Application Note

Pharma & Biopharma

Agilent

Trusted Answers

Characterization of Viral Vector Particles Using the Agilent 6545XT AdvanceBio LC/Q-TOF

Authors

Wendi A. Hale and
Christopher M. Colangelo
Agilent Technologies, Inc.,
Lexington, MA, USA

Roy Hegedus and
Norman Garceau
Lake Pharma,
Worcester, MA, USA

Abstract

This application note describes a workflow for the characterization and determination of critical quality attributes (CQAs) of intact adeno-associated viruses (AAVs), together with post-translational modification (PTM) identification of the capsid proteins. The workflow comprised an Agilent 1290 Infinity II LC coupled to an Agilent 6545XT AdvanceBio LC/Q-TOF, with Agilent MassHunter BioConfirm 10.0 software used for data analysis.

Introduction

AAVs are the main viral vectors for gene therapy and have been successful in treating inherited retinal diseases and spinal muscular atrophy. An AAV is composed of an icosahedral protein shell with a single-stranded genome of approximately 4.7 kb. The intact AAVs act as a vehicle to protect and deliver oligonucleotide therapeutics. There are 13 known serotypes that transduce different cell types, allowing increased selectivity for therapeutics. As AAVs continue to be explored as therapeutic delivery platforms, it is vital to ensure that all the CQAs of the therapeutic product are maintained. Characterizing viral capsid proteins yields several challenges. The protein shell is composed of three capsid proteins, VP1, VP2, and VP3, that assemble into a 3.9 megadalton structure in a ratio of 1:1:10 with 60 capsids per virion. In addition to the low molar ratios of VP1 and VP2, all three proteins have overlapping sequences at the C-terminus. Traditionally, SDS-PAGE is used to establish the molecular weight of the capsid proteins, however, this technique provides an approximate molecular weight and may not be able to distinguish between different serotypes. Mass spectrometry (MS) is a promising method to overcome these challenges and determine CQAs of the capsid proteins. This application note describes a workflow for intact analysis and peptide mapping, including PTM identification of the viral capsid proteins. The tools used for this workflow include a 1290 Infinity II LC coupled to the 6545XT AdvanceBio LC/Q-TOF, using MassHunter BioConfirm 10.0 software for data analysis.

Experimental

Instrumentation

Agilent 1290 Infinity II LC including:

- Agilent 1290 Infinity II high-speed pump (G7120A)
- Agilent 1290 Infinity II multisampler (G7167B) fitted with 20 µL loop for intact analysis, 40 µL loop for peptide-mapping analysis
- Agilent 1290 Infinity II thermostatted column compartment (G7116B)
- Agilent 6545XT AdvanceBio LC/Q-TOF

Materials

AAV8 was produced by Lake Pharma (Worcester, MA, USA). Molecular weight cutoff filters and (*tris*(2-carboxyethyl) phosphine) (TCEP) were purchased from Millipore Sigma. Trypsin and rAsp-N were purchased from Promega.

Sample preparation

For intact analysis, AAVs underwent a buffer exchange three times at 10,000 g with a 10 kDa molecular weight filter. The buffer contained 5 mM TCEP, 80% H_2O, and 20% acetonitrile with 0.1% formic acid (v/v). After the sample was collected, it was incubated at room temperature before injection. For peptide mapping, the AAVs underwent denaturation, reduction, alkylation, and digestion. The enzymes used in this experiment were trypsin and rAsp-N.

LC/MS analysis

LC/MS analysis was performed on a 1290 Infinity II LC coupled to a 6545XT AdvanceBio LC/Q-TOF system with a dual Agilent Jet Stream source. Agilent MassHunter Acquisition (B.09.00) workstation software with the large molecule SWARM autotune feature was used for intact analysis. The instrument was further calibrated and operated in standard mass mode. The iterative MS/MS feature was used for the peptide-mapping workflow.

Data processing

All MS data were processed with MassHunter BioConfirm 10.0 software.

Table 1. Liquid chromatography parameters for intact analysis.

Parameter	Value
Column	Agilent ZORBAX RRHD 300-Diphenyl, 2.1 × 150 mm, 1.8 µm
Mobile Phase A	Water, 0.1% formic acid
Mobile Phase B	Acetonitrile, 0.1% formic acid
Flow Rate	0.4 mL/min
Injection Volume	20 µL
Gradient	0–30 min: 30–40% B; 30–38 min: 40–90% B; 38 –39 min: 90% B; 39–40 min: 90–30% B; 40–45 min: 30% B
Post Time	0 minutes
Column Temperature	60 °C

Table 2. Agilent 6545XT AdvanceBio LC/Q-TOF parameters for intact analysis.

