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Validation of a Quantitative Method for Detection of Adenovirus Aggregation

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Adenoviral vectors have been widely used in gene therapy clinical trials and subjected to rigorous testing to ensure safety and efficacy. Like therapeutic proteins, aggregation of adenoviral vectors needs to be quantified for process consistency and stability monitoring. The sucrose gradient sedimentation method of adenovirus particles using disc centrifugation, which is a modification of a method described by Bondoc and Fitzpatrick, was used.[1] It proved to be quantitative and reproducible in evaluating a variety of samples including deliberately cross-linked adenovirus particles and process development lots of various ages.

This aggregation assay revealed that most aggregates detected in the production lots were dimers, trimers, and tetramers; and the number of these small oligomers was easily reduced with the addition of 300 mM salt, thus demonstrating the reversible nature of a portion of the aggregate population. This method was validated to demonstrate that it was appropriate for final product lot release and stability monitoring.

Four adenovirus production lots with similar biological titers, all in storage at below −60°C, were selected for monitoring. The assay further demonstrated that although each of the four production lots had different levels of aggregation, the aggregation was stable throughout the 18 months they were monitored. Most importantly, data from all adenovirus lots stored at below −60°C and tested by this method revealed that there was no correlation between aggregation levels and the biological activity.

Introduction

Adenoviral vectors have been evaluated in hundreds of clinical trials over the past decade for their efficacy in treating cancer and other diseases.[2] Since most adenoviral vectors used for gene therapy trials are administrated parenterally, adding the process of monitoring adenoviral aggregation to the list of tests is necessary to ensure safety, efficacy, and lot-to-lot consistency.[3-5] Several methods are commonly used to monitor aggregation of therapeutic proteins, such as size-exclusion high pressure liquid chromatography (HPLC), dynamic light scattering, and analytical ultracentrifugation.[6] These methods have provided a good starting point in our search for a test method that could measure adenovirus aggregation.

After a systematic evaluation of available methods, we selected the disc centrifuge "differential sedimentation" method[1] for further characterization. With this technique, adenovirus travels through a sucrose gradient solution located inside a spinning disc specifically designed to allow detection of adenovirus particles by light scattering at a fixed location. The equipment software then calculates the size of the adenovirus particles and aggregates from the theoretical density of a particle, the sedimentation speed, and the mass of these populations by the amount of light scatter. This method appears to provide the aggregation detail, reproducibility, and ease of handling necessary for product monitoring in a quality control laboratory (QCL).

The initial characterization of adenovirus aggregation by disc centrifugation was performed with adenovirus samples subjected to thermal stress or cross-linked with human anti-adenovirus antiserum to produce aggregates. Once the method for detection of aggregates was established, all available process development lots, and GMP lots under normal storage

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conditions (< −60°C), were used to further study the nature of aggregates found in production lots for size, stability, and reversability. In addition, this assay was validated for accuracy and precision. To understand the link between aggregation levels and the quality attributes of adenoviral vectors, all samples were tested for their physical and infectious titers. Here we report our methods, aggregation analysis, assay validation, and the GMP stability monitoring results utilizing disc centrifugation.

Materials and Methods

Adenoviruses

Human serotype 5 oncolytic adenoviruses CG7870, CG8840, and CG0070 were manufactured by Cell Genesys as described previously.[7-9] Selected preparations were further purified by CsCl gradient[7] and the number of particles determined by the anion exchange HPLC method[10] using the RESOURCE™ Q column (GE Healthcare, Piscataway, New Jersey USA). However, the following modification was added to the process: after each injection of adenovirus sample, the column was cleaned with five column volumes of 0.5 N NaOH prior to reconditioning for use with the next sample. The final concentration of these adenovirus preparations was $1–1.5 \times 10^{12}$ viral particles (vp)/mL. All adenovirus lots were stored in a formulation buffer (5% sucrose, 0.05% Tween 80, 10 mM Tris, 1 mM $MgCl_2$, 1% glycine; pH 7.8) at −60°C, except for the thermally-stressed samples. Those samples were generated by storing adenovirus at either 23°C or 30°C, and their infectious titer (plaque forming units [pfu]) were determined as described by Small *et al.*[11]

