ProtaGene
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White Paper

Quality Control of AAV Capsid Loading

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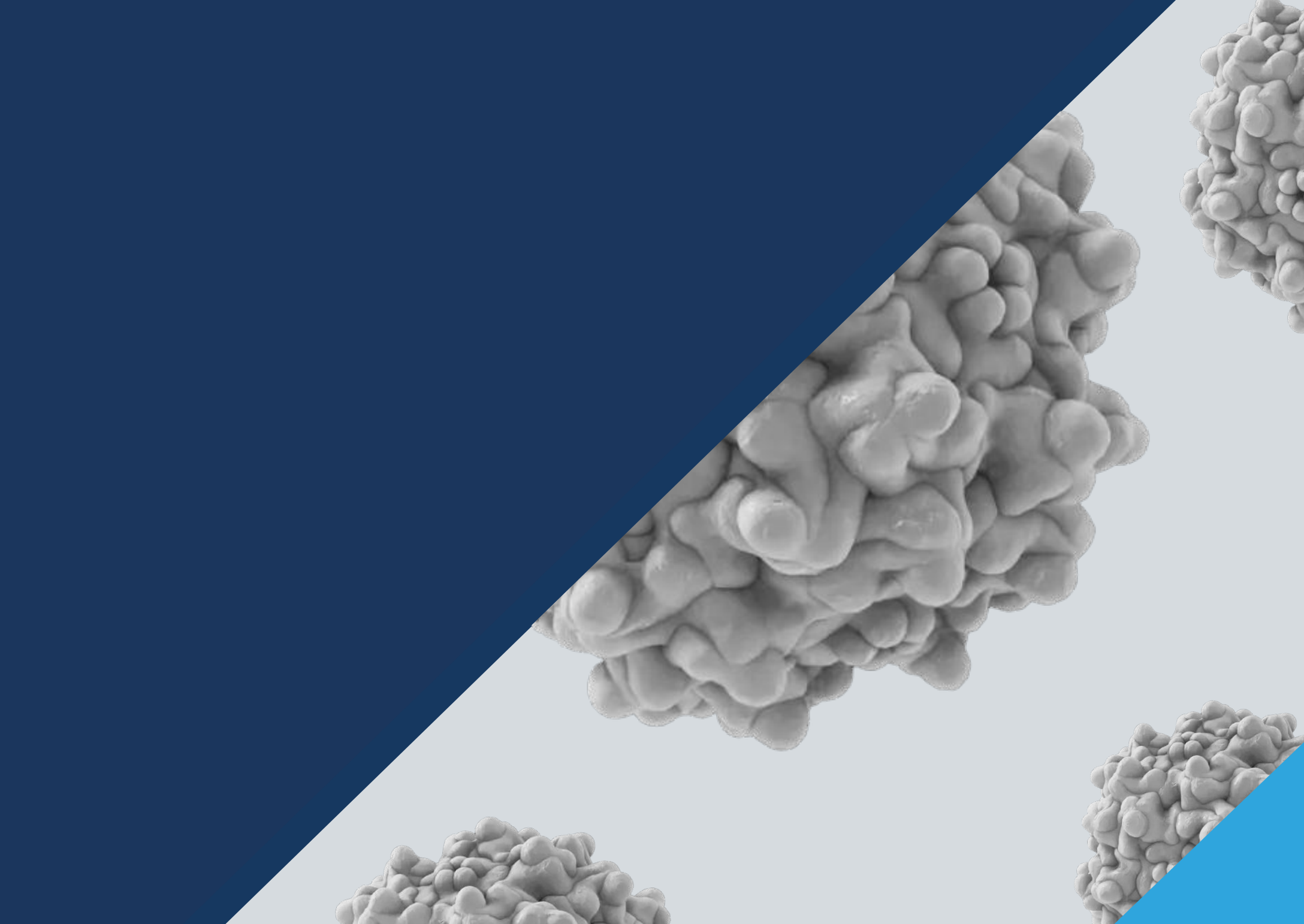

1 Content

2 Abstract

In the rapidly developing field of gene therapy, adeno-associated virus (AAV) represents one of the most prominent and promising platforms for therapeutic applications. Different serotypes of AAVs have demonstrated preferences for targeting specific tissues and represent an auspicious approach for delivering genetic information into patients. A critical quality attribute (CQA) of AAVs is their capsid loading because it influences dose and purity. Capsid loading reflects the efficiency of encapsulation of the genetic material into the viral capsid and is defined as the ratio of full to empty particles. Quantification of capsid loading is an essential aspect of product characterization and can be determined by different analytical techniques, like AUC, SEC-MALLS, and AEX-FLR. AUC remains the gold standard, capable of resolving partially filled capsids in addition to full and empty capsids. SEC-MALLS and AEX-FLR are advantageous because they allow for GMP-compliance and higher throughput.

This paper highlights the importance of quality control strategies for AAVs with state-of-the-art analytical capabilities that can reveal significant variations of the quality of commercially available AAV products. For highly parallelized analysis, DLS is a suitable addition to AUC, SEC-MALLS, and AEX-FLR to tackle quality control aspects of AAVs.

3.1 Emerging Field of Cell and Gene Therapy

Cell and gene therapy is a rapidly evolving field with vast research and development efforts leading to an increasing number of approved products. Its vast growth is fueled by rising investments from industry and funding by national governments. Market assessment studies expect treatment of about 350.000 patients with up to 60 products by 2030 [1].

In general, the aim of human gene therapy is "to modify or manipulate the expression of a gene or to alter the biological properties of living cells for therapeutic use" [2].

Different techniques and types of molecules/delivery vehicles can be used as gene therapy products to cure or treat diseases. The goal of all platforms is to achieve safe and effective target cell specific delivery of the genome altering tool. Adeno-associated viruses (AAVs) have been shown to be a promising delivery system with regard to immunogenicity and bio compatibility of the vector (not associated with any disease symptoms), stability and robustness, and efficacy in clinical trials.

