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Size distribution analysis of recombinant adenovirus using disc centrifugation

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Recombinant adenovirus is one of the primary vectors for human gene therapy. However, the aggregation of unstable virus has been a recurring problem during the production of purified virus for human therapeutics. To facilitate the development of a robust manufacturing process for recombinant adenovirus vectors, a convenient and reliable size distribution analytical assay is necessary and we demonstrate here that disc centrifuge sedimentation is applicable to this purpose. Using the disc centrifuge system and the line start method, the assay can provide particle size distribution of adenovirus samples within 30 min. The assay can detect virus concentrations down to 0.01% (w/v) or 3×10^{11} particles per ml. The apparent hydrodynamic diameter of recombinant adenovirus was determined to be about 0.063 μm. Furthermore, the disc centrifuge analysis was able to detect adenovirus dimers, trimers, and tetramers, consistent with a rigid sphere approximation for adenovirus, as well as a large aggregate of 0.35 μm. The appearance of viral aggregates is confirmed by increased light scattering based on A_{320}/A_{260} ratios. The technique could be useful for monitoring the kinetics of aggregation for adenovirus and other DNA and RNA viruses in the submicron region. Therefore, this novel assay provides a critical tool for purification development of viral vectors for meeting therapeutic and research needs.

Keywords: adenovirus; disc centrifugation; particle size distribution

Adenoviruses are double-stranded DNA viruses that infect a broad range of mammalian cell types, thereby making them potential vectors for human gene therapy [2,7,9,22,25,26]. They are non-enveloped particles of 0.065–0.080 μm diameter and display a regular icosahedral symmetry [3,11,21,23,31]. One of the main challenges in the commercial production of recombinant adenovirus for human gene therapy is to maintain virus infectivity during long-term storage. The instability of a virus is partly caused by the irreversible aggregation of virus particles resulting in precipitation and loss of biological activity. However, the rate determining step in the aggregation pathway is usually dependent on the initial formation of dimers, trimers, and other lower-order oligomers. Therefore, an analytical method that is able to measure and monitor accurately the formation of low-order oligomers in a virus preparation would help to determine the kinetics of the aggregation pathway and facilitate the development and purification of viral vectors.

Lange [13] evaluated several methods that determine particle size distribution in the submicron range for a set of test polymer latexes. Of the methods to determine particle size distributions, ultracentrifugation and, somewhat less, disc centrifugation and electron microscopy with image analysis were the most efficient. Dynamic light scattering only yielded reliable results in the case of small particles with narrow distribution curves; light diffraction and the electrical zone method were less suitable. Other potential techniques that could be used for determining particle size distribution in the submicron range include capillary hydrodynamic fractionation [6] and field flow fractionation, either in the sedimentation, flow, or thermal modes [8]. Gel permeation chromatography [27] is limited by the current exclusion limits of resins. In spite of the availability of many techniques for determining particle size distributions in the submicron regime, no single method has been reported that can identify and quantify the lower-order oligomeric forms of adenovirus or any viral particle which would be useful in predicting and preventing the onset of aggregation.

The technology of modern disc centrifugation, also known as disc centrifuge sedimentation, has extended the range of application of sedimentation particle size analysis to include the submicron range. The technique is based on the measurement of the size-related settling rates of particles dispersed in a fluid and moving under the influence of gravitational or centrifugal forces [19]. The modes of operation of the disc centrifuge can be divided into two: the homogeneous start mode, where the sample is introduced in the form of a dilute dispersion and as sedimentation progresses and the particles move outward, the changing particle concentration in the detection zone is monitored by a detector; and the line start method, where the sample is introduced onto the surface of the spinning liquid and as the sample moves through the spin fluid, the samples are completely separated according to their apparent hydrodynamic diameter before reaching the detector. Detection of the particles can either be optical, with full Mie Theory light scattering corrections, or using lower-sensitivity X-rays; a centrifuge which uses the former mode of detection is called a disc centrifuge photosedimentometer while one

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that uses the latter mode is called an X-ray disc centrifuge [1,19,32].

