Multiple ion activations and proton transfer charge reduction facilitate the characterization of capsid proteoforms of recombinant adeno-associated virus 6 (rAAV6)

Jake T. Kline[1], Kristina Srzentić[2], Katalyn Irvin[3], Christopher Mullen[4], Romain Huguet[4], John EP Syka[4], Luca Fornelli[1]

[1]University of Oklahoma, Norman, OK; [2]Thermo Fisher Scientific, Reinach, Switzerland; [3]Thermo Fisher Scientific, Frederick, MD; [4]Thermo Fisher Scientific, San Jose, CA

ABSTRACT

Here we present our preliminary investigation of the viral proteins of the capsid of adeno-associated viruses. These proteins, named VP1, VP2 and VP3 where chromatographically separated using reversed-phase liquid chromatography and characterized in the gas-phase by high resolution Orbitrap™ Fourier transform mass spectrometry (FTMS). Specifically, these large (59-81 kDa) polypeptide were fragmented using complementary ion activation techniques to improve the final sequence coverage. Importantly, on the most abundant of these subunits the sequencing was also improved by applying ion-ion proton transfer reactions, commercially available on the Orbitrap™ Eclipse™ Tribrid™ as proton transfer charge reduction.

INTRODUCTION

Adeno-associated viruses (AAVs) are a class of small single-stranded DNA viruses. Due to lack of pathogenicity, AAVs represent ideal vectors for gene therapy. There are currently two approved gene therapies that use AAVs as a vector: Luxturna (Spark Therapeutics) for blindness and Zolgensma (Novartis) for spinal muscular dystrophy. Furthermore, the Bill and Melinda Gates Foundation is funding the use of the AAV vector to develop a new COVID-19 vaccine. Structurally, the AAV capsid is composed of three main proteins, namely VP1, VP2 and VP3, with masses spanning from 59 to 81 kDa. The overall capsid mass is 3.7-3.9 MDa, resulting from the combination of about 60 VP proteins. Importantly, 11 serotypes of AAVs have been isolated from human and primate samples. Here we describe the mass spectrometry-based characterization of whole VP proteins isolated from AAV6, a serotype particularly suited for gene therapy directed to the epithelial cells or the respiratory system, using multiple ion dissociation techniques alone and in combination with proton transfer charge reduction (PTCR). In the mid-90's the McLuckey lab first described gas-phase ion-ion reactions that used perfluorinated anions to reduce the charge state of multiply-charged cations[1]. Recently, PTCR has been applied to circumvent the issue of signal overlap that affects MS^2 fragmentation spectra of large proteins. This study builds upon our previous use of PTCR in direct infusion of standard proteins by applying the technique to therapeutically relevant samples on the LC time scale[2].

MATERIALS AND METHODS

Sample Preparation: AAV6 was expressed in HEK293 cells. After enrichment, viral particles were purified using Amicon Ultra 100 kDa molecular weight cut off spin columns (MilleporeSigma) and later disassembled into individual VP proteins by applying denaturing conditions (10% acetic acid).

LC Separations: An Ultimate 3000™ HPLC system (Thermo Scientific) was used for all separations. Mobile phase A was composed of water with 0.1% difluoroacetic acid (DFA) (v/v); mobile phase B was acetonitrile with 0.1% difluoroacetic acid (v/v). Subunit separation was obtained using a Acquity UPLC Protein BEH C4 column (100 mm x 1 mm ID, Waters) and a linear gradient (starting at 20% B, moved to 32% B in 1 min, moved to 36% B in 15 min with following 5.5 min wash at 80% B and 8.5 min re-equilibration again at 20% B) at a 100 µl/min flow rate.

