AAV Application Notebook



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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. As of 2024, the FDA has approved five AAV-based gene therapies for treatment of rare genetic diseases including hemophilia. 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.

Size-exclusion chromatography (SEC) combined with multiple detectors–UV280, UV260, differential refractive index (dRI), and multi-angle light scattering (MALS)– enables multi-attribute quantitation (MAQ) throughout product and process development. From a single assay, three quality attributes (QAs) can be quantified simultaneously in 30 minutes or less: 1) Total number of viral capsid particles (*Cp*); 2) Relative capsid content (i.e., full-to-total ratio *Vg/Cp*); 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 stability⁴. 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

Separation was performed using an HPLC, optimized AAV column, and AAV mobile phase, according the *AAV SOP Guidance Manual* embedded in the ASTRA[™] software. This comprehensive document serves as a complete reference and pathway to customizing, qualifying, and implementing the SEC-MALS AAV method for your specific needs. The flow rate was set to 0.5 mL/min, and the volume of each injection was 10-30 µL. The detection system consisted of the 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's Viral Vector Analysis method. All samples were screened with a DynaProTM Plate Reader for the presence of large aggregates before injecting onto the HPLC system.

AAV8 samples kindly provided by Baylor University were used to illustrate mass recovery from the XBridge^M Premier GTx BEH^M SEC column and the linearity of the total capsid titer (*Cp*) calculation.

AAV9 samples from Virovek, Inc. (https://www.virovek.com/) were used to illustrate SEC-MALS MAQ of relative capsid content (*Vg/Cp*) and aggregate quantitation. 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'.

Results and Discussion

The platform AAV SEC-MALS method provided in *AAV SOP Guidance Manual* provides excellent separation of monomer, dimer, and higher order aggregates while ensuring 100% mass recovery. The method is applicable to both naturally occurring and engineered AAV serotypes. Example 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.



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

Many important biophysical parameters of the AAV samples are obtained from ASTRA's *Viral Vector 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	<i>M</i> _{capsid} [MDa]	<i>М</i> _{DNA} [MDa]	<i>R</i> 9 [nm]	<i>R</i> 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



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

Mass recovery and system suitability

The only assumption required for SEC-MALS MAQ is that all the AAVs injected onto the column elute from the column (i.e., 100% mass recovery). To evaluate mass recovery, multiple AAV sample, along with appropriate BSA controls, were injected on the XBridge Premier GTx BEH SEC column. The UV peak area was compared with the same amounts injected without a column. As shown in Figure 3, the platform SEC-MALS method ensures excellent mass recovery.



Figure 3. Average and standard deviation of three injections with or without the XBridge Premier GTx BEH SEC column, confirm 100% mass recovery and no loss of AAV sample in the column.

As part of the system suitability checks, UV extinction coefficients for the capsid and encapsidated DNA were also determined for each AAV serotype. Seed values are provided in the AAV SOP Guidance Manual along with step-by-step procedures for measuring these parameters using the dRI and UV and extinction coefficient methods built into the ASTRA software. Typical values for extinction coefficients at 260 nm range from 1.2 mL/(mg·cm) to 1.4 mL/(mg·cm) and 23 mL/(mg·cm) to 25 mL/(mg·cm) for the protein and DNA, respectively. Typical extinction coefficients at 280 nm range from 1.9 mL/(mg·cm) to 2.2 mL/(mg·cm) and 14 mL/(mg·cm) to 15 mL/(mg·cm) for proteins and DNA, respectively.

Total particle concentration

The total AAV particle concentration, also referred to as particle titer or capsid concentration, is determined by ASTRA's Viral Vector Analysis method. The measurement requires simultaneous quantitation from two inline concentration detectors: absorbance at two UV wavelengths (260 nm and 280 nm) or UV absorbance at one wavelength combined with dRI detection. These data are combined with the measured extinction coefficients for the capsid and encapsidated DNA and expected capsid monomer molar mass to yield measured Cp.

The sensitivity and linearity of the measured Cp is illustrated for two AAV samples in Figure 4. Sample 1 (blue diamonds) was injected at three concentrations: neat $(5.2 \times 10^{13} \text{ AAV/mL})$, 1:200 dilution $(2.6 \times 10^{11} \text{ AAV/mL})$, and 1:2000 dilution $(2.6 \times 10^{10} \text{ AAV/mL})$. Similarly, Sample 2 (green circle) was injected under the same three conditions: neat $(1 \times 10^{14} \text{ AAV/mL})$, 1:200 dilution, and 1:2000 dilution. Duplicate injections were performed for all six conditions. The total eluting concentration (*Cp*) was measured using UV at 260 nm and 280 nm as the concentration source. The limit of quantitation (LOQ) of 5 x 10^{10} AAV/mL is shown by the blue dotted lines in Figure 4; however, lower concentrations may be measurable, as seen by the 1:2000 dilution of sample 1. The titer measured by SEC-MALS show good comparability with the traditional assays, like ELISA and microBCA^{7–9}. The SEC-MALS method is typically faster and more robust than traditional methods, enabling high-throughput automation, and with no reagents to validate, making it accessible as a QC method⁹.



1.



Figure 4: Linearity and sensitivity of measured Cp

Capsid content

An AAV sample during production often contains both empty and full AAV particles. It is critical to determine the percentages of empty and full AAV particles reliably, 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 AAV SEC-MALS method determines the concentration of full capsids (Vg)

and full-to-total ratio (Vg/Cp) simultaneous with the total capsid titer Cp. This simplified workflow eliminates the need for multiple assays performed in parallel (e.g., ELISA and ddPCR), each with its own reagents and each requiring separate validation. It is important to note that the SEC-MALS method cannot resolve partial AAVs in the sample; the partial AAV is considered as partly empty and partly full according to its DNA MW. However, whether the AAV sample contains partials or not, the correlation of capsid content between SEC-MALS and AUC is known. To validate the Viral Vector Analysis Vg/Cp calculation, Empty and Full samples from Virovek were prepared at the same starting concentration of 5×10^{12} AAV/mL. Aliquots of the empty and full samples were mixed at known ratios from 3% full (expected Vq/Cp = 0.03) to 97% full (expected Vg/Cp = 0.97). The eluting Vg/Cp was measured by SEC-MALS using UV at 260 nm and UV at 280 nm as the concentration source. The results for duplicate injections of all the tested combinations are shown Figure 5. Excellent agreement between measured and expected values was obtained. From these data, we surmise that SEC-MALS would be able to detect changes in Vq/Cp as low as ± 0.03 with LOQ of 0.05.





AAV aggregation

Size exclusion chromatography is well suited for separating AAV monomer from dimers, small oligomers, and large aggregates. Figure 6 shows an example of such aggregate quantitation using ASTRA's *Particle Size and Concentration Analysis* software module. The particle concentration at each eluting slice is measured by MALS (overlaid on the chromatogram) in Figure 6, yielding the following overall composition: 1.1×10^{14} monomers/mL and 2.9×10^{12} aggregates/mL. This type of analysis may provide more robust and accurate quantitation of aggregates as compared to UV or fluorescence detection alone, which can overestimate the quantity of aggregates due to UV scattering effects¹⁰.



Figure 6. Quantitation of particle concentration for AAV monomer and aggregates by SEC-MALS

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 samples¹⁰.