Parameter	Value
Source	Dual Agilent Jet Stream
Gas Temperature	325 °C
Gas Flow	13 L/min
Nebulizer	35 psig
Sheath Gas Temperature	375 °C
Sheath Gas Flow	12 L/min
VCap	5,000 V
Nozzle	500 V
Fragmentor	180 V
Acquisition Rate	1 spec/sec
Reference Mass	922.0098

Table 3. Liquid chromatography parameters for peptide mapping analysis.

Parameter	Value
Column	Agilent AdvanceBio Peptide Mapping, 2.1 × 150 mm
Mobile Phase A	Water, 0.1% formic acid
Mobile Phase B	Acetonitrile, 0.1% formic acid
Flow Rate	0.4 mL/min
Injection Volume	40 µL
Gradient	0–3 min: 3% B; 3–50 min: 3–35% B; 50–60 min: 35–97% B; 60–62 min: 97% B; 62–62.5 min: 97–3% B; 62.5–65 min: 3% B
Post Time	5 minutes
Column Temperature	60 °C

Table 4. Agilent 6545XT AdvanceBio LC/Q-TOF parameters for peptide mapping analysis.

Parameter	Value
Source	Dual Agilent Jet Stream
Gas Temperature	325 °C
Gas Flow	13 L/min
Nebulizer	35 psig
Sheath Gas Temperature	275 °C
Sheath Gas Flow	12 L/min
VCap	4,000 V
Nozzle	0 V
Fragmentor	170 V
Acquisition Rate	5/3 spec/sec for MS and MS/MS
Reference Masses	121.0509, 922.0098

Results and discussion

Intact analysis on the 6545XT AdvanceBio LC/Q-TOF

While SDS-PAGE is a rapid and simple way to verify the molecular weight of AAV capsid proteins, it is not specific enough to resolve different proteoforms, such as acetylation or phosphorylated versus unmodified forms. High-resolution Q-TOF MS provides ample sensitivity, resolves PTMs, and determines accurate intact molecular mass of the proteins. Further aiding this is the large molecule SWARM autotune feature on the 6545XT AdvanceBio LC/Q-TOF, which provides excellent sensitivity for the capsid proteins by improving their transmission throughout the mass spectrometer. In addition, the ultralow TOF vacuum (e^{-8} torr) allows increased spectral clarity due to the increased mean free path of the protein molecules.

Sample preparation before LC/MS is critical for obtaining high-quality mass spectra, and can further be highlighted with our work on AAVs. Figure 1 displays the total ion chromatograms (TICs) and raw mass spectra of the capsid proteins with and without sample preparation. The buffers of the original solution are introducing contaminants into the