MS Instrumentation and Methods: Targeted LC-MS experiments were performed using an Orbitrap Eclipse™ Tribrid™ with HCD, ETD, and PTCR capabilities. Perfluoroperhydrophenanthrene (623 *m/z*, $C_{14}F_{24}$) anion was used as PTCR reagent. All the measurements were performed under "intact protein pressure" settings (3 mTorr in the HCD cell). All FTMS spectra (resolving power: 240,000 at *m/z* 200) were recorded without noise removal using "full profile" mode. MS^2 experiments were performed in a targeted fashion by *m/z* selecting a 12-15 precursor charge states (+80-94 for VP1, +67-82 for VP2, and +58-72 for VP3) with the quadrupole (isolation width: 160, 185, or 210 *m/z* units for VP1, VP2, and VP3, respectively). ETD MS^2 used a 10, 15, or 20 ms duration, for HCD MS^2 normalized collision energies of 40% and 45% were tested. Standard PTCR MS^3 experiments used a single isolation window in the high-pressure region of the LTQ of the dual region RF linear quadrupole ion trap analyzer, and the isolation center was set based upon the MS^2 spectra for the viral particle being analyzed with a width of 1800 *m/z* units. For HCD/ETD/ETHcD-MS^2-PTCR MS^3 experiments, the PTCR duration was set at various ranges from 5 to 40 ms.

Data Analysis: Fragmentation maps were obtained and manually curated using TDValidator (Proteinaceous), which includes an isotope fitting algorithm that matches the experimental ion isotopic *m/z* peak clusters from the original .raw spectrum against theoretical ion isotopic *m/z* peak clusters generated using ions molecular formulae. The signal-to-noise ratio (SNR) threshold for fragment peak picking was set to 7-10. The fragment tolerance was set to 10 ppm, while the inter-isotopic tolerance for a single isotopic cluster was set to 3 ppm. The maximum charge state was set to +20. Fragments meeting the search criteria were manually validated. Plots were generated using GraphPad Prism 9 (GraphPad Software)

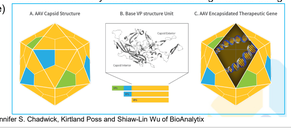

Figure 1. AAV capsid assembly. A) The assembly of viral proteins VP1 (green), VP2 (blue), and VP3 (yellow) as 1:1:10 ratio in the icosahedral capsid structure. B) Each VP has a core folded unit with VP1 and VP2 having N-terminal extensions. C) Therapeutic gene within the capsid core.

Adapted from the White paper by Jennifer S. Chadwick, Kirtland Poss and Shiaw-Lin Wu of BioAnalytix

RESULTS

Due to the narrow retention times for the various viral particle subunits, most of our efforts were focused on VP3. This is due to VP3 being more abundant allowing the collections of more scans for spectral averaging. For example, 21-28 total transients were averaged for VP1, 7-21 transients for VP2, and 35-42 transients for VP3.

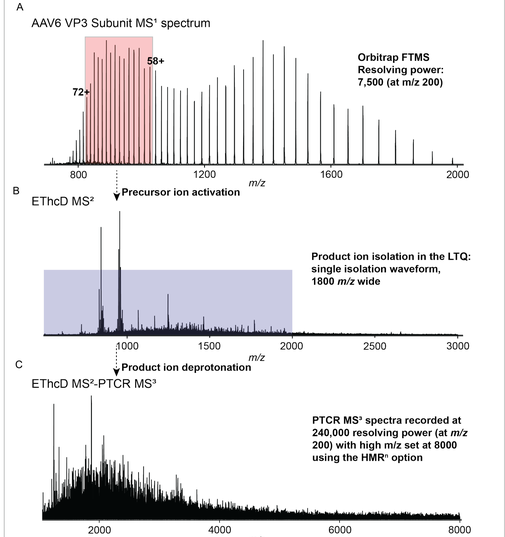

Figure 2. Schematic of data acquisition of AAV6 VP3 subunit. Starting from the MS^1 spectrum (A), multiple precursor charge states are isolated as a group (represented by the red box) and subjected to EThcD MS^2 fragmentation (B). The resulting fragments are then isolated in a single isolation window of 1800 *m/z* units (represented by the blue box) prior to PTCR (C). Deprotonated ions are analyzed in the Orbitrap mass analyzer at 240,000 resolving power (at *m/z* 200) over a 1000-8000 *m/z* window.