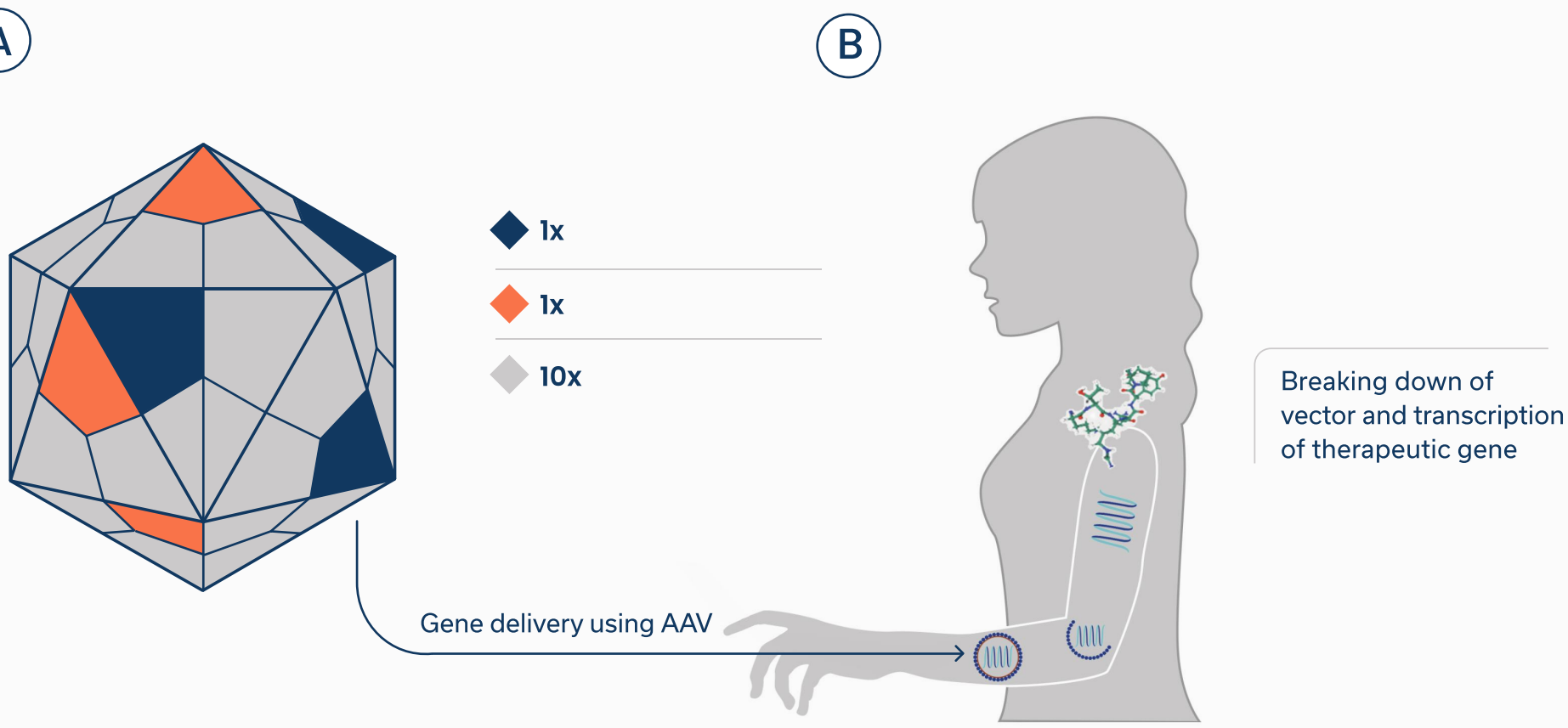

Figure 1: Gene therapy using AAV vector.
A: AAV capsid structure. The icosahedral symmetry retained by VP3 (grey) is intermitted by VP1 (orange) and VP2 (dark blue)[3] B: Gene delivery. The genetic material (light blue) is encapsulated in the AAV viral capsid (red circle decorated with dark blue hexagons). The AAV product is delivered to the patient via IV infusion and taken up by target cells, which then translate the encapsulated genetic material into therapeutic protein (multicolored ball and stick model).

3.2 Capsid Loading – A Critical Quality Attribute

The immune recognition displays one of the most challenging obstacles in development of AAV therapeutics, limiting their use to a single dose andresulting in a requirement for pre-screening patients for existing anti-AAV serotype antibodies. Contamination with high levels of empty AAV particles increases the dose needed to deliver an effective amount of genetic material and thereby increases capsid antigen presentation on AAV-transduced cells. After recognition, these cells are eliminated by capsid-specific cytotoxic T-cell lymphocytes [4]. In contrast, it has been noted that the addition of empty capsids to AAV therapeutics can mitigate the immune response by adsorbing neutralizing antibodies [5]; however, a higher level of AAV can alternatively lead to faster clearance of the product, thereby impacting efficacy, as well as deleterious immune reactions.

To ensure consistent quality, safety, and efficacy of an AAV drug product the "ratio of infectious to non-infectious particles or full to empty particles" has to be known and monitored thoroughly as described in a guidance for industry by the FDA [6]. Further critical quality attributes relate to product or process related impurities and the stability of AAV gene therapy products (Table 1).

Depending on target tissue and disease, different AAV serotypes are predominantly used for gene delivery [7]. This paper focuses on the determination of capsid loading quantified by analytical ultracentrifugation (AUC), size exclusion chromatography with multi-angle laser light scattering (SEC-MALLS) and anion-exchange chromatography with fluorescence detection (AEX-FLR) using AAV8 as reference material.

Table 1: Critical quality attributes of AAV gene therapy products covered by described methods (adapted from FDA guideline [6])

Attribute	Description	Applied Analytical Procedure
Content or Dose (Capsid Loading)	Number of genetically modified vectors in product, copy number of genomes per particle	Full and empty capsids by AUC, SEC-MALLS, AEX-FLR
Purity (Product-Related Impurities)	Defective interfering particles, non-infectious particles, empty capsid particles	Full and empty capsids by AUC, SEC-MALLS, AEX-FLR; HMWS & LMWS by AUC, SEC-MALLS, DLS; Charge variants by AEX-FLR
Purity (Process-Related Impurities)	Residual cell substrate proteins, extraneous nucleic acid sequences, helper virus contaminants (i.e., infectious virus, viral DNA, viral proteins)	HMWS & LMWS by AUC, SEC-MALLS, DLS; Charge variants by AEX-FLR
Stability	Stability-indicating experimental system (e.g., forced degradation studies, accelerated stability studies)	Limits for quality, and purity defined based on AUC, SEC-MALLS, AEX-FLR, DLS

4.1 Evaluation of Capsid-Loading by AUC

Analytical ultracentrifugation (AUC) is considered the gold standard for evaluation of capsid loading and product specific impurity analysis of AAVs. AUC applies a high centrifugal field and monitors the evolution of sample density over time. The experiments can be performed in a wide range of conditions, permitting analysis in formulation buffers of diverse composition and/or under biologically relevant conditions. Additionally, AUC causes minimal disturbance of the sample, and analysis allows recovery of AAV samples after analysis, providing a valuable advantage when sample amounts are limited.

In comparison to SEC-MALLS and AEX (see 4.2 and 4.3), AUC is not suitable for high levels of impurities (e.g., residual DNA). Therefore, contaminated samples have to be purified, prior to the analysis. This purification process is regularly performed for removal of contaminants. Depending on the serotype and buffer a recovery of up to 96% has been achieved [8].

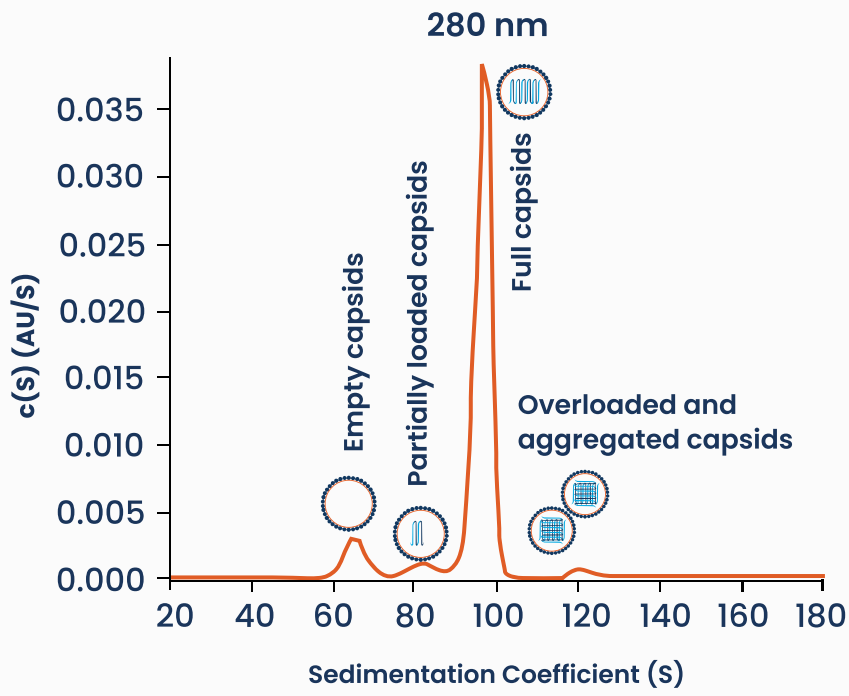

Figure 2: Sedimentation distribution of an AAV sample. AAV8 full sample was diluted to a theoretical concentration of $5x10^{12}$ GC/mL. Sample was filtered using formulation buffer and a 50 kDa MWCO to remove impurities with high absorbance not sedimenting using the applied setup. Data shown was acquired at λ = 280 nm.

