EV-AAV Enhances Transgene Expression Via Partial Protection from Neutralizing Antibodies *In Vitro*

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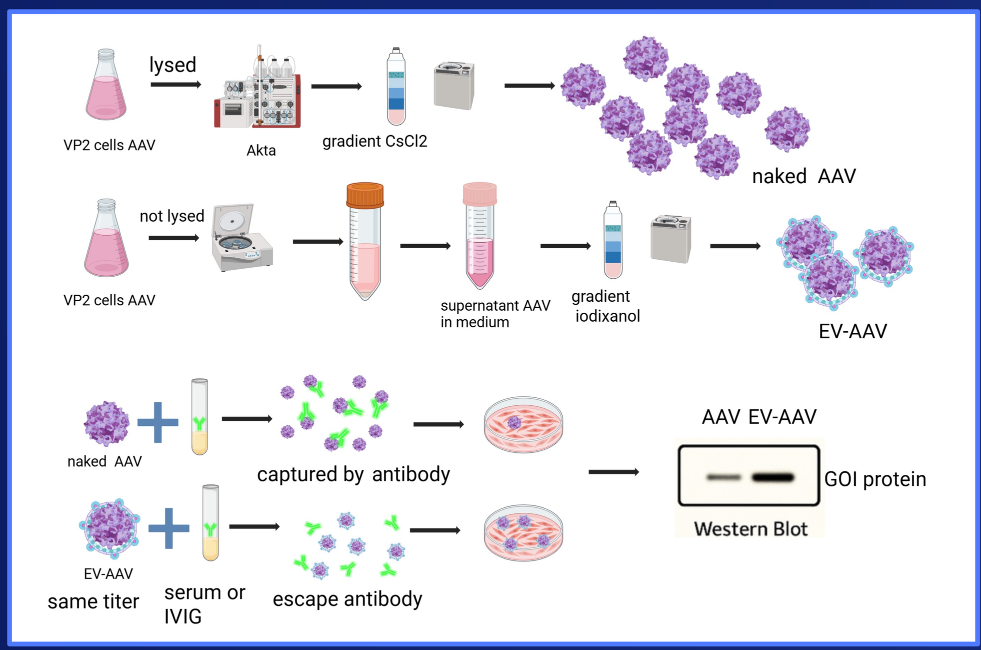
ABSTRACT

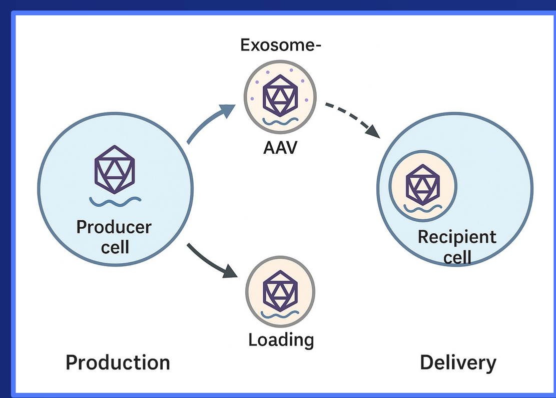
Exosomes are nanosized extracellular vesicles secreted from nearly all known cell types. Exosomes have a membrane-enclosed structure and can carry a range of cargo molecules for efficient intercellular transfer of functional entities. Exosome AAV (EV-AAV) has been detected in the supernatant collected from adherent HEK293T cells. Our objective in this investigation was to determine whether EV-AAV is produced by suspension HEK293 cells and the in vitro potency of the EV-AAV in the presence of neutralizing antibodies. In our research study, we demonstrated EV-AAV is abundant in the supernatant of the suspension culture using particle size and detection of EV membrane protein markers: CD81, CD63, and CD9.

