



Boundary convection during sedimentation velocity in the Optima analytical ultracentrifuge

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ABSTRACT

Analytical ultracentrifugation (AUC) provides the most widely applicable, precise, and accurate means for characterizing solution hydrodynamic and thermodynamic properties. While generally useful, boundary sedimentation velocity AUC (SV-AUC) analysis has become particularly important in assessing protein aggregation, fragmentation and conformational variants in the same solvents used during drug development and production. In early 2017 the only manufacturer of the analytical ultracentrifuge released its newest analytical ultracentrifuge, the Optima, to replace the aging second-generation XLA/I series ultracentrifuges. However, SV-AUC data from four Optima units used in the characterization of adeno-associated virus (AAV) have shown evidence of sample convection. Further investigation reveals this problem arises from the design of the temperature control system, which makes it prone to producing destabilizing temperature-induced density gradients that can lead to density inversions. The problem is intermittent and variable in severity within a given Optima unit and between Optima units. This convection appears to be associated mainly with low rotor speeds and dilute concentration of solvent components, i.e., AAV analysis conditions. Data features diagnostic for this problem and strategies for its elimination or minimization are provided.

1. Introduction

Despite being nearly 100 years old, analytical ultracentrifugation (AUC) continues its role as a useful and important analytical tool in both basic and applied sciences [1–11]. Its longevity and modern capabilities, recently summarized by Peter Schuck and coworkers in a comprehensive three volume series [12–14], is a result of being the most accurate and precise technique for characterizing a solution's thermodynamic and hydrodynamic properties. The availability of absorbance, refractive and fluorescence detection allows AUC's application to a broad range of samples and solvents. In particular, size distribution analysis by boundary sedimentation velocity AUC (SV-AUC) offers access to a wide range of sample characterization information that includes the following: structural heterogeneity (e.g., fragmentation, aggregation and chemical composition) [13], concentration [15], interactions [14], changes in conformation [16] and sample consistency [10] with few solvent limitations, minimum sample manipulation and easy method development [7]. Importantly, SV-AUC offers significant and unique ways to characterize the new classes of very large, complex molecular biopharmaceuticals that are now being actively developed in gene

therapy that involve drug delivery systems such as viral vectors [17,18], liposomes and nanoparticles [19–25].

AUC is a first-principle technique. Therefore, data interpretation does not require comparison with standards. Consequently, to within a ~1%, sedimentation coefficient measurements made by one user should be reproducible by another user with the same type of sample at any other time and on a different machine. Any deviations between the two sets of measurements beyond ~2% reflect sample differences. Clearly, the proper functioning of every component of the machine is required to achieve certainty in the significance of any such differences [26–31].

In an analytical ultracentrifuge there are several critical elements requiring high precision and accuracy: 1) sample cell geometry and alignment in the rotor, 2) machining of the rotor cell-hole positions and sizes, 3) counterbalance machining for radial position calibration, 4) temperature measurement and control, 5) optical system alignment and focusing, 6) rotor speed control and 7) data acquisition timing. Except for the actual cell alignment in the rotor, AUC users depend on the instrument manufacturer's ability to ensure the required accuracy and precision of these critical elements. Temperature accuracy is of central importance for proper SV-AUC data analysis and interpretation due to its

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impact on solvent density and viscosity (at 20 °C, a 1 °C error results in a 4% sedimentation coefficient error). Exceptional temperature control is also required to avoid thermally-induced density gradients that can lead to convection [27–29,32–39].

During sedimentation a stabilizing density gradient (i.e., increasing solution density with radius) builds up due to solvent component (e.g., salts, buffers and sugars) redistribution. If this stabilizing gradient is weak, as occurs with solvents containing dilute concentrations of solvent components at low rotor speeds, dilute samples can experience density inversions that lead to convection. In AUC a key driver of density inversions is temperature gradients where a cooler and denser volume element (dV) is centripetal to a warmer and less-dense volume element (Fig. 1). This situation can occur when there is a positive radial temperature gradient in the AUC cell. In the presence of a gravitational field the higher density region will be driven into the lower density solution resulting in the bulk exchange of material between the adjacent regions (Fig. 1), i.e., turbulent flow (referred to as convection in the AUC community). Historically, proper temperature control has been a challenging part of AUC's development [32–39].

In early 2017 the only manufacturer of analytical ultracentrifuges launched its third-generation analytical ultracentrifuge called the Optima (preceded by the Model E, 1949–1972, and XLA/I, 1990–2017). In upgrading any instrument, it is anticipated that the newest generation instrument will perform as well as, if not better than its predecessors. Today this is more important than ever since AUC is employed in critical decision making in biopharmaceutical development. Unfortunately, early Optima units exhibited a technical problem, see Berkowitz and Philo [10], associated with convective mixing during SV-AUC. This problem was uncovered by one of the authors (S.A. Berkowitz) while collaborating with an early adopter of the Optima. It was observed and reported back to the manufacturer that while normal sedimentation patterns were obtained for a monoclonal antibody (mAb) run at 40K rpm, no sedimentation was observed at lower rotor speeds (10–15K

rpm), even after many hours of centrifugation. The manufacturer determined that poor temperature control was causing thermally-induced convection during centrifugation. To minimize thermal gradients, the manufacturer introduced a thermal shroud within the rotor chamber along with some software changes. With these modifications, it appeared that the Optima thermal convection problem was resolved.

However, subsequent work by S.A. Berkowitz, in collaboration with several other Optima users, revealed intermittent disturbances (artifacts) in data scans during SV-AUC experiments involving adeno-associated virus (AAV). These artifacts are characterized by a spontaneous and unusual sharpening of the leading edge of the fastest moving boundary and appear after the boundary has migrated $\sim 3/4 - 1/2$ of the way down the cell. Data presented in this paper supports the conclusion that these artifacts result from thermally-driven convection in Optima units. The most likely source of this problem (as well as the original temperature problem) arises from the physical location of the Peltier heating/cooling cells at the rotor's edge. By considering the key criteria for achieving convection-free SV-AUC (as discussed in Appendix A. Supplementary data Part 1), recommendations are made for preventing this convection. These recommendations should be considered short-term measures for dealing with the situation until a permanent fix is developed.

2. Material and methods

2.1. Adeno-associated virus

All AAV samples were produced and purified at the site associated with the specific Optima centrifuges outlined in the section heading “Analytical ultracentrifugation” below.

