Biomanufacturing of Recombinant Adeno-associated Virus (rAAV) for Gene Therapy: Production, Purification and Evaluations

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Background and Objective

Gene therapy allows us to replace or inactivate the damaged or mutated genes that are responsible for many diseases such as heart failure, cancers, organ dysfunction or others. In addition to nanoparticle-mediated gene delivery, recombinant adeno-associated virus (rAAV) is actively getting attention as one of the most effective gene therapy vehicles for disease treatment in clinics and basic research. The rAAV is a small (20 nm), replication-defective, non-pathogenic parvovirus containing a single-stranded DNA genome encapsulated in non-enveloped capsid [1]. The advantages of AAV-facilitated gene therapy include low immunogenicity, high transduction efficiency, stable gene expression, and minimal adverse reported in clinics [2]. Due to the broad applications of rAAV in basic research and pre-clinical translational studies, it is highly desired to develop a robust, scalable and Good Lab Practice (GLP) biomanufacturing process of AAV vector. The **objective** of this study was to develop and optimize a new biomanufacturing of rAAV, including scalable high-production production process and high-recovery and -purify purification process. The rAAV quality was be evaluated in both *in vitro* and *in vivo*. The developed bioproduction process can be easily adapted and scaled up to Good Manufacturing Practice (GMP) production and purification.

Materials and Methods

Plasmids for AAV vectors: AAV (2/5/DJ/DJ8) Helper Free Promoterless System (Cell Biolabs) was applied to construct AAVs. The expression pAAV was constructed with our mitochondrial-targeted therapeutic gene [3].

Cell lines, media and culture: Viral Production Cells 2.0 (VPC, Gibco) were maintained in chemical defined viral production medium with 4 mM GlutaMAX in shaker flasks on an orbital shaker at 135 rpm, 37 ºC, and 8% CO_2. U251 cells were maintained in EMEM (Gibco) with 2 mM L-glutamine, 1% non-essential amino acids (NEAA), 1 mM sodium pyruvate, and 10% fetal bovine serum (FBS) in T25 or T75 flasks (Fisher).

rAAV production development and scale up: AAV production process was developed in small-scale shaker flask culture at 37 ºC, 135 rpm and 8% CO_2. Process scale up was performed in 01-0.5-L spinner flask and 2-L stirred-tank bioreactor (Disteck) at 37 ºC, pH 7.0, 210 rpm and DO 40%. The rAAV optimal GLP production parameters are VPC transfection density of 3x10^6 cells/mL, pAAV expression:pAAV Rep-Cap:pHelper of 1:1:3, DNA:cell of 0.5 µg:1 million cells, 10% (v/v) viral-plex buffer, 0.6% AAV-MAX transfection reagent, 0.3% booster, and 1% enhancer. Glucose and glutamax were maintained at 6 g/L and 4 mM, respectively. VPC cells containing AAV were harvested at 72 hrs.

Purification development and scale up: VPC cells were harvested using centrifugation and raw AAV was released using 10% AAV-MAX lysis buffer (Gibco), followed with three freeze-thaw cycles, and incubation with 2 mM $MgCl_2$ and 90 U/mL benzonase (Millipore Sigma) at 37 °C for 60 mins. NGC (Bio-Rad) liquid chromatography (LC) equipped with HiTrap Q Sepharose XL IEX column (Cytiva), HiTrap AVB Sepharose affinity column (Cytivia) and Foresight Nuvia HPQ column (Bio-Rad) was tested to develop AAV purification. Among these columns, HPQ column worked the best for all the four serotypes using equilibration buffer A (25 mM Tris-HCl, 20 mM NaCl, pH 8.0) and gradient (stepwise, 0, 50, 65 and 100%) elution buffers of A and B (25 mM Tris-HCl, 1 M NaOH, pH 8.0). HiTrap Desalting column (Cytiva) was used to desalt and Savant SpeedVac Concentrator (Fisher) was used to concentrate purified AAV.

Titration, Storage, Characterization and Evaluations: The ssDNA of rAAV was extracted and titrated using RT-PCR. Purified AAV was stored in PBS with 5% sorbitol and 350 mmol/L NaCl at -80 ºC for long term. Fluorescent dye sulfo-cyanine 5.5 was used to label AAV to confirm its transfection in U251 cells using confocal microscope (Nikon). IVIS Lumina Series III (PerkinElmer) imaging was taken to confirm the *in vivo* expression of the AAV-delivered gene.