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 prior 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 method¹¹ 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 or request additional information at: www.wyatt.com/request-info

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APPLICATION NOTE

AN2004: Why and how to quantify AAV aggregates by FFF-MALS

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Summary

The percentage of aggregate is a critical quality attribute (CQA) of DNA-based therapeutics delivered by the engineered adeno-associated virus (AAV). This application note compares two platforms for analytical separations size exclusion chromatography (SEC) and field-flow fractionation (FFF)—along with three online detection methods—UV, fluorescence, and multi-angle light scattering (MALS). The results demonstrate that FFF-MALS is the most appropriate method for quantifying all aggregates of AAV-mediated products, from dimer, trimer, and small oligomers to large aggregates.

Introduction

Owing to its recent medical successes, AAV has emerged as the most popular gene vector for delivering small gene therapeutics¹. Ensuring the safety and efficacy of an AAVencapsidated DNA product requires the identification and quantification of its CQAs, which must be monitored throughout the development and production cycle².

AAV aggregation depends on many factors including serotype, capsid titer, empty/full ratio, formulation buffer composition, storage, and stress conditions. Aggregates in an AAV product—just as in protein-based therapeutics may decrease efficacy and increase immunogenicity, which may lead to immune-related adverse effects. Hence the degree of aggregation is one of the CQAs that must be monitored throughout the AAV product lifecycle.^{3,4}.

Size exclusion chromatography with ultra-violet absorption detection (SEC-UV) has been widely used to quantify the aggregation in therapeutic proteins and has been considered for AAV-based gene therapy products as well. For AAVs, the addition of a fluorescence detector (FLD) to the SEC-UV system will enhance detection sensitivity due to intrinsic fluorescence of the analyte. A MALS detector is often added as well, to help understand the aggregation profile and measure other AAV CQAs⁵⁻⁸.

FFF is a size-based separation technique, orthogonal to SEC, that does not incorporate a stationary phase or an affinity-dependent mechanism of action. Combined FFF and SEC data constitutes comprehensive evidence to convince regulatory authorities that all aggregates are detected and quantified in the therapeutic product^{4,8}.

We discuss the strengths and limitations of these separation and detection tools for aggregate quantification and reveal some specific details for correctly quantifying AAV aggregates by MALS.



Materials and Methods

AAV samples were produced in-house at Novartis Gene Therapies and consist of four preparations with descending level of aggregation. The key sample properties are summarized in Table 1.

These preparations were pre-screened by DLS using a DynaPro[™] Plate Reader with DYNAMICS[™] software, to assess size and size distribution, and to confirm measurable differences in aggregation.

Table 1. Details of the AAV samples used in this note.

Sample ID	Type of Capsid	Aggregation level	
Sample A	Empty	Level 4	
Sample B	Empty	Level 3	
Sample C	Empty	Level 2	
Sample D	Full	Level 1	

SEC-MALS-UV-FLD instrumentation

An Acquity[™] UPLC[™] system from Waters[™] Corp., equipped with FLD and PDA UV detectors, was employed for SEC separation and quantification. For some measurements, a DAWN[™] MALS detector was also added for molecular weight (MW) and size analysis. An appropriate SEC column was used to resolve AAV monomer and its oligomers with phosphate buffer saline as the mobile phase. Data from UV absorbance at 280 nm and fluorescence with 280 nm excitation/350 nm emission were collected with Empower software. ASTRA[™] software was used to collect and analyze MALS data.

FFF-MALS-UV-FLD instrumentation

FFF separation was carried out with an Eclipse[™] FFF instrument and separation channel supported by an industry standard HPLC pump and autosampler. Online detectors included a DAWN MALS instrument, an Optilab[™] dRI detector, and industry standard fluorescence detector (FLD)and multi-wavelength UV detector (MWD). The FFF system was controlled by OpenLab with the Eclipse plug-in, while data were collected and processed by the ASTRA software. Separation took place in an Eclipse short channel with a 350 µm spacer and regenerated cellulose (RC) membrane (10 kDa cutoff). Phosphate-buffered saline (PBS) was used as the running buffer at a detector flow of 0.6 mL/min. An optimal FFF separation method was developed. For analysis, at least two injections were made for each sample to assess the reproducibility of the method. The injection amounts were approximately $6x10^{11}$ AAV particles for all SEC and FFF runs. AAV particle concentration and the total volume of particles contained in each peak were calculated from MALS data with ASTRA software's *Number Density* method. The sphere model was used with correction factors applied to account for the non-spherical shape of the different aggregates⁹.

Results and Discussion

Though an AAV, with an approximate radius of 13 nm, is much larger than most proteins, SEC with a large pore size column (e.g., 450 to 2000 Å) is still appropriate for separating AAV monomer, small aggregates, and fragments. However, as we have learned from working with protein therapeutics, large aggregates can be dissociated by column shear, dilution, or solvent exchange, or directly removed by the column acting as a filter^{10,11}. Because of this limitation of SEC, FFF is required to assess aggregation of AAV products, even more so than for protein therapeutics.

SEC removes large AAV aggregates

All four AAV samples were analyzed by SEC and FFF with UV and FLD detectors. The UV and FLD traces from both SEC and FFF of Sample A (the sample containing the most aggregates) are shown in Figure 1. Note that the elution order in FFF is reversed relative to SEC: larger particles elute earlier in SEC but later in FFF. The peak corresponding to large aggregates was only observed in FFF (39 to 46 minutes) and not in SEC (before 10.5 minutes). These results imply that SEC—even using a column packed with large pore sized beads—cannot preserve and properly elute the large aggregates.



Figure 1. SEC-UV (a) and SEC-FLD (b) chromatograms and FFF-UV (c) and FFF-FLD (d) fractograms of AAV Sample A.

We compare the MALS data obtained from SEC and FFF for Samples A, B and C in Figure 2, where the radius determined by MALS is overlaid with the LS fractogram, plotted against elution time. The radius plotted here is the geometric radius, calculated by fitting angular data to ASTRA's sphere model. Under the conditions used for these measurements, SEC provided better resolution than FFF between monomer and dimer.



Figure 2. Radius versus elution time, overlaid with LS traces obtained from SEC-MALS (top) and FFF-MALS (bottom), for Samples A (red), B (blue) and C (gray), with descending degree of aggregation. SEC provides baseline separation of monomer and dimer, whereas FFF does not.

At the same elution time, radii from different samples were different in SEC but similar in FFF. This indicates that eluted aggregates were not as well separated in SEC as in FFF. The apparent large radii of dimer and trimer from Sample A in SEC are due to the co-eluted larger aggregates, which are prominent from 10 to 13 minutes but are believed to bleed into the dimer and trimer peaks. In addition, aggregates with radii greater than 60 nm (which will be referred to as large aggregates, L.A., in this note) were only detected by FFF-MALS, consistent with the other reports that the large aggregates are removed by the column packing or column frits and possibly degraded by shearing⁸.