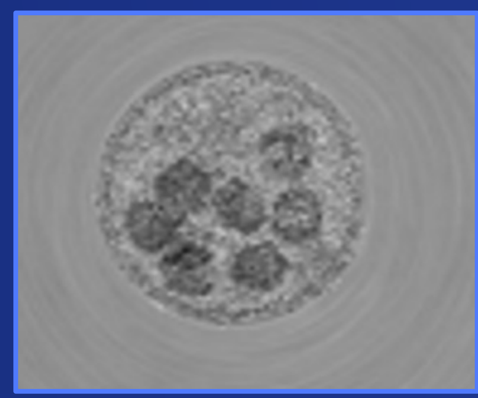
EV-AAV was approximately 30% of the total AAV in the supernatant. An optimized lysis buffer effectively disrupts EVs, resulting in an approximately 3-fold increase in the recovery of naked AAV particles. We next compared EV-AAV with conventional AAV in head-to-head antibody neutralization experiments. C2C12 cells or CHO cells were transduced with equal MOI of conventional AAV or EV-AAV across a range of neutralizing antibody doses and relative expression of a microdystrophin transgene was determined. Remarkably, EV AAV-mediated expression of microdystrophin was enhanced 2-4-fold compared to conventional AAV, across a range of doses of human serum positive for neutralizing antibodies and intravenous immunoglobulin (IVIG). Further, EV-AAV-mediated microdystrophin protein expression was readily detectable by Western blot while not for the conventional AAV, which had been fully neutralized by antibodies at the same concentration. Similar outcomes were repeated using a luciferase reporter approach. These data indicate that AAV packaged in EVs benefited from enhanced cellular uptake and subsequent protection from antibody neutralization. Taken together, the EV-AAV largely enhanced gene expression in muscle cells and CHO cells with resistance to antibody neutralization *in vitro*. Modification to process development to release EV-AAV may represent a transformative platform to improve AAV production. The safety and efficacy of gene transfer may be improved if EVs can be targeted to specific organs *in vivo*.



INTRODUCTION

- **Exosome AAV (EV-AAV)**: refers to AAVs that are naturally or artificially associated with extracellular vesicles, particularly exosomes. These vesicle-associated AAVs can enhance delivery efficiency and immune evasion.
- **Immune Evasion**: The exosome membrane can shield AAV particles from neutralizing antibodies, making EV-AAVs promising for repeat dosing or use in patients with pre-existing immunity
- **Production Method**: EV-AAVs are typically harvested from the supernatant of AAV-producing cells, followed by ultracentrifugation or other purification methods to isolate the vesicle-associated fraction
- **Applications in Gene Therapy**: EV-AAVs are being explored for CNS, muscle, and liver targeting, with potential advantages in crossing biological barriers like the blood-brain barrier

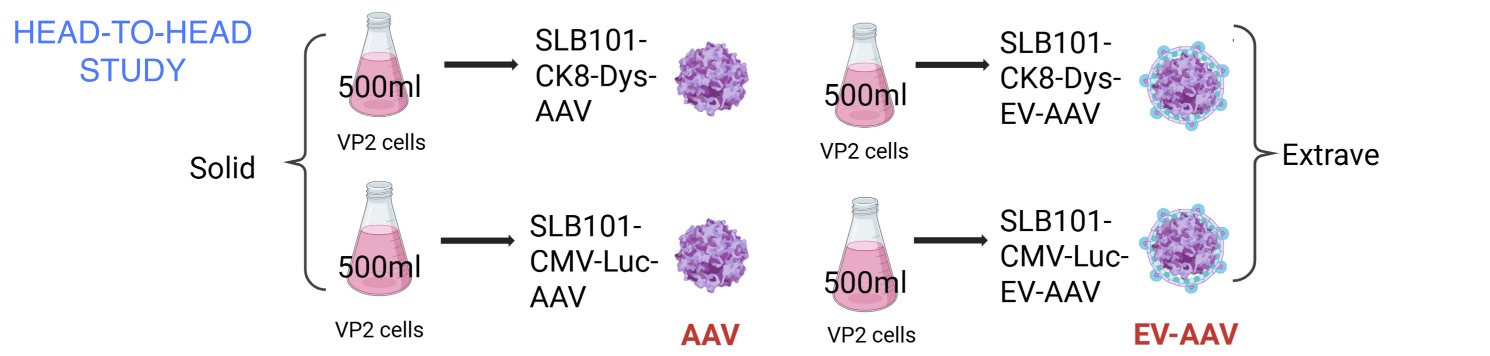




Cryo-EM by Sisaruth Tan and Alain Brisson.

