DEVELOPMENT OF AN ANALYTICAL METHOD FOR AAV CAPSID PROTEINS FOR USE IN STABILITY STUDIES.

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Abstract

Gene therapy based on adeno-associated virus (AAV) vectors is now a reality, with several products already approved by the FDA and EMA (HEMGENIX®, LUXTURNA®, ROCTAVIAN® ZOLGENSMA®,) and some more to come in the next years. In parallel to this evolution, the requirement for fast and reliable characterization of the Critical Quality Attributes (CQAs) to ensure AAV product quality is also increasing. Among those CQAs, the viral capsid proteins (VP) charge distribution is important for the tropism and potency of the different serotypes (1, 2). The capsid protein's charge heterogeneity can account for possible post-translational modifications (PTM) that can happen during different steps of AAV vector manufacturing or storage; some of these modifications can hinder the development of gene therapy products (3). To monitor these modifications, we have developed a method based on imaged capillary isoelectric focusing (icIEF). Since different AAV products can require different formulation buffers which are not always compatible with icIEF analysis, we have developed a procedure that can be theoretically used with any product and then adjusted to obtain the best results in terms of reproducibility. It was previously shown that deamidation of amino acids on the surface of the AAV capsids correlates with charge heterogeneity modifications of the capsid proteins (4) and can have an impact on vector potency. Using icIEF, we show here that the deamidation of AAV9 capsid proteins over time is correlated with the reduction of vector potency. We also checked if this reduced potency was correlated with capsid disassembly. We believe such a method could be particularly useful for the development of improved formulation buffers.

Introduction

The large increase in recent years in the number of gene therapy products developed and the rapid progress from initial clinical studies to authorization and commercialization, have increased the requirements for the characterization of such products. This situation has highlighted the need to develop new analytical and quality control tools that are sufficiently precise, fast, and robust for the comprehensive characterization of the production process and the final product. Among them, the capillary isoelectric focusing (icIEF) method consists of the capillary separation of the macromolecules present in a solution based on their different isoelectric point (pI). This method is used to characterize the heterogeneity of adeno-associated vectors (rAAV) and to guarantee the stability and identity of the product, determining its different components, when the analysis is carried out under denaturing conditions. This work shows the results obtained in the study of the stability of several batches of serotype AAV9 under different conditions.

Methods

AAV vectors production. The AAV vectors were produced through transfection of Pro10™ cells (5), using three plasmids into cell suspension cultures. Cells were harvested after 72 hours and disrupted using non-ionic detergent. AAV particles were purified from clarified lysates through AAV9 affinity chromatography, then full AAV particles were collected from an iodixanol gradient and submitted to ion exchange followed formulation through TFF.

Vector genomes concentration. For vector genome titration, AAV samples were treated with DNase I at 37°C, then digested with proteinase K at 55°C followed by enzyme heat inactivation at 80°C. AAV vectors genome concentrations were measured by droplet digital PCR (ddPCR,Bio-Rad) using primers and probe targeting the AAV2 ITR (6).

Vector capsid concentration. For viral capsid titration, AAV samples were analyzed by ELISA (Enzyme-Linked Immunosorbent Assay). Samples were diluted based on vector genome titers and analyzed using the AAV9 Titration ELISA kit from Progen (PRAAV9)

Viral particle size. For viral particle size determination. Samples were analyzed by DLS (Dynamic Light Scattering) using the Zetasizer-nano-s (Malvern Panalytical).

AAV vector potency determination. To determine the potency of the AAV samples, GM16095A cells (human skin fibroblasts) grown in Eagle´s Minimum Essential Medium (EMEM) supplemented with 10% Fetal Bovine Serum (FBS) were transduced at different multiplicities of infection [MOI: vector genomes (vg)/cell] and the expression of luciferase was analyzed 48 hours post-transduction using the Pierce ™ Firefly Luciferase Glow Assay Kit.

Viral capsid integrity. To determine the capsid integrity, the AAV samples were analyzed by Dot-blot. Samples were incubated in presence/absence of SDS (Sodium Dodecyl Sulfate) and heated or not at 95°C to avoid or force capsid protein denaturation, then transferred on nitrocellulose membranes. Membranes were probed with the ADK9 antibody (Progen, #610162) to identify intact capsids, and with the B1 antibody (Progen, #65158) to detect denatured capsid proteins.

icIEF method. For the analysis of AAV vectors, the method is as follow:

- A master mix is prepared, containing 4% broad-range ampholytes (pH 3–10), 0.35% methylcellulose, two internal pI markers (at pI 5.85 and 8.40), 6M urea, 1% β-mercaptoethanol (BME) and 10 mM Arginine. The AAV sample is heated at 70°C for 5 min and mixed 1/10 with the master mix to reach a final concentration of 7E+12 VP/mL.
- The icIEF protocol is 1 min. pre-focusing at 1,500 V, followed by 2.5 min. focusing at 3,000 V in the Maurice instrument (Bio-Techne).