The high-speed centrifuge has brought the benefits of increased speed, high accuracy, and applicability to a wide variety of materials to the technique [10]. Unfortunately, studies employing the technique have only been done on polymer latexes [18,19,30], clay minerals [19], ceramic powders [1], glass beads, refractory metal oxides [32], pigments [4], and *E. coli* inclusion bodies [20,28,29], but not on viruses. Sedimentation velocity experiments, using an analytical centrifuge and not a disc centrifuge, have been carried out before to determine the molecular weight of viruses but not their molecular dimensions and particle size distribution [5].

In this study, we report the first application of disc sedimentation photosedimentometry in determining the particle size distribution of recombinant adenoviral samples as a means of monitoring the status of viral aggregation.

Materials and methods

Samples

All reagents were of the highest grade available and all manipulations were carried out at 4°C unless otherwise noted. All manipulations of viral samples were carried out under strict compliance with BL-2 (biological safety level 2) requirements. Recombinant adenovirus 5 (rAd5) containing the p53 gene was produced using 293 kidney cells. It was then purified by column chromatography as described previously [12] but substituting size exclusion chromatography instead of zinc metal-chelating chromatography. The rAd5 was comparable in quality to material purified by cesium chloride density gradient centrifugation based on SDS-PAGE, absorbance analysis, anion exchange HPLC, and a bioassay monitoring the expression of p53 gene product and growth inhibition of Saos-2 cells (data not shown). The rAd5 samples were in 20 mM sodium phosphate, 100 mM NaCl, 2 mM $MgCl_2$, 2% sucrose, pH 7.5 and were stored at −80°C until use.

Operation of disc centrifuge

The analytical instrument consisted of a Disc Centrifuge System (Chemical Process Specialists, Gorham, ME, USA) controlled by its Disc Centrifuge Control software (version 6.x) and a Compaq Deskpro computer system. Briefly, the system consisted of an optically clear disc that is mounted vertically onto the shaft of an electric motor and is spun at 10 000 rpm. Particle size distributions were determined using the line start method and allowing the sample to sediment by centrifugation in a sucrose density gradient. The concentration of particles at each size was determined by continuously measuring the turbidity of the fluid near the outer edge of the rotating disc and the turbidity measurements were converted to a weight distribution using Mie Theory light scattering calculations. In practice, the disc was first allowed to reach the desired speed; the sucrose gradient was formed by injecting 1.8 ml of buffer with concentrations of sucrose in the order 6, 5.5, 5, 4.5, 4, 3.5, 3, and 2.5% sucrose in the buffer consisting of 20 mM sodium phosphate, 100 mM NaCl, 2 mM $MgCl_2$, pH 7.5. One milliliter of mineral oil (USP grade) was then added to prevent evaporation of water, which could cause rapid deterioration of the gradient. The sucrose gradient was allowed to run for at least 30 min prior to sample introduction. The accuracy of measured sizes in the range 0.05–3 μm was ensured by calibrating the instrument with a narrow 0.573-μm PVC calibration standard (Chemical Process Specialists) before injecting each sample. Two hundred and fifty-microliter samples were loaded using disposable 1-ml syringes and 20G1 needles (Becton Dickinson & Co, Rutherford, NJ, USA). Typical analysis time per sample was 30 min and the analyses were done at room temperature (22–25°C). The sucrose gradient prepared above could be used for 15–20 samples until the density gradient lost its integrity as determined by comparing the measured peak width of the calibration standard to its known peak width. Relative size distribution patterns were obtained using the Disc Centrifuge Control System software with the operational parameters: particle density = 1.33 g ml^{-1}; refractive index = 1.45; liquid viscosity = 1 centipoise; liquid refractive index = 1.34; liquid density = 1.015 g ml^{-1}.

Other assays and calculations

The number of total virus particles for a sample was calculated as described before [24]. The conversion factor of 1.1×10^{12} particles per A_{260} in the presence of 0.1% SDS [16] was used to calculate particle number. Measurements of A_{260} in the presence or absence of SDS and the A_{320}/A_{260} ratio of rAd5 samples were carried out using an HP 8452A diode array spectrophotometer controlled by a Compaq Deskpro XL 466 computer system. All data were appropriately blank-subtracted and reported as the average of three determinations. For an unaggregated, bioactive rAd5 sample, the A_{320}/A_{260} ratio is typically in the range 0.23–0.27.