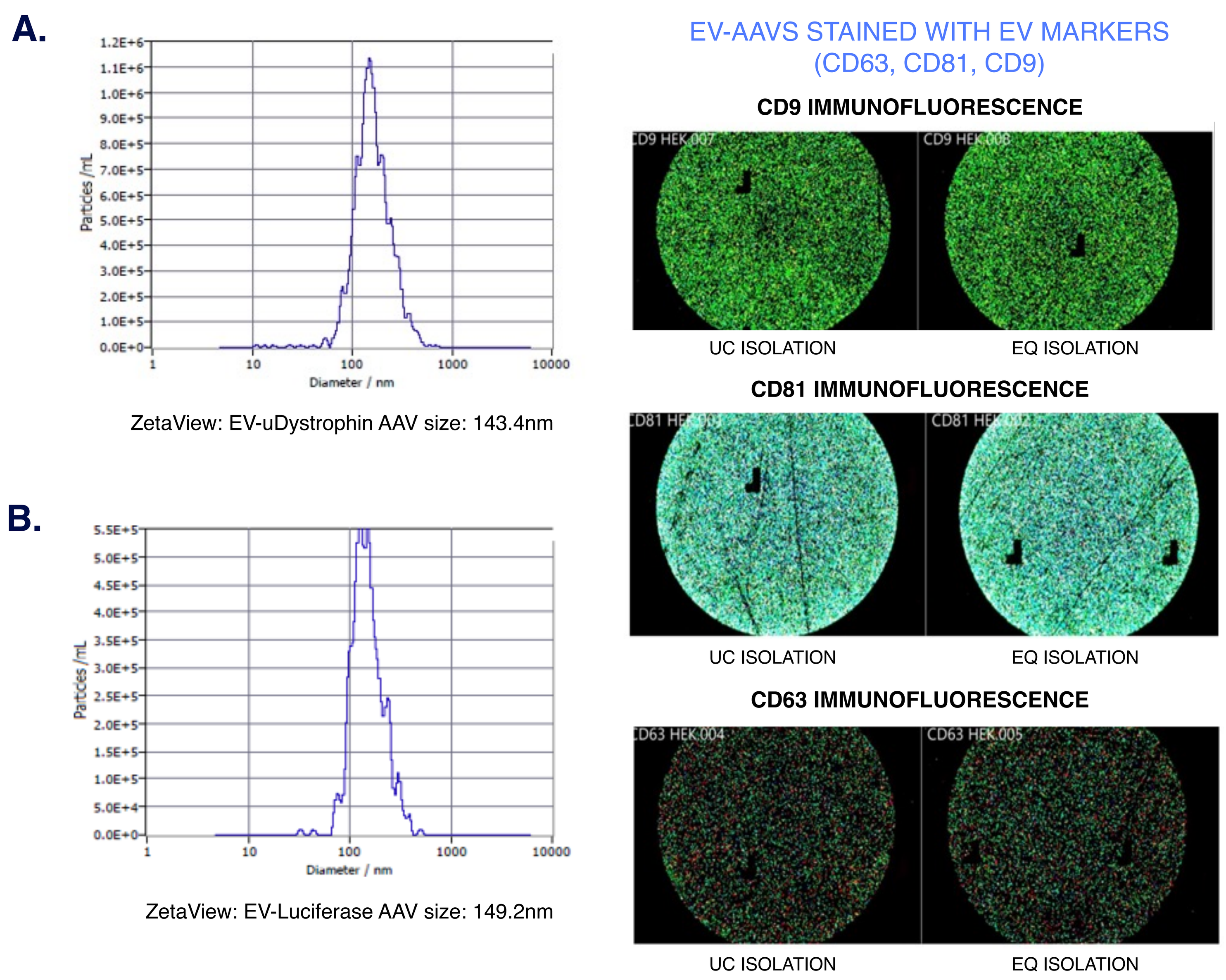
EXPERIMENT DESIGN (1)

Figure 1. Comparing AAV vs EV-AAV



RESULTS

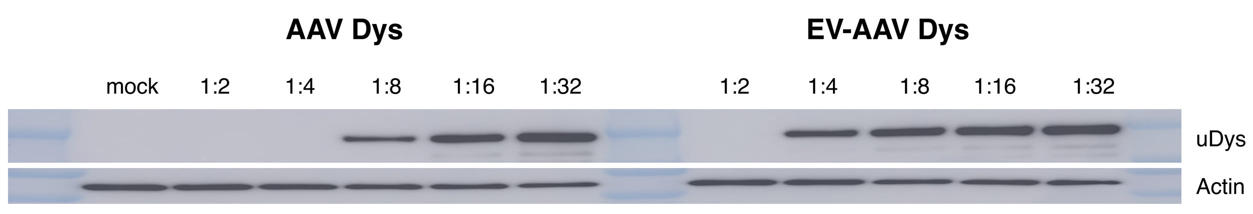
Figure 2. EV-AAV was isolated and characterized