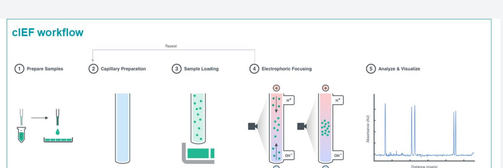

Fig 1. Representation of the icIEF workflow.
The imaged capillary isoelectric focusing (icIEF) method consists in the preparation of the sample in the specific denaturing conditions for the analysis, the capillary conditioning, sample loading, electrophoric focusing based in the isoelectric point of the sample (pI) and the analysis.

Results

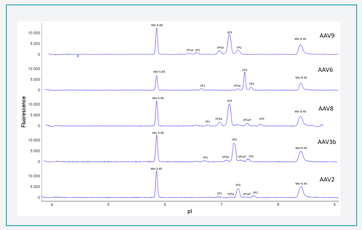

Fig 2. Electropherogram of specific charged species for various AAV serotypes.
An iCIEF method was developed to determine the distribution of charged species for several AAV capsid serotypes. Each serotype has a distinctive charge distribution which can be used to assign capsid identity. The relative abundance of each peak and the pI value obtained in the analysis were used to assign the VP protein distribution. *VPd: deaminated VP protein*.

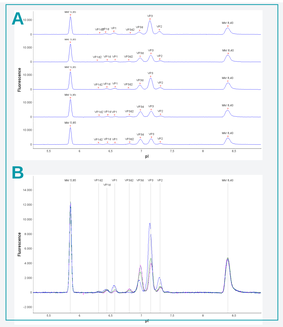

Fig 3. Analysis of different AAV9 lots by iCIEF to check repeatability of the method.
(A) Electropherogram representation of VP charge distribution in different lots of AAV9. (B) Charge distribution overlay of the different AAV9 lots analyzed. The relative abundance of each peak varies between lots, but the charge distribution remains unchanged.

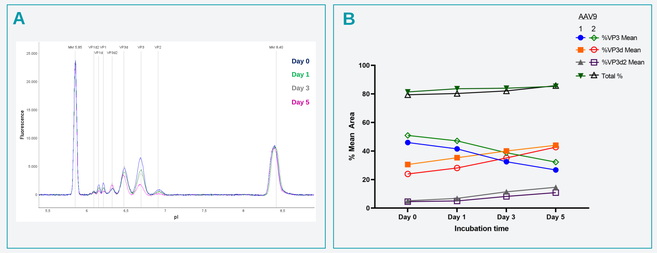

Fig 4. Incubation of two different AAV9 lots at 40°C to promote deamidation.
Two different lots of AAV9 were incubated at 40°C to force a change in the ratio of the different charged species, to study the effect of the theoretical deamidation of the VP proteins in the viability of the AAV9 vector. (A) The icIEF analysis of an AAV9 vector incubated for 0, 1, 3 and 5 days at 40°C. The electropherogram overlay shows a change in the relative abundance of each peak which varies between days of incubation, but the charge distribution remains unchanged. (B) The percentage of the mean peak area of each charged species varies with the incubation time at 40°C. The percentage of the mean VP3 peak decreases over time while the percentage of the mean peak area of VP3 deamidated species (VP3d and VP3d2) increases. The total % accounts for the total amount of VP3, i.e. the sum of %VP3 + %VP3d + %VP3d2 in each sample (two AAV9 lots: AAV9-1 and AAV9-2).

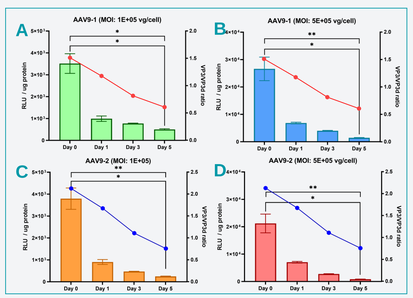

Fig 5. Potency assay of two different AAV9 lots incubated at 40°C to force deamidation.
To determine the effect of the incubation at 40°C in the potency of the AAV9 vector, GM16095A cells were transduced at two different multiplicities of infection (MOI: 1E+05 vg/cell and 5E+05 vg/cell) and expression of the luciferase transgene was analyzed 48 hours post transduction. The figure shows that when the ratio VP3/VP3d is reduced over time, the transduction efficiency decreases. One-way ANOVA followed by Friedman test was used to analyze the data. (A and B) AAV9-1 transduced at MOI 1E+05 and 5E+05 vg/cell (*P=0,0133, **P=0,0017). (C and D) AAV9-2 transduced at MOI 1E+05 and 5E+05 vg/cell (*P=0,0133, **P=0,0017).

