Unlocking Analytics for AAV Gene Therapy Programs: Leveraging Standard Biotherapeutic Strategies to Transform New Modalities

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CASSS MS Presentation 27Sept2022
Overviewing Pfizer’s Extensive Experience in Biotherapeutics

- Pfizer has diverse experience across biotherapeutic modalities.
- Experience from traditional modalities has guided the characterization approach for GTx programs.
Overview of MS Applications for AAV Programs in Pfizer

- Pfizer’s implementation strategy of MS has been adapted from standard biotherapeutics.
- AAV workhorse assays include LC-MS/MS peptide map, MAM (LC-MS), capsid protein analysis (RP-HPLC-MS) and intact capsid analysis (CDMS).
- MS has been a pivotal for AAV programs
  - Product understanding (PQAs/CQAs)
  - Bioprocess and formulation support
  - Routine testing and stability studies
  - Support development of physiochemical methods
  - Support understanding of particle content
  - Aid in product investigations

Overview of the AAV Capsid

<table>
<thead>
<tr>
<th>Capsid Proteins (n=60)</th>
<th>Capsid Information</th>
<th>Genome Information</th>
</tr>
</thead>
<tbody>
<tr>
<td>VP1</td>
<td>~ 20 nm in size</td>
<td>~ 4.7 kb</td>
</tr>
<tr>
<td>VP2</td>
<td>~ 3.5 MDa in mass</td>
<td>~ 1.5 MDa in mass</td>
</tr>
<tr>
<td>VP3</td>
<td>Estimated 1:1:10 capsid protein ratio</td>
<td></td>
</tr>
</tbody>
</table>
## Application of MS for AAV GTx Programs

**Overview of Mass Spec Utilization Rates in Traditional BTx BLAs (2017)**

<table>
<thead>
<tr>
<th>MS attribute</th>
<th>% of MS BLAs</th>
<th>MS attribute (&lt; 10%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amino acid sequence analysis</td>
<td>97.5</td>
<td>Sequence variants (amino acid substitutions)</td>
</tr>
<tr>
<td>Molecular mass</td>
<td>92.4</td>
<td>Covalent dimers</td>
</tr>
<tr>
<td>Disulfide bonds</td>
<td>77.2</td>
<td>Methionine/cysteine formylation</td>
</tr>
<tr>
<td>Glycosylation</td>
<td>70.9</td>
<td>Phosphorylation</td>
</tr>
<tr>
<td>Sequence variants (C-term)</td>
<td>64.6</td>
<td>Truncation</td>
</tr>
<tr>
<td>Sequence variants (N-term)</td>
<td>64.6</td>
<td>Acetylation</td>
</tr>
<tr>
<td>Deamidation</td>
<td>58.2</td>
<td>Aggregation</td>
</tr>
<tr>
<td>Oxidation</td>
<td>57.0</td>
<td>Folding/HOS</td>
</tr>
<tr>
<td>Size variants</td>
<td>27.8</td>
<td>Host cell proteins (HCPs)</td>
</tr>
<tr>
<td>Free thiols</td>
<td>25.3</td>
<td>Partial reduction</td>
</tr>
<tr>
<td>Glycation</td>
<td>22.8</td>
<td>PEylation</td>
</tr>
<tr>
<td>Charge variants</td>
<td>19.0</td>
<td>Translucent particles</td>
</tr>
<tr>
<td>Other impurities</td>
<td>17.7</td>
<td>Zinc</td>
</tr>
<tr>
<td>Proteolysis/fragmentation</td>
<td>13.9</td>
<td>Glutathionylation</td>
</tr>
<tr>
<td>Succinimidation</td>
<td>12.7</td>
<td>Methylation</td>
</tr>
<tr>
<td>Isomerization</td>
<td>10.1</td>
<td>Norleucine incorporation</td>
</tr>
<tr>
<td>Other</td>
<td>10.1</td>
<td>Phosphogluconylation</td>
</tr>
</tbody>
</table>

**Overview of Mass Spec Utilization Rates in AAV Gene Therapy (2022)**

- Particle content (e.g. CDMS): 17%
- Confirmation of primary structure/identity (Peptide mapping): 42%
- Quantification of capsid modifications (Peptide mapping, Multi-Attribute Method): 36%
- Higher order structure (HDX, other): 8%
- Host cell protein: 19%
- Vp ratio: 11%
- Characterization of the packaged genome: 11%
- Other [Please specify in ideas tab]: 3%
- None: 44%