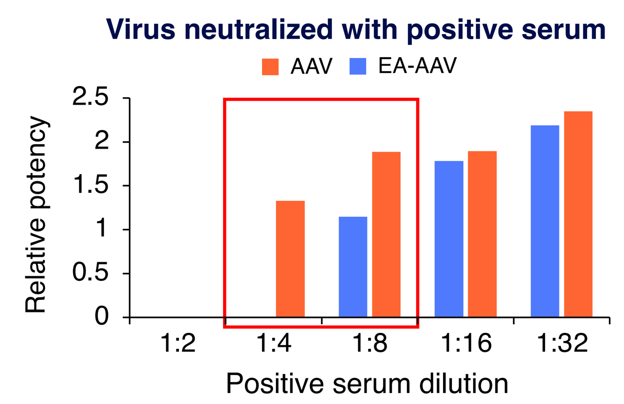


- EV-AAV is abundant in the supernatant of the suspension culture using particle size and detection of EV membrane protein markers: CD81, CD63, and CD9

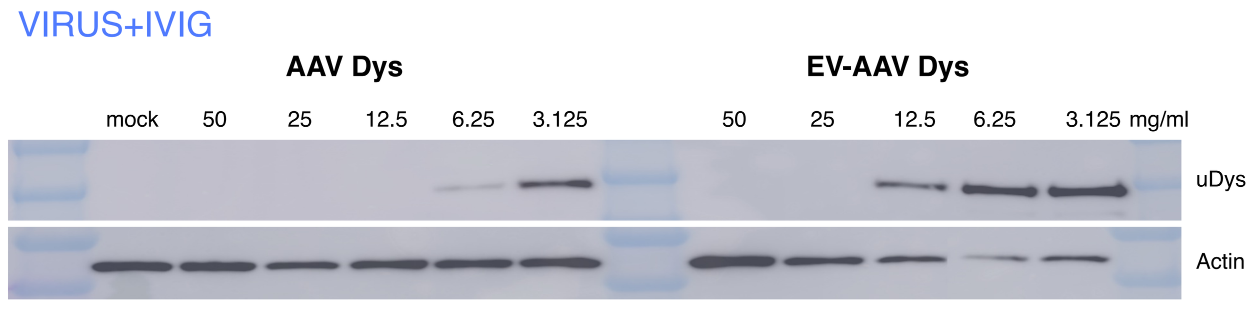
Figure 3. EV-AAV can enhance evasion of anti-AAV antibodies, resulting in increased potency in myotubes

