



Monitoring the homogeneity of adenovirus preparations (a gene therapy delivery system) using analytical ultracentrifugation

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Abstract

This study explores the capability of modern analytical ultracentrifugation (AUC) to characterize the homogeneity, under product formulation conditions, of preparations of adenovirus vectors used in gene therapy and to assess the lot-to-lot consistency of this unique drug product. We demonstrate that a single sedimentation velocity run on an adenovirus sample can detect and accurately quantify a number of different forms of virus particles and subvirus particles. These forms include (a) intact virus monomer particles, (b) virus aggregates, (c) empty capsids (ECs), and (d) smaller assembly intermediates or subparticles formed during normal or aberrant virus assembly (or as a result of damage to the intact adenovirus or EC material during all phases of virus production). This information, which is collected on adenovirus samples under the exact formulation conditions that exist in the adenovirus vial, is obtained by direct boundary modeling of the AUC data generated from refractometric and/or UV detection systems using the computer program SEDFIT developed by Peter Schuck. Although both detectors are useful, refractometric detection using the Rayleigh interferometer offers a key advantage for providing accurate concentration information due to the similar response factors for both protein and DNA and its insensitivity to light scattering effects. Additional AUC data obtained from analytical band sedimentation velocity and density gradient sedimentation equilibrium experiments in CsCl with UV detection were also generated. These results further support conclusions concerning the solution properties of adenovirus, the identity of the different virus species, and the overall capability of boundary sedimentation velocity analysis.

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The concept of delivering genetic material into an organism so that it can direct the expression of a desired protein that replaces a missing protein, enhance the expression of a poorly expressed protein, or add a new protein function to an organism is referred to as gene therapy [1,2]. Such a therapy offers a new medical approach to combat a wide range of human diseases [3–5]. A key feature and challenge in the administration of this type of biopharmaceutical drug is the vehicle or mechanism for delivering the genetic material into

the patient. One approach for solving this problem is the use of specifically engineered viruses that can carry the desired genetic material into the target cell but lack the genetic material to successfully replicate. One virus commonly used for this purpose is adenovirus [6–10]. Hence the production of appropriately engineered adenovirus represents a special class of biopharmaceutical drug products. To achieve drug licensure, drug product quality (with regard to homogeneity and purity) and consistency of manufacturing are of key importance [11]. At present, these attributes of adenovirus vector preparations have been only roughly outlined [5,12–15]. However, there has been no established and agreed-upon approach for assessing the homogeneity of adenovirus

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preparations, which should include measuring both the presence and the fractional content of EC,¹ incomplete or aberrant particles, subparticles, and aggregated material. In fact, no single method capable of measuring all this information has yet been identified. Methods used in the past to assess some of this information have included turbidity [16–19], dynamic [18,20–22] and static [20,23] LS, EM [24,25], IEC-HPLC and RP-HPLC [26–31], FFF [32,33], capillary electrophoresis [34], preparative density gradient sedimentation equilibrium [35], and disk centrifugation [16]. Although these techniques have provided some information, it has been significantly limited in content, scope, and even accuracy. As a result, opportunities exist to find or develop better methods to assess quality and consistency of manufacturing of such large biopharmaceuticals.

One technology that has been used in the past to assess the homogeneity of virus preparations, but has received little attention in the gene therapy area, is AUC [36]. Although commonly used in the 1950s through the 1970s in the characterization of biopolymers and supramolecular structures such as viruses, interest in this technology has diminished as molecular biology techniques evolved, newer and simpler biophysical techniques developed, and the direction in biological research changed over the past several decades. Nevertheless, in recent years a strong need has arisen to provide alternate means to reliably assess aggregation and to cross-validate aggregation information generated by SEC [37,38]. In addition, interest in addressing an array of questions concerning the mechanism and thermodynamics of biological interactions has increased. As a result, AUC has rejoined the arsenal of routine biophysical characterization techniques for studying biological systems [39–47]. Two additional critical drivers in stimulating and allowing this renewed interest in AUC have been (1) improvements in instrument hardware [48,49] and (2) very significant developments in the computational means for extracting information that was always known to be present in sedimentation velocity data but in the past could not be feasibly extracted [50–61]. In light of these developments we have investigated the use of the AUC as a characterization tool to assess the homogeneity of adenovirus preparations under the exact formulation conditions of the final virus drug product.

Initial work in using the AUC to characterize adenovirus preparations focused on developing a simple method to assess the level of EC material present in these preparations. In pursuing this objective we have recognized the broader capabilities of AUC for generating an accurate array of biophysical information about adenovirus preparations under the exact formulation conditions (with regard to both sample matrix and virus concentration) that exist in the adenovirus finished product vial. Information that can be obtained includes the following: (1) amount of intact virus monomer and its heterogeneity, (2) amount of EC material and its heterogeneity, (3) amount of virus aggregation and the distribution of aggregate sizes, (4) detection and quantification of other smaller structural forms of the intact and EC adenovirus particles formed during adenovirus assembly *in vivo* or from damage that may occur after release from infected cells during all phases of virus processing (which would include cell culture production, purification, vialing, or storage), and (5) accurate values for the total concentration of adenovirus material present in a virus sample and a more accurate approach to assess the concentration of physically intact virus monomer particles present in comparison to OD_{260nm} measurements [62].

The above information, it should be noted, can all be extracted from a single sedimentation velocity experiment using the computer software program SEDFIT, developed by Peter Schuck [56,57,60,61]. It should also be noted that these measurements are conducted without the need of any sample preparation steps. In addition, when the refractometric detector (RI) on the analytical ultracentrifuge is used, corrections for differences in the response factors for the different adenovirus species present are not required. This arises because light scattering or chemical composition differences between the different adenovirus species have little to no effect on the RI signal [63,64]. Hence by using the RI the task of achieving accurate quantitative results is greatly simplified in comparison to using UV detection. Nevertheless, the use of UV detection in adenovirus AUC experiments can provide accurate and useful results when appropriate steps are taken to deal with the variability in signal response (extinction coefficient) for different adenovirus species. More importantly, results obtained by UV detection can provide additional information about the nature of the migrating species present through the spectral response and the ratio of UV and RI signals for each species. Hence the use of both detectors during a given AUC run can be very helpful.

In addition to conducting conventional boundary sedimentation velocity experiments, we have explored the use of analytical band sedimentation velocity and density gradient sedimentation equilibrium experiments (in CsCl solutions) in this work. Data generated from these alternative AUC techniques, although not as informative as boundary sedimentation velocity, provide additional unique information that supports our findings with regard

¹ Abbreviations used: (f/f_0), translational frictional coefficient ratio (the ratio of the translational frictional coefficient of a molecule divided by the translational frictional coefficient of the same molecule in the shape of a sphere); IEC, ion-exchange chromatography; RP, reverse phase; AUC, analytical ultracentrifugation; EC, empty capsid; vp, virus particle; LS, light scattering; WFI, water for injection; RI, Rayleigh Interferometer; abs, absolute value; FFF, field-flow fractionation; EM, electron microscopy; SD, standard deviation; RSD, relative standard deviation; $c(s)$, differential sedimentation coefficient distribution (the concentration of material having a sedimentation coefficient, s) corrected for diffusion; $ls^*(s)$, differential sedimentation coefficient distribution not corrected for diffusion; RO/DI, reverse osmosis and deionized water; SEC, size-exclusion chromatography.

to the solution behavior of adenovirus, the identity of various virus species, and the utility of boundary sedimentation velocity analysis on adenovirus samples, especially with refractometric detection. Hence by establishing the ability of the AUC to generate this information in a reliable and reproducible manner we are provided with a uniquely useful characterization tool that can assess adenovirus product quality and manufacturing consistency. More importantly, the coupling of this information with biological data obtained from various *in vivo* assays on these same adenovirus gene therapy preparations should provide insight as to which physico-chemical attributes need to be monitored in developing this new class of biopharmaceuticals into an effective and safe drug product.

Materials and methods

Chemicals

Tris base (ACS reagent) and Tris–HCl (ultrapure bioreagent) were obtained from J. T. Baker while glycerol (certified ACS) and cesium chloride (ultracentrifugation grade) were obtained from Fisher Scientific. Water used to make all solutions was either RO/DI purified or WFI grade.

Adenovirus production

Adenovirus 5 preparations used in this work were purified using methods similar to those published using IEC [27,65,66] or CsCl density gradient sedimentation equilibrium using a combination of discontinuous and self-generating CsCl density gradients [67,68]. Purified virus solutions obtained via these methods were then buffer exchanged using either passive dialysis or diafiltration into 10% w/w glycerol + 20 mM Tris, pH 8.0. This “GT buffer” is somewhat similar to that used for the adenovirus reference material produced under the guidance of the adenovirus reference material working group and U.S. Food and Drug Administration [17] and distributed by American Type Culture Collection.

Analytical ultracentrifuge

AUC work described in this paper was conducted at several sites using Beckman Coulter ProteomeLab XL-A or XL-I centrifuges (equipped with either a 660- or a 675-nm laser). These sites included Alliance Protein Laboratories in Thousand Oaks (CA), Dr. Barbara Imperial’s lab in the Department of Chemistry at MIT in Cambridge (MA), and Biogen Idec in Cambridge (MA). All centrifuge experiments were conducted at 20 °C using four-hole (An-60 Ti) or eight-hole (An-50 Ti) rotors.

Boundary sedimentation velocity

All boundary sedimentation velocity runs were conducted at a rotor speed of 3000 rpm. AUC cells were

typically assembled with 12-mm double-sector charcoal-filled epon centerpieces and sapphire windows, however, on some appropriate occasions 3-mm charcoal-filled epon centerpieces were also used. All samples were run in GT buffer except where otherwise indicated. In nearly all cases samples were run neat, without any sample treatment. However, in some cases adenovirus samples were diluted with GT buffer prior to measurement. Boundary velocity data collected using the XL-I analytical ultracentrifuge were acquired from both the Rayleigh interferometer and the UV detectors at the fastest rate possible, which is limited by the scan rate of the UV detector. To maximize that scan rate the “continuous” scan mode was used with a radial spacing of 0.003 cm setting and one replicate. Data collected using the XL-A were also acquired as fast as possible using the same UV data acquisition protocol indicated for the XL-I.

Data analysis was conducted using the $c(s)$ or $ls-g^*(s)$ method in Peter Shuck’s software program SEDFIT [56,57,60,61]. Unless otherwise noted below, scans covering the full range of boundary movement were analyzed, and the data fitting limits included the entire cell except for regions where the data were strongly influenced by the meniscus or the accumulation of material at the base of the cell. A regularization confidence level of 0.68 was employed.

The weight-average translational frictional coefficient ratio, $(f/f_o)_w$, which provided optimum fits for the $c(s)$ distributions, was 1.87 ± 0.10 (SD). This high value is somewhat surprising since it seems to suggest that adenovirus has a highly asymmetric shape. On the contrary, adenovirus capsid is well known to have an icosahedral shape [69] that can be well approximated by a hydrodynamic sphere having a diameter that is 0.94 times the face-to-face distance of the icosahedron [70]. Hence the f/f_o value for adenovirus should be fairly close to a value of 1.0. This apparent discrepancy between a highly symmetric, nearly spherically shaped particle and the high f/f_o ratio determined by boundary sedimentation velocity in this work is probably due in large part to the 12 spike projections that extend about 30 nm beyond the adenovirus 90-nm-diameter capsid shell [71]. These spike projections must impart a significant amount of frictional drag on the overall adenovirus particle, causing a high f/f_o value, in much the same way reported for the six tail fibers on T2 bacterial virus [72]. Such an explanation, it should be noted, could also account for the previous observation that the hydrodynamic radius of an equivalent sphere for a monodisperse adenovirus sample, as measured by dynamic light scattering [73,23], is much larger than its calculated anhydrous radius or radius measured by EM [73]. Other authors have proposed that the high frictional coefficient of adenovirus is simply due to high hydration; however, this hypothesis requires an amount of bound water far in excess of values quoted for protein-bound water [73].