NIIMBL AAV Analytics Workshop hosted in April of 2022

- MS utilization rates for AAV programs are lower compared to traditional biotherapeutics
MS Applications for AAV:

- Peptide Mapping
- Intact Protein Analysis
- Intact Capsid Analysis

Peptide Mapping by LC-MS(/MS) for AAV

Non-Targeted LC-MS/MS
- Sequence Confirmation
- Attribute Understanding

- Targeted PQA Quantitation
- Routine Monitoring (MAM)
- Primary Structure and Mods
- ID of New Peaks
- Sequence Confirmation
- Attribute Understanding
Mass Spec Enables Superior Attribute Understanding: Linking Capsid Modifications to Potency Data

**Most Sensitive Attributes**

- ✓ Potency (Activity)
- ✓ Infectivity

**No Changes Observed**

- ➢ Titer (genome, capsid)
- ➢ Aggregation
- ➢ Purity

**Investigation needed to understand what is driving potency decrease**

**Capsid modifications are inversely correlated with bioassay results**

In-depth characterization coupled to sensitive bioassays identified a possible cause of the potency drop.
Mass Spec Enables Superior Attribute Understanding: Mutants Confirm Hypothesis from Mass Spec Data

**Single Base Mutations Mimic Deamidation**

- Site X
- Site Y
- Site Z

Individual N to D mutations mimic 100% deamidated capsid

Model of Site Z in the context of the AAV capsid

**Process of Chemical Deamidation**

\[
\text{Asn} \xrightarrow{\text{NH}_3} \text{succinimide} \xrightarrow{\text{H}_2\text{O}} \text{Asp} \xrightarrow{\text{Asp}} \text{isoAsp}
\]

\( D = \text{Aspartic Acid} \)

\( N = \text{Asparagine} \)

% Relative Potency

Mutations mimicking deamidation impact % relative potency

N→D Mutants at Specified Sites

WT

Site Z

N57D

N94D

N263D

100
10
1

Mutations mimicking deamidation impact % relative potency
Multi-Attribute Method Enables Unprecedented Access to Data

The Multi-attribute Method (MAM) can simultaneously detect and quantitate product quality attributes.

- MAM can result in efficiencies for method development and analytical testing
- Method can be platformed and impact early product and process development
- Samples supported include: Batch MFG, stability and forced deg, LPQ/PPQ, comparability, formulation support, etc.

Application of MAM

- Peptide Map Method – Easy and Reproducible Digest
- Targeted Hotspots
- Automated Data Processing

Contrasting Platform Methods for AAV vs. Standard BTx

<table>
<thead>
<tr>
<th>Platform Method</th>
<th>Attribute</th>
</tr>
</thead>
<tbody>
<tr>
<td>ICE or iEX-HPLC</td>
<td>HC CDR deamidation</td>
</tr>
<tr>
<td>ICE or iEX-HPLC</td>
<td>HC CDR isomerization</td>
</tr>
<tr>
<td>Reducing CGE</td>
<td>Fragmentation</td>
</tr>
<tr>
<td>Focused Peptide Mapping</td>
<td>HC Met255 Oxidation</td>
</tr>
<tr>
<td>Glycan Profile (HILIC-FLR)</td>
<td>Glycosylation</td>
</tr>
<tr>
<td>ICE or iEX-HPLC</td>
<td>VS/NK PENNY deamidation</td>
</tr>
<tr>
<td>ICE or iEX-HPLC</td>
<td>C-Terminal Lysine</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Attribute</th>
<th>Platform Method</th>
</tr>
</thead>
<tbody>
<tr>
<td>Deamidation</td>
<td>RP-HPLC or CE</td>
</tr>
<tr>
<td>N-Terminal Heterogeneity</td>
<td></td>
</tr>
<tr>
<td>Clips and Alternative Processing</td>
<td></td>
</tr>
<tr>
<td>Phosphorylation</td>
<td></td>
</tr>
<tr>
<td>Oxidation</td>
<td></td>
</tr>
<tr>
<td>Isomerization</td>
<td></td>
</tr>
</tbody>
</table>
Implementation of MAM – Current and Future Efforts within Pfizer

Advances Enabling MAM Success (AAV and BTx)