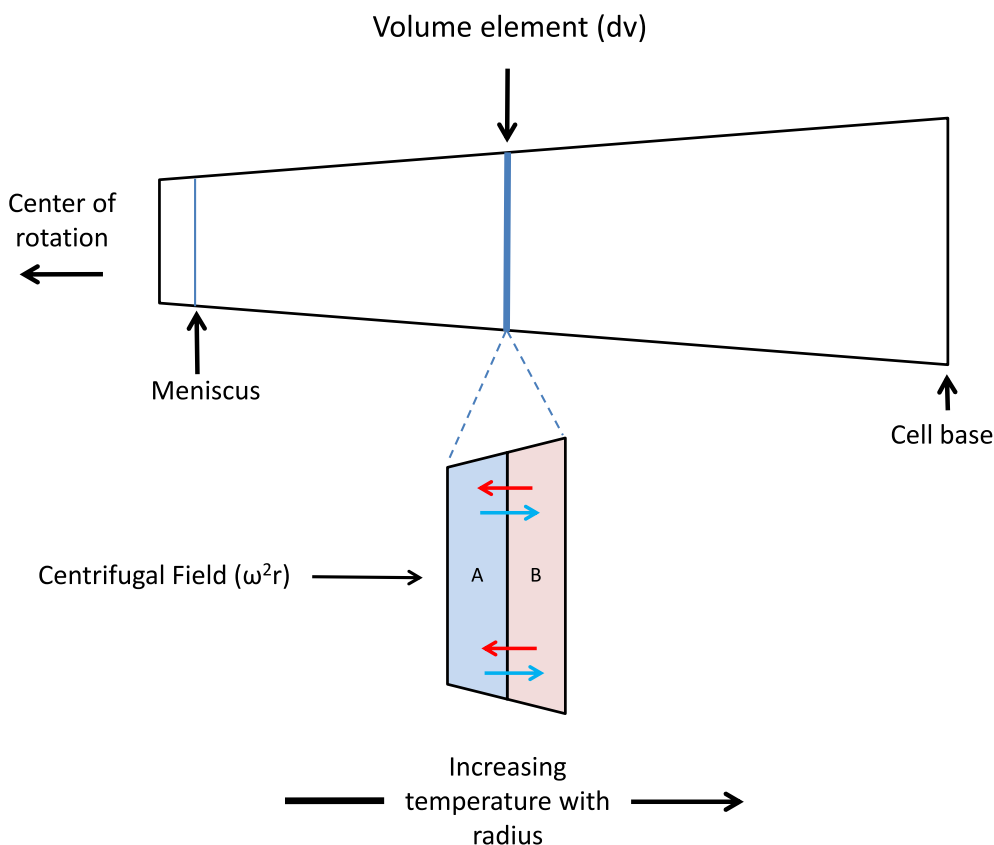


Fig. 1. Illustrates how a positive radial temperature gradient (dT/dr) with increasing radius within the sample sector of an AUC cell can cause convection during SV-AUC. The density of aqueous solutions decreases with increasing temperature at temperatures >4 °C). Hence, a positive dT/dr will result in unfavorable temperature-induced negative radial density gradient, $(dp/dr)_T$. As a result, if the value of dT/dr across volume element dV is > 0 , a higher density region (A) will be located above a lower density region (B). These conditions are unstable in the presence of a gravitational field, and the solutions in A and B will exchange (as indicated by the blue and red arrows), i.e., convective mixing. Nevertheless, if a stabilizing (positive) density gradient can be formed by the sedimentation of solution components (macromolecules, salts, sugars, etc.) that is sufficiently large to overcome the destabilizing negative $(dp/dr)_T$, convective mixing will be avoided. However, this stabilizing density gradient must be formed fast enough, relative to the migrating boundary front of macromolecules; otherwise there can be a radial region in the sample sector where convection can occur. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

2.2. Analytical ultracentrifugation

Optima AAV SV-AUC experiments were carried out on one of four different Optima units, designated as Optima_1, Optima_2, Optima_3 and Optima_4. In addition, at one site AAV experiments were conducted on a XLA/I following the same protocol used with the Optima. All AAV SV-AUC runs were carried out at 20 °C and 18K rpm, using an An-50 Ti 8-hole rotor and at AAV concentrations <1 OD at 230 nm unless otherwise indicated. All AAV samples were in formulation buffers containing concentrations of salt, buffer, etc. totaling between 0.1 and 0.2 M and at a pH near 7. All data analysis was carried out using SEDFIT, version 15.01b [40] and the continuous c(s) distribution model, where it is assumed that all species migrate as independent, ideal components.

Interference data were corrected in SEDFIT for radial- and time-independent (RI and TI) variations. Both corrections also were applied in processing Optima absorbance data to improve the fit to the data, even though RI correction ordinarily is not required or recommended with XLA/I absorbance data. Although it is not entirely clear why the RI correction is needed for the Optima, it may be a consequence of differences in how the data are acquired by the two machines. For the XLA/I absorbance system, data acquisition at a radial position involves first collecting the reference intensity reading, followed immediately by collecting the corresponding sample intensity (note, when using the ‘continuous scan’ mode these intensity readings are taken at slightly different radial positions). In the case of the Optima *all* radial intensity readings from a sample are acquired first, followed by the acquisition of *all* corresponding reference intensity readings. The resulting time delay between sample and reference intensity readings in the Optima may result in slight source intensity shifts to occur (e.g., due to lamp warming) that make the use of RI noise correction useful when analyzing Optima data.

2.3. Simulating SV-AUC experiments

Simulations were made for AAV, mAb and low molecular weight (LMW) formulation buffer using the non-interacting discrete species model in SEDFIT employing the program’s default settings along with user input values for molecular weights, sedimentation coefficients, rotor speed and the data interval of time between data scans. In all cases radial data spacing of 0.001 cm, and S/N of 1 part 10⁴ at a starting arbitrary concentration unit of 1 was used. All simulations include the rotor acceleration phase.

3. Results

3.1. Optima’s abrupt data scan sharpening artifact is associated with convection

SV-AUC experiments on AAV material using Optima 2 (only equipped with absorbance detection) revealed the intermittent appearance of boundary disturbances (Fig. 2A) which appear as a limited, but sequential block of data scans. The presence, absence and intensity of the disturbances varies on a run-to-run basis both for a given Optima and between Optima units. Most often the disturbances are associated with the fastest moving boundary, appearing suddenly after the boundary has migrated $\sim 1/4 - 1/2$ of the way to the bottom of the cell, often with all samples in the SV-AUC run exhibiting similar-looking artifacts. Though some disturbances are irregular in appearance, in most cases the artifact can be summarized as very abrupt boundary sharpening of the leading edge of the fastest boundary that subsequently broadens and disappears over subsequent data scans (Fig. 2A and in Fig. 3).

Analyzing just the scans acquired before the artifact is visible (so-called “partial data scan analysis” of the data in Fig. 2 shown in part B)

