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Optimizing Adeno-Associated Virus (AAV) Capsid Protein Analysis Using UPLC and UPLC-MS

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Abstract

This application note demonstrates the performance of the BioAccord System by developing LC-optical and LC-MS methods for the analysis of intact AAV capsid proteins and to apply these methods to the AAV8 serotype as a case study for the improved characterization of capsid proteins, including their identification, stoichiometry, and post-translational modifications. The developed methods were then applied to additional rAAV serotypes to demonstrate their general applicability for the intact protein analysis of AAV vectors.

Benefits

- An optimized LC-MS solution for the separation and intact mass analysis of viral proteins (VPs) from recombinant AAV vectors to support the characterization and development of gene therapy products
- A sensitive and quantitative LC-FLR method for stoichiometry measurement of AAV capsid proteins

Introduction

Recombinant adeno-associated viruses (rAAVs) are the most widely used vectors in gene therapy development due to their low toxicity and long-term expression ability.[1] With different tissue tropism, a total of 13 common serotypes of AAVs were isolated, and many of them have been explored for the treatment towards multiple diseases.[2] As the protector of the viral genome and mediator of cellular internalization, the AAV capsid (Figure 1) consists of proteins that share high sequence homology across serotypes,[3] requiring reliable and specific methods for vector identification during gene therapy development and commercialization. In addition, the AAV capsid composition is reported to be critical to viral infectivity and gene transduction.[4,5] To ensure the safety and quality of drug products, the structure and properties of the AAV capsid and its constituent proteins need to be well characterized and monitored throughout the gene therapy product development process. Conventional analytical techniques, such as ELISA, Western Blot, or SDS-PAGE, are frequently used to provide basic functional and compositional information, but these techniques are either laborious to deploy and validate or insensitive to AAV serotype.[6] To this end, a robust, specific method that can provide reliable results in a timely fashion is highly desired for identification and characterization of AAV capsid proteins.

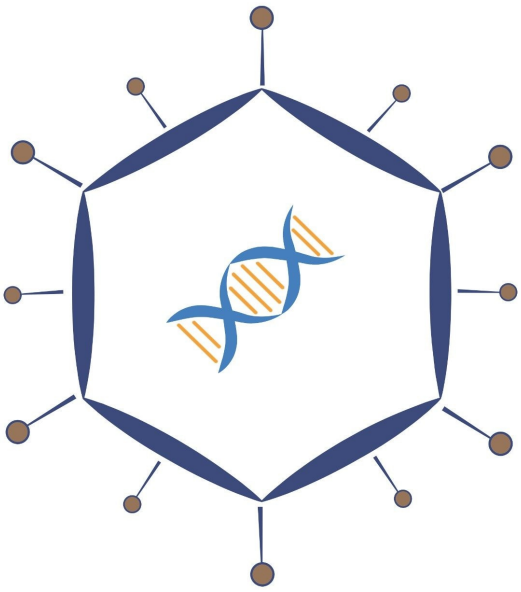

Figure 1. The illustration of an AAV. The structure includes a protein capsid and encapsulated genes.

Mass spectrometry (MS) has been widely adopted for structural analysis of proteins for its sensitivity and specificity. However, extensive training has been traditionally required for users to operate MS instruments and develop methods, limiting the wider deployment of MS technology in the biopharmaceutical industry. Designed as a robust, easy-to-use, and small footprint LC-MS platform, the Waters BioAccord System was developed to deliver fit-for-purpose MS analysis of biotherapeutics accessible to organizations and operators not previously able to deploy LC-MS technologies. As demonstrated in previous publications,[7,8,9] the BioAccord System renders a highly reproducible chromatographic separation and accurate mass measurement in an automated manner through the integration of the ACQUITY UPLC I-Class PLUS System and the ACQUITY RDa Mass Detector. The system, operating under the compliant-ready waters_connect informatics platform is ideally suited for gene therapy product development and commercialization teams to provide structure, composition, and identity information for rAAV capsids.