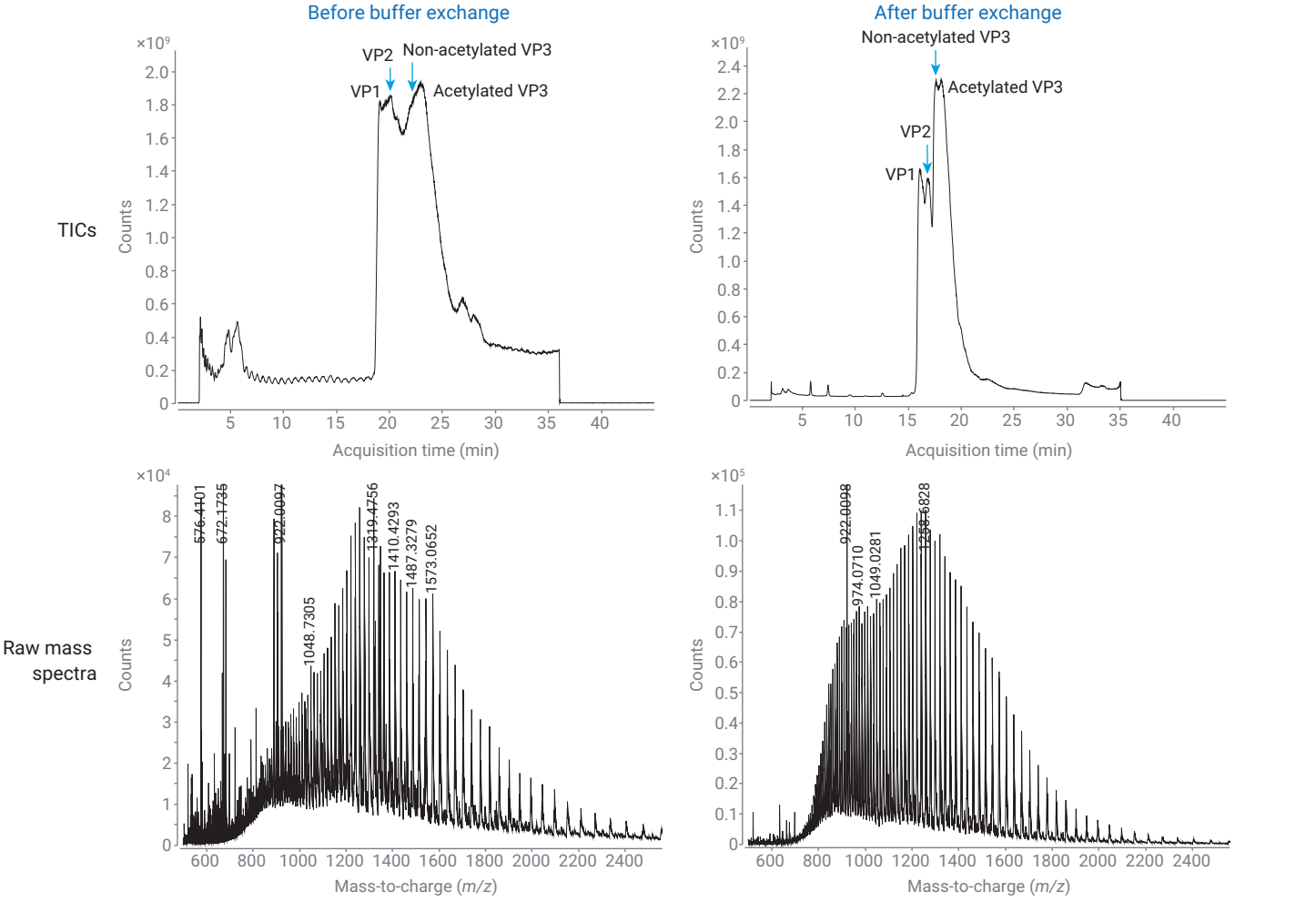

Figure 1. TICs of AAV capsid proteins and raw spectra of VP1 with and without sample preparation.

mass spectrometer. In addition, the separation between the capsid proteins is improved after buffer exchange. The raw data show that the protein has also increased 1.5 times in abundance, and the spectrum is much cleaner. The deconvoluted data are not shown here, but the spectra are clearer due to the lack of sodium and potassium adducts, leaving the interpretation of the data much simpler. Additionally, this will increase the robustness of the workflow, allowing longer times between instrument maintenance.

Figure 2 presents the raw and deconvoluted spectra of VP1. With this workflow, we were able to detect three phosphorylation sites on VP1 with less than 10 ppm error. The accurate mass data confirmed that VP1 is missing its N-terminal amino acid residue and that the new N-terminus is acetylated. There are currently very few reports of PTM analysis on AAV capsid proteins, including phosphorylation on VP1. VP2 is chromatographically separated from VP1. While mass spectrometry can separate these proteins by mass, having chromatographic separation allows less ion suppression of these two low-abundant proteins. The accurate mass data confirm at least two phosphorylation sites on VP2, and likely a third in Figure 3. Figure 4 shows that the unmodified form of VP3 is mostly chromatographically separated from acetylated VP3. Again, the deconvoluted spectra determine that both acetylated and unmodified VP3 are present with high mass accuracy. N-terminal acetylation of proteins is a common PTM and is involved in protein stability, folding, and interactions with other proteins. While VP1 was fully acetylated, approximately 70% of VP3 was acetylated. While it is not clear at this time why VP3 was not fully acetylated, it may affect the overall structure of the capsid shell of the virus. The spectral clarity provided by the improved vacuum on the 6545XT AdvanceBio LC/Q-TOF in combination with the large molecule SWARM autotune feature show all three viral capsid proteins with their PTMs with high mass accuracy, under 10 ppm for all proteoforms.

