



Rapid high-resolution size distribution protocol for adeno-associated virus using high speed SV-AUC

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ABSTRACT

Simulated SV-AUC data for an adeno-associated virus (AAV) sample consisting of four components having closely spaced sedimentation coefficients were used to develop a high-speed protocol that optimized the size distribution analysis resolution. The resulting high speed (45K rpm) SV-AUC (hs-SV-AUC) protocol poses several experimental challenges: 1) the need for rapid data acquisition, 2) increased potential for optical artifacts from steep and fast moving boundaries and 3) the increased potential for convection. To overcome these challenges the protocol uses interference detection at low temperatures and data that are confined to a limited radial-time window. In addition to providing higher resolution AAV SV-AUC data and very short run times (<20 min after temperature equilibration), the need to match the sample and reference solvent composition and meniscus positions is relaxed making interference detection as simple to employ as absorbance detection. Finally, experimental data comparing hs-SV-AUC (at 45K rpm) with standard low-speed (15K rpm) SV-AUC on the same AAV sample demonstrate the size distribution resolution improvement. These experiments also validate the use of a radial-time window and show how quickly data can be acquired using the hs-SV-AUC protocol.

1. Introduction

Sedimentation velocity analytical ultracentrifugation (SV-AUC) can provide the most-accurate, broadest-range, and highest-resolution particle size distribution analysis of any method [1–4]. The size distribution resolving power of SV-AUC is proportional to $rpm^2 \cdot h$, where rpm is the rotor speed and h is the radial distance between the sample meniscus and the cell base [5,6]. For samples having components with sedimentation coefficients values at 20 °C, s_{20} , of ~3–30 Svedberg units (S), experimental protocols typically use rotor speeds of ~30–50K rpm. In these cases, increasing the rotor speed to 60K rpm (the maximum rotor speed of the analytical ultracentrifuge) only improves resolution marginally, while reducing both the total number of useful scans and the total number of samples per run because the 4-hole rotor must be used instead of the 8-hole rotor due to rotor speed limitations. However, in the case of the large (>30 S, at 20 °C), complex supramolecular biopharmaceuticals being developed for gene therapy (e.g. viral vectors and nanoparticles), rotor speeds typically do not exceed 20K rpm [7,8] and can be as low as 3K rpm [9]. As a result, running these latter samples at higher rotor speeds should provide a significant opportunity to achieve

higher-resolved sample size distributions.

To date, little attention has been paid to optimize experimental protocols for SV-AUC analysis of supramolecular biopharmaceuticals. Instead, from the authors' perspective, the focus has been directed towards acquiring more data scans per sample in the hopes of improving the data analysis precision, expanding the range of wavelength that can be used to interrogate samples when using absorbance detection and maximizing the number of samples that can be run in an SV-AUC experiment. In so doing, current SV-AUC protocols use lower rotor speeds and increased run times. These conditions, however, allow diffusion to obscure boundary separation, decreasing the size distribution resolution of any analysis method, regardless of its numerical sophistication. Consequently, a balance must be struck between increased rotor speed, which improves boundary separation and reduces run time, and the time needed to acquire enough data scans per sample to allow effective data analysis.

For the present work, adeno-associated virus (AAV) has been used as a model system to explore how increasing the rotor speed might improve SV-AUC size distribution analysis of gene therapy biopharmaceuticals. The choice of AAV is relevant given its widespread use in gene therapy

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[10–12], coupled with the need to characterize the wide range of drug-related impurities (e.g., empty, partially filled, over filled and aggregated AAV particles) commonly found in AAV preparations [13–16]. Due to diffusion, and the very heterogeneous nature of some of these impurities, size distribution analysis from normal low speed (10–20K rpm [7,8,13,17]) SV-AUC on AAV samples can result in overlapping and misleading size distributions. Consequently, AAV sample characterization may be compromised with respect to assessing its lack of homogeneity, the complexity of its heterogeneity, and thus the accurate quantification of the final AAV drug product potency. Uncertainty in these properties raises questions concerning the accuracy of the dosing solution and its impurity profile being administered to the patient.

An optimized high speed SV-AUC (hs-SV-AUC) protocol for AAV was initially developed by doing simulation work on a hypothetical AAV sample consisting of equal amounts of four homogeneous AAV capsids containing differing amounts of ssDNA in 0.15 M NaCl. The four AAV species differed in their sedimentation coefficients (at 20 °C) by a spacing of 3.7 S and span a range of 89–100 S. SV-AUC simulations at different rotor speeds show a significant resolution improvement is achieved by hs-SV-AUC over lower-speed protocols (10–20K rpm, which we have chosen to be represented by the average rotor speed of 15K rpm).

In addition to improved resolution, hs-SV-AUC results in very short run times (<20 min in the case of AAV). These short run times coupled with the high molecular weight (MW) of particles like AAV yields steep migrating boundaries due to their low diffusion. The presence of these steep, rapidly moving boundaries puts a high demand on the optical detector being used to collect data in terms of having high optical resolution, being insensitive to steep refractive gradients and providing rapid data acquisition. Absorbance detection is too slow (approximately in the range of 14–20 s/cell for the Optima and 30–60 s/cell for the XL-A/I depending on the radial increment and rotor speed), lacks radial resolution (~50 µm) and is too sensitive to schlieren effects that can potentially arise from the steep boundaries [18,19] that hs-SV-AUC can generate. However, the radial resolution (~9 µm), acquisition time (~5 s) and accurate tracking of steep gradients of the interference (refractometric) detector (the Rayleigh interferometer, available and frequently also present on many analytical ultracentrifuges) meet the demands of hs-SV-AUC. Furthermore, the interferometer captures the entire cell image simultaneously (i.e., it does not scan the cell), so there is no data distortion due to the time lag between the acquisition of the first and last radial data points. The interferometer has vastly superior precision, orders of magnitude broader linear concentration response and far lower sensitivity to light scattering and schlieren distortion compared to absorbance detection.

Nevertheless, interferometers are sensitive to the net differential movement of *all* solution components in both the reference and sample sectors. Ordinarily, to prevent solvent component sedimentation from contributing to the interference signal: 1) the solvent composition of components must be matched exactly in the sample and reference sectors and 2) the sample and reference menisci positions also must be matched, making cell filling onerous or necessitating the use of meniscus matching centerpieces which are easily damaged and require more care in filling than simple double sector centerpieces. In cases where the two above conditions cannot be met, attempts at modelling the migration of the solvent salt/buffer components along with the macromolecular components by using water in place of a reference buffer solution in the reference sector have been recommended, but this approach has been shown to have limitations when the typical discrete non-interacting continuous c(s) distribution model in SEDFIT is used [20]. Software, called buffer mismatch model, BMM, has been added to SEDFIT to attempt to account for mismatches by computer modelling [20] (see further discussion concerning BMM at the end of the Discussion section of the paper). Here, however, we show via both computer simulation and actual experiments that by restricting data analysis to a specific

radial and time range (referred to as “radial-time window”), AAV material migration is confined to a relatively large flat salt/buffer refractive index plateau region devoid of contributions from the sedimentation of solvent components, making the c(s) model straight forward to use. Changes that do occur in the solvent plateau, due to solvent radial dilution, are effectively handled by SEDFIT with minimal loss in size distribution resolution. Thus, by choosing an appropriate radial-time window, using interference detection becomes as simple as absorbance detection.

An additional issue surrounding the short run time of the hs-SV-AUC protocol that needs to be dealt with is the absence of a stabilizing solvent density gradient which makes migrating boundaries susceptible to convective boundary distortion, especially at high rotor speeds. To avoid the potential for convection, the hs-SV-AUC protocol should be conducted at a low temperature (e.g. ≤ 10 °C) to take advantage of the unique temperature-dependent density properties of water [21]. In addition, the lower temperature increases solution viscosity, which slows boundary movement, allowing for the acquisition of more data scans.

Finally, AAV samples in AAV formulation buffer were analyzed using both a ‘standard’ low-speed protocol and the new hs-SV-AUC protocol. Results from these experiments provide direct experimental evidence that the AAV hs-SV-AUC protocol results in a significantly higher resolution size distribution analysis. An assessment of the advantages, limitations, and options for overcoming these limitations are discussed.

2. Material & methods

2.1. AAV and other material

All AAV AUC work was carried out using the same AAV sample produced, purified and diafiltered into an AAV formulation buffer at Biogen. AAV stock solutions were provided at concentrations requiring a 10- to 20-fold dilution with buffer to generate two different AAV sample concentrations. For solvent mismatch SV-AUC experiments, either 150 mM NaCl, PBS (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na₂HPO₄, pH 7.4) or AAV formulation buffer were used. The AAV formulation buffer used in this work has NaCl as the main component, along with low levels of a buffer and non-ionic detergent yielding a pH 7 solution.

2.1.1. Hypothetical AAV sample

All AAV simulations used a hypothetical sample consisting of four species (Table 1): a full AAV particle consisting of a protein shell with an assigned MW of 3.90 MDa and a ssDNA genome (DNA payload) with an assigned MW of 1.33 MDa yielding an AAV particle with an assigned sedimentation coefficient at 20 °C under ideal behavior conditions (*s*₂₀^o)

Table 1
Molecular weight (MW) and sedimentation coefficient (s) of the hypothetical AAV sample containing equal amounts of full and three partially filled AAV particles containing 90%, 80% and 70% of the full DNA payload.

^a %DNA	^b AAV particle MW, MDa	^c <i>s</i> ₂₀ ^o , S	^e <i>s</i> ₁₀ ^o , S
100	5.23	^c 100	76.6
90	5.10	^d 96.3	73.8
80	4.98	^d 92.6	71.0
70	4.83	^d 88.9	68.1

^a The % amount of the full DNA payload (of 1.33 MDa DNA MW) in the AAV particle.