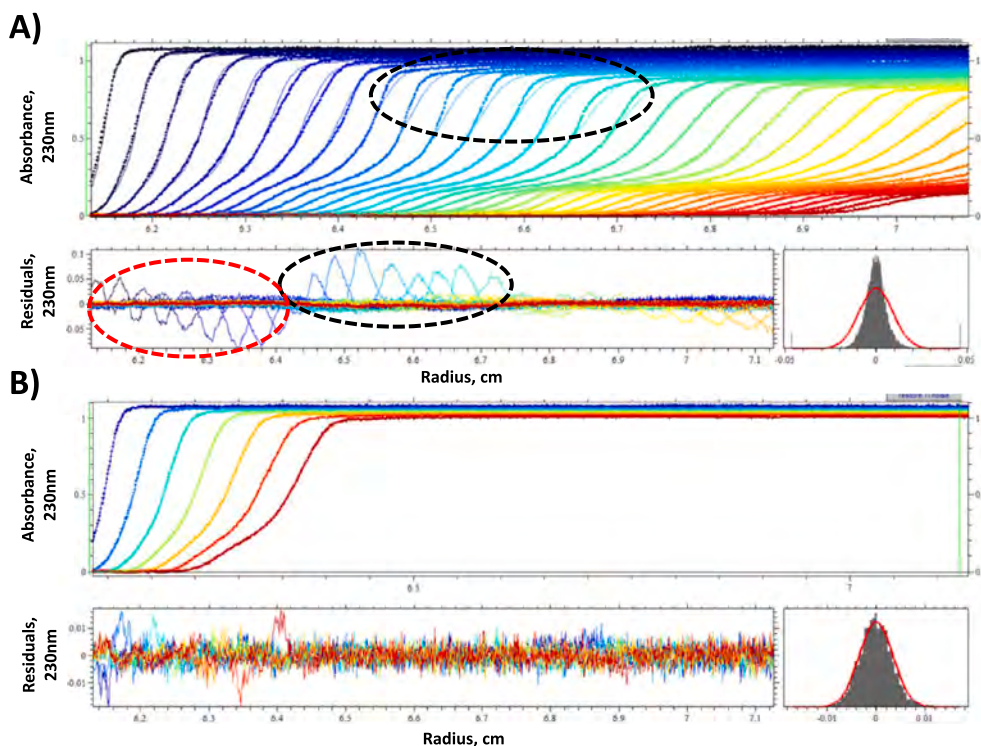


Fig. 2. AAV SV-AUC data obtained from Optima_2 (which only has an absorbance detector) run at 20K rpm and 20 °C showing the presence of the boundary sharpening artifact and evidence for its association with convection. **A)** Top plot is the overlay of the processed absorbance data scans 1-35, which encompasses the entire run, bottom left plot is the resulting residual plot showing the quality of the computed c(s) model fit to the experimental data shown in the top plot and the bottom right plot is the distribution of the experimental residuals (shown in the residual plot) relative to their anticipated normal distribution based on the data’s RMSD, which is outlined in red. The resulting statistical parameters from this c(s) analysis is the following: RMSD = 0.0093, Z-test = 110 and H = 19.9%. The black dashed oval encloses the radial region and block of scans that show the characteristic sharpening and its impact on the fitting residuals. The red dashed oval in the residual plot shows that fitting all of the data “falsely” reports that scans 1-7 do not fit the model, when in fact the poor fit is due to the appearance of convective disturbances in later data scans. **B)** Top plot shows the overlay of processed data scans 1-7 from the same data scan set shown in part A, bottom left is the residual plot for these 7 scans showing the improved fit to the c(s) model and bottom right plot shows the experimental residuals now follow the normal distribution based on the data’s

RMSD, which is outlined in red. Resulting statistical parameters from this c(s) analysis are also significantly improved (over that achieved in part A): RMSD = 0.0034, Z-test = 15 and H = 0.9%. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

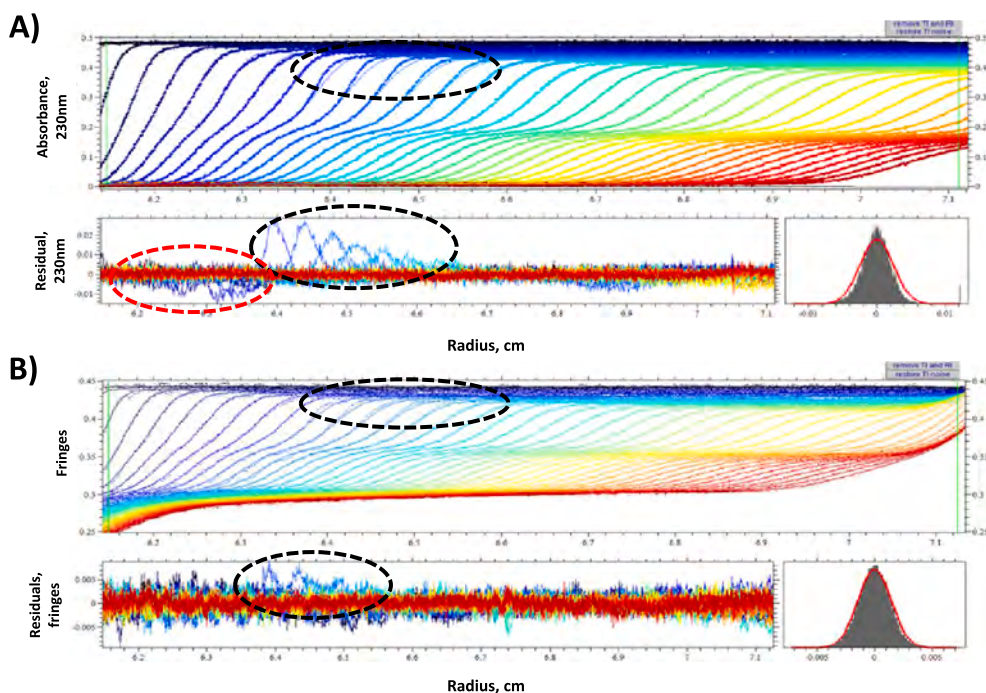


Fig. 3. AAV SV-AUC data obtained from an Optima_1 run at 15K rpm and 20 °C showing the data scan sharpening artifact for data collected concurrently with **A)** absorbance detection (at 230 nm) and **B)** interference detection. In each case the **top** plot corresponds to the overlay of processed experimental data, while the **bottom left** plot is the resulting residual plot and the **bottom right** plot is the distribution of the fitting residuals relative to their anticipated normal distribution based on the data's RMSD, which is outlined in red. Areas highlighted by a black dashed oval indicate the radial region and block of data scans that show the convective artifact. As in Fig. 2, the red dash oval highlights the radial region where the poor fit in the early scans actually is a consequence of convective disturbances in *later* data scans. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

supports the hypothesis that the artifact results from a convective disturbance that developed well after the experiment is initiated. This is shown by comparing the *c(s)* analysis using all 35 data scans (Fig. 2A) with the *c(s)* analysis using just the first 7 data scans (Fig. 2B), which were acquired prior to the onset of convection. When only these early scans are analyzed, the *c(s)* model fits the data well (Fig. 2B) as shown by the absence of systematic residuals, and the low values for the statistical parameters RMSD, H [41] and runs test Z [41,42]. However, when all 35 scans are analyzed (Fig. 2A), the fit is degraded (i.e., by the appearance of significant systematic residual errors and increased RMSD, H and Z values) by the inclusion of the convection-perturbed scans.

In cases where the Optima had absorbance and refractometric detectors (e.g., Optimas_1, _3 and _4), similar disturbances are observed by both detectors when monitoring the same samples, at the same radii and at the same time (Fig. 3A and B). Since these two detectors provide independent, orthogonal sample observations it is *highly* unlikely that the disturbances are a detector artifact. Rather, the disturbances must be due to events occurring *in* the samples.

While concentration-dependent hydrodynamic non-ideality may result in boundary sharpening [12,34,36,43], its effect is observed at concentrations orders of magnitude greater than those employed here (Figs. 2 and 3). Furthermore, concentration-dependent boundary sharpening is observed from the start of an experiment, with boundary steepness increasing progressively over time. In contrast, the Optima convective artifacts appear abruptly long *after* the rotor has reached speed and subsequently disappear (Figs. 2A and 3). Consequently, concentration-dependent hydrodynamic non-ideality cannot explain the data scan sharpening.

Importantly, it should be noted, no such artifacts are observed for AAV samples run under the same experimental conditions using an AUC instrument with a different temperature control design (XLA/I). Even samples that showed the artifact when run on the Optima did not show the artifact when run on the XLA/I. Consequently, the problem is isolated to the Optima. It is important to note that the artifact's appearance is sporadic in terms of frequency and intensity varying both in a particular Optima unit and between Optima units. In some cases, the artifact can be quite subtle. We surmise that slight variations in thermal component properties and/or operation may cause the observed variability.

3.2. Simulations reveal that weak buffer density gradients formed during low-speed SV-AUC

Convection-free sedimentation requires a positive radial density gradient (a "stabilizing" gradient) throughout the sample sector (i.e., $\rho/\rho \geq 0$, preferably > 0 , as outlined in Appendix A. Supplementary data Part 1). Since the AAV concentrations used here are very dilute, sedimentation of the solvent components (e.g., salts, buffer, sugar, etc.) in the formulation buffer must provide the necessary stabilizing density gradient (see discussion concerning eq. 4 in Appendix A. Supplementary data Part 1). However, at the rotor speeds used for AAV SV-AUC analysis, the AAV sediments rapidly compared to the formulation buffer components. As a consequence the AAV boundary moves into regions where $\rho/\rho \approx 0$, i.e., regions where there is insufficient buffer component sedimentation to establish an adequate stabilizing density gradient. Under these conditions, even tiny density disturbances could result in regions where $\rho/\rho < 0$, thus permitting AAV convection.

Since the radial density gradient, ρ/ρ , is directly proportional to the concentration change with radius, dc/dr , (see discussion concerning eq. 4 in Appendix A. Supplementary data Part 1), simulating the sedimentation of AAV and its formulation buffer components is useful. By overlaying the radial concentration profiles of AAV and the buffer components as a function of time, it becomes clear why AAV is vulnerable to convection (Fig. 4).

