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APPLICATION NOTE

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

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Summary

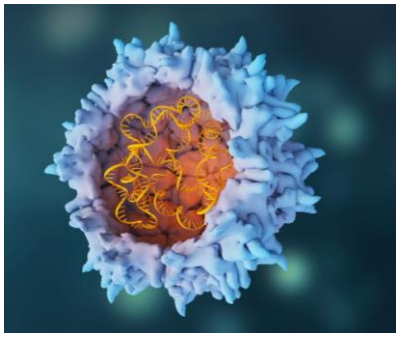
Downstream purification and enrichment of full AAV capsids for gene therapy products is typically accomplished by ion-exchange chromatography (IEX). While the ratio of UV260 to UV280 absorption is often used during IEX as a proxy for the full:total capsid ratio Vg/Cp, this method does not afford process 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 co-expressed. 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 (size-exclusion 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:

1. 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.

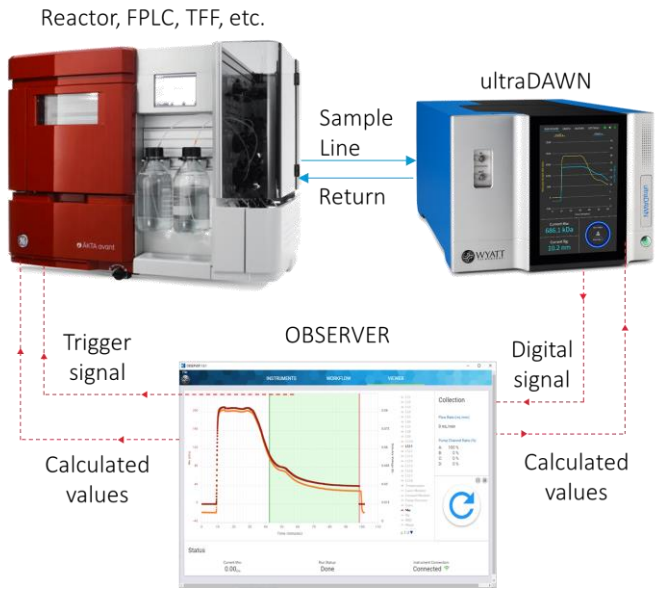


Figure 2. Real-time operation of the ultraDAWN is orchestrated by OBSERVER software.

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 x 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.

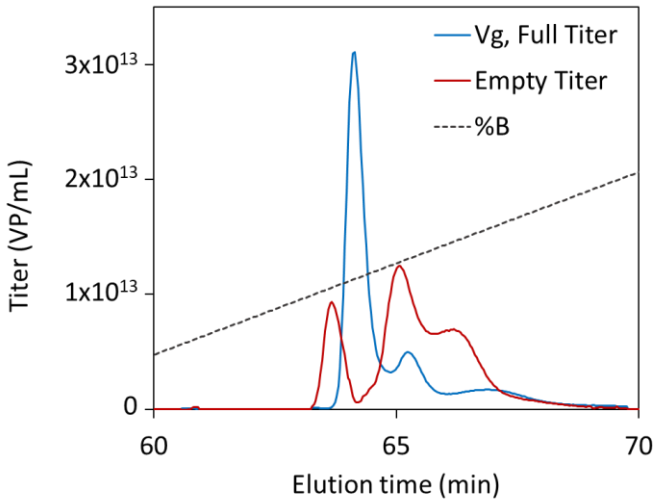


Figure 3. Full and empty capsid elution during a linear gradient.

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.

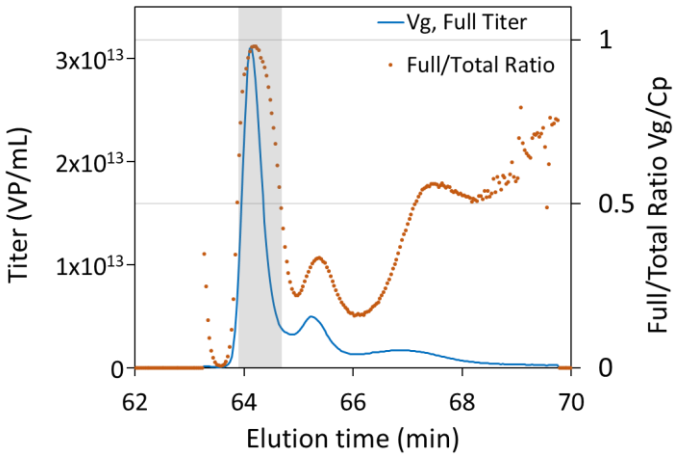


Figure 4. Linear gradient – full/total capsid ratio (orange) and full capsid titer (blue). The shaded area indicates the region of the peak that is collected per the trigger conditions mentioned in the text.

