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Impact of Time Out of Intended Storage and Freeze-thaw Rates on the Stability of Adeno-associated Virus 8 and 9

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ABSTRACT

There are an increasing number of clinical studies evaluating different adeno-associated virus (AAV) serotypes as vectors for gene therapy. Long-term frozen storage can maximize the stability of AAV. Freeze-thaw (F/T) cycles and exposures to room temperature (RT) and refrigerated conditions occur during manufacturing, labeling, and clinical use. In this work we exposed AAV8 and AAV9 at low and high concentrations to five F/T cycles compounded with RT and refrigerated holds in a 'daisy chain' time out of intended storage (TOIS) stability study, which may be a best practice in early development. We also evaluated the impact of 5 F/T cycles for multiple permutations of fast and slow cooling and rewarming rates. The quality attributes of AAV8 and AAV9 remained within acceptable ranges after the daisy chain TOIS and F/T rate studies. Potency and concentration were unchanged within method variability. There was a minor increase in non-encapsidated ('free') DNA released from AAV8 after F/T in a phosphate-buffered saline formulation. DNA release during F/T was minimized in a formulation with a low buffer concentration and was not detected in a formulation containing sucrose. We conclude that AAV8 and AAV9 have stability profiles that are suitable for manufacturing and clinical development.

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Introduction

There are an increasing number of clinical studies evaluating different adeno-associated viruses (AAV) serotypes as vectors for gene therapy.[1] AAV vectors contain a single strand of DNA with up to 4.7 kb contained within an icosahedral capsid comprised of 60 individual proteins. Tissue tropism, transduction efficiency, and pre-existing immunogenicity differ between AAV serotypes and these are factors that may influence the selection of a serotype for a specific clinical indication.[2] Development of a manufacturing process that can produce high yield of AAV with adequate clearance of process and product-related impurities is a challenge.[3-5] Chemical and physical degradation of AAV during manufacturing and storage can result in a decrease in purity and loss of potency.[3]

AAV serotypes have differences in their chemical and physical stability. AAV can lose potency through chemical modification of amino acids, such as the potency loss of AAV8 that occurs upon deamidation.[6] Exposure to elevated temperature initially results in DNA ejection from relatively intact capsids, followed by complete capsid rupture and release of DNA as the temperature is increased further.[7-9] The temperature at which rupture occurs varies by serotype, with AAV2 being one of the least stable and AAV5 one of the most stable.[7-9] The electrostatic and surface charge properties of the capsids are also different between serotypes.[10] This may result in differences in formulation impacts on colloidal stability and aggregation propensity. For example, aggregation of AAV2 at an ionic strength below 200 mM was reported to result in reduced processing yields and efficacy.[11] In another case, a decrease in AAV2 concentration of between 42% and 72% was reported upon freezing and slow thawing in a phosphate-buffered formulation with 0.001% poloxamer 188.[12] In contrast, Gruntman et al. reported that an AAV1 vector formulated in PBS generated functional transgene protein product in mice after exposure to four F/T cycles, after a 12 week hold at 4 °C, and after a 1 week hold at 20 °C.[13] The published literature on the stability of other AAV serotypes has remained relatively limited, particularly for AAV8 and AAV9 in different formulations.

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To minimize chemical and physical degradation, AAV bulk drug substance (BDS) and drug product (DP) are usually frozen at ≤ -60 °C for long-term storage and shipping, sometimes with an allowance for intended short-term storage of DP in a 2-8 °C refrigerator at the clinic. Long-term stability of the formulated AAV and the integrity of the container-closure are both critical aspects for a frozen DP. F/T cycles and short-term exposures to room temperature (RT) and refrigerated conditions occur during manufacturing, labeling, and clinical use. Therefore, the stability of AAV during time out of intended storage (TOIS) exposures is important to evaluate in addition to long-term stability. The stability of AAV to TOIS is expected to vary by AAV serotype and by formulation. During early development, it can be useful to generate data in a streamlined study to confirm that AAV will be stable during manufacturing, labeling, and clinical use. This type of assessment may be a best practice for early stage development of biologics in general.

In this work we consecutively exposed both AAV8 and AAV9 serotypes to five F/T cycles compounded with short-term exposures to RT and refrigerated conditions. The compounded 'daisy chain' stability approach can rapidly provide results with an efficient use of material. Three formulations were studied in this work. A formulation based on phosphate-buffered saline with 0.001% poloxamer 188 (P188) was the initial formulation used for ocular delivery of AAV8 (F1). A later formulation (F3) was identified with a suitable addition of sucrose as a cryoprotectant and a reduction in sodium chloride to adjust the osmolality, which improved the stability to frozen temperature excursions and F/T cycles. F4 is a formulation for AAV9 that was developed to closely mimic the composition of cerebrospinal fluid (with added P188) in order to maximize its tolerability for intrathecal delivery. Both AAV8 and AAV9 were formulated in F3 for ocular delivery. Based on the intended routes of administration, only F1 and F3 were studied for AAV8 and only F4 and F3 were studied for AAV9. In addition, we executed a study focused on the freezing and thawing step. Four permutations of fast (1 hour) and slow (11 h) cooling and rewarming rates were applied to AAV8 and AAV9 for 5 F/T cycles to gain further insight into the robustness of the stability to F/T in different formulations.

Materials and Methods

Production and Formulation of AAV8 and AAV9 Vectors

Adeno-associated virus serotype 8 (AAV8) and serotype 9 (AAV9) were produced by REGENXBIO Inc. In brief, a triple plasmid transfection was used to produce AAV in cell culture, followed by harvest, diafiltration, affinity chromatography, anion exchange chromatography, and diafiltration into the final formulation. The AAV8 and AAV9 studied in this work both contained ssDNA of approximately 4 kb in length. The percent full was approximately 73% for AAV8 and 57% for AAV9 used in the TOIS study.