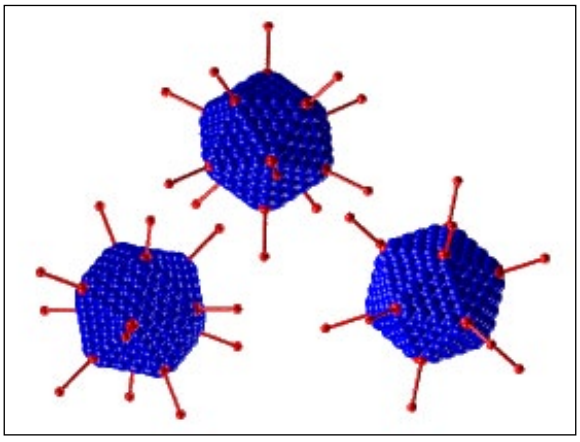
Disc Centrifugation

Disc centrifugation analysis of adenovirus was performed according to Bondoc and Fitzpatrick[1] using a CPS Instruments (Newtown, Pennsylvania USA) model DC24000 with several modifications. Briefly, 50–100 µL of each adenovirus preparation was injected into a spinning disc filled with a continuous 8–24% sucrose gradient (total volume of 16 mL) in 10 mM Tris buffer with 1 mM $MgCl_2$, 1% glycine; pH 7.8, while being centrifuged at 22,000 rpm. The sample parameters (g/mL) used were: 1.24 for particle density, 1.52 for particle refractive index, 0.0 for particle absorption, and 1.0 for non-sphericity factor. The sucrose gradient parameters were: 1.059 g/mL for fluid density, 1.365 g/mL for fluid reflective index, and 1.2 centipoise (cps) for fluid viscosity, based on the sucrose density at the detection window. The data collection range was set for particles from 50–2000 nm. Each analysis typically took 18 minutes to complete. Prior to each injection of adenovirus, the disc centrifuge was calibrated with a 460 nm latex bead standard provided by the manufacturer. For every test result both the light absorption and weight data were evaluated, but only the weight data were used to calculate the aggregate percentage.

The monomeric (single) adenovirus particles usually form a peak centered at 77 nm. Aggregates are defined as adenovirus particles from 86–2000 nm. The weight data was used to calculate the percentage of aggregates by normalizing the mass of the aggregate population against the total detected mass of monomers and aggregates. Every adenovirus sample was analyzed in duplicate with the aggregate percentage data averaged. The variation between duplicate results in reported aggregate percentage values was required to be < 15%, otherwise a third analysis was conducted and used in the calculation of the final results. To study the effect of salt treatment, 50 µL of each adenovirus sample was mixed in an equal volume of either the Tris formulation buffer (for untreated samples) or the same buffer with up to 600 mM of NaCl (for salt-treated samples) added immediately prior to being analyzed.

Adenovirus Treatment With Human Sera

Normal human sera were procured from Stanford Blood Bank (Palo Alto, California USA) and screened for their ability to inhibit adenovirus infectivity in a plaque assay with a 30-minute pre-incubation of the adenovirus sample. The serum lot that demonstrated the highest level of inhibition was used in further studies. Adenovirus was mixed with human serum at different volume ratios and incubated for 15 minutes at 37°C prior to testing.



Adenovirus Structure (courtesy of Wikipedia Commons, http://commons.wikimedia.org)

Analytical Ultracentrifugation for Adenovirus

This analysis was performed by Dr. John Philo at Alliance Protein Laboratories (Camarillo, California USA). The method and data analysis was as described[12] with the following variations: 1) each lot of adenovirus was diluted to 4×10^{11} vp/mL with the formulation buffer; 2) the rotor speed was 3200 rpm when the samples were scanned at 280 nm; and 3) scans were performed every 4.5 minutes for a total of 80 scans per sample.

Results

Evaluation of Disc Centrifuge Method for Detection of Adenovirus Aggregates and Empty Particles

To achieve good resolution between adenovirus monomers, dimers, trimers, and larger aggregates, the disc centrifugation method[1] was optimized for use with our adenovirus production lots. These modifications included the use of a sucrose gradient suitable for the formulation buffer used in the adenovirus GMP lots, and a higher centrifugation speed. A typical result is shown in Figure 1, with the monomer peak detected at 77 nm. This peak is usually the largest in most results, thus the figure is plotted using the height of this peak as the full scale. Six replicate tests of the same sample demonstrated the reproducibility of the method with the exception of the subparticle area (adenovirus particles smaller than monomers) where the overlapping curves had greater variability (Figure 1).

The adenovirus process development lots tested had different levels of aggregates, mostly as dimers and trimers, as well as about 3–8% subparticles (Figure 1). One exception was the lot that was further purified by CsCl gradient centrifugation which had almost no detectable subparticle population and fewer aggregates (data not shown). All light scattering-based methods have better accuracy and precision for larger particles since smaller particles reflect significantly less light, thus resulting in less precise quantitation of the particle mass. This means that the quantification of subparticles (50–70 nm) in this disc centrifugation method will be more variable than the quantification of larger particles (>70 nm). The goal was to accurately and precisely measure the amount of aggregation, so it was decided to exclude the subparticle population and calculate the aggregate percentage by normalizing the mass of the aggregates (particles from 86–2000 nm) against the combined mass values of monomers and aggregates (70–2000 nm).