The objectives of this application note are as follows: To demonstrate the performance of the BioAccord System by developing LC-optical and LC-MS methods for the analysis of intact AAV capsid proteins and to apply these methods to the AAV8 serotype as a case study for the improved characterization of capsid proteins, including

their identification, stoichiometry, and posttranslational modifications. The developed methods were then applied to additional rAAV serotypes to demonstrate their general applicability for the intact protein analysis of AAV vectors.

Experimental

Chemical and reagents

Multiple serotypes of AAV samples were donated by BioReliance (Rockville, MD, USA) or purchased from Vigene Bioscience (Rockville, MD, USA), including AAV1, AAV2, AAV5, AAV6, AAV8, and AAV9. Acetic acid was purchased from Sigma-Aldrich (St. Louis, MO, USA). LC-MS-grade water and acetonitrile were purchased from Honeywell (Charlotte, NC, USA) and used as received. Waters IonHance difluoroacetic acid (DFA) (p/n: 186009201) was used as the additive to prepare the mobile phase.

Sample preparation

AAV samples were diluted with Milli-Q water to a final physical titer of 1 × 1013 GC/mL or used as is if the received sample concentration is lower than 1 × 1013 GC/mL. As previously reported,[6] AAV samples were treated with acetic acid at 10% (v/v) concentration for 15 min, then centrifuged at 12,000 rpm for 5 min. For LC-MS analysis, a 10-µL (~1 µg of proteins) AAV sample was used for each injection. For LC-fluorescence (FLR) analysis, a 1-µL (~0.1 µg of proteins) AAV sample was used, unless stated otherwise.

System Settings

Analytical conditions

Analytical system:	BioAccord incorporating ACQUITY UPLC I-Class PLUS ACQUITY UPLC FLR Detector ACQUITY RDa MS Detector
LC column:	ACQUITY UPLC Protein BEH C_4, 1.7 µm, 300 Å, 2.1 × 100 mm (p/n: 186004496); ACQUITY UPLC BEH

	C_8, 1.7 µm, 130 Å, 2.1 × 100 mm (p/n: 186002878) ACQUITY UPLC Peptide BEH C_{18}, 1.7 µm, 300 Å, 2.1 × 100 mm (p/n: 186003686)
Column temp.:	80 °C
Sample vial:	QuanRecovery with MaxPeak HPS 12 × 32 mm Screw Neck Vial, 300 µL (p/n: 186009186)
Mobile phase A:	LC-MS-grade water with 0.1% DFA
Mobile phase B:	LC-MS-grade acetonitrile with 0.1% DFA

Gradient Table:

Time (min)	Flow rate (mL/min)	%A	%B
Initial	0.200	80.0	20.0
1.00	0.200	68.0	32.0
16.00	0.200	64.0	36.0
20.00	0.200	20.0	80.0
21.50	0.200	20.0	80.0
22.00	0.200	80.0	20.0
30.00	0.200	80.0	20.0

ACQUITY UPLC FLR Detector settings

$\lambda_{excitation}$:	280 nm
$\lambda_{emission}$:	350 nm

Sampling rate:	2 Hz

ACQUITY RDa Mass Detector settings

Mass range:	400–7000 *m/z*
Mode:	ESI positive
Sampling rate:	2 Hz
Cone voltage:	65 V for full scan
Desolvation temp.:	550 °C
Capillary voltage:	1.5 kV
Lock mass:	Leu-enkephalin at 50 fmol/µL in 50/50 water/acetonitrile with 0.1% formic acid
Informatics:	waters_connect platform with UNIFI v1.9.9 intact mass workflow

Results and Discussion

RPLC-MS Method Optimization

AAV capsids are composed of three viral proteins (VP1, VP2, and VP3) at an approximately 1:1:10 ratio with the masses between 50 and 85 kDa. Due to limited sample availability and the difference in protein relative abundances, the characterization of AAV capsid proteins has been challenging through conventional LC-MS-based methods.6 When performing reversed-phase LC-MS analysis of AAV8 proteins using formic acid as