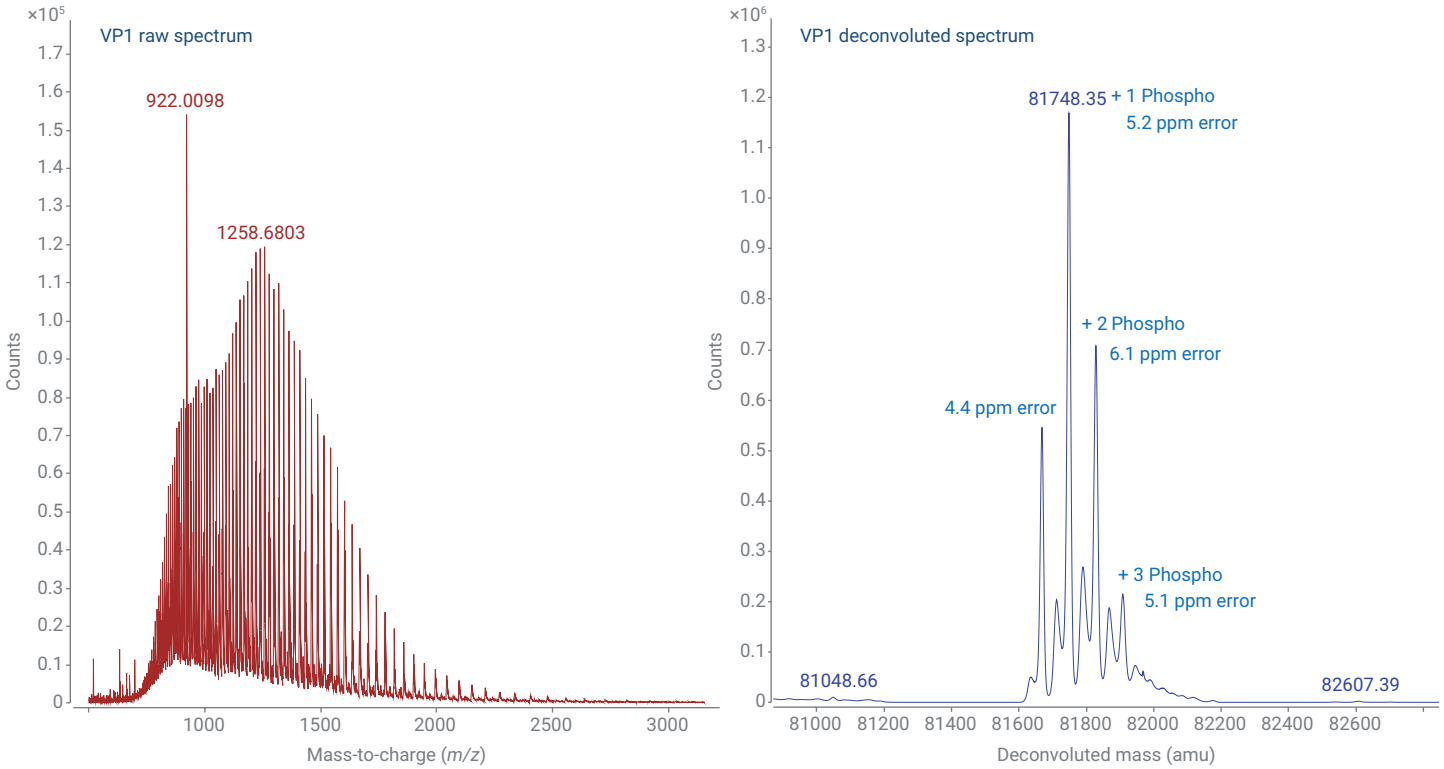

Figure 2. Raw and deconvoluted spectra of VP1 capsid protein. The native and phosphorylated forms of the protein have excellent mass accuracy.

