

Systems Engineering for Viral Vector Manufacturing

by

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Abstract

Gene therapies have emerged in recent years as a great promise for treatments against a variety of otherwise difficult to treat diseases. Recombinant adeno-associated virus (rAAV) is the current vehicle of choice for *in vivo* gene therapy treatments for its reduced toxicity, robust and long-term transgene expression. Rapidly rising interests in both the academic and private sectors have led to increasing demand for gene-therapy products with more than 200 ongoing global clinical trials. The large demand has created a need for manufacturing large quantities of rAAVs. However, current state-of-the-art viral vector manufacturing methods still fall short of meeting current and future demands. Transient transfection via triple transfection of plasmids is the most common manufacturing platform for the clinical stage due to its flexibility in changing the plasmids and transgene.

Triple transfection has low yields and low fraction of full capsids in harvest. The low percentage of full capsids in harvest (1) leads to high doses which could trigger immunogenicity reaction in patient and (2) requires extensive purification which leads to increased development product cost. In order to advance gene therapy development and improve patient accessibility to the therapeutic, manufacturing must be advanced.

At the current state of process development in rAAV vector manufacturing, batch mode is still the method of choice and evaluating the impact of system inputs on the outcome still relies on the empirical approach. Statistical and empirical approaches, such as design of experiment, can be straightforward for optimizing processes but only within a defined parameter space, whereas mechanistic models can provide physical insights and understanding of the process, multivariable interactions and dynamics to guide novel production design, and extrapolated hypotheses that can be tested. While rAAV production is still mostly performed in batch mode, continuous processing can largely reduce footprint of scale-up and provide a much higher degree of flexibility, and remains unexplored for transient transfection to produce rAAV. In this thesis, I presented a step-by-step model-based approach in creating novel methods to produce rAAV vectors, which culminated in a continuous manufacturing platform.

A quantitative analysis of the system via a mechanistic, mathematical model is useful to organize and exploit information obtained from existing data, gain understanding of process dynamics, and predict responses to various process inputs. This thesis constructs a mechanistic, single-cell, triple-transfection model based on AAV replication biology that enables the comprehensive understanding of viral vector production kinetics and facilitates design of processes.

As derived from mechanistic understanding of viral production process, the rapid saturation of the rate of viral capsid synthesis combined with the late onset of viral genome replication could be responsible for the low ratio of full capsids at harvest. This thesis designs a multi-stage transfection strategy in fed-batch that extends the viral capsid production timeline to improve the ratio of full to empty capsids, while keeping the total plasmid dosage constant. The multistage transfection method improved the ratio of full capsids in the harvest while preserving the genome titer.

Continuous processing provides great advantages over batch and fed-batch operations in flexibility and foot-print reduction of scale-up. This thesis develops a perfusion bioreactor platform to produce rAAV at high cell density leveraging understandings from the mechanistic model and multi-stage transfection. The continuous process enables high cell density transfection and reduces genome titer yield per unit weight plasmid.

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Contents

1	Introduction	21
2	Mechanistic Modeling for rAAV Production via Triple Transfection	25
2.1	Introduction	25
2.2	Materials and Methods	27
2.2.1	Materials	27
2.2.2	Cell culture	27
2.2.3	Triple transfection for AAV5 production	28
2.2.4	Measurement of AAV capsids	29
2.2.5	Measurement of intracellular plasmids and AAV genome	29
2.2.6	Measurement of AAV encapsidated genome	31
2.2.7	Mathematical Modeling	31
2.3	Results	43
2.3.1	Dynamics of PEI-mediated delivery of plasmid DNA	43
2.3.2	Dynamics of viral vector synthesis in triple transfection	45
2.3.3	Model validation by varied input plasmid ratio	46
2.3.4	Sensitivity analysis	47
2.4	Discussion	49
2.4.1	Bottlenecks in viral production	49
2.4.2	Limitations and future improvements of the model	51
2.4.3	Conclusion	53

3	Multi-stage transfection of HEK293 cells that improves rAAV full capsids in harvest	55
3.1	Introduction	55
3.2	Materials and Methods	57
3.2.1	Materials	57
3.2.2	Cell maintenance	58
3.2.3	Production of rAAV5 by triple plasmid transfection and sampling	58
3.2.4	Cell count and metabolite analysis	59
3.2.5	Quantification of Rep proteins in cells	60
3.2.6	Quantification of AAV5 capsids in cells and supernatant . . .	61
3.2.7	Quantification of plasmids and replicated AAV DNA in cells .	62
3.2.8	Quantification of genome titer in cells and supernatant	63
3.2.9	Mathematical Modeling	64
3.3	Results	70
3.3.1	Experiments and mechanistic modeling of multi-stage triple transfection of cell culture	70
3.3.2	Cell growth, viability and plasmid uptake are improved in multi-stage transfections	70
3.3.3	Total replicated viral DNA is mostly independent of number of transfection stages	72
3.3.4	Multi-stage transfections increase full to total capsid ratio without compromising genome titer	73
3.4	Discussion	74
4	Perfusion bioreactor for continuous rAAV manufacturing via transient transfection	81
4.1	Introduction	81
4.2	Materials and Methods	82
4.2.1	Cell line, plasmids, media and plasmids	82
4.2.2	Bioreactor system for cultivation of HEK293 cells	82

4.2.3	Perfusion system	83
4.2.4	Triple transfection of HEK293 cells	84
4.2.5	Analytical methods	84
4.2.6	Continuous bioreactor mathematical modeling	85
4.3	Results and Discussion	85
4.3.1	Logic-based re-transfection scheme	85
4.3.2	Capsid titer and full capsid titer dynamics in bioreactor vessel	89
4.3.3	Accumulation of genome titer	90
4.3.4	Conclusion	95
5	Conclusion	97
A	Mechanistic Modeling Supplementary Materials	101
B	Multistage Transfection Supplementary Materials	103
C	Pefusion Bioreactor Supplementary Materials	107

List of Figures

1-1	The manufacturing demand of three rAAV therapies based on the dose requirement and patient prevalence. The estimation of production scale is based on a productivity of 5×10^{14} vg/L and a downstream recovery of 20% [8].	22
1-2	Knowledge pyramid for model-based decision making. Higher layers in the pyramid correspond to greater knowledge, more informed and better reliability of decision making, and lower requirements for regulatory oversight. “First-principles” refers to models involving rather complete mechanistic understanding, e.g., chemical reaction stoichiometry and kinetics; constitutive relationships that define the kinetics of mass, heat, and momentum transfer; and mass, energy, and momentum conservation equations [18].	23

2-1 PEI-mediated plasmid delivery schematic for triple transfection. (A) Triple transfection. (B) Model of plasmid delivery. Non-viral gene delivery kinetics are assumed to be similar across all three different plasmids. The cellular uptake parameter (k_{uptake}) describes the combined process of the extracellular complex (ExComplex) binding to the cell membrane followed by its internalization into the cytosol in the form of endosomal complexes (EndosComplex). Endosomal escape (k_{escape}) then follows and releases the complexes into the cytosol (CytoComplex). PEI complexes in the cytosol can either migrate into the nucleus before unpacking to release plasmids; or they can unpack in the cytosol; all the steps required to deliver the plasmids from inside the cytosol complexes to inside the nucleus are described by an effective kinetic event ($k_{\text{nuclear entry}}$) and the final product is naked plasmid in the nucleus (NuclearPlasmid). In addition, the degradation rates of all plasmid equivalent species in the cytosol are described by one single parameter ($k_{\text{plasmid degrade}}$). This framework was adapted from previous non-viral gene delivery model [29, 30] and simplified ensure model identifiability/observability with available measurement data . 32

2-2	Viral vector synthesis network. Helper plasmid (pHelper) activates expression of the rep/cap gene on the packaging plasmid (pPackaging) and synthesis ($k_{\text{Cap syn}}$ and $k_{\text{Rep syn}}$) of VP (VP) and Rep protein (RepProtein). Capsid proteins are assembled (k_{assembly}) into empty capsids in the nucleus (EmptyCapNuc) and each capsid particle consists of 60 protein subunits. With helper functions from the helper plasmid, Rep protein replicates ($k_{\text{DNA rep}}$) viral DNA (vDNA) from transgene vector plasmid (pVector). Rep proteins dock on empty capsids ($k_{\text{Rep bind capsid}}$) to form intermediate complexes (CapRepcomplex) which then interact with viral DNA and encapsidate them inside capsids($k_{\text{DNA pack}}$) at 1:1 ratio to form full virions inside the nucleus (RepRCcomplex). Regardless of their contents (empty or full), capsids can be secreted out of the nucleus (k_{secrete}) into the cytosol (EmptyCapCyto and FullCapCyto). Rep protein binding ($k_{\text{Rep bind plasmid}}$) forming bounded plasmids (RepRCcomplex) negatively regulates expression of rep/cap gene on the packaging plasmid. Degradation of proteins ($k_{\text{Rep protein degrade}}$ and $k_{\text{VP degrade}}$) is possible during the process. Dotted lines imply that reactants do not get consumed in the reaction.	34
2-3	Dynamics of PEI-mediated gene delivery. (A) Plasmid copies per cell over time. The model was fitted to single transfection data for HEK293-EBNA1 cells from Carpentier et al.18 with initial plasmid input of 2.7×10^5 plasmids/cell. (B) Plasmid content in the nucleus over time. The model prediction was generated using estimated parameters from the Carpentier et al. study and plotted against HeLa cells transfection data from Glover et al. [60] The initial plasmid input was 2.2×10^6 plasmids/cell.	44

2-4	Dynamics of viral DNA and rAAV5 capsid synthesis in in-house triple transfection experiments for HEK293 cells. (A) Dynamics of viral GFP DNA replication. (B) Dynamics of total and full rAAV capsid production. Viral production parameters were fitted to in-house triple transfection experimental data. Measurements of total and full rAAV capsids are per mL culture, harvested from both cell lysate and supernatant, normalized by the cell density at the sampling time. The dotted line indicates time point of media exchange. Initial plasmid input was 7.6×10^4 /cell for each of the three plasmids.	46
2-5	Quantity of full virions produced as a function of the molecular ratios of Helper, Packaging, and Vector plasmids. Data/model simulation were normalized to the maximum value reported/predicted in the four cases. The experimental data were taken from a study by Grieger et al. [22].	47
2-6	Dynamic profiles of the full virion sensitivities (Equation 2-17) for all the model parameters	49
2-7	Dynamic simulation of the (A) species concentrations and (B) reaction fluxes. The values are qualitative. The simulation parameters were according to the in-house experiments described in Materials and Methods.	52

3-1	Multi-stage transfection design and viral vector synthesis schematic. (A) Multi-stage transfection experimental design: one-stage transfection ($N = 1$): 2 μg plasmid/mL culture are transfected in a single dose at the beginning of the culture, or 0 h; two-stage transfection ($N = 2$): 1 μg plasmid/mL culture are transfected at 0 and 24 h; three-stage transfection ($N = 3$): 0.7 μg plasmid/mL culture are transfected at 0, 24, and 48 h. Each culture is performed in replicate ($n = 2$) and each measurement was taken twice. (B) Schematic of rAAV synthesis from transfected plasmids. Numbers in the parentheses refer to corresponding equations in the Mathematical Modeling section. . . .	71
3-2	Cell growth and viability of transfection cultures for one-, two-, and three-stage transfection cultures. (A) Total cell density. (B) Cell viability. Error bars are the average of standard errors and propagated errors from all measurements at one time point for $n = 2$ and duplicate measurements.	72
3-3	Cellular plasmid dynamics for one-, two-, and three-stage transfection cultures. Error bars are the average of standard errors and propagated errors from all measurements at one time point for $n = 2$ and duplicate measurements.	73
3-4	Rep protein and replicated viral DNA dynamics for one-, two-, and three-stage transfection cultures. (A) Rep protein in cells. (B) Replicated viral DNA. Error bars are the average of standard errors and propagated errors from all measurements at one time point.	74
3-5	Capsid titer and genome titer dynamics for one-, two-, and three-stage transfection cultures. (A) Capsid titer inside cells and supernatant per mL of suspension. (B) Genome titer inside cells and supernatant per mL of suspension. (C) Genome titer/replicated viral DNA ratio. (D) Full capsid/total capsid ratio. Error bars are the average of standard errors and propagated errors from for $n = 2$ and duplicate measurements.	75

3-6	Dynamic model simulations of intracellular species. (A) Plasmid per cell. (B) Nuclear plasmid. (C) Rep protein per cell. (D) Viral DNA per cell.	76
4-1	Perfusion bioreactor schematic.	84
4-2	Model simulation of plasmids concentration in the extracellular space and in the cell nucleus.	87
4-3	Model vs. data of cell density and viability. (A) Total cell density. (B) Cell viability. Dotted line represents time of transfection.	88
4-4	Metabolites concentration.	89
4-5	Model v. data of capsid titer and genome titer inside the bioreactor vessel.	90
4-6	Cumulative capsid and genome titer over time. (A) Genome titer in bioreactor, cumulative genome titer in bleed stream, harvest stream, and total genome titer. (B) Capsid titer in bioreactor, cumulative capsid titer in bleed stream, harvest stream, and total capsid titer. . .	92
4-7	Net genome titer for each transfection. (A) Total genome titer (vg). (B) Genome titer per unit weight plasmid (vg/ μ g plasmid DNA). . .	94
4-8	Net genome titer for each transfection. (A) Total genome titer (capsids). (B) Genome titer per unit weight plasmid (capsids/ μ g plasmid DNA).	94
4-9	Percentage of full capsid in harvest in product from each transfection.	95
A-1	Dynamics of nuclear plasmid copies per cell in PEI-mediated transfection of HEK923-EBNA1 cells. The model was fitted to experimental data from Carpentier et al. [45] with initial plasmid input of 2.7×10^5 plasmids/cell.	101
B-1	Quantity of different types of plasmids in cells. Each experiment was performed in duplicate and measured twice; the error was calculated from all four measurements at each time point.	103

B-2	Ammonia concentration in transfection cultures.	104
B-3	Metabolite profiles of the cultures. (A) Glucose concentration [g/L]. (B) Glutamine concentration (mM). (C) Lactate concentration [g/L]. (D) Ammonia concentration (mM). Glucose and glutamine were supplemented at 48 h and 96 h to replenish the levels to 5 g/L and 4 mM respectively.	105
B-4	Distribution of genome capsids in cells and in supernatant per mL of suspension. Error bars are the average of standard errors and propagated errors from for $n = 2$ and duplicate measurements.	106
B-5	Distribution of total capsids in cells and in supernatant per mL of suspension. Error bars are the average of standard errors and propagated errors from for $n = 2$ and duplicate measurements.	106
C-1	Transfection-free perfusion cell density and nutrients. (A) Total cell densities and cell viability. (B) Metabolites concentrations.	108

List of Tables

2.1	Model parameters.	36
3.1	Model parameters.	68
4.1	Bioreactor inner controllers settings	83
4.2	Transfection design.	86
4.3	Viral production kinetic parameters in perfusion experiment.	90
B.1	Fitted cubic polynomial parameters to ammonia concentration profile (Figure B-2).	104
C.1	Perfusion rate at 350 mL working volume inside the bioreactor. . . .	108
C.2	Pump data.	110
C.3	Total AAV5 capsids from ELISA measurement.	110
C.4	Full AAV5 capsids from ELISA measurement.	111

Chapter 1

Introduction

Gene therapies hold great promise as robust and potentially curative treatments against a variety of otherwise difficult to treat diseases [1]. For *in vivo* therapies, recombinant adeno-associated virus (rAAV) is the most commonly used platform [2]. Recombinant AAV is non-pathogenic in humans, replicate-incompetent, can transduce both dividing and non-dividing cells, and can target different tissue types by using varied serotypes, [3, 4, 5, 6]. Gene therapies that are rAAV-based have been developed and approved a wide range of diseases, from rare diseases with patient populations on the order of hundreds such as Leber’s congenital amaurosis, to larger patient populations on the order of millions such as sickle cell anemia and hemophilia [7, 8]. Current production of rAAV is based on cell culture. It has been reported that only 5 to 30% of viral particles in the raw harvest have vector genome of interest [9, 10, 11, 12], and even a smaller portion can effectively transduce cells. The increased dosage associated with having high ratio of empty capsids is more likely to trigger immunogenicity in patients [13, 14]. In a typical batch cell culture, the average yield of rAAV vector genomes (vg) is 10^4 – 10^5 vg/cell from crude harvest [15, 16]. Depending on rAAV potency and whether local or systemic administration is used for patients for a specific indication, the vector dosage per patient ranges between 10^{11} and 10^{15} vg [17]. Assuming a downstream recovery of 20%, tens of millions of liters of culture are required to produce enough to treat a patient population (Figure 1-1) [8]. Thus, it is a timely and necessary challenge to produce rAAV therapy at sufficient scale

and reasonable cost in the future. With the ongoing development of gene therapies, especially for therapies requiring wholebody treatment, such as Duchene Muscular Dystrophy, and for therapies treating patients in millions such as hemophilia and sickle-cell, improved bioprocesses for rAAV are required.