mobile phase modifier, all VP proteins co-eluted as a single TIC peak (Figure 2A). The masses of VP1 and VP2 were not obtained upon the deconvolution of the summed MS spectra (Figure 2A inset), which we attribute to ion suppression from the more abundant VP3 ions during co-elution. To improve the separation of VP proteins, a new RPLC-MS method using difluoroacetic acid as the mobile phase additive was developed on an ACQUITY UPLC BEH C_8 Column. Compared to the conventional mobile phase modifiers, such as formic acid, chromatographic resolution among the capsid proteins was improved while maintaining sufficient MS sensitivity (Figure 2B).[10] The previously co-eluting VP1 and VP2 species were now resolved from VP3, with a third additional peak observed at 12.2 min. This additional peak was assigned as a fragment of VP3 protein (labeled as "VP3 clip") based on intact mass results. To further optimize the separation of the VP proteins, columns packed with BEH particles bearing alternative bonded phases (e.g., C_4 and C_{18} ligands) were evaluated. As shown in Figure 2C, VP1 and VP2 were further resolved on the C_4 column, which can be attributed to the increased selectivity provided by the C_4 bonded phase. The pore size of the packed particles, in addition to selectivity of the surface chemistry, contributed to the improved separation. Compared to the use of the ACQUITY UPLC BEH C_8 Column packaged with particles of 130 Å pore size, the peak width was reduced using the wide pore (300 Å) C_4 column (Figure 2C), resulting in additional enhancement in MS response.