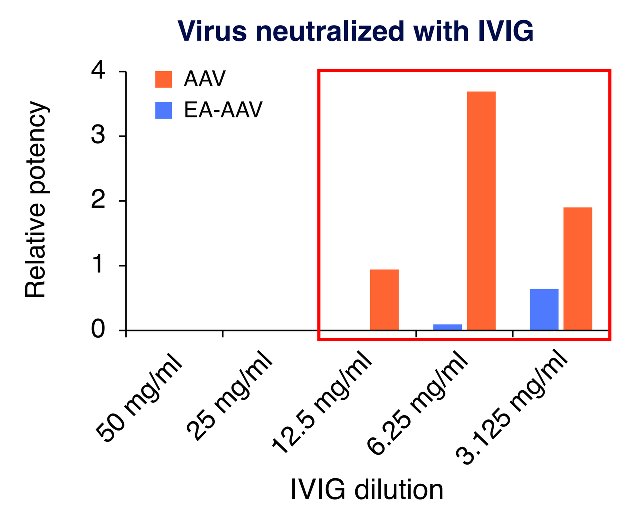
VIRUS+POSITIVE SERUM





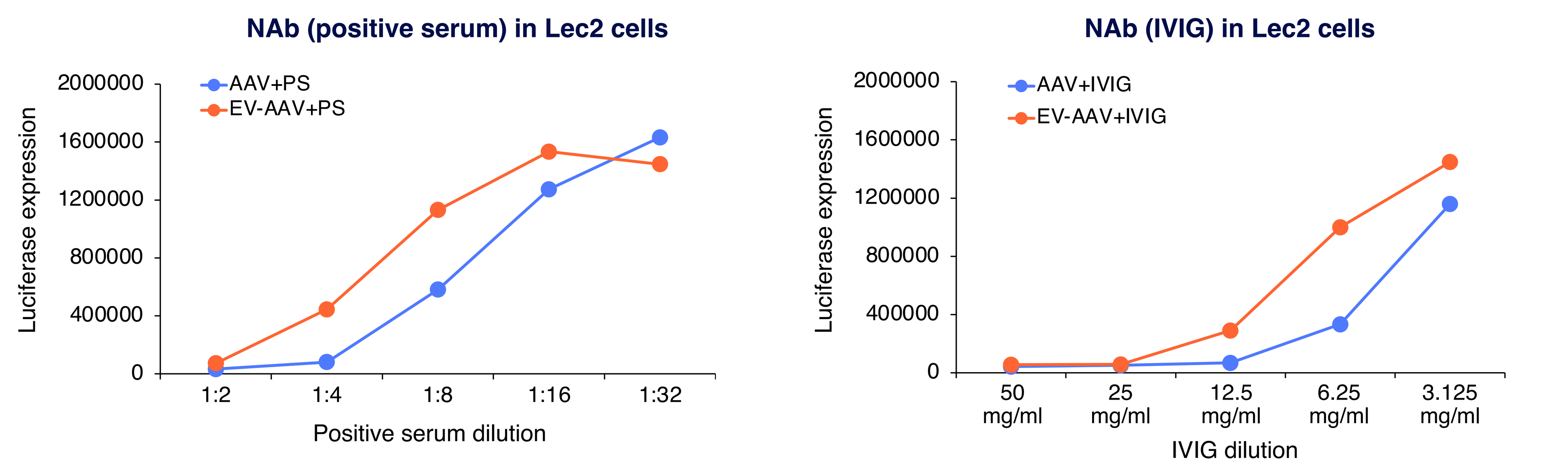
VIRUS+IVIG





- C2C12 cells were transduced with equal multiplicity of infection (MOI = 1E5) using either conventional AAV or EV-AAV, in the presence of varying concentrations of positive human serum or intravenous immunoglobulin (IVIG)
- Expression of the micro-dystrophin (µDys) transgene was assessed by Western blot and quantified using ImageJ

Figure 4. EV-AAV can enhance evasion of anti-AAV antibodies, resulting in increased potency in CHO cells



- CHO (Lec2) cells were transduced with equal MOI (1E5) using either conventional AAV or EV-AAV, in the presence of varying concentrations of human positive serum or IVIG
- Luciferase transgene expression was quantified using a luciferase assay

EXPERIMENT DESIGN (2)