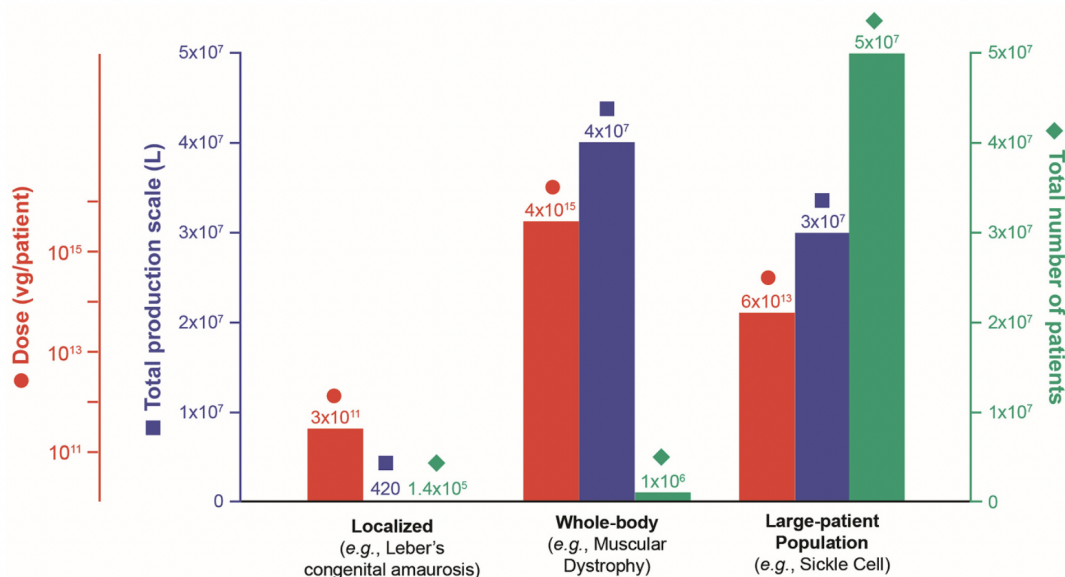


Figure 1-1: The manufacturing demand of three rAAV therapies based on the dose requirement and patient prevalence. The estimation of production scale is based on a productivity of 5×10^{14} vg/L and a downstream recovery of 20% [8].

A quantitative analysis of the system via a mechanistic, mathematical model will be useful to organize and exploit information obtained from existing data, gain understanding of process dynamics, and predict responses to various process inputs (Figure 1-2). This approach to facilitate process development can avoid the high cost of experimentation compared to empirical optimization.

Recent trend in the biopharmaceutical manufacturing industry is the transition of many processes from batch to continuous operation, which mirrors the recent trend for chemically derived pharmaceutical manufacturing (aka “small-molecule drugs”), driven by the goal of reducing manufacturing costs and increasing flexibility and quality. Although continuous perfusion bioreactors have been implemented in commercial biopharmaceutical manufacturing processes for biologics, such as monoclonal

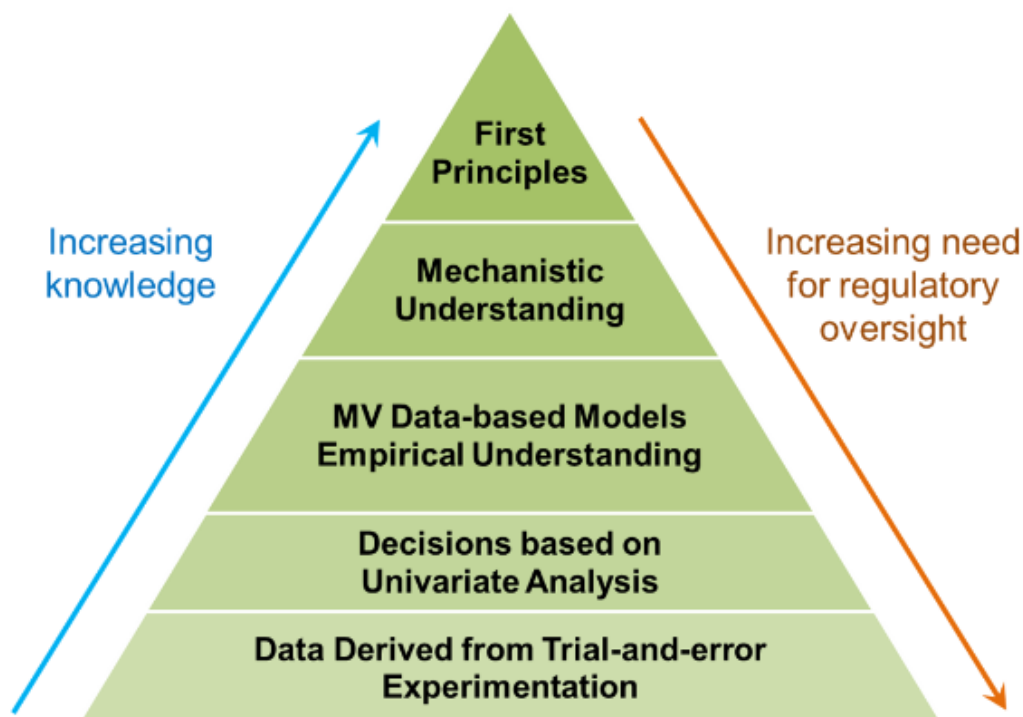


Figure 1-2: Knowledge pyramid for model-based decision making. Higher layers in the pyramid correspond to greater knowledge, more informed and better reliability of decision making, and lower requirements for regulatory oversight. “First-principles” refers to models involving rather complete mechanistic understanding, e.g., chemical reaction stoichiometry and kinetics; constitutive relationships that define the kinetics of mass, heat, and momentum transfer; and mass, energy, and momentum conservation equations [18].

antibodies, transient transfection processes are still carried in batch mode.

To address the challenges associated with rAAV manufacturing, this thesis applies modern system engineering tools to develop advanced methods that improve the yield and product quality of rAAV production via triple transfection.

- *Chapter 2* presents a first-of-its-kind mechanistic model that describes the step-by-step synthesis of rAAV from the delivery of three plasmids, to the synthesis of intermediate components that are Rep protein, viral proteins and replicated transgene, to capsids assembly and packaging of transgene into the premade capsids. Model parameters were estimated using literature data and in-house experimental data. The model was then validated against literature data and

sensitivity analysis was presented. Proposed process bottlenecks derived from model understanding was discussed.

- *Chapter 3* presents a multi-stage transfection method guided by the understanding from the mechanistic model. The total plasmid dosage was divided equally and added to the culture in one, two or three stages. Cultures with increasing number of stages produced products with higher purity, or full capsids in harvest, without compromising the genome titer.
- *Chapter 4* presents a perfusion bioreactor enabled by a tangential flow filter for continuous production of rAAV via triple transfection. Transfections were performed four times over the course of a 15-day culture, and transfection at high cell density was successfully demonstrated. High cell density transfection using the perfusion system produced eight times the amount of conventional batch in the same amount of time, improved the plasmid efficiency by 30% and purity by three times.
- *Chapter 5* presents the conclusion and directions for future works.

Chapter 2

Mechanistic Modeling for rAAV Production via Triple Transfection

2.1 Introduction

Recombinant AAV viral vectors are manufactured by providing mammalian cells or insect cells with genetic elements that express components for rAAV production via infection, transfection, or genetic engineering. In general, the crude harvest of rAAV contains only a small fraction of capsids that contain a therapeutic element, from 5–30% [9, 10, 12], contributing to low viral vector yield and high cost because of the extra purification steps to remove empty particles. Transient transfection of mammalian cells with multiple plasmids is the first and remains the most commonly used method to produce rAAVs [6]. The flexibility and speed of transient transfection are advantageous in early-stage clinical product development compared to other methods of viral vector manufacturing, such as using a stable expressing cell line [19]. In double transfection, the helper virus genes required for viral replication are either provided by a helper virus infection or cloned onto the same plasmid that contains the *rep/cap* genes, whereas in triple transfection, the helper genes are cloned into a third plasmid. Compared to double transfection, triple transfection avoids the potential for incorporation of unwanted viral genomes (vgs) in the product, thus ensuring product safety. However, triple transfection is characterized by low yield. Recent process develop-

ment efforts on rAAV manufacturing have demonstrated transfection in suspension culture that can produce up to 1×10^{14} vg/L after purification [20, 21, 22, 23]. At the current productivity, the total required production of the viral vector, in terms of liters of culture, to treat a population of patients for one disease can be on the order of hundreds of thousands. For instance, the prevalence of Duchenne muscular dystrophy is 19.8 per 100,000 live male births, and each whole-body therapy-treatment requirement of at least 1×10^{15} vg/patient [24]. It follows that the total production needed to treat the whole population for this disorder will be approximately 1×10^5 L of culture per year, with 140 million births per year worldwide [25]. Such demands for viral vectors prompt the need to optimize the production process and increase productivity. A quantitative analysis of the system via a mechanistic, mathematical model will be useful to organize and exploit information obtained from existing data, gain understanding of process dynamics, and predict responses to various process inputs. This approach to facilitate process development can avoid the high cost of experimentation compared to empirical optimization [26].

Before this work was presented, there had been no mechanistic model that captures the triple transfection process as a whole, starting from the delivery of plasmid to the synthesis and final formation of rAAV particles. This thesis chapter presented a mechanistic, single-cell, triple transfection model based on AAV replication biology that includes gene delivery, virion replication, Rep protein and viral protein synthesis, capsid synthesis, and genome encapsidation. Kinetic parameters are identified using data from in-house transfection and literature experiments. The model predictions to varied plasmid ratio inputs are benchmarked against published literature results. Sensitivity analysis and dynamics of intermediate components reveal a possible explanation to the low ratio of full to empty capsid in crude harvest. Transfection strategies are discussed from an operational standpoint that have the potential to overcome the identified bottlenecks. Lastly, model applications in process development and future research for model improvements are presented. This work has been published in *Molecular Therapy - Methods & Clinical Development* [27].

2.2 Materials and Methods

2.2.1 Materials

FreeStyle™ 293-F cells and 293 Expression Medium were purchased from Thermo Fisher Scientific (Waltham, MA). A set of plasmids, including pAAV-GFP (Part No. AAV-400), pRC5 (Part No. VPK-425), and pHelper (Part No. 340202), were purchased from Cell Biolabs (San Diego, CA) for AAV5 production with GFP as the transgene. Plasmid pRC5 is 7.3 kb long with endogenous promoter p5 and p19 for *rep2*, p40 and p81 for *cap5*. Plasmid pHelper is 11.6 kb with endogenous sequences for E2A and E4. Plasmid pAAV-GFP is 5.4 kb long and constructed with a CMV promoter and GFP between two ITR sequences. These plasmids were amplified using One Shot™ Stbl3™ *E. coli* from Thermo Fisher Scientific (Waltham, MA) and purified by the plasmid mega kit from Qiagen (Hilden, Germany). The purified plasmids were further sterilized by 0.22 µm filtration and stored at −20°C. Polyethylenimine transfection agent PEI Max was purchased from Polysciences (Warrington, PA) and dissolved in MilliQ water to make a solution of 1 µg/µL, pH adjusted to 7.0 by adding 1 M HCl. The solution was sterilized by 0.22 µm filtration and stored at 4°C. The AAV5 ELISA kit was from Progen (Wayne, PA). DNA primers were from Integrated DNA Technologies (Coralville, IA). The Monarch® Genomic DNA purification kit, the DNase buffer, DNase I and proteinase K were from New England Biolabs (Ipswich, MA). The iTaq Universal SYBR green supermix was from Bio-Rad Laboratories (Hercules, CA).

2.2.2 Cell culture

A vial of FreeStyle™ 293-F cells was thawed and cultured in FreeStyle™ 293 expression medium. The culture was conducted at 37°C with 135 rpm agitation in a humidified incubator with 5% CO₂. The cells were cultured at 30 mL volume in 125 mL shake flasks (flat-bottomed with vented caps) and subcultures were performed between 0.3 and 3 million cells/mL. At all times, cell viability was maintained above 90%. Samples

of 50 μ L cell culture samples were taken daily from the shake flasks, mixed with 50 μ L trypan blue stain, and analyzed for cell count and viability using the Countess™ II Automated cell counter (Thermo Fisher Scientific, Waltham, MA, USA).

2.2.3 Triple transfection for AAV5 production

Prior to transfection, an aliquot of cells at the exponential growth phase was transferred to new shake flasks and diluted to a cell density of 1 million cells/mL to a total volume of 24 mL by adding pre-warmed medium. Each plasmid solution was quantified using NanoDrop™ spectrophotometer (Thermo Fisher Scientific, Waltham, MA). The three plasmids were mixed at 1:1:1 molar ratio and the total plasmid dosage was 2 μ g plasmid per mL of culture. One milliliter of transfection cocktail was prepared by the sequential addition of the plasmid mix, culture medium, and the PEI Max solution. The amount of PEI Max added was calculated so that the PEI:DNA mass ratio came out to 2:1. The transfection cocktail was vortexed vigorously for 10 seconds, incubated at room temperature for 10 min, and then added to the cell culture. The final volume of the culture in each shake flask was 25 mL. For the control culture, 25 mL culture was set up at 1×10^6 cells/mL without adding transfection cocktail. All the shake flasks were cultured as described above. Media exchange was performed six hours after transfection: the cells were transferred to 50 mL centrifuge tubes and centrifuged at 300 g for 5 min to remove the supernatant, then re-suspended in 25 mL fresh, warmed medium and transferred back to the original shake flasks and cultured as described above. Culture samples of 2 mL were taken at the time points of 10, 24, 34, 46, and 55 h. A volume of 50 μ L culture broth from each sample was used to measure the cell count and viability immediately. The rest of cell culture samples were aliquoted into 500 μ L in each Eppendorf tube and centrifuged at 1000 rpm for 5 min. Supernatant and cells were separated into different tubes and stored at -80°C until the assays below were performed.

2.2.4 Measurement of AAV capsids

The quantity of AAV capsids in the supernatant and cell pellet was measured. To quantify the capsids in the cells, one sample tube containing the cells from 500 μ L culture was thawed on ice. A volume of 100 μ L pre-chilled lysis buffer composed of 150 mM NaCl, 50 mM Tris-HCl, pH 8.5 was added to the cells. The cells were suspended, briefly vortexed, and then processed by a total of three cycles of freezing in an ethanol bath (a mixture of dry ice and 70% ethanol) for 10 min and thawing in a 37°C water bath for 10 min. The sample was clarified by centrifuging at 12100 g, 4°C for 15 min, followed by transferring the supernatant to a new Eppendorf tube and discarding the precipitates. The samples were then analyzed by an AAV5 ELISA kit according to the instruction from the manufacturer (Progen, Wayne, PA). To quantify the capsids in the supernatant, an aliquot of the supernatant saved from cell culture was thawed and further clarified by centrifuging at 12100 g, 4°C for 15 min. The clarified supernatant was then analyzed by the AAV5 ELISA kit. The final absorbance from the ELISA reactions was read by a microplate reader (BioTek Instruments, Winooski, VT). The concentration of capsids in the cell lysate solution was converted to the capsids quantity per cell based on the cell number included in the 500 μ L culture sample.

2.2.5 Measurement of intracellular plasmids and AAV genome

A sum of the quantity of pAAV-GFP and AAV-GFP genome replicated during AAV production was measured from cells. With the procedures described in the following, we lysed the cells without separating the cytosol and nucleus; therefore, the quantified DNA was a population from the entire cells. To begin with, one sample tube containing the cells from 500 μ L culture was thawed on ice. A wash step was performed to cell pellets to remove extracellular plasmids: 1 mL cold $1 \times$ PBS was added to suspend cells; the solution was centrifuged at 1000 rpm and 4°C for 10 min. The supernatant was discarded, and the cell pellets were collected at the bottom of the tubes. DNA was extracted from cell pellets by the Monarch® genomic DNA

purification kit following the instruction from the manufacturer. Monarch® genomic DNA purification kit uses the same principles for extracting and purifying DNA as DNeasy® Blood Tissue kit (Qiagen), which has been used to extract DNA at the range of 5 kb size.[28] In addition, there is an equal chance for the positive (+) or negative (-) sense DNA strand to have been encapsidated in AAV capsids. Once released by proteinase K, these complementary strands can form double-stranded DNA.³⁶ Briefly, cell pellets were suspended in 100 μ L cold PBS, mixed with 1 μ L proteinase K, 100 μ L lysis buffer, and incubated at 56°C for five minutes. Subsequently, the sample was mixed with 400 μ L binding buffer and transferred to a spin column for centrifugation at 1000 g, room temperature for 3 min, followed by centrifugation at 13000 g for 1 min. The column was then placed on a clean tube and washed with 500 μ L wash buffer and a centrifugation at 13000 g for 1 min, followed by one more wash using 500 μ L wash buffer and a centrifugation at 18000 g for 1 min. The spin column was then transferred to a clean tube and mixed with 100 μ L elution buffer that was pre-heated to 60°C, and incubated for 1 min. The spin column was then centrifuged at 13000 g for 1 min. The eluent was collected and ready for qPCR analysis. To perform qPCR analysis, 5 μ L of the extracted DNA was mixed with 2.5 μ L of the 4 μ M forward primer, 2.5 μ L of the 4 μ M reverse primer, and 10 μ L of iTaq 2 $\times$ Universal SYBR green supermix. The quantity of pAAV-GFP was measured in the samples by a LightCycler® 480 System (Roche, Basel, Switzerland) with the following conditions: 95°C for 10 min for the initial denaturation and activation of enzyme; 40 cycles of 95°C for 15 s (for denaturation), 55°C for 15 s (for primer annealing), 68°C for 1 min (for extension). The primers used targeted the GFP sequence. The forward primer was 5' GCAAAGACCCCAACGAGAAG 3' and the reverse primer was 5' TCACGAACTCCAGCAGGACC 3'. The plasmid pAAV-GFP was diluted to a series concentration from 2×10^9 to 2×10^2 molecules/ μ L using DNase-free water and used as the standards. The DNase-free water was used as the non-template control.