To further evaluate the ability of the disc centrifugation method to quantify aggregation levels, adenovirus samples with induced virus aggregation were used. Samples were treated with human anti-adenovirus serum to cross-link adenovirus, thus forming aggregates. All samples were analyzed for infectivity and particle numbers by plaque assay and anion exchange HPLC analysis respectively. In the samples in which adenovirus aggregation was induced by different concentrations of human antiserum, there was a steady decline in biological and physical titers with each increase in antiserum concentration (Figure 2A). These samples also had a steady increase in aggregates with each increase in serum concentration (Figure 2B).

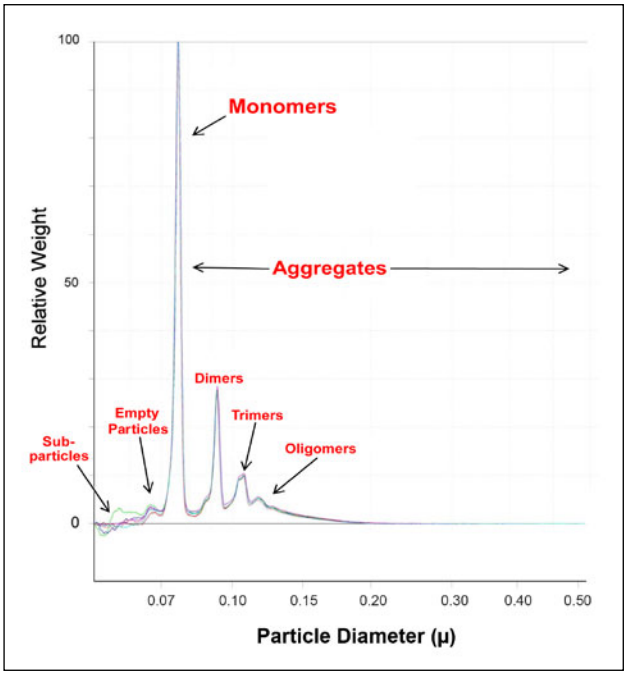


FIGURE 1. Representative disc centrifugation weight curve. The curves represent a single sample assayed six times.

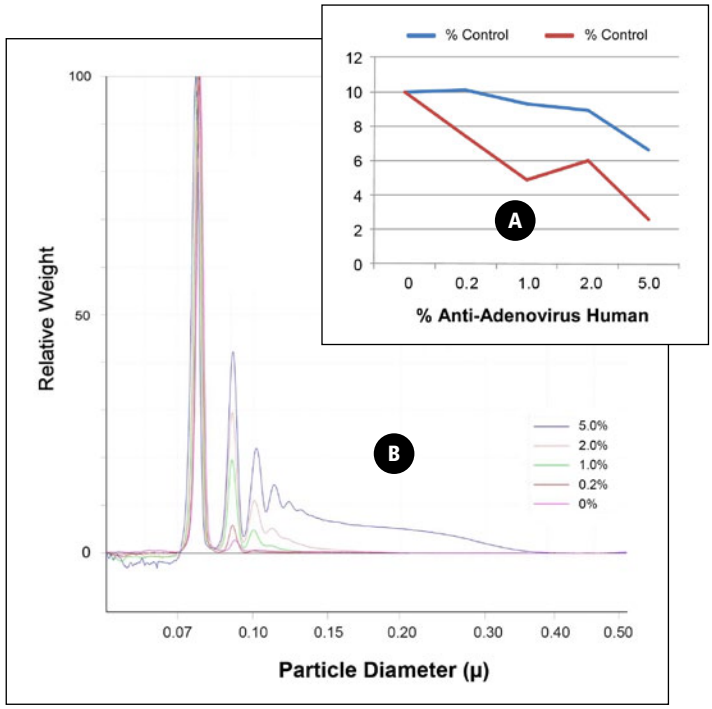


FIGURE 2. Disc centrifugation of antibody treated samples. A: virus particle and plaque titers of anti-adenovirus serum treated samples as percent of day 0 control; B: disc centrifugation results as percent monomers and aggregates for these treated samples.

Adenovirus samples were also thermally stressed by storage at 23°C or 30°C for 24 and 12 days respectively to produce worst-case scenario samples for evaluating the stability-indicating capability of this method (*i.e.*, its ability to recognize severely damaged samples stored under extreme conditions). Since thermal stress at such high temperatures may also physically damage the adenovirus particles along with the visually discernible precipitates accumulated during increased storage time, the evaluation of such samples was considered qualitative. In both sets of samples, rapid declines in viral particle and biological titers were observed within the first several days. When all samples were examined for aggregates by disc centrifugation, every one of them displayed disorganized oligomer peaks that were larger, higher, and wider than seen in unstressed samples (data not shown).

