An Executive Summary

Viral Vector Process Development: Applying CE and MS Techniques for In-Process Testing

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Reliable data for accelerated development of gene therapies

Introduction

Despite its rapid growth, gene therapy development is still hindered by inadequate analytics upon which to base project or manufacturing decisions. The net results are long development times, high production costs, and excessive waste. Thus, there is an urgent need for fast, robust analytics for characterization and impurity determination of viral vectors. SCIEX workflows based on mass spectrometry (MS) and capillary electrophoresis (CE) open up new avenues to enable fast and actionable analytics, thereby accelerating development and commercialization of gene therapies.

Adeno-Associated Virus

Adeno-associated virus (AAV) is one of the more widely used gene delivery vehicles for gene therapy. AAV is non-pathogenic and has low immunogenicity. In addition, different strains of the different serotypes of AAV can be used for multiple cell types. This small virus is approximately 20 nm in diameter with an estimated size of 5.9 megaDaltons (MDa).

The AAV has an outer protein shell called the capsid, which consists of three major types of proteins referred to as VP1, VP2, and VP3. A single strand (ss) of DNA with 4.8 kilobases is encapsulated within the shell. As with any drug product, these viral vectors must be characterized for product safety and efficacy. Three essential analyses evaluate the capsid purity, its contents, and the protein chemistry.

CE-based Workflows

As viral capsids play important roles in the viral life cycle, SCIEX has developed a number of workflow suites that are amenable to viral vector-based gene therapy applications, from assessing raw materials, to identifying product and in-process sample impurities. The PA 800 Plus Pharmaceutical Analysis System can be used for analysis of capsid purity for AAV as well as full versus empty capsid analysis, genome integrity/transgene analysis, plasmid purity analysis, and charge heterogeneity analysis.

Purity analysis. A robust, reproducible, and sensitive method is required for evaluating the purity of the capsid protein. A traditional technique for this application is known as sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). However, SDS-PAGE is a poor technique for protein quantitation because of the inherent sample preparation artifacts, migration time, and staining variability. Reverse-phase high-performance liquid chromatography (HPLC) is also commonly used for assessing

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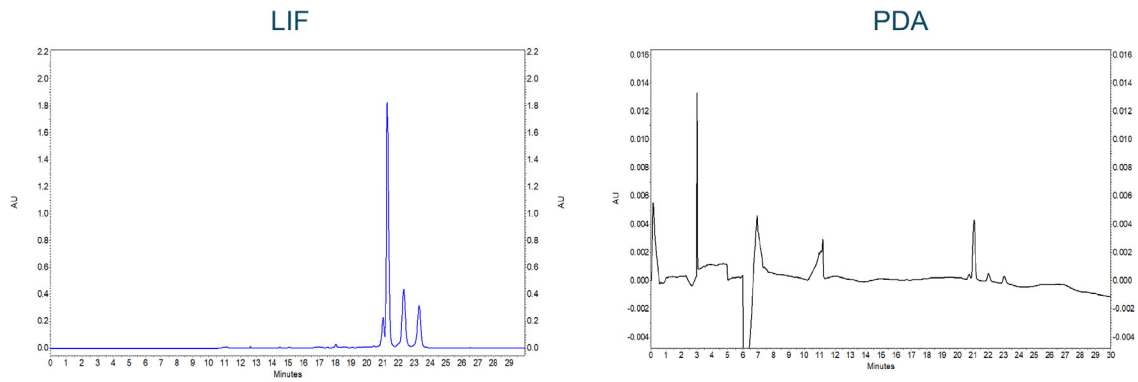
Figure 1: LIF vs PDA detection: Better sensitivity and baseline for AAV8 at 1X10^{13} GC/mL with LIF.



capsid purity. This technique resolves the different types of capsid proteins, but the migration times of the proteins are very dependent upon the serotype and can therefore shift. Because of these drawbacks, a better analytical approach is necessary.

