Application of Mass Spectrometry for AAV-based Gene Therapy Analysis

Yi Pu
CASSS Mass Spec
Sep 17, 2020
**Gene therapy** is the therapeutic delivery of nucleic acid into a patient as a drug to treat disease.

Figure adapted from: Li, et al. 2019, Cell & Gene Therapy Insights 2019; 5(4), 537–547
Wild Type AAV Structural Characteristics and Quality Attributes

Adeno-Associated Virus

- 3.9 MegaDaltons (empty capsids)
- Small icosahedral particles (20-25 nm in diameter)
- Natively package ssDNA to ~ 4.7 kb
- Replication-defective, nonenveloped virus
- Non-pathogenic, mildly immunogenic; Low level integration, maintained episomally
- Many distinct serotypes

Examples of AAV attributes

- Capsid purity
- Capsid identity
- Vector particle titer
- Empty/full capsid

Diagram:
- Structure of Adeno-Associated Virus (AAV)
- Capsid proteins: VP1, VP2, VP3
- VP numbers: VP1 = 5, VP2 = 5, VP3 = 50
- Capsid composition: 1:1:10
- Vector particle titer: 60 subunits
- Inverted terminal repeats (ITR)
Comparing AAV Size with Other Drug Modalities

- **Small Molecule**: < 1 KDa
- **ASO**: < 10 KDa
- **mAb**: ~ 150 KDa
- **AAV**: ~ 3.9 MDa (Capsid) ~ 1.4 MDa (DNA)

**Chemical synthesis vs. Biosynthesis**

ASO: antisense oligonucleotide
mAb: monoclonal antibody
Key Structural Characteristics of AAV Products

Is it the right AAV capsid?
Capsid ID (serotype) and viral protein ratio

Are capsids filled with the transgene?
Empty/partial/full capsid

Does it contain the right transgene?
Gene sequencing and purity

• What modifications could affect the potency/stability?
• Are there size/charge variants?
• How much residual impurities are there after purification?

Deep characterization and HCP/host cell DNA
## Mass Spectrometry (MS) Applications for Gene Therapy Development

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<td><strong>ssDNA characterization</strong></td>
<td>▪ Sequence and size distribution (orthogonal to NGS)</td>
<td>• Negative mode MS</td>
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<td><strong>Structure-function characterization</strong></td>
<td>▪ Critical quality attributes (CQA)</td>
<td>• Custom LC-MS workflow</td>
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Challenges in Gene Therapy Mass Spec Analysis

- **AAV is much larger in size and with complex heterogeneity**
  - Analyzing intact AAV in native state can provide rich information but requires advanced instruments with higher mass range and/or charge detection capability.
  - Heterogeneity could be introduced by capsid purity, genome integrity, and/or packaging behavior, etc.
- **Historical knowledge and literatures are limited**
- **Sample availability is limited, and sample concentration is low**
Case Study 1: AAV Identification by Intact Viral Protein Analysis
## Intact Protein Mass Analysis for AAV Identity

<table>
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<th>AAV Serotype</th>
<th>Viral Proteins (VPs)</th>
<th>Theoretical Mass (Da)</th>
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<tr>
<td>AAV1</td>
<td>Acetyl VP1 (2-736)</td>
<td>81286</td>
</tr>
<tr>
<td></td>
<td>VP2 (139-736)</td>
<td>66093</td>
</tr>
<tr>
<td></td>
<td>Acetyl VP3 (204-736)</td>
<td>59517</td>
</tr>
<tr>
<td>AAV2</td>
<td>Acetyl VP1 (2-735)</td>
<td>81856</td>
</tr>
<tr>
<td></td>
<td>VP2 (139-735)</td>
<td>66488</td>
</tr>
<tr>
<td></td>
<td>Acetyl VP3 (204-735)</td>
<td>59974</td>
</tr>
<tr>
<td>AAV9</td>
<td>Acetyl VP1 (2-736)</td>
<td>81291</td>
</tr>
<tr>
<td></td>
<td>VP2 (139-736)</td>
<td>66210</td>
</tr>
<tr>
<td></td>
<td>Acetyl VP3 (204-736)</td>
<td>59733</td>
</tr>
<tr>
<td>AAVRh10</td>
<td>Acetyl VP1 (2-738)</td>
<td>81455</td>
</tr>
<tr>
<td></td>
<td>VP2 (139-738)</td>
<td>66253</td>
</tr>
<tr>
<td></td>
<td>Acetyl VP3 (204-738)</td>
<td>59634</td>
</tr>
</tbody>
</table>

- The combination of mass measurement of intact VP1, VP2, and VP3 proteins is highly specific as an identity test.
- Potentially transferable to QC.
- The mass differences exist for wild type AAV serotypes from AAV1 to AAV12
ZipChip CE-MS Intact Mass Analysis

- **AAVs**
- **Denaturation**
  - 40 µL is loaded to the sample vial
  - 5 nL for each analysis
- **Autosampler**
- **Microfluidic CE-ESI**
  - On-chip CE separation
- **Orbitrap MS**
  - Mass spectrometry analysis

Data analysis: Protein Deconvolution
ZipChip CE-MS Intact Protein Analysis of AAV2tYF

- The three capsid proteins of AAV2tYF were separated by CE and subsequently identified by MS.
- The method only took 10 min with 5 nL of sample injected.