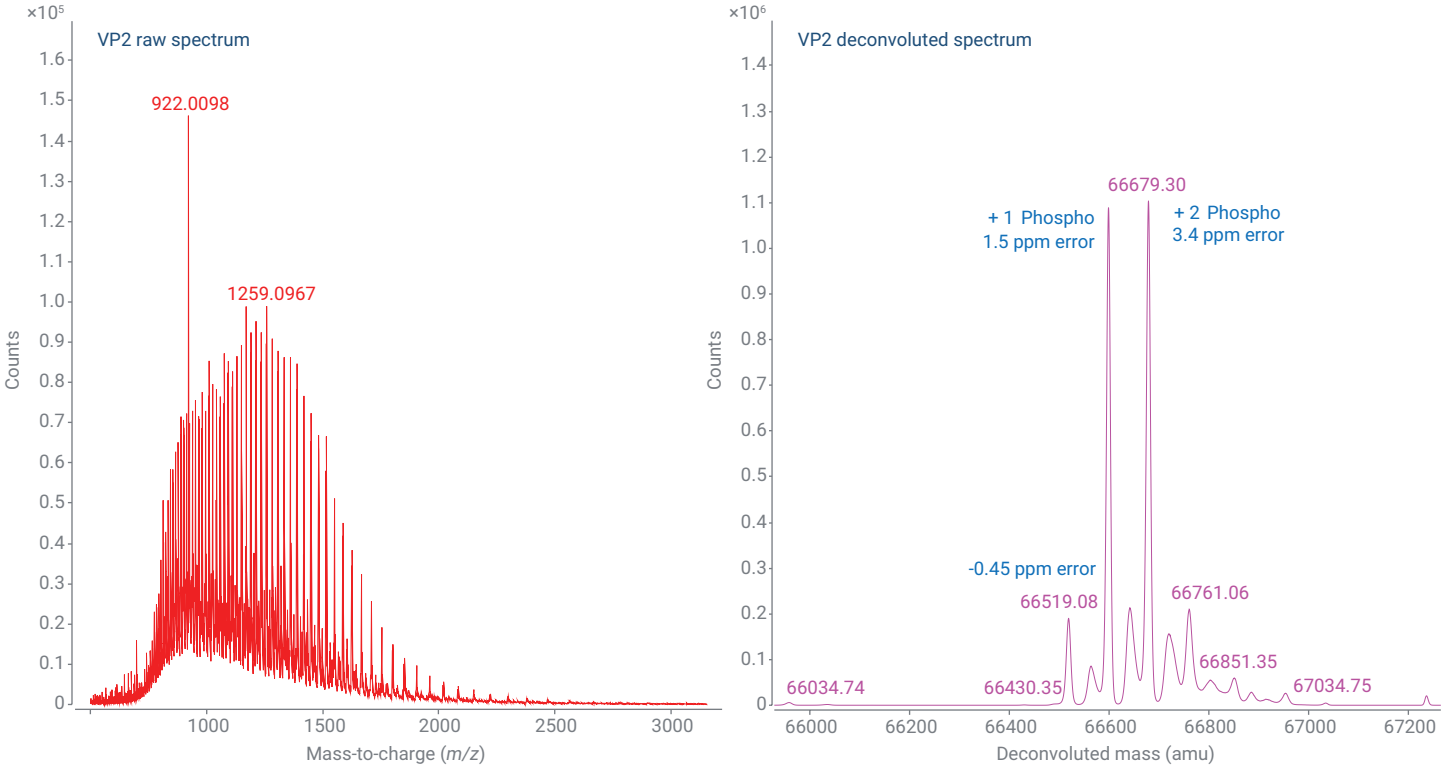

Figure 3. Raw and deconvoluted spectra of VP2 capsid protein. The native and phosphorylated forms of the protein have excellent mass accuracy, all under 5 ppm error.

