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Simplifying Progress

Know Your AAV – Quality Control Starts During Process Development

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Introduction

Adeno-associated viruses (AAV) are used as gene delivery systems for therapeutic applications. Production of AAV as new therapeutic substance is therefore of high priority in the pharmaceutical industry. Suitable analytical procedures for the determination of critical quality attributes (see Figure 1) need to be established. Quality parameters are for example genome and capsid titers as well as the presence of aggregates. The genome packaging of the virus capsids, displayed by the ratio of full and empty capsids (Full/Empty ratio), represents a main criterion of AAV quality.

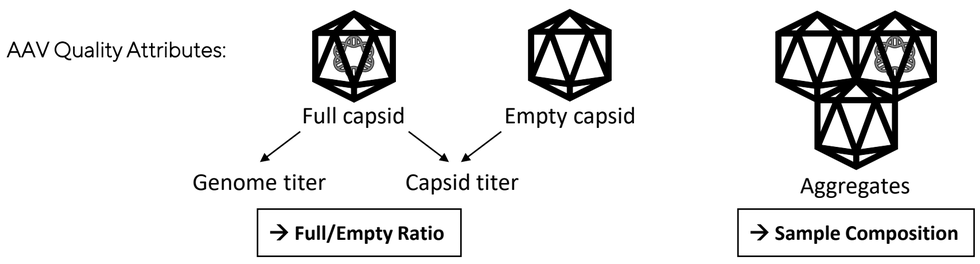


Figure 1: Quality attributes of adeno-associated viruses (AAV).

Common analytical procedures for the analysis of AAV capsid and genome are mostly complex methods as for example enzyme-linked immunosorbent assay (ELISA) and droplet digital PCR (ddPCR) (Gimpel et al. 2021). This calls for novel and simple analytical processes to analyze diverse quality parameters of AAV that can be used especially early on during the upstream process (USP).
Here we show AAVX affinity chromatography using a high-performance liquid chromatography (HPLC) with diode array detector (DAD) and fluorescence detector (FLD) in combination with size-exclusion chromatography followed by multi-angle light scattering (SEC-MALS) analysis for the determination of the Full/Empty ratio during process development of AAV8 as early quality control. SEC-MALS allows the analysis of various parameters, e.g. total particles (capsid titer), full particles (genome titer), empty particles, molar weight and aggregation of AAV in a single run (McIntosh et al. 2022).

1. Experimental Approach

Clean Up of Crude Samples: AAVX Affinity Chromatography

Analytical device:
- HPLC-DAD-FLD (Agilent Technologies)

Mobile phases:
- Loading buffer at neutral pH
- Elution buffer at acid pH

Column: self-made analytical column
- PorosTM CaptureSelectTM AAVX Affinity Resin (Thermo Scientific)

Samples:
- Crude samples containing AAV8

Purification process:
- Loading (see Figure 2) of different sample volume
- Collection of purified AAV fractions and buffer exchange to neutral pH
- Preconcentration of samples (optional)

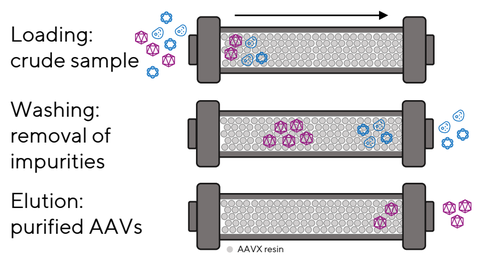


Figure 2: Principle of the purification of crude samples using AAVX affinity chromatography.

SEC-MALS Analysis

Analytical device:
- HPLC-DAD (Agilent Technologies) coupled with MALS-RI (Wyatt Technology) (RI data not shown)

Mobile phase:
- Buffer at neutral pH

Column:
- Wyatt SEC Protein Column 50 NB WTC-050N5, 5 µm, 4.6mm, for MALS

Samples:
- Purified AAV samples after affinity chromatography

Samples Experiment A:

Crude sample A containing AAV8 is split into 4 replicates. For each replicate purification is carried out separately:
- Sample 1 and 2 (concentration factor: 4)
- Sample 3 and 4 (concentration factor: 2)

Samples Experiment B:

Crude sample B containing AAV8 is separated after clean up into:
- Sample 5
- Sample 6 and 7 (stored under harsh conditions)

2. Results – The Need for Affinity Chromatography

SEC-MALS analysis of a crude sample
- Matrix peaks overlap with AAV peaks (see Figure 3)
- No determination of reliable results possible

