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Intact LC-MS analysis and peptide mapping of recombinant adeno associated virus (rAAV) capsid proteins

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ABSTRACT

This poster describes sensitive and easy-to-adopt LC-MS workflows for intact analysis and peptide mapping of rAAV capsid proteins. The ultra-high MS/MS sensitivity offered by the Zeno trap resulted in nearly complete sequence coverage of capsid proteins in a single injection and confident identification of very low abundant modifications despite limited sample quantity. The unique capability of electron activated dissociation (EAD) allowed differentiation between aspartic (Asp) and isoaspartic acid (isoAsp) isomers of deamidation.

INTRODUCTION

rAAVs are widely used vectors in gene therapy because of being non-pathogenic to humans, possessing low immunogenicity and offering long-term gene expression. rAAVs consist of 3 viral proteins (VPs: VP1, VP2 and VP3) assembled in a 1:1:10 ratio. The characterizations of these VPs and their modifications, such as N-terminal acetylation, deamidation and phosphorylation are critical to ensure high product quality of rAAVs.[1] Specifically, deamidation was found to impact transduction efficiency of AAV vectors.[2] However, it is challenging to perform in-depth characterization of VPs in practice due to limited sample quantity, which hampers method optimization and detection of low-abundance species. Additionally, it is not possible to differentiate isomers of deamidation using traditional MS/MS techniques such as collision-induced dissociation (CID) and low-energy electron-based dissociation (ExD).

In this work, an intact and a peptide mapping workflow with easy-to-use analytical flow methods were employed to characterize a rAAV serotype 8 (rAAV8). The highly sensitive data-dependent acquisition (DDA) method with Zeno trap enabled, provided nearly complete sequence coverage of VPs and confident identification of low abundant modifications, such as deamidated species with relative abundance as low as ~0.04%. The differentiation of deamidation isomers was further accomplished by EAD leveraging descriptive *w*-ions.

MATERIALS AND METHODS

Sample preparation: The rAAV serotype 8 samples (rAAV8) were purchased from SignaGen Laboratories and Vigene Biosciences. Each vial (30 µL) of SignaGen rAAV8 contained ~1×10^{13} genome copies per mL (GC/mL), which is equal to ~3×10^{11} viral particles per vial. For intact analysis, the rAAV8 sample was diluted in water and directly analyzed by LC-MS. For peptide mapping, the sample was denatured by guanidine-hydrochloride, reduced with dithiothreitol, and alkylated using iodoacetamide, followed by buffer exchange using Bio-Spin columns (Bio-Rad) and enzymatic digestion (2 hr, 37ºC) using trypsin (Promega). In the heat stress experiment, 1 vial (50 µL) of Vigene rAAV8 sample (1.75×10^{13} GC/mL) was incubated at 37ºC for 24 hrs. The stressed sample was digested using the same procedures as above.

Chromatography: The separation of the intact viral proteins was performed using an ACQUITY UPLC protein BEH C4 column (2.1 mm×50 mm, 1.7 µm, 300 Å, Waters) with a 30-minute gradient. The injection volume for intact analysis was set to 10 µL equaling ~0.3 µg of protein on column. Tryptic peptides were separated using an ACQUITY CSH C18 column (2.1×150 mm, 1.7 µm, 130 Å, Waters) using a 60-minute gradient. For data-dependent CID experiments, 20 µL of the digest from the SignaGen sample were injected, which equals to 0.2 µg on column, assuming a 60% recovery throughout the sample preparation. For data-dependent EAD, 10 µL of the digest from the Vigene Biosciences sample were injected, equaling 0.6 µg on column assuming 60% recovery. LC separation was carried out using an ExionLC system (SCIEX).

Mass spectrometry: LC-MS data were acquired in the SCIEX OS software (SCIEX) using the ZenoTOF 7600 system (SCIEX). The deamidated species in the heat-stressed sample were characterized by a targeted approach using EAD MRM^{HR}.

