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Commentary

Resolving hidden subpopulations of filled AAVs by probing capsid integrity

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Manufacturing recombinant adeno-associated virus (rAAV) is a complex process that hinges on various critical quality attributes (CQAs) to ensure the production of effective therapeutic products. The potency of rAAV products remains an elusive CQA, primarily due to a lack of representative bioassays and the innate complexity of how rAAV interacts with *in vivo* host systems. A traditional indicator of potency is the presence of DNA cargo inside the viral capsid, specifically the quantity of filled capsids (with full transgene cargo), as opposed to the non-potent empty capsids (without cargo). The assessment of the filled-to-empty ratio has heavily relied on bulk measurements, such as the polymerase chain reaction (PCR), which has limited capabilities in resolving the full spectrum of DNA cargo heterogeneity, including the identification of partial capsids. Recent advancements in high-resolution techniques, such as mass photometry (MP) and charge detection mass spectrometry (CDMS),[1] have enhanced our ability to characterize rAAV particles beyond a binary classification of filled or empty. However, DNA cargo cannot fully explain variability in therapeutic potency (Figure 1).

In a study published in this issue of *Molecular Therapy Methods & Clinical Development*, Ebberink and colleagues[2] characterized rAAV structural integrity under thermal stress to resolve previously hidden subpopulations of filled particles with potential implications regarding potency.

Previous approaches to evaluate thermal stability involve thermal melting studies carried out by differential scanning fluorimetry (DSF)[3,4] and CDMS.[5,6] Alternative characterizations include inducing thermal stress and quantifying filled-to-empty ratios using assays such as analytical ultracentrifugation (AUC), transmission electron microscopy (TEM), size-exclusion chromatography with multi-angle light scattering (SEC-MALS), and more recently, MP.[7]

In the study by Ebberink et al., the authors employed a novel combination of MP and nuclease treatment to probe structural integrity during the thermally induced uncoating process of rAAV particles. Their approach introduces a rapid (30 min) and low-volume (15 μL) method to quantitatively characterize the relationship between thermal stress, genome release, and capsid stability. By including a co-incubation procedure with nuclease, this study discovered that filled AAV8 particles consist of three subpopulations with identical mass: "intact" particles that do not respond to thermal stress, "fragile" particles that are prone to disintegration, and "exposed" particles that have partially disintegrated capsids and genomes that are exposed and accessible to nucleases. The exposed particles are at an intermediate stage where upon prolonged exposure to thermal stress, their capsids will be fully disintegrated while releasing the genome or aggregate with other capsids. The exposed rAAV particles, inducible at relatively low temperatures (~45°C), represent a potential source of process impurities that may have been previously overlooked due to their similar mass to intact particles.

To accurately measure the relative abundance of distinct rAAV subpopulations during thermal stress, the authors utilized a double-stranded plasmid, pBR322, as an internal quantitative standard. This plasmid's distinct mass and high thermal stability made it a reliable reference, and its presence did not interfere with the measurement of rAAV particles. With the pBR322 internal standard, the study further validated that empty rAAV capsids are more heat stable compared to their filled counterparts, which disintegrate more rapidly under thermal stress. Moreover, the study found that the disappearance of filled capsids due to heat treatment was not accompanied by an increase in empty capsids, in contrast with what has been proposed in recent studies.[5,8,9]

A major technical advantage of MP-based methods is the rapid time to result and low sample requirements, both of which help reduce the barrier to entry in current analytical laboratory settings. The experimental protocol involves a sample volume of approximately 15 μL, with a concentration range of 1–5 × 10^{12} vector genomes (vg)/mL. The samples were subjected to heat treatment for 15 min, followed by digestion with 100 nM of DNase I and co-incubation for 15 min. This approach required ~30 min for treatment, with a total measurement time of less than an hour. This rapid turnaround time and minimal sample volume make the method highly appealing to biomanufacturing applications. However, to apply this method for in-process or early-stage assessments during upstream cultivation, additional purification and concentration steps may be necessary.