Analysis of AAV can be performed with monitoring at multiple wavelengths, which allows for identification and quantitation of both the protein (280 nm) and DNA (260 nm) components. Simultaneous detection at these two wavelengths reflects the major attributes of empty (protein only) and full (protein plus DNA) to enable calculation of capsid loading for a gene therapy product. Measurement at 230 nm can additionally be executed providing higher sensitivity at low concentrations. Sedimentation distribution analysis on an Optima AUC (Beckman Coulter) using sedimentation velocity evaluation was performed and is depicted exemplarily in Figure 2. Calculated relative areas using ultraviolet (UV) detection for the different analyses with full and empty AAV8 samples are summarized in Table 2. Utilizing the obtained data at 260 nm and 280 nm, the ratio of full to empty capsids was determined (Table 3, Table 5). Additionally, capsids also may be partially loaded, aggregated, or overloaded with DNA, and using the dual wavelength analysis, these species can be identified by AUC.

Table 2: Relative area of different AAV species identified in AUC analysis

Sample	Wavelength	Relative Peak Area of Respective Species [%]		
		Empty	Partial	Full
Full Capsid Sample	UV 260 nm	5.22	3.19	91.58
	UV 280 nm	9.35	4.41	86.24
	UV 230 nm	18.07	4.23	77.70
Empty Capsid Sample	UV 260 nm	97.11	ND	2.89
	UV 280 nm	99.01	ND	0.99
	UV 230 nm	97.27	ND	2.73

Table 3: Capsid loading of AAV8 full sample by AUC

Relative Amount* [%]	
Full	Empty
63	37

**) Mean of 260 nm and 280 nm evaluation, calculated using published response factors for AAV6 [8]*

4.2 Evaluation of Capsid-Loading by SEC-MALLS

Size exclusion chromatography with multi-angle laser light scattering (SEC-MALLS) in combination with UV and refractive index (RI) detection provides information about aggregation and degradation of different AAV species, molecular weight and concentration. This information is applied to calculate capsid loading, purity, and content of impurities (i.e., aggregates and degradation products) as well as size and particle number of AAV samples [10,11].

Separation of the AAV sample on a column is based on the size of the different AAV species. The UV280 signal is used to quantify purity/impurities and combined with the MALLS and RI signals to determine the capsid loading. Alternatively, capsid loading can also be determined by using a combination of the UV280 and UV260 signals. Potential overlap of absorption at UV280 and UV260 is automatically corrected by the data analysis software.

The chromatograms of full and empty AAV particles show well separated AAV species (Figure 3). In this example, the purity of monomeric AAV particles was calculated to be 94.4% for full and 96.1% for empty particles (Table 4). The determined molecular weight for full (4.8 MDa) and empty (4 MDa) capsids is in good agreement with the theoretical capsid molecular weight (Table 5). Importantly, the molecular weight analysis demonstrated the absence of DNA from empty AAV samples (Figure 4).

Analysis of capsid loading showed 0% and 66 ± 2% for empty and full AAV particles, respectively (Table 5).

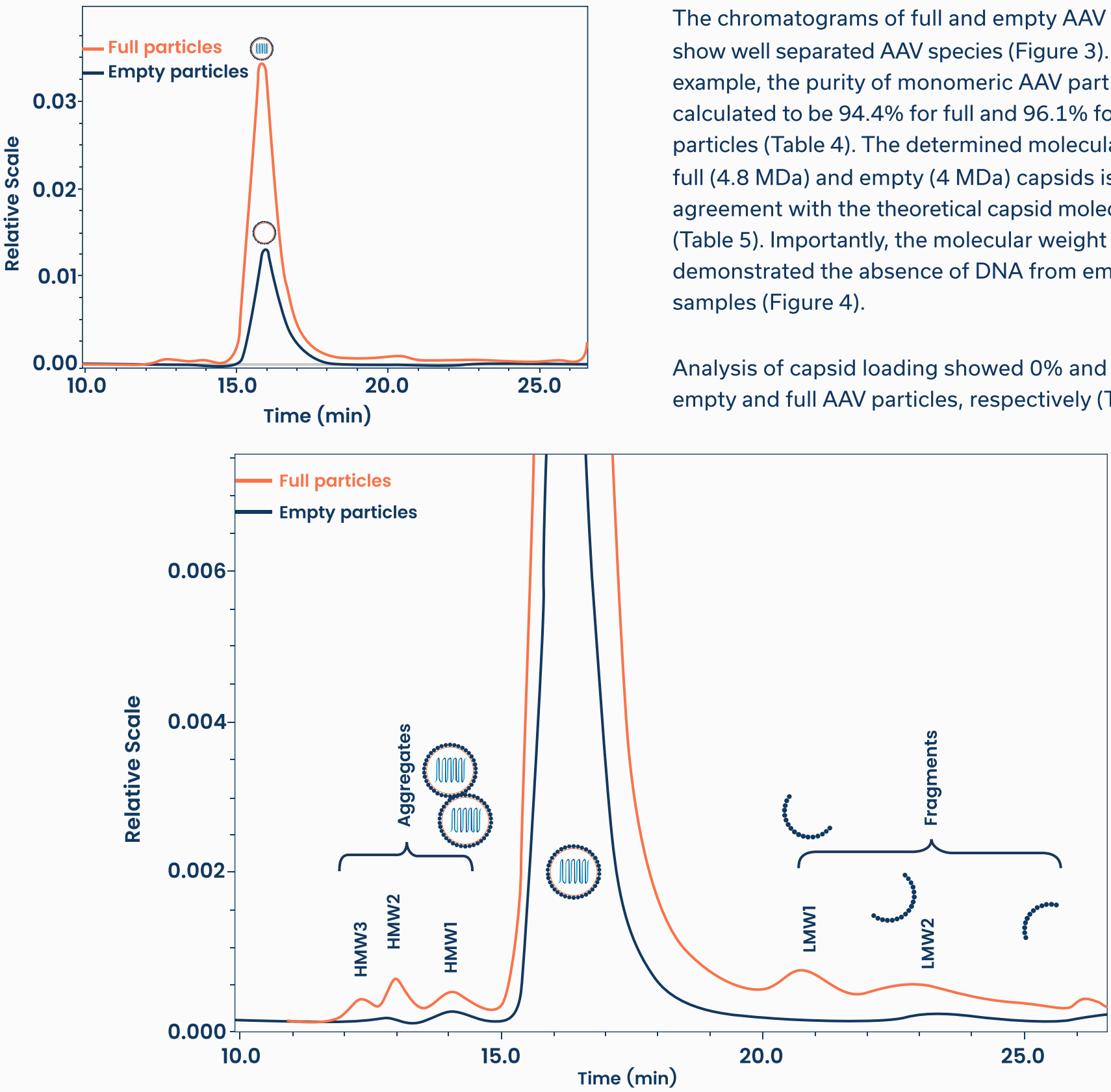

Figure 3: Overlay (upper panel) and zoomed overlay (lower panel) of the UV280 signal of full and empty AAV particles by SEC. Blue: Empty capsid sample, Orange: Full capsid sample

Table 4: Mean relative peak area of full and empty particles

UV_{280} Area %						
Sample	**Monomer***	**HMW1**	**HMW2**	**HMW3**	**LMW1**	**LMW2**
Empty	96.1	1.3	1.2	ND	ND	1.4
Full	94.4	0.9	1.0	0.4	2.3	0.9

