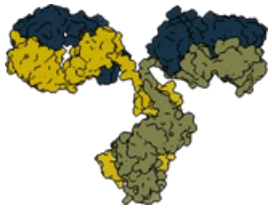
Protein Stable
Applied Innovations in Protein Characterisation

KBI BIOPHARMA



Identification of AAV Serotypes and Characterisation of their Stability by DSF for use in a Gene Therapy Program

Introduction

Gene therapy is a therapeutic technique that modifies a person's genes to treat or cure a disease. This modification can work by replacing a disease-causing gene with a healthy copy of the gene, inactivating a disease-causing gene, or introducing a new gene into the body to help treat a disease. There are a wide variety of gene therapy treatments, but a common technique is to use viral vectors, such as adeno-associated viruses (AAVs), to deliver therapeutic genes into human cells for replication.**[2]**

AAV's have many favourable features including their lack of pathogenicity, replication incompetence, ability to infect non-dividing cells, and their ability to integrate into the host cell genome at a specific site.**[3]** These promising features led to their advancement with early research focusing on a single AAV variant (serotype) AAV2. Since then, new AAV serotypes have been discovered to provide higher transduction efficiencies in certain cells or tissues compared to AAV2. These unique cell tropisms have led to the development of a larger number of AAV serotypes to package different therapeutic genes to treat specific disease.**[4]** The different AAV serotypes not only have different cell tropisms, but they also differ in their structural properties and stability due to their varying amino acid sequences and capsid structures. These differences give each AAV serotype a unique melting temperature.**[4,5]**

To develop efficient AAV therapeutics, it is crucial to characterise key attributes of AAV's during the development process. One key attribute is the thermal stability of the AAV capsid, which can be used for serotype identification, optimising capsid formulation, and for comparison purposes during any process changes.**[6]** Capsid stability can be measured with differential scanning fluorimetry (DSF) by monitoring the change of intrinsic fluorescence from the tryptophan residues within the AAV during a thermal ramp.

In this application note we used the SUPR-DSF to measure the thermal stability of three AAV serotypes: AAV2, AAV5, and AAV6. The SUPR-DSF measured the intrinsic fluorescence of each serotype during a thermal ramp to generate melt curves. In addition, we performed a concentration dilution series on AAV2 and AAV6 to determine the minimal sample requirements. All samples were performed at a well volume of 10µL in triplicate on a single 384-well microplate emphasising the high-throughput possibilities of the instrument.

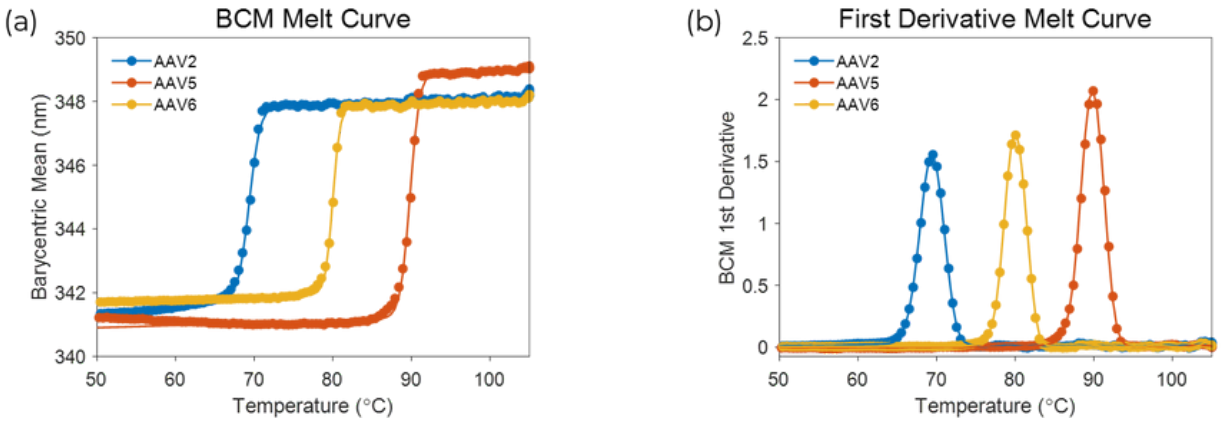


Figure 1 – *(a) Barycentric mean (BCM) melt curves for 3 AAV serotypes: AAV2, AAV5, and AAV6. (b) First derivative representation of BCM melt curve where the peaks correspond to the melting temperatures.*

Table 1 – *Melting temperatures for AAV serotypes determined by fitting a gaussian to the first derivative of the barycentric mean (BCM) melt curve. Standard deviation (SD) values are for the Tm. The errors were found to be the same or less for onset temperatures (Tonset).*

Samples	*Tonset (°C)*	*Tm (°C)*	*SD (°C)*
AAV2	65.8	69.4	0.04
AAV5	86.5	89.9	0.07
AAV6	76.8	80.0	0.01