Using SEDFIT's simulation capabilities, ideal sedimentation data were generated for an AAV sample (containing a 50:50 mix of two key species, empty and full AAV virus particles) and its formulation buffer. In both cases data were computed for the experimental conditions commonly used to conduct AAV SV-AUC analysis (18K rpm and 20 °C) using the molecular weights (MW) and sedimentation coefficient values provided in Fig. 4. The concentration profiles for AAV and the formulation buffer are overlaid in Fig. 4A–F.

These data reveal that early in the SV-AUC run the formulation buffer components in the solvent forms a stabilizing ($\rho/\rho > 0$) gradient immediately adjacent to the sample meniscus and base (Fig. 4A). Over the course of the experiment the solvent density gradient propagates throughout the cell (Fig. 4B–F). However, before this stabilizing density gradient can be fully established there is a period of time where $\rho/\rho \approx 0$ over central portions of the cell (indicated by horizontal bars). The

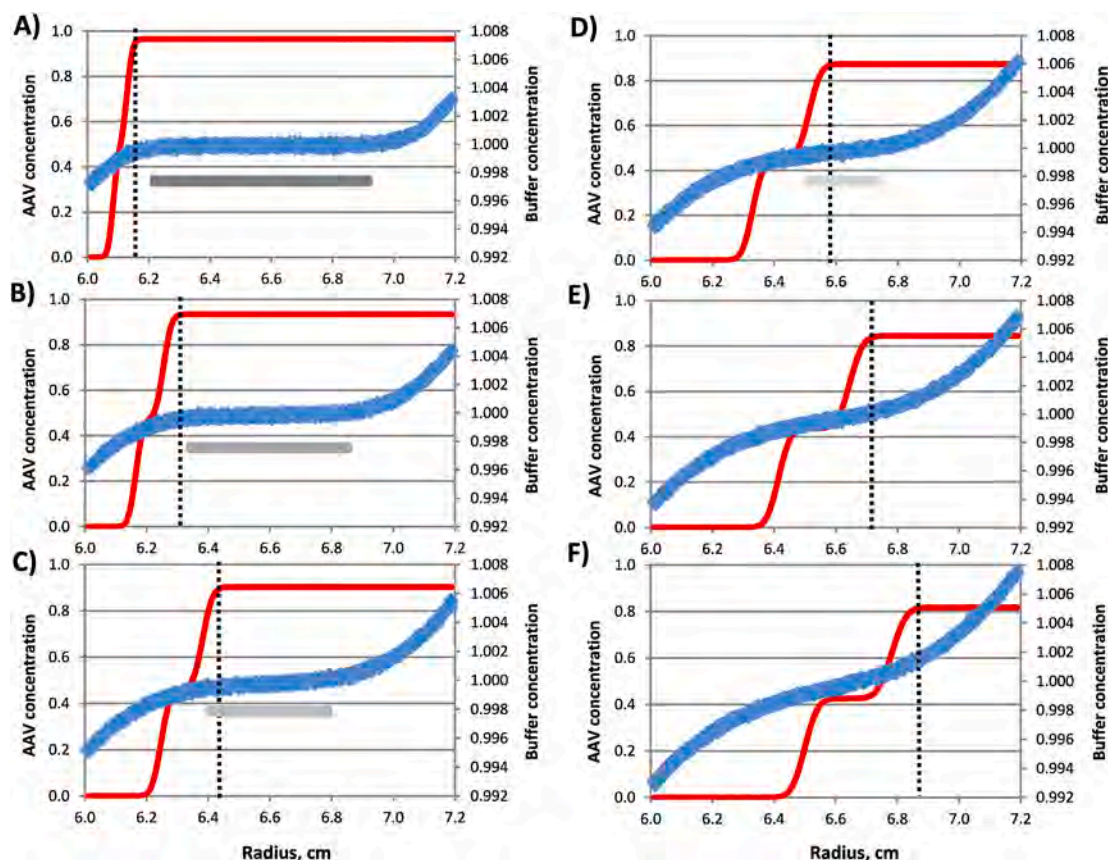


Fig. 4. Simulated SV-AUC data for a hypothetical AAV sample consisting of a 50:50 mixture of empty and full virus particles, having a MW of about 3.9 MDa and a sedimentation coefficient of about 62 S, and a sedimentation coefficient of about 95 S respectively (shown in red), overlaid with the corresponding simulated SV-AUC data for a typical AAV formulation buffer system having a weight-average MW of 100 Da and sedimentation coefficient of 0.2 S (shown in blue), at 18K rpm and 20 °C. Data scans for both the AAV mixture and formulation buffer were calculated for the times intervals of 10 min yielding data scans for the following same time points (from the start of the run): **A)** 10 min, **B)** 20 min, **C)** 30 min, **D)** 40 min, **E)** 50 min and **F)** 60 min for the same radial positions. Black vertical dotted line corresponds to the position of the leading boundary edge of the full AAV material. In both cases the initial starting concentration was an arbitrary concentration of 1. The length of the horizontal grey bars indicates the radial region where the formulation buffer density gradient is the weakest (where the potential for convection is most significant); while the lighter the grey the less prominent is the instability (or weakness) in this density gradient. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

lack of a stabilizing density gradient means that material migrating into these marked regions has very limited protection against transient, thermally-generated destabilizing density gradients. Fig. 4C and D shows that the boundary for full AAV particles is in this poorly stabilized region between 30 and 40 min after the start of the experiment. Qualitatively this corresponds to the observed time-window where convection is seen experimentally (Figs. 2 and 3). Other simulations with smaller biopharmaceutical molecules, e.g., mAbs, (see discussion in Appendix A. Supplementary data Part 2 and Fig. 1S) reveal why convection problem is more of an issue at lower rotor speeds in SV-AUC work on very large molecules where: 1) stabilizing density gradients develops slowly relative to boundary sedimentation of very large molecules and 2) resulting in stabilizing density gradients that are much weaker.

3.3. Comparing the temperature control systems on three generations of analytical ultracentrifuges

If the stabilizing buffer density gradient, $(dp/dr)_B$, is less than a thermally-induced negative radial density gradient, $(dp/dr)_T$, so the sum of these two density gradients is less than zero (i.e., $dp/dr = (dp/dr)_B + (dp/dr)_T < 0$), convection will occur (see discussion concerning eq. (6) in Appendix A. Supplementary data Part 3). Thus one goal of the AUC temperature control system (besides maintaining the running temperature of the AUC experiment) is to prevent situations where $(dp/dr)_T < 0$.

This is more difficult than it may seem, and designing a suitable temperature control systems for analytical ultracentrifuges has been a challenge [27–29,32–39]. Preventing situations where $(dp/dr)_T < 0$ can be achieved by either making certain rotor heating/cooling uniform or by ensuring $(dp/dr)_T > 0$ (i.e., the edge of the rotor is cooler than the center) for all heating/cooling scenarios. Different strategies have been used on the three generations of Beckman analytical ultracentrifuges.

In developing the Model E, temperature control was achieved by constantly cooling a shroud surrounding the rotor while heating was provided by a nichrome heating wire below the rotor and 3 cm out from the rotor center (Fig. 5A). The refrigerated shroud was kept ~ 1 – 2 °C below the desired rotor temperature, and current to the nichrome wire was switched on and off to maintain a thermistor located at the bottom center of the rotor at the desired temperature. This temperature control arrangement ensured that a slight negative dT/dr (i.e., stabilizing) gradient was maintained throughout an experiment. Care had to be taken in establishing the shroud temperature in order to minimize dT/dr , especially if a protocol required using multiple temperatures.