Formulation and Container-closure

All excipients used in the three formulations were multicompendial grade. Three formulations were studied and labeled for consistency with prior work; F1, F3, and F4 (F2 was not studied in this work).

Formulation 1 (F1): Dulbecco's Phosphate Buffered Saline (DPBS) with 0.001% poloxamer 188: 137 mM sodium chloride, 2.70 mM potassium chloride, 8.10 mM sodium phosphate dibasic anhydrous, 1.47 mM potassium phosphate monobasic, 0.001% w/v poloxamer 188, pH 7.4.

F3: 'modified DPBS with sucrose and 0.001% poloxamer 188': 100 mM sodium chloride, 2.70 mM potassium chloride, 8.10 mM sodium phosphate dibasic anhydrous, 1.47 mM potassium

phosphate monobasic, 117 mM sucrose, 0.001% w/v poloxamer 188, pH 7.4. For F3, high-purity sucrose was used (S-124-2-MC, Pfanstiehl, Waukegan, IL).

F4: 'intrathecal formulation': 150 mM sodium chloride, 1.20 mM magnesium chloride 6-hydrate, 0.201 mM sodium phosphate monobasic monohydrate, 0.803 mM sodium phosphate dibasic anhydrous, 3.00 mM potassium chloride, 1.40 mM calcium chloride dihydrate, 4.40 mM dextrose anhydrous, 0.001% w/v poloxamer 188, pH 7.2.

The drug product (DP) vial container closure used was a 2 mL cyclic olefin polymer resin vial (19550057, West Pharmaceutical Services Inc., Exton, PA) with 13 mm chlorobutyl rubber serum stopper with fluoropolymer film barrier (19700302, West), and a 13 mm, aluminum, flip-off seal (54131770, West).

The drug substance (DS) container used for studies of AAV8 was an 8 mL HDPE bottle (342002-9025, ThermoScientific, Waltham, MA).

Compounded ('Daisy-Chain') Freeze-thaw and Time-out-of-intended Storage Studies

Multiple cycles of freeze-thaw, room temperature, and refrigerated hold exposures were consecutively applied to samples in the compounded ('daisy-chain') study.

In the AAV8 studies, samples were exposed to five freeze-thaw (F/T) cycles between approximately -80 °C and room temperature (RT = 22.4 ± 0.6 °C), held at 2-8 °C for 120 h (5 days), and then held in a secondary box (protected from light) at RT for 78 h (3.25 days). During F/T cycles, samples were frozen for at least 1 hour (typically longer depending on scheduling) at -80 °C during the freezing phase and were held for at least 1 hour at RT during the thawing phase of the cycle. The DP vials were filled to 0.9 mL and a parallel study was also performed with 8 mL BDS bottles filled to 1.3 mL.

In the AAV9 studies, DP vials filled to 0.95 mL were exposed to RT (65 h, 2.7 days) cumulatively during the thaw portion of the five F/T cycles, and the 2-8 °C exposure (210 h, 8.75 days) was applied last.

Controlled Rate Freeze-thaw Studies

Controlled cooling and rewarming rate freeze-thaw studies were conducted by placing DP vials filled to 0.5 mL on the temperature-controlled shelf of a lyophilizer (Genesis 25EL, SP Scientific, Stone Ridge, NY). AAV8 was formulated at 1×10^{12} GC/mL in both F1 and F3 and AAV9 was formulated at 3×10^{13} GC/mL in F4 in this study. Thermocouples were centered in four buffer vials for temperature recording. Five cycles were programmed from 25 °C to -60 °C. Separately, the four permutations of fast (1.5 °C/min, taking about 1 hour total) and slow (0.13 °C/min, taking about 11 h) cooling and rewarming rates were applied to the different samples with a 1 hour hold at 25 °C between cycles and at least a 1 hour hold at -60 °C for each cycle.

In Vitro Relative Potency

The *in vitro* bioassay was performed by transducing HEK293 cells and assaying for the protein encoded by the transgene. HEK293 cells were plated onto three 96-well tissue culture plates overnight. The cells were then pre-infected with wild-type human adenovirus serotype 5 virus followed by transduction with three independently prepared serial dilutions of the sample and assay controls.

For the AAV9 assay, the cells were lysed on the second day following transduction and assayed for the expressed transgene protein (tripeptidyl peptidase 1, TPP1) by enzyme activity using a fluorescent peptide substrate.

For the AAV8 assay, the cell culture media was collected from the plates on the third day following transduction. Plates coated with

vascular endothelial growth factor (VEGF) were used to capture the expressed transgene protein (anti-VEGF antigen-binding fragment). A Fab-specific anti-human IgG antibody and horseradish peroxidase (HRP) ELISA assay setup was used to quantitate the anti-VEGF Fab. The HRP substrate solution was added, allowed to develop, stopped with stop buffer, and the plates were read in a plate reader.

For both assays, the signal versus log dilution was analyzed with a four-parameter logistic regression model after passing a parallelism similarity test. The half maximal effective concentration (EC_{50}) ratio of the sample and standard curve was used to report the potency relative to the reference standard.

Vector Genome Concentration

Vector genome concentration (VGC) was determined by droplet digital polymerase chain reaction (ddPCR) on a Bio-Rad Droplet Digital™ PCR System QX200 (Hercules, CA). Samples were treated with DNase I to digest any non-encapsidated vector genomic DNA then mixed with a primer and probe set targeting the transgene sequence of the vector genome. The ddPCR reaction mix was partitioned into ≥ 10,000 droplets, the DNase inactivated by heat, the released DNA amplified by PCR, and read using the Bio-Rad Droplet Reader to determine the VGC.