^b 3.90 MDa protein capsid MW + (%DNA) (1.33 MDa DNA MW).

^c An approximate assigned S value for a full AAV particle containing a 1.33 MDa DNA payload.

^d Computed from the *s*₂₀^o value of the empty AAV of 63 S and the (%DNA) (37 S difference between full and empty *s*₂₀^o), see eq. 1.

^e Computed from data at 20 °C, taking into account the change in temperature on the viscosity of water, see eq. 2.

of 100 S (a reasonable S value for an AAV particle packaged with a DNA molecule of this size) and three additional AAV components consisting of the same intact AAV shell, but are only partially filled, containing 90%, 80% and 70% by mass of the complete AAV DNA payload. The corresponding sedimentation coefficient for these three species was calculated from the difference between the sedimentation coefficient of the full, 100 S, and empty AAV, which was also assigned a reasonable s_{20}^o value of 63 S. The resulting 37 S difference was then multiplied by the percent mass of the full DNA payload (%DNA) present in a given partially filled AAV particle and added to the 63 S value for the empty protein shell as indicated using equation (1) to obtain the s_{20}^o for partially filled AAV (note, this approach is analogous to that used by Burnham et al. [7]):

$$s_{20}^o = 63 + \%DNA * 37$$
 eq. 1

Sedimentation coefficients at 10 °C under ideal behavior conditions (s_{10}^o), Table 1, were calculated using Equation (2):

$$s_{10}^o = s_{20}^o \left[\frac{\eta_{20}}{\eta_{10}} \right]_{water}$$
 eq. 2

Changes in solution density and the partial specific volume of AAV in going from 20 °C to 10 °C were not considered significant in comparison to viscosity (η) changes.

2.2. Analytical ultracentrifugation

All SV-AUC experiments were carried out in the Analytical Development department laboratories at Biogen located in Cambridge, Massachusetts. All AAV and solvent SV-AUC experiments were carried out on an Optima analytical ultracentrifuge equipped with both UV-Vis absorbance and refractometric (Rayleigh interferometer) detection using an An-60 Ti 4-hole rotor at 10 °C. AUC cells employed in these experiments contained 12 mm Epon double sector charcoal-filled centerpieces with sapphire windows. AAV experiments were conducted at either 15 K rpm (a common speed employed when doing AAV SV-AUC), or 45K rpm (hs-SV-AUC), while solvent-only experiments were conducted at 45K rpm (hs-SV-AUC).

2.2.1. AUC cell filling

For hs-SV-AUC to function as intended, sample and reference sectors of the AUC cell must be filled so that the radial difference between the sample and reference menisci is ≤ 0.1 cm and the sample meniscus is at a radial position of about 6.1 cm or less (noting ideally that the reference meniscus radial should be at a value equal or less than the sample meniscus radial position). These filling specifications should be achieved easily in routine practice. Nevertheless, if these limits should be exceeded the resulting impact is very likely not that serious (see Discussion section point 3).

2.2.2. Solvent mismatch SV-AUC experiments

Solvent mismatch hs-SV-AUC experiments for 0.15 M NaCl solution, PBS buffer or AAV formulation buffer were conducted at 45K rpm, at 10 °C. In all cases the sample sector solvent concentration was 5% lower than the reference sector solvent concentration. AUC cell filling followed the protocol stated in section 2.2.1 with the sample meniscus at ~6.1 cm and the reference meniscus at ~6.0 cm (to achieve a menisci difference of about 0.1 cm between reference and sample sectors).

2.3. SV-AUC simulations

All SV-AUC simulations were produced using SEDFIT version 15.01b, for up to four non-interacting discrete (ideal behaving) species. Each simulation consisted of 50 data scans computed using a radial resolution of 0.001 cm, and took into account rotor acceleration at 400 rpm/s.

2.3.1. AAV and NaCl SV-AUC simulations

AAV SV-AUC simulations at 20 °C or 10 °C used the molecular weights (MWs) and appropriate sedimentation coefficient value shown in Table 1, an average partial specific volume, $\bar{v}$, of 0.69 ml/g and a buffer density of 1.0 g/mL. AAV SV-AUC data for each temperature and rotor speed were generated using the time intervals indicated in Table 2 over a radial range of 6.1–7.2 cm with a signal to noise ratio (S/N) value of 10^3 unless stated otherwise. NaCl SV-AUC simulations calculated for each temperature and rotor speed were also generated using the same time intervals indicated in Table 2 over the radial range of 6.0–7.2 cm or 6.1–7.2 cm with a signal to noise ratio (S/N) value of 10^6 using the following parameters: MW = 58.45 Da, $\bar{v}$ = 0.3 mL/g [24], s_{20}^o = 0.1 S (an average of reported literature values [20,22,23]) for simulations at 20 °C and s_{10}^o = 0.077 S (temperature-corrected s_{20}^o value generated using eq. (2)) for simulations at 10 °C.

2.3.2. Simulating the solvent (NaCl) mismatch signal (in fringes) when doing SV-AUC with interference at different rotor speeds

The interference signal contribution arising from solvent mismatch was simulated for 0.15 M NaCl at the rotor speeds and time intervals indicated in Table 2 for 10 °C using a 0.1 cm meniscus position mismatch (6.0 cm for reference sector and 6.1 cm for sample sector) and a 5% NaCl concentration mismatch (5% lower NaCl concentration in the sample sector yielding an actual NaCl concentration of 0.1425 M). These specific levels of mismatch are unlikely to be exceeded by an AUC user and are considered *upper limit* values. Since the fringe displacement output of the interferometer is directly proportional to the concentration (refractive index) difference between the sample and reference sectors, our strategy for simulating the NaCl mismatch signal, given the stated mismatch, was to simulate the sedimentation of the NaCl in each sector independently, then take the difference between the two sectors (see Fig. 1S and 2S, which provides an outline and pictorial overview of the process, respectively). Specifically, NaCl SV-AUC data was simulated separately for the reference sector between 6.0 and 7.2 cm at an initial concentration of 1.00 (arbitrary units) and the sample sector between 6.1 and 7.2 cm at an initial concentration of 0.95 (arbitrary units) as indicated in section 2.3.1. The appropriate resulting NaCl sedimentation scans for both sectors in arbitrary concentration units were then multiplied by the factor 25.5 (the concentration of 0.15 M NaCl in fringes based on a fringe count of 17 fringes at 670 nm obtained from a synthetic boundary experiment for 0.1 M NaCl in a 12 mm centerpiece [20]) to convert them to fringe displacement readings (Fig. 2SB). For each time interval (scan) the expected NaCl interference signal contribution, due to the differential NaCl sedimentation between the reference and sample sectors, was determined by taking the difference between the corresponding reference and sample signals (Fig. 2SC). A “*solvent mismatch interference difference signal plot*” was then generated by taking the difference between the expected NaCl interference signal contribution scans corresponding in time to the first and last scan used in the c(s) analysis of the AAV hs-SV-AUC protocol (Fig. 2SD).

Table 2
Required scan time interval and resulting total run time (t), in seconds, to monitor the complete sedimentation of the full monomeric AAV particle in a total of 50 scans from 6.1 to 7.1 cm as a function of rotor speed and temperature.

Rotor Speed (RPM)	At 20 °C, scan time interval (total time for 50 scans), sec	At 10 °C, scan time interval, (total time for 50 scans), sec
15K	140 (7037)	190 (9537)
30K	34 (1775)	46 (2375)
45K	15 (862)	19 (1062)
60K	7 (550)	10 (650)