Quantification of small oligomers is similar across methods

We then set out to compare the quantity of monomer, dimer, trimer, and oligomers, excluding the large aggregates, as determined by the three different detectors following the SEC and FFF separations. The results are shown in Figure 3, which plots the mass percentage of different AAV oligomeric states obtained under different combinations of separation platform and detection mode. Quantitation of mass percentage by UV and FLD is based on peak area, whereas quantitation by MALS makes use of the total particle volume calculation (provided by ASTRA software), corrected to account for oligomeric shape. Details of the correction will be discussed in the next section. Note that mass percentage and volume percentage are nearly identical quantities (after shape correction). Average values from duplicate injections were found to have a typical relative standard deviation of less than 5%.



Figure 3. Quantitation of AAV oligomeric states in Sample A by SEC (stripped bars) and FFF (solid bars) using UV (red), FLD (blue), and MALS (gray) analyses.

Ignoring large aggregates, the mass percentages of monomer, dimer, and trimer from these five methods agree relatively well. For example, the measured monomer percentage varies across the methods from 74% to 82%, dimer percentage changes from 9% to 12% and trimer percentage from 3% to 4%. However, the mass percentage of oligomers differs noticeably: 3% from FFF-MALS, 4-5% by UV, and 8-10% by FLD. Note that the 'oligomer' peak includes tetramer, pentamer and larger aggregates, constrained to a radius of less than 60 nm. The average radius of the oligomers is about 36 nm, large enough to cause a scattering effect in UV and FLD signals which likely contributed the apparently higher mass percentage from these detectors, relative to the MALS result.¹²

The results in Figure 3 suggest that SEC-UV and SEC-FLD are adequate for quantifying the mass percentage of AAV monomer, dimer, and trimer. They are not appropriate, however, for quantifying larger AAV oligomers due to scattering artifacts. We will discuss similar observations with large aggregates in the next section.

UV and FLD overestimate large aggregates

The complete mass percentage results of all the AAV species—including the large aggregates—are tabulated in Table 2. As discussed above, this table reveals that the large aggregates were not detected when SEC served as the separation platform. Additionally, due to scattering of incident UV light in the respective detectors, quantitation by UV or FLD overestimates the mass percentage of large oligomers and especially of large aggregates.

Table 2. Mass percentage of AAV monomer (M), dimer (D), trimer (T),
oligomer (O), and large aggregates (L.A.) of Sample A obtained from
SEC and FFF with different detectors.

	M%	D%	Т%	0%	L.A.%
SEC-UV	82.0	3.4	3.4	5.1	0.0
SEC-FLD	77.9	10.1	3.9	8.1	0.0
FFF-UV	48.6	7.0	2.1	1.9	40.4
FFF-FLD	61.4	9.1	3.0	6.4	20.1
FFF-MALS	86.8	6.4	1.4	2.2	3.2

To understand these results more fully, we plotted the mass fraction of each aggregate type normalized to monomer mass in Figure 4. It is evident that quantitation with UV and FLD overestimates the amount of aggregates larger than trimer, and greatly overestimates the L.A. fraction. These data and graphs enable us to conclude that FFF-MALS is the only method, among the five methods discussed in this note, that can properly separate and quantify **all** aggregates in an AAV sample.

FFF-MALS is the most appropriate method to quantify all AAV aggregates

As the most appropriate method for quantifying all AAV aggregates, FFF-MALS was then used to determine the mass percentage of each aggregate species in all four AAV samples listed in Table 1. The MALS fractograms from duplicate injections are shown in Figure 5.



Figure 4. Aggregate fraction normalized to monomer mass in Sample A, separated by SEC' (stripped bars) or FFF (solid bars) and analyzed by UV (red), FLD (blue) or MALS (gray).

The perfectly overlapped traces from duplicate injections demonstrate the excellent reproducibility of the FFF-MALS method. The flat trace from a blank injection (injection of mobile phase), which were collected at the end of the data collection sequence, show negligible carryover under the FFF method employed.

Mass percentage is typically used to quantify aggregates. For UV and FLD, mass percentage is based on the areas of monomer and small oligomer peaks. UV and FLD detectors, however, cannot measure reliable mass percentage of large aggregates, as discussed in the previous section. MALS data and ASTRA software determine size, number of particles, and total volume of particles in each of the designated peak regions, enabling the peaks to be both unequivocally identified and quantified in terms of mass percentage.

For FFF-MALS, mass percentage is related to the total volume of particles in each peak, normally calculated under the assumption of uniform spheres with the same densities. However, AAV aggregates, especially small oligomers like dimer and trimer, deviate from a spherical model such that corrections must be applied. The correction factors for each type of aggregate are detailed in Table 3. We applied the shape correction factor to FFF-MALS data from all four AAV, with the results shown in Figure 6.



Figure 5. MALS fractograms of four AAV samples (red, blue, gray, and green traces), 2x each, and a 'blank' injection (black trace). Nearperfectly superimposed fractograms from duplicate injections demonstrate the reproducibility of the method.

Table 3. Correction factor of volume of particle for monomer, dimer, trimer, oligomers, and large aggregates (L.A.).

Aggregation state	Correction factor for volume	
Monomer	1.00	
Dimer	2.22	
Trimer	1.96	
Oligomer	1.39	
L.A.	1.10	

From Figure 6, we readily conclude that the concentration of large aggregates decreases in the order from Sample A to Sample D, consistent with the expected trend. For Sample D, the full capsid sample, there are no measurable large aggregates, and monomer accounts for 98.7% of the total injected mass. The aggregation trend found in the FFF-MALS results are consistent with the one observed from the DLS measurements (not shown).

Though FFF-MALS is the tool of choice for characterizing and quantifying all sizes of AAV aggregates, it may not always be required. When it has been demonstrated that large aggregate formation is not detected under typical storage conditions, SEC-MALS is adequate for routine aggregate monitoring.



Figure 6. Mass percentage (logarithmic scale) of each aggregate type for the four AAV samples.

Conclusions

As more and more AAV-delivered gene therapy products come near to their final stages of clinical trials and begin commercialization, it is critical to accurately assess the amount of all aggregates in these products. Though SEC-UV and SEC-FLD are appropriate for measuring small aggregates such as dimer and trimer, they are inadequate for quantifying large oligomers and larger aggregates that may be present.

Large aggregates are shown to be susceptible to alteration and removal by the SEC column, making FFF the separation tool of choice for quantifying all the aggregates in an AAV sample. Furthermore, UV and FLD are not recommended for quantification by FFF. Due to scattering artifacts in these detectors, they overstate the quantity of larger aggregates. Applying the modified number density analysis to FFF-MALS data is the most appropriate method for measuring the mass percentage of aggregates, from dimer to large aggregates. With aggregates and other critical quality attributes accurately quantified and monitored, the safety and efficacy of AAV products can then be determined with greater certainty.

To learn more about implementing AAV characterization methods, see the AAV Services page or request additional information at: <u>www.wyatt.com/request-info</u>

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APPLICATION NOTE

AN5007: Characterization of AAV-based viral vectors by DynaPro DLS/SLS instruments

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Summary

When developing adeno-associated virus vectors as drug products, multiple quality attributes must be monitored to ensure a safe and efficacious final product. Three such QAs are total AAV particle concentration, aggregate content, and thermal stability. The DynaPro[™] NanoStar[™] and DynaPro[™] Plate Reader enable fast, easy measurements of these quality attributes in batch mode with combined dynamic and static light scattering.