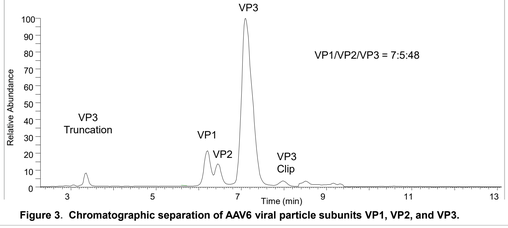

Figure 3. Chromatographic separation of AAV6 viral particle subunits VP1, VP2, and VP3.

Comparison and combination of MS^2 and PTCR MS^3 experiments

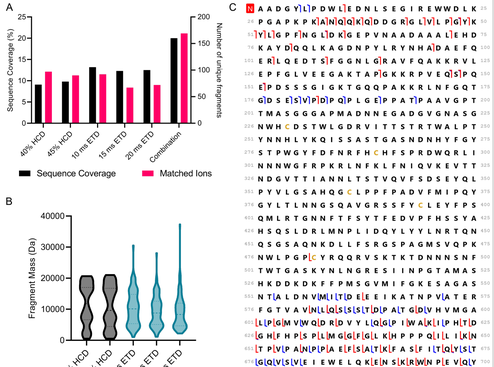

Figure 4. AAV6 VP1 subunit characterization. A single LC run was used for MS^2 fragmentation. Combined refers to the cumulative values of 45% HCD and 10 ms ETD. A) Sequence coverage and number of matched fragment. B) mass distribution for matched fragments. C) Combined HCD and ETD fragmentation map (20% sequence coverage).

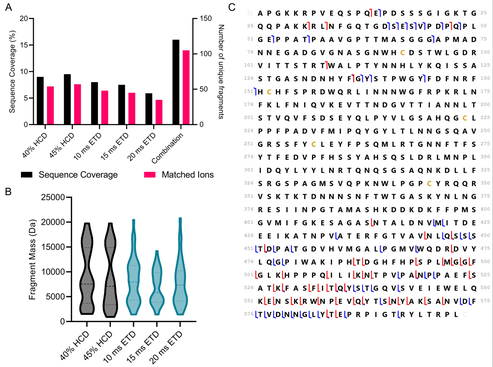

Figure 5. AAV6 VP2 subunit characterization. A single LC run was used for MS^2 fragmentation. Combined refers to the cumulative values of 45% HCD and 10 ms ETD. A) Sequence coverage and number of matched fragment. B) mass distribution for matched fragments. C) Combined HCD and ETD fragmentation map (16% sequence coverage).

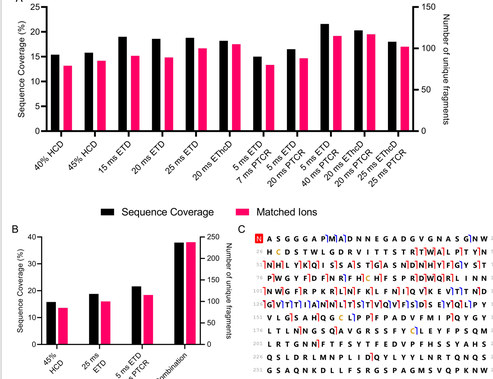

Figure 6. AAV6 VP3 subunit characterization. A single LC run was used for MS^2 fragmentation. A) Sequence coverage and number of matched fragment for all MS^2 and MS^3 experiments. B) Sequence coverage and number of matched fragment for best performing MS^2 and MS^3 experiments. C) Combined HCD MS^2, ETD MS^2, and ETD MS^2-PTCR MS^3 fragmentation map (3 LC runs total). D) Mass distribution for matched fragments for best performing experiments. E) Spectra of indicated MS^2 experiments