Appearance

Color, clarity, and particles were analyzed using a method based on the Ph.Eur. 2.2.2, 2.2.1, USP<790>, and Ph.Eur. 2.9.20 using an A2 liquid viewer lightbox (Pallaypak, Quebeck, Canada).[14-17]

Size Distribution by Dynamic Light Scattering (DLS)

Dynamic light scattering (DLS) was performed on a DynaPro III (Wyatt Technology Corporation, Goleta, CA) using 384 well plates (3540, Corning, Corning, NY) with a 30 μL sample volume. Ten acquisitions each for 10 seconds were collected per replicate and there were three replicate measurements per sample. The solvent viscosity was set to 'PBS' for F1 and F4 and was set to '4% sucrose' for F3. Results not meeting data quality criteria (baseline, noise, visual assessment of data fit) were 'marked' and excluded from the analysis. For the F3 samples, the low delay time cutoff was changed from 1.4 μs to 10 μs to eliminate the impact of the sucrose excipient peak at about 1 nm. The diameter, polydispersity, intensity%, and mass% of each peak were reported. The intensity of light scattered by particles is proportional to the sixth power of their diameter, which is why DLS is very sensitive for detecting large particles. The mass% is proportional to the third power of the particle size and is also reported.

Free (Non-encapsidated) DNA Assay

SYBR® Gold Nucleic Acid Gel Stain (Invitrogen S11494, Thermo-Fisher Scientific, Eugene, OR) was used to quantitate DNA released from AAV capsids exposed to F/T or elevated temperature stress as previously described.[18] In brief, microplates (96 well, 300 μL, black, polypropylene (PP), flat-bottom, part #655209, Greiner Bio-One, Monroe, NC), were loaded with 20 μL of 10X dye, with a volume of 0 (blanks) to 180 μL of sample (and added spike for spike-recovery controls), and formulation buffer to bring the total volume to 200 μL. Fluorescence with excitation at 495 nm and emission at 530 nm was quantitated with a Cytation 5 microplate reader (BioTek, Winooski, VT). A single result for a sample was based on the average of 2 wells. A heat ruptured control with 100% of DNA released from capsids and free in solution was prepared with 10 μL of sample, 5 μL of 1% poloxamer 188, and 85 μL of formulation exposed to 85 °C for 20 min, followed by a 10X dilution in formulation buffer for a total dilution of

100X. The ratio of the sample result to the 100% free DNA control result, corrected for dilution and volume, was used to report free DNA as a percentage of the total DNA (free + inside capsids).To ensure the results were within the assay linear range, and to determine the absolute concentration of DNA for additional information, a standard curve over the range 0.45 to 9 ng/well was prepared using circular M13mp18 ss-DNA (N4040S, New England Biolabs, Ipswich, MA). A sample spiked with standard was included in each plate to confirm recovery accuracy.

Size Exclusion Chromatography

Size exclusion chromatography to separate and quantitate aggregates and free DNA from intact capsids was performed with an SRT SEC-1000, 5 μm, 1000Å 4.6 × 300 mm column (215950P-4630, Sepax Technologies Inc, Newark, DE) at a flow rate of 0.35 mL/minute of 20 mM sodium phosphate, 300 mM sodium chloride, 0.005% w/v poloxamer 188, pH 6.5, with detection performed at 260 nm for free DNA and 214 nm for aggregates. A main peak and a pre-peak at approximately 7.2 min were observed in the samples studied in this work. The pre-peak at 7.2 min was identified as free (non-encapsidated) DNA based on its A260/A280 ratio of approximately 1.8. For additional context we note that two additional pre-peaks eluting closer to the main peak (at 8.0 and 8.7 min) could be resolved by the SEC method for different samples studied during method development. These peaks had an A260/A280 ratio similar to the main peak and were identified as aggregates but were not observed for any of the samples in this work.

Particulate Matter by Light Obscuration

Subvisible particle counts were determined using a HIAC 9703+ instrument equipped with a HRLD150 sensor (HACH, Loveland, CO). Four runs of 0.20 mL each with a 0.20 mL tare were measured. The first run was discarded and the average of 3 runs was reported. The instrument was checked for cleanliness with water and for accuracy with a particle count standard (15 μm Count-Cal, CC15-PK, Thermo-Fisher, Waltham, MA).

pH Measurements

The pH of formulations was measured with a SevenExcellence pH meter (Mettler Toledo, Columbus, OH).

Ultraviolet-visible (UV-Vis) Spectroscopy

UV-Vis data were collected using a Cary 60 UV-Visible spectrometer (Agilent Technologies Inc., Santa Clara, CA). The absorbance at 260 and 280 nm corrected for baseline at 340 nm was used to simultaneously determine the capsid protein and the DNA concentrations and therefore the concentration of full and empty capsids and percent full as described in previous reports.[19,20]

Results

Impact of Compounded Freeze-thaw and Time-out-of-intended-storage Exposures

Table 1 shows the breakdown of the time out of intended storage (TOIS) for a frozen BDS and DP for AAV8 used to design the 'daisy chain' stability study. The TOIS includes allocations for freeze-thaw (F/T), RT, and refrigerated exposures during BDS and DP manufacturing, labeling, and clinical use. The normal operation cumulative total (A) in this illustrative example includes 2 × F/T + 62 h at RT + 22 h at 2-8 °C and represents the maximum normal exposures. Additional

Table 1
Compounded freeze-thaw and time-out-of-intended-storage study design and exposure allocation.