In the XLA/I, both cooling and heating are provided by Peltier cooling/heating modules firmly attached to the bottom of an aluminum thermal shroud and located ~ 6.5 cm from the center of rotation (Fig. 5B). In this design, the high thermal conductance and low thermal inertia of the shroud keeps thermal gradients to a minimum during heating/cooling cycles (i.e., the system is designed to provide a uniform thermal bath). Furthermore, the rotor's high thermal inertia tends to

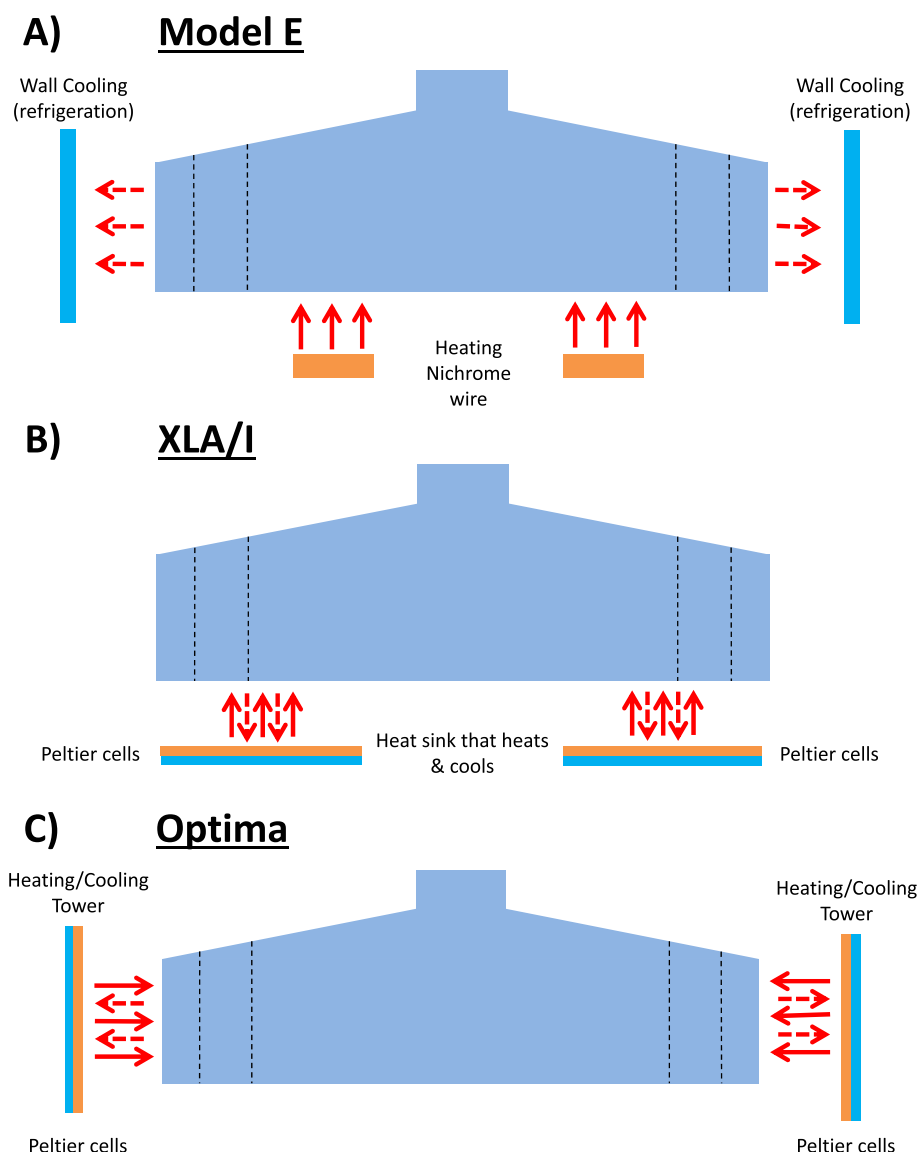


Fig. 5. A schematic view of the layout of the temperature control systems of three different models of analytical ultracentrifuges. **A)** In the Model E analytical ultracentrifuge the bottom area shows the approximate location of the nichrome wire heating element (shown as orange horizontal bars at the bottom of rotor) and the refrigerated cooling coils located along the outside wall of the rotor chamber (shown as blue vertical bars). **B)** In the XLA/I analytical ultracentrifuge the bottom area shows the rough location of the Peltier cooling/heating cells (shown as orange/blue horizontal bars at the bottom of rotor located between the rotor's center of rotation and the radial position of the rotor holes). **C)** In the Optima analytical ultracentrifuge the cooling/heating Peltier cells are located along the outside wall of the rotor chamber (shown as orange/blue vertical bars). In all cases the solid red arrows indicate the directional flow of heat during heating while the dashed red arrows indicate the directional flow of heat during cooling. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

'smooth out' the effects of any small residual thermal gradients in the shroud. As a result, rotor heating and cooling are applied relatively uniformly, especially compared to the Model E.

In the Optima temperature control system the Peltier modules are connected by heat pipes to towers located around the rotor periphery (Fig. 5C). Given, the towers are not attached firmly to the thermal shroud, makes the towers appear to be point heat sources rather than providing a uniform thermal bath, as in the XLA/I design. The Optima design can thus generate destabilizing thermal/density gradients whenever heat is called for. During heating, a thermally-induced, localized density inversion band will propagate from the rotor's edge to its center (see Fig. 3S in Appendix A. Supplementary data). As described above, if sufficiently large these density inversions can cause convection during portions of an SV-AUC experiment, especially where radial density gradient is the weakest (Fig. 4C–D). The Optima temperature control system clearly places *excessive dependence* on the sample generating an adequate positive $(dp/dr)_B$ gradient to override the presence of any negative $(dp/dr)_T$ gradients.

3.4. Reducing the chances for experimental temperature-induced convection in the Optima

For distilled water, change in density as a function of temperature, dp/dT , are greater than 0 over the temperature range of 0–4 °C, however, values for dp/dT are less than 0 for temperatures >4 °C and become increasingly more negative with increasing temperature (see Fig. 2S in Appendix A. Supplementary data). Since $dp/dT < 0$ may lead to convection in the Optima (see discussion concerning eqs. (6) and (7) in Appendix A. Supplementary data Part 3) the more negative dp/dT is the greater the chance for thermal-induced convection. This observation suggests we can take advantage of the variation of dp/dT for water with temperature. At 10 °C the magnitude of dp/dT for water is reduced ~50% of what it is at 20 °C (see Fig. 2S in Appendix A. Supplementary data). Similarly, a comparison of the dp/dT values for water at 20 and 30 °C, nearly the same relationship is observed. As a result, conducting SV-AUC experiments at 30 °C should increase the possibility for convection relative 20 °C, while running at 10 °C should decrease the possibility for convection relative to 20 °C. It should be noted that there are parallel viscosity changes with temperature that also may help limit convection, but viscosity differences are not the driving force for reducing convection.

Based on the above information, AAV SV-AUC experiments were conducted on Optima_4 (which routinely showed convection at 20 °C) at 10 and 30 °C to assess the impact of these changes in temperature on convection, in terms of its presence or absence. Consistent with our model, data acquired at 20 and 30 °C exhibited convection, while data acquired at 10 °C showed no evidence of convection (data not shown). Furthermore, no convection has been detected in any subsequent experiments at 10 °C using Optima_4 and Optima_3. These observations are consistent with our assertion that the Optima temperature control system is the root cause of convective disturbances. The results also suggest that operating the Optima at lower temperatures may provide a route to prevent convection.

