WYATT TECHNOLOGY

APPLICATION NOTE

AN1617: Quantifying quality attributes of AAV gene therapy vectors by SEC-UV-MALS-dRI

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Summary

The adeno-associated virus (AAV) is an attractive delivery vehicle in gene therapy[1,2] attributed to its mild immune response and ability to deliver its genetic payload into a wide range of host cells. The first FDA-approved AAV-based gene therapies are Luxturna® by Spark Therapeutics and Zolgensma® by Novartis for treating a rare genetic eye disease and spinal muscular dystrophy, respectively. With these approvals and many other AAV-mediated *in vivo* gene therapy drug candidates in clinical trials, it is essential that robust and reliable characterization tools are implemented in order to understand the quality attributes of this class of therapeutic products, ensuring their safety and efficacy[3].

A size-exclusion chromatography (SEC) method using triple detection–UV, differential refractive index (dRI), and multi-angle light scattering (MALS)–has been developed to measure the following three important AAV quality attributes (QAs): 1) Total number of viral capsid particles; 2) Relative capsid content (e.g., ratio of empty and full capsids); and 3) Percentage of monomer or aggregates.

Introduction

AAVs are small, single strand DNA viruses from a family of *Parvoviridae* that have become a popular viral vector for gene therapy due to their ability to infect both dividing and quiescent cells, their ability to persist in an extra-chromosomal state, and their absence of pathogenicity to the host target. Because of the stringent requirements imposed by health authorities, the AAV products throughout the manufacturing process need to be exceptionally well characterized. Some of the critical quality attributes (CQAs) of the AAV products include physical viral titer, capsid content, and product stability[4].

The measurement of the aforementioned QAs—especially the viral titer and the vector genome concentration—commonly involves approaches such as ELISA, qPCR, TEM, cryo-EM, analytical ultracentrifugation (AUC), or optical density measurements [5,6]. All these techniques are time-consuming, labor-intensive, and costly; some suffer from data inconsistency and lack of linearity for quantitation. As a result, it is difficult to implement them during the production process of the viral vectors.

In this application note, we present a simple, robust, and direct SEC method with UV-MALS-dRI detection. This method allows rapid sample analysis—with a total run time under 30 minutes per sample. The method can be readily employed to quantify AAV particle concentration, capsid content, and aggregation throughout the AAV product development and manufacturing processes.

Materials and Methods

AAV9 samples were received from Virovek Inc. (https://www.virovek.com/), which specializes in large-scale AAV production. Two samples were used for this application note: an empty AAV (no DNA payload) denoted as ‘Empty’ and a full AAV (a single-stranded DNA of full-length payload) as ‘Full’. Empty and Full AAV samples were mixed at five different ratios (v/v), 1:1, 1:2, 1:3, 1:5, 1:10, for the analysis of relative capsid content. All samples were screened with a DynaPro® Plate Reader for the presence of large aggregates before injecting onto the HPLC system.

An Agilent 1260 Infinity II HPLC system was employed with a Wyatt WTC-050S5 column (7.8 x 300 mm) and the corresponding guard column. Phosphate-buffered saline was used as the mobile phase at a flow rate of 0.5 mL/min. The volume of each injection was 30 µL.

The detection system consisted of the Agilent HPLC's UV-Vis detector measuring at wavelengths of 260 nm and 280 nm, a DAWN® MALS detector with a WyattQELS® embedded online dynamic light scattering (DLS) module, and an Optilab® dRI detector. Data from the MALS, DLS, UV (both wavelengths), and dRI detectors were collected and processed using ASTRA® software.

Results and Discussion

The dRI chromatograms obtained from two injections of the Empty and Full AAV samples are shown in Figure 1. ASTRA data analysis revealed that the aggregates and fragments were well separated from the main monomer peak without observable peak tailing. Excellent reproducibility in retention time and peak area were obtained from duplicate injections, and the peak area is linearly correlated with injection amount. These observations suggest that the SEC method developed for these two AAV samples is optimized and full mass recovery from the SEC column is likely achieved.

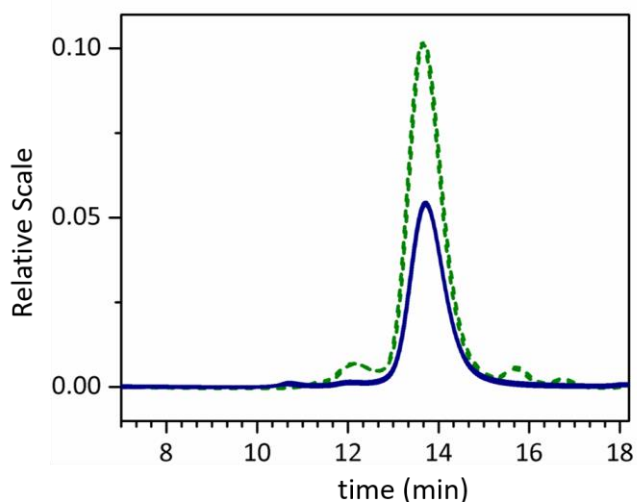

Figure 1. The dRI chromatograms obtained from two injections of Empty (green dashed line) and Full (blue solid line) samples are overlaid.