*) The mean was determined over five independent analyses with a maximum CV for the monomer of 1.7%

Table 5: Capsid loading for full and empty AAV particles

Sample	**MW Capsid [MDA]***	**MW DNA [MDA]**	**Capsid Loading [%]***
Empty	4.046 ± 0.066	ND	1.2
Full	4.780 ±0.180	0.909	66 ±2

*) The mean was determined over five independent analyses with a maximum CV of 3.0%

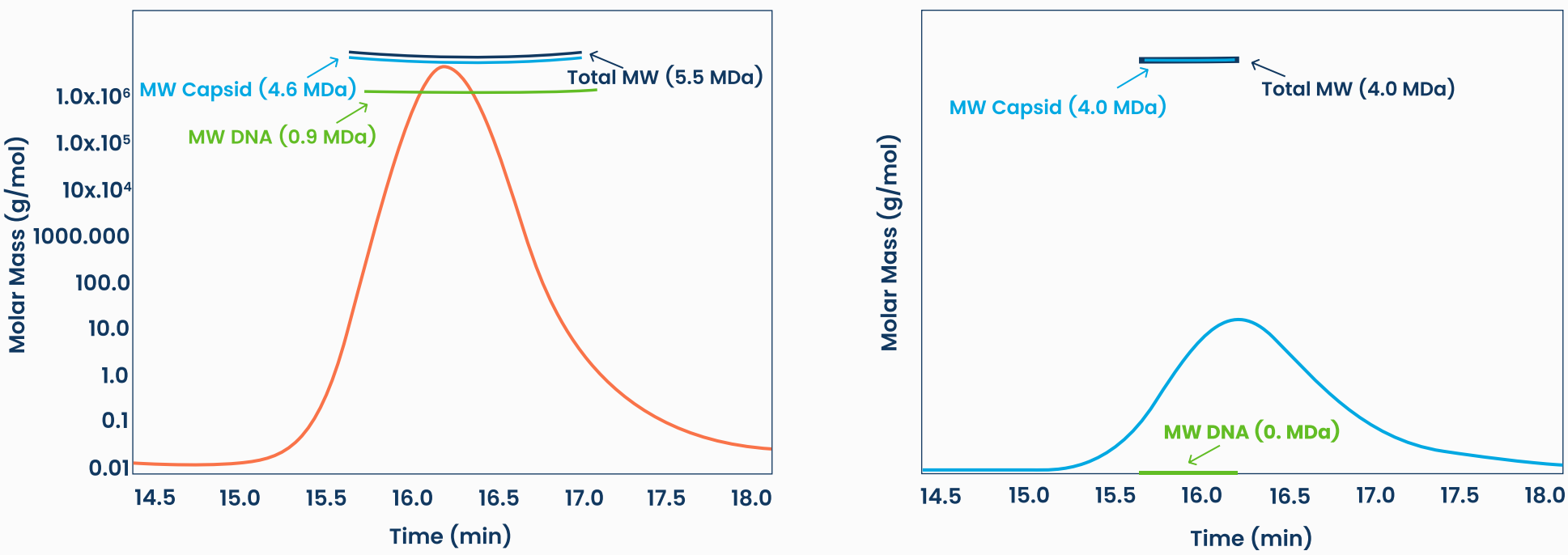

Figure 4: Molecular weight analysis of full (left panel) and empty (right panel) AAV particles by SEC-MALLS.

For AUC analysis, removal of impurities by ultrafiltration prior to the measurement is necessary. SEC-MALLS analysis of the filtered samples indicated that ultrafiltration has no impact on the capsid loading results (data not shown).

4.3 Evaluation of Capsid-Loading by AEX-FLR

Anion-exchange chromatography (AEX) can be used for the analysis of many AAV serotypes, as it is able to separate full and empty capsids based on their different apparent surface charges. The incorporated DNA in the capsid causes a slightly higher negative surface charge compared to the empty capsid such that full capsid selute later from the AEX column than empty capsids. With the applied method, we succeeded in achieving well separated peaks for AAV8 full and empty capsids (Figure 5). The empty sample generated a negligible AEX-FLR signal at the full capsids' peak retention time ofabout 15.0 min, whereas the full sample showed a well detectable empty capsid peak at about 11.5 min of notable size. This points out a deficiency of currently commercially available AAV material and emphasizes the need for proper evaluation of capsid loading. A slight tailing of the full capsid peak was observed. For AUC analysis, removal of impurities by ultrafiltration is necessary. After ultrafiltration and AUC analysis, the same sample was analyzed by AEX-FLR. This procedure did not impair the peak profile and only minor sample loss was observed compared to the original untreated sample, as is shown in Figure 6. Apart from that, impurities detected by AUC and SEC-MALLS did not negatively affect the AEX-FLR analysis (data not shown). The reproducibility of the method was shown by performing six independent analyses yielding CV values for the relative area of the full and empty peak of 0.4% and 3.3% respectively (Table 6).

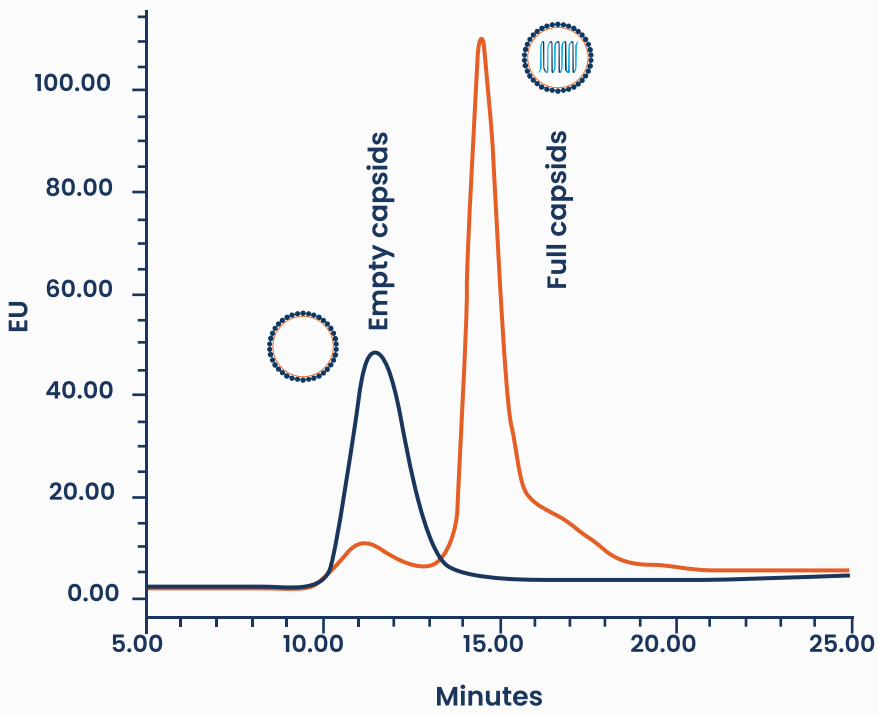

Figure 5: Comparison of AAV8 containing predominantly fullor empty capsids analyzed by AEX-FLR.
Blue: Predominantly empty capsid sample after ultrafiltration
Orange: Predominantly full capsid sample after ultrafiltration