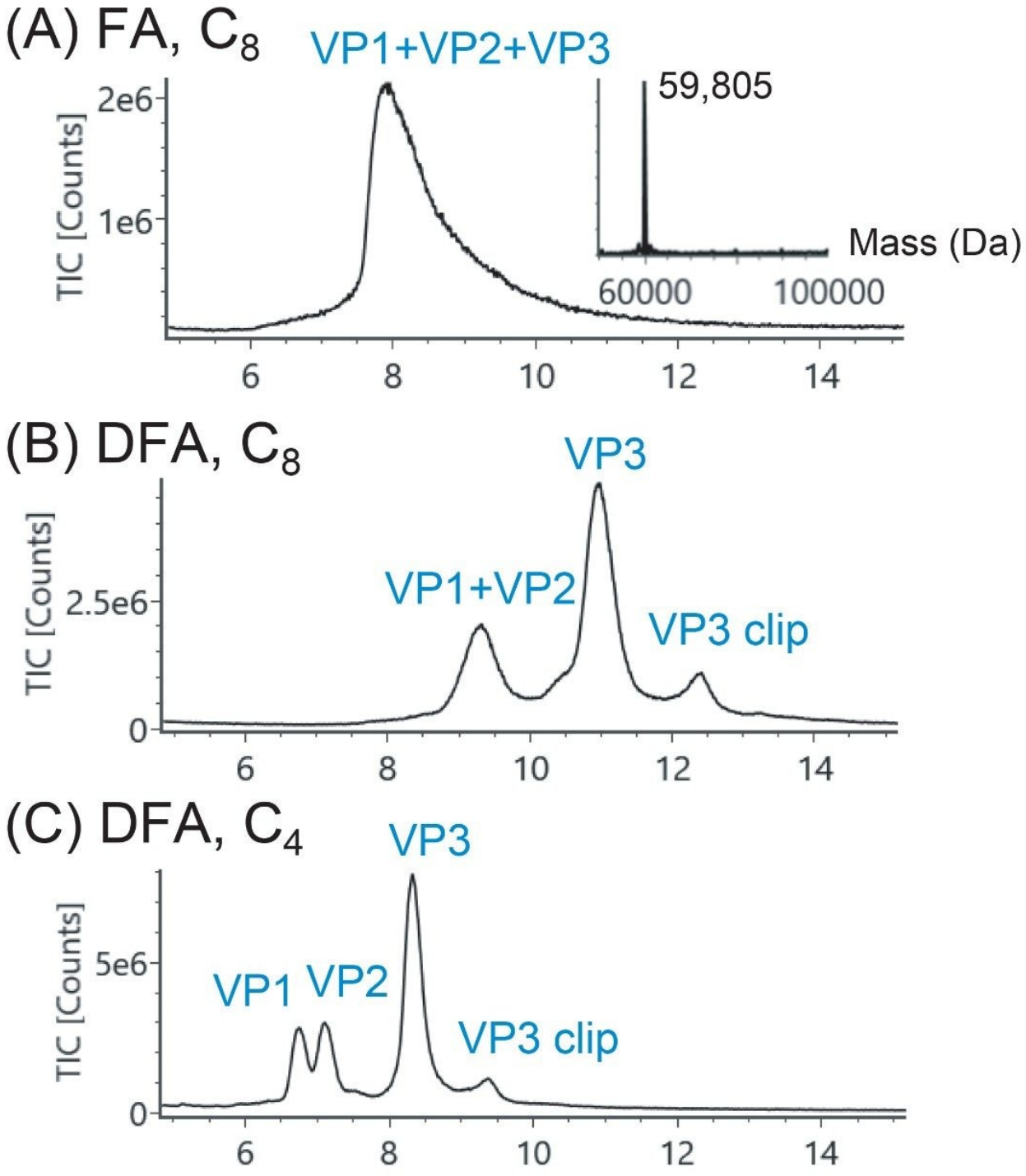

Figure 2. Method development of AAV8 capsid protein analysis. (A) Separation using ACQUITY UPLC BEH C_8

Column and formic acid as mobile phase modifier; (B) separation using the same ACQUITY UPLC BEH C_8 Column with DFA as mobile phase modifier; (C) improved resolution by using an ACQUITY UPLC BEH C_4 Column with DFA as mobile phase modifier. Gradient: (A) 70–62% A in 32 min; (B) 67–63% A in 16 min; (C) 68–64% A in 16 min. Flow rate: 0.2 mL/min.

With the optimized mobile phase and column, the improved chromatographic resolution facilitated detailed MS analysis of individual VP proteins (Figure 3A). Loading 0.5 µg of AAV8, MS data were obtained for all three capsid proteins and their variants (Figure 3B–E). The deconvoluted masses for peaks at 6.74 min, 7.10 min, and 8.32 min were observed to be 81,668 Da, 66,518 Da, and 59,805 Da, respectively. As shown in Table 1, the observed masses of these proteins were consistent with theoretical masses of VP1, VP2, and VP3,6 confirming that the developed method is suited for measuring viral protein masses. In addition, the accurate mass measurement of variants allows assignments of potential post-translational modifications (PTMs) on the VPs, including acetylation and phosphorylation, while confirming removal N-terminal methionine on VP1 and VP3. The later eluting peak at 9.38 min was found to have a mass of 50,592 Da, matching the MW of a VP3 fragment due to labile Asp659-Pro660 bond hydrolysis.[11] Together, these results demonstrated the capability of the method for developmental characterization of AAV-related products.