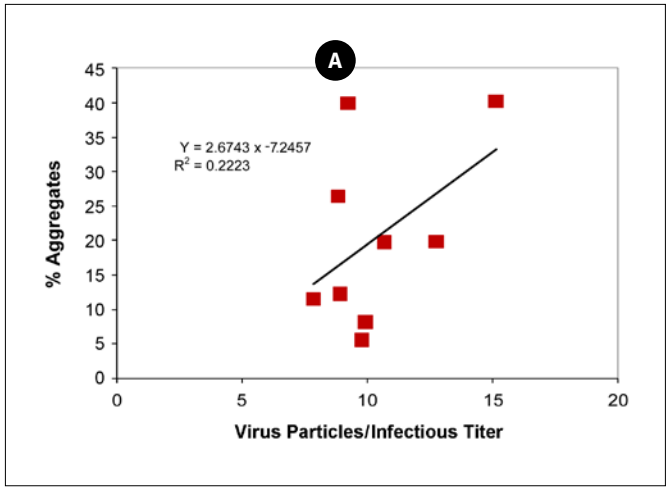
Survey of Adenovirus Aggregates in Frozen Storage

Since our GMP adenovirus lots were stored at <−60°C, it was necessary to survey adenovirus lots stored at this temperature to get an idea of typical aggregate ranges. Nine process development lots that had been stored at <−60°C from several months to three years were assayed by the disc centrifuge method to assess their aggregation levels. These lots had a wide range of aggregation, 8–43% in fact, but all maintained a viral particle-to-infectious titer ratio equal to or less than 15, implying no correlation between infectious titer and aggregation level (Figure 3A). It should also be noted that these samples were tested at various timepoints beyond their production date, and the detected aggregation levels had no relation to time in storage (data not shown).

With such a wide range of aggregation, it was necessary to confirm the disc centrifuge results by another method. Just as analytical ultracentrifugation (AUC) is an orthogonal method to confirm the validity of size-exclusion chromatography (SEC) results for the determination of protein aggregation, it was used here to confirm the disc centrifuge results. The same nine adenovirus lots were analyzed by AUC, and although the AUC aggregate percentage results were higher for all samples, the correlation between the aggregation levels measured by both methods was strong with a correlation coefficient (R) of 0.96, or coefficient of determination (R^2) of 0.927 from the linear regression analysis (Figure 3B).

Impact of Salt Concentration on Detected Adenovirus Aggregation

We evaluated whether the aggregation detected in these process development lots of adenovirus samples was reversible. The hypothesis was that if any aggregates are charge-related and reversible, a high ionic strength condition may be able to dissociate these aggregates. Samples were mixed with an equal volume of the formulation buffer containing salt and tested immediately. Final salt concentrations ranged from 0–1 M NaCl. At 75 mM,



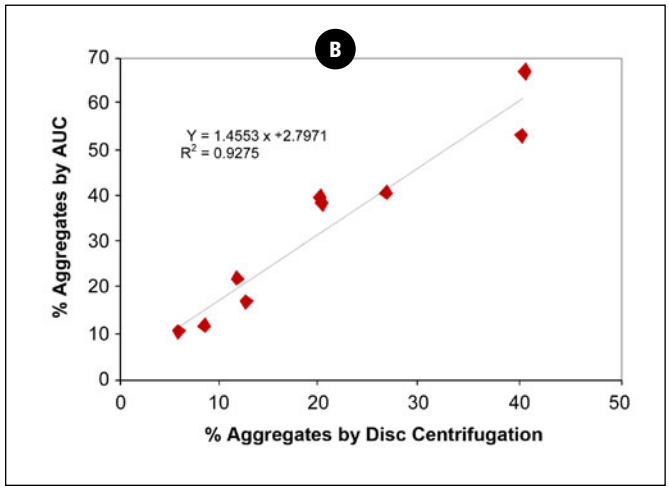


FIGURE 3. Comparison of disc centrifugation with other methods for analyzing process development lots. A: correlation between the aggregation results by disc centrifugation and the ratio between viral particle titers and the infectious titers for nine process development lots; B: correlation between the disc centrifugation and analytical ultracentrifugation in detection of aggregates in nine process development lots.

the percent of aggregates detected decreased modestly. Aggregation continued to decline with increased salt and finally stabilized between 150 and 300 mM (data not shown). Figure 4 shows the overlapped results of an untreated sample with one treated with 300 mM salt. The reduced aggregation in the salt-treated sample included all aggregate species (Figure 4). The effect of salt treatment was rapid, and the decrease was evident when the sample was injected into the disc centrifuge immediately after mixing with the salted buffer. This phenomenon was confirmed by AUC — the sample mixed immediately prior to the AUC analysis with an equal volume of the 600 mM salt solution gave an aggregate percentage of 23.8% versus 71.1% aggregates for the untreated sample analyzed in the same assay.