CE-SDS is a modern-day equivalent of SDS-PAGE. The advantages of using CE-SDS are faster analysis, automation, excellent resolution, and better quantitation of the capsid proteins. As a result, this methodology has become a vital assay for gene therapy development. The SCIEX CE system is particularly useful for this application.

The CE-SDS protocol for purity analysis of capsids begins with sample preparation composed of denaturation with SDS, followed by incubation at 70° C for 5 minutes. The sample is then labeled with the protein labeling dye 3-2-(furoyl quinoline-2-carboxaldehyde), known as FQ. Labeling enables the sample to be detected with a laser-induced fluorescence (LIF) detector. The sample and a capillary cartridge are loaded onto the PA 800 Plus instrument for the analysis. A molecular weight kit is also available.

The sensitive, reproducible results presented on the left side of **Figure 1** show that the three capsid proteins were well-resolved using CE-SDS. Although unlabeled proteins can be observed with a photodiode array (PDA) or UV detector, LIF detection offers an almost 100-fold increase in sensitivity. The right side of the figure demonstrates this difference with the PDA trace exhibiting weak protein signals.

In many cases, especially in processed samples, the concentrations are extremely low and require the analytical technique to have high sensitivity. Even at 1010 genome copies (GC)/mL, the PA 800 Plus signal is strong and reproducible. The results are also consistent across all serotypes, with proven repeatability and linearity over nearly four orders of magnitude. No shifts in migration time or the order of elution are observed.

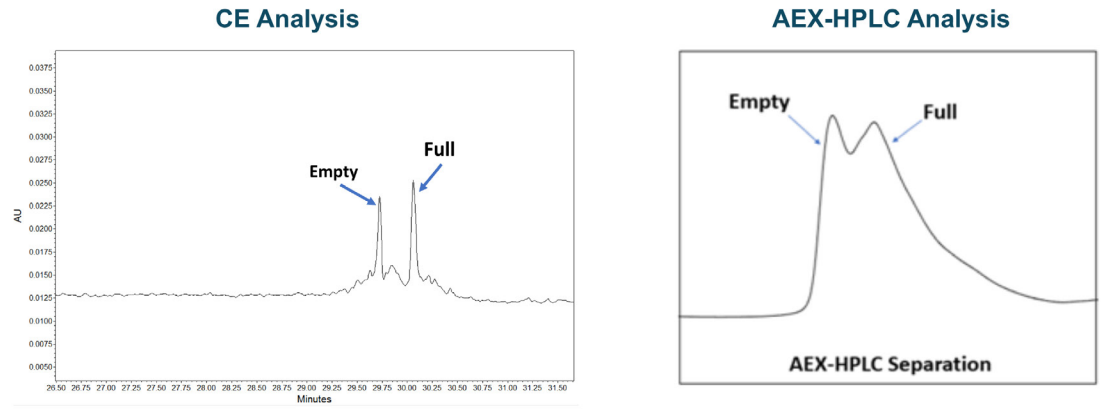
Full/empty capsid analysis. The whole viral vector, which contains both the capsid as well as the transgene (ssDNA), must be analyzed to ensure quality. How the transgene is packaged within the capsid is critical, and different forms can be created during production. In an ideal system, only full capsids would result from the production of the viral vectors, which are characterized by the entire transgene being encapsulated in the capsid. Sometimes, however, the transgene misses and does not get captured by the capsid, resulting in an empty capsid. In other cases, only a fragment becomes encapsulated, forming what is known as a partial capsid. When a capsid accidentally packages extraneous nucleic acids, such as the nucleic acids from the host cell, it is referred to as an “other” capsid. Anything other than a full capsid viral vector is considered a contaminant.

Contamination of packaged genome-related impurities affect the efficacy and safety of the vector product by increasing the potential for immunogenicity. Additionally, these types of undesired products can inhibit transduction of the full capsid by competing for vector binding sites on the target cell. Thus, assessment of contaminants in a sample is imperative.