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. $R(0) > 2.4 \times 10^{-5}$ cm^{-1} where $R(0)$ 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:

- 4.49×10^{13} total capsids at a concentration of 1.94×10^{13} VP/mL
- 4.01×10^{13} full capsids at a concentration of 1.73×10^{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.

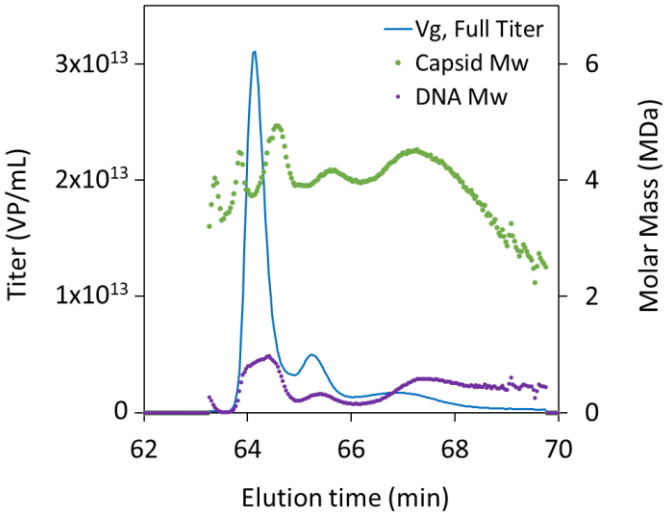


Figure 5. Linear gradient results, indicating capsid and genome molar mass as well as full capsid titer. Capsid MW > ~ 3.7 may be indicative of co-eluting viral aggregates.

- 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.

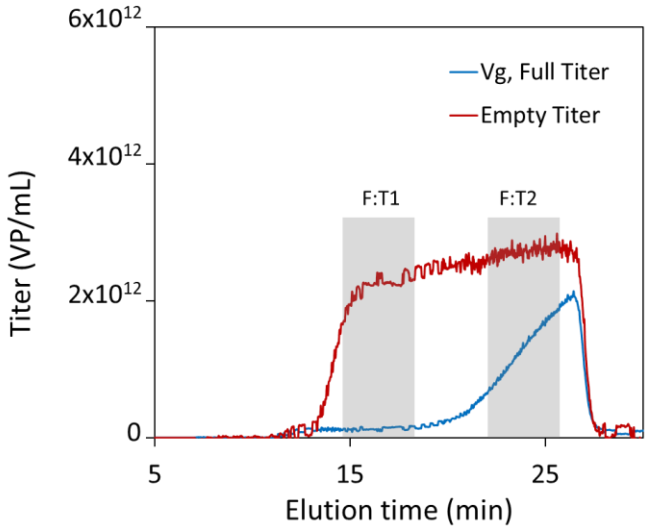


Figure 6. Full and empty AAV titer behavior during column wash in a step gradient with overloading. Breakthrough of full vectors is observed beginning around 21 minutes. F:T1 and F:T2 indicate the fractions analyzed offline for full:total ratio mentioned in the text.

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.

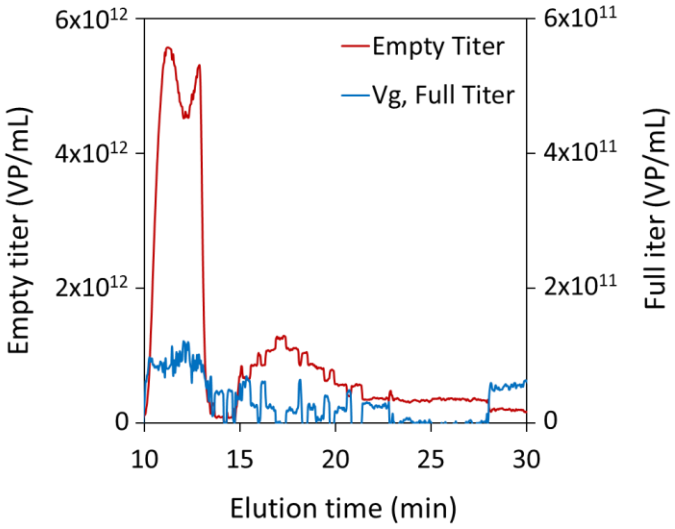


Figure 7. Full and empty titers during column loading (10 - 14 minutes) and wash (14 – 28 minutes) in the final step gradient. Note that the plots are referenced to different vertical axes.