→ Clean up of crude samples is necessary to separate matrix from AAV particles and collection of AAV fractions

Purification of a crude sample
- Separation of AAV (see Figure 4) and matrix
- Loss of AAV during clean up process
  - 60-65% recovery

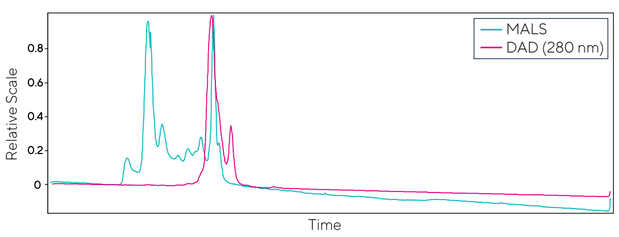


Figure 3: SEC-MALS chromatogram of a crude sample - no evaluation of the AAV peak possible.

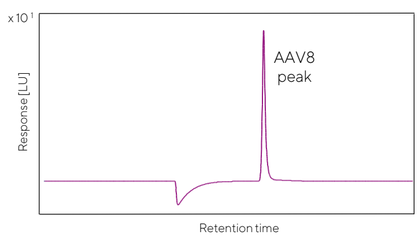


Figure 4: HPLC-FLD chromatogram of the affinity chromatography from a crude sample containing AAV8.

References: Gimpel AL, Katsikis G, Sha S, et al., *Mol Ther Methods Clin Dev.* 2021;20:740-754.; McIntosh NL, Berguig GY, Karim OA, et al., *Sci Rep.* 2022 Nov 17;12(1):19754.

3. Results – AAV Analysis by SEC-MALS

Samples 1-4 originating from the same crude sample A act as 4 replicates and show reproducible results for the different parameters determined by SEC-MALS (please note the different concentration factors, section 1). SEC-MALS results were compared to ELISA and ddPCR for Full/Empty ratio (see Table 1) as well as for capsid and genome titer (see Table 2). SEC-MALS additionally gives values for the AAV's molecular weight (Mw) listed in Table 2.
- SEC-MALS chromatograms with low background (see Figure 5) → distinct monomeric AAV8 peak
- **Full/Empty ratios** acquired by SEC-MALS and ddPCR/ELISA (see Table 1) show different values

Table 1: Full/Empty ratios of the purified samples determined by SEC-MALS and ddPCR/ELISA.

Sample	Full/Empty Ratio	
	(SEC-MALS)	(ddPCR/ELISA)
1	6.1%	4.7%
2	6.1%	4.3%
3	6.3%	4.6%
4	6.4%	4.6%

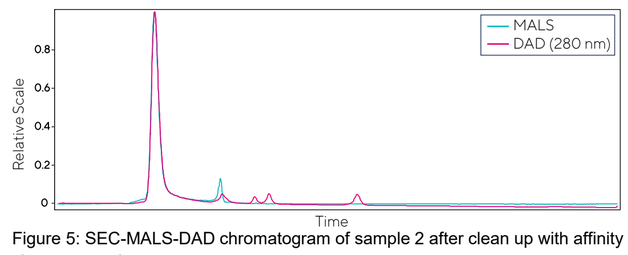


Figure 5: SEC-MALS-DAD chromatogram of sample 2 after clean up with affinity chromatography.

- Capsid titer
  - Deviations between the methods
  - Resulting in different Full/Empty ratios (see Table 1)
- Genome titer
  - Comparable values between SEC-MALS and ddPCR

Table 2: Different parameters for purified AAV8 samples acquired by SEC-MALS in comparison to corresponding ddPCR and ELISA data.

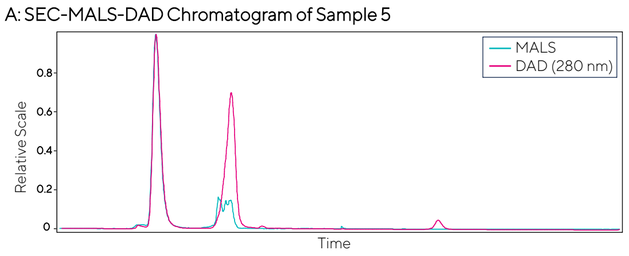
Sample	SEC-MALS			ELISA	ddPCR	Relative Deviation	
	Mw (kDa)	Capsid Titer (cp/mL)	Genome Titer (vg/mL)	Capsid Titer (cp/mL)	Genome Titer (vg/mL)	SEC-MALS / ELISA	SEC-MALS / ddPCR
1	4097.5	5.07E+12	3.09E+11	6.51E+12	3.05E+11	-22.1%	-1.3%
2	4128.3	4.74E+12	2.88E+11	6.81E+12	2.92E+11	-30.4%	1.4%
3	4078.5	2.52E+12	1.59E+11	3.60E+12	1.65E+11	-30.0%	3.8%
4	4109.3	2.16E+12	1.38E+11	3.07E+12	1.42E+11	-29.6%	2.9%