Step	Normal Operation
BDS filtration into bottles and transfer to freezer	RT: 6 h 2-8 °C: 6 h
Shipping and Storage of Frozen DS	*NA (included as part of stability allocation)*
Thawing of BDS bottles	1 × F/T RT: 8 h
DP filling operation, labelling, transfer to freezer	RT: 36 h 2-8 °C: 16 h
Additional labelling operation (if applicable)	*If needed, use part of allowance for excursions (from row B)*
Shipping and Storage of Frozen DP	*NA (included as part of stability allocation)*
Vial thaw at clinical site	1 × F/T
Short-term refrigerated storage at clinical site	*NA (included as part of stability allocation)*
Dose preparation and administration	RT: 12 h
A: Normal Operation Cumulative Total	2 × F/T RT: 62 h 2-8 °C: 22 h
B: Additional Stress Included in Study as an Allowance for Excursions	3 × F/T RT: 16 h 2-8 °C: 98 h
A + B = C: 'Daisy Chain' Study Design Total	5 × F/T RT: 78 h (3.25 days) 2-8 °C: 120 h (5 days)

exposure allowances were included to account for potential additional operations (e.g. additional labeling may involve an additional F/T and 12 h at RT) and excursions (e.g. F/T during shipping, or extra processing time needed during manufacturing). In this example study design, additional stress (3 × F/T + 16 h at RT + 98 h at 2-8 °C) was included as an allowance for excursions and/or additional operational exposures (B). The TOIS exposure allocation does not include long-term storage of frozen DS or DP which are accounted for in the intended storage condition stability program. In some cases, the intended storage may also include time in a refrigerator at a clinical site (e.g. 2 weeks to 2 months) to allow sites to receive frozen DP and thaw and store in their refrigerator for inventory management purposes. The total cumulative F/T, RT, and refrigerated exposure for normal operations (A) with additional allocations for excursions (B) were combined in the daisy-chain study design total (i.e. A+B=C). This is an illustrative example, and the actual time allocation needed could be different than shown here depending upon operational details. The TOIS study duration time allowance design was slightly different for AAV9.

The TOIS study results are compiled in Fig. 1. The columns show results for AAV8 in F1, AAV8 in F3, AAV9 in F3, and AAV9 in F4. The side-by-side results for each assay tested can be compared in each row. Each subpanel shows low concentration results (left pair of bars) and high concentration results (right pair of bars). Each pair of bars are the results for the control (labeled Cont.) and the sample after the cumulative TOIS exposure (labeled TOIS).

The first row of results in Fig. 1 show that there was no change in vector genome concentration (VGC) for both AAV8 (in F1 and F3) and AAV9 (in F3 and F4) at both low and high concentrations after the cumulative 'daisy-chain' TOIS exposures. The VGC results demonstrate that there was no large-scale precipitation or product loss of either AAV8 or AAV9 in the formulations we studied, even for AAV8 in F1 which is a phosphate-buffered formulation.

There was no change to potency within method variability for any of the samples (second row of results in Fig. 1), indicating that the efficacy of the AAV8 and AAV9 will be maintained after the TOIS exposures in the formulations studied.

There were also no signs of large-scale aggregation for any of the samples as measured by the diameter measured by DLS, which

remained consistent after TOIS exposure (Fig. 1, row 3). There was only a single peak for all samples (i.e. no aggregates detected), except for the high concentration AAV9 in F3 after TOIS exposure, for which a peak at 656.6 nm representing <0.1% by mass (1.2% by intensity) was detected. In a long-term stability study (initiated using the exact same AAV9 samples used for the daisy chain study), there was no change in size distribution, and no aggregates were detected (main peak = 100% intensity) by DLS for AAV9 stored at ≤-60 °C for 12 months. These results indicate that the very small aggregate peak detected in this work does not appear to represent a trend that would be of concern on long-term stability and may have been a spurious artifact due to laboratory environmental dust.

For AAV8 in F1, an increase of 2.1% free DNA (0.42% per F/T cycle) at low concentration and 1.1% at high concentration (0.22% per F/T cycle) was observed. There was also an increase of 2.3% free DNA for AAV9 in F4 (0.46% per F/T cycle) at low concentration and 0.8% at high concentration (0.16% per F/T cycle). These changes are larger than the fluorescent dye free DNA method variability (intermediate precision %CV of 6.6%).[18] Given that the difference in concentration of the samples was 10-fold for AAV9 in F1 and 250-fold for AAV9 in F4, the fact that the low concentration samples had approximately twice the release of DNA suggests there may be a weak concentration dependence. In contrast to the results in F1 and F4, there was no change in free DNA for either AAV8 or AAV9 exposed to the compounded TOIS exposures in F3. F3 contains the cryoprotective excipient sucrose, which appears to have protected the AAV capsids from rupture during the 5 F/T cycles in the TOIS study.

Subvisible particulate matter was tested by light obscuration for AAV8 in F1 and F3 (last row in Fig. 1). Counts remained very low (≤78 particles/mL at ≥2 μm, ≤10 particles/mL at ≥10 μm, ≤2 particles/mL at ≥25 μm, and 0 particles/mL at ≥50 μm) after the TOIS exposures. Subvisible particles were not tested for AAV9 in F3 or F4 in this TOIS study but were tested in an associated long-term stability study, where they remained low (≤15 particles/mL at 10 μm) and without any trend for 12 months. The light obscuration method has limitations in that it may undercount translucent proteinaceous/viral aggregate particles. Flow imaging was not used in this work but may be of use in follow-on characterization and stability studies focused on subvisible particle formation.