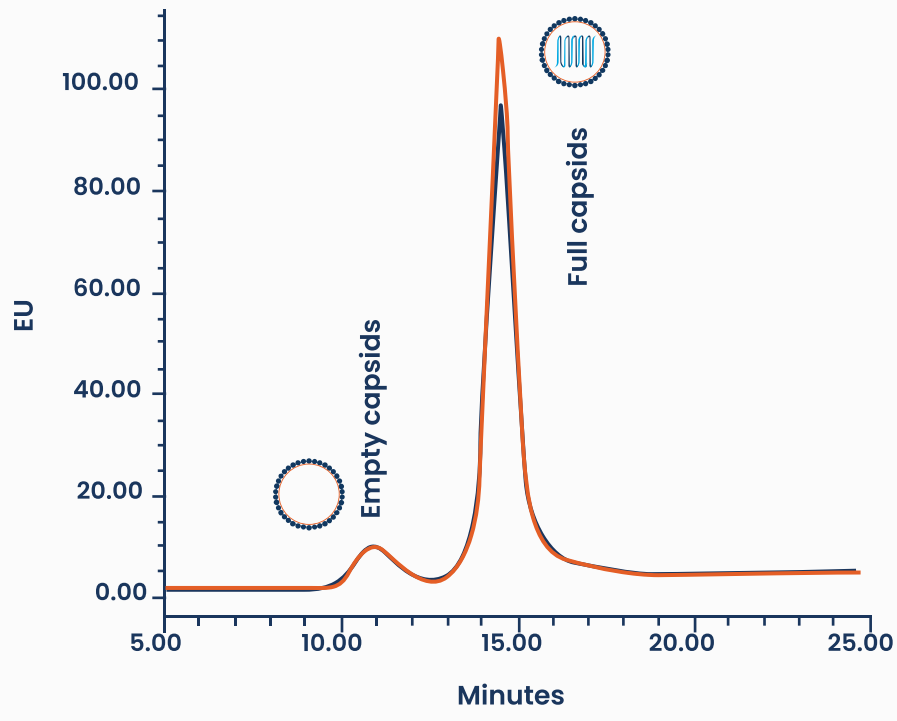

Figure 6: Comparison of ultrafiltration and untreated AAV8 containing predominantly full capsid analyzed by AEX-FLR.
Blue: Predominantly full capsid sample after ultrafiltration
Orange: Predominantly full capsid sample, untreated

Table 6: Capsid loading of AAV8 full sample by AEX-FLR

Predominantly Full AAV-8 After Ultrafiltration			
Area % Full	**Area % Empty**	**% Full**	**% Empty**
89.42	10.58	63	37

Mean of six replicates, correction factor derived from AUC ratios. CV values for the relative area of the full and empty peak were 0.4% and 3.3% respectively.

4.4 Case Study: Quality of Commercial AAV Material

AUC, SEC-MALLS, and AEX-FLR were employed to determine the quality of different AAV material. AAV samples have been purchased from different vendors and solubilized in different formulation buffers. In addition, to give information about capsid loading, the described methods are capable of characterizing the purity and impurities of AAV samples. Besides these methods, dynamic light scattering (DLS) was also applied for a first assessment of the sample's purity.

DLS analysis is based on the determination of the translational diffusion coefficient, which can be further transformed to estimate the size and the size distribution of species in the protein sample. In addition, larger particles scatter light more efficiently than smaller ones, making this method highly sensitive for larger aggregates (>100 nm).

Comparison of the two batches revealed significant differences in their size and size distribution (Figure 7). The one batch showed a strong signal at a hydrodynamic radius of 16 nm for the AAV particle, which is in good correlation to the literature (13-15nm [12]), but also the presence of aggregates with a R_h of ~180 nm.

In contrast, a different size distribution was detected for the other batch. For this sample, a main signal at 217 nm and an additional signal in the larger range (>1 µm) were obtained, indicating high amounts of aggregates.

It must be taken into account that the % Intensity detected by DLS is not quantitative, and as such the particles with larger radius are overrepresented due to their more efficient light scattering. Nonetheless, clear differences in the purity of the two batches were obtained.

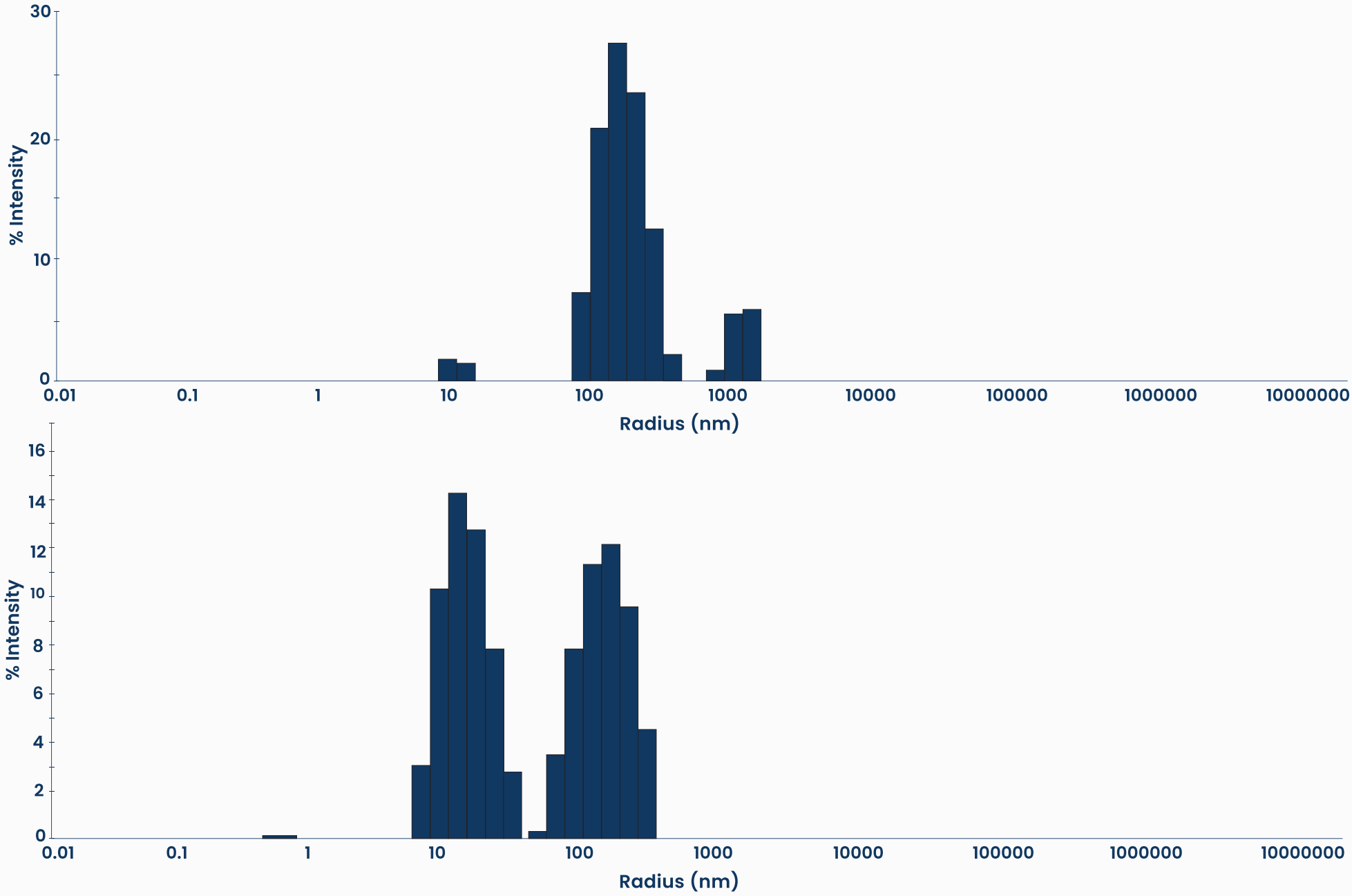

Figure 7: Analysis of different AAV batches of different quality.
Upper panel: AAV8 batch of lower quality at 1×10^{13} GC/mL (concentration according to vendor)
Lower panel: AAV8 batch of higher quality at 1×10^{13} GC/mL (concentration according to vendor)

These high amounts of impurities make samples unsuitable for an AUC-based evaluation without further sample preparation. High absorbance of not sedimenting contaminants makes it impossible to monitor the sedimentation behavior of the AAV capsid by AUC (exemplarily shown in Figure 8).

