Comparative analysis of intact AAV8 capsid proteins derived from SF9 and HEK293 cell lines

Featuring the SCIEX X500B QTOF System and BPV Flex Software 2.1

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Here, the determination of the intact molecular weight of viral capsid proteins (VPs) derived from adeno-associated virus (AAV) samples is presented using liquid chromatography coupled to mass spectrometry (LC-MS). Furthermore, a comparative assessment of post-translational modifications (PTMs) between AAV derived from insect cells (SF9) and human cells (HEK293) was performed. The method presented offers high mass accuracy, sensitivity and robustness for reliable results including throughput capabilities.

Recombinant AAVs are the most widely used vectors in gene therapy due to their low toxicity and ability to induce long-term expression. AAVs are composed of a shell of protein called a capsid encompassing a single stranded DNA. In the case of AAV8, the viral capsid is composed of three viral proteins. All VPs share a common C-terminal sequence. Different AAV production methods might result in different relative expression levels of VP1, VP2 and VP3.[1] Some PTMs of the VPs can impact the viral infectivity and vector potency.[2] Thus, a complete characterization of the constituent AAV proteins, including their sequences and post-translational modifications, is highly recommended to ensure AAV product quality and consistency.

Presented here is a streamlined approach for the determination of the intact molecular weight of viral capsid proteins from AAV8. In addition, PTMs were identified, which can be used for monitoring capsid protein heterogeneity with the benchtop SCIEX X500B QTOF System, powered by SCIEX OS Software, and data processing using BPV Flex Software. The method presented is suitable to support the characterization and development process for AAV samples.

Key features of the SCIEX solution for intact mass analysis of capsid proteins

- Excellent spectral quality resulting in high mass accuracies for confirmation of MW of all capsid proteins offers confidence in product quality of gene therapy products
- Correct and fast identification of PTMs, which can affect the product's potency, with BPV Flex Software can accelerate the development of gene therapy products
- Best results for both expert and non-expert users are ensured through ease-of-use of hardware and software

