Thermal Stability as a Determinant of AAV Serotype Identity

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Currently, there are over 150 ongoing clinical trials utilizing adeno-associated viruses (AAVs) to target various genetic diseases, including hemophilia (AAV2 and AAV8), congenital heart failure (AAV1 and AAV6), cystic fibrosis (AAV2), rheumatoid arthritis (AAV2), and Batten disease (AAVrh.10). Prior to patient administration, AAV vectors must have their serotype, concentration, purity, and stability confirmed. Here, we report the application of differential scanning fluorimetry (DSF) as a good manufacturing practice (GMP) capable of determining the melting temperature ($T_m$) for AAV serotype identification. This is a simple, rapid, cost effective, and robust method utilizing small amounts of purified AAV capsids ($\sim$25 µL of $\sim$10$^{11}$ particles). AAV1-9 and AAVrh.10 exhibit specific $T_m$s in buffer formulations commonly used in clinical trials. Notably, AAV2 and AAV3, which are the least stable, have varied $T_m$s, whereas AAV5, the most stable, has a narrow $T_m$ range in the different buffers, respectively. Vector stability was dictated by VP3 only, specifically, the ratio of basic/acidic amino acids, and was independent of VP1 and VP2 content or the genome packaged. Furthermore, stability of recombinant AAVs differing by a single basic or acidic amino acid residue are distinguishable. Hence, AAV DSF profiles can serve as a robust method for serotype identification of clinical vectors.

INTRODUCTION

Adeno-associated viruses (AAVs) are small, $\sim$260 Å, $T = 1$, icosahedral non-enveloped viruses belonging to the Dependoparvovirinae genus of the Parvoviridae family. Their capsids serve as vehicles to deliver viral-packaged genomes to the nucleus for replication. Currently, 13 human and non-human primate AAV serotypes and over 150 genotypes have been identified.4 The AAV capsid is composed of 60 copies in total of viral protein (VP1 ($\sim$87 kDa), VP2 ($\sim$73 kDa), and VP3 ($\sim$61 kDa) in a population ratio of 1:1:10, respectively.2,3 The AAV VPs share a common VP3 C-terminal sequence, with VP1 and VP2 extended at their N-termini. The VP1 is further N-terminally extended compared to VP2, with the VP1 unique (VP1u) region containing a phospholipase A2 (PLA2) domain, required for infectivity.4,5 Differences in amino acid sequence and capsid structure mediate the interaction of the AAV serotypes with different host cell receptors, leading to alternative cell or tissue tropisms. For example, AAV1 has tropism for skeletal muscle, whereas AAV8 has tropism for the liver (reviewed by Hastie et al.).

AAV gene therapy has developed into one of the lead treatment strategies for various monogenetic diseases. These vectors are recombinant AAVs (rAAVs), with a transgene expression cassette packaged instead of the wild-type (WT) viral genome.6 Early development of the AAV gene therapy system was mostly focused on AAV2.5 However, novel AAV serotypes have been isolated, along with the discovery that some of these viruses exhibit higher transduction efficiencies in certain cells or tissues compared to AAV2. This led to a larger repertoire of available capsids for packaging different therapeutic genes.7,8 Thus, many recent clinical trials have utilized a range of AAV serotypes for therapeutic gene delivery.9–11 Significantly, a rAAV1 vector for treating lipoprotein lipase deficiency is the first approved human gene therapy biologic and was certified by the European Medical Agency.12

The AAVs share high amino acid sequence identity, resulting in a need to develop methods for confirming the serotype in clinical grade vectors prior to administration. However, in contrast to WT AAV packaging unique genomes, the identity of purified rAAV capsids cannot be determined by PCR-based sequencing methods. Furthermore, although mouse monoclonal antibodies are available for some of the AAV serotypes, which can identify their capsids, this is not the case for all available AAVs.16–17 The engineering of AAVs for various reasons, including escape from pre-existing neutralizing host antibodies or improved tissue targeting, which modifies antigenic regions, compounds this problem. An alternative for capsid identification of purified rAAV preparation is mass spectrometry.18 This method has several advantages, including small sample size ($\sim$0.5–5 µg), a low percentage of unique peptide coverage for identification, and single residue variations that can be detected when

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compared to the WT AAV sequence. However, disadvantages include the requirement for serotype-specific proteases, manual manipulation of the sample, and time required to prepare and analyze the sample. This includes running a denatured gel, peptide digest, mass analysis, and database search. Thus, there is a need for new or improved methods for rAAV serotype identification.