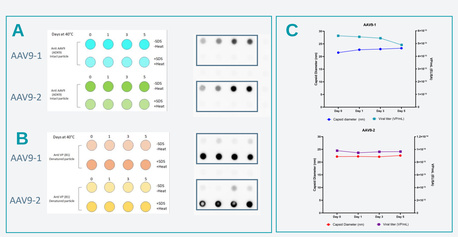

Fig 6. Effect of temperature on assembled capsid stability.
To determine if the reduction in vector potency was due to capsid disassembly, the AAV9 samples incubated at 40°C were analyzed by Dot-Blot (A and B), DLS and ELISA (C). Dot-Blot analysis of untreated (-SDS/-Heat) or denatured (+SDS/+Heat) samples from lots AAV9-1 and AAV9-2 probed with antibody ADK9 (A) or antibody B1 (B). The results show that the incubation at 40 °C does not cause the disassembly of the capsid. (C) Analysis by DLS and ELISA of untreated samples from lots AAV9-1 and AAV9-2. The results show that there is no significant changes in the particle diameter and the capsid titer (VP/mL).

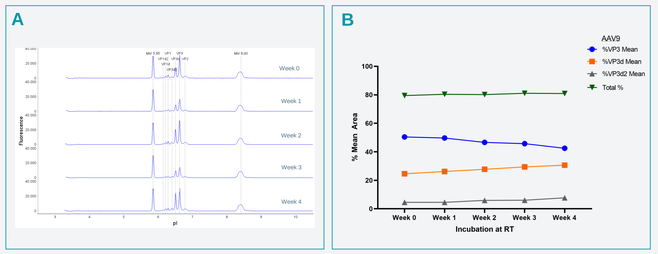

Fig 7. Analysis of AAV9 vector stability at room temperature.
One AAV9 lot was incubated at room temperature (RT) for 0, 1, 2, 3, or 4 weeks and then analyzed by icIEF to check distribution of the different charged species and determine the ratio between VP3 and deamidated VP3 (VP3d and VP3d2). (A) Electropherograms of the AAV9 lot after different incubation times show a small change in the relative abundance of each peak, but the charge distribution remains unchanged. (B) The percentage of the mean peak area of each charged species varies with the incubation of the AAV9 vector. The percentage of the mean VP3 peak decreases over time while the percentage of the mean peak area of VP3 deamidated species (VP3d and VP3d2) increases. The total % accounts for the total amount of VP3 in the sample, i.e. the sum of %VP3 + %VP3d + %VP3d2 in the sample.

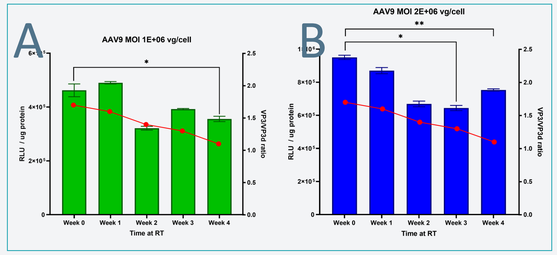

Fig 8. Potency assay of an AAV9 lot to determine vector stability at room temperature.
To determine the effect of the incubation at RT on the stability of an AAV9 vector, GM16095A cells were transduced at two different multiplicities of infection (MOI: 1E+06 vg/cell and 2E+06 vg/cell) and expression of the luciferase transgene was analyzed 48 hours post transduction. The figure shows that when the ratio VP3/VP3d is reduced over time, the transduction efficiency of the vector decreases. This reduction is less pronounced after weeks of incubation at RT compared to days of incubation at 40°C. One-way ANOVA followed by Friedman test was used to analyze the data. (A) AAV9 transduced at MOI 1E+06 (*P=0,0417). (B) AAV9 transduced at MOI 2E+06 vg/cell (*P=0,0456, **P=0,0083).

Discussion

The icIEF analytical method developed can be used to analyze different AAV capsid serotypes and distinguish the specific charge distribution of each of them (Figure 2) which might be used to determine the AAV capsid identity. This method is also reproducible and can be used to determine the mean peak area distribution of different lots from the same vector, as shown here with AAV9 (Figure 3). This analysis shows that different lots can have different peak area distribution depending on, for example, date of manufacturing and storage temperature. To check viability of the method for controlling stability of AAV products, we forced the deamidation of two AAV9 lots through incubation at 40°C up to 5 days, then analyzed the charge distribution and mean peak area, as well as vector potency and capsid stability (Figures 4, 5 and 6). These analyses show that changes in the capsid charge distribution over time (ratio VP3/VP3d) is correlated with lower vector potency.
Since incubation at 40°C is not a condition usually encountered by AAV products, we analyzed AAV9 samples incubated at room temperature for a longer period (0 to 4 weeks). We show that the incubation at room temperature also changes the mean peak area distribution and reduces vector potency, although to a lower extent.

Conclusion

According to our assay development results, icIEF appears as a relevant tool to add to the panel of quality controls for recombinant AAV products, as it could participate in the assessment of several quality attributes, including capsid identity, capsid stability and capsid proteins homogeneity. The assay might be particularly useful in the context of long-term stability studies, as well as for the development of improved formulation buffers.

References

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