Analytical band sedimentation velocity

All band sedimentation velocity runs were conducted at a rotor speed of 10,000 rpm using 12-mm band-forming double-sector charcoal-filled epon centerpieces (initially designed by Vinograd et al. [74] and manufactured by Beckman Coulter). A 15- μ L virus sample was loaded directly into the sample well of a partially assembled AUC cell containing the band-forming centerpiece already in contact with the bottom window. Once the well is carefully filled (avoiding liquid contact with the top surface of the centerpiece so that placement of the top window on the centerpiece does not provide an opportunity to allow capillary flow of the sample onto the centerpiece surface or into the sample sector region) the top window is carefully added to the AUC cell and the assembled cell is then torqued closed. Appropriate volumes of CsCl sedimentation solution are then loaded into both the reference (0.35 mL) and sample (0.33 mL) sectors. The cell's filling holes are quickly sealed and the cell is immediately placed into the rotor. In conducting band sedimentation velocity experiments the acceleration of the rotor to full speed was reduced by using instrument settings of 100 rpm/s and acceleration profile 2. Usable UV scans typically require a 10 to 20 min delay after reaching speed for the initial steep refractive index gradient to be reduced to a level where the light is no longer refracted significantly away from the UV detection system. UV radial scans were acquired as fast as possible using the "step" scan mode with a radial spacing of 0.003 cm setting and one replicate. The integration of individual bands to calculate the weight percentage of EC or aggregated material required correcting each OD value for the baseline offset and the variation of the sector cross-sectional area with radial position [75].

Analytical density gradient sedimentation equilibrium

Density gradient CsCl equilibrium sedimentation was conducted at 15,000 rpm. Runs were continued until stable UV readings were obtained. The starting virus solution density was adjusted to 1.33 g/mL by adding solid CsCl directly to the adenovirus solutions to make a specific weight percentage solution, which from calculation [76] would yield the desired solution density. The reference buffer containing CsCl was made in the same way as indicated for adenovirus samples, but GT buffer was used in place of the virus solution.

Results and discussion

Analytical density gradient sedimentation equilibrium in CsCl

Adenovirus is composed of a number of different proteins and a single double-stranded piece of DNA [77]. These two classes of compounds have very different buoyant densities. Proteins typically have a buoyant density in

CsCl of about 1.3 g/mL while the buoyant density of double-stranded DNA in CsCl is about 1.7 g/mL [78,79]. Hence, if virus particles of different DNA/protein ratios are present in a given adenovirus sample and if they are stable in high-salt solutions, the technique of density gradient (isopycnic) sedimentation equilibrium (which is commonly used for laboratory-scale purifications of viruses, including adenovirus) can be used as a high-resolution analytical method for separating and quantifying these different virus structures [35,67,68]. Since it is well known that EC material is produced during adenovirus production [80–85] and since IEC purification (the key purification step commonly used in the large-scale purification of this virus) is not totally effective in removing this material [35], density gradient sedimentation equilibrium analysis should be capable of providing quantitative information about the residual level of EC material that is present in these adenovirus preparations. Although preparative density gradient sedimentation equilibrium methods have been carried out to quantitatively assess EC levels in adenovirus preparations [35], this technique requires a significant amount of postcentrifugation sample processing, making it labor intensive. In addition, the amount of virus material required is fairly high and some of the high resolution initially achieved via centrifugation is lost during the physical fractionation process of moving the liquid in the centrifuge tube to the UV detector. Hence, we investigated the use of the AUC to perform these same measurements in real time, without the need of any postcentrifugation fractionation, as an alternate approach to quantify EC material. The equilibrium radial scans shown in Fig. 1 clearly indicate the high resolving power of this technique. However, attempts to quantify the main band (intact virus) indicated a nonlinear response with virus load. This artifact is believed to be a result of the hyper-sharp nature of this band, which causes significant refraction effects that distort the optical density readings. Because of this nonlinear response we have investigated the use of an alternative AUC technique called analytical band sedimentation velocity [74] in a solution containing a high concentration of CsCl. By using a CsCl solution having an appropriate solution density this technique can exploit differences in both buoyant density and hydrodynamic properties in EC and intact virus particles to achieve a good separation. Bands generated with this technique do not focus into sharp bands at fixed radial positions (corresponding to their buoyant density), as they do in density gradient sedimentation equilibrium. Rather, the bands formed in band sedimentation velocity experiments actually broaden during the sedimentation process due to diffusion and the increasing magnitude of the centrifugal field with increasing radial position [74]. Hence refractive index artifacts should not be a factor in quantifying the area of these broader bands. Furthermore, the experimental run time of a band sedimentation experiment is significantly reduced, requiring only a few hours in comparison to about 18–24 h required to perform an analytical density gradient sedimentation equilibrium run.

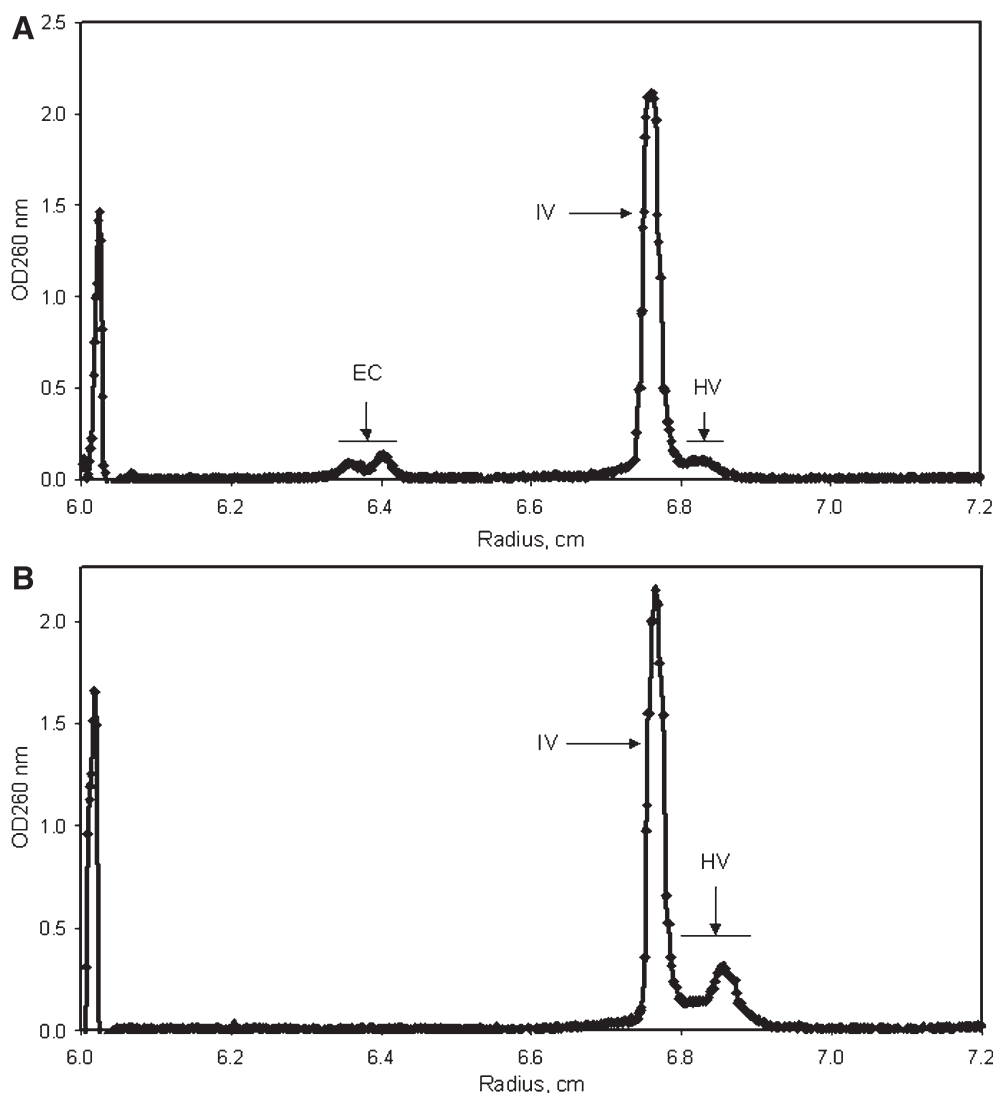


Fig. 1. UV scans at 260 nm of adenovirus AUC profiles at equilibrium in a CsCl density gradient run at 15,000 rpm at a temperature of 20 °C. (A) Adenovirus sample purified by IEC; (B) adenovirus sample purified by preparative CsCl density gradient sedimentation equilibrium. EC, empty capsids; IV, intact virus particles; HV, higher density virus particles.

Analytical band sedimentation velocity in CsCl

In band sedimentation the initial narrow band of sample that is layered onto the top of the CsCl solution at the start of the experiment can be resolved into more than one band as the sample sediments down the column height of the centrifuge cell. The number of bands that appear and their relative intensities are direct measures of the homogeneity of the sample. A key feature in conducting band sedimentation velocity experiments is the requirement for an appropriate stabilizing density gradient to prevent convection [74,86]. The analytical version of this technique, developed by Vinograd and co-workers for AUC [74,86,87], generates this stabilizing density gradient via diffusion by layering a very small sample volume of lower density (typically 10–20 μ L) onto a much larger volume of sedimentation buffer having a higher solution density. (Additional centrifugal density gradient forma-

tion due to CsCl sedimentation can also play a stabilizing role when sufficiently high rotor speeds are used [74,86]. However, for adenovirus the centrifugal field is far too small to be a significant factor in these experiments.) By providing a stabilizing environment for the migrating bands, physical separation can be achieved by taking advantage of differences in the buoyant mass and/or hydrodynamic properties of the various molecular species present in the sample. Band sedimentation velocity experiments conducted in this study were designed to exploit the buoyant density differences between the EC and the intact virus particles. However, differences in their hydrodynamic properties may also play a role in the separation achieved. Since adenovirus EC material has been shown to have buoyant densities that fall within the density range of 1.29–1.32 g/mL [67,68,88–92], which is lower than the buoyant density range of 1.34–1.35 g/mL reported for a mature adenovirus particle [62,67,93], adequate

resolution between these virus particles should be achieved using a CsCl sedimentation buffer matrix having a solution density of 1.29 g/mL. Similar work by Vinograd et al. [74] on the bacterial virus lambda has demonstrated the high resolving power of this approach. Preliminary results obtained for adenovirus are shown in Fig. 2. As expected, complete baseline resolution between intact and EC virus particles was achieved. In addition, analysis conducted on other virus samples (Fig. 2B) has revealed what appears to be multiple species of EC particles. Such EC heterogeneity has been reported previously in the literature [67,68,89,91,93] and can be seen in the isopycnic experiments shown in Fig. 1A. Similar analyses conducted on adenovirus samples purified by preparative CsCl density gradient sedimentation equilibrium instead of IEC, as expected, did not show any signs of EC material (see Fig. 2C) since this material is removed during adenovirus purification. Due to the broader shape of the resulting bands, in comparison to density gradient sedimentation equilibrium, a linear UV signal peak area versus virus load response is obtained with band sedimentation, as shown in Fig. 3. Hence simple peak area integration, taking into account radial dilution effects (see Material and methods under Analytical band sedimentation velocity), allows the percentage of EC material present in a virus preparation to be readily calculated. This approach can be used on virus samples with virus concentrations as low as 1×10^{11} vp/mL if scans are recorded at 230 nm. Quantitative EC levels determined by this technique using UV detection, however, are biased low due to differences in extinction coefficient between EC and intact virus particles as a result of differences in their DNA/protein ratio and possibly also to differences in light scattering properties. At present we have not attempted to apply corrections to account for extinction coefficient differences. Rather, we have investigated the use of classical boundary sedimentation velocity with refractometric detection.