Results

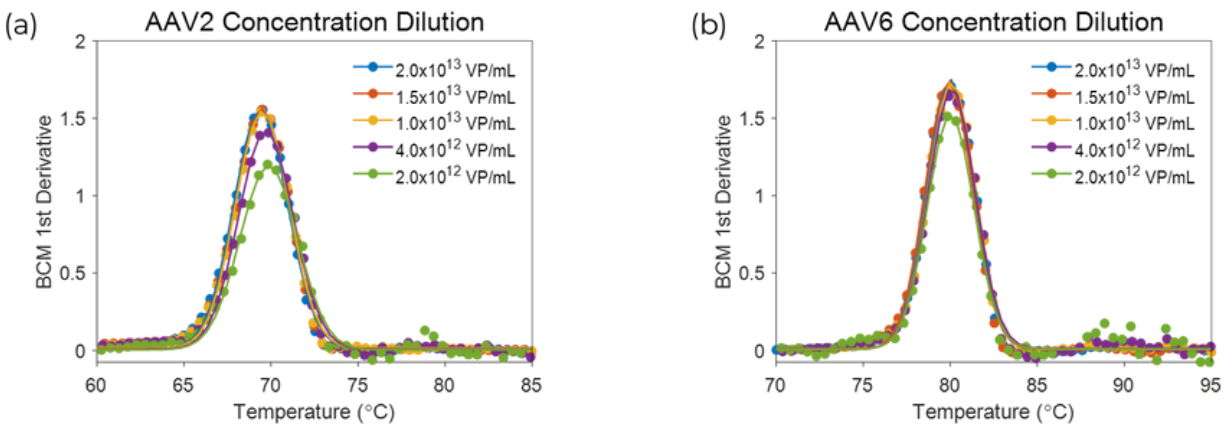
The barycentric mean (BCM) melt curve for AAV2, AAV5,andAA6areshowninFigure 1(a).The AAV serotypes have a very flat pre- and post-transitionbaselineswithasteepinflectionpoint between the baselines. All three AAV serotypes shifttolongerwavelengthduringunfoldingdue to the tryptophan becoming exposed to the aqueousenvironmentaftertheircapsidsbreak.The steep transition, i.e. temperature range it takes tomovefromthefoldedtounfoldedbaseline, implies this happens very quickly. The fast transitionisalsoevidentbylookingattheonset temperature (Tonset), which on average is only 3.4°Cbeforethemeltingtemperature(Tm)forthe three AAVs. The fast unfolding is expected for AAVssincewearemeasuringacapsidbreaking compared to a slower unfolding typically observedwithmostproteinsorantibodies.

Figure 1(b) illustrates the first derivative of the BCMmeltcurvewherethepeakscorrespondto the apparent melting temperatures of the AAV capsids.Thefirstderivativemeltcurveswerefitto a gaussian to obtain the melting temperatures (Tm),whichareshowninTable 1.Thereplicatesfor each AAV agreed very well with SD < 0.1°C and themeltingtemperaturevaluesagreedwithother reported values.[4] The three AAVs had a wide variationinmeltingtemperaturesduetothe differences in their amino acid sequences and capsidstructures.AAV2hadthelowestmelting temperature of 69.4°C, followed by AAV6 at 80.0°C,andthemoststableserotypewasAAV5at 89.9°C. The variation of melting temperatures highlightstheimportanceofmeasuringthermal stability of your AAVs during the entire developmentprocesssinceasmallchangecanleadtoa large impact on product stability.

Next, we looked at a concentration dilution series ofAAV2andAAV6toseewhattheminimal amount of sample is required to accurately measuretheirstability.TheBCMfirstderivativemelt curve for AAV2 **Figure 2(a)** and AAV6 **Figure (2b)** areshownforfivedifferentconcentrations.The fitted melting temperatures for both AAVs are displayedinthe**Figure (2c)** withthereplicatesfor both serotypes agreeing very well with SD < 0.1°C forallconcentrations.BothAAV2andAAV6 produced excellent melt curves for all concentrationstestedshowingwecanmeasurecapsid stability for these two AAV serotypes at 2x1012 VP/mL.

Looking at the results, we can determine the meltingtemperaturesforAAV2begintoshiftto slightly higher temperatures for the two lowest concentrationsamplesversusthehigher concentration samples. This is evident in the **Figure 2(a)**wherethetwolowestconcentration samples have a slightly lower magnitude, and theirpeaksareshiftedslightlytotheright.Onthe other hand, AAV6 showed nearly identical meltingtemperaturesforallconcentrationstested. The lowest concentration had a small magnitudechange,butthisdidn'taffectthemelting temperature.

The differences in stability between AAV2 and AAV6atlowerconcentrationscouldbeduetotheir slight differences in their amino acid compositions.AAV2alsohasaslightlylowerBCM1st derivative amplitude making it more susceptible toeffectsfrombackgroundatlower concentrations. These characteristics can explain thestabilitydifferenceswearesseeingfromthe serotypes and is a key attribute that can be used whiledevelopingyourproduct.Therefore,itis recommended to always use the same concentrationofAAVwhenyouareperformingany comparison studies or formulation screens.