There are several existing technologies for evaluating AAV full/empty viral capsids, but they all have limitations. One such approach is determining the percentage of the full genome in the total capsid. This is done by obtaining the number of genome vectors, which is derived from quantitative polymerase-chain-reaction (qPCR) data, as well as the total capsid numbers obtained from enzyme-linked immunosorbent assay (ELISA) data. This is a traditional method and is easy to use. However, it is limited by insufficient data accuracy and precision.

A spectrophotometric method uses the optical density of AAV samples at 260 nm and 280 nm to determine the protein and DNA content in the sample. This approach is simple, rapid, and easy to operate. Unfortunately,

Figure 2: Analysis of an AAV5 sample.



AAV5 SMP	Empty	Full (Full +Partial for cIEF)
cIEF	33%	67%
HPLC	31%	69%

it requires high purity of the AAV sample to minimize interference of the impurities with UV absorbance at the measured wavelengths, which adversely affect accuracy.

One of the traditional and more popular techniques for empty versus full capsid determination is analytical ultracentrifugation (AUC). This technology is capable of separating full, partial, and empty capsids. However, it has several drawbacks, such as the large sample quantity needed, high cost, lengthy methods, and a need for expert operators.

Electron microscopy (EM) is another frequently used technology, and it is sometimes employed concurrently with AUC. EM can reliably count the full and empty capsids as a population, but it is very difficult to distinguish the partial capsid from the full capsid. It is also quite time consuming for data analysis and is therefore not suitable for a quality control (QC) assay.

Ion exchange chromatography (IEX) is quite simple to use. IEX is used for product purification in the downstream processes, in addition to empty/full viral capsid analysis. The technique suffers from several limitations, however. IEX requires a large number of samples and is serotype dependent. Furthermore, it cannot distinguish a partial capsid from a full or empty capsid. As such, it does not yield well-resolved peaks for the three capsid forms, which can result in inaccurate determination of the full and empty capsid ratios.

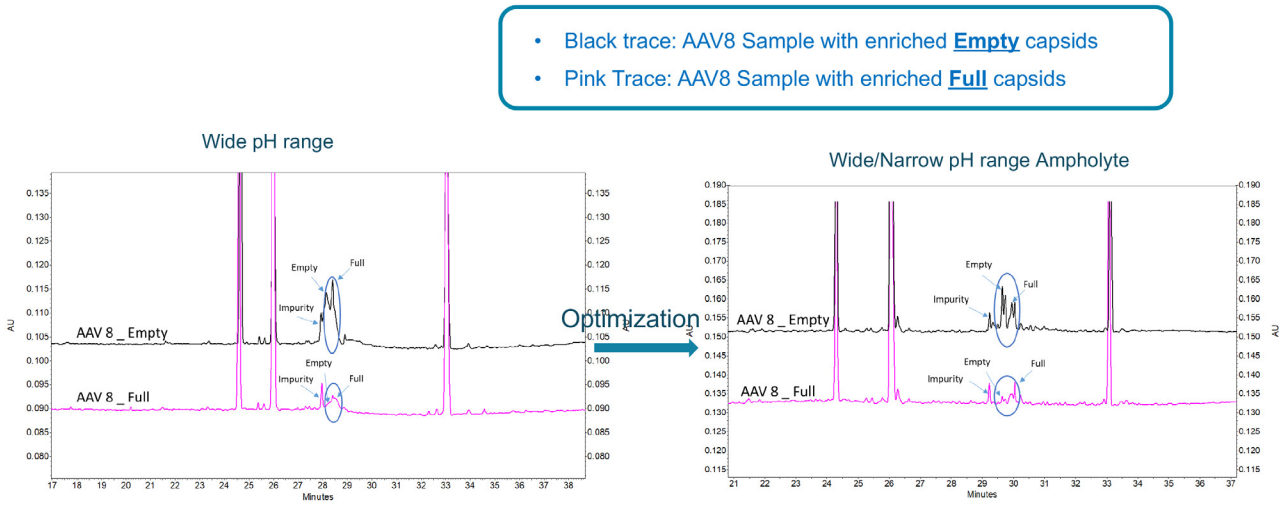
A mass spectrometric approach, referred to as charge detection MS, has recently been shown to separate the full, the partial, and the empty capsid. However, this system is an early prototype instrument that is not yet commercially available.