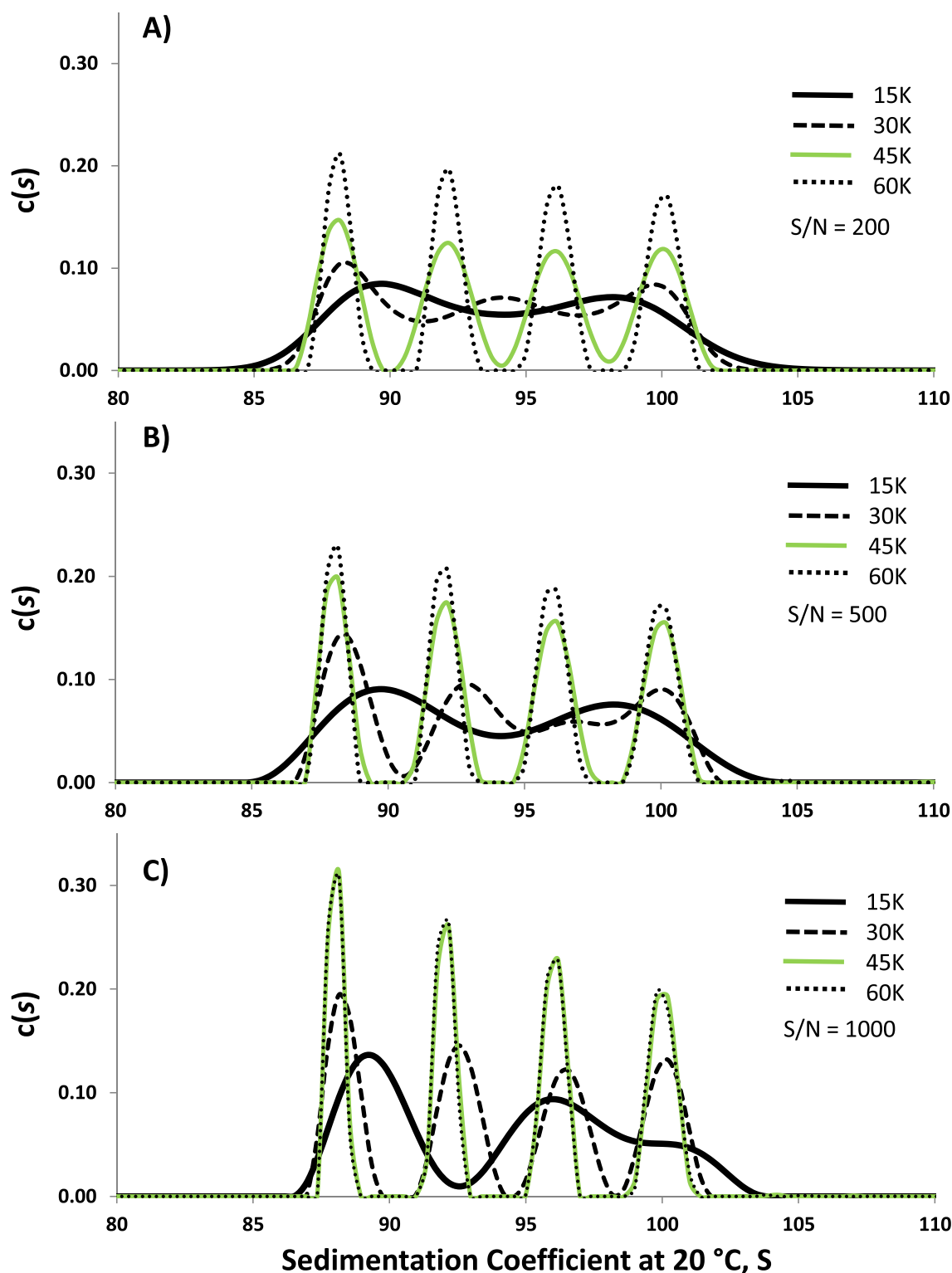


Fig. 1. Computer SV-AUC simulation work on a hypothetical AAV sample (Table 1) in a 0.15 M NaCl solution at 20 °C containing a 1:1:1:1 mix of AAV particles filled with different percentages (100%, 90%, 80% & 70%) of a viral vector DNA payload to assess the impact of rotor speed and S/N on the resolution of the resulting size distribution profile.

2.4. Data analysis on AAV SV-AUC and hs-SV-AUC experiments and solvent mismatch hs-SV-AUC experiments

Data analysis on all AAV sedimentation velocity experiments used the $c(s)$ distribution model in SEDFIT (version 15.01b), which assumes

that all species migrate as discrete independent, ideal components. Low speed (15K rpm, 10 °C, SV-AUC) absorbance and interference data were analyzed using a radial-time window of 6.15–7.15 cm and ~9500 s. High speed (45K rpm, 10 °C, hs-SV-AUC) interference data used a radial-time window of 6.4–7.0 cm and ~1000 s. All $c(s)$ analyses employed

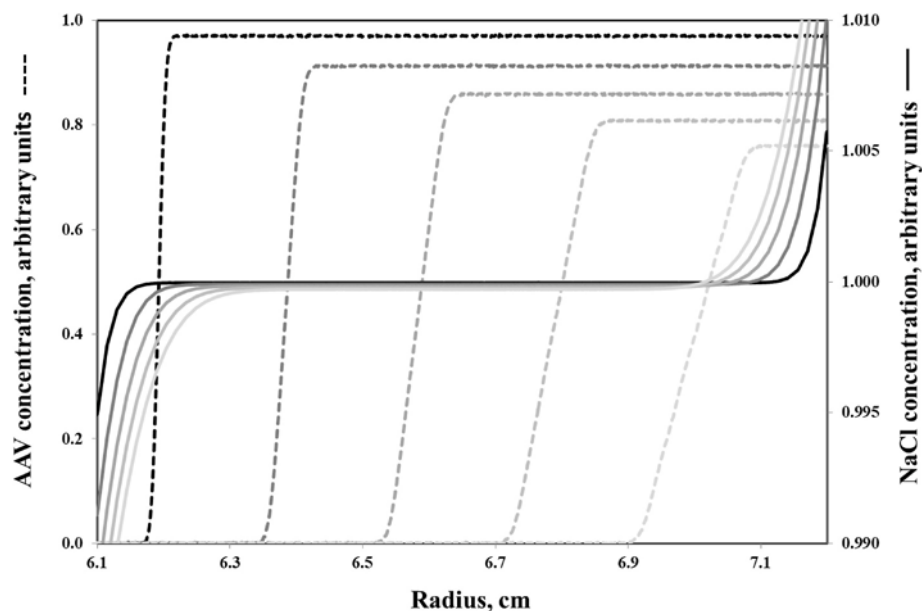


Fig. 2. Overlay of the 1st, 10th, 20th, 30th and 40th data scans of AAV (solid lines) and NaCl (dash lines) from a simulated hs-SV-AUC experiment on a hypothetical AAV sample (Table 1) in a 0.15 M NaCl solution having a meniscus located at a radius (r) of 6.1 cm, run at 45K rpm at 20 °C using a data scan time interval of 19 s. Data scans of AAV and NaCl (based on an initial arbitrary concentration (c) of 1.0 unit) were taken at the same time point have corresponding matching shades of black color (where black corresponds to the 1st scan while the lightest shade of grey corresponds to the 40th data scan). The resulting plot shows that the majority of time AAV migrates through a NaCl solution where there is little to no density (ρ) gradient ($d\rho/dr$) stabilization (noting that $dc/dr \propto d\rho/dr$). As a result, AAV material migrates through a large radial region (from about 6.3 to 7.0 cm) where it is exposed to the potential adverse effects of convection arising from the presence of very weak negative radial density gradients induced, for example, by the presence of increasing temperature with increasing radius.

data processing to remove both radial- and time-independent (RI and TI) noise signals. The steep boundaries encountered for AAV, especially for hs-SV-AUC, challenge finite element modeling [25,26]. Consequently, both maximum entropy and 2nd derivative (Tikhonov-Phillips) with Simplex regularization were used at a confidence level of 0.68. All $c(s)$ vs S distribution plots presented here were obtained using 2nd derivative regularization since it produced fewer erratic fits or extraneous peaks and avoided fusing closely spaced peaks [27]. Resolution was set at 250 and $S_{\min}$ and $S_{\max}$ values were set to 40 S and 130 S, respectively.

To determine the impact of solvent mismatch on AAV hs-SV-AUC data, experimental solvent hs-SV-AUC data were analyzed by generating “normalized solvent mismatch interference difference signal plot” for each solvent by taking the difference between the first and last solvent scans corresponding to the same first and last AAV scan that define the radial-time window used for AAV hs-SV-AUC analysis (i.e. from ~6.4 to 7.0 cm and from the first scan until the last scan acquired at ~1000 s). In order to adjust for the plateau decrease due to salt sedimentation over this time, the readings for the first and last scans were adjusted by subtracting the averaged fringe reading, determined over the radial range of 6.7–6.8 cm. Doing this normalized the scans so that the radial extent of the intrusion of the “salt boundary” into the flat solvent interference signal would stand out allowing for the radial-time window to be more accurately defined.

3. Results

3.1. Effect of rotor speed and detector S/N on $c(s)$ vs S distribution resolution

Generating high-resolution sedimentation coefficient size distribution plots is important for distinguishing the presence of unique components having closely-spaced sedimentation coefficients. AAV samples provide a good example where high-resolution analysis is important. In addition to the fully-intact AAV monomer, AAV samples often contain a variety of other particles, e.g., empty, partially filled, over filled virus

and free DNA and protein components of the viral particle along with a range of potential aggregates of these materials [13–16,28], that can yield closely-spaced sedimentation coefficient profiles (e.g., homogeneous or heterogeneous simple aggregates (dimers) of some of the previously mentioned AAV particles can migrate close to the full monomeric AAV particle). To explore the impact of increasing rotor speed at 20 °C on resolution, SV-AUC data for a hypothetical AAV sample containing four-species (Table 1) were simulated using four different rotor speeds (15K, 30K, 45K and 60K rpm). For each rotor speed, the time interval between scans was chosen (Table 2) to generate 50 data scans [29] enabling the monitoring of the full AAV particle’s sedimentation from meniscus to cell base. Data were generated with stochastic noise levels consistent with the optimal S/N value for the detectors presently available: 200 for the XL-A/I absorbance detector, 500 for the Optima absorbance detector (at ~1 OD signal) and 1000 for the interference detector (at ~2–3 fringes) on either the XL-I or Optima. For each rotor speed, size distributions were assessed as described in Material and Methods. Fig. 1 show the increasing resolution achieved with increasing the rotor speed. While each increasing rotor speed improved the resolution, going from 45K rpm to 60K rpm offers only a marginal gain in resolution while reducing sample throughput by requiring the use of the lower sample capacity 4-hole (An-60 Ti) rotor due to rotor speed limitations on the higher sample capacity 8-hole (An-50 Ti) rotor. Resolution also improved with higher S/N (Fig. 1A–D), but the improvement is not as significant as that achieved by increased rotor speed. We conclude that for AAV doing hs-SV-AUC at 45K rpm is an optimal speed in terms of resolution and sample throughput.