Validation of Assay Performance for the Disc Centrifugation Method

In order for a QCL to use the disc centrifugation method for quantitative measurement of adenovirus aggregates in GMP lots, the assay's key performance characteristics had to be established. The assay needed to be validated for accuracy, specificity, and precision to prove its suitability for monitoring lot-to-lot consistency and stability of aggregates.

Assay Accuracy

The assay's accuracy was proven by a battery of orthogonal methods. First was the strong correlation between the disc centrifuge and the AUC results (Figure 3B). Second, to ensure that the weight signal the software generated from the light scattering data was proportional to the amount of adenovirus injected, a study was performed using increasing viral particle titers in each injection. The calculated weight signal was linearly proportional (with $R^2 = 0.996$) to the injected viral particle titers in the tested range which was from 1×10^{11} to 5×10^{12} vp/mL (Figure 5). Third, the assay's accuracy was further demonstrated by comparing observed values with expected values. Because the aggregation percentage is a normalized value and constrained to a total of 100%, a meaningful comparison should be based on the original weight data of each population. Samples from two adenovirus lots

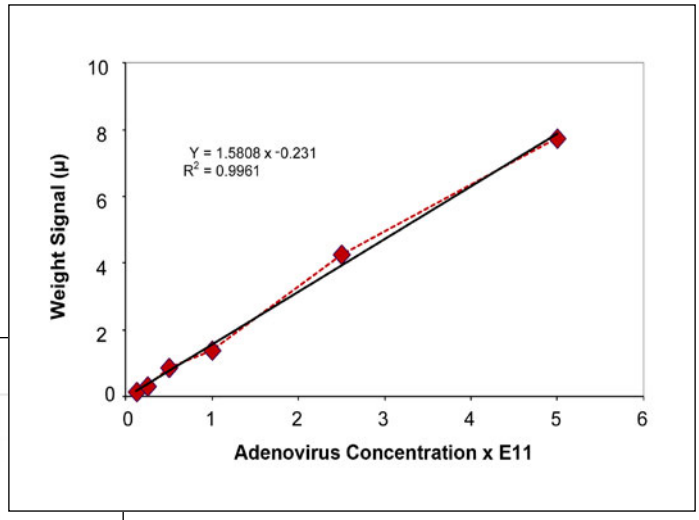


FIGURE 5. Linearity between injected amounts of adenovirus particles and the detected weight signal in the disc centrifugation method.

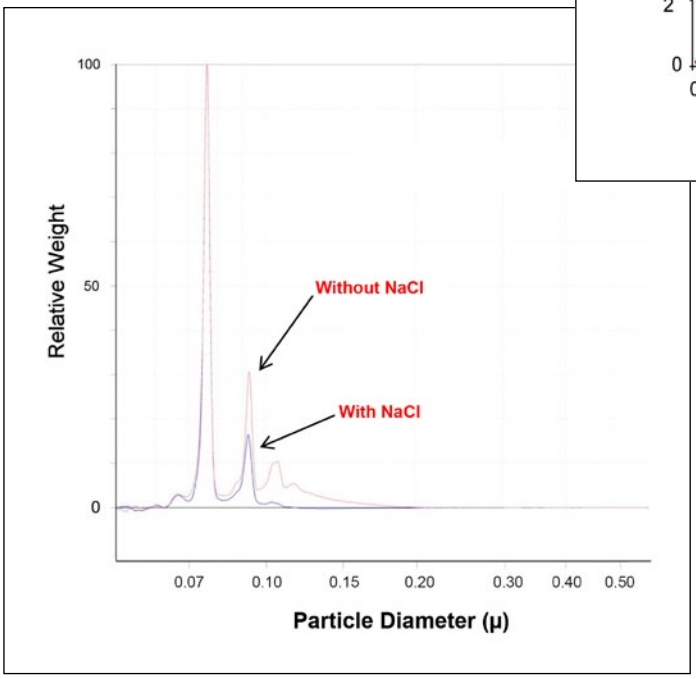


FIGURE 4. Impact of 300 mM NaCl on aggregate levels.

with distinctly different amounts of aggregates by disc centrifugation (averaging 0.72 [14%] and 3.63 [44%] by weight of their respective aggregate amounts in each injection) were mixed at ratios of 20:80, 40:60, 60:40 and 80:20. The expected aggregate weight of each mixed sample was mathematically calculated based on their mix ratio. All mixed samples (N = 24 over three independent assays) showed a recovery of 95.1 ± 8.3% of expected values, further supporting the accuracy of this assay (Table 1).