To meet the challenges of empty versus full capsid analysis, SCIEX has developed a protein separation workflow based on capillary isoelectric focusing (cIEF), which takes advantage of the fact that different molecules have different isoelectric points (pI). Because the full capsid contains a negatively charged ssDNA, the pI value for the full capsid is lower than that of the empty. Accordingly, the pI for the partial capsid falls in between the full and the empty.

The technique is able to separate the different types of capsids across multiple AAV serotypes. In addition, depending on the pI differences between the serotypes, the method can be adjusted by changing the pH range of the ampholyte to achieve the desired resolution. For analysis, the sample is mixed with ampholyte and loaded into the PA 800 Plus instrument. A cIEF kit is available, as is a N-CHO capillary cartridge. Detection is performed at 280 nm.

Figure 2 demonstrates the improved resolution between full and empty capsids obtained by cIEF compared to Ion Exchange chromatography. The two capsid configurations are easily separated by cIEF with a small amount of partial capsid observed in between the two peaks. In contrast, anion exchange chromatography (AEX)-HPLC did not separate the full and empty capsids, and no partial capsid was evident due to the poor resolution. For a quick comparison, cIEF peak areas for the full and partial capsids were compared to that of the full capsid in the AEX-HPLC experiment. The resulting percentages of the full and empty capsids correlated between the two analytical techniques. Other experiments showed agreement of full/empty profiles from cIEF and AUC results as well.

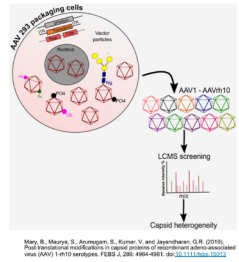
Figure 3: CE separation of an AAV Serotype 8 sample.



Mixed pH ranges ampholyte can help separate molecules with close pI values

Figure 4: Characterization of AAV capsid proteins.

- AAV capsid is the primary interface between host and virus
- Three different capsid proteins VP1, VP2, VP3
- Post-translational modifications (PTMs) are known to impact efficacy
  - Potential for PTM variability in expression systems
  - Understanding and control of PTMs ensures efficacy



The left side of **Figure 3** shows that a wide pH range ampholyte had difficulty resolving the different capsid peaks. However, an ampholyte with a mix of wide and narrow pH ranges was able to achieve good separation, as shown on the right side of the figure. In this manner, pH adjustments can help separate molecules with close pI values. Moreover, different serotypes have distinct ranges of pI values that can be easily measured by cIEF on the PA 800 Plus system. As a result, the technique can be employed to identify AAV serotypes.

MS-based Workflows

The AAV capsid is the primary interface between host and virus, such that any imperfection affects the performance of the viral vectors. The capsid proteins are highly similar and have the same C-terminus. However, they vary in length as well as N-terminus. Interestingly, all of VP3 is contained in VP2 and the entire VP2 is in VP1 (**Figure 4**).

Although there are only three proteins, VP1, VP2, and VP3, they can exist in different forms. Post-translational modifications (PTMs) are known to impact efficacy, and there is potential for PTM variability in expression systems. Thus, understanding and control of modifications can result in the design of a more effective drug. This requires a reliable analytical method for evaluation of capsid proteins.