Advantages in using refractometric detection when conducting boundary sedimentation velocity experiments on samples of adenovirus

Refractometric detection using the RI has significant applications for both boundary sedimentation velocity and sedimentation equilibrium [94,95]. Concentration values obtained with this mode of detection are measured by monitoring changes in refractive index, Δn , by experimentally recording the change in the number of interference fringes, Δj . These signals are directly proportional to the change in a sample's weight concentration, c , [96] as indicated in Eq. (1),

$$c = K \times \Delta n = K^* \times \Delta j, \quad (1)$$

where K and K^* are instrument-dependent calibration constants. Hence values of Δj can be converted to real concentration units using Eq. (2) to calculate the proportionality constant K^* [97],

$$K^* = \lambda / [(\partial n / \partial c)_{\lambda, \mu} \times L], \quad (2)$$

where L is the optical path length of the centerpiece in the centrifuge cell, and $(\partial n / \partial c)_{\lambda, \mu}$ is the specific refractive index increment of the sample at the wavelength, λ , of the laser used in the RI (ideally measured at constant chemical potential, μ). If a sample is composed of i components, then the total concentration, c_0 , of that sample is simply the sum of all components:

$$c_0 = \sum K_i \times \Delta n_i = (\lambda / L) \sum [\Delta j / (\partial n / \partial c)_{\lambda, \mu}]_i. \quad (3)$$

Advances in improving and automating the RI have greatly enhanced its sensitivity and reproducibility in acquiring data that are linear over a very large concentration range [98]. However, for AUC methods such as density gradient sedimentation equilibrium and band sedimentation velocity (discussed above) the large salt and buffer gradient generated during these experiments precludes the use of this mode of detection. Nevertheless, even for normal boundary sedimentation velocity experiments steps still need to be taken to exactly match the buffer matrix composition between the reference and the sample solutions to eliminate artifacts caused by the sedimentation of buffer components [63,64,99].² This matching is typically achieved either by dialysis, by the use of centrifugation spin cartridges (with the appropriate number of buffer exchanges), or by SEC [63,64,99]. In addition to the compositional matching of the reference and sample solutions, careful matching of the solution volumes loaded into the reference and sample sectors is required to further reduce a background signal from the differential sedimentation of buffer components due to differences in the actual column heights of both sectors [63,99]. However, due to the very low centrifugation speed used when conducting boundary sedimentation velocity analysis on adenovirus, sample treatment to achieve buffer compositional matching and precise solution volume loading of the reference and the sample sectors is not required. This is demonstrated in Figs. 4 and 5, where results obtained with water in both the reference and the sample sectors of the cell are compared to results obtained with various artificial buffer solution mismatches. These results show that no significant distortion effects occur when major mismatches in the liquid column height (meniscus position) exist between the reference and the sample sectors (Fig. 4C) or when major buffer compositional differences exist between the reference and the sample solutions (Fig. 5). Although the data in Fig. 5 clearly indicate a very small differential amount of sedimentation of buffer components, in this situation the buffer mismatch is of a gross level. Such an enormous difference (10% glycerol vs water) would never be

² The effect of such mismatches, if small, can to some extent be handled by SEDFIT [125]. Nevertheless, in using refractometric detection, steps to avoid buffer mismatch should always be taken to eliminate or minimize refractometric artifacts that become superimposed on to the RI signal from the migrating macromolecule.

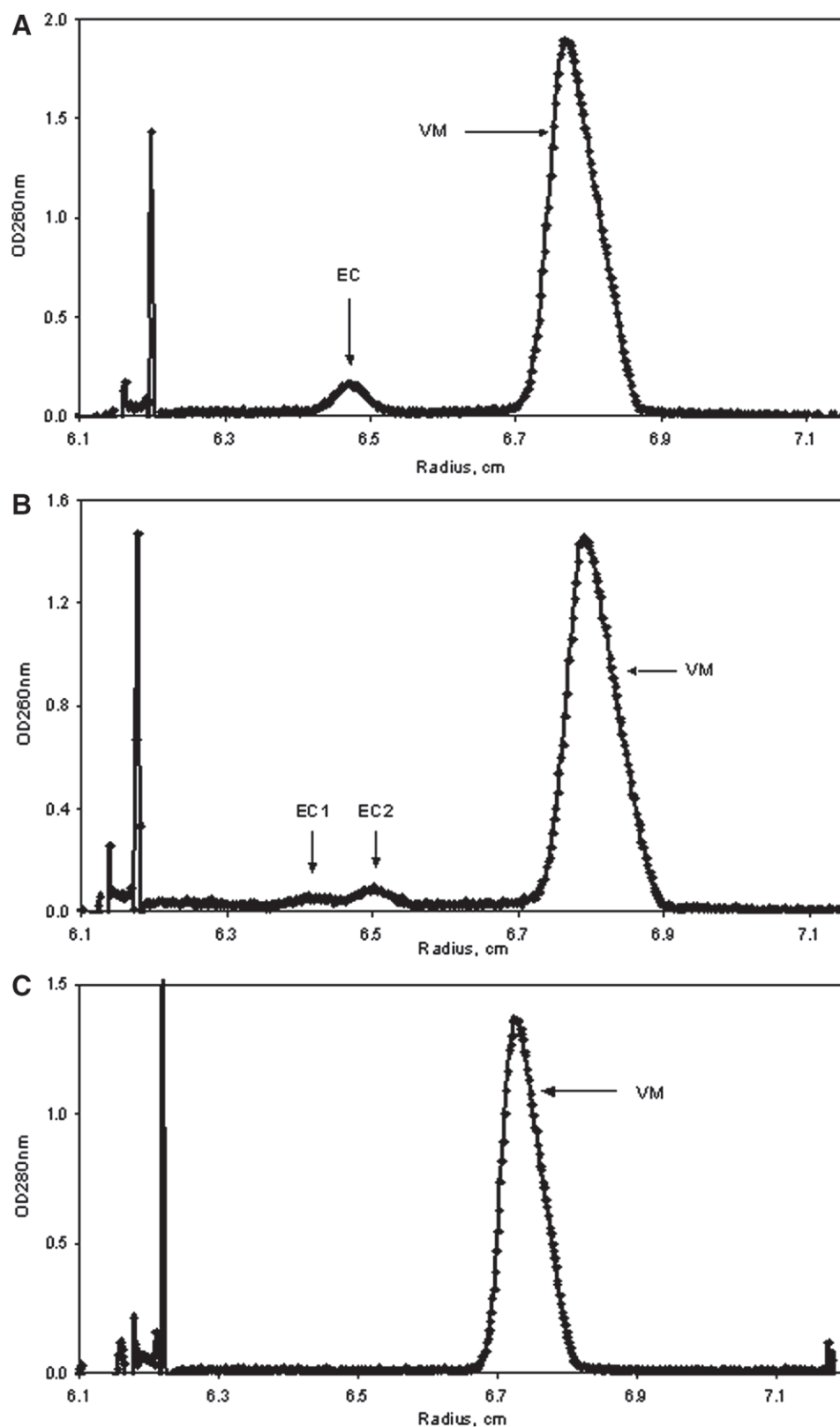


Fig. 2. UV scans at 260 nm of adenovirus AUC profiles generated by conducting analytical band sedimentation velocity in a CsCl density gradient run at 10,000 rpm at a temperature of 20 °C. (A) Adenovirus sample purified by IEC that showed only one EC band, (B) Adenovirus sample obtained from a different virus preparation purified by IEC that showed two EC bands (EC1 and EC2); (C) adenovirus sample purified by preparative CsCl density gradient sedimentation equilibrium that did not contain any EC material. VM, virus monomer band.

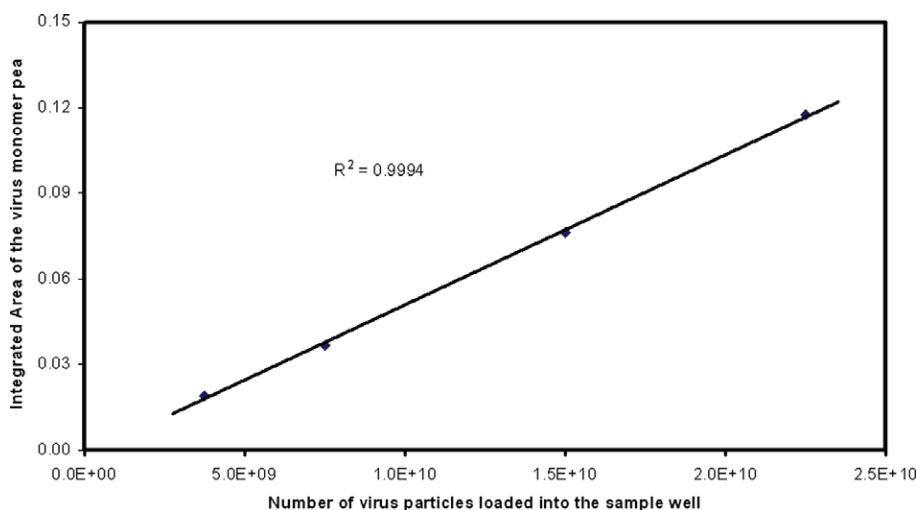


Fig. 3. CsCl analytical band sedimentation velocity calibration curve of the adenovirus monomer band area (obtained from UV scans generated at 260 nm) as a function of virus load (total virus particles) placed into the sample well. All cells were loaded with 15 μ L of appropriately diluted virus sample. All band areas were appropriately weighted to account sector area variation as a function of radius (see Materials and methods).

encountered in a normal AUC experiment. Nonetheless, the sedimentation signals generated by this extreme buffer compositional mismatch represent only a few percent of the total signal that would typically be encountered for an adenovirus sample at the lowest concentration that would be practical to run in an analytical ultracentrifuge using the RI.