The UV extinction coefficients of the protein capsid and DNA payload were determined using the dRI and UV signals measured for the Empty and Full AAV samples along with the known d*n*/d*c* values of the capsid's protein shell (0.185 mL/g) and encapsulated DNA (0.170 mL/g). The extinction coefficients at 260 nm were found to be 1.3 mL/(mg·cm) and 25 mL/(mg·cm) for the protein and DNA, respectively. The extinction coefficients at 280 nm are 2.1 mL/(mg·cm) and 15 mL/(mg·cm) for proteins and DNA, respectively. It should be noted that for different serotypes and mutated capsid proteins, the extinction coefficients of the corresponding capsid may vary slightly.

Many important biophysical parameters of the AAV samples are obtained from ASTRA's *Protein Conjugate Analysis* features. These parameters include molar masses of the capsid and DNA, as well as the root mean square radius (a.k.a. radius of gyration) R_g and hydrodynamic radius R_h, all summarized in Table 1. The molar mass results for the Full AAV samples with respect to elution time are plotted in Figure 2, which illustrate the total molar mass of the Full AAV as well as the molar masses for the protein capsid and the encapsulated full-length DNA molecule.

Table 1. Molar mass and radius results for Empty and Full AAVs.

Sample/ injection	M_{capsid} [MDa]	M_{DNA} [MDa]	R_g [nm]	R_h [nm]
Empty/1	3.76±0.01	0	10.6±0.1	13.3±0.4
Empty/2	3.77±0.01	0	10.6±0.1	13.3±0.4
Full/1	3.77±0.01	1.16±0.01	9.8±0.1	13.4±0.3
Full/2	3.77±0.01	1.16±0.01	9.8±0.1	13.3±0.3

The eluted mass of both the protein capsid and DNA can also be measured by the *Protein Conjugate Analysis*. When combined with molar mass, either measured or theoretical, the mass can be converted to the three important quality attributes of an AAV sample: total particle concentration, relative capsid content, and percentage of aggregation.

Total particle concentration

The total AAV particle concentration, also referred to as particle titer or capsid concentration, is calculated using Equation (1):

$$C_{AAV} = m_P \times N_A / (M_{Capsid} \times v) \quad (1)$$

where C_{AAV} is the total AAV particle concentration, m_P is the total eluted protein mass, N_A is Avogadro's number, M_{Capsid} is the viral capsid's molar mass, and v is the injected volume of the AAV sample. For the Empty and Full

samples used in this study, the total particle concentrations are 8.9×10^{13} mL^{-1} and 4.0×10^{13} mL^{-1}, respectively.

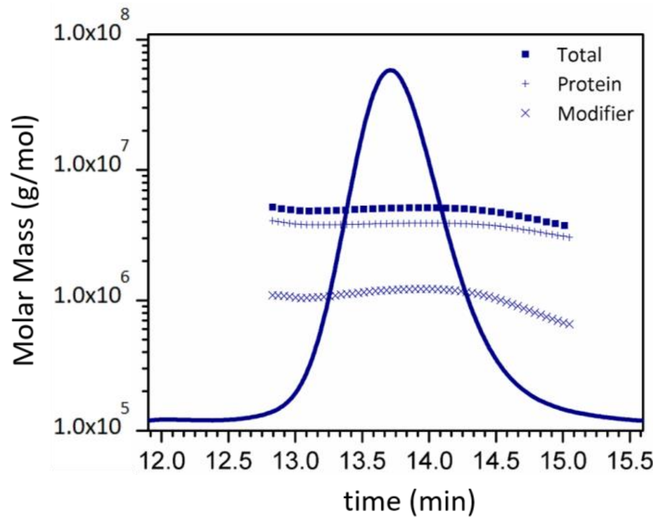

Figure 2. Molar masses for the Full AAV sample (■), protein (+), and DNA (x) are shown here overlaid with the dRI chromatogram.

Capsid content

An AAV sample during production often contains both empty *and* full AAV particles. It is critical to reliably determine the percentages of empty and full AAV particles, i.e., the capsid content in the sample, in order to meet the production and purification goals as well as the final specification of an AAV product. The total AAV concentration can be calculated using Equation (1). The concentration of full AAV particles in the AAV sample, C_{full}, can be calculated in a similar way, shown in Equation (2), by using the total eluted DNA mass, m_{DNA}, and the molar mass of the full-length DNA molecule, M_{Full}.

$$C_{full} = m_{DNA} \times N_A / (M_{Full} \times v) \quad (2)$$

Once the concentrations of the total capsids and full AAV particles are known, the concentration of the empty capsids, C_{empty}, is simply their difference.

$$C_{empty} = C_{AAV} - C_{full} \quad (3)$$

Multiple terms derived from C_{AAV}, C_{full}, and C_{empty} can be used to express the AAV capsid content. These terms include Empty AAV%, Full AAV%, Full/Empty, Empty/Full, C_p/V_g or V_g/C_p, where C_p stands for capsid particle titer and V_g is viral genome titer.

