Comprehensive AAV Characterizations Using HRMS: Peptide Mapping, Capsid Proteins, Intact ssDNA, and Intact Virus

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aaps PharmSci 360

PURPOSE

Analytical regulatory requirements for AAV-based gene therapies are currently not well established.

Monitoring protein and ssDNA heterogeneity for AAV may help define regulated analytics and accelerate AAV clinical and process development.

Developing analytics for AAV include low concentrations, limited quantities, and large analyte masses that challenge commercially available analytical techniques.

Analytical approaches were developed using UPLC-TOF-MS based approaches for characterization of both trypsin digested AAV5 and intact capsid proteins of several AAV serotypes.

Demonstrated the utility of charge-detection MS (CDMS) to analyze ssDNA isolated from AAV9 by anion-exchange chromatography (AEC).

Measured level of AAV8 without its targeted complement ssDNA are compared using orthogonal methods (AEC, SEC and CDMS).

OBJECTIVE(S)

Develop analytical methods meeting regulatory requirement for a comprehensive characterization AAV, including Peptide Mapping, Capsid Proteins, Intact ssDNA, and Intact Virus

METHOD(S)

Samples

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

Method

Tryptic digestion of 1.25 µg of AAV5 capsid protein occurred after surfactant removal using denaturing SEC. UHPLC-TOF-MS with a C18 RP column was used for the peptide analysis. LC-MS with intrinsic fluorescence detection (LC-FLR-MS) was used for the relative quantification and characterization of intact capsid proteins for several serotypes using a C4 RP column and DFA mobile phase modifier. Isolated ssDNA and Intact AAV8 samples were buffer exchanged in 20 mM ammonium acetate prior to nanospray CDMS analysis. ssDNA was isolated from AAV by digestion with proteinase K and purified using AEC. Intact AAV samples were analyzed directly by AEC with intrinsic fluorescence detection and by SEC using UV absorbance at 260 nm and 280 nm.

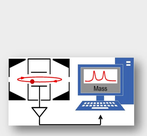
Column Chemistries

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*

Instrumentation:

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

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

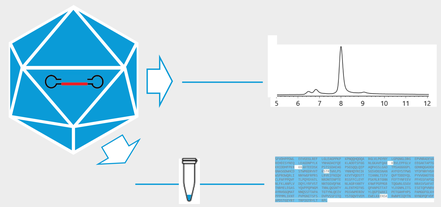


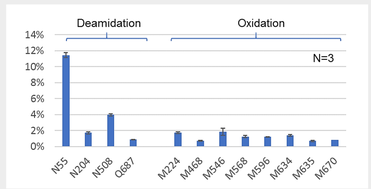
Charge-Detection Mass Spectrometry[1] MegaDalton Solutions. (Empty/Full Capsid Analysis)

Informatics software: UNIFI 1.9.4 and Empower 3.0

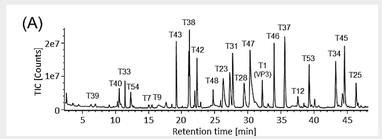
RESULT(S)

Capsid Proteins





Identified PTMs with over 0.5% relative abundance (deamidation and oxidation)



TIC of AAV5 tryptic digest with major peaks annotated with corresponding peptide names

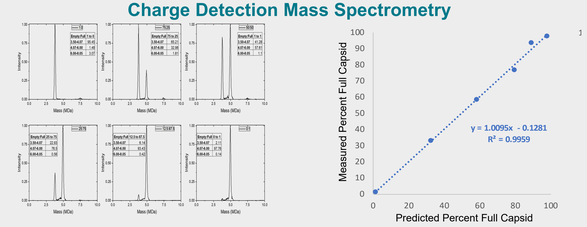
Peptide map showed 98% sequence coverage of AAV5 VP1 with identified peptides highlighted in blue

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]

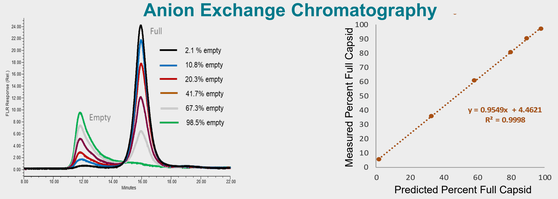
Serotype	VP1			VP2			VP3		
	Observed mass (Da)	AA sequence	Theoretical mass (Da)	Observed mass (Da)	AA sequence	Theoretical mass (Da)	Observed mass (Da)	AA sequence	Theoretical mass (Da)
AAV1	81,289	2(Ac)-736	81,286	66,096	139-736	66,093	59,517	204(Ac)-736	59,517
AAV2	81,854	2(Ac)-735	81,856	66,486	139-735	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

Empty/Full Capsid

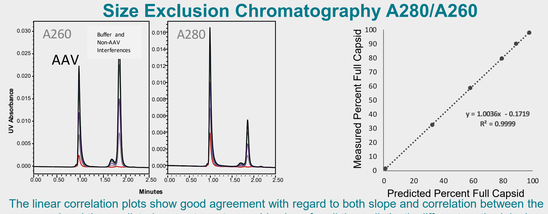
Charge Detection Mass Spectrometry



Anion Exchange Chromatography

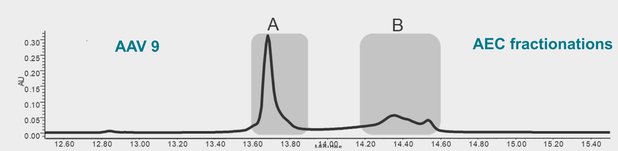


Size Exclusion Chromatography A280/A260

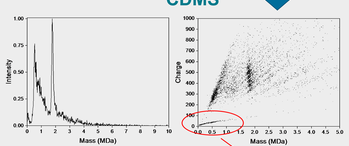


The linear correlation plots show good agreement with regard to both slope and correlation between the measured and the predicted percent empty capsid values for all three distinctly different methodologies.

ssDNA



CDMS

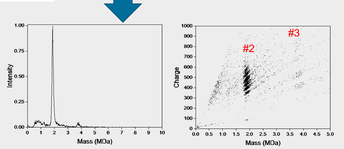


Fraction A	
Buffer	20 mM AmAc
Mass #1	0.53 MDa
Mass #2	1.783 MDa

Low charge species indicate proteins

CDMS

Fraction B contains mainly ssDNA (#2)



The CDMS results indicate that Fraction A contains fragment or degraded DNA (#1), ssDNA (#2), and proteins (oval),

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.

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

CONCLUSION(S)

UHPLC-Tof-MS and Charge Detection Mass Spectrometry (CDMS) along with Anion Exchange Chromatography (AEC) and Size Exclusion Chromatography (SEC) provided in-depth AAV protein characterization and intact AAV integrity information with a few micrograms of sample.

- Methods analysis of both trypsin digested AAV5 and intact capsid proteins of several AAV serotypes were developed on UHPLC-Tof-MS System (BioAccord)
- Demonstrated the utility of charge-detection MS (CDMS) to analyze ssDNA isolated from AAV9 following an anion-exchange chromatography (AEC) based separation of differentially loaded vial particles.
- Empty/Full AAV measurements using orthogonal methods (AEC, SEC and CDMS).
- CDMS also demonstrated a linear response for the level of empty AAV8 in addition to a high correlation with determinations by FLR-AEC and dual wavelength (260 nm and 280 nm) UV-SEC methods.
- For the analysis of ssDNA isolated from AAV9, CDMS data demonstrated that upon release from the capsid the ssDNA partially degraded or paired with its respective complement ssDNA.

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

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