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.

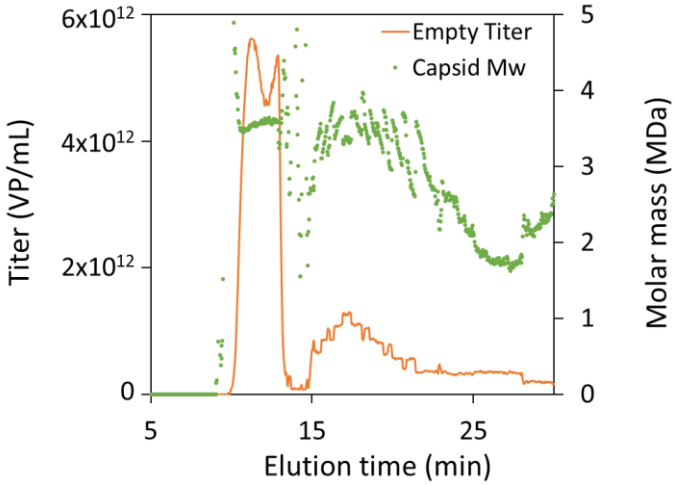


Figure 8. Capsid molar mass overlaid with empty capsid titer.

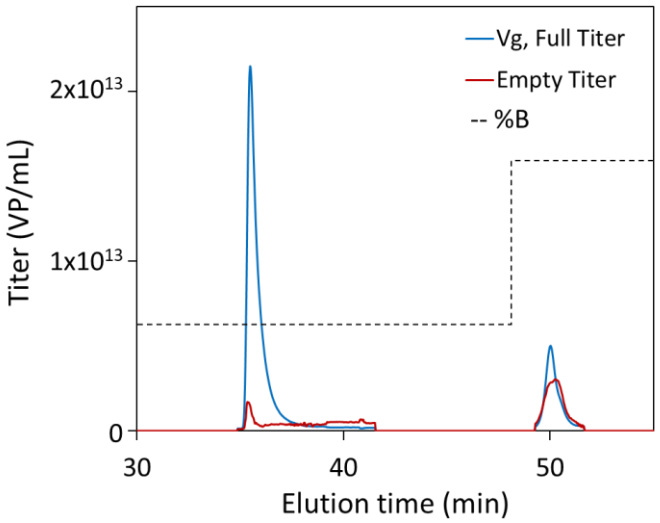


Figure 9. Full (blue) and empty (red) titers determined by RT-MALS during elution (34 – 48 minutes) and strip (> 48 minutes) in the final linear gradient. Buffer ionic strength is represented by the dashed black line.

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 10^{13} total capsids at a concentration of 3.74 x 10^{13} VP/mL
- 4.27 x 10^{13} full capsids at a concentration of 3.31 x 10^{13} 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.

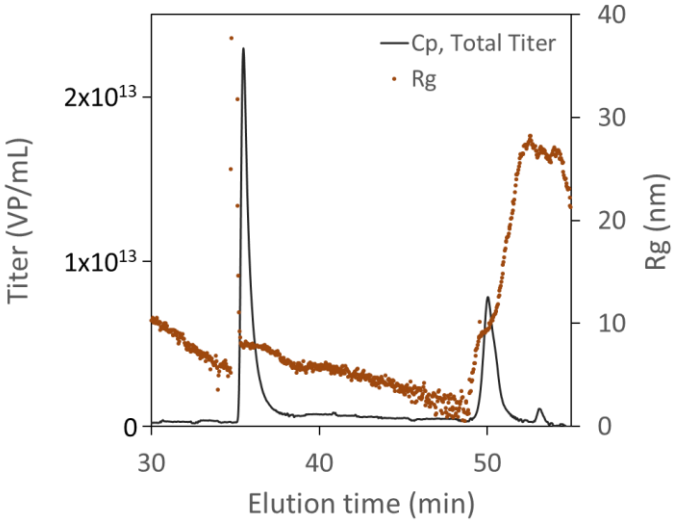


Figure 10. Particle size R_g (bottom), overlaid with total capsid titer during the final linear gradient. The tail of the main peak appears to include free molecules or perhaps capsid fragments, while the strip peak includes aggregates.

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

- 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 quickly and reducing the cost of their development and production.

Request more info

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