Introduction

Adeno-associated virus (AAV) consists of a non-enveloped protein capsid with diameter ~25 nm, packed with singlestranded DNA. With a history of over fifty years of study, AAV has become one of the most popular and well-characterized gene-delivery vectors for clinical applications.^{1,2}

To provide safe and effective gene-therapy products, several quality attributes (QAs) must be quantified throughout the development and manufacturing process. The most common quality attributes relate to stability, purity, and potency of the AAV product.³

A variety of analytical tools are used to monitor QAs of AAV vectors, including analytical ultracentrifugation (AUC) and real-time PCR. However, these techniques can be labor-intensive, costly, and destructive to the sample, making them unsuitable for early-stage, high-throughput screening. SEC-MALS is non-destructive, easier and faster than the aforementioned techniques. The use of SEC-MALS with a DAWN[™] multi-angle light scattering detector to characterize AAV QAs including size, aggregation, concentration and empty:full ratio is discussed in application note AN1617: AAV critical quality attribute analysis by SEC-MALS. SEC-MALS provides detailed analysis, but requires 30 minutes per sample and may not be appropriate for screening of processes and formulations. In contrast, batch light-scattering techniques provide quick, easy, and high-throughput characterization of AAV solutions, albeit with limited resolution and accuracy in comparison. Here, batch static and dynamic light scattering (SLS and DLS) are used to quantify three AAV quality attributes:

- 1. Aggregate content
- 2. Thermal stability
- 3. Total viral particle concentration

This application note highlights all three measurements in both the DynaPro Plate Reader and DynaPro NanoStar DLS/SLS instruments.



DynaPro Plate Reader performs dynamic and static light scattering measurements in standard 96, 384 or 1536 well plates.

Materials and Methods

AAV9 samples (Table 1) were kindly provided by Virovek, Inc. (https://www.virovek.com/), which specializes in large-scale AAV production. Samples S1 and S2 represented purified AAV samples that are either 'empty' (no DNA payload) or 'full' (containing full-length, singlestranded DNA). Sample S3 is an AAV with an unknown amount of DNA payload. Samples S4 through S7 represent investigations of various buffer conditions on the stability and aggregate content of the AAVs.

Batch DLS and SLS measurements were performed with the DynaPro NanoStar and DynaPro Plate Reader as described below. Data acquisition and analysis were performed with DYNAMICS[™] software.

Table 1:	AAV	sample	description
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Sample ID	AAV	Buffer	Note
S1	AAV9	А	Purified, empty
S2	AAV9	А	Purified, full
S3	AAV9	А	Unknown DNA payload
S4	AAV9	В	Formulation testing
S5	AAV9	С	Formulation testing
S6	AAV9	D	Formulation testing
S7	AAV9	E	Formulation testing

DynaPro NanoStar

Low-volume DLS and SLS measurements were performed with a DynaPro NanoStar to assess the size and size distribution for aggregation. For samples S1 through S3, 1.25 μ L of neat AAV solutions were loaded into the quartz cuvette. Each measurement consisted of five 5-second acquisitions. DLS and SLS data were collected to determine hydrodynamic radius (R_h), aggregate content, molar mass, and viral particle concentration.

DynaPro Plate Reader

High-throughput measurements were made using the DynaPro Plate Reader. All measurements were performed in a 384-well microtiter plate (AuroraTM), and each well was loaded with 30 µL solution. The plate was centrifuged at 400 *g* for 1 minute prior to loading into the plate reader.

AAV samples S1 and S2 were diluted 1:10 in buffer. In addition, three mixtures of S1 and S2 were created with

ratios of full:empty AAV corresponding to 1:1, 1:10, and 1:50 (v/v). Each AAV solution was loaded into the microtiter plate in triplicate, and each well was capped with 1-2 drops of silicone oil to prevent evaporation. DLS and SLS data were collected to determine R_h , aggregate content, and viral particle concentration at 25 °C. For thermal stability measurements, the temperature was ramped continuously from 25 °C to 85 °C at a rate of 0.1 °C/min, and R_h was measured throughout the temperature ramp.



DynaPro NanoStar performs dynamic and static light scattering measurements in 1.25 μL quartz or 4 μL disposable cuvettes.

AAV samples S4 through S7 were loaded into single wells without dilution. The wells were sealed with tape (Nunc) to prevent evaporation. The plate was incubated at 37 °C for two hours, and the R_h distribution was measured over time to determine the effect of the formulation buffer.

Results and Discussion

Size and size distribution

Measuring the size and size distribution with batch DLS provides a quick approach to assessing the degree of aggregation in AAV solutions. Among the seven AAV samples tested, DLS revealed clear differences in particle size and aggregate content. Purified AAVs (S1 and S2), exhibited autocorrelation functions (ACF) with smooth, fast decays, characteristic of monodisperse samples of the expected size (Figure 1, top). In contrast, the autocorrelation functions for samples S4 through S7 decayed more slowly, indicative of larger aggregates present in the solution. Fitting the ACFs with a regularization algorithm provides the size distributions shown in the lower graph of Figure 1.

Purified AAV samples S1 and S2 appear to be uniform solutions with $R_h = 14.7 \pm 0.7$ nm and 15.6 ± 0.1 nm, respectively. Furthermore, the weight-average molar mass (M_w) determined by SLS with NanoStar are $3.67 \pm$ 0.01 MDa for S1 and 6.78 ± 0.03 MDa for S2. The molar mass of S1 agrees well with the capsid molar mass measured by SEC-MALS.⁴



Figure 1. Autocorrelation functions (top) and size distribution via regularization (bottom) of AAVs for formulation screening.

The increase in molar mass in S2 is consistent with the incorporation of the DNA into the viral capsid; however, its apparent molar mass is slightly larger than the value of 4.93 ± 0.03 MDa determined by SEC-MALS.⁴ This discrepancy in molar masses determined by (unfractionated) DLS/SLS and (fractionated) SEC-MALS is likely due to the nature of two methods. SEC-MALS separates aggregates from the monomer, and hence reports the molar mass of the monomer separately from oligomers. However, a batch DLS/SLS measurement reports the weight-averaged molar mass of the entire solution including monomer, dimer and higher-molecule-weight species.

Although the autocorrelation function for Sample S3 is only subtly different from samples S1 and S2 (Figure 1, top), the resulting distributions show that Sample S3 contains two size modes (Figure 1, bottom). The smaller mode, at ~ 15 nm, overlaps well with purified AAVs S1 and S2, and is consistent with monomeric AAV or a mixture of monomer and small quantities of oligomers. The second mode contains large aggregates with R_h ~100 nm.

Samples S4 through S7 appear to be highly aggregated (Figure 1). One size mode with average $R_h \sim 30$ nm corresponds to oligomers, and a second, with $R_h \sim 550$ nm, is due to large aggregates. The 30 nm mode appears quite small in terms of %Intensity but actually is dominant when viewed in the %Mass representation. These buffer conditions were thought to mitigate or promote different aggregate content. However, in this study those differences were not apparent at 25 °C, and were only noticeable upon incubation at 37 °C (see below).

Thermal stability

Two tests of thermal stability were performed in this study. First, the hydrodynamic radii of samples S1, S2, and their mixtures were measured throughout a continuous temperature ramp to quantify aggregation onset temperature T_{onset} . In addition, S4 through S7 were incubated at a constant temperature of 37 °C to observe changes in size and size distribution occurring at physiological temperature.