Figure adapted from: Zhang, Y. et al Analytical Biochemistry 555 (2018) 22–25
LC-MS Intact Protein Method

Data analysis: Protein Deconvolution

AAVs

Denaturation

Viral proteins analyzed by LC-MS on orbitrap MS

LC systems to separate viral proteins
RP-C8-MS Intact Mass Analysis of AAV Serotype “A”

Deconvoluted MS

Acetyl VP3

Exp. mass: 59732 Da
Theo. mass: 59733 Da
VP3 (204-736)Ac

Acetyl VP1

Exp. mass: 81376 Da
Theo. mass: 81377 Da
VP1 (2-736)Ac

VP2

Exp. mass: 66238 Da
Theo. mass: 66238 Da
VP2 (139-736)
2D Deconvolution of Intact Protein Analysis (AAV Serotype “A”)

- VP1 is partially overlapped with the pre-peak (peak 1) of VP3.
- VP1 is partially phosphorylated and the phosphorylated species co-elutes with unmodified one.
- VP3 contains two peaks with nearly identical mass, possibly due to presence of deamidated species.
Case Study 2:
Characterization of AAV Empty/Full Capsids by CDMS
Loss of Charge State Resolution of Large Molecules

Conventional $m/z$ spectrum

- Lack of charge state resolution of large molecules caused by heterogeneity
- $m$ could not be determined

Charge detection MS (CDMS)

- Measure $m/z$ and $z$ for each ion
- $m/z \times z \rightarrow m$ for each ion

Figures adapted from: Benjamin E. Draper at Megadalton Solutions
CDMS of AAV

Theoretical Mass of Empty Capsid (1:1:8): 3.75 MDa

- Two primary populations of capsids detected corresponding to empty and full particles
- Some “intermediate” (partially filled) particles observed
- Empty, partial, and full capsids have similar charge characteristics.
- High-molecular-weight (HMW) species could be characterized.

CDMS data provided by Megadalton Solutions
CDMS and Sedimentation Velocity Analytical Ultracentrifugation (SV-AUC)

CDMS

- Simultaneously measure m/z (mass to charge ratio) and z (charge)
  - Resolves intermediate species
  - Provide masses of particles
  - Provide charge for each species
  - Instrument not commercially available yet

SV-AUC

- Separate and quantify based on size, shape and mass
  - Resolves intermediate species
  - Commercial instrument
  - High sample amount required
  - Low throughput
  - Labor intensive

CDMS data provided by Megadalton Solutions
Good correlation between AUC and CDMS for Empty and Full

SV-AUC and CDMS are suitable for quantifying empty and full capsids
Poor correlation between AUC and CDMS for Partial and HMWs

\[ y = 2.1085x - 0.0598 \]
\[ R^2 = 0.4527 \]

\[ y = -0.2687x + 0.0496 \]
\[ R^2 = 0.5895 \]
Case Study 3: Residual Iodixanol Quantification to Support Process Development
Background

- Iodixanol-based density gradient is commonly used for AAV purification.

- However, residue iodixanol, as an in-process impurity, may present a safety concern.

- An analytical method with high sensitivity is essential to ensure sufficient clearance of iodixanol, and hence safety of AAV product.
A RPLC-MS Method for Iodixanol Quantification

iodixanol

(iohexnol (internal standard))

iohexnol

(terminal standard)

iodixanol

(target impurity)

AAV Serotype “A” DS (free of iodixanol)

Formulation blank

AAV Serotype “B” DS (with IS added)

AAVs elute at later washing period

×~10

×~2

Zoomed-in

iohexnol

iodixanol

7

8

9

min
Sensitivity and Linearity

LOQ of 0.01 μg/mL can be achieved.

\[
\text{Area Ratio} = \frac{\text{Peak Area of iodixanol}}{\text{Peak Area of internal standard}}
\]
Application to Analysis of AAV In-process and DS Samples

- A highly efficient purification method was further explored for removal of the residual iodixanol.

- The two AAV batches after purification showed residual iodixanol levels well below the recommended safety threshold.
Conclusions

• Mass spectrometry (MS) is a powerful analytical tool that shows great promise in AAV-based gene therapy development.

• The combination of *intact mass measurement* of VP1, VP2, and VP3 proteins is highly specific as an identity test using CE-MS or LC-MS.

• SV-AUC and **CDMS** are suitable for characterizing empty and full capsids.

• A **MS-based method** for iodixanol quantification was successfully developed and applied in support of process development.
Acknowledgements

**Analytical Development**
- Hui-wen Liu
- Dana Tribby
- Vinay Bhatt
- Rachel Chen
- Wei Zhang
- Zoran Sosic
- Svetlana Bergelson
- Bernice Yeung
- Brian Fahie

**Gene Therapy-Process Development**
- Russell Katz

**Research**
- Vic Kostrubsky
- Pete Clarner
- Joyce Lo