3.2. Achieving convection-free sedimentation with hs-SV-AUC

Because AAV migration outruns the formation of a stabilizing solvent density gradient at 45K rpm and 20 °C, as illustrated in the case of a 0.15 M NaCl solvent in Fig. 2, the AAV hs-SV-AUC protocol is susceptible to sample convection due to thermal fluctuations [21]. This can be of

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particular concern during the adiabatic cooling of the rotor on accelerating it to run speed [21,30]. Even very small temperature-induced negative density gradients can cause convection due to the amplifying effect of the high gravitational field [30]. Lowering the rotor temperature from 20 °C to around 4 °C, where $\frac{\partial \rho}{\partial T} \approx 0$, should mitigate this problem. However, because rotor temperature equilibration can take a long time at 4 °C we have initially investigated using 10 °C for hs-SV-AUC, where $\frac{\partial \rho}{\partial T}$ is $\sim \frac{1}{2}$ the value at 20 °C (see Fig. 2S in reference [21]). Pre-equilibration of the rotor and samples to the lower temperature significantly reduces the equilibration time. However, to prevent sample leaks, cells should be re-torqued before running if they were loaded at room temperature.

3.3. Limitations of absorbance detection when doing hs-SV-AUC

To acquire 50 scans for AAV hs-SV-AUC analysis at 45K rpm (which enables the complete sedimentation of the AAV full particle) requires that all cells are scanned every 19 s or less (Table 2). Coupling this requirement with the scanning time limit of about 30–60 and 17–20 s/cell (depending on radial step size), for the XL-A/I and Optima respectively, means only the Optima could be used to acquire data on one sample and one wavelength using normal absorbance data acquisition, or two samples using pseudo-absorbance [31]. However, using pseudo-absorbance excludes the use of interference detection. Furthermore, even at the fastest scan rate there will be significant boundary movement during a scan. While it is possible to account for the distorting effect of boundary movement during the scanning process, by assigning a unique time point to each radial reading during a given scan [32], the resulting increase in data analysis complexity exposes the analysis to opportunities for inaccuracies and added statistical uncertainties. Also, the sharp boundaries (or concentration gradient) encountered in hs-SV-AUC can result in schlieren distortion of the absorbance readings [33], which when coupled with the absorbance system optical resolution on both the XL-A/I and Optima of $\sim 50 \mu\text{m}$ [34] can limit the absorbance system's ability to accurately monitor sharp boundaries.

3.4. hs-SV-AUC using interferometry

The Rayleigh interferometer, a refractive detector, is capable of acquiring data from all samples in a rotor in ~ 5 s, capturing the entire image of each cell in a fraction of a second (i.e. no scanning) with a radial resolution of $\sim 9 \mu\text{m}$ [18,34]. The detector also has comparable sensitivity to the absorbance detector (~ 3.3 fringes/mg/ml versus ~ 1 OD_{280 nm}/mg/ml or ~ 5 OD_{230 nm}/mg/ml for proteins). In the case of the Optima's interferometer, uncertainty in fringe displacement measurements can approach RMSD values of ± 0.001 fringes (see Fig. 3S), which for a 0.2 fringe sample yields a S/N that is nearly comparable to the absorbance detector precision at 230 nm. In addition to having a much wider dynamic range, the signal vs concentration linearity of the interferometer is superior to the absorbance system, which is subject to stray light limitations at both low and high concentrations [18,35]. Its combination of rapid data acquisition, higher radial resolution, greater accuracy in tracing steep boundaries, superior S/N at higher concentration (at or above 1 fringe ~ 5 OD_{230 nm} for AAV) and lower sensitivity to light scattering signal (that can lead to inflated absorbance concentration readings) makes a properly focused interference system [36] the detector of choice for AAV hs-SV-AUC experiments.

However, the interference system is sensitive to all solution components, including the solvent salts and buffers and other formulation-added excipients. Hence any difference in the solvent component sedimentation between the reference and sample sectors will give rise to refractive index changes that will contribute a signal to the total signal output from the interferometer. Even the slightest mismatch in the solvent component concentrations and difference in meniscus position between both sectors (due to slight difference in filling volume) will result in the superposition of the solvent component sedimentation with the macromolecular component signals of interest, distorting and complicating the data analysis leading to what we call “the interference solvent mismatch problem”. However, the short run time of hs-SV-AUC can mitigate this problem by limiting data analysis to a radial-time window that is unperturbed by the redistribution of solvent boundaries. Only the flat decreasing solvent plateau signal with time (due to radial dilution) is present in the analysis region. The contribution to the plateau signal resulting from the solvent mismatch appears to be

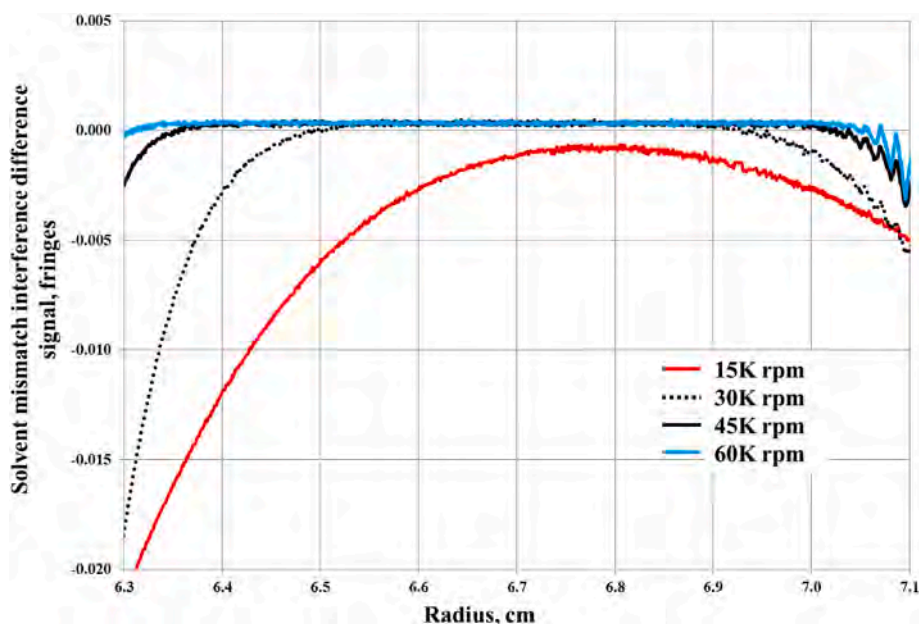


Fig. 3. Overlay of the simulated “solvent mismatch interference difference signal plots” (see section 2.3.2) for 0.15 M NaCl (a proxy AAV formulation buffer or solvent) due to a 5% solvent compositional mismatch between the reference (0.15 M NaCl) and sample (0.1425 M NaCl) sectors of an AUC cell along with a 0.1 cm mismatch in menisci positions between the reference (6.0 cm) and sample (6.1 cm) sectors (due to a difference in solvent filling volumes) as a function of rotor speed at 10 °C: 15K (red), 30K (black, solid), 45K (black, dashed) and 60K (light blue) rpm.

radially-independent systematic noise and is removed during the analysis.

3.5. *hs-SV-AUC overcomes “the interference solvent mismatch problem”*

Solvent contributions to the interference signal were simulated for different rotor speeds with a 0.1 cm meniscus mismatch and 5% solvent concentration mismatch as described in Materials and Methods (and Fig. 1S and 2S) using 0.15 M NaCl as a proxy solvent. The resulting overlay of the simulated “*solvent mismatch interference difference signal plot*” at each speed over the time range that allows the complete migration of the full AAV particle (Fig. 3) indicate that AAV *hs-SV-AUC* experiments are unaffected by solvent sedimentation at 45K rpm and 10 °C, when limiting the radial-time analysis window to 6.4–7.0 cm and the first 1000 s. Thus, the AAV *hs-SV-AUC* protocol alleviates the need for extensive dialysis and meniscus matching. The question arises, though, whether the subset of data acquired using this radial-time window (Fig. 4A) diminishes the resolution of the size distribution analysis. Comparison of data analysis constrained to the AAV *hs-SV-AUC* protocol’s radial-time window (6.4–7.0 cm and 1000 s) to the analysis that encompasses the complete data range (6.15–7.15 cm and ~1100 s) shows minimal resolution reduction (Fig. 4B) when analyzing simulated data using our hypothetical AAV test sample (Table 1). This question is also addressed more critically in terms of differences in the operation of the interferometers on the XL-I and Optima in Fig. 4S. The simplicity (no dialysis, meniscus matching or additional modelling), increased resolution (Fig. 1) and greatly shortened run time (<20 min versus hours) makes AAV analysis by the *hs-SV-AUC* protocol an attractive alternate approach to AAV SV-AUC conducted at much lower rotor speeds.

3.6. *Experimental verification of the solvent (NaCl) mismatch simulations*

Experimental data acquired for three solvents using the *hs-SV-AUC* protocol and analyzed as described in Materials and Methods, section 2.4, to generate “*normalized solvent mismatch interference difference signal plots*” are shown in Fig. 5. For all three solvents, using the *upper limit* meniscus and composition mismatches used in the solvent (0.15 M NaCl) mismatch simulation work (Materials and Methods section 2.2.2), the resulting experimental plots are flat, in reasonably good agreement with our solvent simulation work at 45K rpm when confined to the radial-time window of the AAV *hs-SV-AUC* protocol. Consequently, the solvent interference signal contribution arising from solvent mismatch levels that are at or below what we have defined as *upper limits* are handled as radially-independent noise by SEDFIT yielding AAV sedimentation data as though there were no mismatch.