Figure 2. Aggregation screening of AAVs upon thermal ramping.

Both full and empty AAVs appeared to have the same T_{onset} , suggesting incorporation of the DNA payload does not change the thermal stability of the capsid. Figure 2 shows R_h as a function of temperature for empty AAV (S1), full AAV (S2), and three different mixtures. The size remains constant for all the samples until the temperature is increased beyond 60 °C. A sharp increase in R_h was then observed, growing from 14 nm to 300 nm, indicating the formation of aggregates. Onset analysis in DYNAMICS was used to determine T_{onset} and the corresponding radius for each sample. The onset temperature was the same across all samples at 62.5 ± 0.5 °C, and its corresponding onset R_h was 18.3 ± 1.1 nm.

The response of aggregated AAV samples (S4 through S7) to physiological thermal conditions was examined at a fixed incubation temperature of 37 °C. As shown in Figure 3, all four AAV solutions exhibit a decrease in R_h as a function of incubation time. At t = 0, the mean size of all four samples ranged from 450 nm to 530 nm, with S5 being the largest and S4 being the smallest. The rate of aggregate dissolution varied as a consequence of the

different formulation additives, resulting in final mean sizes of 140 nm to 400 nm.

*R*_h decreased the most for S6, which was formulated with the highest concentration of a particular buffer ingredient. It was hypothesized that this component could prevent aggregation of AAVs, and may also enable recovery of monomers from aggregated samples.



Figure 3. Top: R_h versus time upon incubation at 37 °C for AAVs in different formulations. Bottom: An example of evolving size distribution during incubation, sampled at t = 0 and t=2 hours.

Table 2. Size distribution and particle concentration by regularization in DYNAMICS for Sample S1, S2 and S3.

	Monomer (r	main species)	Aggregates		
	Radius Particle concentration (nm) (mL ⁻¹)		Radius (nm)	Particle concentration (mL^{-1})	
S1	14.7 ± 0.7	$(8.2 \pm 0.6) \times 10^{13}$	-	-	
S2	15.6 ± 0.1	$(2.7 \pm 0.1) \times 10^{13}$	-	-	
S3	14.2 ± 0.2	$(6.9 \pm 0.1) \times 10^{13}$	118.2 ± 4.2	$(1.3 \pm 0.1) \times 10^7$	

Particle concentration

Batch SLS and DLS measurements with the NanoStar and Plate Reader enable rapid guantitation of the particle concentration for both monomodal and multimodal systems. Determining particle concentration requires knowledge of the particle shape—in this case a sphere. In addition, the refractive index (RI) of the particles and buffer must be specified. DYNAMICS comes pre-loaded with a library of RI values for common materials, and when these are not appropriate, users may specify custom RI values for complex materials like AAVs. RI values of 1.43 and 1.48 were assigned to S1 and S2, respectively, based on protein and nucleic acid content and empirically validated. With these inputs, the R_h measured by DLS and the static light scattering intensity are used together to provide particle concentration. For typical AAV samples, the concentration measurable by this technique ranges from $^{6}\times10^{10}$ mL⁻¹ to $^{1}\times10^{15}$ mL⁻¹.

Figure 4 compares the particle concentrations for S1 and S2, obtained with NanoStar and DynaPro Plate Reader, to values determined by the SEC-MALS AAV method described in AN1617. These samples had previously been quantified by SEC-MALS to determine the molar mass, dimer and aggregate content, and the concentration of each species.⁴ The SEC-MALS method utilizes a well-validated analysis which is highly accurate and orthogonal to the DLS/SLS method. DYNAMICS reports concentrations of $(8.2 \pm 0.6) \times 10^{13}$ mL⁻¹ for S1 and $(2.7 \pm 0.1) \times 10^{13}$ mL⁻¹ for S2, respectively. Both NanoStar and DynaPro Plate Reader determined comparable result, within 22% of the SEC-MALS values. This excellent level of agreement means that batch measurements can be used to quickly screen AAVs for capsid concentration.

DYNAMICS can also determine the particle concentrations of multimodal samples, such as sample S3, as shown in Table 2. The monomer concentration of S3 is $(6.9 \pm 0.1) \times 10^{13}$ mL⁻¹ and the concentration of larger aggregates was measured as $(1.3 \pm 0.1) \times 10^7$ mL⁻¹. Since the degree of DNA loading was unknown, the average RI value of empty and full AAVs was used -1.46.

Three main limitations are encountered for batch concentration measurements by DLS/SLS relative to separation-based techniques such as SEC-MALS or FFF-MALS (FFF is appropriate for particles that are too large for SEC):

- Oligomers: In DLS/SLS the measured R_h value is approximately the z-average of all species present, while the scattered intensity is their weight average. Thus the presence of oligomers leads to underestimation of the concentration. In most cases the accuracy is much better than an order of magnitude.
- Size limit: the upper limit of R_h for concentration measurement is 165 nm for DynaPro Plate Reader and 175 nm for DynaPro NanoStar. This range covers AAVs and just about all viral and non-viral vectors.
- 3. RI dependence: The choice of refractive index significantly impacts the calculation. RI values of 1.43 and 1.48 have been confirmed empirically for empty and full AAVs, respectively, and agree with more rigorous characterization by SEC-MALS.⁴ Where the DNA loading is unknown, an average RI of 1.46 may be applied, consistent with literature.⁵ However, if 1.46 is selected for the analysis, while the AAVs are in actuality all full and the true RI is 1.48, this error of 1.5% in refractive index leads to an error of 33% in concentration. For screening purposes this discrepancy is usually considered acceptable. Notably, the effect on absolute accuracy does not impact linearity of the analysis for samples with identical composition.



Figure 4. Comparison of viral particle concentration determined in DYNAMICS with those determined by ASTRA's AAV method.

Conclusions

The DynaPro NanoStar and DynaPro Plate Reader offer rapid, low-volume screening of AAV quality attributes via batch static and dynamic light scattering. Both instruments characterize particle size and size distribution, thermal and colloidal stability, and total capsid concentration. These methods are non-destructive and require no method development, making them ideal for incorporation into multiple areas of AAV drug development, process development and quality control.

For more information about how to implement AAV characterization methods, see the AAV Services page.

Click the button below to request information on the NanoStar and DynaPro Plate Reader instruments or visit: <u>www.wyatt.com/request-info</u>

Request information

Acknowledgements

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APPLICATION NOTE

AN8008: Real-time monitoring and control of AAV chromatographic enrichment with RT-MALS

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Summary

Downstream purification and enrichment of full AAV capsids for gene therapy products is typically accomplished by ion-exchange chromatography (IEX). While the ratio of UV260 to UV280 absorption is often used during IEX as a proxy for the full:total capsid ratio Vg/Cp, this method does not afford process developers deep insight into accurate empty and full titers, or the presence of product-related impurities. Only when detailed offline analysis of fractions is complete does that information make its way back to process developers or manufacturing teams.

Real-time multi-angle light scattering (RT-MALS) operates in-line with bench-scale FPLC systems to monitor and quantify critical quality attributes (CQA) and identify impurities. RT-MALS provides immediate results for pool CQA values and enables optimized control over the collection of purified product. In addition, with no additional effort, RT-MALS supports the acquisition of invaluable process knowledge.