The ease with which the RI can be used for adenovirus detection in the AUC provides an opportunity to exploit a key advantage of refractometric detection over UV detection when measuring adenovirus sample concentration. This advantage arises from the fact that the RI has a similar response factor for all of the various species that might be found in an adenovirus sample, including the intact virus, EC, subassemblies or fragments of virus or EC material, free proteins, and even free DNA. This similarity in response factors for refractometric detection of these different adenovirus species is due to the relatively small difference in the $(\partial n/\partial c)_{\lambda,\mu}$ values for the two different chemical compounds (protein and DNA) that make up all the different adenovirus-related species, see Table 1. Hence a single value for $(\partial n/\partial c)_{\lambda,\mu}$ can be used to calculate accurate concentrations for each adenovirus species present and thus yield an accurate total concentration (e.g., mg/mL) value for any adenovirus sample. In this work a value for $(\partial n/\partial c)_{\text{virus}}$ of 0.180 mL/g was used.³ This value is based on linear addition of the average $(\partial n/\partial c)_{\lambda,\mu}$ values for protein and DNA weighted by the weight percentage compo-

sition data of these two compounds for the adenovirus (see Table 1). A relative estimate of the percentage difference in adenovirus concentration data that would be obtained if the value of $(\partial n/\partial c)_{\text{protein}}$ or $(\partial n/\partial c)_{\text{DNA}}$ was used instead of $(\partial n/\partial c)_{\text{virus}}$ can be assessed from Eqs. (4) and (5), respectively:

$$[\% \text{ difference}]_{\text{protein}} = 100[\text{abs}((\partial n/\partial c)_{\text{virus}} - (\partial n/\partial c)_{\text{protein}})/(\partial n/\partial c)_{\text{virus}}] \approx 1\% \quad (4)$$

and

$$[\% \text{ difference}]_{\text{DNA}} = 100[\text{abs}((\partial n/\partial c)_{\text{virus}} - (\partial n/\partial c)_{\text{DNA}})/(\partial n/\partial c)_{\text{virus}}] \approx 9\% \quad (5)$$

These small percentage differences justify the use of a single $(\partial n/\partial c)_{\text{virus}}$ value for conducting all our boundary sedimentation velocity calculations with the RI to obtain fairly accurate weight fraction data for all adenovirus species and the total concentration, c_o , values for adenovirus samples. In contrast, for the same major classes of adenovirus species large variations in the range of values are encountered for their response factors when using UV detection, see data summarized in Table 1. These results indicate that corrections are required to obtain accurate information with UV detection. Hence boundary sedimentation velocity using the RI can be conducted without any sample preparation steps or the need to take into account differences in response factors for the different adenovirus species present.

Boundary sedimentation velocity analysis

Boundary sedimentation velocity RI profiles, corrected for radial- and time-dependent noise, obtained for

³ AUC measurements were conducted using two different XL-I analytical ultracentrifuges, which differed in the wavelength of the laser used in the RI (660 or 675 nm). Hence two different $(\partial n/\partial c)_{\text{virus}}$ should be used, one for 660 nm and one for 675 nm. However the expected difference in this parameter for these wavelengths differs by only a few tenths of a percent. Hence an average value of 0.180 mL/g was used in all calculations.

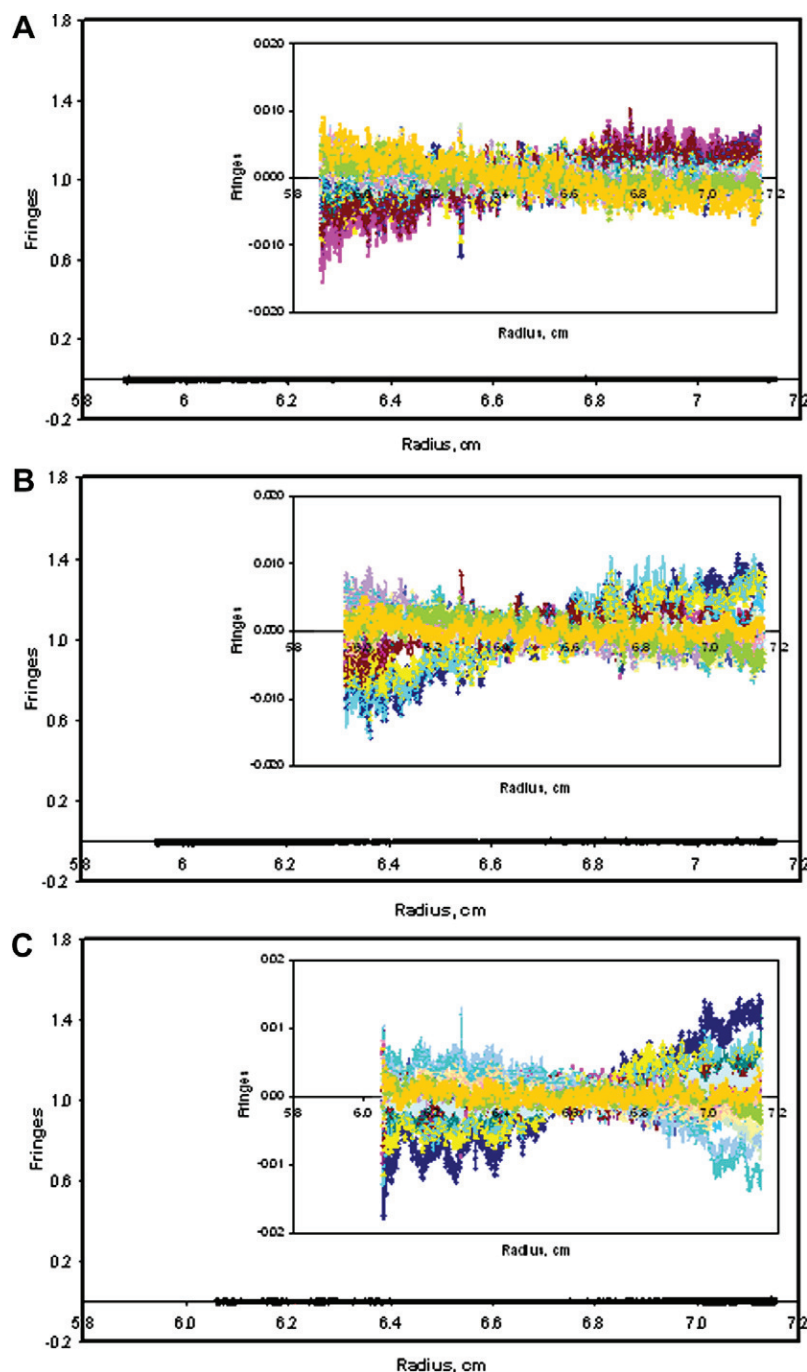


Fig. 4. Effect of having different solution volumes in the sample and reference sectors of an analytical ultracentrifuge cell on the overlay of boundary sedimentation velocity RI profiles, corrected for radial- and time-independent noise using SEDFIT. (A) Cell loaded with 440 μL of water in reference sector and 430 μL of water in the sample sector; (B) cell loaded with 440 μL of GT buffer in reference sector and 430 μL of GT buffer in the sample sector; (C) cell loaded with 440 μL of GT buffer in reference sector and 390 μL of GT buffer in the sample sector. The run was conducted at 3000 rpm at a temperature of 20 $^{\circ}\text{C}$ over a period of about 18 h. Profiles from the RI were overlaid using a y axis scale corresponding to a virus sample concentration of about 2×10^{12} vp/mL (note insets in each plot show an expanded view in the y axis).

two different preparations of adenovirus purified by IEC are shown in Figs. 6A and B. Each plot contains an overlay of corrected radial profiles recorded at different times during the velocity experiment. Simple visual inspection of these plots provides a coarse, but

important initial assessment of the sample's homogeneity, revealing the following key observations: (1) a single sharp major boundary, labeled "I," (2) a second, smaller, slower moving boundary, labeled "II," and (3) the appearance in some cases of a significant

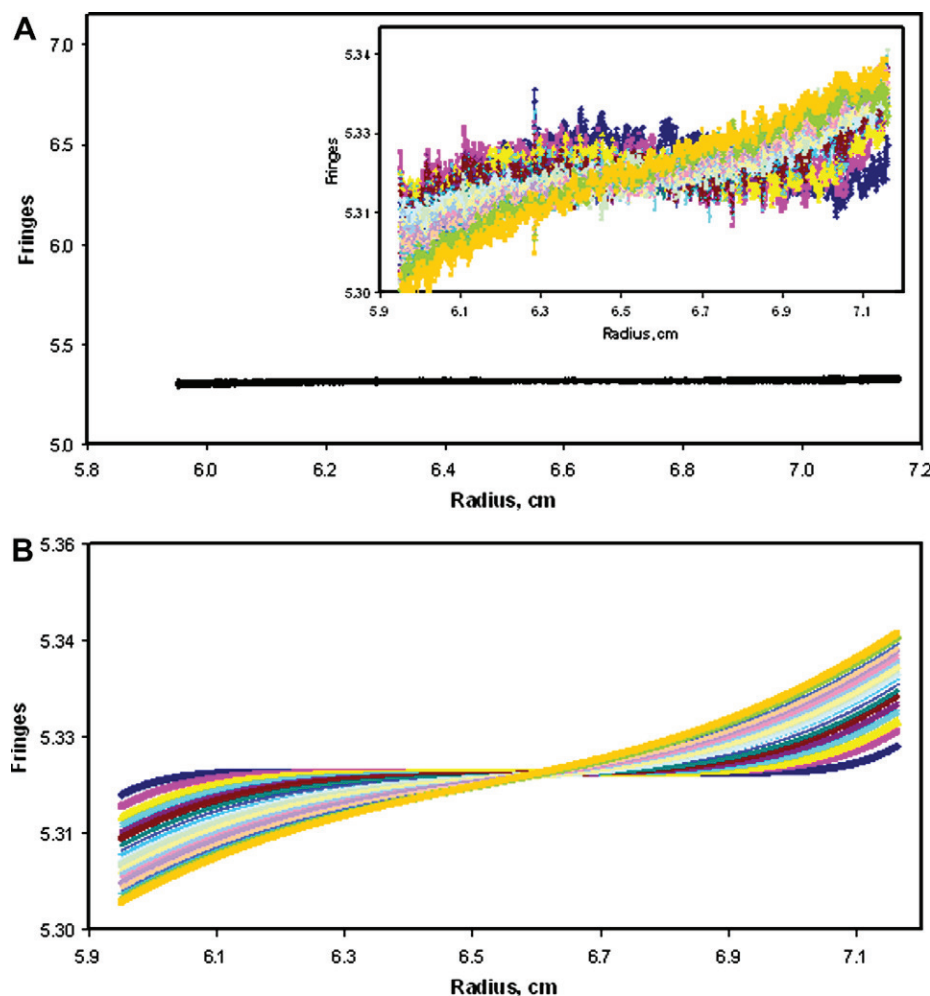


Fig. 5. Effect of having solutions of different composition in the sample and reference sectors of an analytical ultracentrifuge cell on the overlay of boundary sedimentation velocity RI profiles. (A) Cell loaded with 440 μ L of water in the reference sector and 430 μ L of GT buffer in sample sector (note the inset plot represents an expanded view in the y axis of the collected data); (B) corresponding modeled fit data to the experimental data shown in (A) obtained using SEDFIT. The AUC run was conducted at 3000 rpm at a temperature of 20 $^{\circ}$ C over a period of about 18 h. RI profiles were overlaid using a y-axis scale corresponding to a virus sample concentration of about 2×10^{12} vp/mL.

sloping plateau region (as shown in Fig. 6B, see area labeled “III” relative to the similar sedimentation plots shown in Fig. 6A).