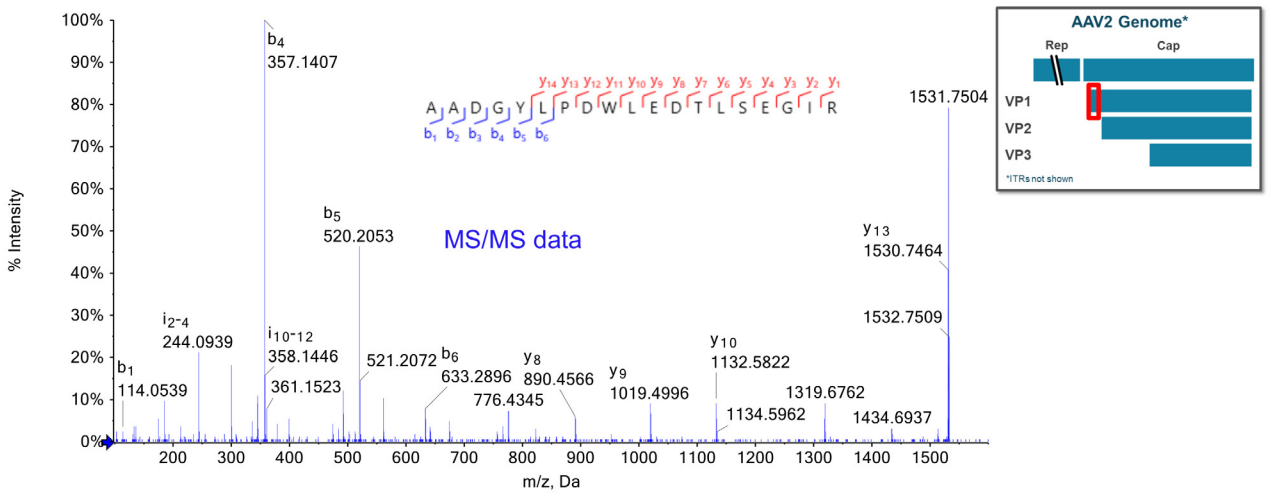
LC-MS assays can be used for characterizing the capsid sequence for PTMs with the SCIEX X500B QTOF System. To prepare the sample, it is first denatured and reduced to inhibit any potential disulfide bonds. This step breaks the capsid apart so that all of the three different capsid proteins are present independently from one another. An alkylation step follows to ensure that no cysteines reform and build disulfide bonds. Then the proteins are digested with trypsin and the resulting peptides are subjected to LC-MS analysis using a C18 column. After the data is acquired, it is processed with BPV Flex Software 2.1 dedicated biopharma software.

The software is focused on ease of use for analysts of any skill level. **Figure 5** shows a screenshot of the various functionalities, including sequence coverage on the top left. An overview of the sequence of one of the capsid proteins is depicted, in which the yellow highlighted portion is the sequence which was confirmed by the MS data. The blue and red hash marks indicate fragmentation information that was obtained with MS/MS. Each green bar represents a peptide that was identified, while the magenta highlights denote modified amino acids. This graphical representation provides users with a simple way to quickly identify modifications. This is especially useful, as the PTMs have an impact on the efficacy of the gene therapy product.

All of the information depicted in the sequence can also be found in the table below it. This offers the ability to sort and filter for specific information of interest. In addition,

Figure 5: BPV Flex Software is an easy-to-use solution for capsid analysis.

Figure 6: VP1 N-terminal sequence confirmation.