Modeling of the aggregation pattern for a deformable or rigid sphere was carried out using the equations relating the predicted hydrodynamic diameter of an aggregate with N particles ($D_{app,N}$) with the apparent hydrodynamic diameter of the monomeric sphere (D_0). For a completely deformable or liquid sphere, the equations are: $D_{app,1} = D_0$; $D_{app,2} = 1.26\ D_0$; $D_{app,3} = 1.44\ D_0$; $D_{app,4} = 1.59\ D_0$. For a completely rigid sphere, the equations are: $D_{app,1} = D_0$; $D_{app,2} = 1.20\ D_0$; $D_{app,3} = 1.35\ D_0$; $D_{app,4} = 1.48\ D_0$ [30].

Results and discussion

Human gene therapy using adenovirus vectors is typically designed such that targeted expression of a missing or nonfunctional protein results in amelioration of a certain disease state. The mature infectious recombinant virion enters a target cell according to specific mechanisms [33]. This leads eventually to the expression of a protein product whose presence results in the desired therapeutic response. Unfortunately, under certain circumstances, the virus would irreversibly aggregate leading to precipitation of material and complete loss of biological activity. We initially developed the use of the A_{320}/A_{260} ratio as a measure of adenovirus aggregation, but it was used only qualitatively for noting relative aggregation among various samples. Prior to the formation of large aggregates, however, the accumulation of virus dimers, trimers, and other lower-order oligomers would provide an early indication of virus

instability; since adenoviruses are particles of 0.065–0.080 μm in diameter [3,11,21,23,31], employing a particle-sizing technique applicable to the submicron region would aid in identifying those circumstances that would predictably lead to aggregation.

Given the availability of particle-sizing techniques in the submicron regime but the dearth of applications for viruses, it was a challenge to come up with a sensitive and reproducible assay for adenovirus analysis. The major problem in the development of disc centrifuge sedimentation analysis of adenovirus was to achieve the sensitivity suitable for analyzing typical concentrations of average viral preparations. Sample concentration was not a serious issue in analyzing non-virus materials using the Brookhaven Disk Centrifuge Photosedimentometer [30] or the Joyce-Loebl disk centrifuge [28] because it was easy to obtain the desired concentrations of 0.05–1% (w/v). In contrast, virus samples had lower concentrations, normally 0.01–0.03% (w/v), and this concentration restriction discouraged us in pursuing this technique. To develop a working disc centrifuge method, sensitivity and signal to noise ratio had to be improved by a factor of 10. We used several well-characterized, narrow polymer latex samples as models for the virus during methods development. These latexes were diluted to very low concentrations ($<$0.001% w/v) to simulate the expected optical density range of virus samples. Improvements were made in amplifier gain and shielding and analog signal cable shielding; these gave more stable and reproducible analog digital signals. Improvements were also made in software-based digital noise filtration, to further improve the signal-to-noise ratio. When the customized analyzer was used for virus samples, the result was an instrument that could meet the requirements for the analysis of recombinant virus.

Figure 1 shows that this technique is capable of detecting recombinant adenovirus in the 0.03–3.0 μm size region. The distributions of particle sizes are reported as relative weight percentages where the highest peak is assigned 100%. Figures 1a–c all manifest a single main peak at 0.062–0.064 μm which would correspond to the apparent hydrodynamic diameter of recombinant adenovirus. The adenovirion consists of a protein capsid that displays a regular icosahedral symmetry with fibers sticking out at its 12 vertices. Since most of the mass of the virus is concentrated on what is contained in the capsid, the 'diameter' of a virus particle in a five-fold symmetry orientation was determined to be 0.073 μm based on electron microscopic measurements of wild-type adenovirus 5 [21]. The slightly smaller particle size obtained for the recombinant virus using disc centrifugation analysis compared to that measured by electron microscopy of the wild-type adenovirus could be due to several considerations: (1) the probable variation of virus particle density value that is inputted to calculate the size distribution; (2) the recombinant adenovirus samples being devoid of protein IX, a major surface structure-stabilizing protein present in the wild-type version of the virus; and (3) the probable difference in the flow properties between a sphere and an icosahedron and noting that the size calculations are based on particles being assumed as spheres. Disc centrifugation analysis could be used with other viruses with diameters in the submicron range including DNA viruses like poxvirus (0.1–0.3 μm), iridovirus (0.18–0.20 μm), herpesvirus (0.14–0.16 μm), and papovavirus (0.05–0.06 μm) and the RNA viruses such as paramyxovirus (0.1–0.3 μm), orthomyxovirus (0.08–0.12 μm), retrovirus (0.1–0.2 μm), and rotavirus (0.07–0.08 μm).