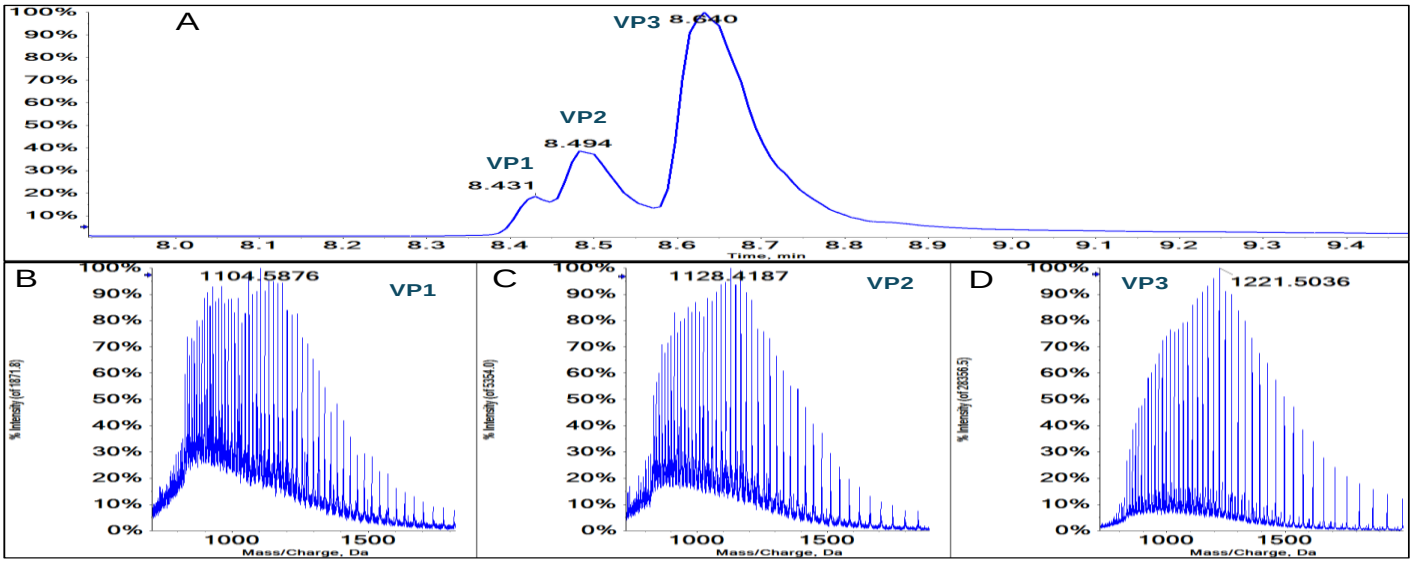

Figure 1. Intact mass analysis of AAV8 capsid protein. Total ion chromatogram (TIC) for VP1-3 from AAV8 (top pane) and respective TOF-MS spectra (bottom panes).

Methods

Sample preparation: 100 µL of AAV virions at a concentration of 1E12 gene copies (GC) per mL were first concentrated with an Amicon Ultra filter (0.5 mL, 10 kDa MWCO) and then buffer exchanged with 25 mM of Tris, pH 8.0 (0.5 mL; three times) at 4,000×g. The concentrated AAV virions (30 µL) were denatured by adding 70 µL of 10% acetic acid.

Chromatography: The separation was accomplished using a SCIEX ExionLC™ system fitted with a Waters BEH C8 column (2.1×100 mm, 1.7 µm,1 30Å). Mobile phase A consisted of 0.1% formic acid in water while mobile phase B was 0.1% formic acid in acetonitrile. A flow rate of 250 µL/min was used with the gradient shown in Table 1. The column temperature was held at 80 °C. The injection volume was set to 50 µL.

Table 1. Chromatography for intact mass analysis.

Time [min]	Mobile phase A [%]	Mobile phase B [%]
Initial	*80*	*20*
2.0	*80*	*20*
10	*20*	*80*
12	*0.0*	*100*
18	*0.0*	*100*
20	*80*	*20*
30	*80*	*20*

Mass spectrometry: Data were acquired using a SCIEX X500B QTOF System operated with SCIEX OS Software. Detailed MS settings can be found in Table 2.

Table 2. MS parameters for intact mass analysis.

Parameter	setting
Scan mode	*TOF-MS*
Polarity	*positive*
Intact protein mode	*ON*
Gas 1	*50 psi*
Gas 2	*65 psi*
Curtain gas	*35*
Temperature	*450 °C*
Ion spray voltage	*5500 V*
CAD gas	*7*
Time bins to sum	*120*
Accumulation time	*0.5 s*
Start mass	*600 m/z*
Stop mass	*3,500 m/z*
Declustering potential	*150 V*
Collision energy	*10 V*

Data processing: Data were visualized and processed using SCIEX OS Software with the Bio Tool Kit add-on, as well as BPV Flex Software.