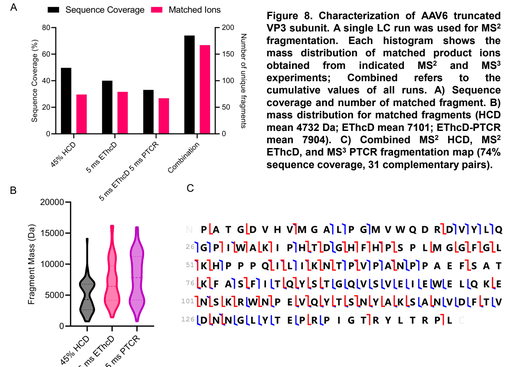

Figure 8. Characterization of AAV6 truncated VP3 subunit. A single LC run was used for MS^2 fragmentation. Each histogram shows the mass distribution of matched product ions obtained from indicated MS^2 and MS^3 experiments; Combined refers to the cumulative values of all runs. A) Sequence coverage and number of matched fragment. B) mass distribution for matched fragments (HCD mean 4732 Da; EThcD mean 7101; EThcD-PTCR mean 7904). C) Combined MS^2 HCD, MS^2 EThcD, and MS^3 PTCR fragmentation map (74% sequence coverage, 31 complementary pairs).

CONCLUSIONS

Our results demonstrate that combining multiple complementary fragmentation techniques at the MS^2 and MS^3 levels is required to more accurately characterize the subunits of AAV6. For the 81 kDa VP1 subunit the combination of HCD MS^2 and ETD MS^2 increased sequence coverage to 20%; for VP2 a similar combination increased sequence coverage from 9.5% to 16%. The higher abundance of VP3 allowed the use of spectral averaging with ETD MS^2-PTCR MS^3 activation which when paired with HCD MS^2 and ETD MS^2 improved sequence coverage to 37.9% by enabling the detection of larger fragments due to the reduction of spectral congestion. We observed that for the VP1 and VP2 were poor candidates for PTCR activation for two reasons: low abundance and overlapping elution windows. The low abundance limited the signal intensity and PTCR exacerbated the issue leading to difficulties with signal-to-noise ratio. The overlapping elution profiles reduced the potential for spectral averaging which compounded the signal-to-noise ratio reduction due to the low abundance. Recently, hydrophobic interaction liquid chromatography has recently been applied to AAV vector system and is a potential avenue to improve separation of VP1 and VP2 for future experiments[3].

REFERENCES

1. Herron, W. J.; Goeringer, D. E.; McLuckey, S. A. Gas-Phase Electron Transfer Reactions from Multiply-Charged Anions to Rare Gas Cations. *J. Am. Chem. Soc.* **1995**, *117* (46), 11555–11562. https://doi.org/10.1021/ja00151a024.
2. Kline, J. T.; Mullen, C.; Durbin, K. R.; Oates, R. N.; Huguet, R.; Syka, J. E. P.; Fornelli, L. Sequential Ion–Ion Reactions for Enhanced Gas-Phase Sequencing of Large Intact Proteins in a Tribrid Orbitrap Mass Spectrometer. *J. Am. Soc. Mass Spectrom.* **2021**, jasms.1c00062. https://doi.org/10.1021/jasms.1c00062.
3. Liu, A. P.; Patel, S. K.; Xing, T.; Yan, Y.; Wang, S.; Li, N. Characterization of Adeno-Associated Virus Capsid Proteins Using Hydrophilic Interaction Chromatography Coupled with Mass Spectrometry. J. Pharm. Biomed. Anal. 2020, 189, 113481. https://doi.org/10.1016/j.jpba.2020.113481.

Acknowledgements

The Fornelli lab would like to acknowledge the University of Oklahoma for startup funds and thank Dr. Jennifer Sutton for her support with sample preparation.

Poster presented at ASMS 2020 Reboot