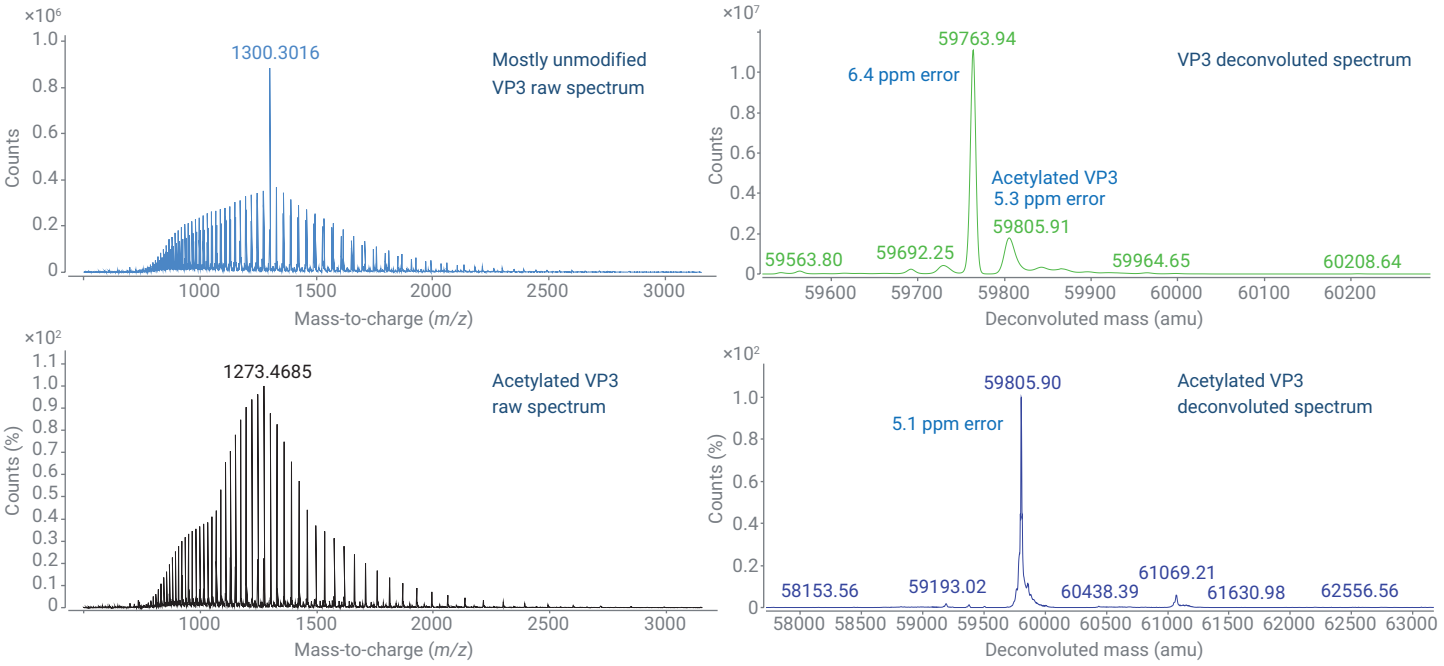

Figure 4. Raw and deconvoluted spectra of VP3 capsid protein. The unmodified and acetylated forms are mostly chromatographically separated, with excellent mass accuracy for each proteoform.

Peptide mapping on the 6545XT AdvanceBio LC/Q-TOF

Peptide mapping of biotherapeutics is an essential method to determine protein sequence and PTMs, required by the ICH, FDA, and other regulatory agencies. Although gene therapy via AAV is an emerging field, it is conceivable to imagine a future requirement for peptide mapping of the capsid proteins. As of January 2020, the FDA recommends providing information regarding primary and secondary structure including PTMs for human gene therapy drug substances. The 6545XT AdvanceBio LC/Q-TOF's iterative MS/MS feature excludes peptides from all previous runs for isolation and fragmentation, allowing selection and detection of low-abundant peptides. In addition, MassHunter BioConfirm 10.0 allows multiple runs to be selected to give a total sequence coverage. This feature is useful for combining results from iterative MS/MS runs as well as using multiple enzymes.

Determining identity of PTMs such as oxidation and deamidation is vital in determining protein stability. To have confidence with peptide mapping, all identified peptides had less than 10 ppm error and at least one MS/MS spectrum to confirm peptide sequence and to localize PTMs. Furthermore, the false discovery rate was set to 1%. AAV8's sequence has several regions where there are frequent lysine and arginine residues, rendering it difficult to obtain full sequence with trypsin alone. Therefore, rAsp-N was used to complete sequence coverage.

The largest protein, VP1, had a total sequence coverage of 97.7%, as shown in Figure 5. The solid lines represent identification of the peptide by MS/MS. The blue and green lines come from two iterative runs of the tryptic digestion, while the black and red lines come from two iterative runs of the rAsp-N digestion. MS/MS data confirm site-specific phosphorylation as shown in Figure 6. The red annotations display peptide fragments that contain the phosphorylated serine. Other common PTMs such as asparagine deamidation and methionine oxidation are present, but in low abundance, as expected. Figure 7 shows examples of these low-level modifications with the relative quantitation feature in BioConfirm 10.0. VP2 and VP3 had 98.5 and 100.0% sequence coverage, respectively. While there have been reports of N-glycosylation in AAV8, no N-glycosylation was found. This discrepancy may be due to the differences in vector expression systems.