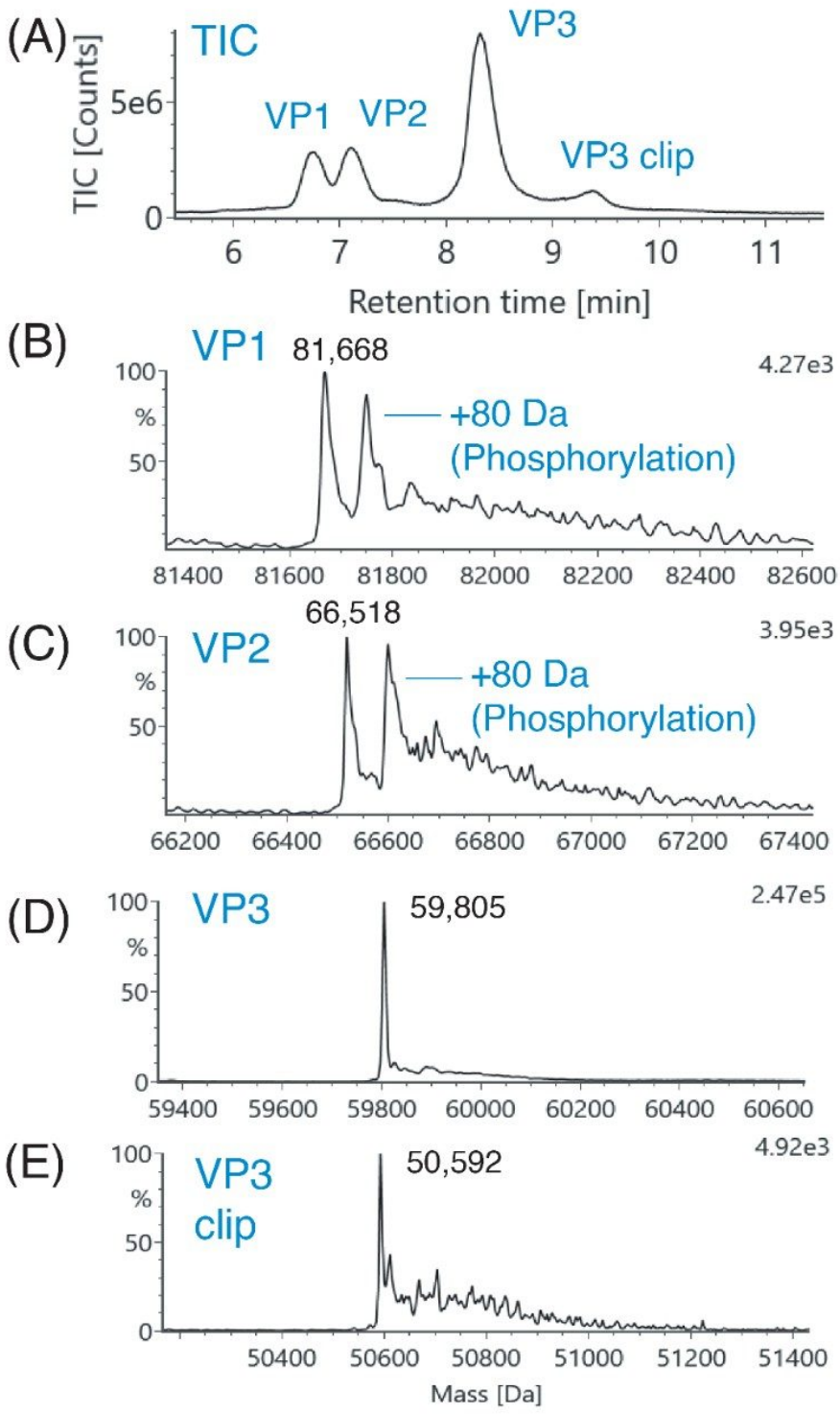

Figure 3. RPLC-MS of AAV capsid proteins with the optimized condition, incluing (A) TIC and deconvoluted MS

spectra of (B) VP1, (C) VP2, (D) VP3, and (E) the VP3 variant that fragmented at Asp-Pro bond (labeled as "VP3 clip"). Phosphorylated forms were detected for VP1 and VP2. Column: ACQUITY BEH C_4, 300 Å, 2.1 × 100 mm, 1.7 µm.

Peak	Tentative identity	AA sequence	Observed mass (Da)	Theoretical mass (Da)
1	VP1	2(Ac)-738	81,668	81,667
2	VP2	139-738	66,518	66,519
3	VP3	205(Ac)-738	59,805	59,805
4	VP3 clip	205(Ac)-659	50,592	50,593

Table 1. Assignments of AAV8 capsid proteins based on theoretical average mass of the resolved peaks in Figure 3A.

While the theoretical ratio of VP1:VP2:VP3 is at 1:1:10, it has been reported that the production process can impact the relative abundances of VP proteins.[12] The stoichiometry of VP proteins can affect vector potency,[13] making it necessary to develop a routine and reliable method to measure the ratio for the production of AAV-related drugs. The increased resolution achieved by the developed RPLC method allows the use of optical signals, such as UV or fluorescence (FLR), to directly measure the abundance of individual VP and calculate the VP1:VP2:VP3 ratio using the integrated peak areas.