3.7. *Experimental comparison of the same AAV sample run at 45K rpm (hs-SV-AUC) vs 15K rpm (SV-AUC) at 10 °C*

Using a single AAV sample at two concentrations (0.2 and 0.45 fringes), three separate experimental runs were conducted using the AAV *hs-SV-AUC* protocol (45K rpm at 10 °C) involving a total of 4 cells. Results from these 4 cells are shown in Fig. 6 (see Fig. 5S, for an overlay of these data). The same AAV sample was also analyzed in two separate SV-AUC runs at 15K rpm and 10 °C using absorbance and interference detection (at an AAV concentration of about 2 OD_{230nm} ≈ 0.4 fringes) involving a total of two cells. Results from these 2 cells are shown in Fig. 7A and B reveal a reduced level of complexity in the *c(s)* distribution relative to the data generated using the *hs-SV-AUC* protocol at the same temperature. This difference is more visible in the overlay plot shown in Fig. 7C where the data have been normalized for plot area. Here the higher complexity and amount of material involved in this complexity, afforded by *hs-SV-AUC*, is revealed in terms of the higher complexity of the distribution of AAV material between the empty and filled AAV particles and in particular on either side of the full AAV particle.

A more quantitative comparison of data acquired with the same AAV

sample analyzed at 45K and 15K rpm is provided in Table 3. Results show excellent agreement between the two sets of data for the sedimentation coefficients of the empty and full AAV particles, as well as the fractional amount of empty AAV particles. However, the percentages for partially filled (defined as the AAV material located between the AAV empty and full peaks), full, and aggregated material (corresponding to the AAV moving faster than full AAV peak, which may possibly include over filled intact AAV particles [14,37]) showed noticeable differences. Some of this deviation could result from the different response factors (extinction coefficient at 230 nm and refractive index increment at 670 nm) that translate the concentration of the different chemical components of AAV particles (protein and DNA) into detector signal. However, given the response constants for protein and DNA absorbance at 230 nm (extinction coefficient) and refractive index at 670 nm (refractive index increment) are similar [38], the enhanced resolution of material on either side of the full AAV particle peak at higher rotor speed (Fig. 7C) is more likely the major contributing factor for the observed differences. The data in Table 3 suggest that SV-AUC runs at lower speeds (i.e., at 15K rpm) might lead to an overestimate of the fractional amount of full particles present in the AAV sample, since higher speed data show that other AAV species are present with sedimentation coefficients that are close to that of the full AAV particle.

The results shown in Figs. 6 and 7 clearly demonstrate the ability of *hs-SV-AUC* to provide a more detailed characterization picture of the AAV heterogeneity and thus a potentially more accurate concentration assessment of the active pharmaceutical ingredient, API. The higher resolution *hs-SV-AUC* protocol also reveals a higher sample complexity which we speculate is due to partially filled and over filled AAV particles. Nevertheless, this assignment is based solely on the AAV material’s sedimentation coefficient, not their molecular weight, since accurate MWs for highly heterogeneous samples like AAV materials cannot be determined accurately by SEDFIT [39]. As a result, these assignments should be considered tentative and will require correlation with other methods.

An example of AAV *hs-SV-AUC* data quality along with some statistical indicators on the quality of our model’s fit to experimental data can be found in Fig. 3S.

4. Discussion

Robust methods for determining the size heterogeneity of the large (>30 S), complex supramolecular biopharmaceuticals being developed for gene therapy (e.g. viral vectors and nanoparticles) are needed. When optimized, SV-AUC provides the most-accurate, broadest-range, and highest-resolution size distribution analysis of any method [1–4]. Here, we describe the development of a high speed protocol, called *hs-SV-AUC*, that improves the size distribution analysis of large supramolecular materials (in this case AAV viral vectors) using a high rotor speed (45K rpm), low temperature (10 °C) and interference optics. Both simulations and actual experimental AAV data reveal the following attributes of *hs-SV-AUC*:

1. *Improves particle size distribution resolution.* Since SV-AUC’s resolving power is proportional to rpm², increasing the rotor speed used for AAV analysis from 15K rpm to 45K rpm improves component peak resolution. Higher resolution reveals important insights into AAV drug product consistency, homogeneity and drug product related impurities, which are critical quality attributes (CQAs) in developing biopharmaceuticals. These CQAs may also shed light on the *in vivo* virus assembly process important in developing scalable economical production processes for the manufacturing of these drugs and in expanding our general knowledge concerning viral biological processes. Based on the data we have generated we presently recommend doing AAV *hs-SV-AUC* at 45K rpm and 10 °C, although future work could indicate that running at 5 °C might be needed to provide a more robust AAV *hs-SV-AUC* protocol free of convection.

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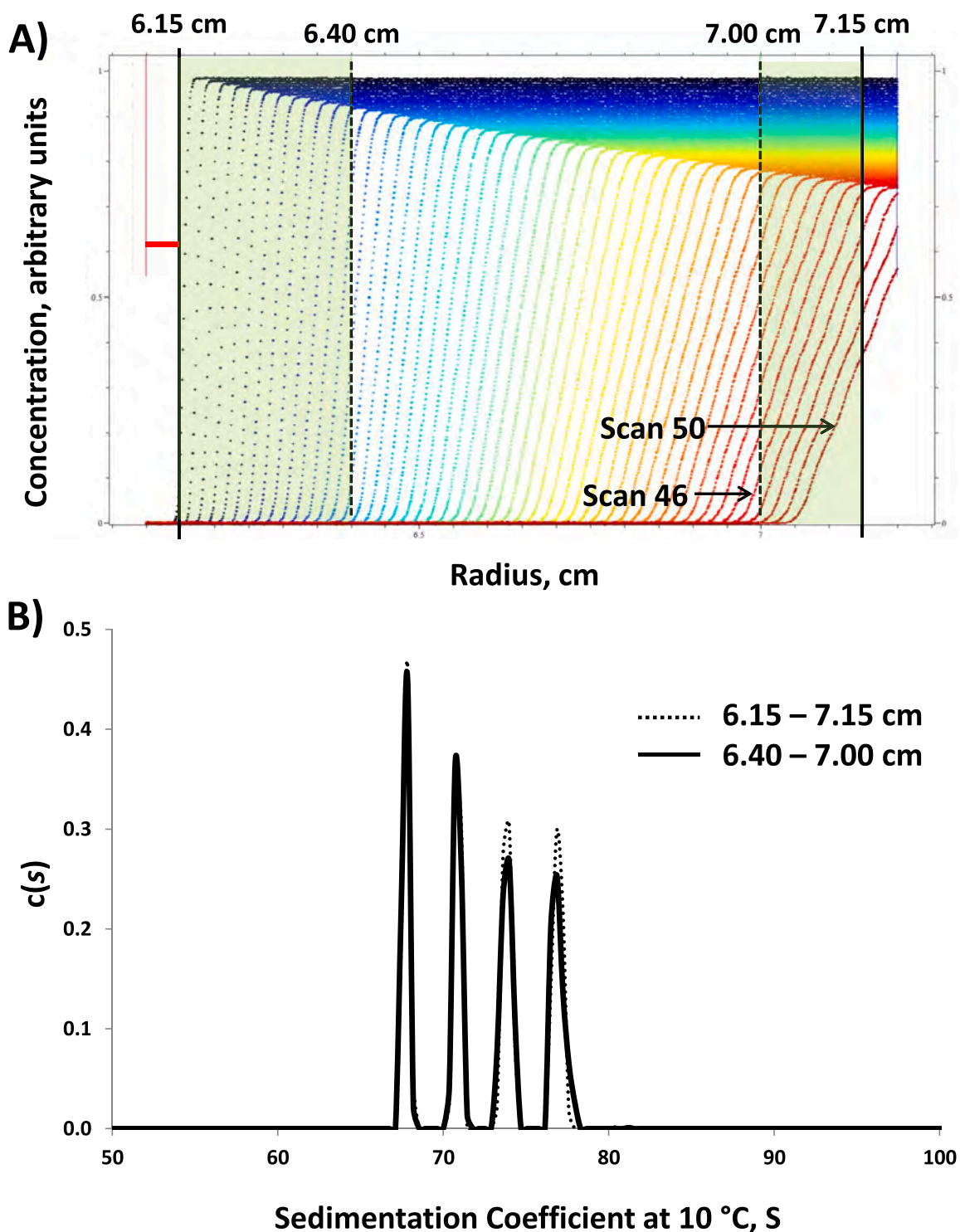


Fig. 4. Comparison of the full (6.15–7.15 cm, ending with the 50th data scan acquired at about 1100 s from the start of the AAV hs-SV-AUC run) vs restricted (6.4–7.0 cm, ending with the 46th data scan acquired at about 1000 s from the start of the AAV hs-SV-AUC run) radial-time windows on the resulting computed AAV $c(s)$ vs S distribution plot obtained from the same simulated AAV hs-SV-AUC data in 0.15 M NaCl at 45k rpm and 10 °C using a data scan time interval of 19 sec. **A)** Simulated 50 AAV hs-SV-AUC data scans used when doing AAV $c(s)$ data analysis on the full radial-time window vs the restricted radial-time window where only data scans 1–46 are used and in terms of radius, data highlighted in light green are excluded. **B)** Resulting overlay of the computed AAV $c(s)$ vs S distribution plots using the full radial-time window, dotted black line, vs the restricted radial-time window, solid black line. The horizontal light red line in “A” indicates sedimentation data lost due to the time delay needed for the rotor to reach running speed (which for an acceleration rate of 400 rpm/s amounts to about 120 s for the An-60 Ti rotor).