Introduction

Adeno-associated virus (AAV) is one of the primary modalities for therapeutic gene delivery. During upstream processing in the bioreactor, capsid proteins, the genome to be delivered, and in some cases, helper viruses are coexpressed. The viral capsid form and must encapsidate or be transfected with the genome. Typically, this process is inefficient; a significant portion of the capsids contain no genomic material or perhaps only a partial genome. Empty and partially filled capsids are considered impurities which must be removed in downstream purification.

In small quantities, effective enrichment of full capsids (i.e., removal of empty and partially filled viral vectors) may be accomplished by ultracentrifugation in a cesium chloride or iodixanol density gradient, which has the benefit of being serotype-independent. However, ultracentrifugation is not suitable for capsid enrichment at commercial/GMP scales where chromatographic separations are preferred.



AAV enrichment by ion-exchange chromatography

Ion-exchange chromatography has emerged as the primary commercial AAV enrichment process, taking advantage of the fact that empty capsids elute at lower ionic strength than full capsids, while aggregates elute later. Bench-scale development of AAV enrichment processes utilizes common FPLC systems such as ÄKTA[™] avant. FPLC offers a clear pathway to scaling up to larger chromatography skids.

AAV enrichment by IEX does pose certain challenges.

- The separation is usually not perfect, so empty and full capsid peaks overlap in elution time.
- Optimal IEX gradient methods may vary with AAV serotype and gene of interest (GOI), requiring renewed method development with each serotype, engineered variant or GOI.
- Variability in the buffer, column aging and differences in sample loading and content may lead to variability in elution and separation properties.

Process control in IEX enrichment

Due to these hurdles and the high value of purified viral vectors, close control of product pooling is imperative. The common approach makes use of the FPLC's UV detector: the difference in DNA content between empty and full capsids leads to different degrees of absorption at 260 nm and 280 nm. In the simplest control scheme, the eluting solution is discarded when the 260 nm signal is lower than the 280 nm signal, and the pool is collected when the opposite is true.

The UV260/280 crossover control method suffers from several deficiencies.

- No positive identification: it does not distinguish between empty or full capsids on the one hand and free protein or DNA on the other.
- Sample-dependent: the empty-full capsid ratio at the UV260/280 crossover point differs depending on serotype and GOI size.
- Aggregate-blind: it gives no indication if the eluting viral vectors are monomeric or aggregated.

These deficiencies are overcome by adding an ultraDAWN[™] RT-MALS instrument in line with the FPLC system. RT-MALS provides positive identification of AAVs, readily accounts for different serotypes, and gives direct and quantitative information on capsid loading and aggregate levels. All of these occur in real time and can be used for downstream process control and feedback/feed-forward modeling.

RT-MALS in-line monitoring of AAVs is closely related to offline analysis by SEC-MALS [1-3] or IEX-MALS [4], which predate the application of RT-MALS to AAV purification. In the way of an introduction to the method, let us first review the offline analyses.

Offline analysis of AAVs by MALS

The combination of analytical chromatography (sizeexclusion or ion-exchange) with detection by MALS has emerged as one of the most effective, sensitive and robust methods for the determination of three AAV CQAs: capsid ratio (Vg/Cp), titer (Cp) and aggregate content. The method further provides extended characterization, including capsid molar mass, particle size, and extinction coefficients of the capsid and genome. Analysis is generally serotype-independent and, as a chromatographic method, fully automated with no need for special reagents.

In MALS-based analysis of AAVs, the online MALS signals are accompanied by two distinct concentration-dependent signals – UV absorption and/or differential refractometry (dRI).

- MALS + two UV wavelengths (260 nm and 280 nm) is more sensitive and suitable for IEX as well as SEC, but requires knowledge of specific extinction coefficients for the most accurate analysis of different serotypes.
- MALS + one UV wavelength + dRI is less dependent on serotype, and can determine UV extinction coefficients directly, but is less sensitive. Due to the effect of variations in salt concentration on the RI signal, when used with IEX this method may require special algorithmic means such as 'baseline subtraction' wherein measurements taken of a run with no viral vectors are subtracted from the run with viral vectors.

Scattered intensity data measured by MALS are combined with the two concentration signals to calculate the molar masses of the capsid, M_{capsid} , and entrained DNA, M_{DNA} , as well as total eluting capsid mass, m_{capsid} , and DNA mass, m_{DNA} , for every data slice (usually every 0.5 or 1 second for HP-SEC or every 0.1 second for UHP-SEC).

Vg/Cp analysis: The value of M_{genome} determined from MALS is compared to the expected genome molar mass calculated from the genome's sequence, and the ratio is taken as Vg/Cp. Since the analysis averages the values of all capsids present in the flow cell during a given data slice, it will not discriminate between mixtures of empty and full capsids versus partially full capsids or cases where DNA is bound externally to an empty capsid.

Titer: m_{capsid} is used along with the expected monomeric capsid molar mass to determine the capsid concentration in each data slice and integrated over the peak to determine overall capsid titer Cp. Empty and full titers are calculated from Cp and Vg/Cp.

Aggregation: If the virus is aggregated, M_{capsid} will be an integer multiple of the expected capsid molar mass. Oligomers may be fully separated from monomers such that the monomer molar mass is directly determined and can be applied to interpreting the aggregate peaks in terms of oligomeric state. If monomers and aggregates co-elute, they may exhibit a gradually increasing M_{capsid} value as the eluting aggregate content increases.

More information on the characterization of AAV may be found in AN1617: AAV critical quality attribute analysis by SEC-MALS and in references 1-4.

The angular scattering data from MALS alone (without concentration data) are analyzed to determine the particle size R_g , the radius of gyration. Monomers and aggregates exhibit distinct R_g values, providing further insight on aggregation levels. More information on the use of MALS to quantify aggregates small and large may be found in AN2004: Why and how to quantify AAV aggregates by FFF-MALS.

Analytical SEC-MALS and IEX-MALS utilize Wyatt's DAWN[™] MALS instrument and Optilab[™] dRI detector along with HPLC separation, for example by a Waters Arc[™] Premier system. microDAWN[™] and microOptilab[™], respectively, are used with Waters' Acquity[™] for UPLC[™] separations. Wyatt's ASTRA[™] software controls the run, and data are analyzed in ASTRA's *Viral Vector Analysis* module.

Real-time AAV monitoring in prep-IEX

The analysis applied to MALS + UV260/UV280 is just as applicable to prep-scale separations as to analytical separations, and all the same information may be obtained. Unlike offline analysis, though, in process chromatography it is desirable to have the results made available in real-time. For these reasons, the analytical setup does not meet the needs of process:

- The DAWN instrument is designed for low-volume, low-flow-rate analytical separations and is not suitable for preparative scale separations – the backpressure produced by the instrument at flow above ~ 1 mL/min would exceed the FPLC's and IEX column's maximum pressure rating.
- 2. ASTRA software does not provide real-time output. Data processing only occurs after the run is complete.

Wyatt's real-time MALS instrument and software are, respectively, ultraDAWN and OBSERVER™.

ultraDAWN: MALS as PAT

While operating on the same physical principles as Wyatt's other MALS instruments, the ultraDAWN is

adapted for use in-line, with bench-scale chromatographic processes, as well as online, with scaled-up processes that operate at higher flow rates.