Figure 4 shows the calculated relative abundance for each resolved chromatographic peak. The sensitivity of the method can be enhanced by selecting the protein intrinsic fluorescence response ($\lambda_{excitation}$: 280 nm, $\lambda_{emission}$: 350 nm) as the detection signal. With FLR detection (Figure 4B), the signal-to-noise ratio of VP3 was enhanced by approximately 50-fold compared to UV detection (Figure 4A). The use of fluorescence detection facilitates highly sensitive (50 ng) and reliable peak integration for AAV capsid proteins. To this end, the developed LC-FLR method can be used for rAAV samples with lower concentration to check the sample purity or serve as a serotype identity test for product release.

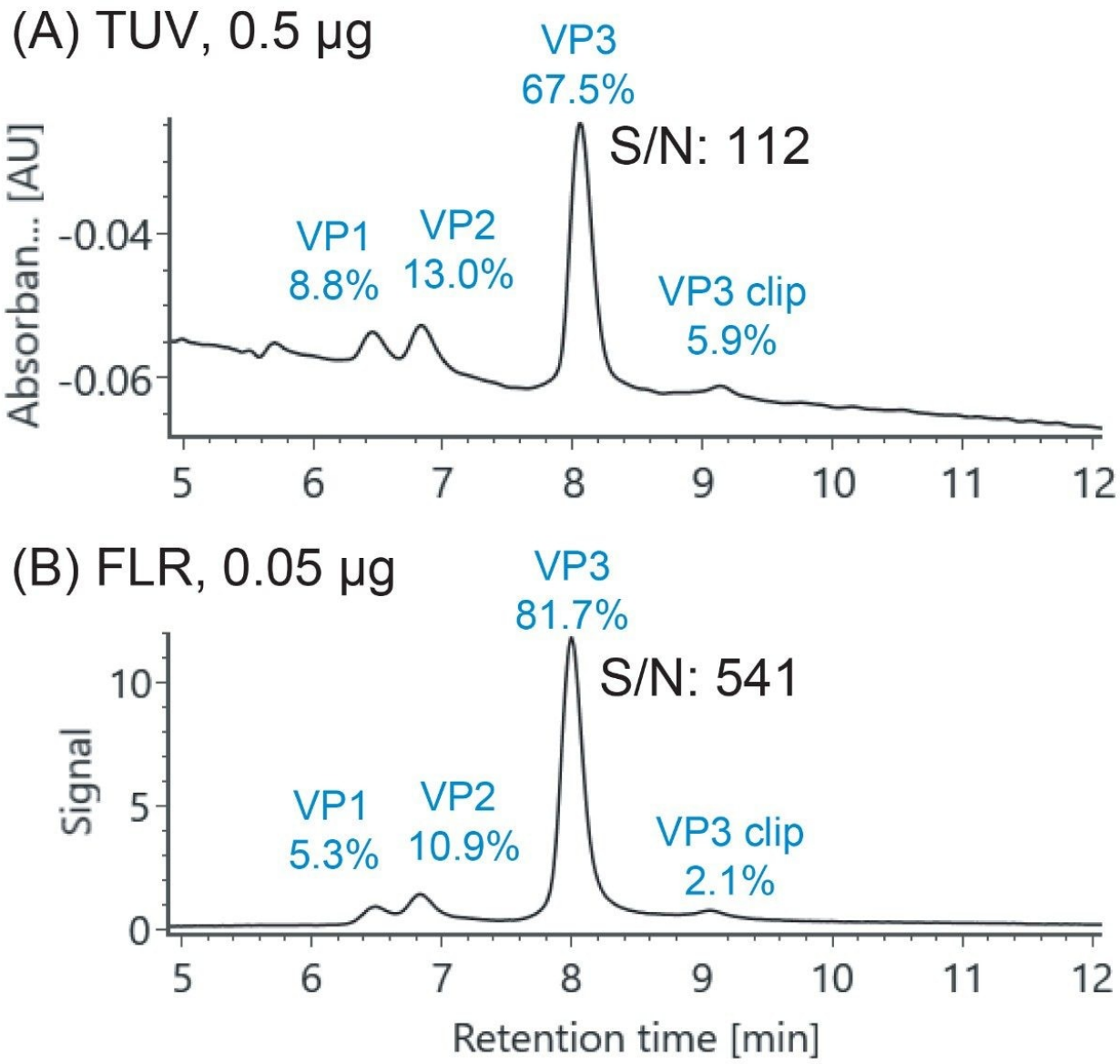

Figure 4. Relative quantification of VP proteins was measured by optical detection, including (A) UV and (B) Fluorescence (FLR). Peak annotation shows the assignment and calculated relative abundance of the detected components. With FLR detection, the S/N of VP3 is almost five times higher than the S/N of using UV detection with a 10-fold higher mass load, suggesting an approximately 50-fold improvement in sensitivity.