Figure 5. Optimizing EV-AAV may increase AAV yield

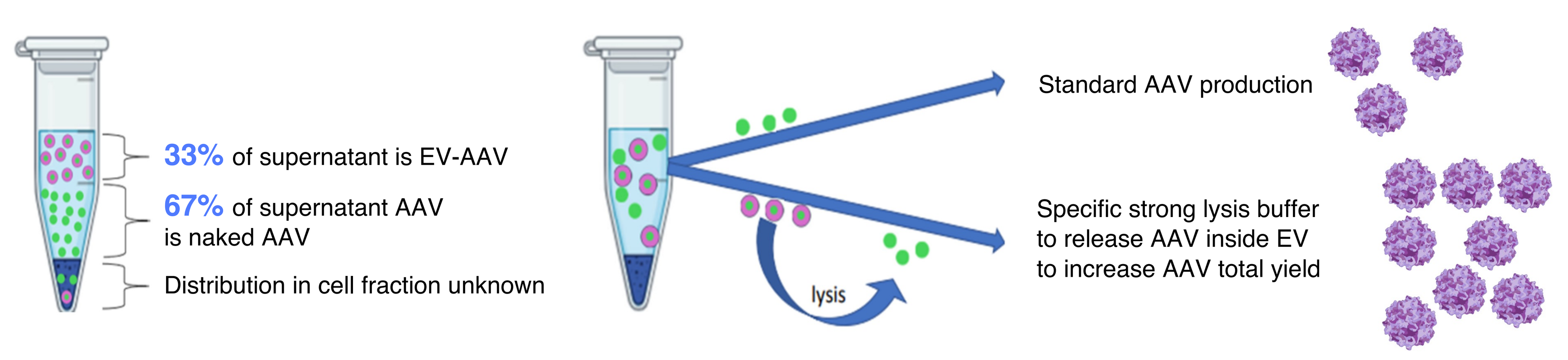
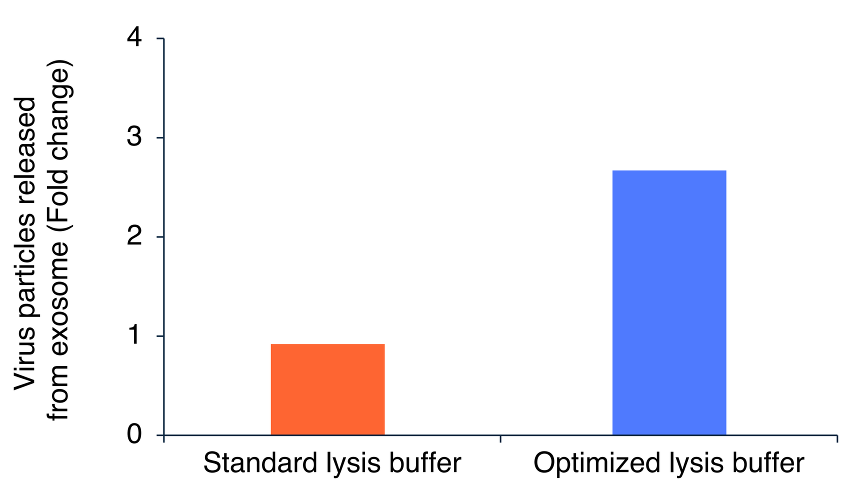


Table 1. The number of naked AAV within EV

	AAV/EV
EV Dys AAV	13
EV Luc AAV	3

- EV-AAV was approximately 33% of the total AAV in the supernatant
- Optimized lysis buffer effectively disrupts EV, leading to an approximate 3-fold increase in the recovery of naked AAV particles (n=1)
- The number of AAV in individual EVs may be varied

Figure 6. Releasing AAVs sequestered within EVs can increase the yield of naked AAV particles



CONCLUSIONS

- **EV-AAVs are efficiently produced** in the supernatant of suspension HEK293 cell cultures, representing approximately 30% of total AAV yield in the supernatant
- **Exosome markers (CD81, CD63, CD9)** confirmed the presence of EV-AAVs and optimized lysis conditions significantly increased AAV recovery
- **EV-AAVs demonstrated superior resistance** to neutralizing antibodies compared to conventional AAVs, maintaining transgene expression even in the presence of human positive serum or IVIG
- **Enhanced transgene delivery** was observed in both C2C12 and CHO cells, with 2- to 4-fold higher expression of micro-dystrophin and luciferase from EV-AAVs
- **Western blot analysis confirmed** detectable protein expression from EV-AAVs, while conventional AAVs were fully neutralized under the same conditions
- **These findings support EV-AAVs** as a promising platform for improving AAV-based gene therapy, particularly in immune-challenged environments
- **Process development** may need to consider to optimize out EV-AAV with Good Manufacturing Practice grade detergents

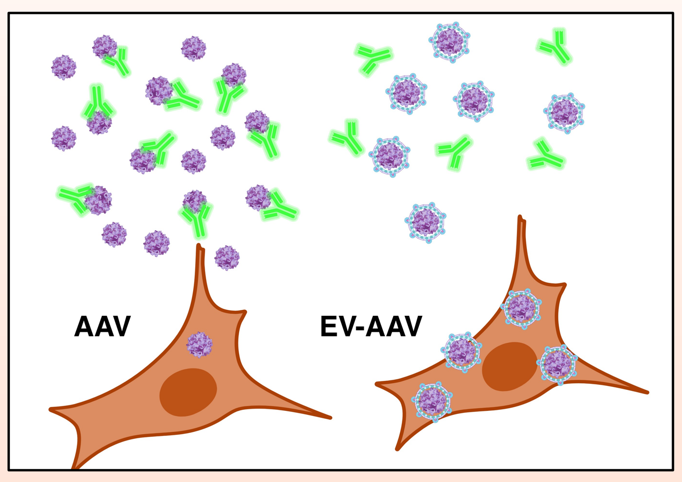


Image generated by BioRender.

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DISCLOSURES

XE: No conflicts of interest.

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