- ultraDAWN integrates in-line with FPLC at flow rates up to at least 150 mL/min, limited mainly by the FPLC's backpressure tolerance level. If the process equipment can tolerate higher backpressures, the flow rate may exceed 150 mL/min. Future models will accommodate higher inline flow rates.
- When inline operation is not feasible, a precise pump draws sample continuously at a low flow rate from the main process and supplies it to the ultraDAWN.
- ultraDAWN can import analog UV signals as well as digital timing pulses from the FPLC, and export signals to the FPLC that are useful for synchronization and process control.
- For larger viral vectors such as adenovirus and lentivirus, or very high AAV titers, ultraDAWN can accommodate scattered intensities that would saturate standard MALS detectors.



Figure 1. ultraDAWN real-time MALS instrument. Real-time data such as size, molar mass, capsid ratio or titer are displayed on the front panel and exported in analog format from rear-panel connectors.

ultraDAWN may be utilized as a process analytical technology (PAT) in a variety of downstream biologics applications including purification, UF/DF and homogenization of proteins, nucleic acids and viral vectors. Other applications in biopharma include lipid nanoparticle production and the depolymerization or conjugation of polysaccharides. For more information on ultraDAWN, see www.wyatt.com/ultraDAWN.

OBSERVER: real-time MALS analysis

OBSERVER is the real-time software that accompanies ultraDAWN. Designed to provide a simple, intuitive and unobtrusive user interface, OBSERVER is a good fit for process development labs that need to focus efforts on primary software such as UNICORN[™] in order to optimize the process, rather than on the PAT software. OBSERVER runs on a Windows PC.

- OBSERVER takes the raw light scattering and UV data and computes product attributes up to 5 times per second.
- Results are displayed on the computer screen and output to the ultraDAWN for display and analog transmission to the FPLC.
- A 'trigger' condition may be set up to indicate when the product is in spec and should be pooled, or is out of spec and should be discarded. For example, the trigger condition for AAV enrichment can be Vg/Cp > 0.5, or $R_g < 15$ nm to eliminate aggregates (R_g of an AAV monomer is 10- 11 nm). When the trigger is 'on', an analog signal provided by the ultraDAWN switches to +2 V, and when it is 'off' the signal switches back to zero. This trigger signal can be read by the process control software, e.g., UNICORN, to control pooling or fraction collection.
- At the end of the run, OBSERVER calculates average or total attribute values in the pool, assuming the trigger signal was in fact used to control pooling.

OBSERVER's *Inline AAV* workflow is specifically designed to integrate with an FPLC system for AAV attribute monitoring and process control. Exchange of digital pulses and analog signals enables synchronization and signaling with the FPLC software. This workflow calculates and displays:

- total, full and empty titer
- full:total capsid ratio Vg/Cp
- total, capsid and DNA molar masses
- particle radius R_g.

The final report includes averages of the above attributes over the pool, as well as the total numbers of empty and full virions in the pool.

GMP operation

For GMP and commercial-scale PAT, OBSERVER communicates via OPC-UA with a user-supplied client program that controls the run, collects the data and monitors the trigger. The client program can ensure 21 CFR Part 11 compliance such as audit trails, secure database and electronic signatures, and provide real-time or post-process data to PAT software. When under OPC-UA control, OBSERVER's UI is locked to manual intervention for maintaining regulatory compliance.



Figure 2. Real-time operation of the ultraDAWN is orchestrated by OBSERVER software.

For more information on OBSERVER, see www.wyatt.com/OBSERVER. To learn more about RT-MALS, see www.wyatt.com/RT-MALS.

The remainder of this application note describes RT-MALS results from prep-scale AAV separations, obtained with ultraDAWN and OBSERVER's *Inline AAV* workflow.

Materials and Methods

AAV: the AAV8 serotype was employed, encapsidating a modified green fluorescent protein gene with expected MW of 0.86 MDa. Starting material consisted of 53% empty capsids, 44% full and 3% ambiguous as determined by mass photometry. The viral genome concentration of the starting material as determined by ddPCR was 3.3×10^{13} vg/mL, corresponding to an overall capsid concentration of 7.3 x 10^{13} AAV/mL.

FPLC: the chromatographic system comprised an ÄKTA[™] avant operated with a 1 mL IEX column, running under UNICORN 7, at a flow rate of 3 mL/min. Elution methods consisted of either a linear or a step gradient, mixing Buffer A (low ionic strength) and Buffer B (moderately high ionic strength).

UV: Analog UV absorption data at 260 nm and 280 nm, measured by the ÄKTA's triple-wavelength detector configured with a 2 mm path length flow cell, were exported to the ultraDAWN via an ÄKTA E9 I/O Box.

MALS: an ultraDAWN was placed in-line with the ÄKTA unit, plumbed between the UV and conductivity detectors. OBSERVER 1.5 RT-MALS software was configured to acquire MALS and UV data at 2 second intervals under the *Inline AAV* workflow. Baseline data were acquired prior to elution: the column valve was set to bypass and a buffer mixture corresponding to the primary elution condition was run.

Data analysis: UV260 and UV280 extinction coefficients for capsid and DNA were obtained by direct measurements using SEC-MALS with ASTRA software's *Viral Vector Analysis* module as described in ASTRA's "SOP Guidance Manual: Critical Quality Attributes of AAV by SEC-MALS".

The trigger signal and the value of Vg/Cp were streamed in real-time, via analog voltage, from the ultraDAWN to the ÄKTA avant's I/O Box. The RT-MALS data stream was read by UNICORN for display, recording and potentially for use in a watch condition.

Offline analysis: Fractions were collected and analyzed offline by means of several methods including ddPCR and mass photometry.

Results and Discussion

Two gradient types were employed to develop the AAV capsid enrichment method:

- A linear gradient was used to gain an initial understanding of elution behavior with respect to buffer composition.
- Step gradients were iterated to optimize recovery, trading off yield against quality.

Linear gradient

Process knowledge

The elution behavior against buffer condition (%B) is shown in Figure 3 (ionic strength, dashed gray line). Fortuitously, it is seen that the apex of the full virion peak (blue) elutes at the trough of the empty virion peak (red). The knowledge gained here – buffer compositions leading to full or empty capsid elution – is fed into the design of the step gradient used in the next stage of process development.





Process control

While a linear gradient is not generally used for production processes, it is instructive to consider how one might use RT-MALS to set up effective AAV enrichment by means of a linear gradient, with almost no process optimization. OBSERVER software includes a 'trigger' function for automated process control as described earlier. For AAV, the most desirable criterion for cutting pools is based on Vg/Cp, the full:total capsid ratio, which should be greater than, e.g., 0.5. As seen in Figure 4, most of the main full capsid peak occurs at high Vg/Cp and can be separated from the high empty capsid peaks by the trigger functionality.



Figure 4. Linear gradient – full/total capsid ratio (orange) and full capsid titer (blue). The shaded area indicates the region of the peak that is collected per the trigger conditions mentioned in the text.