Quantitative analysis conducted on these two experiments using the direct boundary modeling software program SEDFIT is shown in Figs. 6C and D. This analysis yields the differential sedimentation coefficient distribution, $c(s)$, for each sample. This $c(s)$ distribution is similar to a chromatogram: each species produces a peak at its characteristic sedimentation coefficient, and the area under each peak gives the concentration of that species (in signal units of OD or fringes). A key assumption in this analysis is that all components present in solution behave as a simple mixture of independently sedimenting species. Hence both chemical changes due to association or dissociation reactions (within the time scale of the AUC experiment) and solution nonideality (“molecular crowding” and electrostatic) effects are assumed to be absent. Plots of the sedimentation coefficient distribution for these two adenovirus preparations,

and corresponding data for a number of other adenovirus preparations purified by IEC indicate the presence of a single major component (labeled “I” in Figs. 6C and D) that migrates with an apparent sedimentation coefficient of about 470 ± 20^4 S in GT buffer. Conversion of these data to a molecular weight distribution using the appropriate buffer viscosity and density along with a

⁴ It should be noted that, due to the presence of relatively high glycerol concentration in GT buffer, even a small error in the preparation of adenovirus matrix buffer (GT buffer) can lead to noticeable shifts in the measured sedimentation coefficients of all adenovirus species. This effect is due to the strong contribution of glycerol to the bulk solution viscosity, which significantly affects the rate of migration of all species. Hence a small error in making up GT buffer degrades the normally very high precision and accuracy in determining sedimentation coefficients via sedimentation velocity analysis. In addition, since adenovirus samples run at lower concentrations bracket the 470 S value reported as the sedimentation coefficient of this major component we consider the 470 S value effectively equivalent to the value one would obtain at zero concentration in this buffer system.

Table 1
Refractometric and UV response factors for protein, DNA, and adenovirus

Material	$(\partial n/\partial c)_{\lambda,\mu}$, mL/g	Extinction coefficient (260 nm), OD/mg/mL	Extinction coefficient (280 nm), OD/mg/mL
Protein	0.183 ^a	0.25–1.0 ^c	0.5–2.0 ^b
DNA	0.164 ^a	20 ^f	10 ^c
Adenovirus	0.180 ^{a,d,e}	3.2–3.9 ^{d,e}	1.9–3.2 ^{d,e}

^a Based on average literature $(\partial n/\partial c)_{\lambda,\mu}$ values at 546 nm, for protein [127] and DNA [128,129] which typically vary less than $\pm 5\%$. These values were then corrected to 660 or 675 nm using the dispersion equation employed by Pearlmann and Longworth [130]. Values for the coefficients used in this equation were the average of the values reported by Pearlmann and Longworth [130] and Camerini-Otero et. al. [131]. Since the computed $(\partial n/\partial c)_{\lambda,\mu}$ values at 660 and 675 nm differed by only a few tenths of a percent the values reported in the table are simply the average of these two calculated values.

^b Based on published literature values for proteins where over 80% of the listed values [132–134] fell within the stated range.

^c Estimate from typical protein or DNA spectrum, of a factor of 2 reduction from OD_{280 nm} values for proteins and from OD_{260 nm} values for DNA.

^d Data calculated assuming linear additivity of refraction or absorbance from its components, protein, and DNA (indicated in the above columns), appropriately weighted on the basis of chemical composition. Chemical compositional data used in these calculations was 85% protein and 15% DNA [100].

^e Contributions from light scattering to UV extinction coefficient not included. Light scattering makes no contribution to refractometric measurements.

^f Based on the commonly used extinction coefficient for dsDNA of 1 OD_{260 nm} = 50 µg/mL.

calculated adenovirus partial specific volume of 0.69 mL/g [73] gives an average molar mass of 154 MDa for this peak⁵, which agrees well with reported values of 150×10^6 Da for a complete adenovirus [77,100–102].⁶ Hence this major species is assumed to correspond to the intact virus monomer.

A second common feature seen in adenovirus sedimentation coefficient distributions (for samples purified by IEC) is the presence of material migrating with sedimentation coefficient values from 350 to 420 S (labeled “II” in Figs. 6C and D) that corresponds to intact EC particles.

⁵ It should be noted that the accuracy of this value is most likely no better than $\pm 10\%$ [60] due to sample heterogeneity. Hence samples containing the highest homogeneity were used to reduce the uncertainty in the calculated M .

⁶ Although this molecular weight value has been specifically cited as a lower limit due to the incomplete accounting for minor proteins and the absence of complete protein maturation (lack of complete hydrolytic processing of proteins), estimates that take these factors into account have indicated that the total mass of the adenovirus would increase at most only few percent [100]. This molecular weight is based on the following accurate information: (a) sequence data for the different adenovirus proteins [100] derived from experimental adenovirus DNA sequence data [111], which is also in good agreement with modern more recent sequence data [112,113], (b) molecular weight of the adenovirus DNA molecule from the same DNA sequence data [111–113], and (c) adenovirus capsid structural data [77,100,109] that provide accurate copy numbers for the major proteins present in the complete adenovirus particle.

A slower sedimentation is anticipated for EC particles due to their lower buoyant mass relative to that of an intact virus particle (although EC particles have essentially the same hydrodynamic friction as an intact virus, because the friction is determined by the outer capsid structure, the absence or lower DNA content yields a particle that is lower in mass and more buoyant). Further, we have directly determined that this slow-sedimenting component has a lower OD_{260 nm}/OD_{280 nm} ratio relative to the main intact virus peak via UV detection at these wavelengths. Finally, the assignment as EC particles is consistent with the fact that when one conducts AUC analysis with both UV and refractometric detection the calculated fraction of EC material is always significantly lower with UV data relative to the results obtained with refractometric detection (a consequence of the absence or low level of DNA in EC particles).

Material migrating faster than the intact adenovirus monomer boundary in Figs. 6C and D should correspond to long-lived adenovirus aggregates. (Note that virus oligomers that display rapidly reversible concentration-dependent association/dissociation properties, with lifetimes short compared to the time scale of the separation, would typically not be resolved as separate peaks). When present at a significant level this faster-migrating material can be detected visually in the data profiles (as mentioned above after correction for systematic noise) by the presence of a sloping plateau region, as seen in the region labeled “III” in Figs. 6A and B. However, the peak at about 530 S, and other material migrating in the region of 500–600 S (which is seen in nearly all adenovirus preparations irrespective of their mode of purification), may actually correspond to altered forms of the adenovirus monomer rather than to virus aggregates. This latter conclusion is based on hydrodynamic calculations for oligomers of spherical particles [72,103] and the fact that the icosahedral shape of viruses, such as adenovirus, is well approximated by a sphere [70,104], as discussed under *Materials and methods* under *Boundary sedimentation velocity*. As a result the smallest aggregate of adenovirus, a dimer, should sediment about 1.50 [72,103] times faster than an intact adenovirus monomer or about 680–740 S under these conditions. The large discrepancy between the experimental 530 S value for the peak migrating just ahead of the major adenovirus monomer peak and the presence of additional components detected between 530 and 600 S in comparison to the theoretical 680–740 S value for an adenovirus dimer plus the additional supporting data discussed below under *Type and sources of heterogeneity in adenovirus preparations* all indicate a need for an alternative explanation for these species. Consequently hereafter the material migrating faster than the intact adenovirus monomer boundary will be referred to as “apparent aggregates.”

Boundary velocity sedimentation data obtained for a third adenovirus preparation shown in Fig. 7 reveal some notable differences in the visual sedimentation pattern in comparison to the sedimentation pattern for virus samples

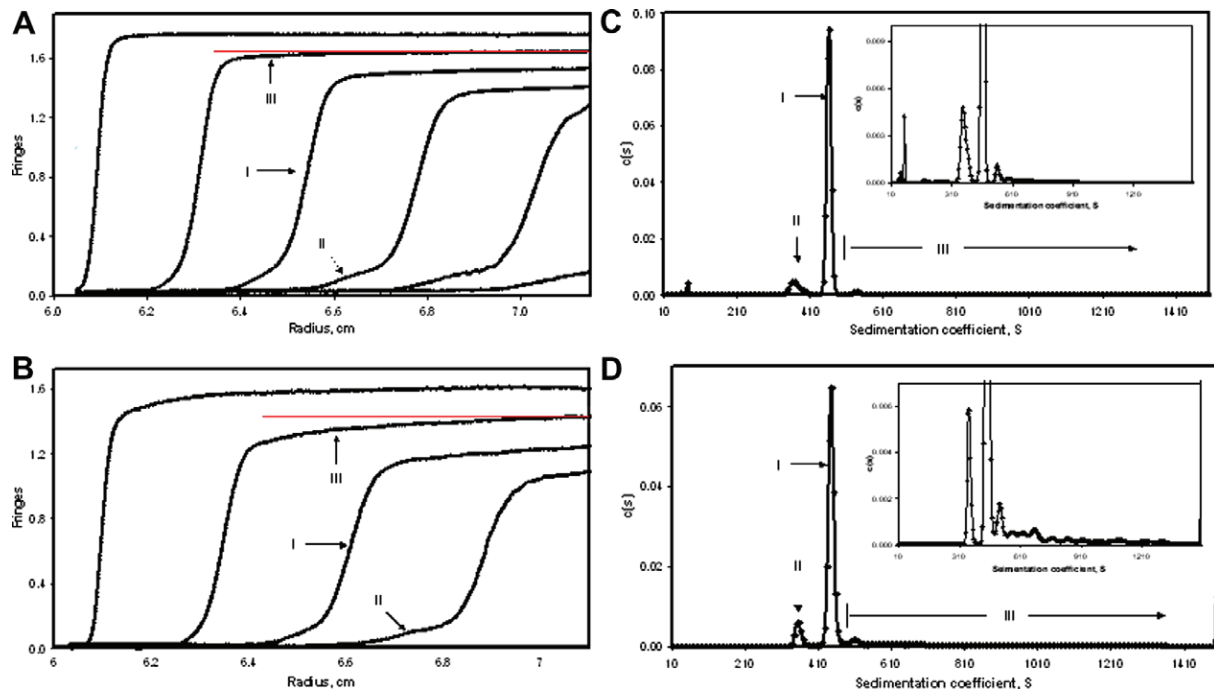


Fig. 6. Overlay of boundary sedimentation velocity RI profiles, corrected for radial- and time-independent noise using SEDFIT, for a sample of adenovirus in GT buffer that had (A) a low amount of apparent aggregation and (B) a high amount of apparent aggregation. Three general areas are indicated in each figure. They correspond to areas associated with the virus monomer (I), EC material (II), and material migrating faster than the virus monomer that includes virus aggregates (III). The red horizontal line indicated in the 2nd profile shown in each plot corresponds to the plateau region near the cell base for each RI profile. This plateau was then extended forward roughly to the position corresponding to the main boundary. Curvature and the amount of curvature of the RI profile in the region indicated by "III" from this plateau is a direct visual indicator of the presence and the amount of faster migrating material (e.g., aggregation) in the sample. Data shown in (A) and (B) were then processed using SEDFIT to calculate the sedimentation coefficient distribution plots shown in (C) and (D), respectively. Regions I, II, and III indicated in these plots correspond to the same regions indicated in (A) and (B). The inset that appears in each plot shown in (C) and (D) is an expanded view in the y axis of the distribution of sedimentation coefficients plot.

shown in Fig. 6. These differences include a slower moving third boundary (labeled "IV") located very close to the meniscus and a very broad boundary "II." When the data in Fig. 7A are analyzed via SEDFIT the resulting sedimentation coefficient distribution, shown in Fig. 7B, indicates that this sample contains a larger fraction of a very heterogeneous collection of species that migrate much slower than the intact virus particle. This slow-migrating material includes the EC particles and additional material with sedimentation coefficients less than 100 S. This very-slow-migrating material most likely corresponds to smaller fragments of EC and virus particles and possibly aggregates of viral proteins that are still relatively small. This material may have been generated during cellular production of the virus particles but might also have occurred from degradation of the intact virus or/and EC particles during various processing steps required to make the adenovirus biopharmaceutical, such as column purification, diafiltration, filtration, vialing, and freeze/thaw cycling. It should be noted that degradation is a particular concern for EC particles since this material is known to be unstable [105–107]. The presence of this heterogeneous and much lower molecular weight material in this sample was also confirmed by analytical band velocity experiments, as shown in Fig. 7C.