Although the majority of the virus population appeared to be a single particle, Figures 1a and 1b also show several minor peaks with apparent particle hydrodynamic diameters greater than the main adenovirus peak. Both samples have the 0.073–0.076 μm peak as well as the 0.084-μm peak; sample (b) has an additional recognizable 0.094-μm peak. Partial aggregation of narrow unimodal particles produces a size distribution which consists of a series of relatively narrow peaks. The ratios between the apparent hydrodynamic diameters of these peaks and the original particle size depends upon the extent to which the particles can deform and coalesce following aggregation. One extreme is when the individual particles are completely deformable, like liquid droplets; the hydrodynamic diameter of an aggregate of N completely deformable particles is equal to $N^{1/3}D_o$, where D_o is the diameter of the original particle. The expected ratios of sizes for aggregates of 2, 3, and 4 particles are 1.26, 1.44, and 1.59 times the original particle diameter. The other extreme is when the individual particles are completely rigid and cannot deform following aggregation. There is no theoretical method for calculating the hydrodynamic diameters of aggregates of rigid particles. However, partially aggregated rigid spheres, such as PVC and polystyrene latexes, show a consistent pattern of hydrodynamic diameters for aggregates. For clusters of N individual rigid particles, the apparent hydrodynamic diameter (D_{app}) is given by the empirical model: $D_{app} = (0.518 + 0.418\ N^{1/2})D_0$, which gives 1.20 D_0, 1.35 D_0, and 1.48 D_0, for aggregates of 2, 3, and 4 particles, respectively. This empirical model gives r^2 values of $>$0.999 (least-squares fit) with rigid polymer particles and N of 6 or less. Using 0.064 μm as the apparent hydrodynamic diameter for a single particle, theoretical calculations for particle hydrodynamic diameters corresponding to given oligomers were carried out for two spherical models, ie the deformable sphere and the rigid sphere (Table 1). The measured peak sizes of adenovirus particle aggregates are in close agreement with the predicted sizes from the empirical model for rigid spheres. Therefore, the three distinguished peaks of 0.073–0.076 μm, 0.084 μm, and 0.094 μm correspond to the adenovirus dimer, trimer, and tetramer, respectively.

Figures 1a–c also indicate that with increasing light scattering based on the A_{320}/A_{260} ratio measured prior to disc centrifuge analysis, there was a considerable increase in the area and multiplicity of the peaks greater than 0.070 μm. Sample (c) which had more aggregates showed poor resolution of the dimer, trimer, and tetramer peaks (Figure 1c). The A_{320}/A_{260} ratio is an arbitrary but reliable measure of scattering and is very sensitive to aggregation. Furthermore, the disc centrifuge analysis apparently elaborates more on the size and distribution of oligomers and viral aggregates responsible for the increase in scattering. It is therefore a useful tool for monitoring the kinetics of particle aggregation. The ability to monitor the formation of virus aggregation would certainly aid in mapping out the aggregation pathway for adenovirus and thereby predict the immediate