The full set of data for all assays tested is provided in tabulated form in supplemental information. The results of a parallel study of AAV8 in both F1 and F3 in the BDS bottle container were similar to the results for the DP container (see supplemental data).

As a follow-on to these daisy chain studies, additional freeze-thaw rate studies described below were designed to look more closely at whether different rates of F/T can impact the potency, aggregation, or release of DNA during F/T.

Impact of the Freeze-thaw Rate on the Stability of AAV

A follow-on study was designed to assess whether different rates of F/T can impact the potency, aggregation, or release of free DNA during F/T. All four permutations of fast (over 1 hour) and slow (over 11 h) freeze and thaw combinations were studied. An example of the temperature profile for the controlled rate slow freeze-fast thaw cycling study arm is shown in Figure S1. The measured freezing profiles of the vials for slow and fast cooling are shown in Figure S2. The freezing time was estimated from the temperature profiles according to Kasper et al.[21] The freezing time for slow cooling was estimated to be 10-15 min, 2 to 3 times longer than the fast cooling rate freezing time of approximately 5 min. As a reference benchmark, the cooling and rewarming times of actual vials and bottles were measured. The uncontrolled cooling time was 0.5 h and the rewarming time was between 0.8 and 1 hour for a 0.9 mL fill in a 2 mL DP vial (Figure S4). The uncontrolled cooling time was 2 h and the rewarming time was
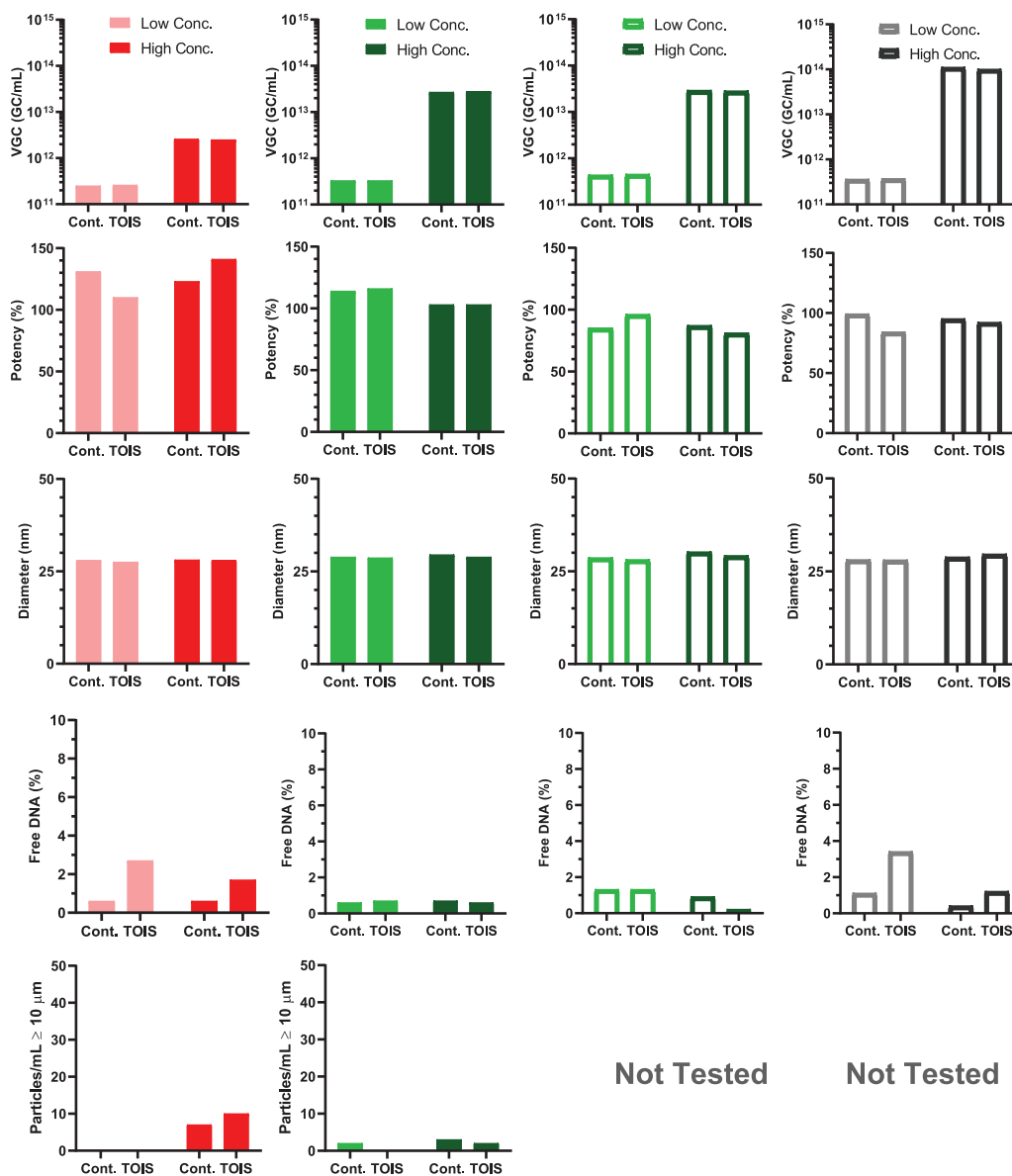
Figure 1. Impact of Compounded F/T and TOIS on the Stability of AAV8 and AAV9. The results are arranged in columns by serotype and formulation with each row showing side-by-side results for a different assay. Column 1: AAV8 in F1, Column 2: AAV8 in F3, Column 3: AAV9 in F3, Column 4: AAV9 in F4. Each subpanel shows low concentration results (left pair of bars) and high concentration results (right pair of bars). Each pair of bars are the results for the control (labeled Cont.) and the sample after the cumulative TOIS exposure (labeled TOIS).

between 3.5 and 3.8 h for a 150 mL fill in a HDPE BDS bottle (Figure S5). The cooling and rewarming rates in the controlled rate studies (1 to 11 h) were approximately similar to the actual small vial fill case (fast) and were slower than the actual larger BDS bottle case (slow).