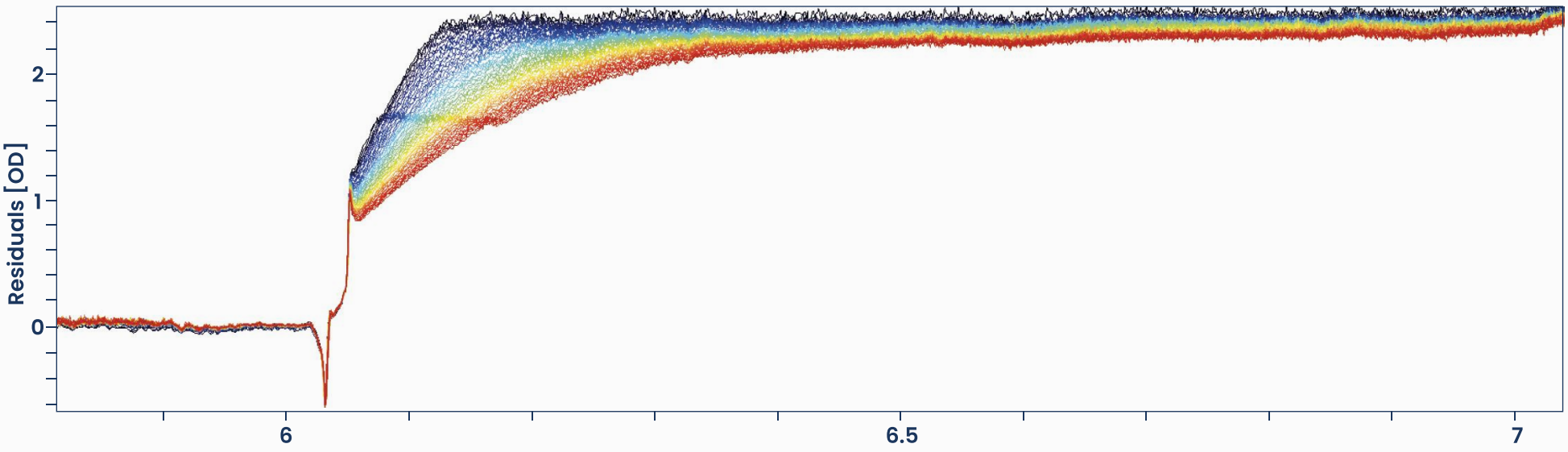

Figure 8: Exemplary absorbance scans (λ = 280 nm) of an AAV of lower quality.
AAV8 full sample diluted to a theoretical concentration of 5×10^{12} GC/mL. Due to the high amounts of impurities, no data evaluation was possible.

After depletion of contaminants, different amounts of LMWS were detected by AUC in the two characterized AAV samples (Figure 9).

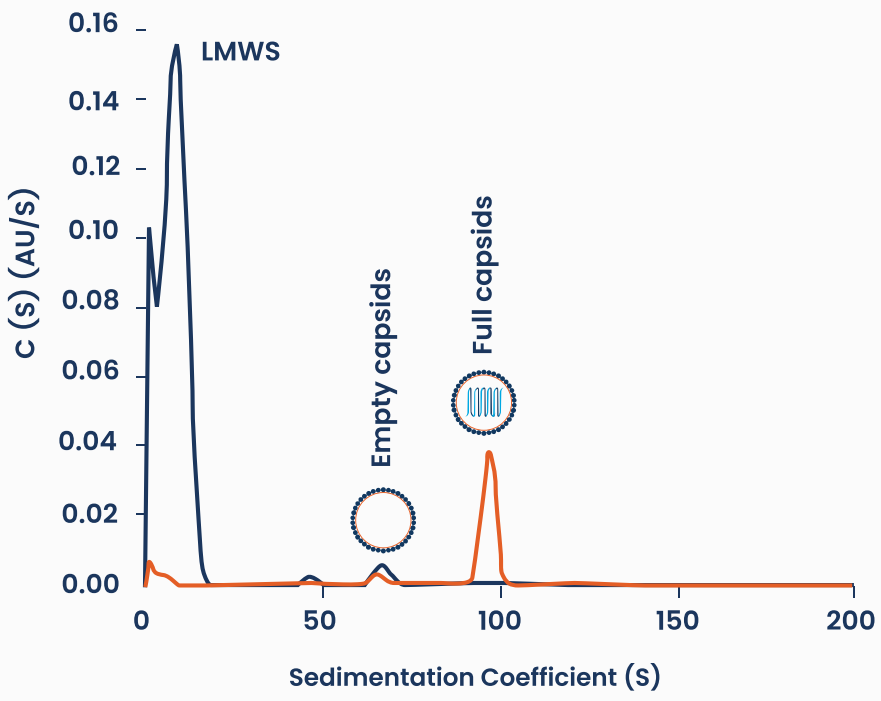

Figure 9: Comparison of sedimentation distribution.
(λ = 280 nm) of AAV batches of different quality by AUC
Blue: AAV8 batch of lower quality
Orange: Purified AAV8 batch of higher quality

Similarly, significant differences in the quality of AAV samples were demonstrated between these two different batches by SEC-MALLS (Figure 10). Degradation of the AAV particles is clearly detected with occurrence of single capsid proteins (likely VP3) and various aggregation species of capsid proteins.