- Automated Preparation
- Demonstrated Method Performance (Qual/Val)
- Generic Report
- Data Access – LIMS Compatibility
- Data Trending: Spotfire

Implementation of High-Throughput Automation Enables Routine MAM Support

Enhancing the multi-attribute method through an automated and high-throughput sample preparation

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\textsuperscript{a} Integrated Micro-Chromatography Systems, Inc., Irmo, SC, USA \textsuperscript{b} Pfizer, Inc., Chesterfield, MO, USA

Automation performance mirrors manual sample preparation but at greater efficiency
Implementation of MAM – Current and Future Efforts within Pfizer

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Evaluating MAM Method Performance

- Intermediate Precision: RSD < 20%*
- Oxidation

- Linearity

➢ Method evaluated for specificity, system precision, linearity/range, LOQ, sample stability, accuracy, and robustness

* Excludes selected oxidized peptides related to column fouling
Enhancing MAM Implementation and Data Documentation Practices are Key Enablers to the Technology Advances Enabling MAM Success (AAV and BTx)

- Automated Preparation
- Demonstrated Method Performance (Qual/Val)
- Generic Report
- Data Access – LIMS Compatibility
- Data Trending: Spotfire

Enhancing MAM Quality and Access

- Universal Report – No changes needed across methods!
- Criteria (SST, AC) Specified in Processing Method
- Automatic Integration Visualization
- Assessment of Composite Scoring Criteria
- Automatic Export
- Exported Data compatible with LIMS

➢ Enhancing MAM Implementation and Data Documentation Practices are Key Enablers to the Technology
Implementation of MAM – Current and Future Efforts within Pfizer

**Advances Enabling MAM Success (AAV and BTx)**

- Automated Preparation
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**Enhancing MAM Quality and Access**

- Spotfire Improves Access and Trending of Data

<table>
<thead>
<tr>
<th>Attribute 1 (%)</th>
<th>Attribute 2 (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Sample Number</th>
<th>Relative % Abundance</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td></td>
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<td>7</td>
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<td>9</td>
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</tr>
<tr>
<td>10</td>
<td></td>
</tr>
<tr>
<td>11</td>
<td></td>
</tr>
<tr>
<td>12</td>
<td></td>
</tr>
</tbody>
</table>

Attribute 1

Attribute 2

Attribute 3

Attribute 4

Attribute 5

Graph showing trends over sample number.
MS Applications for AAV:

Intact Capsid Protein Analysis by LC-MS for AAV:
- Primary Structure and Mods
- Purity Assessment
- Support Complementary Separation Methods
Capsid Protein Analysis Supports Product Quality Understanding

Characterizing AAV Capsid Proteins by LC-MS

Deconvoluted Mass Spectra

AAV Serotype “A”

VP2: 66328.5 Da

VP3: 59826.2 Da

VP1: 81709.6 Da

Understanding Capsid Heterogeneity

➢ Approach can support VP ratio

➢ Multiple modifications can be identified: N-term heterogeneity, phosphorylation, oxidation, deamidation, clips and alternative processing sites, etc.
Mass Spec Supports Development of Quality Analytics:

Advances Enabling MAM Success (AAV and BTx)

- Supporting Method Development
- ID of Unknowns
- Next Generation Methods

Understanding Capsid Heterogeneity

- Incorporating MS into method development ensures that peaks are understood before optimizing methods around them.
Mass Spec Supports Identification of Unknowns

Advances Enabling MAM Success (AAV and BTx)

Supporting Method Development → ID of Unknowns → Next Generation Methods

Understanding Capsid Heterogeneity

- As unknown impurities appear, having a method that can directly couple to the MS is valuable
- RP-HPLC/MS has also been used to support impurities that were observed by CGE purity assessments
Mass Spec Supports Next Generation Separation Methods

Advances Enabling MAM Success (AAV and BTx)

Supporting Method Development ➔ ID of Unknowns ➔ Next Generation Methods

Understanding Capsid Heterogeneity

Baseline Separation

Enhanced RP Separation

Enhanced HILIC Separation

➢ More selective HPLC methods are being evaluated that have utility in separating PQAs. Coupling MS to the separation enables understanding to support method development.
MS Applications for AAV:

Intact Capsid Protein Analysis

Peptide Mapping

Intact Protein Analysis

Capsid Mass

Particle Content

Assessment of Packaged Genome
Overview of “Particle Content” in the AAV GTx Space:

Possible Impurities in AAV Materials

➢ Cellular, Tissue and Gene Therapies Advisory Committee (CTGTAC – Sept 2021) highlighted enhanced scrutiny on AAV impurities and a concern around the level of empty/partially-filled capsids

➢ Committee recommended additional characterization techniques for AAV empty/full capsids
Evaluating CDMS Performance

Comparison of analytical techniques to quantitate the capsid content of adeno-associated viral vectors

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*A work in conjunction with the Jarrold Lab at Indiana University
**MS Reveals Additional Information on Capsid Composition**

**Particle Content Evaluation on Alternate System**

- **Count**
  - m/z
  - 4
  - 6

- **Mass [MDa]**
  - 100%
  - 50%
  - 0%

**UHMR-MS Documents Unique Capsid Heterogeneity**

- **REP**
- **CAP**
- ITR
- 1
- 130
- 203
- 736
- wild type AAV1
- 3.622±0.2 kDa
- 3.626±0.4 kDa
- 3.637±0.3 kDa


- *Work in conjunction with the Heck Lab at Utrecht University*
CDMS Supports Characterization of the Packaged Genome: Assessing the Assembled Capsid and Extracted Genome

Assessment of AAV Capsids with Various Genome Sizes

DNA Mass

y = 1.041x - 0.016


*Work in conjunction with the Jarrold Lab at Indiana University
Mass spec remains a unique and key assay for AAV products. Novel applications continue to offer significant potential for GTx development.

➢ At Pfizer, mass spectrometry is an important tool in AAV analytical toolbox, where utilization ranges from characterization testing to impurity identification to routine testing support.
➢ Mass spectrometry has enabled superior product understanding by identifying stability indicating attributes, degradation pathways, and unique capsid and protein heterogeneity.
➢ Mass spectrometry has aided in the development of novel, non-MS separation methods, including RP-HPLC and HILIC-HPLC.
➢ Mass spectrometry can provide confirmation of results from orthogonal and complementary methods, including AUC for particle content.
Acknowledgements

**Pfizer AAV Team:**
Herb Runnels
Amanda Werle
Phoebie Baldus
Vess Mitaksoy
Leah Wang
Jim Zobel
Caitlin Wappelhorst
Courtney Sloan
Andrew Wolf
Sharee Adams-Hall
Daniel Ryan
Sai Srinivasan
Mark Chipley
Ting Chen

**Pfizer MAM Efforts:**
Halyna Narepekha
Viktoriya Dilay
Vamsi Kandhi
Natalia Kozlova
Zhenjiu Liu
Beth McCoy
Tiffany Medwid
Nataliya Parahuz
Yuting Huang
Bradley Bare

**Pfizer Leadership:**
Meg Ruesch
Tom Lerch
Jason Rouse
Olga Friese

**Indiana University:**
Martin Jarrold
Nicholas Lytkey
Benjamin Draper
Lauren Barnes

**Utrecht University:**
Albert Heck
Joost Snijder
Tobias Worner
Sana Habka

**IMCS:**
Andrew Lee
Nikki Sitasuwan
Abstract

Mass spectrometry is a crucial component of the analytical toolbox used to support traditional biotherapeutics. While utilization rates of mass spectrometry for gene therapy programs may not match the utilization rates for traditional biotherapeutics, the technology offers unique insights into AAV gene therapy programs. The current presentation aims to outline several orthogonal mass spectrometry-based approaches that can be used to look wholistically at the AAV product, from bottom-up analysis to the assessment of intact AAV capsids. By leveraging the array of mass spectrometry-based methods for AAV characterization, mass spectrometry can be used to support a wide range of analytical studies. To date, mass spectrometry has been successfully implemented to better understand critical quality attributes (CQAs), support investigations, support routine manufacturing testing, aid in method development, and provide supportive data to complementary technologies.

While the application of mass spectrometry to AAV is still relatively new, the characterization of AAV products has been enabled by cutting edge movements in the field of mass spectrometry, including innovations in the multi-attribute method (MAM), charge detection mass spectrometry (CDMS), and ultra-high mass range (UHMR) systems. Importantly mass spectrometry has been useful in identifying new quality attributes and support methods in place that monitor already established CQAs. The current presentation details cutting edge research that has been implemented to AAV gene therapy programs.