Here, we report the application of differential scanning fluorimetry (DSF), capable of accurate melting temperature (Tm) determination, for AAV serotype identification. This approach was developed and applied to the characterization of AAV capsid stability as previously described. This is a simple, cost effective, rapid, and robust method utilizing small amounts of purified rAAV capsids (~10^11 particles) and a dye (SYPRO Orange). This study analyzed AAV1–AAV9 and AAVrh.10, where AAV3 refers specifically to AAV3b. The AAV serotypes displayed specific capsid stabilities in buffers commonly used for their formulation and storage, with a single amino acid difference being detectable. The Tm is dictated by the VP3 sequence alone, and not VP1, VP2, or the packaged genome, making it an ideal method for clinical sample authentication.

RESULTS

Stability of rAAV1–rAAV9 and rAAVrh.10 in PBS Buffer Shows Disparate Profiles

The stability of rAAV1–rAAV9 and rAAVrh.10 in PBS, the most commonly utilized AAV formulation and storage buffer, was analyzed using DSF. The AAV samples were produced using the Baculovirus/Spodoptera frugiperda 9 (Sf9) cell expression system or in HEK293 cells, and purified by AVB affinity column chromatography, with genome-containing (full) and empty capsids separated by sucrose sedimentation gradient, as previously described. Sample purity and capsid integrity were confirmed by SDS-PAGE and negative-stain electron microscopy (EM), respectively (Figure 1A). The Tm for the purified full and empty capsids, diluted into PBS buffer, was determined by DSF, as described in Materials and Methods. The Tm is the temperature at which 50% of the sample is unfolded and bound with dye. The thermal profiles show Tm,s ranging from 66.5°C to 89.5°C ± 0.5°C, with rAAV2 being the least stable and rAAV5 being the most stable, respectively (Figure 1B; Table 1). The Tm of each AAV was unique, with the exception of AAV7, AAV9, and rAAVrh.10 at 77.0°C ± 0.1°C–0.8°C, which could not be distinguished within experimental error (Figure 1B; Table 1). Significantly, the full and empty capsid Tms were similar for all the AAVs tested (Table 1).

Comparative Stability Analysis of rAAV1–rAAV9 and rAAVrh.10 in Commonly Used Formulation and Storage Buffers Reveals Serotype-Specific Stabilities

The observation that rAAV9 and rAAVrh.10 exhibited experimentally the same Tm in PBS buffer, and the knowledge that the composition, ionic strength, and pH of a buffer could have an impact on Tm, led to further investigation of thermal stability profiles in other commonly used AAV formulation and storage buffers (Table 1). The stability of purified full and empty capsids for rAAV1–AAV9 and rAAVrh.10, diluted 10x into five additional (to PBS) common buffers and universal buffer (UB), developed to be insensitive to pH changes during thermal melting experiments, was tested. The Tm,s for each AAV in the buffers aligned into three groups: group I was AAVs with Tm,s varying by ~15°C–20°C, AAV2 and AAV3; group II was AAVs with Tm,s varying by ~3°C–6°C, AAV1, AAV4, AAV6, AAV7, AAV8, and AAVrh.10; and group III was AAVs with Tm,s varying by <2.0°C, AAV5 and AAV9 (Figures 2A–2J; Table 1). Comparative analysis of the AAVs in the different buffers showed that each buffer had a different effect on each serotype, i.e., no buffer caused a consistent stabilization or destabilization of all ten viruses tested (Figure 2K; Table 1). Of note, only minor Tm fluctuations, ~1°C–2°C, were observed between full and empty capsids of a few AAVs, whereas most showed no detectable Tm difference (Figure 3; Table 1). For Tm,s showing minor differences, there was no trend of full or empty capsids being more or less stable.