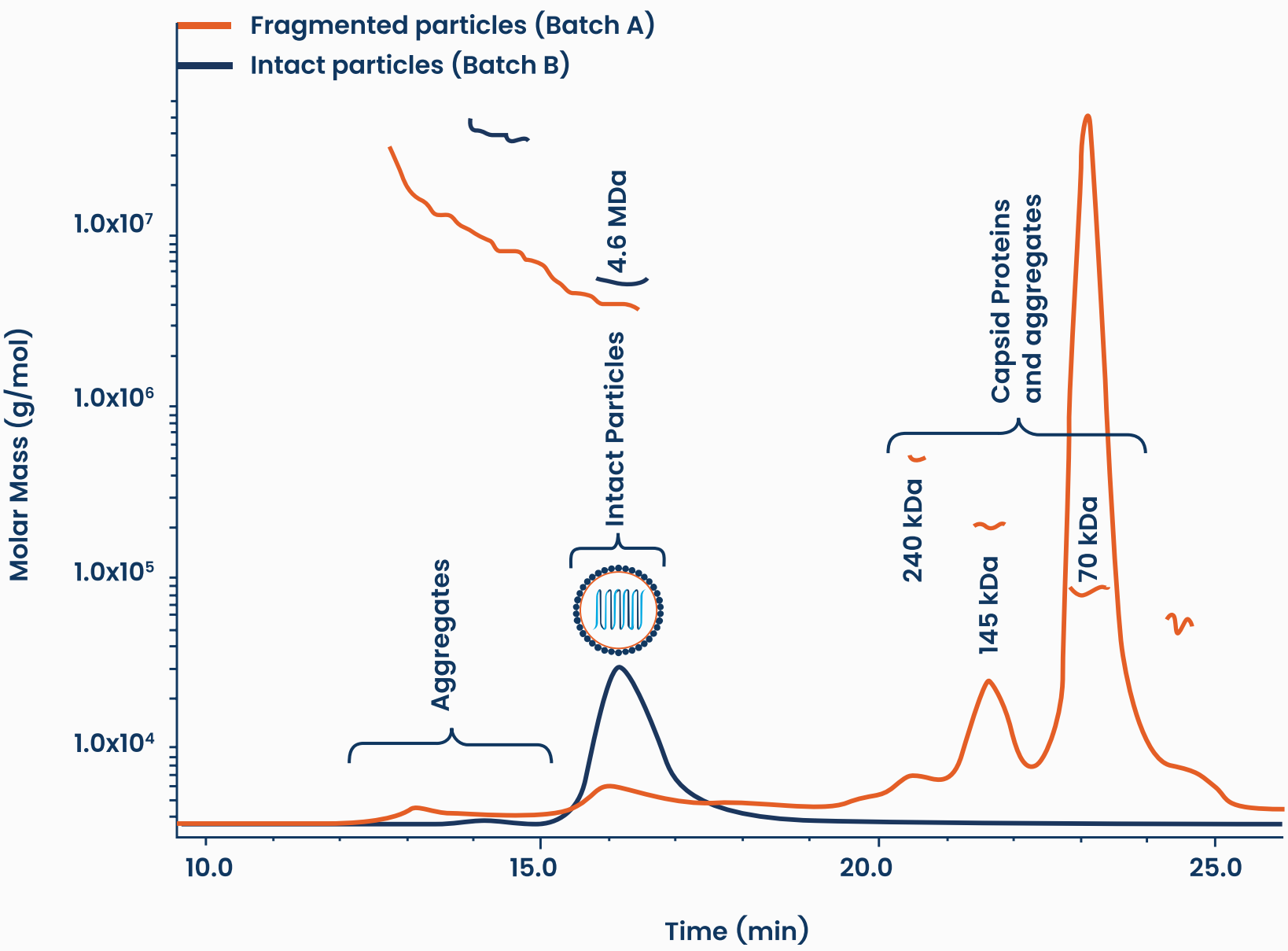

Figure 10: Overlay showing different AAV quality of intact particles and degraded particles in different batches.

In samples with high amounts of impurities, AEX-FLR likewise detected high amounts of additional charge variants (data not shown).

Taken together, these examples demonstrate that an initial quality testing step to determine the purity of the AAV sample is essential. Sub-optimal formulation conditions can affect the stability of the sample, e.g., for freeze/thaw cycles or result in general instability during storage or shipping. These instabilities might lead to an impurity level that is not comparable to the initial quality control performed by the vendor and makes it necessary to recheck the purity of the samples. But it also highlights the importance of stability testing for identifying suitable formulation conditions and maintaining the viability of the AAV product.

5 Discussion

A combined analysis, using the described methods allows a comprehensive characterization of AAV therapeutics. Contaminants, product and process related impurities can be identified on the basis of size/molecular weight and charge. As presented in this paper, all three methods were capable of determining the capsid loading with highly comparable results (see Figure 11, Table 7). Therefore, the combination of AUC with SEC and AEX is very well suited to ensure consistent quality and efficacy of an AAV drug product.

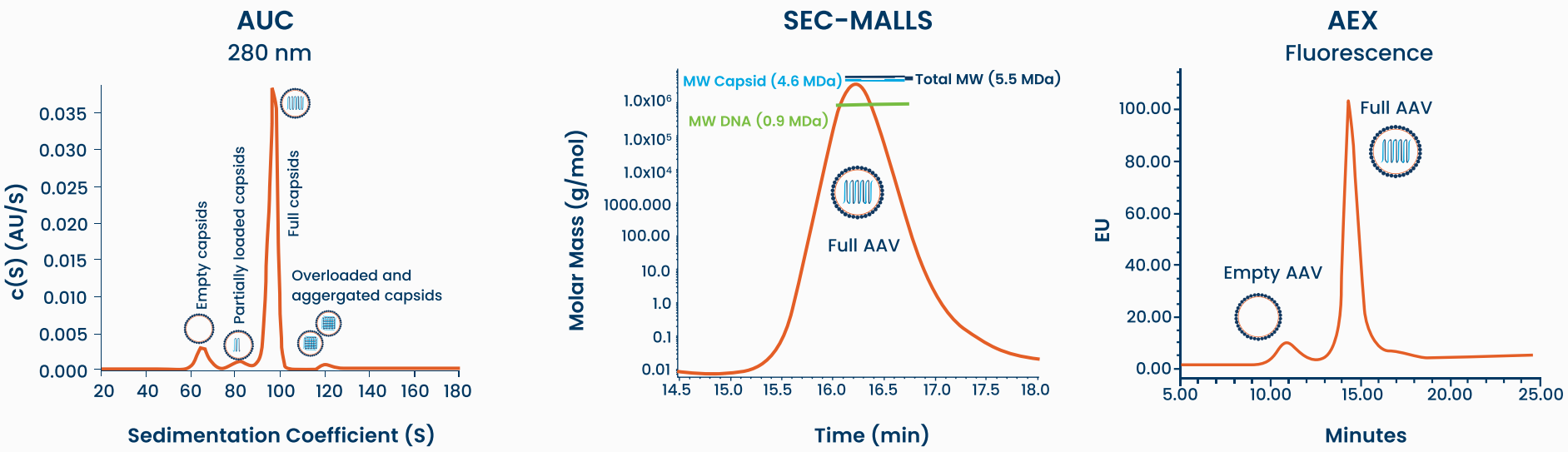

Figure 11: Comparison of AAV analysis by AUC, SEC-MALLS, and AEX-FLR.

Table 7: Capsid loading of AAV8 full sample by AUC, SEC-MALLS, and AEX-FLR

Method	Relative Amount [%]	
	Full	Empty
AUC	63	37
AEX-FLR	66	34
AEX-FLR	63	37

Of the methods described here, AUC provides the best resolution between full and empty capsids and is capable of separating partially loaded vectors. Monitoring the amount of partially loaded capsids is an important feature. Their actual composition is scarcely known but they could pose a high risk for immuno- and genotoxicity, especially if oncogenes are encapsulated [13]. Because high-throughput analyses typically are performed by SEC or AEX, an initial characterization by AUC is worthwhile to include as well to confirm the level of partially full species in a sample, which if high can then trigger further analysis of potential genetic contaminants using additional analytical approaches. The upside of SEC and AEX-FLR compared to AUC is the lower sample amount and that less time is needed for analysis as well as the availability of a GMP compliant software (see Table 8).

The advantage of fluorescence detection in AEX analysis, in contrast to UV 260 nm and UV 280 nm, is the high sensitivity for AAV, resulting in substantially less sample amount needed compared to AUC and SEC-MALLS analysis (about 100 times and 5 times lower, respectively). This is crucial, as the production of AAV is expensive and the available material is usually very limited, especially in early development stages. Moreover, the FLR signal is known to be much less biased by the presence of the DNA in the full capsids as it is the case for UV 260nm and UV 280 nm, where the number of full capsids is massively overestimated [9, 14]. Nevertheless, the signal bias between full and empty capsid peaks is most likely not the same for all AAV subtypes and also depends on the DNA sequence. Here, we propose a correction of the AEX-FLR signals by calibration with the gold standard AUC. The same sample is measured with both methods (AUC and AEX-FLR) and the ratio of full and empty capsids from AUC is taken as reference. Using this correction factor, the peak areas obtained in the AEX-FLR analysis are converted to the actual ratio of full and empty capsids (see equation [4,15]) in further routine analyses with no need for further AUC measurements. The described AEX approach provides a comparably quick and straight forward way to obtain quantification of full and empty particles by combining the accuracy of AUC with the easy handling and low material consumption of AEX-FLR. Thereby labor-intensive cleanup of full and empty standard samples is not necessary. However, the AEX method alone without signal correction can be used for a fast and robust screening of AAV samples for presence of undesired empty capsids giving a precise qualitative yes/no response. This is of high interest for, e.g., clone selection and quality control of AAV drug products. AAV samples down to a loading amount of 1×10^{10} GC can be directly analyzed by AEX without prior sample preparation or rebuffing, so that direct AEX analysis is possible vastly independent of the AAV formulation. Due to this, AEX is the method of choice for fast, robust and minimal sample consuming analysis and screening of AAV samples regarding the packaging of capsids. Another asset of this method is, that it can be validated for its use in QC labs and GMP compliant batch release of AAV products in the future.