Applying the Developed Methods to other rAAV Serotypes

To evaluate the broader applicability of the developed methods for intact mass and stoichiometry of other rAAV serotypes, six serotypes of AAV (1, 2, 5, 6, 8, and 9) obtained from a different supplier were analyzed. Figure 5 shows the majority of these AAV serotypes (AAV1, AAV6, AAV8, and AAV9) have a comparable chromatographic profile. Furthermore, a front shoulder to the VP3 peak with the same mass as VP3 was observed (peak annotated with *) on these serotypes, which could be a structural isomer of VP3. Phosphorylation was observed for VP1 and VP2, while acetylation was also detected on VP1 and VP3 on all AAV serotypes. Table 2 lists the observed and theoretical masses of the VP proteins as well as the assignment based on the matches of these values. These data illustrate that the developed method has broader applicability for efficient separation and mass measurement of VPs across AAV serotypes.

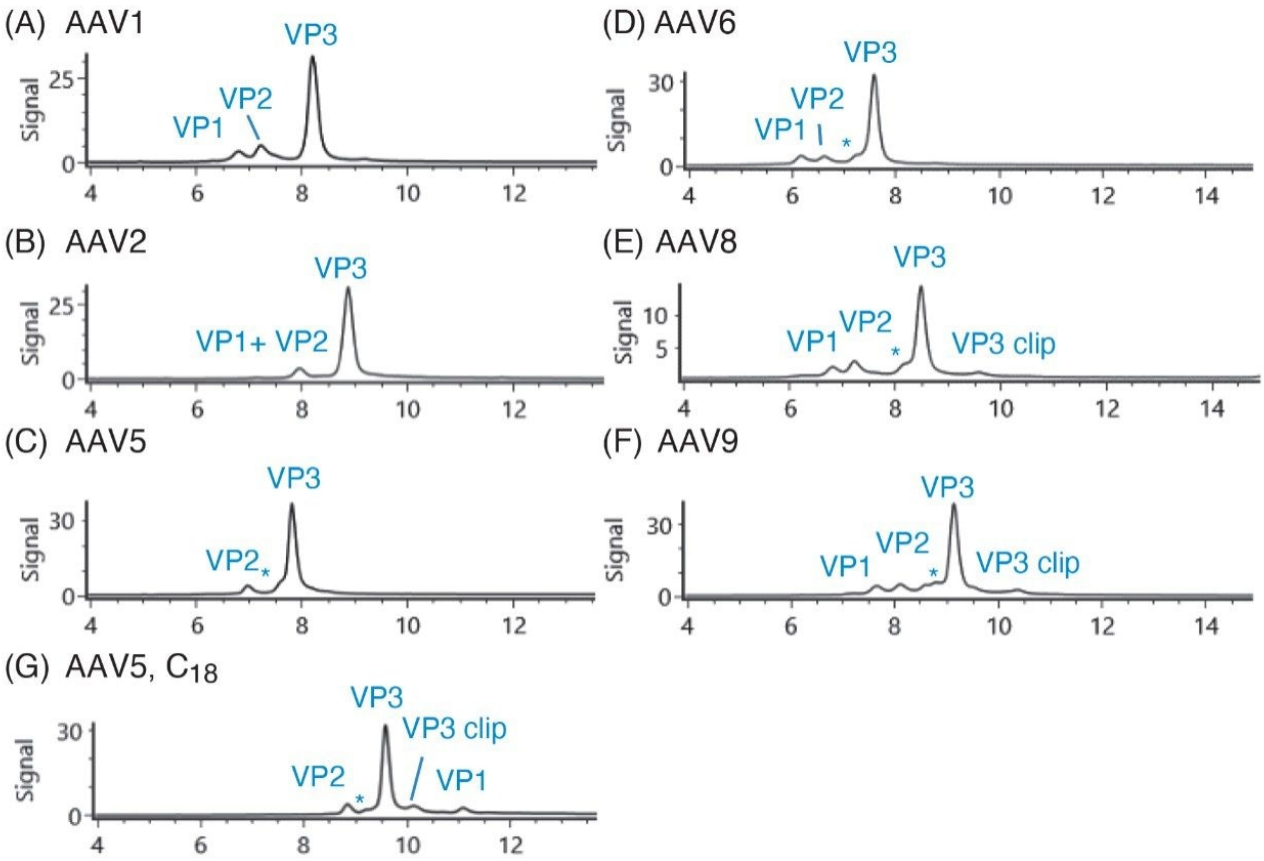

*Figure 5. Separation of six AAV serotypes, including (A) TIC, and deconvoluted MS spectra of (B) VP1, (C) VP2, (D) VP3, and (E) VP3 clip. Peaks annotated with * are observed variants with the same mass of VP3 in each AAV sample.*

Serotype	VP1			VP2			VP3		
	Observed mass (Da)	AA sequence	Theoretical mass (Da)	Observed mass (Da)	AA sequence	Theoretical mass (Da)	Observed mass (Da)	AA sequence	Theoretical mass (Da)
AAV1	81,289	2(Ac)-736	81,286	66,096	139–736	66,093	59,517	204(Ac)-736	59,517
AAV2	81,854	2(Ac)-735	81,856	66,486	139–735	66,488	59,974	204(Ac)-735	59,974
AAV5	80,336	2(Ac)-724	80,336	65,283	139–724	65,283	59,463	199(Ac)-724	59,463
AAV6	81,324	2(Ac)-736	81,322	66,094	139–736	66,096	59,518	204(Ac)-736	59,519
AAV8	81,668	2(Ac)-738	81,667	66,519	139–738	66,519	59,805	205(Ac)-738	59,805
AAV9	81,292	2(Ac)-736	81,291	66,210	139–736	66,210	59,732	204(Ac)-736	59,733

Table 2. Assignments of the capsid proteins from six AAV serotypes based on theoretical average mass and noted modifications.