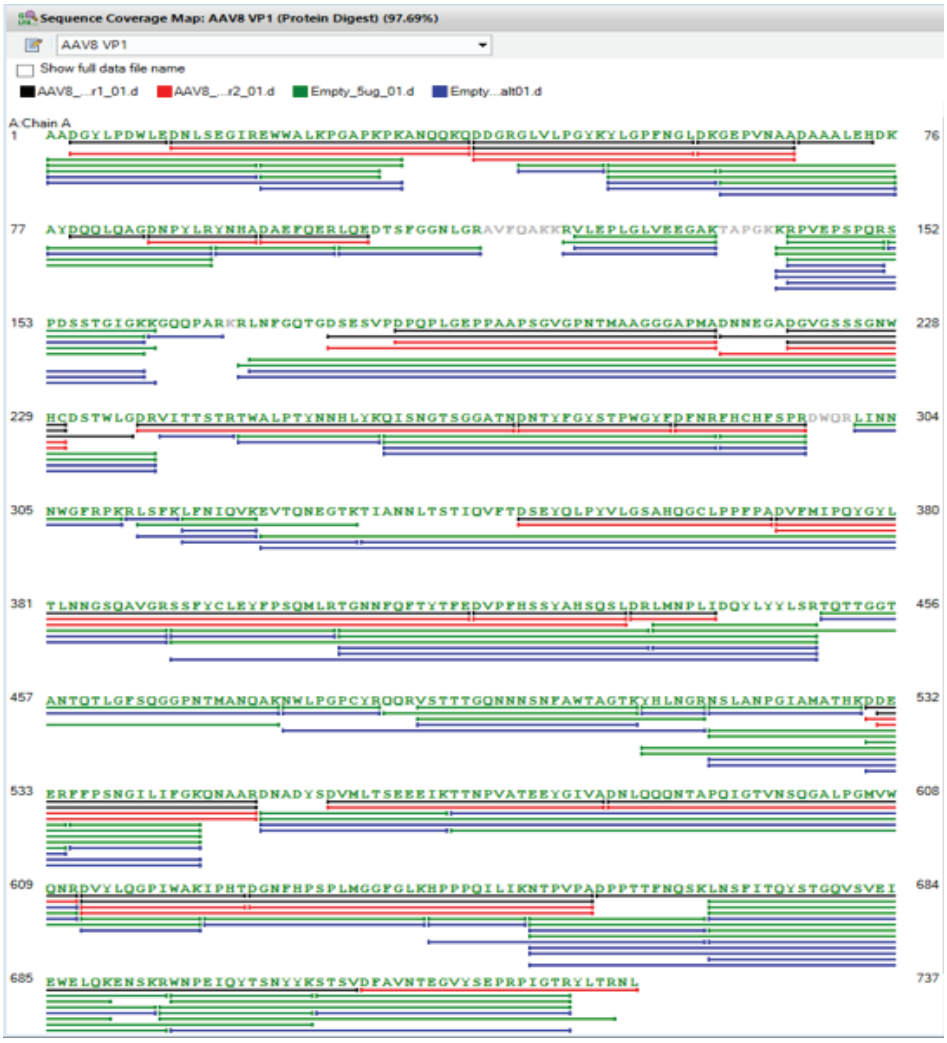

Figure 5. Agilent MassHunter BioConfirm 10.0 screenshot showing sequence coverage of VP1 with iterative MS/MS and using trypsin and rAsp-N as complementary enzymes. This protein has 97.7% sequence coverage.

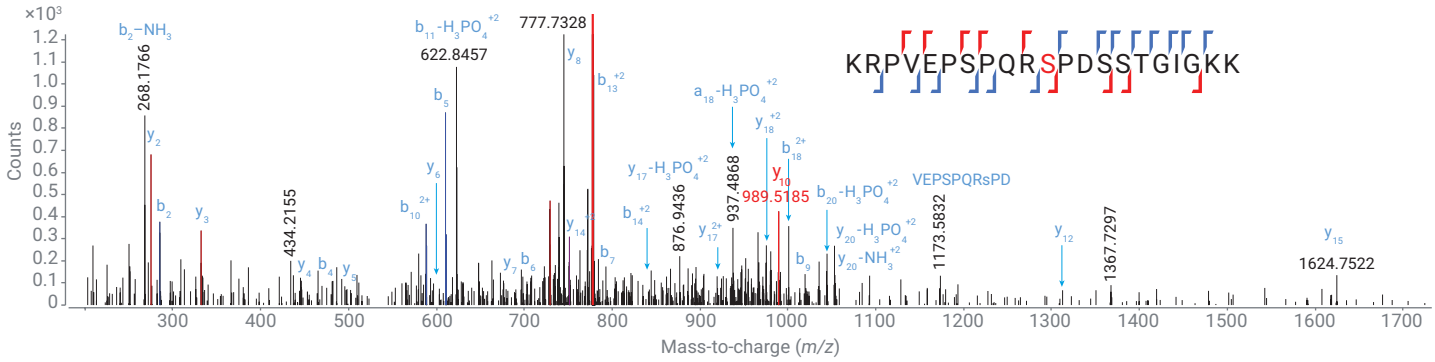

Figure 6. One example of site-specific phosphorylation with MS/MS confirmation. The annotated peptide has red markings when it contains the phosphorylated serine residue.