2.2.6 Measurement of AAV encapsidated genome

To measure the copy number of AAV genome that was encapsidated in AAV particles, one sample tube containing the cells from 500 μL culture was thawed and lysed by three cycles of freeze and thaw, following the same procedures described in the 2.2.4. In the 100 μL lysate, 10 μL of 10x DNase buffer and 5 μL DNase (10 U) were added. The mixture was incubated at 37°C for 1 h, followed by a heating step at 75°C for 10 min to deactivate the DNase. The solution was then cooled down on ice. A 2 μL of proteinase K (1.6 U) was subsequently added to the solution, incubated at 50°C for 1 h. The solution was then heated at 95°C to deactivate the proteinase. Then, the encapsidated genome was quantified from the samples using the primers targeting GFP and the qPCR procedures as described in 2.2.5.

2.2.7 Mathematical Modeling

Model formulation

The mechanistic model describes kinetic behavior of transient transfection at the subcellular level and is separated into two parts: the trafficking of plasmid from the supernatant to the nucleus and the viral genome replication process. Schematics of the two parts of the model are shown in Figures 2-1 and 2-2. The model has a total of 21 species and 14 parameters.

The mass balances of the species in the first part of the model are

$$\frac{d(\text{ExComplex})}{dt} = -k_{\text{uptake}(\text{ExComplex})}, \quad (2.1)$$

$$\begin{aligned} \frac{d(\text{EndosComplex})}{dt} = & k_{\text{uptake}(\text{ExComplex})} - \\ & (k_{\text{escape}} - k_{\text{plasmid degrade} + \mu})(\text{EndosComplex}), \end{aligned} \quad (2.2)$$

$$\begin{aligned} \frac{d(\text{CytoComplex})}{dt} = & k_{\text{escape}}(\text{EndosComplex}) - \\ & (k_{\text{nuclear entry}} + k_{\text{plasmid degrade}} + \mu)(\text{CytoComplex}) \end{aligned} \quad (2.3)$$

$$\frac{d\text{NuclearPlasmid}}{dt} = k_{\text{nuclear entry}}(\text{CytoComplex}) -$$

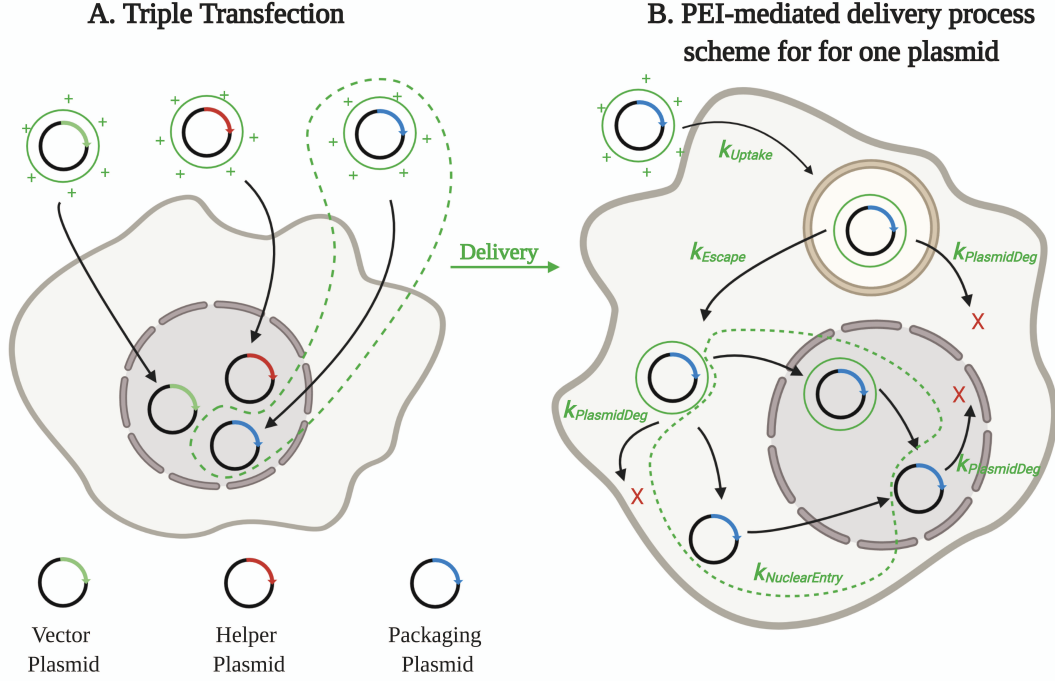


Figure 2-1: PEI-mediated plasmid delivery schematic for triple transfection. (A) Triple transfection. (B) Model of plasmid delivery. Non-viral gene delivery kinetics are assumed to be similar across all three different plasmids. The cellular uptake parameter (k_{uptake}) describes the combined process of the extracellular complex (Ex-Complex) binding to the cell membrane followed by its internalization into the cytosol in the form of endosomal complexes (EndosComplex). Endosomal escape (k_{escape}) then follows and releases the complexes into the cytosol (CytoComplex). PEI complexes in the cytosol can either migrate into the nucleus before unpacking to release plasmids; or they can unpack in the cytosol; all the steps required to deliver the plasmids from inside the cytosol complexes to inside the nucleus are described by an effective kinetic event ($k_{\text{nuclear entry}}$) and the final product is naked plasmid in the nucleus (NuclearPlasmid). In addition, the degradation rates of all plasmid equivalent species in the cytosol are described by one single parameter ($k_{\text{plasmid degrade}}$). This framework was adapted from previous non-viral gene delivery model [29, 30] and simplified ensure model identifiability/observability with available measurement data

$$(k_{\text{plasmid degrade}} + \mu)(\text{NuclearPlasmid}). \quad (2.4)$$

These equations include four major steps, namely the uptake of PEI/plasmid complex from culture medium into the cytosol (k_{uptake}) in the form of endosomal vesicles,

the escape of complexes from endosomal vesicles into the cytosol (k_{escape}), the entry of plasmid/complex from the cytosol to inside the nucleus $k_{\text{nuclear entry}}$, and the overall degradation of plasmid-associated species (k_{degrade}), which describes the combined effect of possible degradation of vesicles containing complexes and uncomplexed plasmids due to various factors such as nucleases in the cytosol [31]. In addition, intracellular species dilution due to cell division is described by a mutual cell growth parameter (μ).

In the second part of the model, the knowledge of rAAV production mechanism [32] was adapted to construct a viral vector synthesis network from the three plasmids once they are successfully delivered inside the cell nucleus. The main steps capturing viral production are described in Figure 2-2, including Rep protein and viral protein (VP) synthesis, capsid assembly, viral DNA replication, DNA packaging into the capsids, and capsid secretion from the nucleus into the cytosol. The model does not include the secretion of viral particles from the cytosol to the supernatant; this step can be added and is expected to vary for different serotypes.

The mass balances of the species in the second part of the model are

$$\begin{aligned} \frac{d(\text{RepProtein})}{dt} = & k_{\text{Rep syn}}(\text{pPackaging})(\text{pHelper}) \\ & - k_{\text{Rep bind plasmid}}(\text{RepProtein})(\text{pPackaging}) \\ & - k_{\text{Rep bind capsid}}(\text{RepProtein})(\text{EmptyCapNuc}) \\ & + k_{\text{DNA pack}}(\text{CapRepComplex})(\text{vDNA}) \\ & - (k_{\text{Rep protein degrade}} + \mu)(\text{RepProtein}), \end{aligned} \quad (2.5)$$

$$\begin{aligned} \frac{d(\text{VP})}{dt} = & k_{\text{Cap syn}}(\text{pPackaging})(\text{pHelper}) \\ & - (k_{\text{VP degrade}} + \mu)(\text{VP}) - 60k_{\text{assembly}}(\text{VP}), \end{aligned} \quad (2.6)$$

$$\begin{aligned} \frac{d(\text{vDNA})}{dt} = & k_{\text{DNA rep}}(\text{RepProtein})(\text{pHelper})(\text{pVector}) \\ & - k_{\text{DNA pack}}(\text{CapRepComplex})(\text{vDNA}) - \mu(\text{vDNA}), \end{aligned} \quad (2.7)$$

$$\begin{aligned} \frac{d(\text{EmptyCapNuc})}{dt} = & k_{\text{assembly}}(\text{VP}) - k_{\text{Rep bind capsid}}(\text{RepProtein})(\text{EmptyCapNuc}) \\ & - (k_{\text{secrete}} + \mu)(\text{EmptyCapNuc}), \end{aligned} \quad (2.8)$$

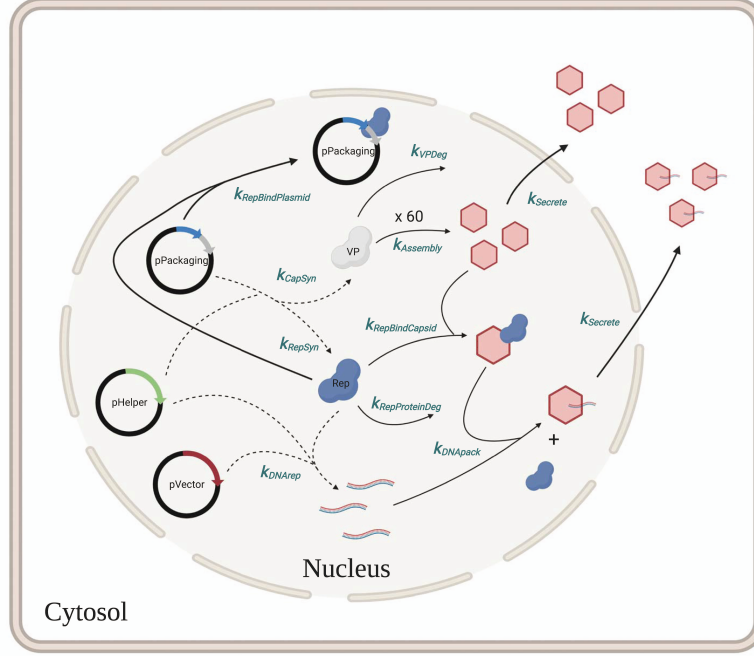


Figure 2-2: Viral vector synthesis network. Helper plasmid (pHelper) activates expression of the rep/cap gene on the packaging plasmid (pPackaging) and synthesis ($k_{\text{Cap syn}}$ and $k_{\text{Rep syn}}$) of VP (VP) and Rep protein (RepProtein). Capsid proteins are assembled (k_{assembly}) into empty capsids in the nucleus (EmptyCapNuc) and each capsid particle consists of 60 protein subunits. With helper functions from the helper plasmid, Rep protein replicates ($k_{\text{DNA rep}}$) viral DNA (vDNA) from transgene vector plasmid (pVector). Rep proteins dock on empty capsids ($k_{\text{Rep bind capsid}}$) to form intermediate complexes (CapRepcomplex) which then interact with viral DNA and encapsidate them inside capsids ($k_{\text{DNA pack}}$) at 1:1 ratio to form full virions inside the nucleus (RepRCcomplex). Regardless of their contents (empty or full), capsids can be secreted out of the nucleus (k_{secrete}) into the cytosol (EmptyCapCyto and FullCapCyto). Rep protein binding ($k_{\text{Rep bind plasmid}}$) forming bounded plasmids (RepRC-complex) negatively regulates expression of rep/cap gene on the packaging plasmid. Degradation of proteins ($k_{\text{Rep protein degrade}}$ and $k_{\text{VP degrade}}$) is possible during the process. Dotted lines imply that reactants do not get consumed in the reaction.

$$\begin{aligned} \frac{d(\text{FullCapNuc})}{dt} &= k_{\text{Rep bind capsid}}(\text{RepProtein})(\text{EmptyCapNuc}) \\ &\quad - (k_{\text{secrete}} + \mu)(\text{FullCapNuc}), \end{aligned} \quad (2.9)$$

$$\frac{d(\text{EmptyCapCyto})}{dt} = k_{\text{secrete}}(\text{EmptyCapNuc}) - \mu(\text{EmptyCapCyto}), \quad (2.10)$$

$$\frac{d(\text{FullCapCyto})}{dt} = k_{\text{secrete}}(\text{FullCapNuc}) - \mu(\text{FullCapCyto}), \quad (2.11)$$

$$\frac{d(\text{RepRCcomplex})}{dt} = k_{\text{Rep bind plasmid}}(\text{RepProtein})(\text{pPackaging}), \quad (2.12)$$

$$\begin{aligned} \frac{d(\text{CapRepComplex})}{dt} = & k_{\text{Rep bind capsid}}(\text{RepProtein})(\text{EmptyCapNuc}) \\ & - k_{\text{DNA pack}}(\text{CapRepComplex})(\text{vDNA}). \end{aligned} \quad (2.13)$$

The cellular process corresponding to each kinetic constant is shown in Table 1.

Table 2.1: Model parameters.

Parameter	Process	Value	Unit	Evaluating Method	Literature
		(95% Confidence Interval)			Range
k_{uptake}	DNA uptake from medium into cytosol	2.78×10^{-2}	h-1	Fit to literature measurement of total plasmid.	Exponential decay of 9×10^4
		$(2.06 \times 10^{-2}, 3.75 \times 10^{-2})$			
		1.19×10^{-3}			
		$(1.00 \times 10^{-3}, 1.40 \times 10^{-3})$		Fit to in-house measurement of vDNA	6.527×10^{-3} – 4.2×10^{-1}
k_{escape}	Endosomal escape	6×10^{-1}	h-1	Parameter for PEI 25K	–
$k_{\text{nuclear entry}}$	Nuclear entry	4.3×10^{-3}	h-1	Fit to literature measurement of nuclear plasmid	8.75×10^{-2} – 3.54
		$(2.4 \times 10^{-3}, 7.7 \times 10^{-3})$			
$k_{\text{plasmid degrade}}$	Plasmid degradation	1.95×10^{-2}	h-1	Fit to literature measurement of total plasmid	0.4621–0.8318
		$(1.09 \times 10^{-2}, 3.48 \times 10^{-2})$			

$k_{\text{Cap syn}}$	Capsid protein synthesis	6.50×10^4	molecule/	Fit to in-house
		$(5.28 \times 10^4, 8.00 \times 10^4)$	cell/h	measurement of vDNA, total capsid and full virion
k_{Rep}	Rep protein synthesis			
$k_{\text{VP degrade}}$	VP degradation	2.77×10^{-1}	h-1	Literature
$k_{\text{Rep protein degrade}}$	Rep protein degradation	2.45×10^{-1}	h-1	Literature
k_{assembly}	Capsid assembly	1.00×10^5	h-1	Fast step assumption
k_{secrete}	Capsid secretion from the nucleus to cytosol	1×10^5	h-1	Fast step assumption
$k_{\text{DNA pack}}$	Viral DNA encapsidation	8.18×10^4 $(1.10 \times 10^{-9}, 6.08 \times 10^6)$	molecule/ cell/h	Fit to in-house measurement of full virion
$k_{\text{Rep bind plasmid}}$	Binding of Rep protein on packaging plasmid	3.38×10^{-5}	molecule/ cell/h	Literature
$k_{\text{Rep bind capsid}}$	Binding of Rep protein on packaging plasmid	5.70×10^{-3} $(4.10 \times 10^{-3}, 8 \times 10^{-3})$	molecule/ cell/h	Fit to in-house measurement of total capsid and full virion

The model assumptions and simplifications of the actual events are:

1. One kinetic rate is used to describe the combined kinetics of transcription and translation and do not consider the transportation of mRNA into the cytosol and protein into the nucleus after synthesis;
2. The synthesis rates of Rep protein and VP are described by bilinear kinetics to capture the role of the helper plasmid, specifically the E2A gene, in activation of rep/cap gene expression⁴¹ (Equations (2.5) and (2.6)). Although the helper protein E1A is also crucial in transcription activation and repression relief [33, 34], this protein is stably expressed in HEK293 cells [6, 35, 36] so its effects on capsid production are not explicitly modeled;
3. Viral DNA requires both AAV helper functions of the E4 protein [37] and the Rep protein to replicate [38], and the replication kinetics are formulated as trilinear reactions, including both helper plasmid and Rep protein as reactants (Equation 2.7);
4. Capsid proteins are viral proteins of three different types (VP1, VP2, and VP3), but are denoted as one species and assumed to have the same relative synthesis rates;
5. The Rep protein has been reported to negatively regulate the promoter p5 to the *rep* gene as well as a translational inhibitor of p40 mRNA in AAV2 [39, 40]. Although the plasmid used for in-house experiments has the cap gene from AAV5, it has the same promoter p40 and the Rep protein regulatory function remains the same in our case. The Rep protein regulation is captured via inhibitive binding to the packaging plasmid ($k_{\text{Rep bind plasmid}}$), which effectively inactivates that plasmid for gene expression after binding. The binding of such Rep protein to the packaging plasmid does not reduce the quantity of plasmid present, but rather simulates the effect of reduced transcription and translation rates; the bound plasmids are still present but no longer active in protein synthesis activities;

6. Packaging of the viral genome into preformed capsids requires the helicase activity of Rep protein as well as the presence of Rep protein/capsid complex intermediates [41, 42, 43], thus viral DNA encapsidation is formulated as a two-step process, in which Rep protein first docks on empty capsids ($k_{\text{rep bind capsid}}$) and DNA is packaged via strand displacement synthesis ($k_{\text{DNA pack}}$). The Rep protein is postulated to detach from the capsid after packaging, as there has been evidence that Rep proteins accumulate mostly inside the nucleus [42].