Assay Specificity

Assay specificity was demonstrated by a variety of methods. First, a sample was assayed by disc centrifugation before and after CsCl gradient centrifugation to further purify the monomeric viral particles. The assay detected a reduction in aggregates post-centrifugation. Second, the disc centrifugation of samples treated with human anti-adenovirus antibodies (described above) demonstrated an increase in aggregates with each increase in serum concentration, as expected. And finally, the mixing study for assay accuracy also implied the assay specificity by the fact that the signal was proportional to the amount of the target analyte, which in this case were aggregates.

Assay Precision

The assay precision was measured in terms of repeatability and intermediate precision. Repeatability was demonstrated by testing six replicates of a sample in one assay—the results showed a relative standard deviation (RSD), calculated as standard deviation over the mean, of 1.4%. One of these assays is demonstrated in Figure 1. The intermediate precision for the same sample tested over six independent assays, with duplicate injections per assay, had a RSD of 11.2% (or 13.59 ± 1.52% aggregates) for one lot and 5.0% RSD (or 43.57 ± 2.2% aggregates) for a second lot (Table 2).

TABLE 2. Intermediate precision of aggregation measurement. Two different samples, A and B, were assayed in duplicate in six independent assays.

Sample	Assays Performed	Detected Aggregation	RSD
A	12	13.59% ± 1.52%	11.2%
B	12	43.57% ± 2.20%	5.0%

TABLE 1. Accuracy of aggregate measurement. The expected weight values for aggregates of the mixed samples were calculated mathematically from the original materials in each assay. The recovery was calculated by comparing the observed results against expected results.

Day	Mix Ratio	Expected	Observed	Recovery (%)
1	0	0.74	0.73	—
			0.75	—
	20	1.27	1.36	107.10
			1.15	90.55
	40	1.82	1.75	96.15
			1.72	94.51
	60	2.41	2.41	100.00
			2.29	95.02
	80	3.02	2.79	92.38
			2.93	97.02
	100	3.67	3.67	—
			3.67	—
2	0	0.71	0.74	—
			0.67	—
	20	1.2	1.21	100.80
			1.30	108.30
	40	1.73	1.60	92.49
			1.80	104.10
	60	2.28	2.17	95.18
			2.31	101.30
	80	2.86	2.73	95.45
			2.68	93.71
	100	3.48	3.47	—
			3.49	—
3	0	0.72	0.73	—
			0.70	—
	20	1.24	1.00	80.65
			0.90	72.58
	40	1.79	1.48	82.68
			1.99	106.10
	60	2.37	2.19	88.61
			2.32	97.89
	80	2.98	2.73	91.61
			2.94	98.66
	100	3.63	3.80	—
			3.45	—

The accuracy, specificity, and precision of this disc centrifugation method indicated that it is appropriate for monitoring lot-to-lot consistency and stability of aggregates in GMP production lots.

Stability Study of Four GMP Lots

Four GMP lots were monitored for their aggregation levels (total and irreversible) by the disc centrifugation approach. Because the method was validated and ready several months after these lots were manufactured, the stability monitoring started at the six-month timepoint. Figure 6 shows the stability (in total aggregates) from six to 24 months. Each lot started with different amounts of aggregation and maintained that level throughout the stability study. These aggregates were mostly dimer, trimer, and tetramers, and the sum of these three oligomers usually accounted for 99% of the detected aggregates (data not shown). Not only did these lots have similar biological and particle titers immediately after manufacturing — but most importantly — there were no changes in titers during this stability study (data not shown).

Discussion

In the course of establishing this assay, a large number of methods for measuring aggregates were evaluated. The most widely used method for quantifying protein aggregation is SEC. Because adenovirus are large (1.5×10^6 Da),[13] SEC does not effectively distinguish adenovirus from its aggregates.[14-15] Dynamic or static light scattering is capable of instantaneously providing aggregation information for particles, including viruses and liposomes, in the format of relative size distribution. The disadvantages of using this method to measure adenovirus aggregation have been discussed elsewhere.[16] While the average hydrodynamic radius of the entire population is reported, the size and quantity of each aggregate population is less used[17] likely because the aggregated populations are heterogeneous over a wide range of sizes. The estimated overall size from light scattering techniques is indicative of the extent of aggregation. In a feasibility study, we found that our formulation gave a particle population around 30 nm with unknown identity (data not shown). The presence of