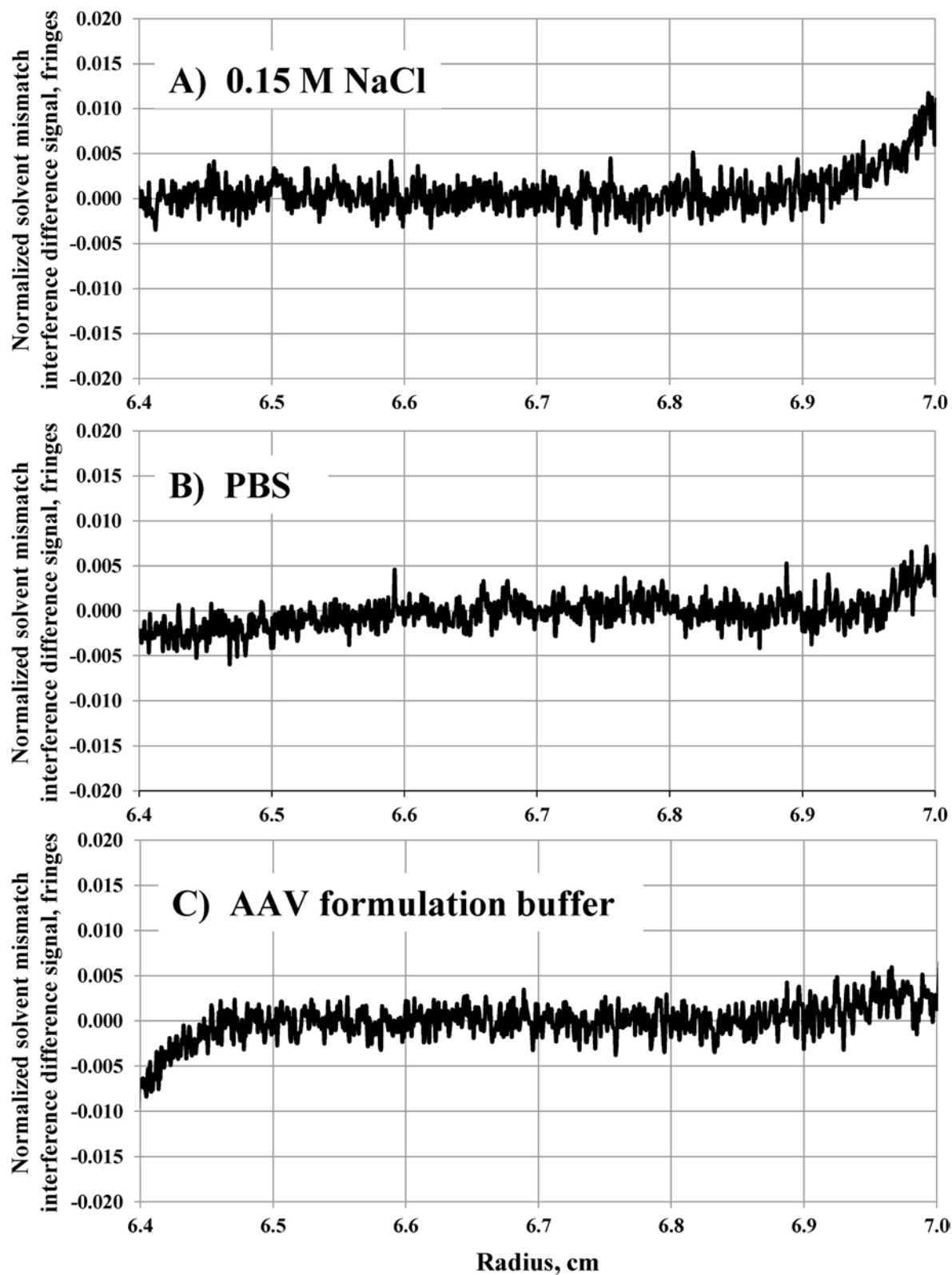


Fig. 5. Experimental “normalized solvent mismatch interference difference signal plot” (see section 2.4) for three different solvents, generated using the AAV hs-SV-AUV protocol (45K rpm, 10 °C and the restricted radial-time window used in Fig. 4). In all cases the cells were filled so the reference sector meniscus was at or near 6.0 cm, while the sample meniscus was at or near 6.1 cm (i.e. 0.1 cm mismatch), having a compositional mismatch of 5% (solvent composition was 5% lower in the sample sector than the reference sector): **A)** 0.15 M NaCl, **B)** PBS and **C)** AAV formulation buffer.

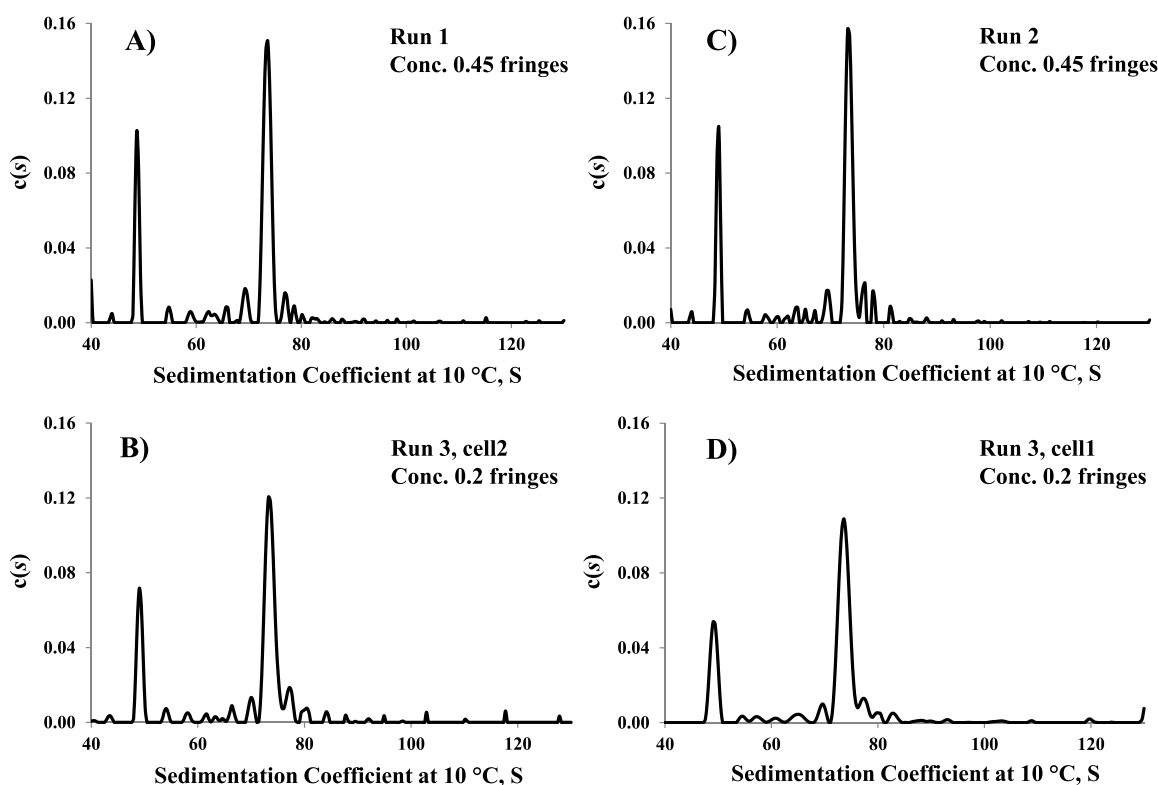


Fig. 6. AAV $c(s)$ vs S distribution plots obtained from three separate AAV hs-SV-AUC experimental runs each conducted on different days on the same AAV sample (at concentrations indicated in units of fringes) at 45K rpm and 10 °C resulting in data from a total of 4 separate AUC cells A-D. It should be noted that plots B-D were normalized to the total fringe area in plot “A”. An overlay plot of these four area normalized AAV $c(s)$ vs S distributions is shown in Fig. 4S.

2. *Mandates the use of interference (refractometric) detection for best results.* AAV component boundaries sediment quickly at 45K rpm potentially making the absorbance detector scan time interval too long to be useful for hs-SV-AUC, especially when attempting to analyze a number of samples in a given rotor. Not only will there be too few scans for analysis, for more than one or possibly two samples, absorbance data may be distorted by the resulting steep boundaries, the movement of the boundaries during the scanning process and the detector’s reduced spatial resolution. On the other hand the Rayleigh interferometer acquire the entire cell image in a fraction of a second, resulting in no discernible boundary movement during data acquisition and can achieve data scan time intervals that enable the acquisition/storage process of all cells, in a either a 4-hole or 8-hole rotor, that approach ~5 s, allowing a sufficient number of data scans to be acquired per cell per run. Furthermore, the sensitivity of the interferometer, coupled with its excellent S/N characteristics and wide dynamic range, allows the analysis of relatively dilute AAV solutions (a 1 OD_{230nm} sample in a 12 mm centerpiece provides a 0.2 fringe signal with a S/N of ~200 using an Optima, Fig. 3S). It is also worth noting that the interferometer may be used with solvents that are opaque at the wavelengths ordinarily used for AAV SV-AUC absorbance analysis (230 nm). As a result, refractometric detection using the Rayleigh interferometer is the recommended detector of choice when doing hs-SV-AUC.

3. *Avoids the annoying challenges associated with solvent mismatch signal contributions to interference detection.* Interference detection is sensitive to the sedimentation of solvent components. Ordinarily, the need to match the solvent component concentrations in the reference and sample sector solutions requires exhaustive dialysis or special size exclusion chromatography, SEC, spin cartridges and meniscus-matching centerpieces. However, for AAV and other large supramolecular materials, confining data analysis to a radial region and run time window, which in the case of AAV at 45K rpm and 10 °C is

defined by 6.4–7.0 cm and the first 1000 s, results in a flat solvent plateau region through which the AAV boundaries are unaffected by solvent sedimentation. As a result, the AAV hs-SV-AUC protocol avoids the need for preforming additional experimental or data processing steps when using interference detection so long as the mismatch in sample and reference sector meniscus position and formulation buffer composition does not exceed 0.1 cm (keeping in mind that ideally the reference meniscus should be at the same or shorter radial position) and 5%, respectively (see Fig. 5S in the Supplementary Material concerning the robustness of the hs-SV-AUC protocol if these specific solvent mismatch limits are exceeded). Nonetheless, when first establishing a hs-SV-AUC method, after identifying the required sedimentation time needed to monitor the supramolecular structure of interest, it is recommended that a solvent mismatch experiment, as described in section 2.2.2 and shown in Fig. 5, be conducted so that the appropriate radial-time window can be determined for the specific solvent-sample combination.