Determination of gene delivery parameters

Existing models for this process contain parameters that do not pertain to the cell line that was used in in-house transfection experiment [30, 29] or were only validated for shorter time courses (24 hours or less) [30, 29, 44] than the length of transfection considered (48 hours or more). In addition, uptake kinetics can vary between laboratories depending on transfection conditions. Therefore, kinetic parameters were re-estimated to ensure accuracy of the plasmid delivery dynamics. While the quantitative dynamic measurement of intracellular plasmid content including both cytosolic and nuclear plasmids, has been reported in the literature, the corresponding measurement of plasmid content in the nucleus alone, is rarely reported considering the often laborious protocol of nuclei isolation. Since the viral vector production requires component synthesis from all three plasmids, quantifying the plasmid copy number in the nucleus relative to the intracellular amount is necessary to constrain plasmid delivery kinetic constants, specifically nuclear entry rate constant ($k_{\text{nuclear entry}}$). In this work, the data of a single transfection reported by Carpentier et al. [45] was leveraged to estimate the gene delivery parameters because (1) they reported quantitative measurements of plasmids both in the cell and the nucleus; and (2) the cell line and transfection used in the experiment, single transfection of HEK293 with PEI40K, were the most similar to our system. Some data were extracted from the figures [45] using WebPlotDigitizer. Although the measurement of plasmids both in the cell and the nucleus cannot uniquely determine endosomal escape and nuclear entry constants, we assume prior knowledge on the endosomal escape constant using results from a

previous study [29].

Estimates of delivery parameters can vary between laboratories due to differences in transfection conditions. The plasmid uptake rate constant (k_{uptake}), which directly affects transfection efficiency (percentage of cells effectively transfected), can change depending on experimental conditions, including the chemical structure and molecular weight of transfection agent, media composition, cell density, cell health, cell line, complex incubation times, and plasmid dosage. In particular, utilizing HEK293-EBNA1 cells that express the EBNA1 that enhances oriP plasmids will lead to higher plasmid copies overall, and will affect the estimated value of k_{uptake} [46, 47]. Therefore, the uptake rate constant was re-estimated to fit in-house experimental data. However, the feature of this cell line will not affect the nuclear entry barrier and the related nuclear entry rate parameter ($k_{\text{nuclear entry}}$). Loss of intracellular plasmid content (μ) also depends on the doubling time of cells in each experiment. Cell cultures in the literature mentioned above had a faster doubling time (18 h) compared to our in-house experiments (~ 30 h on average), suggesting that the plasmid dilution rate would be lower in our case. However, it is reasonable to assume that the rates of endosomal escape and nuclear entry are similar for the same cell line and for the same transfection reagent. Although PEI 40K was used in in-house experiment instead of PEI 25K in the study by Carpentier et al. [45], data provided from the manufacturer [48] suggests that the two reagents are close in transfection efficiency as measured in level of protein expression, thus making it reasonable to apply the endosomal escape constant (k_{escape}) for PEI 25K previously reported [29] and the nuclear entry constant ($k_{\text{nuclear entry}}$) estimated from the data by Carpentier et al. [45] to estimate model parameters with in-house experimental data.

Determination of viral production parameters

Due to the intricate viral synthesis network, limited availability of dynamic data and the lack of prior knowledge on some parameter values, steps are taken to ensure that all parameter values estimated were unique and make physical sense. We leveraged structural analysis and prior parameter knowledge to make assumptions based on

observations from literature in order to reduce the number of degrees of freedom and estimate a unique set of parameters.

Protein synthesis constants: Myers and Carter reported the dynamics of Rep protein and VP during wild-type AAV production by co-infection with Adenovirus and showed that the synthesis rates of Rep protein and VP are comparable [49]. Therefore, the same value was used for both constants $k_{\text{Rep syn}}$ and $k_{\text{Cap syn}}$ in the estimation.

Protein degradation constants: while assembled capsids are stable and do not degrade easily, Grosse et al. [50] found that unassembled capsid proteins are prone to degradation and have a half-life of 2 to 3 hours, whose first-order kinetic constant equivalence is 0.23 to 0.35 h^{-1} . On the contrary, Redemann et al. [51] reported that Rep proteins are more stable and have a half-life of at least 15 hours, which translates to a first-order degradation rate constant of 0.0462 h^{-1} or lower. The viral protein degradation constants in mammalian cells including GFP protein in HEK293 cells, as reported in literature, fall within the 0.01 to 0.06 h^{-1} range [52, 53]. The protein degradation constant can be assumed to be symmetrically and normally distributed in $\log_{10}$ scale and that its range contains 95% confidence intervals. Therefore, the degradation constants are fixed at 0.2773 h^{-1} for VP and 0.0245 h^{-1} for Rep protein.

Rep protein binding constant: To our knowledge, the association rate constants of the Rep protein to the respective promoter and capsid have not been reported. Transcription factor binding constants *in vitro* have been reported to be on the order of 106 (insect) to 108 (bacteria) $\text{M}^{-1}\text{s}^{-1}$ [54]. However, the binding constants observed in *in vivo* systems can be two orders of magnitude lower than *in vitro* as a result of intracellular diffusion and other proteins crowding around binding sites [55, 56]. Because mammalian cells and insect cells are both eukaryotic while bacteria cells are prokaryotic, the binding rate constant $k_{\text{Rep bind plasmid}}$ is fixed at the lower limit adjusted by a factor of 100, at 104 $\text{M}^{-1}\text{s}^{-1}$. Unit conversion to $(\text{molecule/cell})^{-1}\text{h}^{-1}$ was performed using 15 μm as the average diameter of HEK nucleus [57].

Capsid assembly constant: Myers and Carter’s study [49] suggested that capsid proteins are assembled into viral capsids rapidly following synthesis. Therefore, fast

kinetics is assumed based on this observation and k_{assembly} is fixed to be at least 1000 times faster than the rate of VP synthesis.

Packing and secretion constants: The relationship between DNA packing rates and capsid secretion rates dictate full:empty capsid ratios in the final product, as viral DNA encapsidation into viral capsids only happens inside the nucleus in the case of parvovirus. The total full virions in in-house experimental data does not distinguish between capsids inside or outside the nucleus. The majority of harvested rAAV particles are empty as reported in literature and observed in experimental data, suggesting that secretion of capsids from the nucleus might be much faster than DNA packing. Accordingly, secretion is assumed to have fast kinetics and k_{secrete} was set to be arbitrarily large while estimating $k_{\text{DNA pack}}$ and $k_{\text{Rep bind capsid}}$.

Computational methods

Variances of parameters predetermined by the literature ($k_{\text{Rep protein degrade}}$, $k_{\text{VP degrade}}$) or estimated from gene delivery data ($k_{\text{nuclear entry}}$) will affect the determination of the remaining viral production parameters (k_{uptake} , $k_{\text{Rep syn}}$, $k_{\text{Cap syn}}$, $k_{\text{DNA rep}}$, $k_{\text{DNA pack}}$, $k_{\text{Rep bind plasmid}}$, $k_{\text{Rep bind capsid}}$). Maximum likelihood (ML) estimation, i.e.,

$$\min_{\mathbf{k}} = (\mathbf{y} - \mathbf{u}(\mathbf{k}, \mathbf{k}_0^*))^T \mathbf{V}_y^{-1} (\mathbf{y} - \mathbf{u}(\mathbf{k}, \mathbf{k}_0^*)), \quad (2.14)$$

$$\mathbf{V}_y = \left. \frac{\partial \mathbf{u}}{\partial \mathbf{k}_0} \right|_{\mathbf{k}_0^*} \mathbf{V}_k \left. \frac{\partial \mathbf{u}}{\partial \mathbf{k}_0} \right|_{\mathbf{k}_0^*}^T + \mathbf{V}_\epsilon, \quad (2.15)$$

was used to capture the effect of predetermined parameter variances in the objective function, where $\mathbf{k}$ is the vector of parameter estimates, $\mathbf{k}_0$ is the vector of predetermined parameters, $\mathbf{y}$ is the vector of experimental observations, $\mathbf{u}$ is the vector of model predictions, and $\mathbf{V}_y$ is the matrix of experimental data variance taking into account the predetermined parameter variance $\mathbf{V}_k$ and the normal noise $\mathbf{V}_\epsilon$. Detailed derivations can be found in the Methods section in a past work by our laboratory [58].

Simulation and optimization were performed entirely in MATLAB (MathWorks®), Waltham, MA). The differential equations were solved using a stiff ODE solver

(ode15s). The best fit parameters were computed using a multi-start approach with interior-point algorithm to look for the global minima by minimizing the objective function locally from 1,000 random starting points. Since the values of the parameters span a large range in order of magnitude, the optimization was performed over $\log_{10} \mathbf{k}$ to search over a larger numerical space, facilitate convergence, and ensure that the estimated confidence intervals are positive.

The sensitivities of model species with respect to the model parameters $\frac{\partial \mathbf{u}}{\partial \mathbf{k}}$ were calculated by integrating the sensitivity equations along with the state equations [59]. Let $\mathbf{u}$ denote the vector of state variables, $\mathbf{f}$ denote the vector of kinetic equations, $\mathbf{W}$ denote the sensitivity matrix, and $\mathbf{J}_{\mathbf{u}}$ and $\mathbf{J}_{\mathbf{k}}$ denote the Jacobian matrices with respect to the states and parameters, respectively. Then

$$\mathbf{W}(t) = [\mathbf{W}_1(t) | \cdots | \mathbf{W}_{11}(t)], \quad (2.16)$$

$$\mathbf{W}'(t) = \mathbf{J}_{\mathbf{u}} \mathbf{W} + \mathbf{J}_{\mathbf{k}}, \quad (2.17)$$

where $\mathbf{W}_i(t) = \partial \mathbf{u} / \partial k_i$.

The Jacobian matrices are defined $\mathbf{J}_{\mathbf{u}} = \partial \mathbf{f} / \partial \mathbf{u}$ and $\mathbf{J}_{\mathbf{k}} = \partial \mathbf{f} / \partial \mathbf{k}$ and the partial derivatives can be calculated for each time step by using the auto differentiator ADiGator.⁶⁵ Since we optimize over $\log_{10} \mathbf{k}$, the sensitivities are adjusted accordingly:

$$\mathbf{W}_{\log_{10} k_i} = \frac{\partial \mathbf{u}}{\partial \log_{10} k_i} = (\ln 10) k_i \mathbf{W}_i, \quad (2.18)$$

$$\mathbf{V}_{\log_{10} \mathbf{k}}^{-1} = ((\ln 10) k_i \frac{\partial \mathbf{f}}{\partial \mathbf{k}})^{\top} \mathbf{V}_{\mathbf{y}}^{-1} ((\ln 10) k_i \frac{\partial \mathbf{f}}{\partial \mathbf{k}}). \quad (2.19)$$

2.3 Results

2.3.1 Dynamics of PEI-mediated delivery of plasmid DNA

Model simulation with optimized gene delivery parameter values shows good agreement with the data used for fitting from Carpentier et al [45]. (Figure 2-3A and Supplementary Figure A-1). Simulation results show total plasmid uptake peaked

at 20 hours post transfection (hpt) (Figure 2-3A), and plasmid copies in the nucleus peaked later at 45 hpt (Supplementary Figure A-1). Only 5% of the initial plasmid added to the cell culture (total plasmid input) are transfected into the cells, and the amount of plasmid that are eventually trafficked inside the nucleus is much smaller, around 0.6% of the total plasmid input. To validate the dynamic trend of nuclear plasmid copies in PEI-mediated gene delivery, model simulation with the estimated parameters was generated and compared against the data from the study by Glover et al. (Figure 2-3B) [60]. The model was able to describe the dynamic trend of the nuclear plasmid copies up to 24 hpt. The slight overprediction of the model can be attributed to the differences in cell lines used in the studies: (1) HEK293 cells have been shown to have better transfectability than HeLa cells at lower doses with calcium phosphate [61] and (2) the study by Carpentier et al. [45] used HEK293-EBNA1 cells that express EBNA1 protein for episomal replication of oriP-harboring plasmid, leading to overall higher plasmid copies [46, 47].

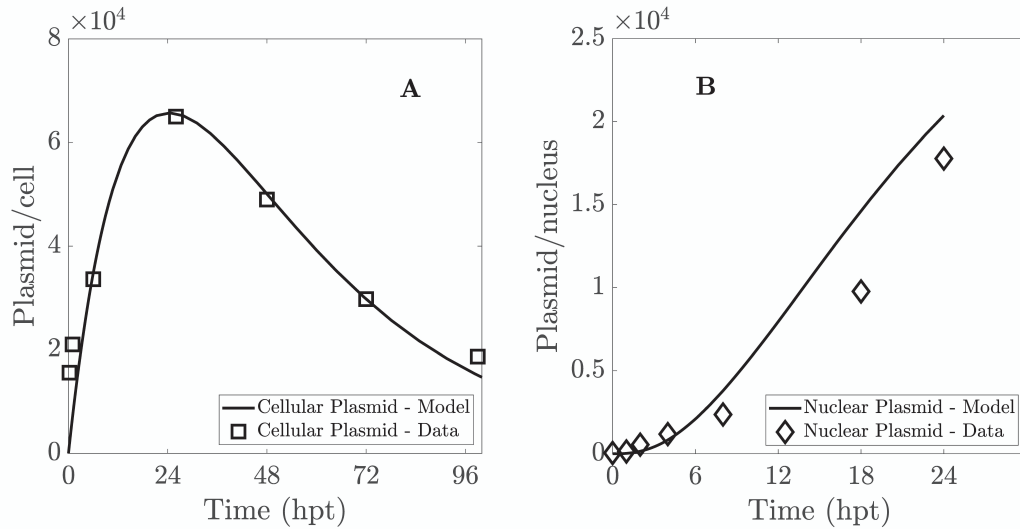


Figure 2-3: Dynamics of PEI-mediated gene delivery. (A) Plasmid copies per cell over time. The model was fitted to single transfection data for HEK293-EBNA1 cells from Carpentier et al. [45] with initial plasmid input of 2.7×10^5 plasmids/cell. (B) Plasmid content in the nucleus over time. The model prediction was generated using estimated parameters from the Carpentier et al. study and plotted against HeLa cells transfection data from Glover et al. [60]. The initial plasmid input was 2.2×10^6 plasmids/cell.

Parameter estimates are provided in Table 2-1. The uptake rate constant is higher in the case estimated by the literature than the value estimated from in-house experiment. The phenomenon is to be expected since Carpentier et al. performed transfection in HEK293-EBNA1 cells that express EBNA1 proteins that enhances replication of oriP-harboring plasmid [45, 46, 47]. The estimated degradation constant of plasmid-associated species in the cytosol is 0.0195 h^{-1} ; this rate is lower than the intrinsic instability constant of uncomplexed plasmid ($0.4621\text{--}0.8318 \text{ h}^{-1}$) found in other literature [31]. The estimated value is reasonable as plasmids associated with complexes are expected to be more stable than uncomplexed plasmids. The smaller value of our plasmid nuclear entry constant ($k_{\text{nuclear entry}}$) compared to the range found in the literature is because several steps were combined into one parameter, effectively prolonging the timescale.

2.3.2 Dynamics of viral vector synthesis in triple transfection

Model simulation with estimated parameters provides a good fit with experimental data (Figure 2-4). Interestingly, the plasmid uptake rate constant (k_{uptake}) estimated from in-house experimental data is significantly lower than that estimated from literature data. In-house experimental data shows that viral DNA started replicating around 12 hpt and doubled every ~ 12 hours (Figure 2-4A). The total amount of capsid produced per cell increased sharply at first, with $\sim 80\%$ of total capsids produced within the first 24 hours, and then plateaued. Total capsid particles and full virion production displays a similar trend and saturates at a slightly later time point between 24 and 36 hours; this trend is consistent with data from another transfection study where the majority of full virions were produced in the first 24 hours [20]. Given the assumption that newly synthesized capsid proteins are rapidly assembled, the plateau in capsid particle production would be caused by saturation in VP production regulated by total Rep protein production. The amount of full virion only accounts for a consistent 2–3% of the total capsid produced (Figure 2-4B). Although most of viral DNA were encapsidated at 24 hpt, and DNA replication continued at an exponential rate, the rate of encapsidated DNA did not follow DNA replication and

decreased significantly beyond that time point. By 48 hpt, only 26% of total viral DNA made it inside capsid particles (Figure 2-4A).