4. Results – Quality Assessment by SEC-MALS

Crude sample B containing AAV8 was purified by affinity chromatography and then split into 3 replicates (5-7). Samples 6 and 7 were stored under harsh conditions. Analysis of all samples was carried out by SEC-MALS and ELISA.

- Sample 5 with distinct monomeric AAV peak
- Samples 6 and 7 show large AAV8 aggregate peaks (see Figure 6, data of sample 7 not shown)
- Measured capsid titers appear too low (see Table 3)
- Aggregates cannot be quantified neither by SEC-MALS nor by ELISA (undefined number of AAV in aggregates)
- SEC-MALS visualizes aggregation, the reason for the low capsid titers, whereas ELISA only shows a titer

A: SEC-MALS-DAD Chromatogram of Sample 5



B: SEC-MALS-DAD Chromatogram of Sample 6

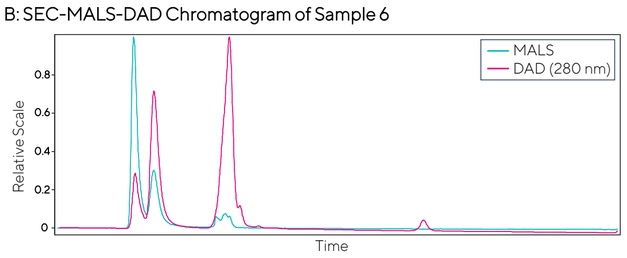


Figure 6: SEC-MALS-DAD chromatograms of sample 5 (A) and sample 6 (B). Sample 6 was stored under harsh conditions before measurement. A: Chromatogram with monomeric AAV peak and matrix signal. B: Chromatogram with aggregate peak, monomeric AAV peak and matrix signal (from left to right).

Table 3: Comparison of capsid titers measured by SEC-MALS and ELISA before and after sample storage under harsh conditions.

Sample	Time of Analysis	Capsid Titer (cp/mL)	
		SEC-MALS	ELISA
5	Before storage	8.29E+12	9.42E+12
6	After storage	3.61E+12	5.72E+12
7		3.97E+12	5.79E+12

5. Discussion

For the analysis of crude samples by SEC-MALS, purification is necessary because of high backgrounds from the sample matrix. After affinity chromatography the chromatograms of purified samples contain clear monomeric AAV8 peaks, enabling the evaluation of crude AAV samples by SEC-MALS. Unfortunately, the recovery after affinity chromatography is not optimal and optimization is needed. Yet, main quality attributes, like aggregation, Full/Empty ratio or molecular weight become accessible.
In this study the values for the genome titer acquired by ddPCR and SEC-MALS are comparable. On the contrary, the capsid titers measured by SEC-MALS are lower than the corresponding ELISA data. Both methods are influenced by various factors. ELISA results might be affected by, for example, detection of damaged capsids. For the analysis by SEC-MALS extinction coefficients are used, which impact the results. To improve the results, the respective coefficients should be determined per serotype. Additionally, SEC-MALS only takes intact monomeric capsids into account, which might also differ from ELISA.
Regarding the Full/Empty ratio by ddPCR/ELISA, two different procedures are combined, each adding its own errors, thus increasing the deviation of the calculated Full/Empty ratio. A big advantage of SEC-MALS is that all parameters are received by the same method in a single run. This decreases the approaches variability and makes the results more reliable. Also, there is no need for external calibration, therefore SEC-MALS is independent from reference material.
The visualization of AAV, aggregates and matrix (Figure 6) gives valuable insights into the sample's composition and purity, which is not possible using for example ELISA. This makes SEC-MALS a suitable method to monitor several critical quality attributes.

6. Conclusion

We show an effective protocol to analyze the Full/Empty ratio from crude samples containing AAV8 during USP. SEC-MALS offers several advantages compared to other analytical methods, above all the ability to determine different quality attributes (capsid titer, genome titer, molar mass and aggregation of AAV particles) in a single measurement. There is no introduction of variability through the combination of different methods or external references. All things considered SEC-MALS is an effective method for AAV analysis even in early developments during USP.