Molecular weight confirmation and PTM identification

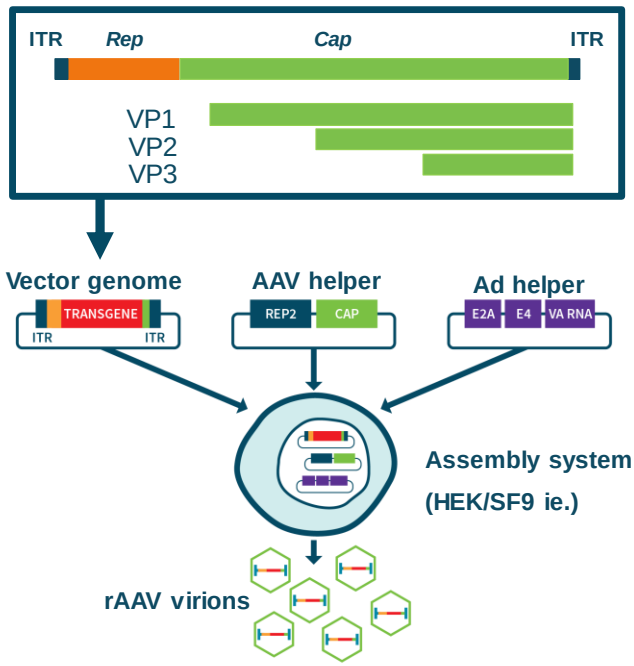

Figure 2. Overview of AAV virons. Schematics of gene map for VP1-3 derived from Cap gene (top). Therapeutic AAVs usually contain three plasmids: a vector genome containing the transgene, an AAV helper plasmid coding for VP proteins and an AD helper plasmid coding for needed enzymes and others (bottom).

Two AAV8 samples derived from insect and human cell lines (SF9 and HEK293) were analyzed. All VPs share a common C-terminal sequence and the entire VP3 is contained in VP2, and all of the VP2 sequence is contained within VP1 (Figure 2).The expected molecular weights for the three proteins forming the capsid are approximately 81 kD (VP1), 66 kD (VP2) and 60 kD (VP3). Intact protein mass analysis of VP proteins was performed using an ExionLC system coupled to a SCIEX X500B QTOF System. The VP1, VP2 and VP3 were separated (Figure 1A) and high-quality charge state envelopes were detected for each of the proteins (Figure 1B, C, D). Upon reconstruction of the TOF-MS raw data (Figure 3), the experimentally determined masses were compared with the theoretical molecular weights allowing for the assignment of VP1-3 to the chromatographic peaks (Figure 1A). Excellent mass accuracies for all protein forms were achieved (example for SF9 in Table 3). Data sets were processed using BPV Flex software to confirm the MW and determine the presence of PTMs such as glycosylations, acetylations, phosphorylations and disulfide linkages. The experimental determined mass of 81666 Da could be matched to the expected sequence for VP1 (amino acid 2-737), whereas VP2 and VP3 could be matched to 66518 Da (amino acid 139-737) 59762 Da (amino acid 205-737), respectively. The analysis further confirmed that there were no glycosylations present in any in the viral capsid proteins, even though several consensus sequences for *N*-linked glycosylation exist. However, a 42 Da mass shift, indicating an acetylation was found for VP1 and VP3 (Figure 3 and Table 3). No evidence of disulfide linkages has been reported in the literature for AAV8, which is in alignment with the experimentally determined masses for all capsid proteins analyzed here. The intact mass analysis of VP1 and VP2 showed significant peaks with the mass shift of ~79 Da compared to the theoretical sequence. Since sulfation can be ruled out based on no tyrosine being present in the sequence, this mass shift indicated phosphorylation events. To confirm that theory, the samples were treated with alkaline phosphatase. The data before and after phosphatase treatment is shown in Figure 4. The absence of the mass shift of ~79 Da for VP1 and VP2 in the samples treated with phosphatase confirmed the presence of this post translational modification in the untreated samples (Figure 4).

Table 3. Results obtained from intact mass analysis of SF9.

Protein	Theoretical MW [Da]	Experimental MW [Da]	Mass error [ppm]
VP3	*59762.44*	*59762.40*	*-0.7*
VP3, acetylated	*59804.48*	*59804.70*	*3.7*
VP2	*66517.87*	*66518.10*	*3.5*
VP2, phosphorylated	*66597.85*	*66597.80*	*-0.8*
VP2, diphosphorylated	*66677.83*	*66677.80*	*-0.4*
VP1, acetylated	*81666.43*	*81666.30*	*-1.6*
VP1,acetylated, phosphorylated	*81746.41*	*81746.20*	*-2.6*
VP1, acetylated, diphosphorylated	*81826.39*	*81826.80*	*5.0*