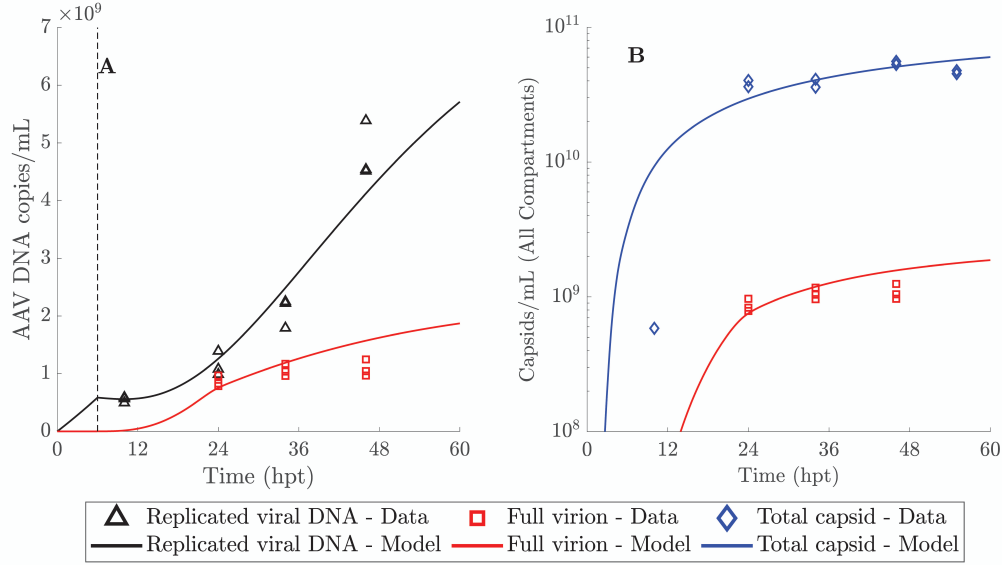


Figure 2-4: Dynamics of viral DNA and rAAV5 capsid synthesis in in-house triple transfection experiments for HEK293 cells. (A) Dynamics of viral GFP DNA replication. (B) Dynamics of total and full rAAV capsid production. Viral production parameters were fitted to in-house triple transfection experimental data. Measurements of total and full rAAV capsids are per mL culture, harvested from both cell lysate and supernatant, normalized by the cell density at the sampling time. The dotted line indicates time point of media exchange. Initial plasmid input was 7.6×10^4 /cell for each of the three plasmids.

2.3.3 Model validation by varied input plasmid ratio

The model was validated by comparing its predictions using the estimated parameters for various plasmid ratio inputs against data published in literature by Grieger et al (Figure 2-5) [22]. Data from triple transfection experiments using the same PEI:DNA mass ratio of 2:1 as in-house experiments was used. Model outcomes for the four plasmid ratios experimentally tested in the aforementioned study were produced and plotted side by side. Data were normalized to the maximal value reported, and model predictions were normalized to the maximal value predicted among four cases. The model captured the relative change in full virion quantity as input plasmid

ratio varied: case 2, where both helper and packaging plasmids ratios are increased, gives the highest amount of product, whereas case 4, in which only vector plasmid ratio is increased, gives the lowest yield of all. The observation suggests that optimal productivity requires a balance between the packaging gene and helper gene expression, and that increasing the copies of therapeutic gene plasmid does not improve production. This result also verifies the capability of our model to capture the interactions between components during the viral production process.

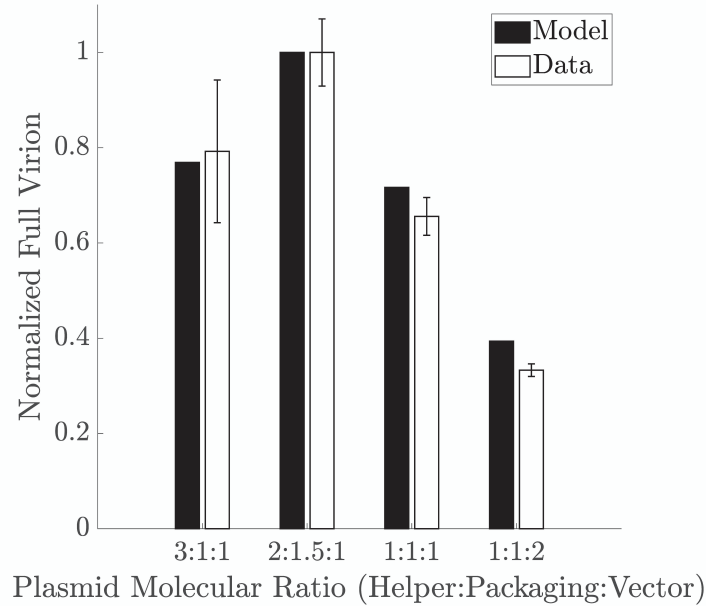


Figure 2-5: Quantity of full virions produced as a function of the molecular ratios of Helper, Packaging, and Vector plasmids. Data/model simulation were normalized to the maximum value reported/predicted in the four cases. The experimental data were taken from a study by Grieger et al. [22].

2.3.4 Sensitivity analysis

The dynamic sensitivity profile of the optimized parameters was computed to evaluate their impacts on the final quantity of full virions as production progresses (Figure 2-6). Plasmid delivery parameters (k_{uptake} , $k_{\text{nuclear entry}}$, k_{escape}) consistently display positive effects on the quantity of full virion. Degradation constants of plasmid-associated species ($k_{\text{plasmid degrade}}$) and Rep protein ($k_{\text{Rep protein degrade}}$) exhibit consistent inverse

effects on full virion production. The assembly and secretion constants (k_{assembly} and k_{secrete}) have little impact on full virion production, as expected from the fast step assumptions we made in model formulation. Binding rate constant ($k_{\text{Rep bind plasmid}}$) has the largest negative impact on productivity. Interestingly, the sensitivity of full virion to the DNA replication rate constant ($k_{\text{DNA rep}}$) and packaging constant ($k_{\text{DNA pack}}$) switches from positive to negative at approximately 25 to 26 hpt. Packaging of DNA simultaneously releases the Rep protein to the free Rep protein pool as formulated in the model, and these Rep proteins can bind onto the packaging plasmid, limiting further *rep/cap* gene expression. On the other hand, the binding of Rep protein on empty capsid ($k_{\text{Rep bind capsid}}$) as the first step to packing process has positive impact on the full virion formation, and its sensitivity dominates the sensitivity of the packaging constant ($k_{\text{DNA pack}}$). The observation explains the wide confidence of the packing constant estimated. The analysis suggested factors that significantly impact viral production process include (1) plasmid trafficking, (2) Rep proteins function as a repressor to gene expression and catalyst to DNA packaging, and (3) DNA replication during first 24 hpt.

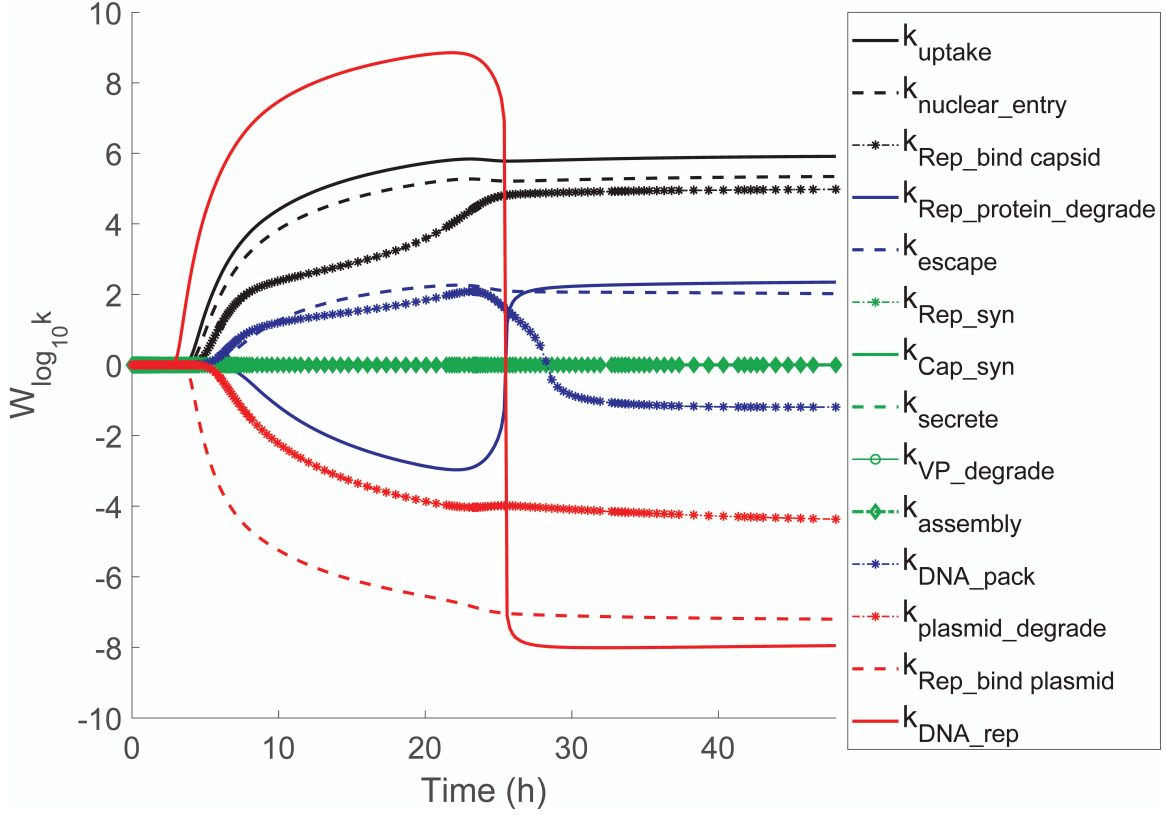


Figure 2-6: Dynamic profiles of the full virion sensitivities (Equation 2-17) for all the model parameters

2.4 Discussion

2.4.1 Bottlenecks in viral production

Gene delivery efficiency

Transient transfection at its current state is not an efficient process. Although transfection efficiency could be as high as 60% as seen in this experiment and in the literature [62], plasmid copies in the nucleus after overcoming trafficking barriers are very low, as observed in most studies regarding gene delivery via non-viral transfection. Data from Carpentier et al. and in-house experiment show that only a maximal of 5% of initial plasmid input are taken up inside the cells and less than 1% of initial plasmid input were trafficked inside the nucleus [45]. This result is also consistent

with the conclusion from the study by Pollard et al. [63]. Model sensitivity analysis indicates that plasmid delivery parameters are limiting to full virion production, therefore it is necessary to engineer a better transfection reagent or devise a delivery strategy that allows more plasmids to enter the nucleus. Varga et al. performed a quantitative analysis on the performance of transfection reagents and found that PEI performs better than lipofectamine [29]. Within the class of polymer transfection reagents, compared to PEI Max that we used, there are other transfection reagents such as PEI Pro and FectorVIR-AAV (Polyplus-transfection®), New York, NY) that boast increased production in functional viral titers in suspension cell culture. There has been evidence that an increase in PEI/DNA complex size over longer incubation time with cells had an adverse effect in uptake process efficiency [64], thus another strategy to get more plasmids inside the cells can be temporal dosing of cell cultures at lower dosage to prevent complexes from getting too large to enter the cells.

Saturation of capsid production and delayed DNA replication result in empty capsid

We visualized the reaction fluxes simulated by the model using in-house experimental settings (Figure 2-7B) to better understand the impact of their dynamics in full virion production. Rep protein (r_{RepSyn}) and VP production (r_{CapSyn}) begins shortly after plasmids successfully enter the nucleus at around 6 hpt, peaks around 12 hpt and declines shortly after. Capsid particles are synthesized with the same timeline as Rep protein and VP since the assembly is assumed to have fast kinetic; the majority of capsids are shown to assemble ($r_{Assembly}$) during the first 24 hpt. Beyond this point, unbound packaging plasmids that are active for protein synthesis are significantly reduced due to inhibitive binding of Rep protein (Figure 2-7A). Analysis of reaction fluxes and intermediate species concentrations also reveals a possible explanation as to why the majority of capsids in harvest are usually empty. Viral DNA start replicating later than capsid synthesis, around 12 hpt, peaks at ~ 24 hpt and the trend remains steady until at least 48 hpt as simulated by the model (Figure 2-7B), and also observed in our data (Figure 2-4). As mentioned above, capsid production is

significantly reduced by 24 hpt, and as postulated in the model formulation, replicated DNA in later phases cannot be packed due to the lack of new capsid production as previously made empty capsid are already secreted out to the cytosol without DNA. This also explains why enhancing the viral DNA replication kinetics at an earlier time point will have a positive effect on full virion production, as indicated in our sensitivity analysis. DNA replication and capsid synthesis dynamics depends largely on Rep protein regulations, which simultaneously negatively and positively affect gene expression. Optimization of rAAV production will require balancing *rep/cap* gene with helper genes expression as evident in Figure 2-5. An essential strategy to enable viral DNA replication before or in parallel with capsid production is early expression of Rep proteins, which is a required machinery. With the current method, Rep protein and VP are linked on the same plasmid. Decoupling the expression of Rep protein and VP by expressing Rep protein from a separate plasmid from the *cap* gene will give better control of genes expression levels. In fact, a study by Emmerling et al. (2016) employed a four-plasmid transfection strategy by splitting *rep/cap* gene on two different plasmids and achieved higher rAAV yield that corresponded to an increase in VP production [65]. Our analysis should also motivate a transfection strategy that alleviates protein production stress on cellular machinery as well as syncs the timelines of viral DNA and capsid production. From a process operating standpoint, temporal dosing of plasmid at different time points is a potential solution, although it will be of concern whether the ability of the culture to uptake plasmids decreases over time.

2.4.2 Limitations and future improvements of the model

Currently, all three plasmids are assumed to have the same kinetics; however, the reality could be different: nonlinear effects of circular DNA length on plasmid transfection efficiency have been reported in literature [66]. Therefore, it is important to determine whether there is a significant difference in the kinetics across different plasmid uptake rates. Besides the assumption of similar uptake kinetics for all three different plasmids, several other simplifying assumptions were made to accommodate

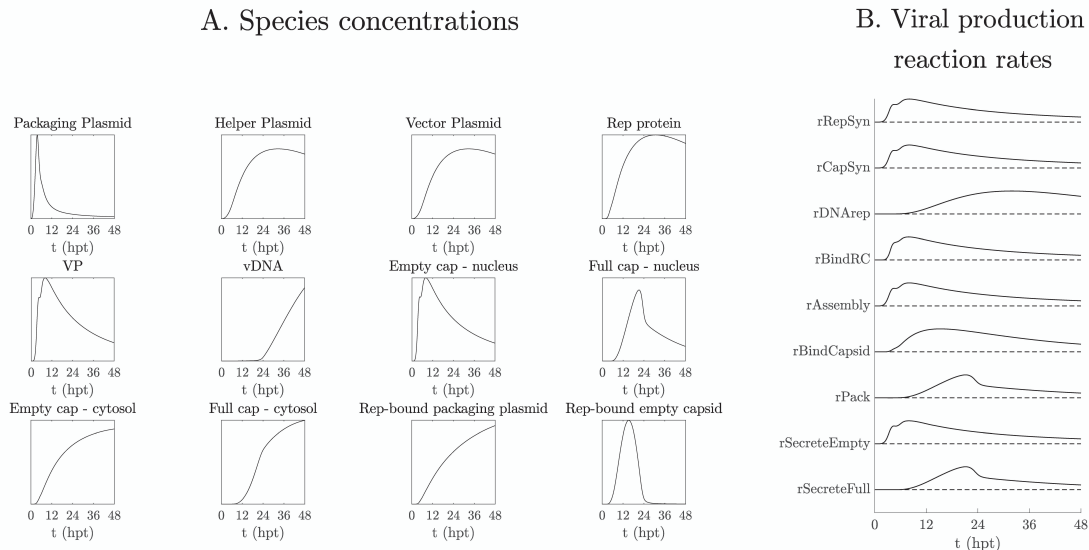


Figure 2-7: Dynamic simulation of the (A) species concentrations and (B) reaction fluxes. The values are qualitative. The simulation parameters were according to the in-house experiments described in Materials and Methods.

limited data availability: (1) transcription and translation steps were combined into one kinetic step; (2) capsid assembly and secretion of capsid from the nucleus into the cytosol were assumed to have fast kinetics; (3) degradation of all plasmid-associated species were described by one parameter; and (4) the DNA encapsidation rate was assumed to only depend on free replicated viral DNA and empty capsid in the nucleus. Experiments that elucidate these intermediate steps have potential for future work and improvements upon the model.

Although the developed model enables the prediction of changes in rAAV production in response to several process parameters such as cell growth rate and plasmid quantity, it has not yet captured the effects of many other process parameters that are commonly manipulated during process development, such as cell density, pH, dissolved oxygen, and media component concentrations on rAAV production. As an example, cell density at the time of transfection has also been reported to influence final product quantity through an undefined mechanism [20, 22]. Enhancing the media with sodium butyrate and certain peptones, which are known to increase recombinant protein expression, can have positive effect on the recombinant viral vec-

tor productivity [21, 67, 68]. Therefore, the model can be improved by correlating the physiological state of the cell culture to its viral vector productivity. Extension of the model to include such correlations will allow predictions from more process production parameters for further support in process development.

2.4.3 Conclusion

At the current state of process development in viral vector manufacturing, evaluating the impact of system inputs on the outcome still relies on the empirical approach. Statistical and empirical approaches, such as design-of-experiment (DoE) done by Zhao et al. [21], can be straightforward for optimizing processes but only within a defined parameter space, while mechanistic models can provide physical insights and understanding of the process, multivariable interactions and dynamics to guide novel production design and provide extrapolated hypotheses that can be tested [69, 58]. In this study, a mechanistic model was developed for triple transfection production platform to link transfection inputs to outputs, and estimated the relevant parameters. Since the biological mechanism of rAAV synthesis will remain largely the same for other transfection methods and even productive infection or stable production cell line, the model can be easily adapted to other production methods and serotypes with small modifications. For instance, a double transfection can be derived by removing one set of gene delivery parameters and species and re-estimating protein synthesis constants. Transfection production data from Chahal et al. (2014) showed that the quantities of genome-containing particles of different serotypes produced in the same platform are comparable [20]; therefore, the proposed mechanistic model can be adapted to the production of various rAAV serotypes with slight parameter adjustments. This mechanistic model can provide valuable insights to experimental data, identify process bottlenecks and guide future research. Leveraging the model in a quality-by-design implementation has great potential to facilitate process development and advance gene therapy manufacturing [70].