Reproducibility of EC and apparent aggregation measurements and general observations concerning the solution behavior of adenovirus from AUC analysis

For any new analytical approach it is obviously important to establish its precision and reproducibility. In particular, the complexity of the sedimentation coefficient distributions in Figs. 6 and 7 naturally raises questions about whether one can reproducibly quantify quality characteristics such as fraction EC and fraction of apparent aggregates present in adenovirus samples by this approach. Since preliminary studies seem to indicate a substantial variation in aggregation content for different vials from the same frozen virus preparation, the precision and reproducibility of boundary sedimentation velocity results were assessed by creating a large pool of virus by combining material from several vials from the same virus preparation. Replicate samples from this pool were then run on the same day in the same AUC run to measure the EC and aggregation levels. In addition, we measured this same adenovirus preparation a number of times over different days using a freshly thawed vial for each experiment.

One additional potential concern about the reliability of the data analysis is that these samples might exhibit substantial solution nonideality, especially because of the

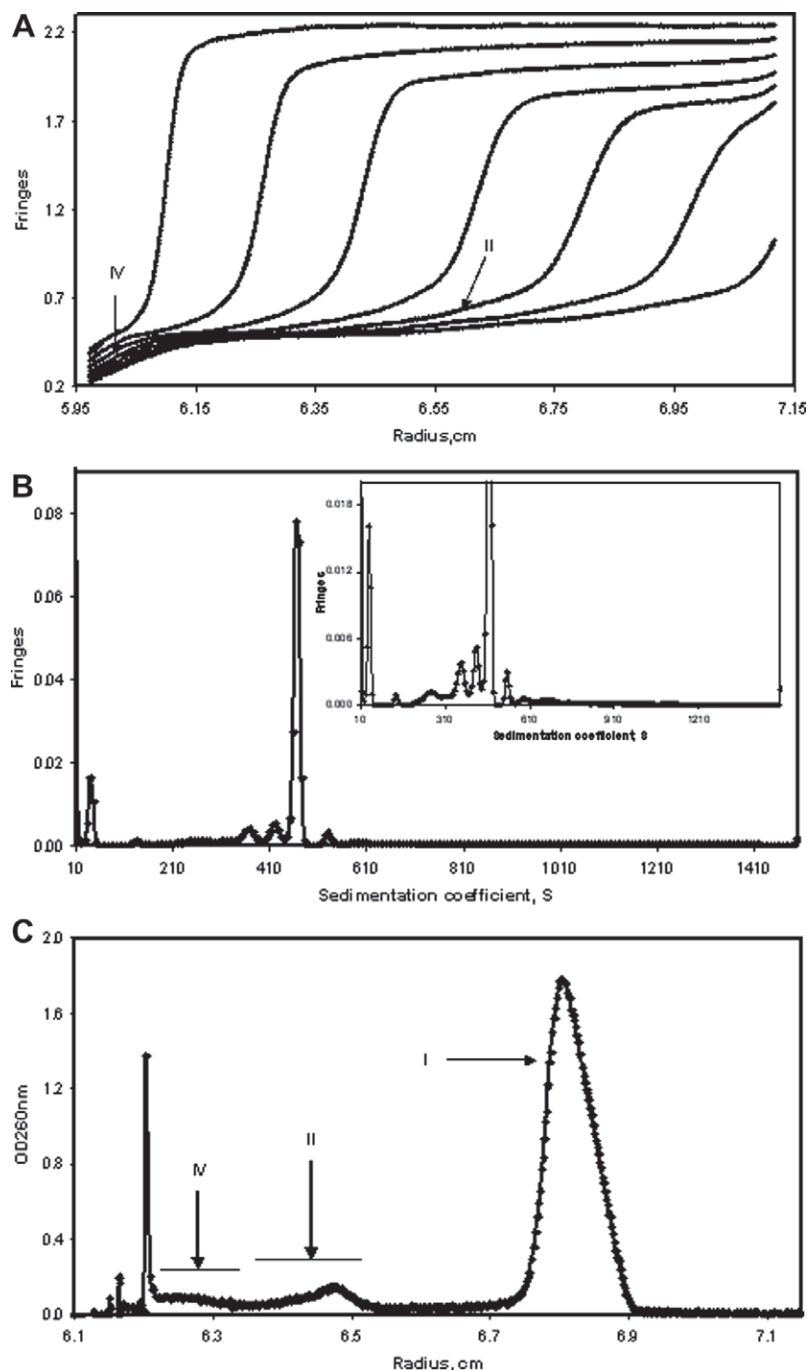


Fig. 7. (A) Overlay of boundary sedimentation velocity RI profiles, corrected for radial- and time-independent noise using SEDFIT, for a sample of adenovirus in GT buffer that has an altered region “II” in comparison to the results shown in Fig. 6 and that shows an additional new region indicated by a very-slow-moving boundary labeled “IV.” (B) Data shown in (A) were processed using SEDFIT to calculate the sedimentation coefficient distribution. The regions labeled “II” and “IV” correspond to the same regions indicated in (A). (C) A band sedimentation velocity scan, recorded at 260 nm, in CsCl for the same adenovirus sample run in (A). Results show a very heterogeneous empty capsid region, “II,” and a region containing very low molecular weight material “IV” near the sample meniscus. The band labeled “I” corresponds to the intact virus monomer band.

low ionic strength of the GT buffer. The data analysis method that we have employed assumes that the sedimentation of each component is ideal (the macrosolutes do not occupy a significant fraction of the volume and there are no significant long-range interactions between them). However, with little ionic strength to suppress electrostatic effects, the observed sedimentation of adenovirus in GT

buffer could be significantly retarded by the primary charge effect. This would also distort the shape of the sedimentation profiles, which in turn could potentially produce artifacts such as false peaks when the data are analyzed using a model that does not account for the nonideal sedimentation. During the course of our preliminary studies we did not observe any obvious variation of the virus monomer

sedimentation coefficient with virus concentration, which suggests that solution nonideality is not significant. Nonetheless to more directly assess this issue we spiked a portion of the pooled adenovirus stock (mentioned above in the previous paragraph) with NaCl to raise the ionic strength approximately five-fold to determine whether this would substantially increase the sedimentation coefficient of the adenovirus monomer peak in comparison to the monomer peak in the unspiked pooled adenovirus sample. Thus for the pooled adenovirus experiment a total of five replicate samples were run, three without added NaCl (unspiked) and two spiked with GT buffer containing 1 M NaCl to give a final NaCl concentration of 50 mM in the GT buffer.

The measured mean monomer sedimentation coefficients for the spiked and unspiked pooled samples were 459.0 ± 1.1 S and 462.2 ± 0.5 S, respectively. Allowing for the increased density and viscosity from the NaCl, those values imply standardized $s_{20,w}$ values of 632.2 ± 1.5 S and 630.8 ± 0.7 S, respectively. The agreement between these two values demonstrates that the nonideality arising from electric charge effects is not significant. The total concentration, percentage empty capsids, and percentage apparent aggregate values obtained from the pooled vial experiments and from the individual vial experiments are summarized in Table 2. The data for the three unspiked samples show good reproducibility of the total concentration (1.0% RSD) and good reproducibility of the EC content at (9.5 ± 0.5) but larger variability in the apparent aggregate content (which is fairly low for this virus preparation) at (7.2 ± 3.1)%. The exact reason for the higher variability of aggregate content is unclear especially when considered in relation to the much lower variability recorded for EC level determined on the same adenovirus samples (see discussion below). Since the results for the two pooled samples that were spiked with NaCl are not obviously different from the results for the unspiked pooled samples (although the concentrations may differ slightly due to dilution errors), Table 2 also summarizes the mean concentration, EC, apparent aggregation behavior, and their variability by considering all five pooled samples to be equivalent.

The EC content obtained by measuring either 10 individual vials or pooled vials from the same virus preparation demonstrates that a reproducible value can be obtained for this parameter by AUC. However, the apparent aggregation values obtained from the individual vials are quite variable, ranging from only a few percent to as high as 23%. Nevertheless, the mean value is similar to the mean value computed from the pooled samples. This variation in aggregation is clearly much higher for the individual vials in comparison to when multiple vials were pooled and then sampled, and as already mentioned far higher than the variability for the EC levels. This higher variability for aggregate levels is also seen when different vials from the same preparation are measured on the same day, in a single run, so this difference is not simply due to problems with run-to-run or day-to-day assay reproducibility. We therefore conclude that the major source of aggregation variability is not a consequence of assay

Table 2

Repetitive measurements of the total concentration, % empty capsids, and % apparent aggregation from individual or pooled vials from the same adenovirus lot

Sample	Total concentration, mg/mL	% Empty capsids	% Apparent aggregate
<i>Measurements from pooled vials^a</i>			
1	0.540	9.2	9.4
2	0.538	9.2	3.6
3	0.548	10.1	8.5
Mean $\pm$ SD	0.542 ± 0.005	9.5 ± 0.5	7.2 ± 3.1
RSD	1.0%	5.3%	43.4%
<i>Measurements from pooled vials spiked with 50 mM NaCl^a</i>			
1	0.528	8.9	7.1
2	0.529	7.6	9.4
Mean $\pm$ SD	0.529 ± 0.001	8.3 ± 0.9	8.3 ± 1.6
<i>Summary of data from pooled vials (unspiked and spiked)^a</i>			
Mean ($n = 5$) $\pm$ SD	0.536 ± 0.008	9.0 ± 1.0	7.6 ± 2.6
RSD	1.5%	11.3%	33.6%
<i>Measurements from individual vials</i>			
1	0.550	8.2	23.0
2	0.541	8.2	10.8
3	0.550	9.1	8.1
4	0.549	9.4	3.9
5	0.525	7.8	6.1
6	0.527	9.2	3.9
7	0.542	9.1	5.5
8	0.553	8.3	14.0
9	0.538	8.6	3.0
10	0.534	8.5	3.9
Mean $\pm$ SD	0.541 ± 0.010	8.6 ± 0.5	8.2 ± 6.3
RSD	1.4%	6.1%	76.1%

^a See section entitled *Reproducibility of EC and apparent aggregation measurements and general observations concerning the solution behavior of adenovirus from AUC analysis* for description of pooling and spiking experiment.

variability but rather is due to a real variation in aggregation from one vial to another. Moreover, the absence of aggregates in the analytical band velocity sedimentation experiment shown in Fig. 2A, which was conducted on exactly the same adenovirus preparation that showed aggregation by boundary sedimentation velocity (Fig. 6B), has led us to the following hypothesis for the variability of adenovirus aggregation seen in GT buffer. Since the nature of the sedimentation buffer used in boundary and band sedimentation velocity experiments differs greatly, it would appear that the aggregates seen in boundary velocity sedimentation experiments (in GT buffer) can be readily dissociated by the high ionic strength and/or reduction/removal of glycerol that occurs during band sedimentation velocity. This hypothesis is supported by the data shown in Fig. 8. In this experiment a preparation of adenovirus purified by preparative CsCl centrifugation techniques was dialyzed into GT buffer at a concentration of approximately 10^{13} vp/mL and stored frozen at -70 °C. Subsequent characterization of this adenovirus material by boundary sedimentation velocity