4. Discussion

Recent developments in gene therapy over the last decade or so has drawn the renewed attention of many researchers and companies to the use of viral vectors (using various virus systems, e.g., adenovirus [44–48] and AAV [49–51]), along with completely synthetic particles, involving liposomes and nanoparticles [52–57], as gene therapy drug (nucleic acid)/delivery systems. Initially, interest in this area was predominately concerned with the delivery of DNA therapeutic material; however, interest in gene therapy has widened to include the use of RNA therapeutics [58] such as interference ribonucleic acids, RNAi [59,60], and messenger ribonucleic acids, mRNA [61,62], along with gene editing payload material [63–66] as the active pharmaceutical ingredient (API). Given the success and potential offered by these new classes of biopharmaceuticals, they are revolutionizing drug development. This has been clearly demonstrated by the incredible rapid development of vaccines and therapeutic agents for dealing with the recent COVID-19 pandemic [67–70]. These and other events are driving the future appearance of a whole new array of more effective therapeutics. As a result, drug development is progressing rapidly into producing much larger and more complex biopharmaceuticals [71]. These developments will require analytical tools capable of characterizing their homogeneity with respect to incomplete assembly, fragmentation, drug content and aggregation. Such information is important in making sound and timely decisions in the development of these new drugs, as well as in assessing their manufacturing consistency. As of today the AUC can meet these challenges, in some cases uniquely so [17,18,25]. It is a golden opportunity for this instrument to shine and fill an important analytical void.

In this paper, work carried out on a number of different Optima units has exposed a performance issue that appears to be a remnant temperature problem uncovered when the Optima was first released 3+ years ago. Our analysis shows that the problem is a consequence of a design flaw in the Optima temperature control system.

Other than rotor cooling and heating via the Optima's temperature control system, other sources of heating and cooling can be encountered during AUC include:

1. The continuous heat transfer from the drive shaft to the rotor [32–34, 36].
2. Cooling from the adiabatic rotor stretch during acceleration [27,35, 36].
3. Heating due to adiabatic fluid compression during acceleration [36, 72] and rotor compression at the outer edge of the sample cell during the acceleration [36].
4. Frictional heating of the rotor due to the residual air still present inside the rotor chamber [32,33,36].

Case 1 leads to $(dp/dr)_T > 0$, which provides a stabilizing density gradient against convection. Taken together, Cases 2 and 3 could lead to situations where $(dp/dr)_T < 0$, thus producing a destabilizing density gradient [36]. However, the low rotor speed (coupled with the low compressibility of the materials involved) in the examples provided here make this an unlikely contributor to convection. For Case 4 the high

vacuum used in AUC prevents rotor warming from frictional heating. Finally, it is important to recognize that *all* these heating and cooling sources exist for the XL series analytical ultracentrifuge where no similar problem was seen or has been reported.

Consequently, the Optima's temperature-induced convection problem appears to be rooted in the design of the Optima's temperature control system. Specifically, the physical location of the Optima's cooling/heating elements, and inadequate coupling to the thermal shroud, leads to the generation of adverse positive radial dT/dr gradients and thus negative radial $(dp/dr)_T$ gradients (see discussion concerning eq. (5a) in Appendix A. Supplementary data Part 3) when heating the rotor. If $(dp/dr)_T$ is sufficiently large (see discussion concerning eq. (6) in Appendix A. Supplementary data Part 3), convection will occur.

It should be noted that the Optima's convection problem becomes apparent under conditions that are particularly susceptible to convection (i.e., analysis of dilute solutions of very HMW solutes and formulation buffer components at low rotor speeds [36,73–76]). In these cases the stabilizing density gradient, generated by the sedimentation of solvent buffer components, is absent or very weak at best over large portions of the sample in the AUC cell (Fig. 4A–D). Should the HWM sample boundary move into this radial region or “zone”, any weak destabilizing perturbations can give rise to convection. In short, whether convection is possible is controlled by the rate of (HMW) boundary movement *relative* to the rate of formation of an adequate stabilizing density gradient. Simply raising the rotor speed may actually increase the chances for convection since the (HMW) boundary can move into an even more unstable (weaker density gradient) zone faster (see Fig. 4S and discussion in Appendix A. Supplementary data Part 4). It is recommended that sedimentation simulations be conducted for the macromolecule and solvent components to determine conditions that will minimize the time the HWM sample material being studied spends in the unstable zone.

4.1. Experimental approaches for avoiding convection in the Optima

Interim (though dissatisfying) methods to minimize the chances for convection include:

1. Add a solute such as sucrose [76,77], to strengthen the stabilizing properties of $(dp/dr)_B$.
2. Intentionally induce a small negative temperature gradient across the rotor in order to generate a positive (stabilizing) density gradient. This might be achieved by initially choosing an equilibration temperature that is slightly higher than the desired final run temperature, which is then dropped to the desired final running temperature shortly before starting the SV-AUC run.
3. Decrease the experimental temperature to take advantage of the reduced dp/dT at lower temperatures (see Fig. 2S and part B in Fig. 3S in Appendix A. Supplementary data).

Method 1 negates one of the greatest strengths of AUC, which is its ability to characterize a sample in its *native* solution. Such changes can potentially alter the physicochemical properties of the sample and thus provide misleading information, especially about aggregation. However, if this approach is pursued, the added solute(s) should have a high density increment, dp/dc , to achieve the highest radial density gradient (see discussion in Appendix A. Supplementary data Part 1). Sucrose is a good option.

Method 2 actually takes advantage of the Optima's thermal control system to generate a small negative temperature gradient across the rotor by equilibrating the rotor at a temperature setting that is slightly higher than the desired running temperature, which on the initiation of the run is reduced to the actual desired running temperature. This approach is similar to that used on the Model E. However, this approach adds uncertainty to the experiment's accuracy and may result in analysis problems. At present, this approach has not been investigated and is

fraught with potential problems.

Method 3 constitutes the simplest and most practical approach for dealing with the Optima's temperature control problem, by conducting SV-AUC experiments at a lower temperature ($\leq 10^\circ\text{C}$). However, it should be noted that the temperature change may impact data interpretation, particularly when comparing data acquired at different temperatures.

5. Conclusion

Analytical ultracentrifugation provides the most powerful means for solution size distribution analysis. The Optima analytical ultracentrifuge has a thermal control problem that can negatively impact the quality of the data generated from this instrument. Such attributes can limit its utility in the critical and rapidly-evolving field of biopharmaceutical development and in other scientific areas where high quality data is needed to make critical assessments. The problem has been defined, the cause found and possible temporary workarounds (conducting experiments at lower temperatures, adding a stabilizing solute) have been suggested. Nevertheless, the manufacturer needs to provide a permanent fix to this problem. To do less is a disservice to the instrument, to the users and to science.

Author contributions

Both authors have contributed equally to the interpretation of the data collected and the writing of this paper.

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Appendix A. Supplementary data