Chapter 3

Multi-stage transfection of HEK293 cells that improves rAAV full capsids in harvest

3.1 Introduction

Recombinant adeno-associated virus (rAAV) is the most widely used *in vivo* gene therapy due to its non-pathogenicity, long-term expression, and ability to target different tissue types [71]. To date, two rAAV-based therapies LUXTRNA[®] and ZOLGENSMA[®], respectively treating Leber’s congenital amaurosis and spinal muscular atrophy, have been approved by the U.S. Food and Drugs Administration and the European Medicines Agency [71, 72, 73]. In addition, nearly two hundred rAAV-based therapies are in clinical trials to treat various diseases, some of which treat large patient populations, such as with hemophilia, or require high vector dosage per patient, such as with Duchene Muscular Dystrophy [74]. The pace of clinical development and commercialization of rAAV therapy drives the demand for rAAV viral vector manufacturing, and therefore the need for rAAV process development.

Several cell culture-based methods have been developed for rAAV production [8], including transient transfection of HEK293 cells using multiple plasmids [75, 76,

77], stable integration of rAAV genes into HEK293 cell lines [78, 79], infection of Sf9 cells with recombinant baculovirus [15], and infection of baby hamster kidney cells with Herpes Simplex Virus [80]. Transient transfection of HEK293 cells using three plasmids remains the most flexible method to produce different serotypes of rAAV and is widely used to produce clinical material [74, 6]. However, these cell cultures typically yield between 1 and 30% full capsids in the crude harvest with the remaining capsids lacking the therapeutic transgene [81]. Empty viral capsids are one of the major impurities in AAV gene therapies: the excessive presence of empty particles increases the total capsid exposure, potentially triggers adaptive immune response, and inhibits transduction efficiency [14]. Additionally, removal of empty capsid impurities is challenging due to the structural similarities between full and empty capsids, resulting in product loss and separation inefficiencies [82].

The conventional triple transfection method is performed in batch with all three plasmids added at the start of the process. In Chapter 2, I developed a mechanistic model based on experimental data from a triple transfection process. The rapid saturation of the rate of viral capsid synthesis combined with the late onset of viral genome replication could be responsible for the low ratio of full capsids at harvest. Additionally, low plasmid availability in later stages of cell culture inhibited capsid production activity [27]. In this chapter, a multi-stage transfection strategy is investigated — this protocol extends the viral capsid production timeline to improve the ratio of full to empty capsids. Keeping the total plasmid used constant, the ratio of full capsids in the harvest was improved while quantity of full capsids, or genome titer, was preserved. In parallel, the mechanistic model of rAAV production was further generalized through molecular analysis. Model analysis provided insights into the dynamics of rAAV synthetic pathways, including plasmid uptake, capsid synthesis, viral DNA replication, and genome encapsidation, that allow rational experimental design for further improvements of rAAV production. This work has been submitted to *Molecular Therapy - Clinical & Methods Development*.

3.2 Materials and Methods

3.2.1 Materials

FreeStyle™ 293-F cells, culture medium FreeStyle™ F17 Expression Medium, and Gibco™ l-glutamine (200 mM) were purchased from Thermo Fisher Scientific (Waltham, MA, USA). A set of plasmids for AAV5 production, including pAAV-GFP (Part No. AAV-400), pRC5 (Part No. VPK-425) and pHelper (Part No. 340202), were purchased from Cell Biolabs (San Diego, CA, USA). These plasmids were amplified in-house using the One Shot™ Stbl3™ *E. coli* bought from Thermo Fisher Scientific (Waltham, MA, USA) and purified by the plasmid mega kit bought from Qiagen (Hilden, Germany, USA). The purified plasmids were further sterilized by 0.22 µm filter made of polyvinylidene fluoride (PVDF), quantified using NanoDrop™ spectrophotometers (Thermo Fisher Scientific, Waltham, MA), and stored at -20°C . The transfection reagent PEI Max™ is the transfection-grade linear polyethylenimine hydrochloride with molecular weight of 40,000 and was purchased from Polysciences (Warrington, PA, USA). To prepare the transfection reagent, PEI Max™ was dissolved in Milli-Q water to make a solution of 1 g/L and adjusted to pH 7.0 by adding 1M HCl. The solution was sterilized by passing 0.22 µm filter and stored at 4°C . The ELISA kits for AAV5 capsids were bought from Progen (Wayne, PA, USA) and the kits for AAV Rep protein were bought from Cellbiolabs (San Diego, CA, USA). The DNase I and the Monarch® Genomic DNA purification kits, which includes the DNase buffer and proteinase K, were purchased from New England Biolabs (Ipswich, MA, USA). The ddPCR EvaGreen supermix and ddPCR related reagents were from Bio-Rad Laboratories (Hercules, CA, USA). DNA primers were synthesized by Integrated DNA Technologies (Coralville, IA, USA) and dissolved in nuclease-free water as instructed by the manufacturer.

3.2.2 Cell maintenance

A vial of FreeStyle™ 293-F cells stored in liquid nitrogen was thawed in a 37°C water bath and transferred to 30 mL FreeStyle™ F17 expression medium supplemented with 4 mM glutamine in a 150 mL shake flask. The culture was conducted at 37°C in a humidified incubator with 5% CO₂ and 135 rpm agitation. Sub-cultures were performed between 0.3–3 million cells/mL. At all times, cell viability was maintained above 90%. The seed culture was expanded prior to transfection according to the volume needed for the experimental cultures.

3.2.3 Production of rAAV5 by triple plasmid transfection and sampling

Prior to transfection, the seed cells must be at the exponential phase and the cell density should be between 1 to 2 million cells/mL. Based on the total number of cells required in the experiment, the corresponding volume of the seed culture was transferred to 50 mL centrifuge tubes and centrifuged at 1000 rpm for 5 min. After discarding the supernatant, the pelleted cells were suspended by fresh medium (FreeStyle™ F17 expression medium supplemented with 4 mM glutamine). A set of new 125 mL shake flasks were prepared. In each shake flask, 20 mL of cells were added to target 1 million cells/mL cell density. Two replicate cultures were set up for each experimental condition.

At each point of transfection as outlined in Figure 3-1, a transfection cocktail was prepared with 1:1:1 pAAV-GFP:pRC5:pHelper molar ratio. The dosage was added as indicated in the transfection schemes (2, 1, or 0.7 µg/mL). The PEI:DNA plasmid mass ratio was 2:1. The volume of a transfection cocktail was 5% of the volume of the culture. The remaining volume other than plasmid mix and PEI solution was FreeStyle™ F17 expression medium. The transfection cocktail was prepared by adding the three components in the order of plasmids, culture medium, and PEI Max solution. The transfection cocktail was vortexed vigorously for 10 seconds, incubated at room temperature for 10 min, and added to the cell culture. For the control culture,

20 mL culture was set up at 1 million cells/mL without adding the transfection cocktail. All the shake flasks were cultured in the same setting as seed cultures in the incubator for 120 h (5 days). At 48 and 96 h, the metabolic profiles of the supernatant were measured to enable a feeding procedure. The required volumes of glucose (300 g/L stock) and glutamine (200 mM stock) were supplemented to the culture to replenish the glucose concentration to 5 g/L and glutamine concentration to 4 mM.

A total of 2.2 mL culture sample was taken from cultures every 24 h from day 0 to day 5. The culture in each shake flask was first homogenized by using a serological pipet to mix up and down three times. A volume of 50 μ L culture broth was immediately used for measuring cell count and viability (see Section 3.2.4. Cell count and metabolite analysis). The rest of cell culture samples were aliquoted, 500 μ L in each microtube and centrifuged at 1000 rpm for 5 min. Supernatant was removed from cell pellets to clean tubes. The cell pellets were washed by adding 500 μ L cold PBS to suspend cells; the solution was centrifuged at 1000 rpm and 4°C for 10 min. One aliquot reserved for intracellular plasmid and replicated DNA measurement was washed by additional 500 μ L cold PBS to reduce potential contamination to intracellular DNA caused by the highly abundant extracellular plasmids. The washes were intended to decrease such contamination. The samples of supernatant and cell pellets were stored at -20°C and -80°C respectively until performing the assays.

3.2.4 Cell count and metabolite analysis

To measure cell count and viability, a volume of 50 μ L culture broth was mixed with 50 μ L trypan blue stain and proceeded to the measurement by the Countess™ II Automated cell counter (Thermo Fisher Scientific, Waltham, MA). To analyze the metabolic profiles, the supernatant samples were thawed and measured by BioProfile™ FLEX2 (Nova Biomedical, Waltham, MA).

3.2.5 Quantification of Rep proteins in cells

One vial of cell pellets containing the cells from 500 μ L culture was thawed on ice. The lysis buffer (RIPA buffer and 10% proteinase inhibitor cocktail) was added using wide-bore tips to the cell pellets. The ratio between the lysis buffer and the cell pellets was 100 μ L for 1 million cells. The cell pellets were suspended, mixed up and down a few times, and incubated at 4°C for 30 min. The solution was centrifuged at 4°C at 12000 rpm for 20 min. The supernatant was transferred to clean tubes and analyzed with Rep protein ELISA kit according to the manufacturer's instruction. Briefly, samples were diluted by the diluent provided by the kit. AAV Rep2 standards provided by the kit were diluted to a range of 2 to 0.03 ng/mL. Two replicates of 100 μ L diluted standards or samples were loaded onto the antibody-coated plate and incubated at room temperature for 1 h on an orbital shaker at 120 rpm. The plate was then washed by 250 μ L 1 $\times$ wash buffer three times, followed by incubation with 100 μ L of diluted biotinylated anti-AAV rep antibody at room temperature for 1 h on an orbital shaker at 120 rpm. The plate was washed by 250 μ L 1 $\times$ wash buffer three times and followed by further incubation with 100 μ L diluted strep-enzyme on an orbital shaker at 120 rpm for 1 h. After the last three washes using 250 μ L 1 $\times$ wash buffer, 100 μ L substrate solution was added, incubated at room temperature on an orbital shaker. The 100 μ L stop solution was added after the reaction color on the plate changed. The final absorbance from the ELISA reactions was read by a microplate reader at 450 nm (BioTek Instruments, Winooski, VT, USA). According to the manufacture, the standard Rep protein used in the kit is a subset of Rep proteins and has molecular weight of 17 kDa. To quantify the copies of Rep proteins in samples, the concentrations of rep standards were converted to copy/mL by applying the molecular weight and Avogadro's number. The standard curve was plotted with a linear correlation between the signals and the standard concentrations. The concentrations of rep in samples (copy/mL) was subsequently calculated according to the standard curve.

3.2.6 Quantification of AAV5 capsids in cells and supernatant

The quantity of AAV capsids was measured both in the cells and the supernatant. To quantify the capsids in the cells, one sample tube containing the cells from 500 μ L culture was thawed on ice. The pre-chilled lysis buffer (150 mM NaCl, 50 mM Tris-HCl, pH 8.5) was added to the cells. The ratio between the lysis buffer and the cell pellets was 100 μ L for 1 million cells. The cells were suspended, briefly vortexed, and processed by a total of three cycles of freezing at ethanol bath (mixed by dry ice and 70% ethanol) for 10 min and thawing at 37°C water bath for 10 min. The sample was clarified by centrifuging at 12100 $\times$ g, 4°C for 15 min. The supernatant was transferred to clean microtubes and followed by an AAV5 ELISA analysis. To quantify the capsids in the supernatant, an aliquot of the supernatant saved from cell culture was thawed and further clarified by centrifuging at 12100 $\times$ g, 4°C for 15 min. The clarified supernatant was then analyzed. Samples from either the cell lysate or the supernatant were diluted by the diluent buffer provided by the kit. AAV5 capsid standards provided by the ELISA kit were diluted to a range of 1.20×10^{10} to 1.88×10^8 capsids/mL. Two replicates of 100 μ L diluted standards or samples were loaded onto the antibody-coated plate and incubated at 37°C for 1 h. The plate was then washed by 200 μ L 1 $\times$ wash buffer three times, followed by incubation with 100 μ L of diluted biotinylated anti-capsid rep antibody at 37°C for 1 h. The plate was washed by 200 μ L 1 $\times$ wash buffer three times and followed by further incubation with 100 μ L diluted strep-enzyme at 37°C for 1 h. After the last three washes using 200 μ L 1 $\times$ wash buffer, 100 μ L substrate solution was added, incubated at room temperature for 15 min. The 100 μ L stop solution was subsequently added. The final absorbance from the ELISA reactions was read by a microplate reader at 450 nm (BioTek Instruments, Winooski, VT, USA). The standard curve was plotted using four-parameter logistic (4PL) curve between the signals and the standard concentrations. The concentrations of capsids in samples (copy/mL) was subsequently calculated according to the standard curve.

3.2.7 Quantification of plasmids and replicated AAV DNA in cells

The quantity of pAAV-GFP, pRC5, and pHelper during AAV production was measured from cells. One sample tube containing the cells from 500 μ L culture (with two PBS washes) was thawed on ice. The supernatant was discarded, and the cell pellets were collected at the bottom of the tubes. DNA was extracted from cell pellets by the MonarchTM genomic DNA purification kit following the instruction from the manufacturer. Briefly, cell pellets were suspended in 100 μ L cold PBS, mixed with 1 μ L proteinase K, 100 μ L lysis buffer, and incubated at 56°C for five minutes. Subsequently, the sample was mixed with 400 μ L binding buffer and transferred to a spin column and centrifuged at 1000 $\times$ g, room temperature for 3 min, followed by centrifugation at 13000 $\times$ g for 1 min. The column was then placed on a clean tube and washed with 500 μ L wash buffer and a centrifugation at 13000 $\times$ g for 1 min, followed by one more wash using 500 μ L wash buffer and a centrifugation at 18000 $\times$ g for 1 min. The spin column was then transferred to a clean tube and mixed with 100 μ L elution buffer that was pre-heated at 60°C, and incubated for 1 min. The spin column was then centrifuged at 13000 $\times$ g for 1 min. The eluent was collected and ready for ddPCR analysis.

Each sample was measured by four primers—GFP, Rep2, Helper, AMPR listed below—targeting the sequences on the GFP, rep2, helper, and backbone, respectively:

GFP (forward) 5' GCAAAGACCCCAACGAGAAG 3'

GFP (reverse) 5' TCACGAACTCCAGCAGGACC 3'

Rep2 (forward) 5' AACTCGCGGTCCCAAATCAA 3'

Rep2 (reverse) 5' GCCGCATATTGGGGATCGTA 3'

Helper (forward) 5' CACGCCCACGAGATTAGGTT 3'

Helper (reverse) 5' GAAACTCTTGGCGGGCTTTG 3'

AMPR (forward) 5' CAACGTTGTTGCCATTGCT 3'

AMPR (reverse) 5' GCGGCCAACTTACTTCTGAC 3'

The samples were diluted using nuclease-free water to a concentration below 10⁴

gc/ μ M. The DNase-free water was used as the non-template control. The forward and reverse primers were mixed and diluted to a concentration of 10 μ M. In each well, the reaction mixture was composed of 11 μ L 2 $\times$ EvaGreen supermix, 0.22 μ L of 10 μ M primer, 8.6 μ L water, and 2.2 μ L sample. Droplet droplets were formed on QX200TM droplet generator (Bio-Rad Laboratories, Hercules, CA, USA). The plate was loaded on PCR using the program of 95°C for 5 min for the initial denaturation and activation of enzyme; 40 cycles of 95°C for 30 sec (for denaturation), and 60°C for 1 min (for primer annealing and DNA extension). The plate was finally loaded on the QX200TM droplet reader and analyzed by QuantaSoft software (Bio-Rad Laboratories, Hercules, CA, USA).

The copy numbers of pRC5 and pHelper were directly measured. The copy number of pAAV-GFP was calculated by

$$\text{pAAV-GFP} = \text{pAMPR} - \text{pRC5} - \text{pHelper}$$

The copy number of replicated DNA is then calculated by subtracting the GFP plasmid copies from the total GFP copies:

$$\text{Replicated DNA} = \text{GFP}_{\text{total}} - \text{pAAV-GFP}$$

3.2.8 Quantification of genome titer in cells and supernatant

To measure the copy number of AAV genome that was encapsidated in AAV particles remaining in cell pellets, one sample tube containing the cells from 500 μ L culture was thawed and lysed by three cycles of freeze and thaw, following the same procedures described in Section 3.2.7. To measure the copy number of AAV genome that was encapsidated in AAV particles that had been secreted to supernatant, the clarified supernatant could be directly used. For a 50 μ L reaction, 2 μ L of sample, 5 μ L of 10x DNase buffer, 5 μ L of DNase I (10 U), and 38 μ L DNase-free water were mixed. The samples were incubated at 37°C for 2 h. A 50 μ L volume of 10 mM EDTA was added to the reaction to inhibit the activity of DNase I. The samples were diluted to a concentration below 10⁴ gc/ μ M and quantified using the primers targeting GFP and the ddPCR procedures as described in 3.2.7.