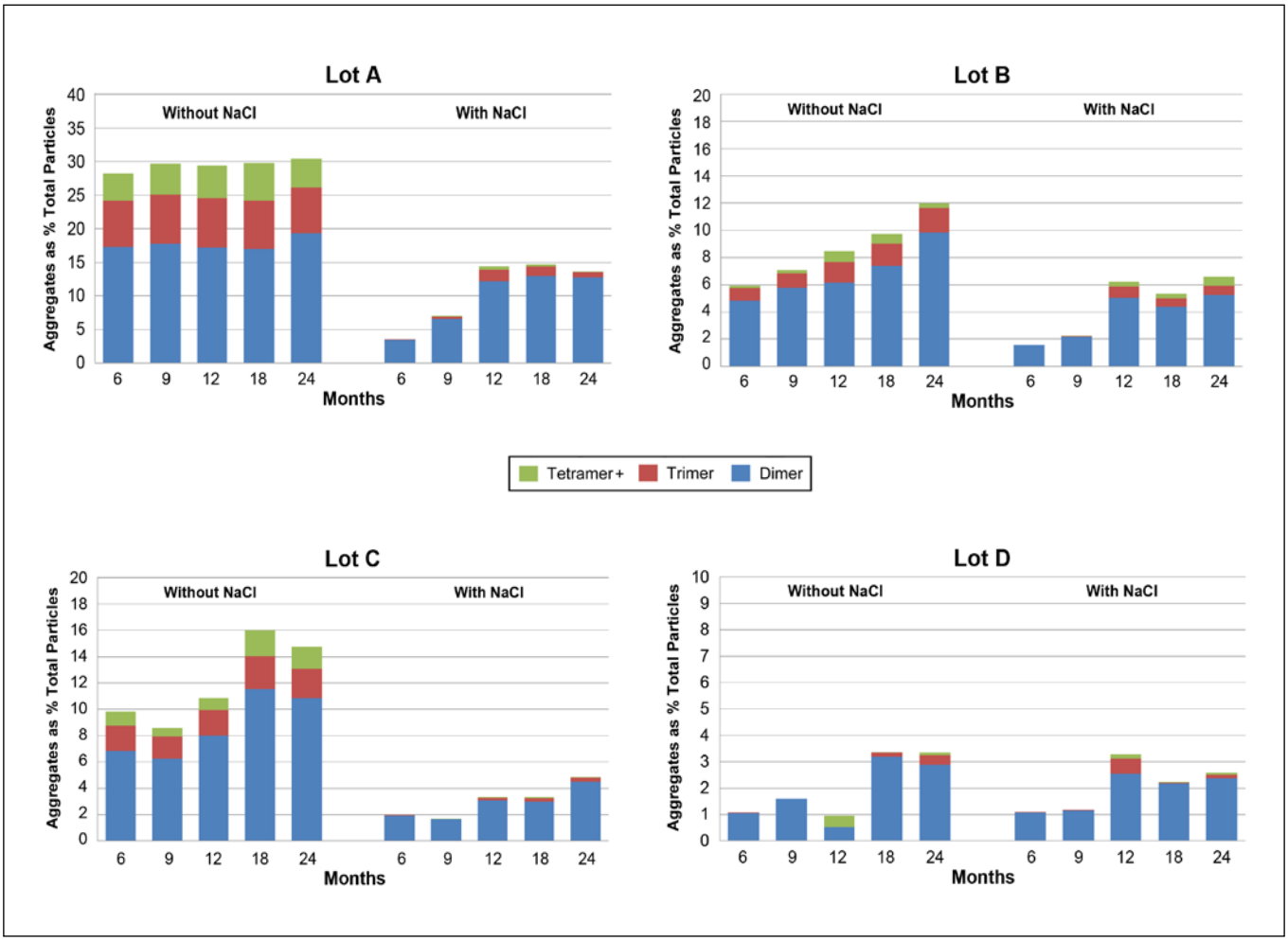


FIGURE 6.
Stability of aggregates in four adenovirus GMP lots with and without salt treatment.

this population would impact the accuracy in quantifying adenovirus aggregates, at least for the current formulation.

AUC is an orthogonal aggregation detection method to SEC because it measures the aggregates in their natural state (for review) while in solution.[6] This method provides both aggregate size and the percentage of the total for each aggregate population for adenovirus samples, and it doesn't usually require further manipulation of samples (*e.g.*, as extensive dilution) that could affect the aggregation levels detected.[12] However, AUC is usually not the method of choice for QCLs, even for therapeutic proteins, due to the complexity of instrument operation and data analysis as well as the concern about whether the method and the software can be validated for cGMP use.[6]