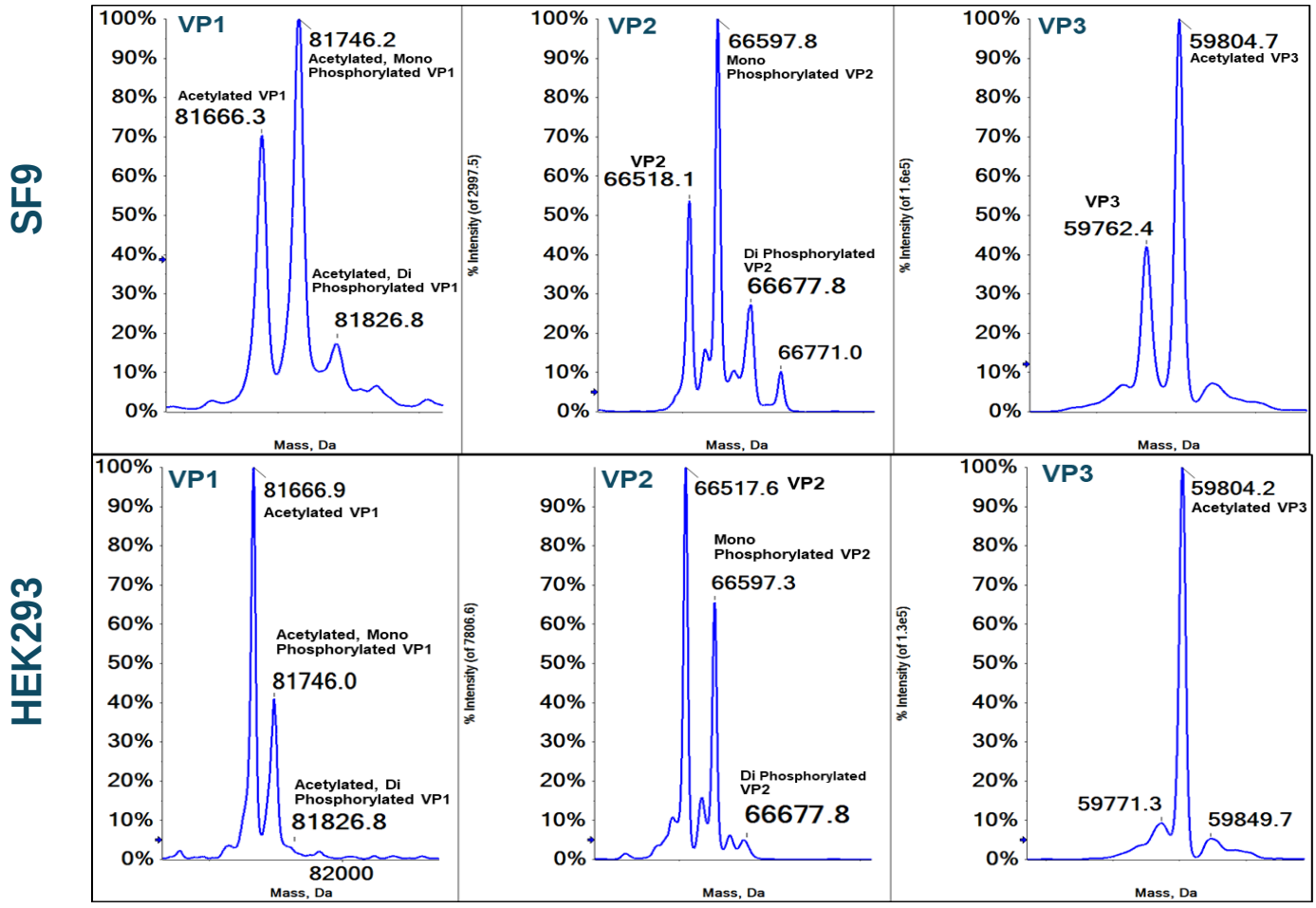

Figure 3. Reconstructed masses for VP proteins from AAV8 assembled in SF9 and HEK293 cells. Top pane: VP1, VP2 and VP3 proteins of AAV8 from SF9 cells. Bottom pane: VP1, VP2 and VP3 proteins of AAV8 from HEK293 cells.