Small Volume and Low Concentration Requirement of AAV Samples for DSF Analysis

An important aspect of developing DSF profiling as a method for determining capsid stability and serotype identity was to determine the minimal amount of AAV required for the assay. Thus, purified full and empty capsids of AAV5, the most stable serotype, were tested at starting concentrations of 5 × 10^13 viral genome (vg)/mL and 3.4 × 10^13 particles/mL, respectively. The samples serially diluted 10–100x into PBS and followed by Tm,s determination identified a minimal concentration at which signal could be measured. The lowest detectable concentrations for the full and empty capsids were 5 × 10^11 vg/mL and 3.4 × 10^11 particles/mL, respectively, tested in 25 μL (Figures 4A and 4B).

AAV VP3 Is the Determinant of Capsid Stability

Following the observation that the genome had only a minor effect on the AAV stability, the role of VP1, VP2, and VP3 on capsid stability was analyzed for AAV2, the serotype exhibiting the lowest capsid stability (Figure 1B; Table 1). AAV2 virus-like particles (VLPs) assembled from (1) VP1, VP2, and VP3 (AAV2-VP123); (2) VP1 and VP3 only (AAV2-VP13); (3) VP2 and VP3 only (AAV2-VP23), and (4) VP3 only (AAV2-VP3) were generated by the site-directed mutagenesis of the start codon of the respective VP to be eliminated in the pFFBDVPm11 plasmid. The VLPs were produced and purified, as described in Materials and Methods, and diluted into PBS for Tm analysis by DSF. SDS-PAGE (silver stained) and negative-stain EM showed the correct VP content for each construct and intact capsid assembly (Figure 5A). The Tm was ~67.5°C ± 0.5°C (Figure 5B). In this assay, AAV5 VLPs were included as a control sample and its Tm was measured at 90°C ± 0.5°C, which is within experimental error of the value obtained from the samples produced in the OneBac system and used for the assays reported above (Figure 5B). These data showed that VP3 is sufficient to assemble the virus capsid, as previously reported, and that its sequence is the sole determinant of the capsid stability.
The observation that VP3 was the determinant of AAV capsid stability initiated a comparative analysis of the sequences of the AAVs. The sequence identity between the ten AAVs was calculated using the Clustal Omega application\(^\text{28}\) (data not shown), and calculation of the isoelectric point (pI) and analysis of the number of basic and acidic residues were conducted using the ExPASy application.\(^\text{29}\) The sequence identity between the AAVs range from \(55\%\) between AAV4 and AAV5 and between them and the other AAVs, to \(99\%\) between AAV1 and AAV6 (data not shown), consistent with previous reports.\(^\text{1,18}\) A reversed relationship was observed between the \(T_m\), of each AAV in PBS and UB when plotted with the calculated pI, with the exceptions being AAV4 and AAV6 (Figure 6). These viruses both had a higher pI than was expected in the overall trend, in which AAV5, with the lowest calculated pI, was the most stable serotype. The data indicated a potential role of the acidic and basic residues (reflected in the pI) in dictating the stability of the AAV capsid.

**Tm Distinguishes a Single Acidic or Basic Amino Acid Residue Difference in an AAV Variant**

The validity of the prediction that VP3 sequence and pI, particularly the ratio of basic to acidic residues, is a determinant of AAV stability, was tested by comparing AAV1 and AAV6, which differ by six amino acids, including one acidic/basic amino acid, E531K. Reciprocal variants, AAV1-E531K and AAV6-K531E\(^\text{30}\) and AAV1 and AAV6 packing the luciferase gene were analyzed by DSF. The \(T_{m}\)s for AAV1, AAV1-K531, AAV6, and AAV6-E531 were \(84.0^\circ\text{C},\)
78.0°C, 79.0°C, and 83.5°C, respectively. The Tm for AAV1-K531 was similar to that of AAV6, whereas that of AAV6-E531 was similar to that of AAV1 (Figure 5C). Thus, mutation of AAV1 residue E531 to K resulted in destabilization, whereas mutation of AAV6 K531 to E resulted in stabilization (Figure 5C).