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References

- [1] S.E. Harding, A.J. Rowe, J.C. Horton (Eds.), *Analytical Ultracentrifugation in Biochemistry and Polymer Science*, The Royal Society of Chemistry, Cambridge, 1992.
- [2] K.E. van Holde, J.C. Hansen, *Analytical ultracentrifugation from 1924 to the present: a remarkable history*, *Chemtracts* 11 (1998) 933–943.
- [3] T.M. Laue, W.F. Stafford, *Modern application of analytical ultracentrifugation*, *Annu. Rev. Biophys. Biomol. Struct.* 28 (1999) 75–100.
- [4] *Analytical ultracentrifugation V*, in: H. Colfen (Ed.), *Progress in Colloid and Polymer Science*, vol. 113, Springer-Verlag, Berlin, 1999.
- [5] J. Lebowitz, M.S. Lewis, P. Schuck, *Modern analytical ultracentrifugation in protein science: a tutorial review*, *Protein Sci.* 11 (2002) 2067–2079.
- [6] T. Laue, *Analytical ultracentrifugation: a powerful 'new' technology in the drug discovery*, *Drug Discov. Today Technol.* 1 (3) (2004) 309–315.
- [7] S.A. Berkowitz, *Role of analytical ultracentrifugation in assessing the aggregation of protein biopharmaceuticals*, *AAPS J.* 8 (3) (2006) E590–E605.
- [8] *Analytical Ultracentrifugation*, 1st, in: J.L. Cole (Ed.), *Methods Enzymology*, vol. 562, Academic Press, 2015.
- [9] S. Uchiyama, M. Noda, E. Krayukhina, *Sedimentation velocity analytical ultracentrifugation for characterization of therapeutic antibodies*, *Biophys. J. Rev.* 10 (2018) 259–269.
- [10] S.A. Berkowitz, J.S. Philo, *Characterizing biopharmaceuticals using analytical ultracentrifugation*, in: D.J. Houde, S.A. Berkowitz (Eds.), *Biophysical Characterization of Protein in Developing Biopharmaceuticals*, second ed., Elsevier, Amsterdam, 2019, pp. 225–283.
- [11] R.C.J. Dobson, T.R. Patel, *Analytical ultracentrifugation: still the gold standard that offers multiple solutions*, *Eur. Biophys. J.* 49 (2020) 673–676.
- [12] P. Schuck, H. Zhao, C.A. Brautigam, R. Ghirlando, *Basic of Analytical Ultracentrifugation*, CRC Press, Boca Raton, 2016.
- [13] P. Schuck, *Sedimentation Velocity Analytical Ultracentrifugation: Discrete Species and Size-Distributions of Macromolecules and Particles*, CRC Press, Boca Raton, 2016.
- [14] P. Schuck, H. Zhao, *Sedimentation Velocity Analytical Ultracentrifugation: Interacting Systems*, CRC Press, Boca Raton, 2018.
- [15] S.A. Berkowitz, *Determining the concentration and the absorptivity factor at 260 nm in sodium dodecyl sulfate of the adenovirus reference material using analytical ultracentrifugation*, *Anal. Biochem.* 380 (2008) 152–154.
- [16] C.A. Brautigam, S.-C. Tso, R.K. Deka, W.Z. Liu, M.V. Norgard, *Using modern approaches to sedimentation velocity to detect conformational changes in proteins*, *Eur. Biophys. J.* 49 (8) (2020) 729–743.
- [17] S.A. Berkowitz, J.S. Philo, *Monitoring the homogeneity of adenovirus preparations (a gene therapy delivery system) using analytical ultracentrifugation*, *Anal. Biochem.* 362 (2007) 16–37.
- [18] B. Burnham, S. Nass, E. Kong, M. Mattingly, D. Woodcock, A. Song, S. Wadsworth, *Analytical ultracentrifugation as an approach to characterize recombinant adeno-associated viral vectors*, *Hum. Gene Ther. Methods* 26 (6) (2015) 228–242.
- [19] R.P. Carney, J.Y. Kim, H. Qian, R. Jin, H. Mehenni, F. Stellacci, O.M. Bakr, *Determination of nanoparticles size distribution together with density or molecular weight by 2D analytical ultracentrifugation*, *Nat. Commun.* 2 (2011) 335, <https://doi.org/10.1028/ncomms1338>.
- [20] J. Walter, K. Lohr, E. Karabudak, W. Reis, J. Mikhael, W. Peukert, H. Colfen, *Multidimensional analysis of nanoparticles with multiwavelength analytical ultracentrifugation*, *ACS Nano* 8 (9) (2014) 8871–8886.
- [21] S. Inagaki, R. Ghirlando, *Nanodisc characterization by analytical ultracentrifugation*, *Nanotechnol. Rev.* 6 (1) (2017) 3–14.
- [22] D. Mehn, P. Lavicoli, N. Cabaleiro, S.E. Borgos, F. Caputo, O. Geiss, L. Calzolari, F. Rossi, D. Gilliland, *Analytical ultracentrifugation for analysis of doxorubicin loaded liposomes*, *Int. J. Pharm.* 523 (2017) 320–326.
- [23] D. Mehn, R. Capomaccio, S. Gioria, D. Gilliland, L. Calzolari, *Analytical ultracentrifugation for measuring drug distribution of doxorubicin loaded liposomes in human serum*, *J. Nano Res.* 22 (2020) 158, <https://doi.org/10.1007/s11051-020-04843-5>.
- [24] M. Chen, S. Wang, B. Hu, *Revealing the formation of well-dispersed polystyrene@ZIF-8 core-shell nanoparticles by analytical ultracentrifugation*, *Langmuir* 36 (2020) 8589–8596.
- [25] A. Henrickson, J.A. Kulkarni, J. Zaifman, G.E. Gorbet, P.R. Cullis, B. Demeler, *Density matching multi-wavelength analytical ultracentrifugation to measure drug loading of lipid nanoparticle formulations*, *ACS Nano* 15 (3) (2021) 5068–5076, <https://doi.org/10.1021/acsnano.0c10069>.
- [26] H. Zhao, R. Ghirlando, G. Piszczek, U. Curth, C.A. Brautigam, *Recorded scan time can limit accuracy of sedimentation coefficients in analytical ultracentrifugation*, *Anal. Biochem.* 437 (1) (2013) 104–108.
- [27] R. Ghirlando, A. Balbo, G. Piszczek, P.H. Brown, M.S. Lewis, C.A. Brautigam, P. Schuck, H. Zhao, *Improving the thermal, radial, and temporal accuracy of the analytical ultracentrifuge through external references*, *Anal. Biochem.* 440 (2013) 81–95.
- [28] H. Zhao, A. Balbo, H. Metger, R. Clary, R. Ghirlando, P. Schuck, *Improved measurement of the rotor temperature in analytical ultracentrifugation*, *Anal. Biochem.* 451 (2014) 69–75.
- [29] H. Zhao, R. Ghirlando, C. Alfonso, F. Arisaka, I. Attali, D.L. Bain, M.M. Bakhtina, D. F. Becker, et al., *A multilaboratory comparison of calibration accuracy and performance of external references in analytical ultracentrifugation*, *PLoS One* (2015), <https://doi.org/10.1371/journal.pone.0126420>.
- [30] M. Stoutjesdyk, A. Henrickson, G. Minors, B. Demeler, *A calibration disk for the correction of radial errors from chromatic aberration and rotor stretch in the Optima AUC analytical ultracentrifuge*, *Eur. Biophys. J.* 49 (8) (2020) 701–709.
- [31] H. Zhao, A. Nguyen, S.C. To, P. Schuck, *Calibrating analytical ultracentrifuges*, *Eur. Biophys. J.* (2021), <https://doi.org/10.1007/s00249-020-01485-2>.
- [32] T. Svedberg, H. Rinde, *The ultracentrifuge, a new instrument for the determination of size and distribution of size of particle in amicroscopic colloids*, *J.A.C.S.* 46 (12) (1924) 2677–2693.
- [33] T. Svedberg, K.O. Pedersen, *The Ultracentrifuge*, Oxford University Press, London, 1940.
- [34] E.G. Pickels, *The Ultracentrifuge: practical aspects of the ultracentrifugal analysis of proteins*, *Chem. Rev.* 30 (3) (1942) 341–355.
- [35] D.F. Waugh, D.A. Yphantis, *Rotor temperature measurement and control in the ultracentrifuge*, *Rev. Sci. Instrum.* 23 (1952) 609–614.
- [36] H.K. Schachman, *Ultracentrifugation in Biochemistry*, Academic Press, New York, 1959, pp. 13–62.
- [37] L. Gropper, W. Boyd, *Temperature measurement and control of analytical rotors in the ultracentrifuge*, *Anal. Biochem.* 11 (1965) 238–245.
- [38] S.J. Fabricant, A.A. Windsor, F.T. Lindgren, *Ultracentrifuge rotor temperature measurement and control*, *Rev. Sci. Instrum.* 37 (1966) 495, <https://doi.org/10.1063/1.1720224>.
- [39] J.E. Hearst, H.B. Gray Jr., *Titanium centerpieces and a modified temperature control system for the Spinco analytical ultracentrifuge*, *Anal. Biochem.* 24 (1968) 70–79.
- [40] P. Schuck, *Size-distribution analysis of macromolecules by sedimentation velocity ultracentrifugation and Lamm equation modeling*, *Biophys. J.* 78 (2000) 1606–1619.
- [41] J. Ma, H. Zhao, P. Schuck, *A histogram approach to the quality of fit in sedimentation velocity*, *Anal. Biochem.* 483 (2015) 1–3.
- [42] M. Straume, M.L. Johnson, *Analysis of residuals: criteria for determining goodness-of-fit*, *Methods Enzymol.* 210 (1992) 87–105.
- [43] S.K. Chaturvedi, J. Ma, P.H. Brown, H. Zhao, P. Schuck, *Measuring macromolecular size distributions and interactions at high concentrations by sedimentation velocity*, *Nat. Commun.* 9 (2018) 4415, <https://doi.org/10.1038/s41467-018-06902-x>.
- [44] D. T. Curiel (Ed.), *Adenoviral Vectors for Gene Therapy*, second ed., Elsevier, Amsterdam, 2016.