Results and Discussion

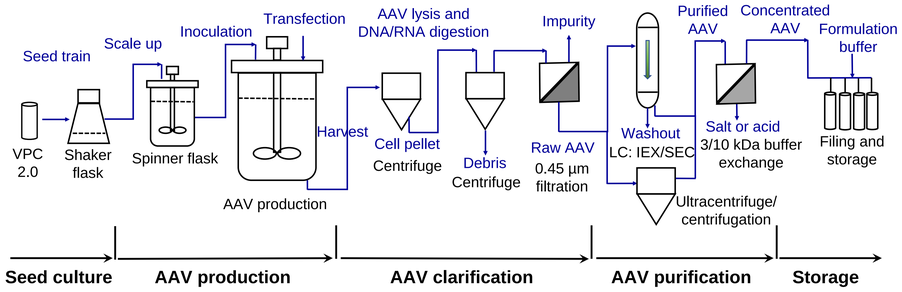

Figure 1. Process flow diagram (PFD) of the developed scalable, suspensive, high-production biomanufacturing of rAAV, including seed train, production, scale up, clarification, purification (ion-exchange/size exclusion: IEX/SEC), desalting, buffer exchange, concentration, and storage.

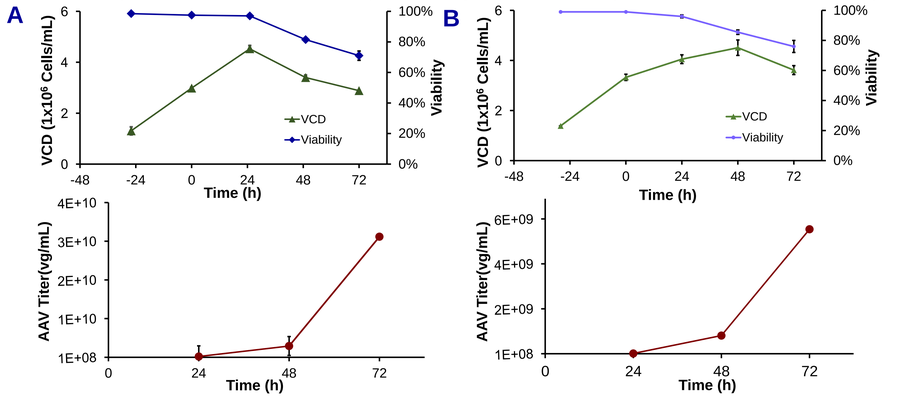

Figure 2. Development and optimization of rAAV production. The transfection cell density, plasmid amount, ratio of three plasmids, transfection formulation, and nutrients were optimized. (A) Small-scale AAV production in shaker flask. 30-60 mL, 37 ºC, 135 rpm and 8% CO_2. (B) Process scale up to spinner flask. 0.1-0.5 L, 37 ºC, 210 rpm and 8% CO_2.

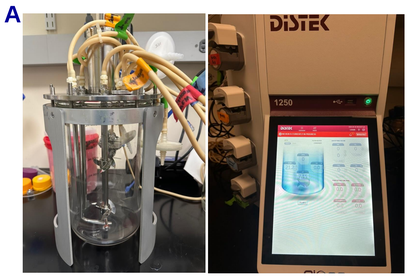

Figure 3. (A) The optimized suspensive AAV production scale up to 2-L stirred-tank bioreactor : VPC cells, viral production medium, 37 ºC, pH 7.0, 210 rpm and DO 40%.

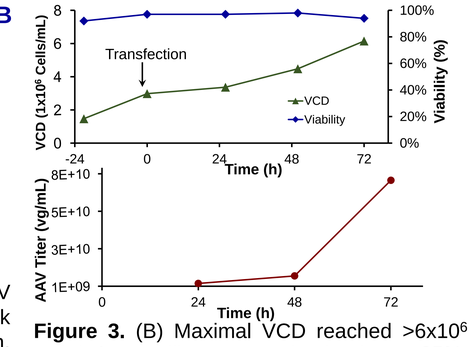

Figure 3. (B) Maximal VCD reached >6x10^6 cells/mL; viability was maintained at >95%; and AAV titer was 7.2x10^{10} vg/mL.