### Capsid Stability in Different Buffer Formulations Shows No Significant Effect on AAV Transduction Efficiency

To determine if the difference in stability observed in different buffer formulations translated to transduction efficiency, luciferase gene expression by four AAV serotypes belonging to group I (AAV2), II (AAV1 and AAV8), and III (AAV5), diluted into the different buffers, was tested in HEK293 cells. These four serotypes were selected not only because they represent the range of sequence identity and structural diversity between the AAV serotypes, AAV2 transduces HEK293 more efficiently than AAV1 (23% of AAV2), AAV5 (2% of AAV2), and AAV8 (2% of AAV2) based on mean relative light unit (RLU). Comparison of the transduction efficiency (TE) of each serotype in the respective buffers (Figure 7). Similarly, there was no correlation between the Tm and TE of each AAV in the different buffers, except for AAV8, which displayed a minor upward trend (Figure 7). This observation is consistent with the cell culture media, in which the viruses, previously exposed to different buffers, are diluted by 10X, serving as the determinant of the virus properties and not the formulation buffers. This observation was anticipated because dilution of the viruses from Tris-HCl, utilized for neutralization after purification, into the different formulation buffers dictated their thermostability, as presented above. Thus, when working at a physiological or close to physiological pH, AAV vector storage buffer formulations dictate stability. However, the extracellular environment would dictate rAAV vector performance in vitro and likely in vivo.

### Discussion

The use of AAVs as a clinical gene therapy vector requires good manufacturing practice (GMP), with the characterization of the final product to include certification of purity, stability, genome titer, infectious titer, and absence of endotoxins, according to Food and Drug Administration (FDA) requirements (reviewed by Wright31). In this study, we developed a DSF protocol that determines the thermal profile or Tm of AAV capsids as a method for serotype identification. The reported dosage of AAVs utilized in several clinical and preclinical trials is 1012–1013 vg/kg or 1011–1014 vg/patient.32–34 The application of DSF for serotype identification required minimal samples of ~1011 vg/mL or particles/mL for full and empty capsids, respectively, in 22.5 μL of total volume to obtain a measurable fluorescence enabling Tm calculation (Figures 4A and 4B). This amount of material is easily attainable in therapeutic samples, making DSF a suitable approach for the proposed serotype identification.

Significantly, the distinct Tm of each AAV serotype in different buffer formulations enables their identification in ~1 hr. The Tm of AAV1–AAV9 and AAVrh.10 was a unique identifier for each serotype, with the exception of AAV7, AAV9, and AAVrh.10 in PBS, which were similar (Figure 1B; Table 1). These serotypes could, however, be distinguished based on their Tm measured in other buffers (Figure 2; Table 1). For example, AAV9 showed almost no variation in Tm in a different buffer formulation, whereas AAV7 showed a 3°C variation of Tm in the same buffers (Figures 2G and 2I), providing additional means for distinguishing the two viruses. A previous comparative