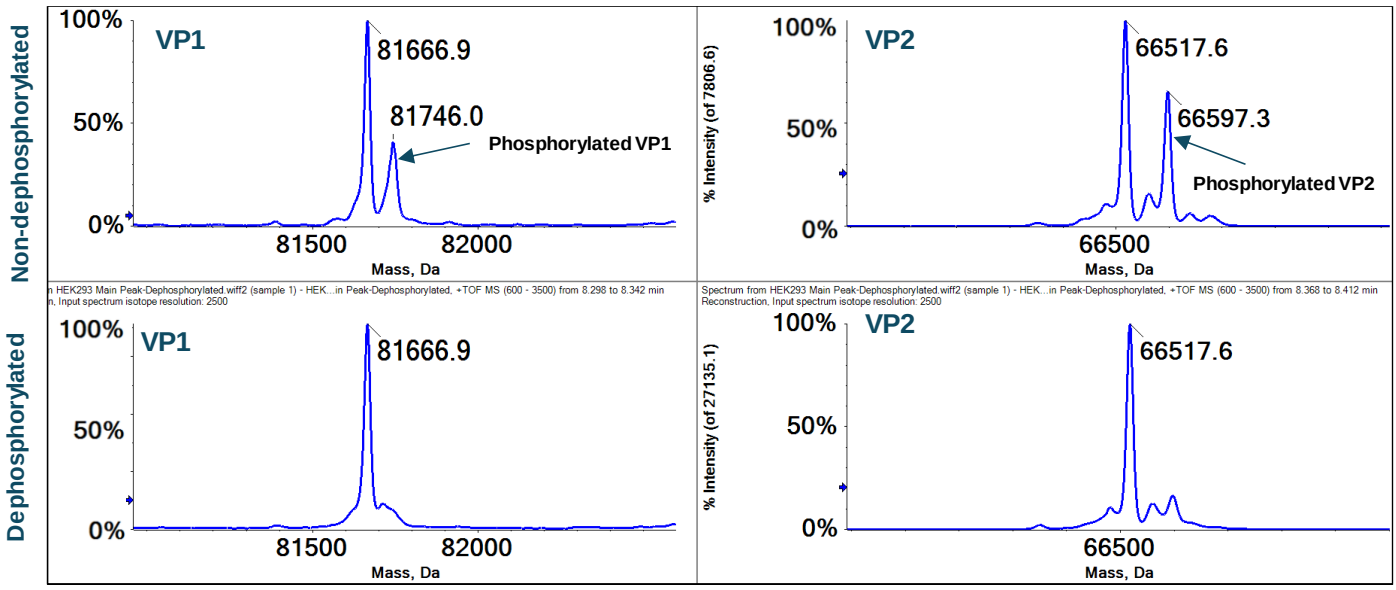

Figure 4. Reconstructed masses of VP1 and VP2 from HEK293 cells with and without phosphatase treatment. Top panes show VP1 and VP2 proteins and their potentially phosphorylated forms. Bottom panes show reconstructed data from the sample treated with alkaline phosphatase in order to remove phosphorylation.