Determination of capsid loading by SEC-MALLS is a strong orthogonal approach to AUC analysis 16. As MALLS is a technique known for the absolute determination of molecular weight, no correction factor is needed as it is the case for e.g., AEX. The parameter needed for calculation (dn/dc of capsid / DNA; $_{\varepsilon280}$ capsid /$_{\varepsilon280}$ DNA) are publicly accessible or can be determined. Further, the detection mode can be switched from either UV_{280} + RI or UV_{280} + UV_{260}, yielding independent confirmation of the measured capsid loading data. As SEC-MALLS is typically known as a high throughput method, the analysis time of 30 min for AAV samples is compatible with high throughput testing. Despite the need of more sample material compared to AEX, SEC-MALLS is a valuable and robust tool for the determination of AAV capsid loading.

In addition, a significant advantage of SEC-MALLS is the capability to determine the purity/impurities of an AAV sample with high resolution. Even the different capsid proteins (VP1, VP2, VP3) can be resolved. Compared to AUC, samples do not need any preparation step before measurement. As impurities often provide a source for potential immunological response, it is important to determine not only the amount of empty or partially loaded capsids but also of capsid proteins and their aggregates [17,18]. Even though the range of SEC-MALLS for the assessment of higher aggregates is limited by the column pore size it provides a valuable tool for determination of AAV purity.

In addition to the before mentioned methods, DLS allows a quick assessment of the sample's purity. Major benefits are short analysis time, no sample preparation, low sample amount and large size range. Therefore, DLS is an ideal method for a first quality control of AAV samples before running them in cost intensive and time-consuming analytical methods. In addition, DLS is a non-destructive method and, if sample amount is a limiting factor, the sample can be removed after DLS analysis and reused for further analytical methods.

6 Material and Methods

A predominantly empty and a predominantly full capsid sample of AAV8 containing a GFP gene were purchased from different vendors. The concentrations given by the manufacturer were $1x10^{13}$ GC /mL, respectively capsids/mL, determined by qPCR for the full sample and by ELISA for the empty sample.

In the applied setup, the Optima AUC (Beckman Coulter) was used in sedimentation velocity experiments at 15.000 rpm and 20°C. Sedimentation was monitored at 230 nm, 260 nm, and 280 nm. Data analysis was performed using the SEDFIT software [19]. Samples were purified using centrifugal concentrators (VS0231, MWCO 50 kDa, Sartorius) and the respective formulation buffers.

AAV samples were injected undiluted on an Agilent 1260 Infinity II HPLC system with an UV, MALLS, and RI detector. The separation was performed on a WTC 050S5 column (7.8 x 300 mm, 5 µm, 500 Å) with the corresponding guard column and a flow rate of 0.5 mL min^{-1}. 1XPBS was used as the mobile phase. 45 µL of each full and empty AAV sample were injected. Five independent analyses were performed. Commercially available MW standard was used for calibration. Data evaluation was performed with Wyatt ASTRA® software.

For the determination of capsid loading, information on molecular weight and concentration for both the capsid and the DNA packed inside is needed. This information is obtained by measuring the Rayleigh ratio at multiple angles (via MALLS) and the change of the refractive index (dRI). Using publicly accessible refractive index increment values for the capsid (0.185 mL g^{-1}) and for DNA (0.170 mL g^{-1}) as well as UV280 extinction coefficients (2.1 mL*(mg cm) $^{-1}$ for the capsid, 15 mL*(mg cm)$^{-1}$ for DNA) yields the eluted masses of both the capsid and DNA. Given that the theoretical molecular weight of both the capsid and the DNA is known, the capsid particle concentration can be determined according to equation (1):

$$(1) \qquad c_{Capsid} = \frac{m_{Capsid} \cdot N_A}{V_{Injection} \cdot Mw_{Capsid}}$$

c_{Capsid}: Total concentration of capsid
m_{Capsid}: Mass of capsid
$V_{Injection:}$ Injection volume
Mw_{Capsid}: Molecular weight of capsid
N_A: Avogadro's constant (6.022×10^{23} mol^{-1})

The DNA-containing particle concentration can be determined similarly by exchanging capsid-specific values for DNA-specific values. Finally, the capsid loading is calculated according to equation (2):

$$(2) \qquad Capsid\ Loading = \frac{c_{DNA}}{c_{Capsid}}$$

6.4 Evaluation of Capsid-Loading by AEX-FLR

AEX analysis of AAV samples was performed using a Protein-Pak Hi Res Q column (Waters) and an Acquity UPLC H-Class Bio (Waters). As liquid phase 70 mM bistris propane, pH 9.0 was used with a gradient of 100 mM to 300 mM tetramethylammonium chloride. A column temperature of 30 °C and a flow rate of 0.4 mL/min were applied. Signals were detected by fluorescence with excitation at 280 nm and emission at 340 nm. Data was evaluated using Empower 3 Software (Waters). The correction factor applied to the relative areas of the full and empty capsid peak in AEX-FLR is determined according to equation:

$$(3) \qquad CF = \frac{E_{AUC\%} \cdot E_{AEX-Area}}{F_{AUC\%} \cdot F_{AEX-Area}}$$

$$(4) \qquad E_{AEX\%} = 100 \cdot \frac{E_{AEX-Area}}{E_{AEX-Area} \cdot \frac{F_{AEX-Area}}{CF}}$$

$F_{AUC\%}$: Percentage of full capsids determined by AUC
$E_{AEX\%}$: Percentage of empty capsids determined by AUC
$F_{EX\%}$: Percentage of full capsids determined by AEX-FLR
$E_{AEX\%}$: Percentage of full capsids determined by AEX-FLR
$F_{AEX\text{-}Area}$: Area of full capsid peak determined by AEX-FLR
$E_{AEX\text{-}Area}$: Area of empty capsid peak determined by AEX-FLR
CF: Correction Factor

6.5 Evaluation of Impurities by DLS

AAV samples were loaded undiluted into a quartz cuvette and DLS batch measurements were performed using a DynaPro NanoStar (Wyatt Technology). The analysistemperature was set to 25°C, acquisition time was set to 5 sec and 20 scans were acquired for one sample. Hydrodynamic radii were evaluated by regularization fit using Dynamics Software (Wyatt Technology).

7 Acknowledgment

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8 Terms / Abbreviations

AAV	Adeno-associated virus
AEX	Anion-exchange chromatography
AUC	Analytical ultracentrifugation
DLS	Dynamic light scattering
FLR	Fluorescence
GC	Gene copies
GMP	Good manufacturing practice
HMWS	High molecular weight species
LMWS	Low molecular weight species
MALLS	Multiangle laser light scattering
MWCO	Molecular weight cutoff
RI	Refractive index
R_h	Hydrodynamic radius
SEC	Size exclusion chromatography

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