3.2.9 Mathematical Modeling

A mechanistic model was previously developed and validated in Chapter 1 that describes the viral production process via triple transfection [27]. Included in the reaction network were the non-viral gene delivery pathway and synthesis of viral particle components from plasmid DNA. With the availability of more data, both in terms of dynamics and types of measurements presented above in this study, the model was extended to include another layer of information, and more kinetic details to better reflect the viral production dynamics. Cell growth, plasmid uptake, and effect of plasmid uptake on cell growth are modeled by

$$r_{\text{uptake,PEI}} = k_{\text{uptake,plasmid}}[\text{Plasmid}]_{\text{extracellular}} \frac{X_v}{K_{\text{uptake,density}} + X_v}, \quad (3.1)$$

$$\frac{dX_v}{dt} = \mu_{\text{max}} \frac{K_{i,\text{plasmid}}}{K_{i,\text{plasmid}} + \frac{[\text{Plasmid}]_{\text{live cell}}}{X_v}} X_v, \quad (3.2)$$

$$\frac{dX_d}{dt} = k_d \frac{[\text{Plasmid}]_{\text{live cell}}}{X_v} X_d, \quad (3.3)$$

$$\frac{d[\text{Plasmid}]_{\text{live cell}}}{dt} = r_{\text{uptake,PEI}} - k_{\text{degrade,plasmid}}[\text{Plasmid}]_{\text{live cell}} - \frac{[\text{Plasmid}]_{\text{live cell}}}{X_v} \frac{dX_d}{dt}, \quad (3.4)$$

$$\frac{d[\text{Plasmid}]_{\text{cell}}}{dt} = r_{\text{uptake,PEI}} - k_{\text{degrade,plasmid}}[\text{Plasmid}]_{\text{cell}}, \quad (3.5)$$

$$\frac{d[\text{Plasmid}]_{\text{extracellular}}}{dt} = -r_{\text{uptake,PEI}}, \quad (3.6)$$

in which the effects of PEI-induced cytotoxicity on cell growth and death.

Plasmid trafficking is modeled by

$$\frac{d[\text{Plasmid}]_{\text{endosomal}}}{dt} = \frac{1}{3} \frac{r_{\text{uptake,PEI}}}{X_v} - (k_{\text{escape}} + k_{\text{degrade,plasmid}} + \mu)[\text{Plasmid}]_{\text{endosomal}}, \quad (3.7)$$

$$\begin{aligned} \frac{d[\text{Plasmid}]_{\text{cytosol}}}{dt} = & k_{\text{escape}}[\text{Plasmid}]_{\text{endosomal}} + k_{\text{expel}}[\text{Plasmid}]_{\text{nucleus}} \\ & - (k_{\text{entry,nuclear}} + k_{\text{degrade,plasmid}} + \mu)[\text{Plasmid}]_{\text{cytosol}}, \end{aligned} \quad (3.8)$$

$$\begin{aligned} \frac{d[\text{Plasmid}]_{\text{nucleus}}}{dt} &= k_{\text{entry,nuclear}}[\text{Plasmid}]_{\text{cytosol}} \\ &\quad - (k_{\text{expel}} + k_{\text{degrade,plasmid}} + \mu)[\text{Plasmid}]_{\text{nucleus}}, \end{aligned} \quad (3.9)$$

where $\mu = \mu_{\text{max}} \frac{K_{\text{i,plasmid}}}{K_{\text{i,plasmid}} + \frac{[\text{Plasmid}]_{\text{live cell}}}{X_v}}$ is the effective growth/dilution rate. Trafficking kinetics is identical for all three plasmid (pRC5, pHelper, pAAV-GFP). This assumption is supported by the measurement of each of the different plasmids in cells following transfection; there was not a significant difference of trafficking kinetic between different plasmids (Figure B-1). In addition, it has been reported that plasmid in the nucleus can be expelled to the cytoplasm upon mitosis [83]. Therefore, the kinetics of plasmid translocation from the nucleus to the cytoplasm was added to the model and described by the parameter k_{expel} .

The reaction network describing the synthesis of viral DNA, empty capsid, and genome capsid from nuclear plasmids is structurally similar to the previous version of the model:

$$r_{\text{synthesis,Rep}} = \frac{k_{\text{synthesis,Rep}}}{k_{\text{Amn}}^2 + [\text{Amn}]^2} \frac{[\text{Plasmid,RC}]_{\text{nucleus}}}{\frac{[\text{Plasmid,Helper}]_{\text{nucleus}}}{K_{\text{Rep synthesis,pHelper}} + [\text{Plasmid,Helper}]_{\text{nucleus}}}}, \quad (3.10)$$

$$r_{\text{synthesis,VP}} = \frac{k_{\text{synthesis,VP}}}{k_{\text{Amn}}^2 + [\text{Amn}]^2} [\text{Plasmid,RC}]_{\text{nucleus}} [\text{Rep}]^{1/2}, \quad (3.11)$$

$$r_{\text{replication,DNA}} = k_{\text{replication,DNA}} \frac{[\text{Rep}]}{K_{\text{DNA rep,Rep}} + [\text{Rep}]} \frac{[\text{Plasmid,Helper}]_{\text{nucleus}}}{K_{\text{DNA rep,pHelper}} + [\text{Plasmid,Helper}]_{\text{nucleus}}}, \quad (3.12)$$

$$r_{\text{pack,DNA}} = k_{\text{pack,DNA}} [\text{DNA}]_{\text{cell}} \frac{[\text{Rep}]}{K_{\text{pack,Rep}} + [\text{Rep}]}, \quad (3.13)$$

$$\frac{d[\text{Rep}]}{dt} = r_{\text{synthesis,Rep}} - (k_{\text{degrade,Rep}} + \mu)[\text{Rep}], \quad (3.14)$$

$$\frac{d[\text{VP}]}{dt} = r_{\text{synthesis,VP}} - 60k_{\text{assembly}}[\text{VP}] - (k_{\text{degrade,VP}} + \mu)[\text{VP}], \quad (3.15)$$

$$\begin{aligned} \frac{d[\text{EmptyCap}]_{\text{nucleus}}}{dt} &= k_{\text{assembly}}[\text{VP}] - k_{\text{secrete}}[\text{EmptyCap}]_{\text{nucleus}} \\ &\quad - r_{\text{pack,DNA}} - \mu[\text{EmptyCap}]_{\text{nucleus}}, \end{aligned} \quad (3.16)$$

$$\begin{aligned} \frac{d[\text{EmptyCap}]_{\text{cyt}}}{dt} &= k_{\text{secrete}}[\text{EmptyCap}]_{\text{nucleus}} \\ &\quad - (k_{\text{secrete}} + k_{\text{degrade,cap}} + \mu)[\text{EmptyCap}]_{\text{cyt}}, \end{aligned} \quad (3.17)$$

$$\frac{d[\text{DNA}]_{\text{cell}}}{dt} = r_{\text{replication,DNA}} - r_{\text{pack,DNA}} - k_{\text{degrade,DNA}}[\text{DNA}]_{\text{cell}}, \quad (3.18)$$

$$\frac{d[\text{FullCap}]_{\text{nucleus}}}{dt} = r_{\text{pack,DNA}} - (k_{\text{secrete}} + \mu)[\text{FullCap}]_{\text{nucleus}}, \quad (3.19)$$

$$\begin{aligned} \frac{d[\text{FullCap}]_{\text{cyt}}}{dt} &= k_{\text{secrete}}[\text{FullCap}]_{\text{nucleus}} \\ &\quad - (k_{\text{secrete}} + k_{\text{degrade,cap}} + \mu)[\text{FullCap}]_{\text{cyt}}. \end{aligned} \quad (3.20)$$

As the experiments in this study were performed over a longer duration (120 hours) compared to the previous experiments [27], saturation kinetics have been incorporated into the model equations to describe the dynamics evolution over the entire time course of reactions. The reaction of viral protein synthesis (Equation 3.11) is also modeled to include upregulation by Rep protein [39]. In addition, the inhibition effect of ammonia in the media to structural and non-structural protein synthesis was correlated (Equations 3.10 and 3.11). The ammonia production dynamics (Figure B-2) was not explicitly modeled and was fitted as a cubic function of time to the measured concentration using the BioProfile[®] FLEX2 (Nova Biomedical, Waltham, MA). Since the reaction network in Equations 3.14–20 is in single cell, the production rates have to be converted to whole cell population per mL culture before being fitted to the data. The concentration of Rep protein, replicated DNA, total capsid and genome capsid in the whole cell population are modeled in Equations 3.21–25 under the assumptions that (1) protein synthesis and degradation due to proteasome activities only occur in live cells, (2) DNA copies encapsidated in capsids are not susceptible to degradation, (3) DNA also degrades as genome capsid degrades, and (4) capsid secretion to the extracellular media only occurs in live cells:

$$\frac{d[\text{Rep}]_{\text{culture}}}{dt} = r_{\text{synthesis,Rep}}X_v - k_{\text{degrade,Rep}}[\text{Rep}]_{\text{culture}}, \quad (3.21)$$

$$\begin{aligned} \frac{d[\text{DNA}]_{\text{culture}}}{dt} &= r_{\text{replication,DNA}} X_v - k_{\text{degrade,DNA}} ([\text{DNA}]_{\text{culture}} - [\text{FullCap}]_{\text{culture}}) \\ &\quad - k_{\text{degrade,capsid}} [\text{FullCap}]_{\text{cyt}} X_v, \end{aligned} \quad (3.22)$$

$$\frac{d[\text{TotalCap}]_{\text{culture}}}{dt} = r_{\text{assembly}} X_v - k_{\text{degrade,capsid}} ([\text{EmptyCap}]_{\text{cyt}} + [\text{FullCap}]_{\text{cyt}}) X_v, \quad (3.23)$$

$$\frac{d[\text{TotalCap}]_{\text{media}}}{dt} = k_{\text{secrete}} [\text{FullCap}]_{\text{cyt}} X_v, \quad (3.24)$$

$$\frac{d[\text{FullCap}]_{\text{culture}}}{dt} = r_{\text{pack,DNA}} X_v - k_{\text{degrade,capsid}} [\text{FullCap}]_{\text{cyt}} X_v. \quad (3.25)$$

Parameters were estimated using maximum likelihood estimation in the log space, i.e.,

$$\min_{\mathbf{k}} = (\mathbf{y} - \mathbf{u}(\mathbf{k}, \mathbf{k}_0^*))^\top \mathbf{V}_y^{-1} (\mathbf{y} - \mathbf{u}(\mathbf{k}, \mathbf{k}_0^*)), \quad (3.26)$$

$$\mathbf{V}_y = \left. \frac{\partial \mathbf{u}}{\partial \mathbf{k}_0} \right|_{\mathbf{k}_0^*}^\top \mathbf{V}_k \left. \frac{\partial \mathbf{u}}{\partial \mathbf{k}_0} \right|_{\mathbf{k}_0^*} + \mathbf{V}_\varepsilon, \quad (3.27)$$

$$\mathbf{V}_{\log_{10} \mathbf{k}}^{-1} = \left. \frac{\partial \mathbf{u}}{\partial \log_{10} \mathbf{k}} \right|_{\mathbf{k}^*}^\top \mathbf{V}_y^{-1} \left. \frac{\partial \mathbf{u}}{\partial \log_{10} \mathbf{k}} \right|_{\mathbf{k}^*}, \quad (3.28)$$

$$\frac{\partial \mathbf{u}}{\partial \log \mathbf{k}} = (\ln 10) \frac{\partial \mathbf{u}}{\partial \mathbf{k}} \begin{pmatrix} k_1 & & \\ & \ddots & \\ & & k_i \end{pmatrix}. \quad (3.29)$$

The model parameters are summarized in Table 3-1.

Table 3.1: Model parameters.

Parameter	Value	Confidence Intervals	Unit	Method
$\mu_{\max}$	2.71E-01	(1.88E-01, 3.90E-01)	h^{-1}	Fitted to data
k_d	2.62E-07	(2.44E-07, 2.82E-07)	h^{-1}	Fitted to data
$k_{\text{uptake,plasmid}}$	1.91E-02	(1.37E-02, 2.67E-02)	h^{-1}	Fitted to data
$k_{\text{degrade,plasmid}}$	2.66E-02	(2.38E-02, 2.98E-02)	h^{-1}	Fitted to data
$K_{i,\text{plasmid}}$	3.23E+02	(2.07E+02, 5.04E+02)	plasmid/cell	Fitted to data
$K_{\text{uptake,density}}$	2.70E+06	(1.58E+06, 4.63E+06)	viable cell/mL	Fitted to data
k_{escape}	6.00E-01		h^{-1}	Fixed to literature value [29]
$k_{\text{entry,nuclear}}$	4.30E-03	(2.40E-03, 7.70E-03)	h^{-1}	Fixed to literature value [27]
$k_{\text{synthesis,Rep}}$	6.50E+04	(5.28E+04, 8.00E+04)	(Rep protein)(mM) ² (cell)/(plasmid)	Fixed to literature value [27]
k_{expel}	1.80E+00		h^{-1}	Fixed to literature value [84]
$k_{\text{degrade,Rep}}$	7.45E-02	(5.24E-02, 1.00E-03)	h^{-1}	Fitted to data
k_{Amn}	9.98E-01	(6.17E-01, 1.61)	mM	Fitted to data
$K_{\text{Rep synthesis,pHelper}}$	1.10E+01		plasmid/cell	Fixed to literature value [85]
$k_{\text{synthesis,VP}}$	1.53E+02	(1.43E+02, 1.65E+02)	mM ² /(plasmid/cell)/ (Rep protein/cell) ^{1/2}	Fitted to data

k_{assembly}	7.50E-03	(3.10E-02, 1.80E-02)	capsid/ (VP protein/cell)/h	Fitted to literature data [86]
$k_{\text{degrade,VP}}$	2.70E-01	(2.3E-01, 3.5E-01)	h ⁻¹	Fixed to literature value31
$k_{\text{degrade,cap}}; k_{\text{edegrade,DNA}}$	6.28E-02	(5.79E-02, 6.82E-02)	h ⁻¹	Fitted to data
$k_{\text{secrete,cyto}}; k_{\text{expel}}$	1.80E+00		h ⁻¹	Fixed to literature value42
$k_{\text{secrete,media}}$	1.00E-02	(9.18E-03, 1.11E-02)	h ⁻¹	Fitted to data
$k_{\text{replication,DNA}}$	9.53E+02	(8.69E+02, 1.04E+03)	DNA/cell/h	Fitted to data
$K_{\text{DNA rep,Rep}}; K_{\text{pack,Rep}}$	6.11E+05	(5.25E+05, 7.11E+05)	Rep protein/cell	Fitted to data
$K_{\text{pack,DNA}}$	2.06E-01	(1.78E-01, 2.38E-01)	capsid/DNA/h	Fitted to data

3.3 Results

3.3.1 Experiments and mechanistic modeling of multi-stage triple transfection of cell culture

HEK293 cells were transfected with plasmids (pAAV-GFP, pRC5, and pHelper at a 1:1:1 molar ratio) in three different experimental conditions. In each condition, the total plasmid input remained constant in one, two, or three-stage transfection ($N = 1, 2, 3$, respectively), but was evenly divided between stages, each 24 hours apart (Figure 3-1A). Regardless of the number of stages, the ratio of three plasmids (pAAV-GFP, pRC5, pHelper) was kept constant at a 1:1:1 molar ratio. The 24 h interval between stages of plasmid additions was chosen for two reasons: to promote plasmid uptake into cells as it has been shown to peak 24 h after transfection [45], and to enable a practical implementation of the experimental design.

In addition to the experiments, a mechanistic model was developed that describes the viral production process during triple transfection, extending from the previous mathematical model developed in Chapter 2 [27]. The mechanistic model was based on a reaction network starting from transfection-mediated plasmid DNA delivery into the cell nucleus, leading to the synthesis of viral particles, and their release into media (Figure 3-1B). The set of uncertain parameters were fitted to experimental data from discrete time points aiming to capture the trends observed from the cell culture.

3.3.2 Cell growth, viability and plasmid uptake are improved in multi-stage transfections

The impact of multi-stage transfection ($N > 1$) on the cell density and viability was first evaluated. Both cell density and viability improved for $N > 1$ with gradual plasmid dosage; $N = 3$ showed the highest cell density and viability, while $N = 1$ showed the lowest (Figure 3-2). On the other hand, $N = 1$ resulted in higher uptake of plasmids at the beginning of the culture although $N > 1$ eventually exceeded $N = 1$ in cellular plasmid content (Figure 3-3). Notably, it was experimentally observed that

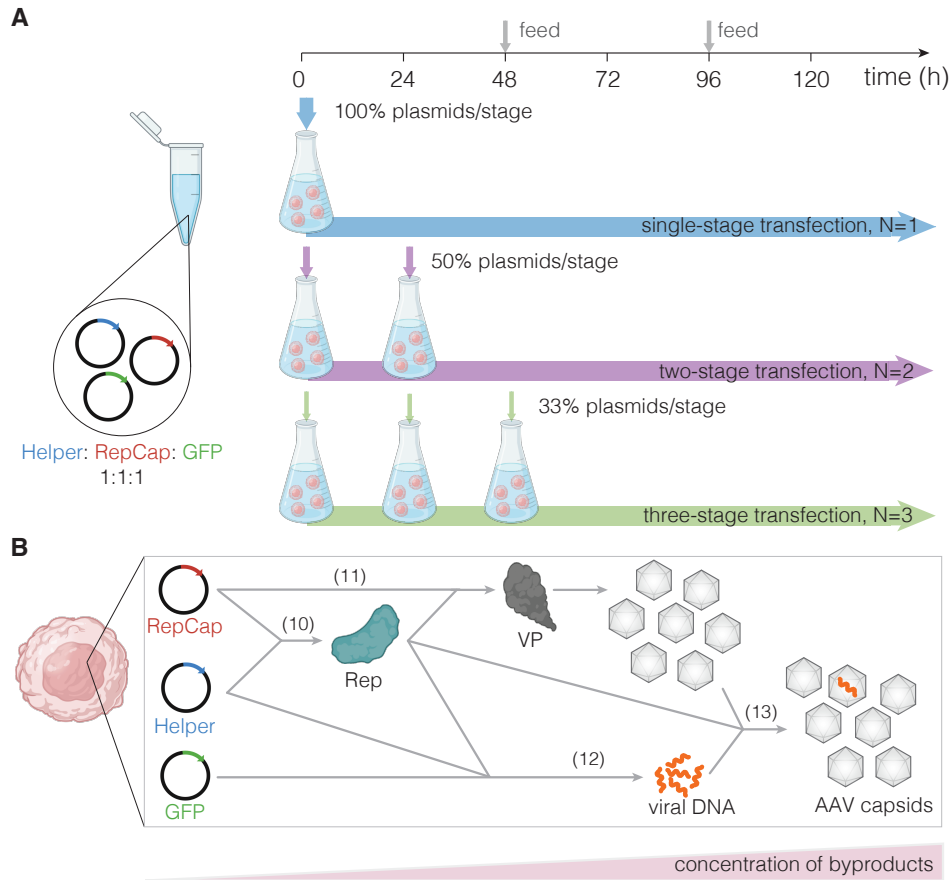


Figure 3-1: Multi-stage transfection design and viral vector synthesis schematic. (A) Multi-stage transfection experimental design: one-stage transfection ($N = 1$): 2 μg plasmid/mL culture are transfected in a single dose at the beginning of the culture, or 0 h; two-stage transfection ($N = 2$): 1 μg plasmid/mL culture are transfected at 0 and 24 h; three-stage transfection ($N = 3$): 0.7 μg plasmid/mL culture are transfected at 0, 24, and 48 h. Each culture is performed in replicate ($n = 2$) and each measurement was taken twice. (B) Schematic of rAAV synthesis from transfected plasmids. Numbers in the parentheses refer to corresponding equations in the Mathematical Modeling section.

there was no significant difference between the uptake of different types of plasmids into cells, suggesting that their kinetics are similar (Figure B-1).