### Table 1. Tm of AAV1–AAV9 and AAVrh.10 Full and Empty Capsids in Different Buffers

| Buffer   | AAV1 Full | AAV1 Empty | AAV2 Full | AAV2 Empty | AAV3 Full | AAV3 Empty | AAV4 Full | AAV4 Empty | AAV5 Full | AAV5 Empty | AAV6 Full | AAV6 Empty | AAV7 Full | AAV7 Empty | AAV8 Full | AAV8 Empty | AAV9 Full | AAV9 Empty | AAV10 Full | AAV10 Empty |
|----------|-----------|------------|-----------|------------|-----------|------------|-----------|------------|-----------|------------|-----------|------------|-----------|------------|-----------|------------|-----------|------------|-----------|------------|
| PBS      | 83.5 ± 0.1| 83.8 ± 0.3 | 68.2 ± 0.6| 67.3 ± 0.8 | 71.7 ± 1.0| 71.2 ± 1.2 | 75.0 ± 0.7 | 75.0 ± 0.5 | 89.2 ± 0.1| 89.2 ± 0.3 | 85.0 ± 0.9 | 85.2 ± 0.6  | 67.2 ± 0.6 | 65.5 ± 0.9 | 67.8 ± 0.8 | 82.7 ± 0.6 | 83.0 ± 0.1 | 77.0 ± 0.7 | 76.0 ± 0.5 | 88.7 ± 0.1 | 88.7 ± 0.3 |
| GIP04    | 83.0 ± 0.1| 83.2 ± 0.6 | 68.1 ± 0.9 | 67.8 ± 0.3 | 71.9 ± 0.3 | 80.2 ± 0.3 | 86.8 ± 0.3 | 86.8 ± 0.1 | 76.5 ± 2.1 | 77.5 ± 0.5 | 89.0 ± 0.1 | 84.7 ± 0.6  | 75.2 ± 0.3 | 76.0 ± 1.8 | 78.5 ± 4.0 | 79.0 ± 2.5 | 75.3 ± 0.4 | 75.5 ± 0.5 | 89.0 ± 0.1 | 89.0 ± 0.1 |
| HEPES    | 85.0 ± 0.1| 85.0 ± 0.9 | 76.8 ± 0.3 | 77.5 ± 0.5 | 82.7 ± 0.6 | 83.0 ± 0.1 | 77.0 ± 0.7 | 76.0 ± 0.5 | 88.7 ± 0.1 | 88.7 ± 0.3 | 84.2 ± 0.3 | 71.2 ± 0.6  | 70.7 ± 1.8 | 76.3 ± 1.2 | 76.8 ± 0.3 | 75.5 ± 0.7 | 76.0 ± 0.5 | 89.3 ± 0.1 | 89.3 ± 0.3 | 89.0 ± 0.1 |
| Tris     | 86.0 ± 0.1| 85.0 ± 2.2 | 79.7 ± 0.3 | 80.2 ± 0.3 | 86.8 ± 0.3 | 86.8 ± 0.1 | 76.5 ± 2.1 | 77.5 ± 0.5 | 88.7 ± 0.3 | 88.7 ± 0.3 | 85.0 ± 0.5 | 76.3 ± 0.3 | 75.0 ± 1.3 | 81.0 ± 0.9 | 82.5 ± 2.8 | 77.8 ± 0.4 | 77.5 ± 0.5 | 89.0 ± 0.1 | 89.0 ± 0.1 |
| LR       | 84.5 ± 0.9| 84.7 ± 0.6 | 75.2 ± 0.3 | 76.0 ± 1.8 | 78.5 ± 4.0 | 79.0 ± 2.5 | 75.3 ± 0.4 | 75.5 ± 0.5 | 89.0 ± 0.1 | 89.0 ± 0.1 | 84.2 ± 0.3 | 71.2 ± 0.6  | 70.7 ± 1.8 | 76.3 ± 1.2 | 76.8 ± 0.3 | 75.5 ± 0.7 | 76.0 ± 0.5 | 89.3 ± 0.1 | 89.3 ± 0.3 | 89.0 ± 0.1 |
| BSS      | 84.3 ± 0.3| 84.2 ± 0.3 | 71.2 ± 0.6 | 70.7 ± 1.8 | 76.3 ± 1.2 | 76.8 ± 0.3 | 75.5 ± 0.7 | 76.0 ± 0.5 | 89.3 ± 0.1 | 89.3 ± 0.3 | 85.0 ± 0.5 | 76.3 ± 0.3 | 75.0 ± 1.3 | 81.0 ± 0.9 | 82.5 ± 2.8 | 77.8 ± 0.4 | 77.5 ± 0.5 | 89.0 ± 0.1 | 89.0 ± 0.1 |
| UB       | 85.3 ± 0.3| 85.0 ± 0.5 | 76.3 ± 0.3 | 75.0 ± 1.3 | 81.0 ± 0.9 | 82.5 ± 2.8 | 77.8 ± 0.4 | 77.5 ± 0.5 | 89.0 ± 0.1 | 89.0 ± 0.1 | 80.8 ± 3.0 | 78.5 ± 0.1 | 77.2 ± 0.3 | 73.3 ± 0.8 | 73.0 ± 0.1 | 77.5 ± 0.3 | 78.5 ± 0.9 | 78.5 ± 0.9 | 78.5 ± 0.6 |

Buffer abbreviations and formulations are provided in the Materials and Methods.
analysis of AAV capsid stability and capsid dynamics for AAV1, AAV2, AAV5, and AAV8 introduced DSF as a method for distinguishing AAV serotypes based on stability. The Tm obtained for AAV1, AAV2, AAV5, and AAV8 in this previous study in the PBS buffer used is consistent with our observations. In other studies of AAV2 and AAV5 in the lactate ringer (LR) and balanced salt solution (BSS) buffers, similar Tm values to those obtained in our study were reported. More recently, another group utilized this DSF approach...
to identify AAV1, AAV2, AAV5, AAV6-K531E, AAV8, and AAV9.38 Significantly, these previous studies used different biological “replicates” that were produced and purified using different protocols. The similarity of $T_m$s in the different buffers supports the validity of the DSF approach for serotype identification.

