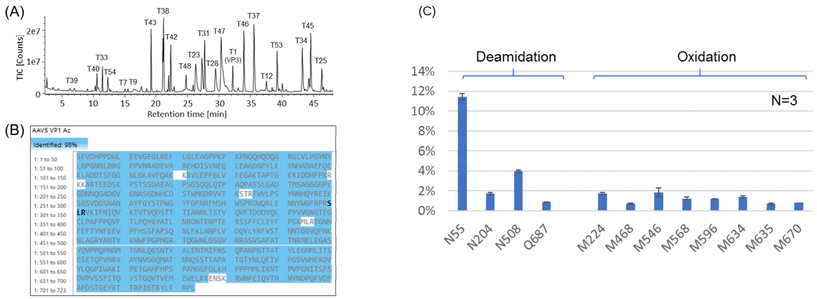


Figure 2. Peptide analysis of AAV5 VPs using approximately 1.25 µg proteins as the starting material in enzymatic digestion. (A) TIC of AAV5 tryptic digest with major peaks annotated with corresponding peptide names. (B) Peptide map showed 98% sequence coverage of AAV5 VP1 with identified peptides highlighted in blue. (C) Identified PTMs with over 0.5% relative abundance including deamidation on N55, N204, N508, Q687; and oxidation on M224, M468, M546, M508, M596, M635, M670. The error bars represent the standard deviation (less than 0.5%) of %PTMs from three separate sample preparations.[2]

Empty/Full Capsid

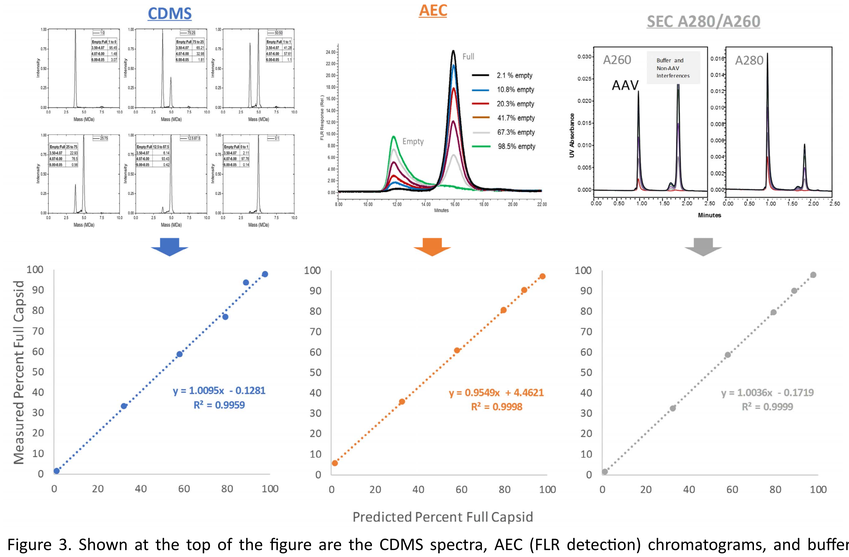
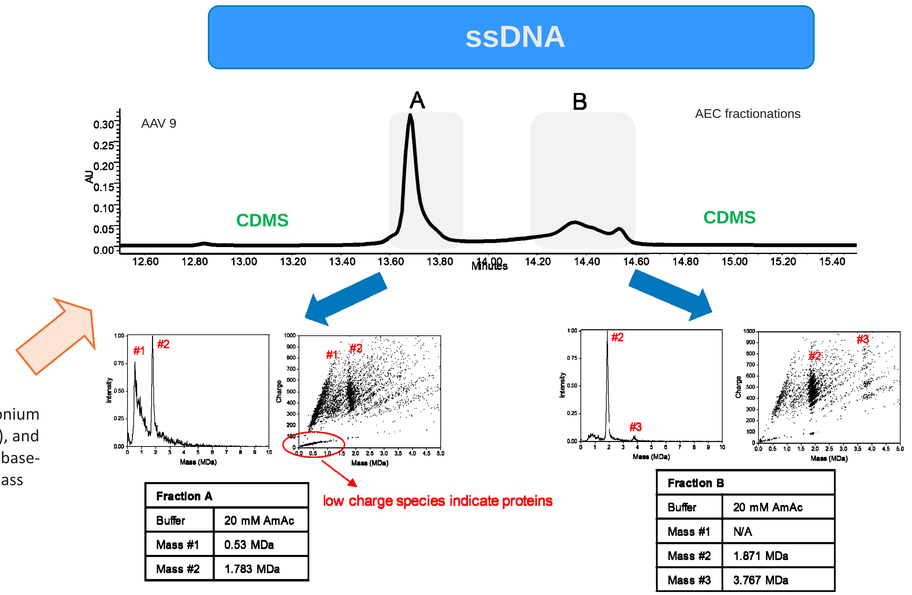


Figure 3. Shown at the top of the figure are the CDMS spectra, AEC (FLR detection) chromatograms, and buffer exchange SEC chromatograms (UV Absorbance at 280 nm and 260 nm) for a series of rAAV8 samples. Samples were prepared from serial dilutions of two samples with estimated concentrations of 2.4 $X10^{12}$ and 1.67 $X10^{12}$ capsids/mL and percentages of empty capsid of 2.1 % and 98.5 %, respectively as determined by CDMS. The linear correlation plots show good agreement with regard to both slope and correlation between the measured and the predicted percent empty capsid values for all three distinctly different methodologies.



Fraction A	
Buffer	20 mM AmAc
Mass #1	0.53 MDa
Mass #2	1.783 MDa

Fraction B	
Buffer	20 mM AmAc
Mass #1	N/A
Mass #2	1.871 MDa
Mass #3	3.767 MDa

Figure 4. Single-Stranded DNA (ssDNA) was released from AAV9 capsid by Proteinase K digestion. The digested sample was injected onto the Protein-Pak Hi Res Q column and the fractions were collected. The buffer was exchanged into 20 mM ammonium acetate before CDMS analysis. The CDMS results indicate that Fraction A contains fragment or degraded DNA (#1), ssDNA (#2), and proteins (oval), while Fraction B contains mainly ssDNA (#2). The expected mass of ssDNA is ~ 0.9 MDa. Since ssDNA tends to base-pair with each other in solution to form dsDNA, the mass is doubled. Therefore, Mass #2 indicates the existence of ssDNA. Mass #3 is the dimer of the dsDNA.

Reference:
1. J. Mol Biol. 2016 January 29; 428(2 Pt A): 292–300. doi:10.1016/j.jmb.2015.06.019.
2. Optimized reversed phase LC/MS methods for intact protein analysis and peptide mapping of adeno-associated virus (AAV) proteins. https://doi.org/10.1089/hum.2021.046