(c)

Sample (VP/mL)	AAV2 Tm (°C)	SD (°C)	AAV6 Tm (°C)	SD (°C)
2.0 x 1013	69.4	0.04	80.0	0.01
1.5 x 1013	69.5	0.06	79.9	0.01
1.0 x 1013	69.5	0.01	80.1	0.01
4.0 x 1012	69.7	0.01	80.1	0.03
2.0 x 1012	69.9	0.07	80.0	0.04

Figure 2 – *BCM first derivative melt curves for a concentration dilution series for (a) AAV2 and (b) AAV6. (c)Table of melting temperatures with errors for AAV2 and AAV6 concentration dilution series.*

Conclusion

The SUPR-DSF from Protein Stable demonstrates high data quality, capable ofquantifyingstability of AAV capsids in a 384-well microplate at a well volume of 10µL. Three differentAAVserotypes were characterised (AAV2, AAV5, and AAV6), and the melting temperaturesobtainedfromthe BCM first derivative melt curves agreed very well with reported values.**[4]** Themeltcurveswere observed to have very fast transitions, which aligns well with the expectedbehaviourofAAV capsids breaking during a thermal ramp. The melting temperatures variedby20°C,highlighting the large differences in stability among the various AAV serotypes. Theselargedifferencesin stability enable scientists to use DSF measurements to identify serotypesandaddstothe importance of measuring AAV product stability all along the developmentprocess.

The concentration dilution series of AAV2 and AAV6 exhibited concentrationvariationsbetween the serotypes. AAV2 has a small concentration effect, with lower concentrationsampleshavinga slightly higher melting temperature compared to the control. On theotherhand,all concentrations for AAV6 had nearly identical melting temperatures. ThedifferencesinAAV2 stability demonstrate why it is important to use the same concentrationofsamplewhen characterising AAV samples during development. The results from the concentrationstudies indicate a concentration of 2x1012 VP/mL is sufficient to measure the stabilityofyourAAVcapsids safely and accurately.

The experiments demonstrated here show how the SUPR-DSF can be a valuabletoolin characterising AAVs during the development process in a gene therapy program.TheSUPR-DSF can determine the AAVs' stability by measuring their intrinsic fluorescence duringathermal ramp, eliminating the need to contaminate your samples with extrinsic dyes. Pairingthiswith the ease of use of 384-well microplates enables easy workflow scaling up to largerscale experiments that require increased number of samples.

Methodology

Sample Preparation

The AAVs were sourced from Virovek. AAV5 and AAV6 were formulated in PBS with 0.001% Poloxamer 188 (P188). AAV2 was formulated in PBS with 100 mM citrate and 0.001% P188. Samples were manually pipetted in triplicate into a 384-well plate at a 10µL well volume. After dispensing, the plate was sealed with an optically clear adhesive film. The plate was then immediately transferred to the SUPR-DSF for measurement.**[1]**

Thermal Denaturation Measurement

The SUPR-DSF was set-up to measure the fluorescence spectra of the AAV samples from 20°C to 105°C with a 1°C per minute ramp rate. Quantification of the spectral shift, that is indicative of protein denaturation, was determined via the barycentric mean (BCM) calculation. Background subtraction was done on all samples by subtracting the PBS control wells from the sample data. The BCM data was analysed as a function of temperature and the first derivative was determined from the BCM melt curve. Melting temperatures were calculated from the peaks of the derivative.

Methods and results for all samples are available on request.

References

[1] – KBIBiopharma (Louisville, CO) sourced all the AAVs, performed the sample preparation, and data acquisition with the SUPR-DSF. https://www.kbibiopharma.com

[2] – "What is Gene Therapy". U.S. Food & Drug. https://www.fda.gov/vaccines-blood-biologics/cellular-gene-therapy-products/what-gene-therapy. Accessed April 2024.

[3] – Kotin RM, Siniscalco M, Samulski RJ, Zhu XD, Hunter L, Laughlin CA, McLaughlin S, Muzyczka N, Rocchi M, Berns KI. Site-specific integration by adeno-associated virus. Proc Natl Acad Sci U S A. 1990, Vol 87, p. 2211-2215.

[4] – Bennet A, Patel S, Mietzsch M, Jose A, Lins-Austin B, Yu J, Bothner B, McKenna R, Agbandje-Mckenna M. Thermal Stability as a Determinant of AAV Serotype Identity. Molecular Therapy Methods & Clinical Development, 2017, Vol 6, p. 171-182.

[5] – Rieser R, Penaud-Budloo M, Bouzelha M, Rossi A, Menzen T, Biel M, Büning H, Ayuso E, Winter G, Michalakis S. Intrinsic Differential Scanning Fluorimetry for Fast and Easy Identification of Adeno-Associated Virus Serotypes. Journal of Pharmaceutical Sciences, 2020, Vol 109 (1), p 854-862.

[6] - Cole, L, Fernandes, D, Hussain, M, Kaszuba, M, Stenson, J, Markova, N. Characterization of Recombinant Adeno-Associated Viruses (rAAVs) for Gene Therapy Using Orthogonal Techniques. Pharmaceutics 2021, 13, 586.

Protein Stable

21Mole BusinessPark
Leatherhead
Surrey
KT22 7BA
United Kingdom