Capillary electrophoresis may eventually be useful to assess adenovirus aggregation,[18] but based on a preliminary evaluation, additional work will be required in order for the method to be suitable for our formulation (data not shown). In addition, published reports indicate that turbidity[1, 17, 19] and agarose gel electrophoresis[20] can detect either virus aggregation or virus assembly processes. Turbidity has been used to study protein stability and aggregation[21] most commonly as the optical absorbance at 340–360 nm using a spectrophotometer, and is also described as being useful for measuring adenovirus aggregation.[1, 21] We used a Hach (Düsseldorf, Germany) 2100AN portable laboratory turbidimeter that provided a fast but crude estimate of changes in aggregation status or any drastic change in adenovirus concentration. We found that it could prove useful in confirming visual evidence of significant aggregation when an immediate result was more important than getting quantitative information (*e.g.*, during in-process monitoring). Another use might be in formulation development for adenoviral vectors when a quick verification of increased aggregation in accelerated stability studies is needed. However, because we did not find a way to translate its results into quantitative extent of aggregation, it was not pursued further.

Native agarose gel electrophoresis has been used to study virus assembly, such as for bacteriophages.[20] This simple gel electrophoresis method was once intended for use in non-analytical laboratories for verifying adenovirus quality without involving HPLC or other complex instrumentation. We evaluated it and found the thermally-stressed and human antiserum-treated samples had abnormal mobility in native gels (0.5% agarose) when compared to untreated control samples, thus proving that this method could provide unique information about the status of adenovirus aggregation. Most importantly, the altered migration results in this native gel system correlated well with significant reduction in both viral particle and infectious titers (data not shown). Although the quantity of adenovirus aggregates and single, intact adenovirus particles may be quantitated by densitometer scanning and band intensity analysis, we did not pursue such an approach since the protocol would require a large number of steps and sample handling for a QCL.

We briefly evaluated the feasibility using field flow fractionation (FFF)[22] to quantify adenovirus aggregation. It was another simple and quantitative method but it reported significantly fewer aggregates than detected by AUC or disc centrifugation for reasons that remain to be explored (data not shown). Because of its significantly different results, we discontinued the evaluation.

Finally, Bondac and Fitzpatrick[1] described a disc centrifugation method to determine adenovirus aggregation utilizing a simple centrifuge instrument that has also been used in the pigment and semiconductor industries for particle size monitoring. Of the methods evaluated, this was the method that proved to be quantitative, consistent, and easy to perform. Thus, it was a suitable analytical method for the QC group to monitor aggregation in adenovirus products. This method is also able to detect particles smaller than monomeric adenovirus particles (subparticles) such as empty particles. Because it was calibrated to collect data for particles ranging from 50–2000 nm, only subparticles larger than 50 nm (or particles migrating through the sucrose gradient like 50 nm or larger) could be detected. The quantification of subparticles is more variable and sensitive to baseline drifts than the quantification of larger particles because they scatter much less light than larger particles. As a result, when used under the current conditions, the disc centrifugation method was able to detect subparticles but could not quantify them as reliably as it did with adenovirus monomers and aggregates. Since the changes in aggregation are more critical for lot-to-lot consistency and stability monitoring than those in the subparticle population, the data from 50–70 nm were collected but not used in the calculation of aggregate percentage. Thus, should there be the need somewhere down the line, the current assay would require further optimization to reliably quantify empty particles.

It was not clear why the AUC method detected higher amounts of aggregates than the disc centrifugation method. The passage through the 8–24% sucrose gradient in disc centrifugation might have dissolved certain aggregates, resulting in fewer aggregates being detected. However, the difference in the aggregate percentage detected by AUC and disc centrifuge method was consistent across the aggregation levels tested. Furthermore, both methods showed that salt treatment caused a significant reduction in aggregates, probably by dissolving reversible aggregates. Overall, these two methods demonstrated a good correlation in detecting adenovirus aggregation and supported the validity of disc centrifugation as a method for routine monitoring of adenoviral vector lots.

The reduction in detected amounts of adenovirus aggregates by treatment with high concentrations of salt implies that a portion of the adenovirus aggregation is reversible to single particles. Every lot had a different level of reduction in detected aggregation upon salt treatment—there was no fixed percentage or amount of reduction. The reversible nature of some aggregates might explain why lots with 40% aggregates still maintained good biological

activity (a good ratio of viral particles-to-infectious titer). The aggregates might revert back to single particles during assays for viral particles and infectious titer. However, this hypothesis requires further study.

Although adenovirus samples thermally-stressed or treated with anti-adenovirus sera exhibited a relationship between increased aggregation and loss of viral particle and infectious titers, the storage of these samples was not representative of conditions for clinical lots of adenovirus typically stored below −60°C. The aggregates in properly stored GMP lots of adenovirus were dimers and trimers. These results suggest that the smaller aggregates do not have a strong negative impact on product quality. In the nine development and four GMP lots we studied, there was no correlation between aggregation levels and biological quality attributes. However, this observation needs more supporting evidence, preferably from multiple laboratories with different manufacturing processes.

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