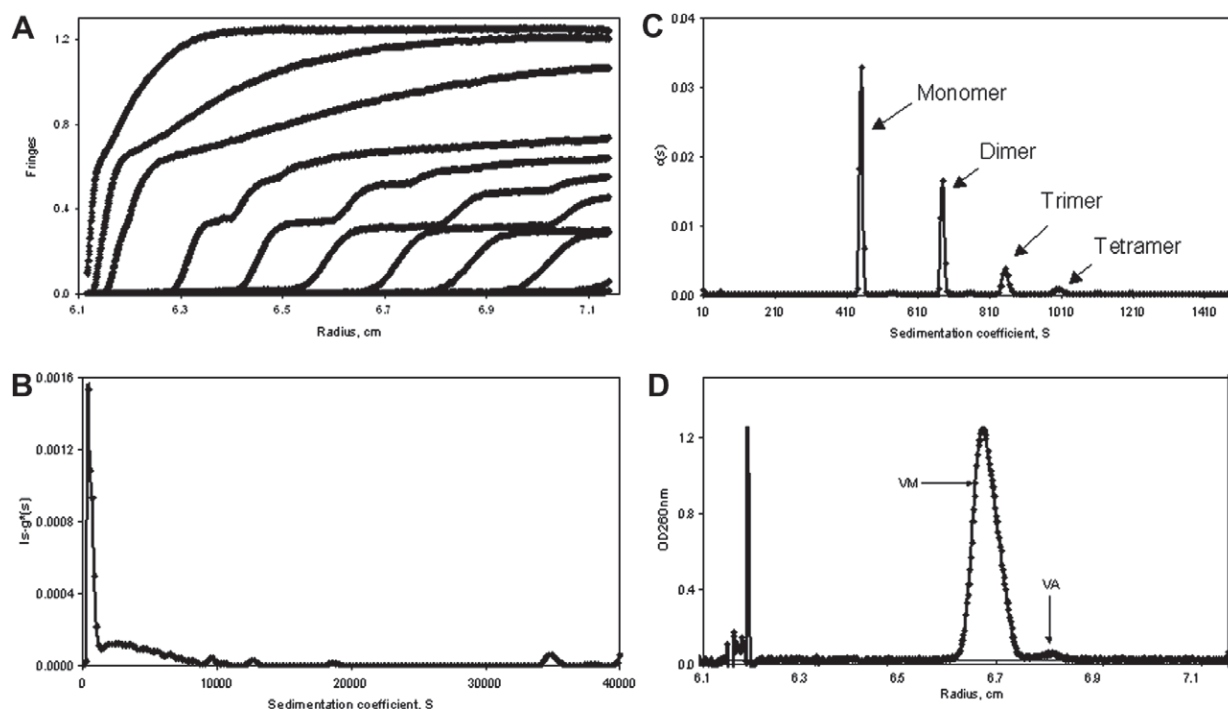


Fig. 8. (A) Overlay of boundary sedimentation velocity RI profiles, corrected for radial- and time-independent noise using SEDFIT, for a sample of adenovirus in GT buffer that is highly aggregated. (B) Resulting plot of the distribution of sedimentation coefficients obtained with SEDFIT from data shown in (A) using only the early RI profiles (1–24) that contain the bulk of rapidly migrating and very heterogeneous virus material. In this case the distribution of sedimentation coefficients was calculated using the $ls-g^*(s)$ method, which does not take into account boundary spreading due to diffusion, due to the more focused interest in the very largest particles (which contribute little to the diffusional boundary broadening) and to the large range of f/f_0 values that might be present in this sample, which could negatively impact the accuracy of $c(s)$ analysis. The fit was conducted over the range of S values from 100 to 40,000 S using a resolution of 250. (C) Resulting distribution of sedimentation coefficients calculated with SEDFIT using $c(s)$ analysis for the remaining sample after the bulk of the rapidly migrating and very heterogeneous virus material had been pelleted to the bottom of the centrifuge cell. The calculated distribution shown was obtained using scans 25–95 and was conducted over the range from 10 to 1500 S with a resolution of 250. The sedimentation coefficient ranges used in (B) and (C) were chosen to include the bulk of material that was sedimenting significantly over the range of scans that was analyzed. (D) Analytical band sedimentation velocity run in CsCl on the same adenovirus sample shown in (A). Results indicate that level of aggregation is almost completely eliminated. The amount of aggregation detected was only about 3%, which most likely corresponded to a dimer aggregate.

(after thawing and dilution to about 10^{12} vp/mL with GT buffer) showed (see Figs. 8A–C) that this preparation was highly aggregated (about 70% of the material was aggregated). However, when the same sample was analyzed by analytical band sedimentation velocity in CsCl (see Fig. 8D) only 3% of the preparation was found aggregated. The fact that nearly all of the material migrating faster than the adenovirus monomer in boundary sedimentation velocity experiments is not observed when the same samples are analyzed by CsCl analytical band sedimentation velocity⁷ indicates that

⁷ It should be noted that in band sedimentation velocity the determination of how many species are present is made by visual observations rather than by numerical analysis as done in boundary sedimentation velocity with SEDFIT. Hence it is possible that the 500–600 S material seen in boundary experiments may actually be present on the leading edge of the large adenovirus band in band velocity sedimentation experiments. Although SEDFIT has the capability to carry out numerical analysis to compute $c(s)$ values for band sedimentation, the fact that the density of the sedimentation buffer is very close to the buoyant density of adenovirus, coupled with the dynamic changes in the buffer density and viscosity as a function of time and radial position during these band sedimentation experiments, makes the accuracy of $c(s)$ calculations highly questionable.

adenovirus aggregation can be reversed by changing buffer conditions. Hence avoiding changes to the formulation conditions of an adenovirus sample is an important consideration in assessing its true level of aggregation. Similarly, these results also indicate the need to assess the impact of different solution diluents and in vivo solution conditions on aggregation when making and administering adenovirus dosing solutions. Hence under appropriate buffer conditions (low ionic strength and high concentration of glycerol), and possibly in combination with relatively high virus concentrations, virus particles may weakly associate to form aggregates. This association may involve physical entanglements between the fiber spikes from different virus particles and may display slow kinetics of dissociation. Furthermore, these weak associations and/or slow dissociation events may also be influenced by subtle differences in vial-to-vial history such as the rates of freezing and thawing. Such subtle differences could be the cause for the observed vial-to-vial variability in aggregation detected by AUC. We hope further work in this area will confirm our initial observations and provide a more detailed elucidation of the physical basis for this variability.

Types and sources of heterogeneity in adenovirus preparations

In characterizing samples of adenovirus by AUC we have placed all adenovirus material, other than the major peak corresponding to the intact virus monomer, into two general categories. The first category represents all adenovirus-related material migrating slower than the adenovirus monomer. In most cases this material corresponds primarily to EC, but this category may also include viral assembly intermediates and/or damaged virus or EC particles. In particular, since EC particles are fragile [105–107] in comparison to complete intact virus particles they are more prone to alterations and degradation during normal adenovirus processing and as a result may more likely be responsible for the variations seen in the sedimentation profiles of the slower-migrating material, as shown in Fig. 7. At this time the significance of EC material and other heterogeneous viral material that sediments slower than the intact adenovirus monomer on the therapeutic properties of adenovirus gene therapy drugs is not understood. Nevertheless, the ability to monitor this material by AUC and to assess lot-to-lot variations offers the future possibility of better assessing its importance.

The second category of adenovirus material consists of all species migrating faster than the intact adenovirus monomer. This material includes aggregated adenovirus species (virus oligomers). However, as discussed previously under Boundary sedimentation velocity analysis, the material migrating with sedimentation coefficients in the range of 500–600 S may not correspond to adenovirus aggregates. Additional data that support this conclusion can be found in Fig. 8C. In this highly aggregated adenovirus sample a series of major aggregate peaks appears at 669, 846, and 996 S (note that the boundaries corresponding to the 669 and 846 S species are easily visible in Fig. 8A). These sedimentation coefficients are actually fairly close to the theoretical values one would expect for oligomers (dimer, trimer, and tetramer) of the adenovirus monomer, based on calculations published for oligomers of spherical particles [72,103]. The validity of applying these calculations to adenovirus is based not only on the similarity of the icosahedral viral capsid of adenovirus to a sphere but also on the resemblance of adenovirus aggregates to oligomers of spheres as seen in electron micrographs [108]. The comparison of the experimental sedimentation coefficients to those calculated based on oligomers of spheres is summarized in Table 3. It should be noted that peaks with similar sedimentation coefficient ratios (relative to monomer) have also been observed in a number of different adenovirus lots with much lower aggregate content than the sample shown in Fig. 8C. For nine samples showing the putative dimer peak (samples in addition to those described in Figs. 6–8 or Table 2), that peak moved on average 1.48 ± 0.05 times faster than the monomer. Five of these samples also showed peaks consistent with a trimer that moved 1.90 ± 0.12 times faster than the monomer, and

Table 3

Comparison of theoretically calculated sedimentation coefficients for aggregated spheres^a in which the monomer sphere is the size of an adenovirus monomer to experimental adenovirus sedimentation coefficients determined for a series of aggregated peaks shown in Fig. 8C

Number of monomer units	Monomer, $n = 1$	Dimer, $n = 2$	Trimer, $n = 3$	Tetramer, $n = 4$
Configuration			Linear	Linear
Ratio S_n/S_1	1.00	1.50	1.80	2.04
Calculated sedimentation coefficient		665	797	904
Configuration			Triangle	Square
Ratio S_n/S_1			2.00	2.32
Calculated sedimentation coefficient			886	1028
Configuration				Tetrahedron
Ratio S_n/S_1				2.44
Calculated sedimentation coefficient				1081
Theoretical S_n value ^b		665	842	1004
Experimental sedimentation coefficients (S_w) for peaks identified in Fig. 8C	443	669	846	996

^a S_n/S_1 values obtained from the work of Jose Garcia de la Torre [72,103], where S_1 = sedimentation coefficient for the spherical monomer and S_n = sedimentation coefficient for the aggregate containing n spherical monomers.

^b All values are based on the experimental value for the monomer of 443 S. In the cases $n = 3$ (trimer) and $n = 4$ (tetramer), all configurations were considered equally probable, hence a simple average was calculated.

two showed a putative tetramer peak that moved 2.21 ± 0.06 times faster than the monomer. Hence these observations provide additional support for our conclusion that the adenovirus material migrating with sedimentation coefficients in the range of 500–600 S, seen in virtually all AUC boundary experiments on adenovirus preparations, probably does not correspond to an aggregate of two intact adenovirus monomers.

Hydrodynamic considerations have also led us to identify the adenovirus material migrating between 500 and 600 S as possibly representing adenovirus monomers that have lost their fiber spikes, which extend more than 30 nm beyond the virus capsid on each of 12 vertices of the intact adenovirus particle [71,101,102,109]. Such a particle would have nearly the same molecular mass as an intact virus monomer but significantly reduced hydrodynamic drag and therefore a higher sedimentation coefficient than the intact adenovirus particle. Similar effects have been observed for the bacterial virus T2 with regard to an extended versus compact geometric arrangement of its tail spikes or fibers [72]. In addition, particles with missing spikes would also have a slightly higher DNA to protein ratio and therefore have a slightly higher buoyant density

than the intact adenovirus particle. The fact that such higher buoyant density material is actually detected in adenovirus preparations (see material labeled HV in Figs. 1A and B and references [67,89]) further supports this hypothesis. Alternatively, a second explanation is that the 500–600 S species could be particles composed of an intact adenovirus monomer particle associated with smaller virus fragments. In either case, self-association or/and hetero-association of this material with intact adenovirus particles would give rise to a large number of different species whose sedimentation coefficients would most likely overlap to some degree with those for oligomers of intact virus particles.