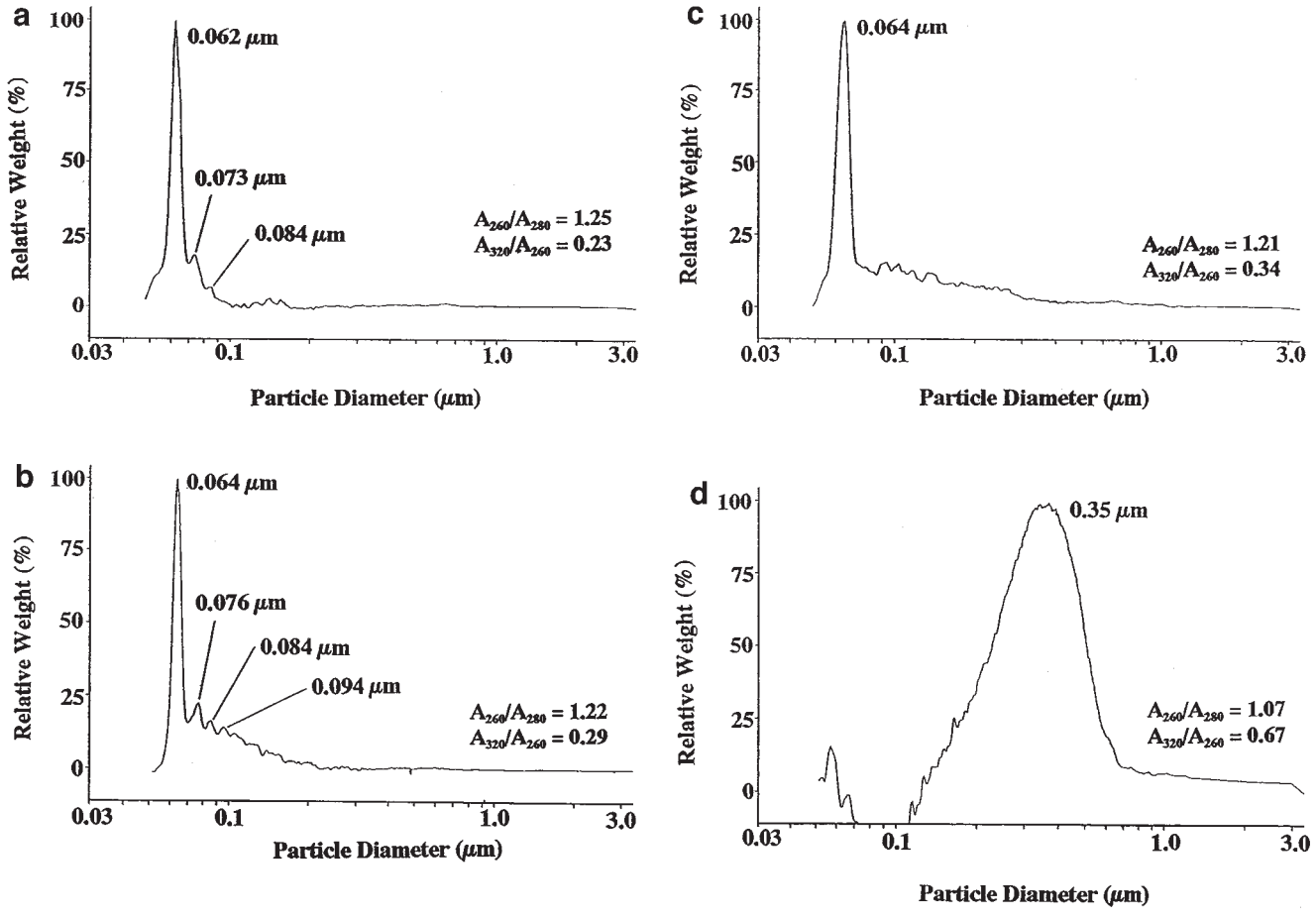

Figure 1 Particle size distribution for four recombinant adenovirus samples (a–d) with various scattering ratios. Representative samples were arbitrarily chosen to reflect differences in A_{260}/A_{280} and A_{320}/A_{260} ratios; differences were generated through prolonged storage at 4°C. Two hundred and fifty microliters of each sample were subjected to disc centrifugation analysis; the actual amount of virus loaded was similar for all samples (concentration of *ca* 0.3–1 × 10^{12} particles per ml or 0.01–0.03% (w/v)) based on particle number derived from absorbance information. Triplicate injections of the same sample yielded almost identical results. The indicated absorbance ratios were determined in the absence of SDS and were measured prior to subjecting the sample to disc centrifuge analysis. The distributions are represented as plots of relative weight % (100% corresponding to the major peak) *vs* apparent particle hydrodynamic diameter in μm as automatically calculated by the software.

Table 1 Modeling of adenovirus oligomerization

n-mer	Calculated $D_{app,N}$ (μm)[a]		Adenovirus apparent hydrodynamic diameter (μm)[b]
	Deformable sphere	Rigid sphere	
Monomer	0.064	0.064	0.064
Dimer	0.081	0.077	0.076
Trimer	0.092	0.086	0.084
Tetramer	0.102	0.095	0.094

[a]$D_{app,N}$ = predicted hydrodynamic diameter of an aggregate with *N* particles; the apparent hydrodynamic diameter of a single particle (0.064 μm) was derived from Figure 1b. See Materials and Methods for calculations.
[b]Data derived from Figure 1b.

fate of a particular preparation given the size distribution data.