In addition to there being no significant difference between full and empty capsids (Figure 3; Table 1), the $T_m$s for full capsids packaging GFP or luciferase, e.g., AAV1 and AAV6, were the same in PBS (Figures 2A, 2F, and 5C). These data support the conclusion that the encapsulated genome is not a determinant of AAV capsid thermostability. It was recently reported, based on atomic force microscopy analysis of AAV2 empty capsids and those packaging single-stranded DNA (ssDNA) or self complementary DNA (scDNA) genomes, that the scDNA-containing capsid had greater resistance to deep deformation.39 This information was revealed by quantitative statistical analysis because the observable correlations with genome content were minimal.39 This observation implies that only a packaged

Figure 3. Comparative Analysis of the $T_m$ of Full, Packaging GFP Transgene, and Empty Capsids in the Different AAV Formulation and Storage Buffers
Plots show $T_m$ (°C) versus buffer (x-axis) for two selected rAAV serotypes from the buffer stability groupings. Group I was AAVs with $T_m$s varying by −15°C–20°C, AAV2 and AAV3 (top); group II was AAVs with $T_m$s varying by −3°C–6°C, AAV8 and AAVrh.10 (middle); and group III was AAVs with $T_m$s varying by <2.0°C, AAV5 and AAV9. The $T_m$ of the full rAAV capsids is shown in a black line, and the empty capsids are shown in a gray line.
Double-stranded genome imparted mechanical AAV capsid stability, not ssDNA, compared to empty capsids, as reported here.

The observation that VP3 content alone is the determinant of AAV capsid stability ensures that vectors generated, with modified quantities of VP1 and VP2, can be analyzed by DSF (Figures 5A and 5B). The VP3 observation is consistent with previous reports, in which AAV5 containing a reduced VP1 content mixed with AAV2 with a higher VP1 content analyzed by DSF resulted in two distinct Tms, corresponding to the Tm of AAV2 and AAV5 when tested alone. The reversibility of the AAV1 and AAV6 Tms by the single E531K difference (Figure 5C) confirmed this observation. The Tm for the AAV6-E531 variant is higher than that of AAV6 and equivalent to that of AAV1, whereas the Tm for the AAV1-K531 variant is equivalent to that of AAV6 within the ±0.5°C error associated with these measurements. Thus, DSF has the ability to differentiate among AAVs that differ by a single amino acid, provided the amino acid difference causes a change in the overall charge and pI of the virus capsid.

Previous analysis of the association energy and buried surface area for the symmetry-related VP:VP interactions of AAV1, AAV2, AAV5, and AAV8 capsids, based on the crystal structures, did not show any correlation with AAV capsid stability. Data reported here indicate that AAV serotype stability is based on the amino acid sequence of VP3 and is governed by the ratio of basic:acidic residues, which is correlated to the pI of the capsids (Figure 6). The reversibility of the AAV1 and AAV6 Tms by the single E531K difference (Figure 5C) confirmed this observation. The Tm for the AAV6-E531 variant is higher than that of AAV6 and equivalent to that of AAV1, whereas the Tm for the AAV1-K531 variant is equivalent to that of AAV6 within the ±0.5°C error associated with these measurements. Thus, DSF has the ability to differentiate among AAVs that differ by a single amino acid, provided the amino acid difference causes a change in the overall charge and pI of the virus capsid.

The group I AAV serotypes, with highly variable Tms in the different buffers, can have a broad tropism (e.g., AAV2) or limited host tropism, for example, AAV3B. The group II AAV serotypes, with intermediate variability in stability, can have limited tissue tropism, e.g., AAV1 for skeletal muscle and AAV8, which targets the liver. Finally, group III AAV serotypes, displaying little to no change in Tm in the different buffers, could also have broad (e.g., AAV9) or limited tissue tropism (e.g., AAV5). These observations show that storage buffer is not a determinant of tissue or cellular tropism, as was observed in the lack of any significant correlation with the level of gene expression in HEK293 cells (Figure 7). This suggests that the cellular or tissue environment encountered during infection dictates each AAV’s transduction efficiency, consistent with observed differences in cell or tissue tropism during comparative infection analysis.