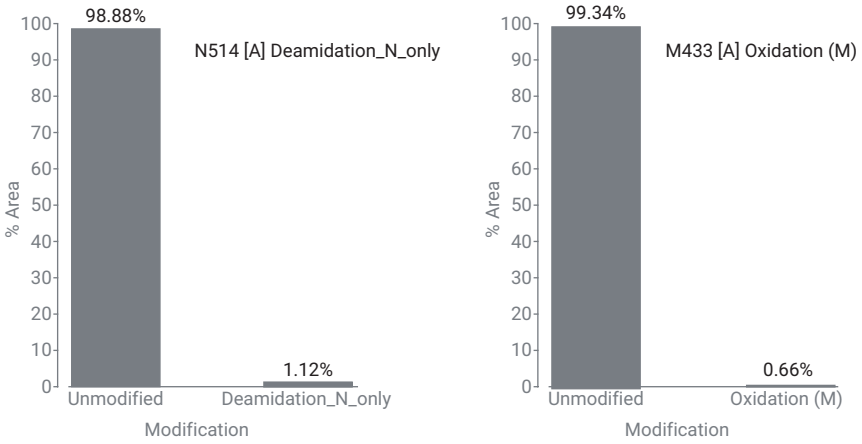

Figure 7. Examples of common PTMs in proteins: methionine oxidation and asparagine deamidation. Both peptides are minimally modified, as expected.

Conclusion

The use of AAV particles as vehicles for gene therapy has shown great promise, making characterization of the capsid proteins CQAs vital to the drug approval process. Here, we show a workflow from sample preparation through data analysis that determines the accurate mass of the capsid proteins and identifies PTMs. A 1290 Infinity II LC coupled to an 6545XT AdvanceBio LC/Q-TOF with MassHunter BioConfirm 10.0 provides a reliable and accurate solution for analysis of AAV capsid proteins.

References

1. Dalkara, D. *et al. In vivo*–Directed Evolution of a New Adeno-Associated Virus for Therapeutic Outer Retinal Gene Delivery from the Vitreous. *Sci. Transl. Med.* **2013**, *5(189)*, 189ra76-189ra76.
2. Xie, Q. *et al.* The Atomic Structure of Adeno-Associated Virus (AAV-2), a Vector for Human Gene Therapy. **2002**, *99(16)*, 10405–10410.
3. Wu, Z.; Asokan, A.; Samulski, R. J. Adeno-Associated Virus Serotypes: Vector Toolkit for Human Gene Therapy. *Mol. Ther.* **2006**, *14(3)*, 316–327.
4. Bui, H. *et al.* (**2014**) ASMS Poster WP-681.
5. Jin. X. *et al.* Direct Liquid Chromatography/Mass Spectrometry Analysis for Complete Characterization of Recombinant Adeno-Associated Virus Capsid Proteins. *Hum. Gene Ther. Methods* **2017**, *28(5)*, 255–267.
6. Giles, A. R. *et al.* Deamidation of Amino Acids on the Surface of Adeno-Associated Virus Capsids Leads to Charge Heterogeneity and Altered Vector Function. *Mol. Ther.* **2018**, *26(12)*, 2848–2862.
7. Van Vliet, K. *et al.* Adeno-associated virus capsid serotype identification: Analytical methods development and application. J. Virol. Methods **2009**, *159(2)*, 167–177.
8. Arruda, V. R. *et al.* It's All About the Clothing: Capsid Domination in the Adeno-Associated Viral Vector World. *J. Thromb. Haemost.* **2007**, *5(1)*, 12–15.
9. Office of Medical Products and Tobacco, Center for Biologics Evaluation and Research. *Chemistry, Manufacturing, and Control (CMC) Information for Human Gene Therapy Investigational New Drug Applications (INDs)*. Silver Spring, MD. **2020**, 28–29.

Acknowledgements

Agilent would like to thank Dominique Garceau, Tristan Cano, Caitlin Jaeger, and William Hermans from Lake Pharma for being involved in sample production. An additional thanks to Brian Liau and Ravindra Gudihal of Agilent Singapore for sharing ideas.

www.agilent.com/chem

DE.4113541667

Printed in the USA, July 23, 2020
5994-1980EN

Agilent
Trusted Answers