Data processing: Intact and peptide mapping data were processed using the default workflow templates in the Biologics Explorer software (SCIEX). The MRM^{HR} data were analyzed using the Explorer module within SCIEX OS software.

RESULTS

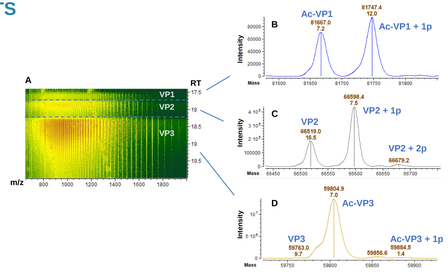


Figure 1. Intact analysis of rAAV8 capsid proteins (VP1–VP3) using the Biologics Explorer software. The ion map (A) of the Biologics Explorer software offers excellent visualization of the VPs separated chromatographically. The protein mapping results confirmed acetylation of VP1 (B) and VP3 (D) and phosphorylation of VP1 (B) and VP1 and VP2 (C). Minor species, including non-acetylated (~0.4%) and singly phosphorylated VP3 (~1.5%) as well as doubly phosphorylated VP2 (~2.3%), were also detected. All the VP peaks detected were measured with very high mass accuracies (<20 ppm). Ac = acetyl, p = phosphate. Deconvoluted masses and mass errors in ppm are indicated for every peak.

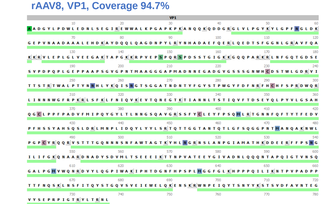


Figure 3. Sequence coverage of rAAV8 VP1. A nearly complete sequence coverage (94.7%) was obtained from a single injection of the trypsin digest of rAAV8 using a highly sensitive data dependent CID method with the Zeno trap. Most of the sequences not covered correspond to short tryptic peptides containing ≤5 amino acid residues. These short peptides were not well retained with the reversed phase C18 column. In addition, they predominantly formed singly charged species that were not targeted in this study.

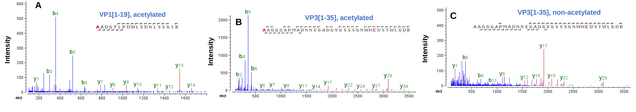


Figure 4. Identification of N-terminal peptides of VP1 and VP3. The high-quality CID MS/MS spectra led to confident identification of acetylated N-terminal peptides of VP1 (A) and VP3 (B). In addition, low-abundance VP3[1–35] without acetylation (~0.4%) was identified with high confidence.

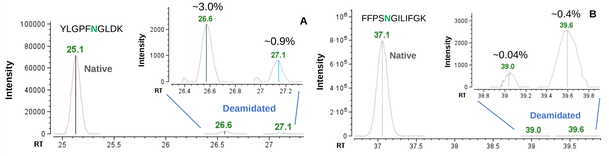


Figure 4. XICs of native and deamidated peptide species. A: YLGPF**N**GLDK and B: FFPS**N**GILIFGK. The deamidations with abundance as low as ~0.04% were confidently identified using the MS/MS spectra derived from the highly sensitive CID method, as displayed in Figure 5 below.

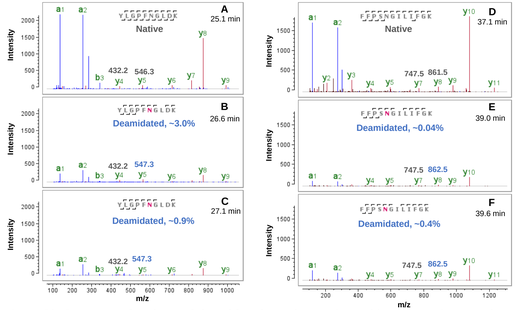


Figure 5. CID MS/MS spectra of native and deamidated species. A–C: peptide YLGPFNGLDK and D–F: peptide FFPSNGILIFGK. Confident identification was achieved for the deamidated forms present at very low level (~0.9% for YLGPFNGLDK and ~0.04% for FFPSNGILIFGK). The *m/z* values of the signature fragments (y_4/y_5 for YLGPFNGLDK and y_7/y_8 for FFPSNGILIFGK) that indicate the position of deamidation are shown in each spectrum.