Figure 1d shows the profile of a highly aggregated virus sample, which has a high light scattering ratio of 0.67. There is essentially complete disappearance of the adenovirus peak and the appearance of a broad peak centered at about 0.35 μm. The absence of monomeric virus particles in samples with high light scattering is confirmed by anion-exchange HPLC analysis which does not detect aggregated virus (data not shown). Also, it is possible that there are even aggregates larger than 3 μm that quickly sediment and are thus not picked up by the predefined measurement size range of 0.05–3 μm. For this or any size aggregate, the number of particles (assumed to be rigid spheres) can be estimated by rearranging the empirical equation for apparent hydrodynamic diameter to solve for *N* in terms of D_{app} and D_0: $N = \{2.08(D_{app}/D_0) - 1.08\}^2$ where *N* is the estimated number of particles in the aggregate. Thus, for the observed aggregate where $D_{app} = 0.35$ μm and $D_0 = 0.064$ μm, the number of particles in the aggregate is about 106.

It should be noted that determination of the particle size distribution is of paramount importance rather than mere determination of average particle size, for the relative amounts of the lower-order oligomers would determine the direction and extent of the aggregation pathway. Thus, the techniques of turbidimetry, dynamic light scattering, and static light scattering would not be effective in generating

size distributions. On the other hand, electron microscopic analysis is a labor-intensive and time-consuming technique which could introduce some artifacts because of the nature of the sample preparation methods; furthermore, the measurement of a large number of particles which accurately represent the whole sample is of great concern and is not always achieved. The light diffraction and electrical zone methods are, unfortunately, limited by their detection capabilities in the submicron range [13]. For gel permeation chromatography [27], the useful working range for separating globular molecules based on size is only up to an MW of 20 M Daltons compared to the MW of adenovirus which is 180 M [17]. Asymmetrical flow field-flow fractionation has been reported to separate and quantitate monoclonal antibody aggregates [15] and proteins, nucleic acids, and viruses such as the 1.8 M-Da satellite tobacco necrosis virus and the 50 M-Da Semliki forest virus [14]. Although the technique has yet to be employed for adenovirus, the size range of interest falls at the juncture of the two modes of operation of the technique (ie normal *vs* hyperlayer mode) which could complicate the interpretation of the results [8]. Finally, ultracentrifugation [13] and capillary hydrodynamic fractionation [6] could be applicable to size analysis in the submicron range but they have yet to be tried for adenovirus samples.

The disc centrifugation technique is a sensitive and convenient assay for adenovirus analysis. The volume requirement per sample is small, *ca* 100–300 μl. Although analysis time per sample is *ca* 30 min with the disc running at 10 000 rpm, preliminary studies with disc speeds of 18 000 rpm reduced the measurement time to *ca* 10 min without compromising sensitivity and accuracy (data not shown). Unlike the operation of the Brookhaven and Joyce-Loebl disk centrifuges, the CPS system requires the injection of a standard prior to an actual sample to confirm the integrity of the density gradient and to adjust size calculations based on the sedimentation time of the standard. In analyzing samples, the line start method of operation was used instead of the homogeneous start method because measurement in the former method is a differential one which is inherently of higher resolution than the latter method which uses cumulative measurement. Moreover, efforts will be made to incorporate features such as automated analysis and reporting absolute amounts into the system. The reporting of absolute amounts is critical to figure out the mass balances in processing biological samples.

In conclusion, we have shown for the first time that disc centrifuge sedimentation is applicable to the study of recombinant adenovirus samples. Using the CPS system and the line start method of operation, the assay provides a fast particle size distribution analysis of adenovirus samples, using minimal sample volumes, and with concentrations in the 0.01–0.03% (w/v) concentration range. The assay could detect adenovirus dimers, trimers, and tetramers as well as a large aggregate of 0.35 μm. Also, the particle distribution data seem to correlate well with the A_{320}/A_{260} scattering ratio and HPLC analysis. Although confirmation of the observed adenovirus oligomers by other physicochemical techniques and determination of the bioactivity of these oligomers have yet to be done, the technique is useful for studying the kinetics of aggregation of adenovirus and other DNA and RNA viruses in the submicron region.

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