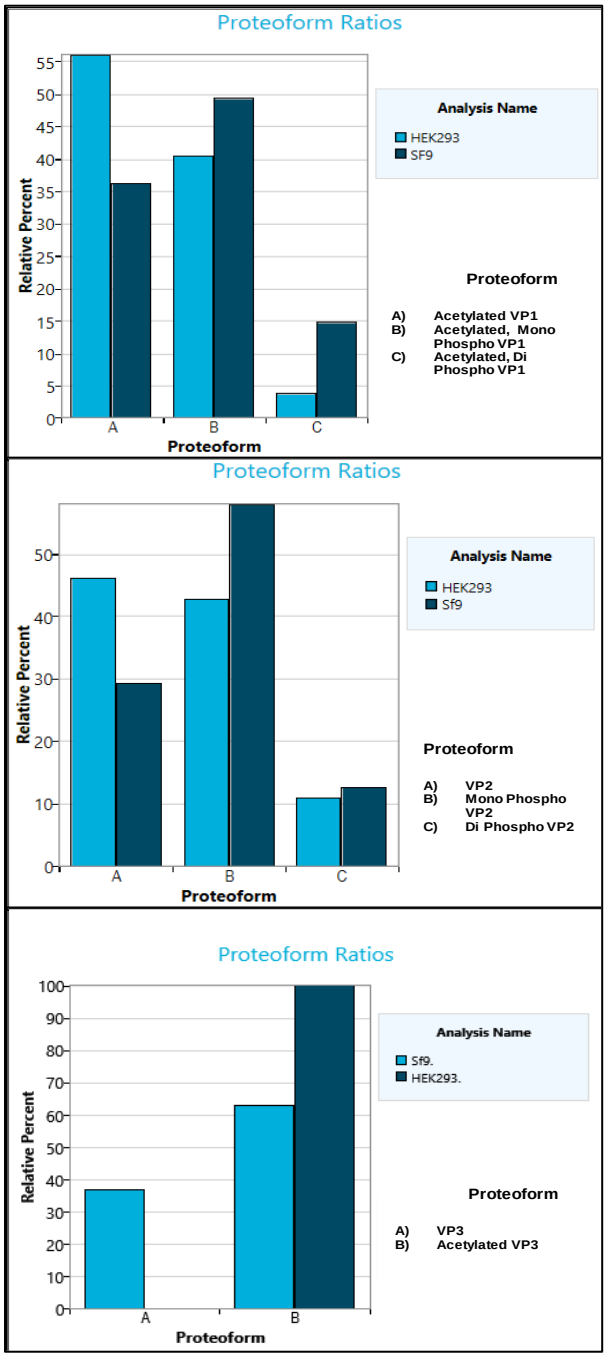

Figure 5. Proteoform distribution of VP proteins. Comparison of SF9 (light blue) and HEK293 (dark blue) derived AAV samples in BPV Flex Software VP1 (A), VP2 (B) and VP3 (C).

The abundances of the different PTMs were compared for both cell systems across all three capsid proteins. The degree of relative (mono- and diphosphorylation) on VP1 and VP2 was higher for the SF9 compared to the HEK293 cell system (Figure 5). VP3 was found to have significantly higher acetylation for capsids derived from HEK293 compared to SF9, whereas VP1 showed no difference in acetylation for both cell systems. Especially modifications on the capsid proteins, which lead to a change in a protein's charge heterogeneity (such as phosphorylation) are known to impact the effectiveness of the virus.[2] To assist with the development of AAVs and to monitor the quality of AAV products, the presented LC-MS workflow can be used as a fast and reliable tool by confirming molecular weights and identifying modifications on the intact protein level. In addition to minimizing sample preparation time, the workflow also minimizes the risk of introducing artifacts by avoiding lengthy sample preparation.

Conclusions

- All capsid proteins were separated, detected with excellent spectral quality despite the minimal sample amount available and molecular weights were confirmed using the SCIEX X500B QTOF System and BPV Flex Software
- The high spectral quality of the raw and reconstructed data enabled the identification of the PTMs such as acetylation and phosphorylation
- Easy to review plots, automatically generated in BPV Flex Software, revealed differences in the modification levels of VP1, VP2 and VP3 for SF9 and HEK293T cell systems
- The development of AAV-based therapeutics can be accelerated with the usage of additional information derived from the SCIEX X500B QTOF System, an LC-MS system accessible for every user level

References

1. Xiaoying Jin, Lin Liu, Shelley Nass et al. (2017). Direct liquid chromatography/mass spectrometry analysis for complete characterization of recombinant adeno-associated virus capsid proteins. *Human Gene Ther Methods*. **28**(5):255-267.
2. Bertin Mary, Shubham Maurya, Sathyathithan Arumugam et al. (2019). Post-translational modifications in capsid proteins of recombinant adeno-associated virus (AAV) 1-rh10 serotypes. *FEBS* **286**(24):4964-4981.

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