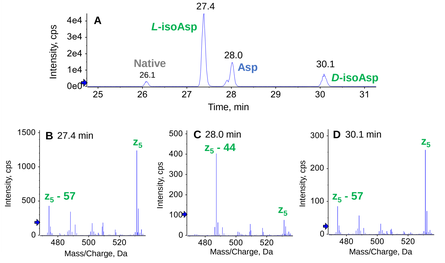
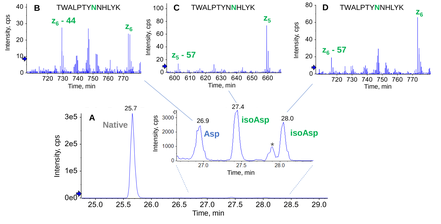


Figure 6. Signature EAD fragments of three deamidated species of peptide YLGPFNGLDK (*z* = 2+). The three deamidated species of YLGPFNGLDK in the XIC (A) were identified as the Asp or isoAsp isomers based on the detection of their respective signature fragments, for example z_5 - 57 for isoAsp (B and D) and z_5 - 44 for Asp
(C). The formation of two isoAsp species can be attributed to racemization from naturally occurring L- to D-form in lower abundance.

Figure 7. Signature EAD fragments of three deamidated species of TWALPTYNNHLYK (*z* = 3+). The 2 deamidated species at RT = 26.9 min and 28.0 min (A) were assigned as the Asp and isoAsp isomers of N8-deamidated TWALPTY**N**NHLYK, respectively, based on the detection of the signature fragments z_6 – 44 (B) and z_5 – 57 (D). The peak at RT= 27.4 minutes corresponds to the isoAsp isomer of N9-deamidated TWALPTY**N**NHLYK due to the presence of a z_5 – 57 fragments. *unrelated *z* = 4+ species.



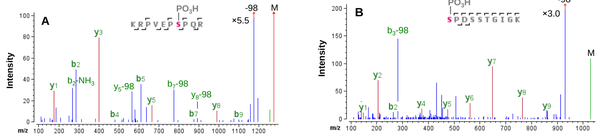


Figure 8. CID MS/MS spectra of 2 serine phosphopeptides. A: KRPVEP**S**PQR and B: S**P**DSSTGIGK. The detection of CID fragments b_2 and y_6 for KRPVEPSPQR (A) and b_2 for SPDSSTGIGK (B) allowed confident localization of the phosphate group in these two peptides.

CONCLUSIONS

- The presence of 3 capsid proteins, acetylation for VP1 and VP3 and phosphorylation for all VPs was confirmed for rAAV8 by high-quality accurate intact mass analysis
- Nearly complete sequence coverage was achieved for rAAV8 VPs in a single injection using the highly sensitive CID method with enabled Zeno trap
- Confident identification of deamidated species at extremely low-abundance (as low as ~0.04%) was enabled by excellent MS/MS sensitivity offered by the Zeno trap
- Unambiguous differentiation between Asp and isoAsp isomers, which is critical to quality assessment and stability studies of rAAV products, was enabled by EAD

REFERENCES

1 Bertin Mary *et al.* (2019) Post-translational modifications in capsid proteins of recombinant adeno-associated virus (AAV) 1-rh10 serotypes. *The FEBS J.* **286**:4964-4981.
2 April Giles *et al.* (2018) Deamidation of amino acids on the surface of adeno-associated virus capsids leads to charge heterogeneity and altered vector function. *Mol. Ther.* **26**(12):2848-2862.

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