- [45] A.R. Shaw, M. Suzuki, Immunology of adenoviral vectors in cancer therapy, *Mol. Ther. Methods Clin. Dev.* 15 (2019) 418–429.
- [46] M.A. Barry, J.D. Rubin, S.-C. Lu, Retargeting adenoviruses for therapeutic applications and vaccines, *FEBS Lett.* 594 (2020) 1918–1946.
- [47] P. Boucher, X. Cui, D.T. Curiel, Adenoviral vectors for in vivo delivery of CRISPR-Cas gene, *J. Contr. Release* 327 (2020) 788–800.
- [48] A. Ricobaraza, M. Gonzalez-Aparicio, L. Mora-Jimenez, S. Lumbrales, R. Hernandez-Alcoceba, High-capacity adenoviral vectors: expanding the scope of gene therapy, *Int. J. Mol. Sci.* 21 (2020) 3643, <https://doi.org/10.3390/ijms21103643>.
- [49] D. Wang, P.W.L. Tai, G. Gao, Adeno-associated virus vector as a platform to gene therapy, *Nat. Rev. Drug Discov.* 18 (5) (2019) 358–378.
- [50] C. Li, R.J. Samulski, Engineering adeno-associated virus vectors for gene therapy, *Nat. Rev. Genet.* 21 (2020) 255–272.
- [51] C.M. Brommel, A.L. Cooney, P.L. Sinn, Adeno-associated virus-based gene therapy for lifelong correction of genetic disease, *Hum. Gene Ther.* 31 (17–18) (2020) 985–995.
- [52] C. Lui, N. Zhang, Nanoparticles in gene therapy: principles, prospects, and challenges, *Progress Mol Biol and Transl* 104 (2011) 509–562.
- [53] H. Yin, R.L. Kanasty, A.A. Eltoukhy, A.J. Vegas, J.R. Dorkin, D.G. Anderson, Non-viral vectors for gene-based therapy, *Nat. Rev. Genet.* 15 (8) (2014) 541–555.
- [54] A.M. Reichmuth, M.A. Oberli, A. Jaklenec, R. Langer, D. Blankschtein, mRNA vaccine delivery using lipid nanoparticles, *Ther. Deliv.* 7 (5) (2016) 319–334.
- [55] C. Zylberberg, K. Gaskill, S. Pasley, S. Matosevic, Engineering liposomal nanoparticles for targeted gene therapy, *Gene Ther.* 24 (2017) 441–452.
- [56] K.H. Moss, P. Popova, S.R. Hadrup, K. Astakhova, M. Taskova, Lipid nanoparticles for the delivery of therapeutic RNA oligonucleotides, *Mol. Pharm.* 16 (2019) 2265–2277.
- [57] C. Liu, L. Zhang, W. Zhu, R. Guo, H. Sun, X. Chen, N. Deng, Barriers and strategies of cationic liposomes for cancer gene therapy, *Mol. Ther. Methods Clin. Dev.* 18 (2020) 751–764.
- [58] N. Dammes, D. Peer, Paving the road for RNA therapeutics, *Trends Pharmacol. Sci.* 41 (10) (2020) 755–775.
- [59] R.L. Setten, J.J. Rossi, S.-P. Han, The current and future direction of RNAi-based therapeutics, *Nat. Rev. Drug Discov.* 18 (2019) 421–446.
- [60] Y. Weng, H. Xiao, J. Zhang, X.-J. Liang, Y. Huang, RNAi therapeutics and its biotechnological evolution, *Biotechnol. Adv.* 37 (5) (2019) 801–825.
- [61] D. Weissman, mRNA transcript therapy, *Expert Rev. Vaccines* 14 (2) (2015) 265–281.
- [62] N. Pardi, M.J. Hogan, D. Weissman, Recent advances in mRNA vaccine technology, *Curr. Opin. Immunol.* 65 (2020) 14–20.
- [63] Y. Rui, D.R. Wilson, J.J. Green, Non-vial delivery to enable genome editing, *Trends Biotechnol.* 37 (3) (2019) 281–293.
- [64] H. Ates, T. Rathbone, C. Stuart, P.H. Bridges, R.N. Cottle, Delivery approaches for therapeutic genome editing and challenges, *Genes* 11 (10) (2020) 1113, <https://doi.org/10.3390/genes11101113>.
- [65] J.V. Haasteren, J. Li, O.J. Scheidele, N. Murthy, D.V. Schaffer, The delivery challenge: fulfilling the promise of therapeutic genome editing, *Nat. Biotechnol.* 38 (2020) 845–855.
- [66] L.S. Tay, N. Palmer, R. Panwala, W.L. Chew, P. Mali, Translating CRISPR-Cas therapeutic: approaches and challenges, *The CRISPR J* 3 (4) (2020) 253–275.
- [67] D. van Riel, E. de Wit, Next-generation vaccine platforms for COVID-19, *Nat. Mater.* 19 (2020) 810–820.
- [68] K.S. Park, X. Sun, M.E. Aikins, J.J. Moon, Non-viral COVID-19 vaccine delivery systems, *Adv. Drug Deliv. Rev.* 169 (2021) 137–151.
- [69] G. Chauhan, M.J. Madou, S. Kalra, V. Chopra, D. Ghosh, Nanotechnology for COVID-19: therapeutics and vaccine research, *ACS Nano* 14 (2020) 7760–7782.
- [70] R. Medhi, P. Srinoi, N. Ngo, H.-V. Tran, T.R. Lee, Nanoparticle-based strategies to combat COVID-19, *ACS Appl. Nano Mater.* 3 (2020) 8557–8580.
- [71] S.A. Berkowitz, Challenges with the biophysical characterization of complex (multi-chain assembly, chemically modified, big and high concentration) biopharmaceuticals, in: D.J. Houde, S.A. Berkowitz (Eds.), *Biophysical Characterization of Protein in Developing Biopharmaceuticals*, second ed., Elsevier, Amsterdam, 2019, pp. 459–479.
- [72] P.F. Mijnlief, P.V. Es, W.J.M. Jaspers, Temperature gradient in ultracentrifuge cells due to adiabatic volume changes, *Recueil des Travaux Chimiques des Pays-Bas* 88 (1969) 220–224.
- [73] V.N. Schumaker, H.K. Schachman, Ultracentrifugal analysis of dilute solutions, *Biochim. Biophys. Acta* 23 (1957) 628–639.
- [74] T.G. Scholte, B.J. Rietveld, Convection disturbances in sedimentation-equilibrium experiments with the Spinco-E-ultracentrifuge, *Makromol. Chem.* 94 (1966) 60–67.
- [75] H. Triebel, Convection-free boundary sedimentation, *Anal. Biochem.* 49 (1972) 198–207.
- [76] D.B. Millar, J.P. Christopher, Coping with convection in the ultracentrifuge, *Anal. Biochem.* 90 (1978) 33–40.
- [77] D.A. Yphantis, Equilibrium ultracentrifugation of dilute solutions, *Biochemistry* 3 (1964) 297–317.