In summary, DSF offers a rapid, cost effective, and robust method for AAV serotype identification in specific buffers because of their unique thermal stability profiles in the commonly utilized formulation and storage buffers. Table 1 reported here can serve as a “look up” reference table for the AAV serotypes tested because the data presented are reproducible, regardless of the source of sample production and method utilized for purification. The observation that capsid stability is based primarily on the charged VP3 residues ensures that Tm trends can be predicted for novel capsid variants compared to the WT virus. These data presented lead us to propose DSF as a method to be utilized for AAV vector identification post-storage and pre-administration as part of the quality control protocol for preclinical and clinical samples. Investigators should be able to add to the look up table for additional AAV serotypes or novel vectors of interest to expand it for all subsequent sample preparations.

MATERIALS AND METHODS

AAV Production

OneBac:Sf9 rep/cap producer cell lines of serotypes AAV1–AAV6, AAV8, AAV9, and AAVrh.10 were grown at 28°C in suspension culture under constant agitation to 1 L at 1.0 × 10^8 cells/mL. The infected
cells were harvested 72 hr post infection by centrifugation at 1.5K rpm in a JA-20 rotor. A successful infection was characterized by a bright green pellet due to intense GFP expression. The pellets were resuspended in TD buffer (1x PBS, 1 mM MgCl₂, and 2.5 mM KCl), and the supernatant was precipitated with 10% (w/v) PEG8000, as previously described. The cell pellets were subjected to three freeze-thaw cycles in a dry ice/ethanol freezing bath and a 37°C water bath. After the third freeze-thaw cycle, the cell pellet and resuspended polyethylene glycol (PEG) pellet were treated with Benzonase (250 U) and incubated at 37°C for 30 min. Both solutions were clarified by centrifugation at 10,000 x g at 4°C for 20 min for purification (see below).

rAAV luciferase (rAAV1-luc, rAAV2-luc, rAAV5-luc, rAAV8-luc, rAAV7-luc, rAAV6-luc, rAAV6-E531-luc, and rAAV1-K531-luc) packaging vectors were produced by triple transfection in HEK293 cells. For this purpose, cells were seeded on 15-cm cell culture dishes. At a confluence of 75%, the old growth medium was replaced with 15 mL of fresh DMEM. The three plasmids pHelper, pTR-UF3-luc, and pXRAAV (for the respective virus as listed above) were mixed at an equimolar ratio (total: 40 mg to be used per 15-cm plate) and diluted in OptiMEM (Gibco) to a volume of 1 mL (per 15-cm plate). After adding 125 µL of polyethyleneimine (1 mg/mL), the solution was mixed, incubated at room temperature (RT) for 15 min, and added dropwise to the HEK293 cells. The following day, 5 mL of fresh DMEM were added to each plate. The cells were harvested after 72 hr and treated as described above for the Sf9 cells.

The AAV2 VP variant VLPs were generated by site-directed mutagenesis (Agilent) of the AAV2 plasmid pFBDVPM11. The VP1
and VP2 start codons were mutated to GCG to generate AAV2-VP13, AAV2-VP23, and AAV2-VP3 only constructs. The WT and mutant plasmids were used to generate recombinant AAV2 VLPs in Sf9 cells by the Bac-to-Bac expression system (Invitrogen) according to the manufacturer’s protocol. The infected S9 cells were harvested by centrifugation at 1,100 × g for 20 min in a JA10 rotor. The pellets were resuspended and prepared for purification as described above for the samples produced in the S9 cell lines.

Figure 7. Transduction Efficiency of rAAV Diluted into Different Buffers
The normalized relative fluorescence (RFU) in HEK293 cells is shown for rAAV1-luc, rAAV2-luc, rAAV5-luc, and rAAV8-luc post incubation in the different buffers (as indicated on the right-hand side) above a plot of the Tm (left-hand side) and TE (right-hand side) against the same buffers. A similar trend in stability and TE is only observed for AAV8.
AAV Purification by AVB and Capture Select Affinity Ligand Column Chromatography Followed by Sucrose Gradient