The results of the F/T rate studies for AAV8 in F1 and F3 and AAV9 in F4 are summarized in Fig. 2. There was no change to potency (Fig. 2, panel A) and no change to the diameter or aggregation measured by DLS (Fig. 2, panel B) for any of the four permutations of fast and slow F/T cycles for all three studies. A minor aggregate peak with a size of 101 nm at 2.4% by intensity (<0.1% by mass) was detected in one DLS sample of AAV8 in F3 (5 cycles of slow freeze-fast thaw). Long-term stability studies of AAV8 in F3 at ≤-60 °C for over 2 years have shown no trend in the size distribution or aggregation by DLS and the size distribution of AAV8 in F3 by DLS was consistent for all the other samples and no aggregate peaks were detected. This aggregate peak may therefore be related to method variability or dust.

Aggregates were not detected by SEC for any of the samples. Example SEC chromatograms for AAV8 in F1 are given in supplemental information Figure S3. Aggregates detected by SEC are smaller (<100 nm) whereas DLS typically resolves larger aggregates (100−1000 nm).

There was an increase in free DNA for AAV8 in F1 after 5 F/T cycles of between 3.3% and 4.0% (0.66% to 0.80% per cycle) by the fluorescent dye method (Fig. 2, panel C). There was also an increase in the pre-peak at 7.2 min attributed to free DNA of between 1.3% and 2.4% (0.26% to 0.48% per cycle) by the SEC method (Fig. 2, panel D). We note that the quantitation of free DNA by the SEC method was slightly lower than the dye method for these samples. It is not uncommon that there are differences in absolute results for two methods based on different principles. In a larger data set comparing the two methods, we found that they were well-correlated; the slope of the SEC pre-peak attributed to free DNA (at 7.2 min) peak area percent versus free DNA was 0.83 for 61 samples with between 1% and 20% free DNA (not shown). One explanation for this is that the SEC peak areas at
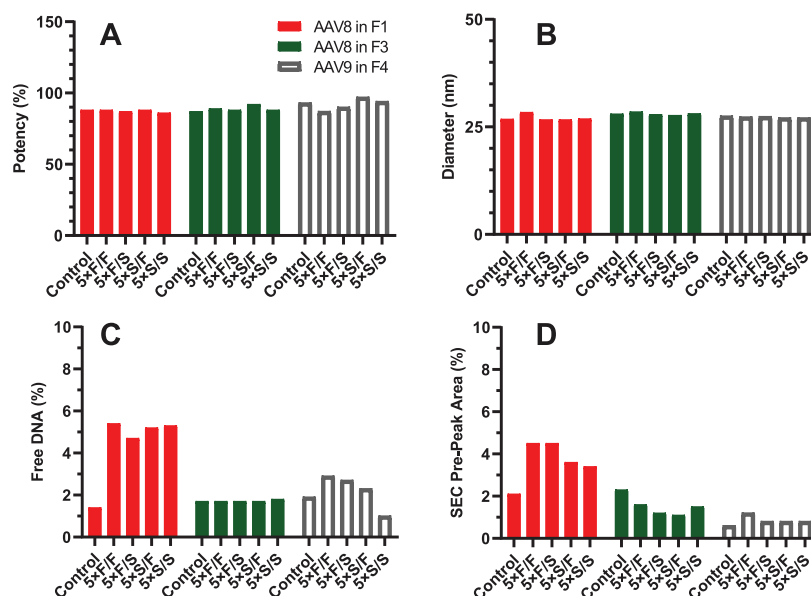
Figure 2. Impact of Different Rates of Freeze-Thaw Cycling on the Stability of AAV8 in F1 and F3 and AAV9 in F4. Results are grouped by serotype and formulation: AAV8 in F1 (red bars), AAV8 in F3 (green bars), AAV9 in F4 (open gray bars). The first bar in each group is the control, followed by the results of 5 F/T cycles for the different permutations of fast (F) and slow (S) freezing and thawing rates. Panel A: potency, panel B: DLS diameter, panel C: free DNA, panel D: SEC pre-peak area %.

260 nm were used to calculate the free DNA which can lead to a slight under-quantitation of free DNA because the main peak also includes A260 signal from capsid proteins in addition to DNA absorbance. There could also be DNA associated with an intact capsid (surface bound or partially ejected) that elutes under the main SEC peak and is therefore not quantitated as part of the free DNA in pre-peak.

The broad trend was the same for both methods in that the free DNA by the dye method and SEC method were both higher for all samples of AAV8 in F1. To a lesser extent, with some variability observed, the results were slightly higher for most of the samples of AAV9 in F4. As with the daisy chain study, AAV8 was robustly stable in F3 for all the fast and slow permutations of the 5 F/T cycles. There was no increase in free DNA by either the dye or SEC method for AAV8 in F3. The SEC control result for AAV8 in F3 had the largest pre-peak area percent and the other samples had noticeable variability that is likely a result of variability in the SEC method itself (e.g. integration settings, baseline noise etc). For AAV8 in F1, the two fast freezing samples had very slightly higher SEC pre-peak areas than the other samples, but the difference was very small, and no difference was observed in the dye-based method free DNA results. The difference in the SEC results for F1 can therefore be reasonably attributed to SEC method variability. There was also an increase in free DNA for AAV9 in F4, but it was much smaller than for AAV8 in F1. In this case there was no difference in the SEC results but there was a slightly higher free DNA by the dye method for the two fast freeze conditions. Again, the difference was small and could be a result of overall variability in the study due to, for instance, the stochastic nature of ice nucleation for individual vials or other uncontrolled factors related to the F/T process. Overall, none of the individual permutations of freezing and thawing rates resulted in a large difference in both free DNA and the SEC pre-peak compared to the other conditions. If there was a truly greater impact of, for example fast freezing, then it was very small compared to all sources of variability in this study. A greater number of F/T cycles and replicate samples would be required to unambiguously separate out any meaningful difference, if it exists, from the overall variability of the results in the study.