4. *Uses a low temperature (10 °C):* Using a low temperature for AAV analysis has two benefits: 1) the higher viscosity of aqueous solutions at 10 °C versus 20 °C slows sedimentation such that the sedimentation coefficient at 10 °C, s_{10} , equals (0.766) s_{20} . The slower sedimentation allows for more useful scans to be acquired at higher rotor speeds, and 2) the lower temperature reduces the chance for thermally-induced convection by reducing the change in water’s density due to a change in temperature. At present a temperature of 10 °C seems to be adequate, but lower temperatures are even less prone to convection [21]. Thermally induced convection is most frequently encountered during or shortly after rotor acceleration when adiabatic cooling resulting from rotor stretch at lower radii, coupled with compressional rotor heating at the base of the cell (Fig. 7S) produces a thermal gradient that is prone to induce convection. Since the radiometer senses the temperature over a radial area defined by the sensor location and its field of view that

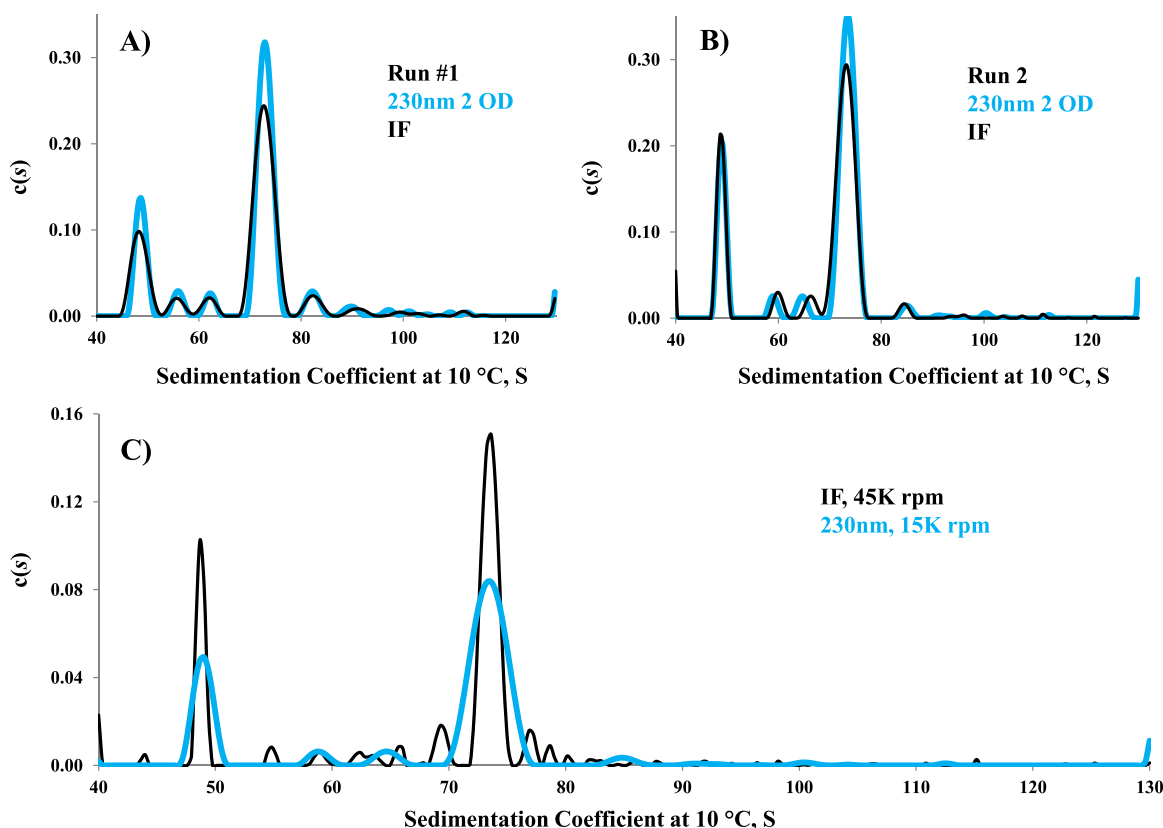


Fig. 7. The overlay AAV c(s) vs S distribution plots in A) and B) were each obtained from two separate SV-AUC experimental runs conducted on different days at 15K rpm and 10 °C on the same AAV sample used in Fig. 6 using absorbance, OD_{230nm}, (light blue) and interference (black) detection. For each run the AAV c(s) vs S distribution plot from both detectors are provided as area normalized overlay plots (interference data was normalized to absorbance data). C) The resulting overlay of the AAV absorbance c(s) vs S distribution plot obtained at 15K rpm and 10 °C (light blue), shown in “part B”, with the AAV c(s) vs S distribution plot at 45K rpm and 10 °C (black), shown in Fig. 6A, for the same AAV sample at about the same concentration (where absorbance data was normalized to interference data on the basis of total area).

encompasses the adiabatically cooled rotor region, it is possible that it will call for more heat during acceleration. For the Optima, where the heating/cooling towers are towards the rotor's edge, this warming exacerbates the thermal gradient during acceleration [21, 30]. The extent and rate of the temperature change is controlled by the acceleration rate and the final speed of the rotor. Both the XL-I and Optima have a maximum acceleration rate of 400 rpm/s (which for the former is the default setting). As a result, a slower rotor acceleration rate (200 rpm/s or possibly even 100 rpm/s) might allow sufficient heat flow within the rotor to limit the temperature changes during rotor acceleration and, thus, avoid convection. For the XL-I the user has some level of control over rotor acceleration, while in the case of the Optima this capability is not available except by stepping up the rotor speed in small increments [40,41]. The tradeoff, of course, is that it will take longer to reach speed, thus lengthening a run and narrowing the radial-time window.

5. **Improves sample throughput.** Due to the high rotor speed employed with hs-SV-AUC, run times are very short, typically < 20 min at 10 °C. This allows for two or three hs-SV-AUC runs to be conducted in single day, with each run carrying three to four (4-hole rotor) or seven to eight (8-hole rotor) cells depending on whether an Optima or XL-I is being used. This sample throughput is significantly greater than that offered by a typical SV-AUC low-speed protocol. Having a second set of AUC cells available that can be cleaned, filled and stored in a refrigerator facilitates higher throughput, particularly if the cells are kept at or just below the running temperature and re-torqued at the lower temperature. Once the rotor is removed from

the centrifuge, the cell replacement process should be conducted as quickly as possible to minimize rotor temperature change and water condensation on the rotor. Just prior to placing the rotor in the chamber, remove any condensation by wiping with a lint-free cloth. By minimizing temperature changes and condensation, the time needed for pump-down and thermal equilibration will be minimized. Having a second rotor at or just below temperature and filled with properly torqued cells would reduce the turn-around time to just a minute or two (noting despite common practice, there is no need nor any justification for radial recalibration when switching rotors or counterbalance). Once the rotor has reached the run temperature, allow ~2 h to assure thermal equilibrium before starting the next run. During this interval, cells from the first run can be prepared for the next run.

Although we have focused on the application of hs-SV-AUC to AAV characterization, the hs-SV-AUC protocol can be applied to a wide range of other gene therapy products (e.g., other viral vectors, virus-like particles (VLPs) and nanoparticles) of similar or greater size and sedimentation coefficient values. Likewise, hs-SV-AUC should also find useful applications in the polymer and material science area where a broader range of large more complex supramolecular structures exists.

Nevertheless, in developing a hs-SV-AUC protocol for other biological or non-biological systems there are considerations that the AUC user needs to be aware of, particularly concerning the radial-time window and its coupling with the operation of either the XL-I or Optima interferometer. These considerations play out in impacting the upper and lower range of S values that can be covered during a given hs-SV-AUC

Table 3
Quantitative analyses of the same AAV sample via hs-SV-AUC, 45K rpm (Fig. 6A–D), vs low speed SV-AUC, 15K rpm (Fig. 7A and B), at 10 °C.