A validation test on C_p/V_g was carried out by testing five mixtures of the Empty and Full AAV samples at different ratios as shown in Figure 3. Excellent agreement between measured and expected values was obtained as seen from the plot.

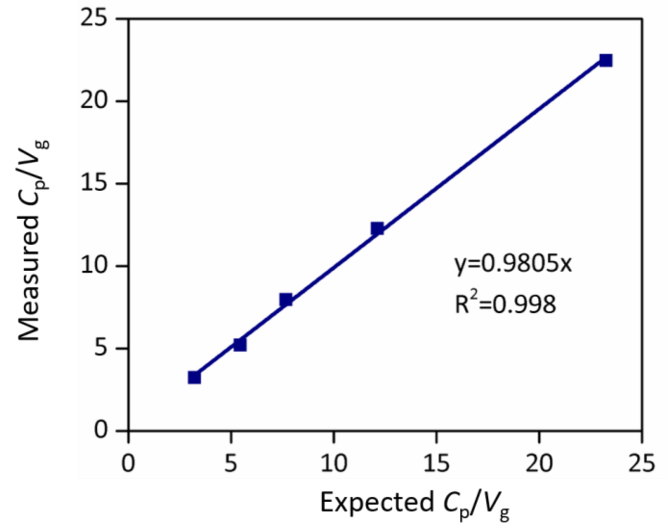

Figure 3. Plot of measured C_p/V_g from five mixtures of the Empty and Full samples at different ratios against the expected C_p/V_g values.

AAV aggregation

Using the equations from the previous sections, we calculated capsid concentrations of the monomer peak and the entire AAV peak to obtain the monomer percentage and aggregate percentage for both the Empty and Full AAV samples. The monomer in the Empty and Full sample is approximately 92% and 99%, respectively.

Large aggregates can be altered or removed by the SEC column separation mechanism. For AAV samples containing large aggregates, field-flow fractionation (FFF) employing an Eclipse™ system is used as an alternative or orthogonal tool for separating and quantifying aggregation, since FFF has no stationary phase that can interact with or damage the AAV samples.

These quality attributes were also measured on different SEC-MALS setups and by two different analysts. The results show that the method is highly robust and consistent when the SEC conditions are optimized.

Conclusions

The SEC-UV-MALS-dRI method measured particle concentration, relative capsid content, aggregation, and other quality attributes of AAV-based gene therapy vectors reproducibly and consistently. No a priori knowledge about the AAV structure or content is required. Similar methods incorporating these instruments have been validated and used in regulatory filings, manufacturing, and quality

control for other biologics. As a result, we believe this method can be implemented in the AAV manufacturing process and serve as a release assay for different production lots.

To learn more about implementing AAV characterization methods, see the AAV Services page.

Click the button below to request information on the DAWN and Optilab instruments.

Request information

Acknowledgments

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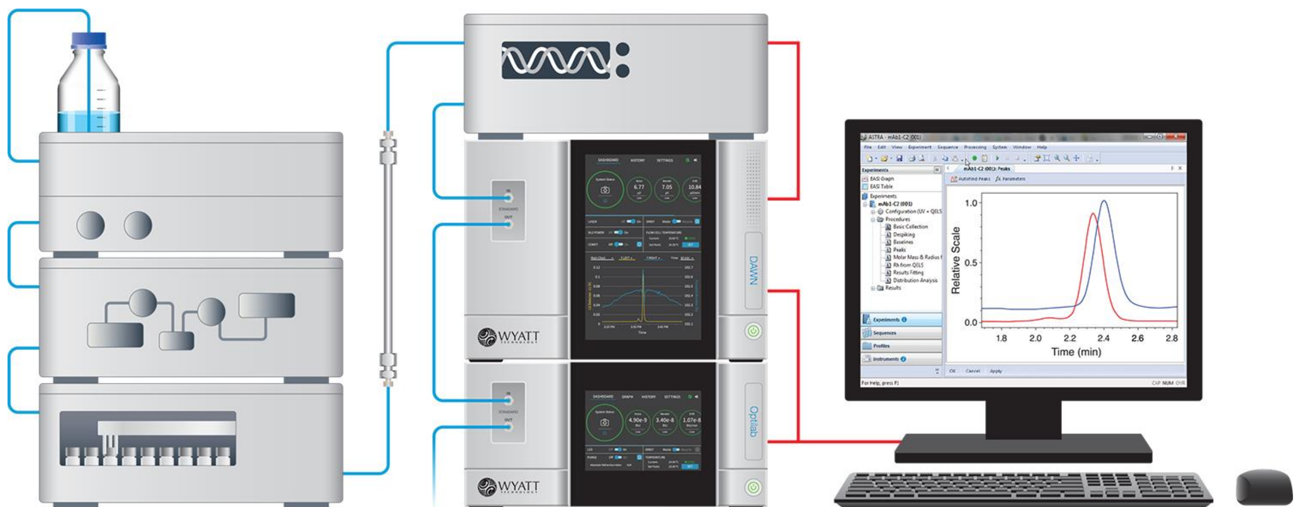

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