Assessing the total concentration of material in a adenovirus sample and transforming it into vp/mL

The total concentration, c_o , values (obtained by integrating the entire area under the sedimentation coefficient distribution plots and converting the total fringe readings to mg/mL using Eqs. (1) and (2)) for a single adenovirus lot summarized in Table 2 show that this value can be measured with good precision in an analytical ultracentrifuge. Using the measured average weight concentration determined from these measurements (expressed in g/mL) and the correct molar and the correct molar mass for the intact virus particle, Eq. (6) can be used to calculate the number concentration of virus particles for this sample as the number of “equivalent intact monomer” vp/mL, which will be simply referred to as apparent vp/mL (due to the heterogeneous nature of adenovirus preparations):

$$\text{vp/mL} = (c_o/M) \times \text{Avogadro's number.} \quad (6)$$

Since the most accurate estimate for the value of M for adenovirus is 150×10^6 Da (as stated earlier and discussed in Footnote 6) the calculated apparent vp/mL value for the adenovirus preparation in Table 2 is 2.2×10^{12} . The apparent vp/mL determined for this same adenovirus preparation from absorbance measurements at 260 nm in SDS and the absorptivity of adenovirus reported as 1.1×10^{12} vp/mL per OD_{260nm} (measured in a 1-cm-path-length cell) by Maizel et al. [62],⁸ gives an average apparent vp/mL value of $1.9 \pm 0.2 \times 10^{12}$. As discussed in Footnote 8 Maizel’s reported adenovirus absorptivity is based on an adenovirus DNA composition of 13% [110]. Unfortunately, more recent data indicate that a DNA composition of adenovirus of 15% (calculated from the protein mass of the adenovirus and the mass of adenovirus DNA [77,100,111–113]) is more accurate. Application of these newer DNA compositional data to Maizel’s earlier results causes the vp/mL per OD_{260nm} to increase by about 15%

⁸ This absorptivity value is based on Lowry protein measurements using bovine serum albumin as a protein standard, a virus protein and DNA composition of 87 and 13% [110], respectively, an adenovirus DNA molecular mass of 23×10^6 Da [126], and OD_{260nm} measurements conducted on adenovirus purified by CsCl gradient (and therefore free of EC material) in a SDS solution.

to a value of 1.27×10^{12} . This correction would in turn cause the vp/mL value for our sample calculated from OD_{260nm} measurements in SDS to increase to about 2.2×10^{12} vp/mL, which is in excellent agreement with the AUC result. Hence our AUC data indicate the need for only a small correction to Maizel’s original reported adenovirus absorptivity, which is due solely to the small error in the DNA composition of adenovirus used in Maizel’s calculations. In addition, it should be noted that the AUC concentration values could be further corrected to a more accurate value for the number of intact monomer vp/mL by accounting for the fraction of EC material present in an adenovirus sample. In the case of the adenovirus results given in Table 2 the total concentration, c_o , would be lowered by 8.6%, resulting in an apparent vp/mL value of 2.0×10^{12} . A correction for the presence of apparent aggregates (which would further lower the vp/mL value) is unfortunately not feasible due to the following: (a) the amount of aggregates and possibly altered virus monomer seems to vary from vial to vial, (b) the aggregates themselves may disassociate over time or under appropriate solution conditions to yield potentially intact (and presumably biologically active) monomer particles, and (c) some or all of the altered virus monomer material may still be infectious and biologically active. Hence the concentration of apparent aggregates detected by AUC has been converted to “equivalent intact monomer” virus particles.

It should be noted that adenovirus concentration information provided by AUC is completely orthogonal to that provided by UV spectroscopy (62). As a result it avoids issues associated with light scattering and the necessity for virus disruption procedures that could cause variability in the degree of disruption and/or alteration of DNA (melting and reannealing) and therefore lead to variability in OD_{260nm} readings [114] and hence inaccurate adenovirus concentration values. The key source of uncertainty in AUC concentration values resides in the value used for $(\partial n/\partial c)_{\text{virus}}$. At present, we estimate the RSD in this calculated parameter to be about $\pm 5\%$.

General comments on the capabilities and advantages of AUC to assess the homogeneity of adenovirus preparations in comparison to other commonly used methods

Boundary sedimentation velocity measurements using refractometric detection on adenovirus samples can be achieved using sample volumes as low as 70–100 μL with adenovirus sample concentrations as low as 8×10^{11} vp/mL when using a 3-mm centerpiece or as low as 300–400 μL with adenovirus sample concentrations as low as 2×10^{11} vp/mL when using a 12-mm centerpiece. This information can be obtained without any sample preparation, in the exact buffer matrix, and at the same virus concentration that exists in the adenovirus vial. Variations in chemical composition and in the amount of light scattered by different virus species do not produce any significant errors when computing the weight fractions of each of

the adenovirus species present. Hence AUC experiments on adenovirus using the RI offer a significant advantage in comparison to AUC analysis of adenovirus with UV detection. Nevertheless, AUC analysis using UV detection is still very useful. This is especially true for situations when the analytical ultracentrifuge does not contain a RI or when the adenovirus concentrations are too low for refractometric detection (since UV detection can extend AUC analysis on adenovirus to concentrations two to four times lower than is feasible with refractometric detection by using wavelengths of 230 nm or lower, if the sample matrix is sufficiently transparent at these lower wavelengths).

One of the key advantages of AUC analysis on adenovirus (and biopharmaceuticals in general) is due to the minimal contact of the sample with surfaces in comparison to chromatographic methods such as SEC or techniques that use membranes, such as FFF. In both FFF and SEC especially the enormous surface area to which virus test samples are exposed provides significant opportunities for surface adsorption and removal of virus or virus-related materials from the solution, which are the same issues that arise in applications to protein-based pharmaceuticals [38,115,116]. As a result extensive method development studies are frequently required to find mobile phases that will minimize these interactions. A common outcome from this method development is that the mobile phase used in SEC or FFF differs from the test sample matrix. In addition, during the normal running of SEC and FFF significant sample concentration changes occur throughout the separation process [38,115]. Such changes in the bulk solution matrix and the sample concentration can cause changes in the actual homogeneity of the sample, as illustrated in Fig. 8. In contrast, in AUC the samples come in contact with only a relatively small area of the sapphire or quartz windows and the charcoal-filled epon centerpieces, analysis is typically conducted directly in the sample matrix, and sample dilution is typically only 30%. Alternative techniques such as disk centrifugation or EM also significantly alter the sample's bulk solution matrix and virus concentration during normal operation and hence can also cause changes in the actual homogeneity of a sample. Therefore data obtained via these techniques may not reflect the true physical state of the virus, as it initially exists in the sample vial.

Although light scattering techniques can allow analysis to be conducted under the exact conditions that exist in the initial virus sample (same formulation matrix and virus concentration), such approaches also have significant drawbacks. One problem is the difficulty of detecting and quantifying small viral fragments or other low-mass species in the presence of very large virus particles and viral aggregates. Additional issues are the complex nature and strong angular dependence of light scattering for particles the size of adenovirus and its aggregates [117,118], the “dust” problem associated with light scattering [119], and its low resolution [120,121]. These problems all significantly reduce the accuracy and limit the amount of information

that light scattering techniques can provide. In comparison, the simple and straightforward experimental execution of a boundary sedimentation velocity experiment leads to minimal experimental method development and minimal opportunity for the introduction of artifacts or perturbations of the system while offering the ability to extract a wealth of information.

Several methods for assessing the level of EC material present in large-scale adenovirus virus preparations have previously been investigated. In one approach SDS-PAGE or RP-HPLC was used to measure a specific marker band or peak associated with EC material [35,122]. However, a recent study [93] has shown that this approach has limited accuracy due to the complex nature of EC material (not all EC particles contain this protein marker). In the case of preparative density gradient equilibrium sedimentation in CsCl [35] the limitations discussed above under *Analytical density gradient sedimentation equilibrium in CsCl* make the use of this technique unattractive. In the present study the ability of AUC to assess the weight fraction of EC in adenovirus samples has indicated that at an EC level of about 9% the RSD of this value is just over $\pm 6\%$. This precision has been established when analyzing adenovirus samples at particle concentrations of about 2×10^{12} vp/mL. Furthermore, none of these alternative methods for measuring EC levels can provide the additional information that can be generated from one boundary sedimentation velocity run using the RI or the combined use of the RI and UV detectors. Even AUC measurements made using just the UV detector, although lacking to some extent in accuracy, are still very useful for detecting differences between adenovirus preparations.

Aggregate characterization for adenovirus gene therapy products

At present, the assessment of aggregation in adenovirus gene therapy drug products is absent as a release criterion. In contrast, aggregate content is a release specification used for virtually all protein-based pharmaceuticals. This situation is most likely due to the inability to use SEC (the standard method in the biopharmaceutical industry to assess this quality attribute) due to pore size limitations of SEC chromatography media that limit its ability to separate large molecular structures such as adenovirus and its aggregates and to the lack of any appropriate simple method that can be validated to replace SEC. Nevertheless, in generating adenovirus gene therapy preparations, characterization information related to adenovirus aggregation is typically provided by using one of the methods mentioned in the introduction of this paper, with dynamic light scattering being the most common. Unfortunately, as discussed above, the utility of the information provided by light scattering and other methods is limited and they may yield results that do not necessarily reflect the true state of aggregation of the virus in the vial. In contrast, AUC results presented in this paper have clearly demonstrated its new

capabilities to generate detailed characterization information concerning the homogeneity of adenovirus preparations in their final formulated state. While our data to date indicate that the precision of AUC for aggregate content assessment is lower than that for EC content and lower than that often obtained for protein-based pharmaceuticals via this approach, the aggregation information from boundary sedimentation velocity is nonetheless far more detailed and precise than that from light scattering [23]. Such improved information should provide further insight into the complex nature of adenovirus solution behavior and the physical attributes of adenovirus gene therapy drug products that need to be monitored to better assess their quality and lot-to-lot consistency. Hence the analytical ultracentrifuge can play an important role in developing appropriate tissue culture growth conditions, purification processes, and effective formulations that will lead to safe and effective gene therapy drugs.

Conclusions

In developing an effective AUC method to accurately assess the level of EC material present in preparations of adenovirus we have found that boundary sedimentation velocity with refractometric detection can, in a single experiment, provide additional important information about the homogeneity of the virus sample. We have also demonstrated that the total concentration of virus material (mg/mL) in a given adenovirus sample can be accurately assessed and converted to vp/mL, independent of the presence of UV contamination [123], variations, artifacts, or inconsistencies associated with the adenovirus disruption procedures required by the OD_{260nm} SDS method [114] developed by Maizel et al. [62]. The concentration measured via AUC is also independent of concerns about the accuracy of Maizel's reported adenovirus UV extinction coefficient [124]. Furthermore, the accuracy of adenovirus concentration data can be improved by using AUC information to account for the presence of EC, and other adenovirus material that does not represent intact virus particles.

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