Overall, the data in Fig. 2 shows there was no impact to potency, diameter or aggregation by DLS, or aggregation by SEC for AAV8 in F1 and F3 and AAV9 in F4 subjected all variations of fast or slow F/T.

Capsid rupture with release of free DNA during F/T was the most sensitive formulation-dependent degradation pathway that could be detected for AAV8 in F1 and to a much lesser extent for AAV9 in F4 after 5 F/T cycles. From a practical perspective, the results indicate that there would not be any meaningful reduction in release of DNA by altering the freezing profile based on these study results. There will be only 2 or 3 F/T cycles that will occur during normal operations, which is lower than the 5 conducted for this study. The highest increase of free DNA from this study projected for F1 is ≤0.8% per F/T cycle and for F4 is ≤0.2% per F/T cycle, which is very low.

Discussion

The use of AAV vectors for gene therapy is a relatively new field and there has been a rapid increase in the number of clinical trials.[1] There is relatively limited information published on degradation pathways of AAV compared to more established biologics, such as monoclonal antibodies. Activity loss of AAV after freezing and thawing, aggregation at low ionic strength, and aggregation/precipitation during fill/finish operations have been reported.[3] Currently there is limited information published about the stability of AAV8 and AAV9, especially with practical relevance to time out of intended storage (TOIS). In this work we found that three formulations of AAV8 and AAV9 were stable after exposure to the cumulative F/T, RT, and refrigerated conditions that will occur during manufacturing and clinical use, with additional allowances for excursions. In addition, AAV8 and AAV9 remained stable when subjected to five cycles of F/T executed with all four permutations of fast (over 1 hour) and slow (over 11 h) cooling and rewarming rates.

The compounded 'daisy chain' time out of intended storage (TOIS) stability study conducted in this work could be considered a best practice for early AAV development and for development of biologics in general. This type of study is valuable in that it can be used to provide assurance that the AAV will be stable during manufacturing, labeling, and clinical use. One future improvement to the TOIS study is to include an excursion and hold at -20 °C during the F/T portion to provide supporting data for freezer and shipping temperature excursions. In the case where unacceptable instability is detected after the compounded 'daisy chain' study, then F/T cycles and exposure to RT

will need to be studied separately. Alternatively, if there is enough material, then samples could be taken after each stage of the compounded stress (i.e. 5 F/T, 5 F/T + 3d at RT, 5 F/T + 3d at RT + 5d at 2-8 °C) to isolate the stress that results in degradation. For some biologics the intended storage condition may be as a liquid at 2-8 °C, so the analogous study might include RT exposure and F/T cycles and be referred to as a compounded time-out-of-refrigeration (TOR) study.

The rate of F/T could also be a factor in the stability of AAV. In this work we performed a follow-on study focused on the rates of freezing and thawing and did not find any permutation that resulted in a higher amount of degradation that would be meaningful in practice. There was some variability in the amount of free DNA detected after F/T that is likely in part related to the stochastic nature of the freezing process for individual vials, in part related to method variability, and with potentially unknown/uncontrolled sources of variation also contributing. Additional insights into the F/T stability may be gained as further studies are performed during ongoing development of AAV therapies.

There are multiple factors that may play a role in stability of a final formulated AAV drug product, such as the serotype, the length of encapsided DNA, the formulation, the container-closure, the AAV concentration, product-related impurities, and process-related impurities.

Differences in the thermal melting temperature,[7-9] and charge properties,[10] are expected to differentially impact the colloidal stability and aggregation propensity of different serotypes. In this work we found that AAV8 and AAV9 were stable to F/T and TOIS in formulations with ionic strengths (F1 = 166 mM, F3 = 129 mM and F4 = 163 mM) that are lower than the 200 mM that has been reported necessary to stabilize AAV2 against aggregation.[11] An advantage of a lower ionic strength needed to inhibit aggregation is that the formulation design-space is less restrictive. For instance, in this work we were able to closely match the composition of cerebrospinal fluid for the AAV9 formulation (F4) and we were able to generate a formulation with a balance of salt to inhibit aggregation and sucrose as a cryoprotectant while maintaining an acceptable tonicity (F3).

Genome length can vary for any given AAV vector and may be a factor, such that shorter genomes have been reported to be generally more thermally stable when encapsided than those vectors containing longer genomes.[9] The AAV8 and AAV9 we studied contained ssDNA with approximately 4 kb, which could be one factor contributing to their relatively robust stability. The impact of genome length on stability would require additional investigation.