While the developed method is effective in the separation of most AAV serotypes in the study, the chromatographic separation profiles differ for AAV2 and AAV5 samples. Co-elution of VP1 and VP2 of AAV2 was observed (Figure 5B), where a shallower gradient might be needed to fully resolve the peaks. VPs from serotype AAV 5 (Figure 5C) display a similar chromatographic profile as AAV2 although the deconvoluted MS spectra for the peak at 6.95 min did not show VP1. This errant behavior prompted us to explore additional experimental conditions to improve the analysis. An alternative column chemistry, an ACQUITY UPLC BEH C_{18} Column, was then evaluated to see if more desirable results can be obtained. Figure 5H shows a total ion chromatogram from the analysis of the AAV5 sample using the ACQUITY UPLC BEH C_{18} Column under the optimized gradient condition. The use of the ACQUITY UPLC BEH C_{18} Column leads to an increased recovery of VP1 protein, which now elutes later than VP3 (Figure 5H). This change in the elution order suggests the higher hydrophobicity of VP1 in AAV5, which is also suggested by the more hydrophobic residues (such as proline and phenylalanine) in its amino acid composition. Collectively, these results demonstrated that the developed LC-FLR/MS-based method is effective for the measurement of rAAV capsid proteins from a range of AAV serotypes that are commonly seen in gene therapy development.

Conclusion

In this work, an optimized RPLC method was developed using DFA as a mobile phase modifier for improved separation of AAV capsid proteins, while retaining adequate mass spectrometry sensitivity. The combination of the mobile phases and judicious selection of column chemistry results in a chromatographic separation that

facilitates intact mass measurement of individual VP isoforms and their variants. The developed method also renders a means to quantify VP stoichiometry through LC-optical response at sub-microgram levels. Overall, results acquired on the BioAccord System show that this platform can deliver the structural information necessary to improve the understanding of gene therapy products throughout product development and commercialization.

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