However, there are regions in the tail that also exhibit high Vg/Cp, above 0.5. As will be seen below, the tail region is not desirable for pooling and should be excluded. In order to exclude those regions, OBSERVER can be programmed to collect regions that meet two conditions simultaneously:

- 1. Vg/Cp > 0.5
- 2. $R(O) > 2.4 \times 10^{-5} \text{ cm}^{-1}$ where R(O) is the MALS intensity extrapolated to 0° .

The latter condition ensures that the tail region, which has low titer and therefore low LS intensity, will not be included in the pool. R(0) can be displayed in OBSERVER and overlaid with the attributes to identify the desired cutoff value.

Assuming that the FPLC does use the trigger signal to start and end product pooling as shown in Figure 4, OBSERVER calculates the pool to contain:

- 4.49 x 10¹³ total capsids at a concentration of 1.94 x 10¹³ VP/mL
- 4.01 x 10^{13} full capsids at a concentration of 1.73 x 10^{13} VP/mL
- Overall Vg/Cp of 0.89

The full capsid yield (assuming pooling during the trigger period) is 62% of the total eluting full capsids, which is comparable to the yield and quality obtained with the fully optimized step gradient (described below).

The data collected by OBSERVER may be re-analyzed under different trigger conditions, without additional test runs in order to find the optimal balance between yield and quality.

Digging deeper into the data

Further identification of the peaks is determined by examining attributes like capsid MW (M_{capsid}) and genome MW (M_{genome}). These are shown in Figure 5, overlaying the full capsid titer plot.



Figure 5. Linear gradient results, indicating capsid and genome molar mass as well as full capsid titer. Capsid MW > ~ 3.7 may be indicative of co-eluting viral aggregates.

- Values of M_{capsid} above the monomer MW (~3.7 MDa) are indicative of co-elution of aggregates. In fact, the region shown in Figure 4 between 67 and 68 minutes—with Vg/Cp > 0.5—is seen in Figure 5 to have a high M_{capsid} and therefore is suspect of including aggregates. This justifies its exclusion from the product pool as mentioned in the previous section.
- Values with low M_{capsid} that maintain high M_{genome} such as the region beyond 68 minutes—suggest co-elution of free DNA.

Step gradient

Typically, AAV enrichment processes in final production consist of step gradients rather than linear gradients. Enrichment consists of two primary steps.

- Column loading and wash: the buffer is selected to cause most full AAV monomers to adhere to the column, while most empty capsids and aggregates will pass through to waste.
- Elution: here the buffer is selected to provide complete elution of the loaded full capsids as well as good separation from the residual eluting empty capsids and impurities.

In the absence of RT-MALS, real-time monitoring of the apparent full:total capsid ratio for process control is usually accomplished via the UV260/UV280 ratio, and the cutoff point is optimized via offline fraction analysis. The UV ratio, however, is a limited predictor of AAV attributes because, among other things, it does not discriminate between monomeric AAVs and impurities such as aggregates or free DNA.

The results below illustrate 1) an initial, sub-optimal condition, and 2) the final process that was identified following several iterations of adjusting the column loading quantity, column loading buffer and elution buffer.

Column loading and wash

Column overloading

The first example, presented in Figure 6, is representative of overloading the column. Initially only empty AAVs wash through the column but beginning around the elution time of 21 minutes the full titer plot begins rising. Thus the buffer condition is appropriate in that full vectors do bind to the column and empty viruses wash through. However, some precious full vectors are lost, so in the following iterations the load amount was reduced.

Comparison with offline analytics

During the column load, fractions were collected during the times depicted in Figure 6 as shaded rectangles and analyzed offline by mass photometry to determine the full:total ratio F:T. The results were F:T1- 5% ; F:T2 – 37%, matching the values found by in-line RT-MALS for these fractions, 6% and 32% respectively.



Figure 6. Full and empty AAV titer behavior during column wash in a step gradient with overloading. Breakthrough of full vectors is observed beginning around 21 minutes. F:T1 and F:T2 indicate the fractions analyzed offline for full:total ratio mentioned in the text.

Final step gradient condition

The second example, presented in Figure 7, pertains to an optimized set of conditions. The solution coming through the column is found by both in-line RT-MALS and off-line mass photometry to include ~ 98% empty capsids, indicating that the loading buffer does in fact preferentially capture full AAVs while allowing empty capsids to pass through to waste. Almost no full vectors are lost during the loading step.



Figure 7. Full and empty titers during column loading (10 - 14 minutes) and wash (14 - 28 minutes) in the final step gradient. Note that the plots are referenced to different vertical axes.

The material coming off the column during the wash phase, between 15 and 20 minutes, consists of empty AAV, as determined from MW, shown in Figure 8 (~ 3.7 MDa, similar to capsid MW during the loading phase). The decreasing molar mass from 20 minutes suggests that most likely the capsids are mixed with impurities like free DNA or host cell proteins, that continue to wash off until about 30 minutes.



Figure 8. Capsid molar mass overlaid with empty capsid titer.



Figure 9. Full (blue) and empty (red) titers determined by RT-MALS during elution (34 – 48 minutes) and strip (> 48 minutes) in the final linear gradient. Buffer ionic strength is represented by the dashed black line.

Elution

Figure 9 presents empty and full titers during the elution and strip steps of Run 8. The vast majority of full capsids elute between 35 and 37 minutes, where Vg/Cp is almost 100%. Hence collecting this peak would produce a pool containing:

- 4.83 x 10¹³ total capsids at a concentration of 3.74 x 10¹³ VP/mL
- 4.27 x 10¹³ full capsids at a concentration of 3.31 x 10¹³ VP/mL
- Overall Vg/Cp of 0.88, which matched the offline measurement of ~ 86% by mass photometry

The full capsid yield is 61% of the total full capsid amount eluting during the elution and strip phases.

Final performance assessment

Comparing the titer values integrated over column load and over the elution phase, we find that a minority, 31%, of the empty capsids and other low-DNA species (e.g., host cell proteins) adhere to the column, with the remainder discarded. Conversely, only 3% of the full capsids are discarded during column load and the remainder adhere to the column. Hence the column loading step is very effective in enrichment, simplifying pooling during the elution step.



Figure 10. Particle size R_g (bottom), overlaid with total capsid titer during the final linear gradient. The tail of the main peak appears to include free molecules or perhaps capsid fragments, while the strip peak includes aggregates.

For deeper characterization, it is useful to review R_{g} , shown in Figure 10. Some useful information that can be gleaned is:

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- The tail of the main elution peak exhibits decreasing size and probably includes free molecules or capsid fragments.
- The strip peak consists mostly of monomers (peak at 50 minutes) and some aggregates and/or particulates (tail and secondary peak).

Conclusions

Considering the typical workflow for developing and optimizing the AAV polishing step, which requires collecting and analyzing many fractions offline by multiple techniques, RT-MALS provides clear benefits. Critical data such as the full capsid ratio, empty and full titer are determined continuously and immediately with high data density. Column loading, wash, elution and strip phases are all readily monitored, accelerating optimization of this process step. In fact, RT-MALS is seen to provide a means of obtaining high-yield, high-quality pools even with a simple linear gradient. Additional quality attributes such as aggregation indicators are available in the RT-MALS data.

Implementation of RT-MALS is not limited to bench scale FPLC – it can be applied to larger scales and other downstream unit operations. This unique technology is essential for bringing AAV-based gene therapies to market

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Drug Nanocarriers

Non-viral Vectors



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