The formulation appears to be able to dramatically impact the stability of AAV vectors. For example, AAV1 was reported to have good stability in phosphate-buffered saline.[13] In contrast, massive product loss was reported for AAV2 in a phosphate-buffered formulation with 0.001% poloxamer 188, and greater stability was observed in a TRIS buffer.[12] The phosphate-buffered formulations with 0.001% w/v poloxamer 188 studied in this work were found to confer robust stability to AAV8 (F1 and F3) and AAV9 (F3 and F4). A formulation containing sucrose as a cryoprotectant was particularly stabilizing (F3). Both F1 and F4 contained similar levels of salt yet there was a much greater release of DNA from ruptured AAV8 capsids subjected to F/T in F1 relative to AAV9 in F4. Both F1 and F4 were phosphate-buffered, but F4 contained a minimal level of 1 mM whereas the phosphate level in F1 was 9.6 mM. The phosphate buffer level and the sucrose level therefore appear to play a role in the degree of capsid rupture that occurs during F/T. Additional studies to further investigate the robustness of these formulations to different excipient levels may be of value.

The AAV serotype, genome length, and formulation could all impact the solubility of AAV vectors. We found that AAV8 was stable from 3×10^{11} to 3×10^{13} GC/mL and AAV9 was stable from 4×10^{11} to 1×10^{14} GC/mL, suggesting that concentration

was not a critical factor impacting stability, at least for the conditions studied in this work.

It is known that AAV can adsorb to certain materials, such as glass.[3] We did not observe any product losses due to adsorption to the COP vial with a fluoropolymer-coated rubber closure used in this work.

Product-related impurities or substances could impact stability. A common impurity in AAV preparations are empty capsids, but little has been reported about their impact on stability of AAV DP. The percent full was 73% for AAV8 and 57% for AAV9, and both had robust stability to TOIS. We did not study the impact of the %full on stability but this may become increasingly important to understand with the current drive to increase the %full in AAV DPs.

Finally, process-related impurities may impact the stability of AAV DPs. In one report, residual DNA (from host-cell and plasmid) was found to enhance aggregation of AAV2.[11] There is the potential for unexpected stability impacts of host cell impurities, metal ions, or leachables from process equipment.

The results of this particular study are not meant to be generally representative of the stability of other AAV serotypes - more investigation is required. However, it seems reasonable that we might expect similar TOIS stability results for other vectors of the AAV8 and AAV9 serotype with similar genome lengths and in the same or similar formulations.

We did note a practical method interpretation challenge. In isolated instances, an aggregate peak representing a very small mass (<0.1%) was detected by DLS. Such a small quantity of aggregates is not expected to have an impact on safety or efficacy. In situations where an aggregate peak is not consistently detected in repeated studies or on long-term stability, it may be a spurious result due to factors such as environmental dust or cuvette cleanliness. We suggest that potential for spurious peaks be considered when evaluating DLS data. On the other hand, the repeated and consistent detection of an aggregate peak by DLS may indicate the result is real and warrant additional studies. Use of orthogonal submicron particle characterization methods (e.g. nanoparticle tracking, resonant mass measurement, resistive pulse sensing, or other methods) may be of value in cases where aggregates are detected by DLS. However, there is the ongoing challenge that in many cases that AAV samples are limited based on small yields and batch sizes, and some characterization methods can consume relatively large amounts of material.

Out of all the attributes tested, the greatest change was a minor increase in the level of free DNA from capsids ruptured during F/T. It is known that DNA release from ruptured capsids occurs at high temperature.[7-9] We previously reported that trace levels of DNA can be released from AAV capsids ruptured during F/T cycles.[18] In this work, the release of DNA from capsids ruptured during F/T was a minor but detectable degradation for AAV8 in F1. Release of DNA was not detected for AAV8 in F3 and was low for AAV9 in F4.

Overall, the different rates of freezing and thawing did not result in a meaningful differential impact to the stability of AAV8 and AAV9, suggesting that these serotypes have robust stability in the formulations tested.

Exposure to surfaces of ice, crystalline excipients or air bubbles, pH shifts during freezing, cryoconcentration of excipients, devitrification events, or cold denaturation are factors that could be related to degradation of biologics during F/T.[22] One or more of these factors could be a cause of the small amount of DNA released from ruptured AAV capsids during F/T. Some of these factors can be stochastic or variable in nature (e.g. ice nucleation) or depend upon the scale (i.e. time taken for the freezing phase to complete during cooling). The freezing time for slow cooling was estimated to be 10-15 min, 2 to 3 times longer than the fast cooling freezing time of approximately 5 min in this study. The cooling and rewarming rates could impact various factors such as the time spent in the transition during freezing, the size of ice crystals, exposure to the ice surface, the

completeness of crystallization of excipients, exposure to air bubbles, and devitrification of amorphous excipients/water. F3 contains the cryoprotective excipient, sucrose, and there was no detectable increase in free DNA in this formulation after F/T cycling. There are several ways that sucrose can stabilize biologics to F/T stress.[22,23] As ice crystalizes, the AAV in the freeze concentrate will be surrounded by an increasingly high concentration of sucrose which can be conformationally stabilizing. The highly viscous amorphous environment sucrose inhibits buffer crystallization and the associated exposure to crystal surfaces or low pH shifts. Future studies could focus on a mechanistic assessment of the factors that play a role in release of DNA during F/T and how the formulation can modulate the F/T stability of AAV8 and AAV9.

Conclusions

The compounded 'daisy chain' time out of intended storage (TOIS) stability study conducted in this work could be considered a best practice for early AAV development and for development of biologics in general. Overall, the time out of intended storage (TOIS) daisy chain stability and F/T rate studies demonstrated that AAV8 and AAV9 product quality attributes remained within acceptable ranges. These results demonstrate that AAV8 and AAV9 have a stability profile suitable for manufacturing and clinical operations, with additional allowances for excursions.

Declaration of Competing Interest

All authors are employees of REGENXBIO Inc., which is a clinical-stage AAV gene therapy company. The authors declare no other conflicts of interest.

Acknowledgments

Supplementary Materials

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.xphs.2022.01.002.

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