Speed, Detection	Sample ID	Empty (%)	Empty s_{10} (S)	^a Partials (%)	Full (%)	Full s_{10} (S)	^b Agg (%)
45K, IF	^c Run 1	20.6	48.7	11.6	58.0	73.6	8.3
45K, IF	^c Run 2	21.2	48.9	11.0	57.4	73.5	9.3
45K, IF	Run 3_cell1	20.5	49.2	9.0	58.6	73.6	11.8
45K, IF	Run 3_cell2	20.5	49.1	10.2	56.7	73.5	12.1
	Average	20.7	49.0	10.4	57.7	73.5	10.4
	Std. Dev.	0.3	0.2	1.1	0.8	0.1	1.8
	^c CV%	1.6	0.4	10.9	1.4	0.1	18
15K, Abs	^c Run 1	20.1	48.7	7.8	61.0	72.8	11.5
15K, Abs	^c Run 2	22.5	49.0	6.3	67.7	73.1	3.6
	Average	21.3	48.8	7.0	64.3	73.0	7.6
	Std. Dev.	1.6	0.3	1.1	4.7	0.3	5.6
	CV%	7.7	0.5	15.0	7.3	0.3	74
15K, IF	^c Run 1	19.2	48.7	9.8	61.7	72.8	9.4
15K, IF	^c Run 2	23.2	48.7	8.7	64.3	73.1	3.6
	Average	21.2	48.7	9.2	63.0	73.0	6.5
	Std. Dev.	2.8	0.0	0.8	1.8	0.2	4.1
	CV%	13.3	0.0	8.8	2.9	0.3	63
^d 15K, Abs & IF	Average	21.2	48.8	8.1	63.6	73.0	7.0
	Std. Dev.	1.9	0.2	1.5	3.0	0.2	4.0
	CV%	8.9	0.3	18.3	4.8	0.3	58

^a Corresponds to AAV material with S values located between the empty and full AAV peaks.
^b Corresponds to AAV material migrating faster than the intact full AAV peak. As a result this may include monomeric over filled AAV particles.
^c Sample concentration in this run is about twice that of samples in Run 3.
^d Combined analysis of both absorbance (Abs) and interference (IF) 15K data.
^e Coefficient of variance.

experiment as follows:

In the case of the XL-I: laser’s timing and other associated settings that control the interference pattern image for each rotor hole are typically done manually in a separate SV-AUC run at the running speed of the desired experiment (45K rpm in the case of the AAV hs-SV-AUC protocol). As a result data acquisition for AAV hs-SV-AUC using a XL-I will typically start as soon as the rotor reaches running speed, which for 45K rpm and an acceleration rate of 400 rpm/s results in a time delay before the 1st data scan is acquired of about 120 s. Given the radial-time window for AAV hs-SV-AUC is 6.4–7.0 cm and ~1000 s an upper value limit for s_{10} was estimated from simulations on an AAV sample consisting of equal amounts of intact full AAV monomer and several aggregates of this monomer (Fig. 8SA). Data from this simulation work shows that AAV aggregates having s_{10} values in excess of 300 S can easily be captured in the 50 collected data scans at scan rate of 19 s. In the case of the lower s_{10} limit, AAV hs-SV-AUC simulation work using a hypothetical monoclonal antibody sample consisting of equal amounts of a monomeric mAb and several of its aggregates (Fig. 8SC) indicated none of the mAb boundaries would have entered the radial-time window of the AAV hs-SV-AUC experiment before date acquisition had terminated at ~1000 s. Based on this information AAV material migrating with s_{10} values < ~25 S will not be detected using the XL-I.

In the case of the Optima: laser’s timing and other associated settings that control the interference pattern image for each rotor hole are done *automatically* during the actual AAV hs-SV-AUC run after reaching final running speed. As a result, data acquisition for an AAV hs-SV-AUC run using an Optima is delayed until this automated procedure is completed. While convenient when doing interferometry for ordinary SV-AUC, the

automated setup takes several minutes, which will reduce the amount of data that can be acquired during AAV hs-SV-AUC. While the simplification in using interferometry is appreciated, given the operation of the Optima, *every time* the Optima interference detector is used this laser setup procedure is automatically conducted before data acquisition starts. Unfortunately, the ability to turn off the automated procedure and enable manual laser setup is not available presently. From our experimental AAV hs-SV-AUC work we have observed that total delay time (time to reach speed plus time to complete laser setup) averages about 400 s before the 1st data scan is acquired, although it should be noted this total delay can vary somewhat. Fortunately, it should also be noted that difference in laser setup time when using seven cells versus only one cell increases this automated setup time by only ~40–50 s. Again, given the radial-time window for AAV hs-SV-AUC (of 6.4–7.0 cm and ~1000 s) an upper s_{10} limit for the Optima was estimated from simulations on the same AAV sample consisting of equal amounts of intact full AAV monomer and several aggregates of this monomer used for the XL-I. Data from this simulation work (Fig. 8SB), using the average delay time of 400 s, shows that AAV aggregates 3 & 4 have passed through the radial-window before data acquisition is initiated. Based on this data using the Optima, the upper s_{10} limit appears to approach a value of ~150 S. In the case of the lower s_{10} limit when using the Optima to do AAV hs-SV-AUC, simulation work using the same hypothetical monoclonal antibody sample used for the XL-I also shows (Fig. 8SD) that material having s_{10} values < ~25 S will not enter the radial-time window to be reliably monitored before acquisition has terminated at ~1000 s.

In using the AAV hs-SV-AUC protocol any concerns about the

presence of significant amounts of AAV material being present in a sample with s_{10} values that are *outside* the above mentioned upper and lower s_{10} limits, $> \sim 300$ S or $< \sim 25$ S in the case of the XL-I or $> \sim 150$ S or $< \sim 25$ S in the case of the Optima, it is recommended that an initial standard 2–3 h, lower-speed (15K rpm) run be conducted before 45K rpm is used for routine analysis. Nevertheless, if there is material outside this range, it should be noted that opportunities for extending some of these limits for the AAV hs-SV-AUC do exist.

To increase the upper s_{10} limit of the AAV hs-SV-AUC protocol four strategies can be implemented, which include the following:

- 1) Increase the frequency of data acquisition by reducing the time interval between scans, while simultaneously increasing the number of data scans collected to fill the hs-SV-AUC radial-time window (~ 1000 s). Unfortunately, the ability to implement this approach on the Optima is hampered by the slow automated setup of its interferometer. Consequently, this strategy is most effective when using the XL-I.
- 2) Slow down the sedimentation process by reducing the temperature from 10 to 5 °C. Reducing the temperature not only allows capturing the sedimentation of the larger species, but also further reduce the potential for encountering convection and from preliminary simulation solvent mismatch hs-SV-AUC work, may even expand the radial-time window slightly.
- 3) Perform a multi-speed AAV hs-SV-AUC [40,41] experiment in which the rotor is set to collect data at different speeds during different stages of the same SV AUC run. However, simulation and actual solvent hs-SV-AUC experiments should be carried out to assess the impact of solvent mismatch contribution to the final interference signal at each of the rotor speeds.
- 4) Make use of the BMM software package in SEDFIT [20], to expand the restricted radial-time window to allow a large portion of the earlier parts of the interference data to be incorporated into the data analysis. Unfortunately, the ability to implement this approach on the Optima is hampered by the slow automated setup of its interferometer. Consequently this strategy is most effective when using the XL-I. In addition, the implementation of BMM comes with the introduction of additional fitting parameters, that may be responsible for the slight reduction in the quality of the analysis (see Table 1S) and which results in much longer data processing times.

To decrease the lower s_{10} limit of the AAV hs-SV-AUC protocol, two approaches are available. In the first approach the dual independent operation of the Optima's interferometer and absorbance detectors is exploited. In this scenario AAV material with high s_{10} values would be monitored using the interferometer, adhering to the AAV hs-SV-AUC radial-time window, while the AAV material with low s_{10} values would be monitored using the absorbance detector, which is free to operate independent of the interference radial-time window. This dual-detector arrangement can reduce the lower s_{10} limit of the AAV hs-SV-AUC protocol down to ~ 1 S, although doing so will significantly increase the run time from ~ 20 min. In the second approach, the strategy used in point "4" to increase the upper s_{10} limit using BMM software can also decrease the low s_{10} limit. In fact this is the primary benefit of employing BMM since it allows later scans to be incorporated into the data analysis to provide information on the slower moving AAV sample components.

5. Conclusion

The size distribution of a biopharmaceutical is an important CQA for assessing the purity and the manufacturing consistency of these drugs, which translates into their therapeutic dosing accuracy and safety. There are other biophysical techniques that can provide size distribution information on AAV, and other viruses and nanoparticles [17,42,43]. Some of these can do so with higher sample throughput and/or requiring

very small amounts of sample material. However, when properly optimized SV-AUC provides the broadest, most accurate and most detailed size distribution on an AAV sample in its *native state* of any method [1–4]. In this paper we show AAV, hs-SV-AUC can further improve this capability of SV-AUC to provide even higher-resolution size distribution information and allows for a higher throughput by eliminating sample preparation steps (such as dialysis) and minimizing the experiment run time (< 20 min).

In terms of our association and identification of the AAV peaks revealed by hs-SV-AUC we wish to reiterate that at present they are speculative and will require the use of orthogonal methods for corroboration. In addition, closer scrutiny of data analysis software is needed to better understand the peak variability seen in $c(s)$ distributions.

While the experimental parameters determined in this manuscript were optimized for doing hs-SV-AUC experiments on our AAV samples (45K rpm, 10 °C, interference detection, etc.) and should very likely be capable of being used for the analysis of most AAV samples, the numbers are not meant to be prescriptive. Rather, the authors have aimed to provide AUC users with the strategies required to develop and optimize their own hs-SV-AUC method, or at a minimum, verify that the method parameters determined here are suitable for their buffer system (mismatch issues) when analyzing their AAV material. Other variations of the hs-SV-AUC experiment are possible and have been mentioned in this work. Regardless of the intended use, hs-SV-AUC offers multiple advantages over standard SV-AUC experiments commonly conducted on AAV samples and other large complex supramolecular structures and should become an alternative method of high interest when analyzing these materials.

CRedit authorship contribution statement

Steven A. Berkowitz: Conceptualization, Data curation, Formal analysis, Writing – original draft, Writing – review & editing. **Nicholas Larson:** Investigation, Resources, Writing – review & editing. **George Bou-Assaf:** Resources, Writing – review & editing. **Thomas Laue:** Writing – review & editing.

Declaration of competing interest

None.

Data availability

Data will be made available on request.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.ab.2024.115482>.

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