The clarified supernatants from the various virus production protocols described above were diluted 1:1 with TD buffer and loaded onto a pre-packed 1-mL AVB Sepharose HP column (GE Healthcare) (for AAV1–AAV8 and AAVrh.10) or a gravity flow column packaged from the capture select affinity ligand for AAV9 (CSA9) at a flow rate of 0.5 mL/min. After loading, the column was washed with 20 mL of TD buffer. The virus was eluted with elution buffer (0.1 M NaAc and 0.75 M NaCl, pH 2.5), and the peak fractions were collected and neutralized with 1 M Tris-HCl, pH 10 (neutralization buffer), at a 1:10 ratio. The elution fractions that showed VP1, VP2, and VP3 on an SDS-PAGE gel were pooled, and the sample was concentrated to a volume of 500 μL and loaded onto a 5%–40% step sucrose gradient. Empty and full virus capsids were separated by centrifugation at 35K rpm in an SW41 rotor for 3 hr at 4°C. The empty capsids were extracted with a syringe from the 20%–25% sucrose fraction and the full capsids from the 30% to 35% fraction. The samples were buffer exchanged into 20 mM Tris-HCl, pH 7.4, and 150 mM NaCl and concentrated by pelleting at 50K rpm in an SW51 rotor. The pellet was resuspended in the Tris-HCl buffer, and the sample concentrations were determined by UV spectrometry for the empty capsids (E = 1.7 for concentration in mg/mL) and titers were determined by qPCR for full capsids, as previously described.19

Determination of the Thermal Stability of AAV Capsids by DSF

This method monitors the binding of the SYPRO Orange dye to hydrophobic regions of proteins that are exposed during unfolding. For the comparative analysis of full and empty capsids, 2.5 μL of each AAV serotype, at a concentration of 1 × 10^14 vg/mL or 1 mg/mL (1 × 10^{13} particles), respectively, were added to 20 μL of each of the six commonly used buffer formulations for AAV vector storage and gene therapy applications and a newly developed buffer called UB (see formulation below). This buffer was created to overcome potential effects of temperature on pH when thermal stability assays are conducted at low pH and was included here as a control. For the comparative analysis of full and empty capsids, 2.5 μL of each AAV serotype, at a concentration of 1 × 10^14 vg/mL or 1 mg/mL (1 × 10^{13} particles), respectively, were serially diluted 100x in PBS, and 22.5 μL of each dilution was used for the DSF analysis, as described above. To determine the role of the AAV VPs in capsid stability, WT AAV2 and VP variant VLPs were assayed, also in PBS, at a concentration of 1 × 10^13 particles/mL, as described above, but without dilution because the samples were already in the assay buffer. To determine the role of basic and acidic residues in capsid stability, rAAV1-luc and rAAV6-luc and their variants, rAAV1-K531-luc and rAAV6-E531, at concentrations of ~1 × 10^{11}–1 × 10^{12} vg/mL in PBS, were assayed by DSF, as also described above.

Transduction of HEK293 Cells by rAAV-luc Vectors in Different Buffer Formulations

The genome-containing titer of rAAV1-luc, rAAV2-luc, rAAV5-luc, and rAAV8-luc vectors, with a packaged luciferase transgene, were determined by qPCR, as previously described.17 For the transduction assay, ~2.5 × 10^6 HEK293 cells were seeded in a 96-well plate 1 day prior to infection. On the day of infection, the rAAV vectors were diluted into the six common AAV buffers plus UB (total volume: 10 μL) to infect cells at an MOI of 10^4 for AAV2 or 10^5 for AAV1, AAV5, and AAV8. The difference in the MOI used for AAV2 and the other AAVs stems from the higher transduction level observed for AAV2 in vitro compared to the other AAV serotypes tested.1 The AAV-buffer mixtures were incubated at 4°C for 1 hr. Prior to infection, 90 μL of DMEM supplemented with 2% FBS and 1% antibiotic and antifungal (ABAM) was added to the mixtures. The old media on the cells was discarded, and the virus-buffer-medium mixture (total volume: 100 μL) was added to the cells. After incubating the cells at 37°C and 5% CO2 for 48 hr, the medium was removed and the cells were washed with PBS. For the luciferase assay, the cells were lysed and the luminescence was determined using the Luciferase Assay Kit (Promega), according to the manufacturer’s protocol, on a Synergy HTX plate reader (BioTek).

AUTHOR CONTRIBUTIONS

A.B. and M.M. contributed to study conception, Figures 1–7, and manuscript preparation; S.P., A.J., and J.C.Y. contributed to Figures 1 and 2; B.L. and B.B. contributed to study conception; R.M. and...
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