Evaluating the potency of each subpopulation of filled rAAV with variable integrity (namely intact, fragile, and exposed) is crucial, as they may be misclassified as intact particles and remain in the final formulation. This evaluation can be practically achieved by analyzing existing retain samples with matching potency assay readouts using MP in combination with nuclease treatment. A study by Rossi and colleagues[10] has shown that in dendritic cells, rAAV vectors with reduced thermal stability have enhanced transduction efficiencies due to improved uncoating in the nucleus. Therefore, the non-intact subpopulations

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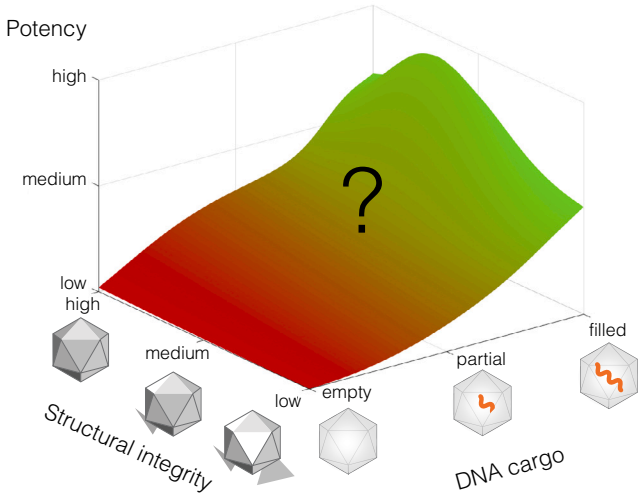

Figure 1. Conceptual plot of potency as a function of not only DNA cargo but also structural integrity of capsids

The potency of rAAV products remains an elusive CQA.

discovered in the present study may be more prone to undergo uncoating after nuclear entry, which could potentially enhance transduction efficiency in the host cells.

The long-term stability of the newly identified subpopulations is also worth further investigation. The present study focuses on short-term temperature changes, but evaluating the stability of these particles during long-term storage is essential, as short-term responses may not predict long-term behavior. Depending on whether the structural integrity affects potency and stability over time, future processes could be optimized to enrich the fragile or exposed subpopulations. Additionally, due to variability in their relative abundance across different rAAV serotypes (AAV2, -8, and -9) and packaging cell types (HEK293 and an insect cell line), a broader range of serotypes should be examined to understand the prevalence and implications of this intermediate stage in the rAAV uncoating process.

Additionally, employing orthogonal methods, such as atomic force microscopy (AFM), cryo-electron tomography (cryo-ET), and charge-based separation techniques, could provide insights into other physical characteristics of the filled but structurally variable particles. These insights might facilitate a purification strategy that removes undesired subpopulations. It would be worth combining size- and charge-based techniques to explore the separation of non-intact particles from the intact particles. The separation of these subpopulations will enable more specific characterization of capsid composition, probing what drives the difference in capsid integrity. A potential factor for differing capsid integrity is post-translational modifications of the capsid proteins, which can be profiled by Mass Spectrometry (MS). Once these links are identified, they will provide valuable insights for vector optimization.

The integration of MP-nuclease treatment into formulation development and optimization may provide additional insights into thermo-stability stress testing. In addition, implementing this approach in early product development stages could also facilitate developability screening by capturing a more in-depth characterization of stability profiles, particularly given the high variability observed among different rAAV serotypes.

The study raises an important question about the factors driving the differences between rAAV particles of variable structural integrity. Integrating these relevant assays into real-time monitoring during the cultivation process may offer valuable insights into the production kinetics of particles that are more prone to enter a less "fragile" or intermediate uncoating stage. This information might correlate with higher stability and *in vivo* potency, potentially guiding process optimization for better therapeutic outcomes.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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