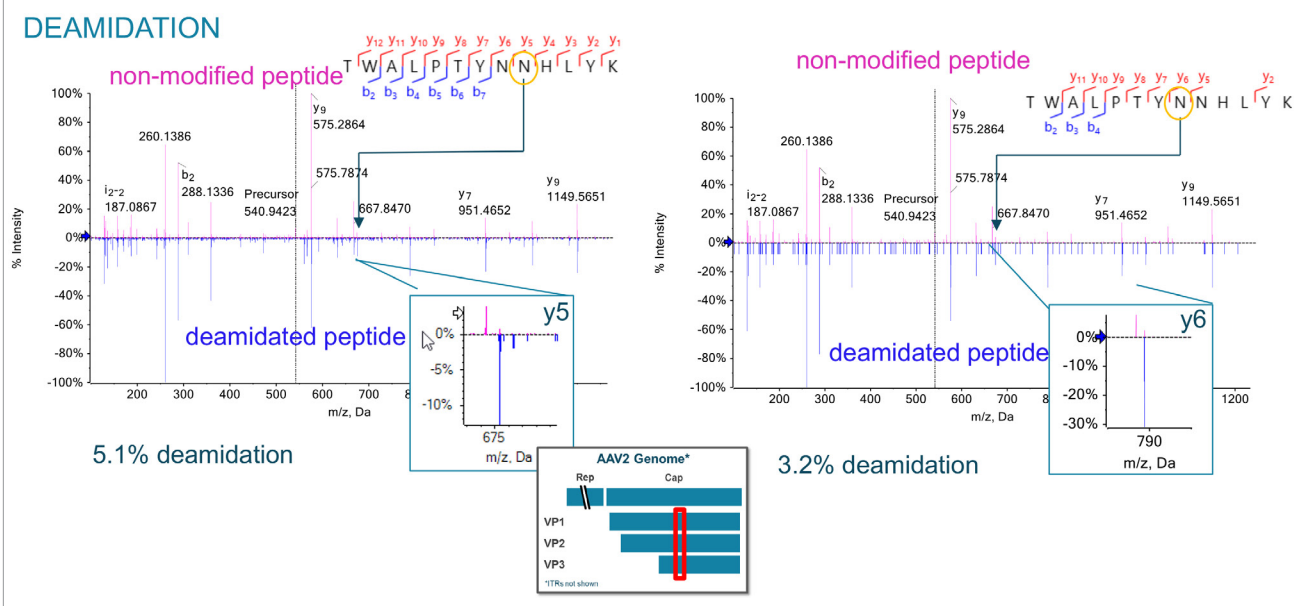


the MS and MS/MS information on the right side of the figure is directly linked to the table. Clicking on a specific peptide brings up its raw data for perusal. Thus, the BPV Flex Software delivers comprehensive data in a flexible single interface.

Analysis of the VP1 protein achieved 100% sequence coverage of the N-terminal peptide, as shown in **Figure 6**. The MS/MS data identified every fragment and confirmed 100% acetylation of the N-terminal alanine residue. Information on oxidation, deamidation, and other modifications can be readily acquired as well, including their location, based on mass shifts and fragmentation data.

An example of deamidation is illustrated in **Figure 7**. This peptide is of particular interest because it contains two deamidation sites in close proximity to each other, as two asparagines are in direct contact. The X500B QTOF System was able to automatically identify the modification and specify its location using tandem mass spectrometry. The non-modified peptide is shown on the upper trace in pink; the deamidated peptide is in blue. On the left side, there is a mass shift at the Y5, which

Figure 7: Identification and positional confirmation deamidation.



indicates that the modification is present at the circled amino acid. The right side of the figure shows a mass shift at the Y6, which is clear evidence of deamidation at that particular amino acid.

Deamidation can be specifically challenging for MS analysis because the hydrophobicity does not change as much as oxidation, making the separation more difficult via reverse-phase HPLC column. In addition, the mass shift is only 1 Dalton, which makes it less noticeable. In spite of this, the deamidation sites in the example were readily detected and located, and the software automatically provided their quantitation.

Tabulated results in the BPV Flex Software can be easily grouped, filtered, and sorted to make it simple for users to find relevant information. For example, data can be harvested for a certain peptide and modifications. Identification of PTMs is provided, including automatic, relative quantitation. Every aspect of the software design was developed with simplicity in mind.

Conclusion

SCIEX offers proven CE and LC-MS workflows that are well-suited for AAV capsid protein product quality assessment and characterization. The PA 800 Plus CE-based solutions feature excellent baseline resolution and sensitivity of intact AAV viral proteins VP1, VP2, and VP3 down to the in-process product analysis requirement level of 1×10^{10} GC/mL. The validated cIEF-based method is suitable for QC testing has the capability to perform optimal separation of full, partial, and empty capsids in AAV samples of different serotypes.

The X500B QTOF System with BPV Flex Software enables even non-MS experts to achieve high quality data. The workflow affords in-depth characterization of capsid proteins on the peptide level, including confirmation of both C and N-termini and identification of modifications, along with their localization and relative quantitation, automatically. These robust analytics deliver rapid, accurate results to strengthen gene therapy development and commercialization.