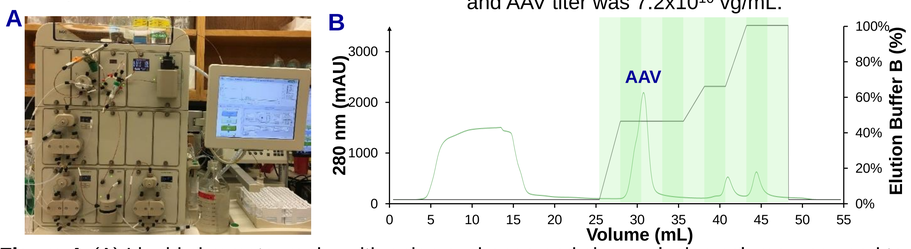

Figure 4. (A) Liquid chromatography with anion exchange and size exclusion column was used to develop AAV purification. Buffer A: 25 mM Tris-HCl, 20 mM NaCl, pH 8.0; Buffer B: 25 mM Tris-HCl, 1 M NaOH, pH 8.0. (B) Stepwise gradient elution (0, 50, 65 and 100%) was developed to primarily purify all four serotypes of AAV, followed with 100 kDa size exclusion purification, buffer exchange, desalting and concentration. Purification was scaled up from 5 to 25-100 mL columns.

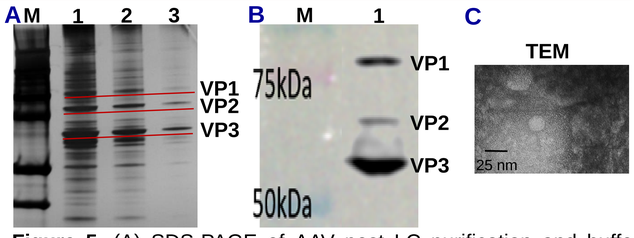

Figure 5. (A) SDS-PAGE of AAV post LC purification and buffer exchange. M: marker; 1: 5X; 2: 10X; 3: 100X. (B) Western blot confirmed three AAV capsid proteins: VP1 (87 kDa), VP2 (73 kDa) and VP3 (62 kDa). (C) TEM image of AAV.

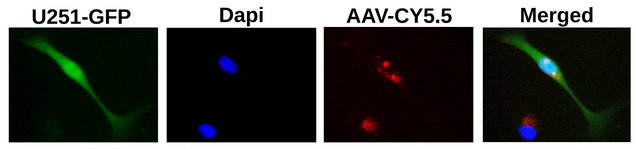

Figure 6. Confocal microscope demosntrated high transfection of AAV, revealed by co-localization of green GFP (U251 cells), blue DAPI (nucleus), and red Sulfo-cyanine 5.5 (AAV). MOI = 5,000.

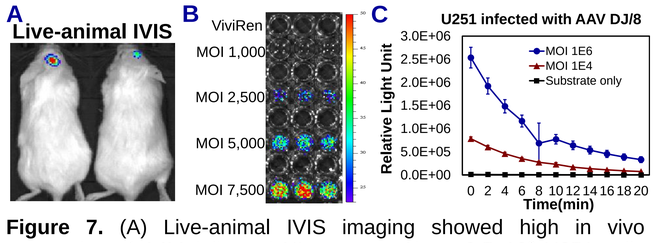

Figure 7. (A) Live-animal IVIS imaging showed high in vivo expression of AAV-delivered NLuc gene. About 0.5x10^6 U251 cells were intracranially injected to NSG mice using stereotactic instrument to develop glioblastoma xenografted models. AAV (1x10^{11} vg) and ViviRen (3.7 µg) were injected into each mouse. (B) The *in vitro* AAV gene expression is dosage (multiplicity of infection, MOI)-dependent. (C) *In vitro* AAV gene expression correlates to MOI, which was measured by i3x plate reader (Spectramax).

Summary and Future Work

- ❑ A scalable, suspensive, high-production, stirred-tank bioreactor-based fed-batch rAAV production process was successful developed and optimized.
- ❑ The liquid chromatography equipped with ion-exchange and size exclusion columns was developed and scaled up for rAAV purification.
- ❑ High quality of rAAV was confirmed with high transfection, infection, expression and function.
- ❑ The rAAV produced from the developed biomanufacturing will be used to produce gene therapy for pre-clinical studies and translational studies.

References

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Acknowledgement

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