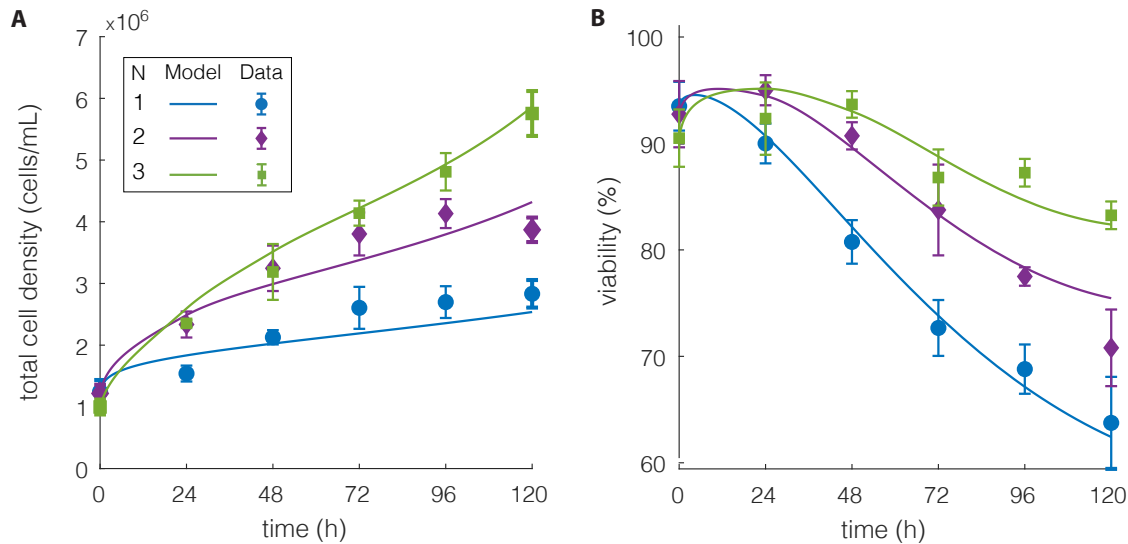


Figure 3-2: Cell growth and viability of transfection cultures for one-, two-, and three-stage transfection cultures. (A) Total cell density. (B) Cell viability. Error bars are the average of standard errors and propagated errors from all measurements at one time point for $n = 2$ and duplicate measurements.

3.3.3 Total replicated viral DNA is mostly independent of number of transfection stages

Next, the expression of Rep protein was studied, which is a critical step in the rAAV production process (Figure 3-1B) following the uptake of plasmids. Rep is a non-structural protein that plays a critical role in AAV DNA replication [38] and DNA packaging into AAV capsids [41, 42, 43]. Here, the total expression of all four Rep protein isoforms (Rep 78, 68, 52, and 40) was quantified as a unified Rep component over the course of viral production. The quantity of Rep reached a maximum approximately 48 h after the last plasmid dose, independent of N (Figure 3-4A). Interestingly, the maximum concentrations of Rep for $N > 1$ were lower than $N = 1$ (Figure 3-4A), despite the higher number of intracellular plasmids (Figure 3-3). However, replicated viral DNA, which uses Rep as a catalyst (Figure 3-4B), displayed relatively similar trends for different transfection stages (Figure 3-4B). This observation suggests DNA synthesis only requires a threshold concentration of Rep protein, then becomes limited by another biological mechanism.

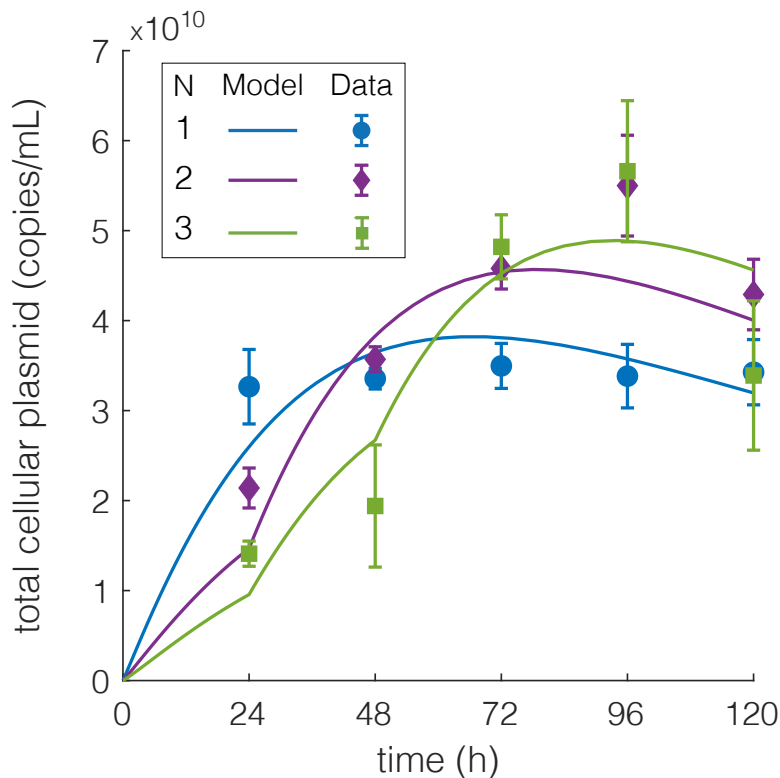


Figure 3-3: Cellular plasmid dynamics for one-, two-, and three-stage transfection cultures. Error bars are the average of standard errors and propagated errors from all measurements at one time point for $n = 2$ and duplicate measurements.

3.3.4 Multi-stage transfections increase full to total capsid ratio without compromising genome titer

The Rep proteins also drive the synthesis of viral proteins, which are then assembled into AAV capsids (Figure 3-1B). The dynamics of AAV capsid production (Figure 3-5A) resembled that of Rep protein synthesis (Figure 3-4A). Similar to the trend seen with Rep (Figure 3-4A), capsid titer was highest for $N = 1$, followed by $N = 2$ and $N = 3$. In fact, the production of capsids is three times higher for $N = 1$ than $N = 3$. In comparison, the encapsidated genome copies, or genome titer, is comparable for all N at the end of the culture (Figure 3-5B). Furthermore, the majority (60–80%) of replicated viral DNA was encapsidated in viral capsids as soon as 48 h after the first transfection for all N (Figure 3-5C). This result suggests that the dynamics of genome titer is more dependent on viral DNA replication than capsid production.

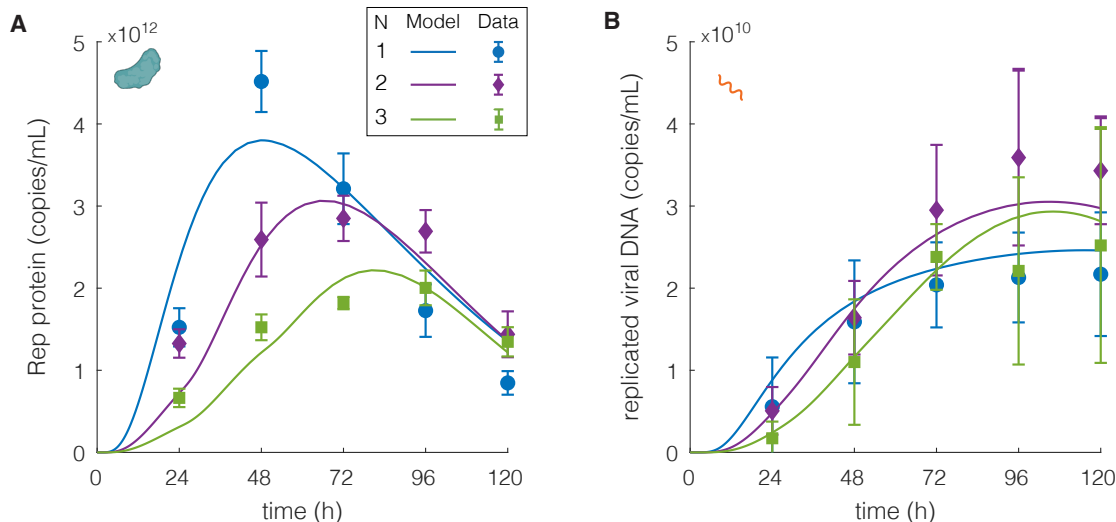


Figure 3-4: Rep protein and replicated viral DNA dynamics for one-, two-, and three-stage transfection cultures. (A) Rep protein in cells. (B) Replicated viral DNA. Error bars are the average of standard errors and propagated errors from all measurements at one time point.

The ratio of full AAV capsids increased over time for all numbers of transfection stages (Figure 3-5D). Furthermore, $N > 1$ yielded higher ratios of full capsids (10 and 15% respectively for $N = 2$ and 3) than $N = 1$ (6%) as the capsid titer was significantly higher for $N = 1$ (Figure 3-5A) while the genome titer was comparable for all N (Figure 3-5B).

3.4 Discussion

To understand the effect of multi-stage transfection on rAAV production, the dynamics of the critical components during the process was examined (Figure 3-1B), including plasmid uptake, Rep protein synthesis, capsid assembly, viral DNA replication, and genome packaging via molecular analysis and mechanistic model (Figure 3-6).

Cell growth and viability increased when using multi-stage ($N > 1$) transfections (Figure 3-2B). For $N > 1$, the DNA plasmids complexed with cytotoxic cationic polymer (PEI) [87, 88] were spread out over multiple stages, thus improving cell

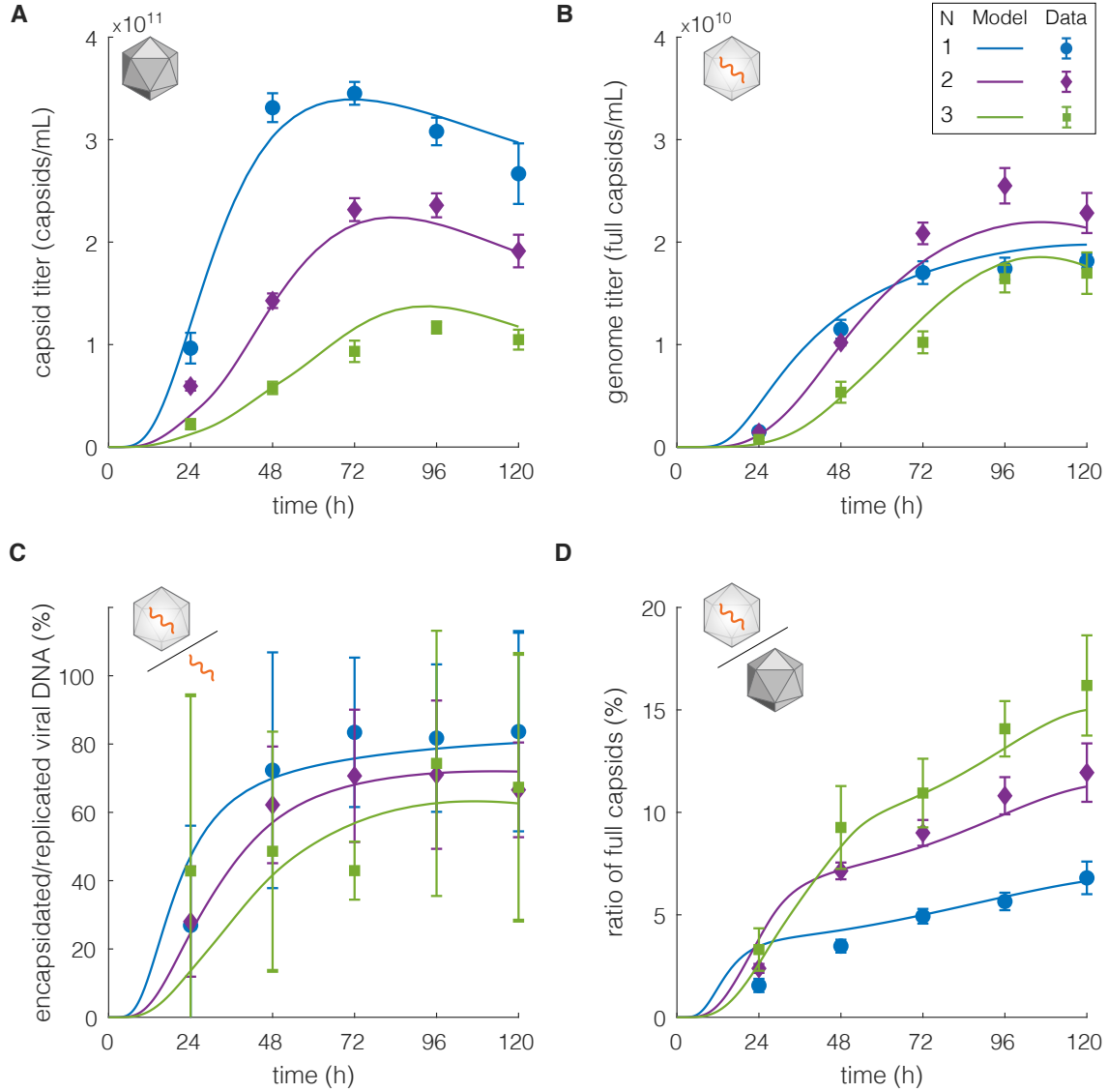


Figure 3-5: Capsid titer and genome titer dynamics for one-, two-, and three-stage transfection cultures. (A) Capsid titer inside cells and supernatant per mL of suspension. (B) Genome titer inside cells and supernatant per mL of suspension. (C) Genome titer/replicated viral DNA ratio. (D) Full capsid/total capsid ratio. Error bars are the average of standard errors and propagated errors from for $n = 2$ and duplicate measurements.

viability compared to $N = 1$ where PEI was added at once. Cell viability is critical as structural and non-structural components of rAAV synthesis require active host cell resources for translation and transcription.[89] An increase in the total number of plasmids taken up by cells in later phase was observed (after 48 h) with the subsequent

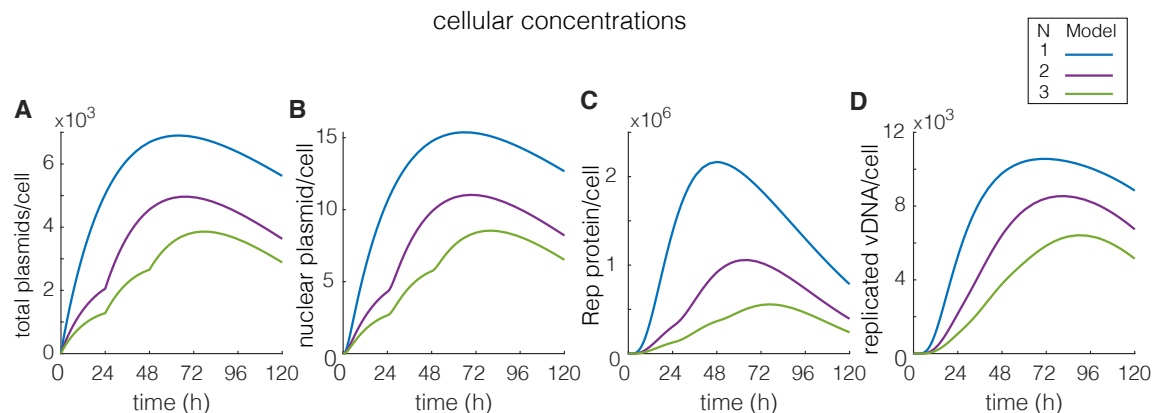


Figure 3-6: Dynamic model simulations of intracellular species. (A) Plasmid per cell. (B) Nuclear plasmid. (C) Rep protein per cell. (D) Viral DNA per cell.

transfections for $N > 1$ due to higher cell densities and viability (Figure 3-3). Model simulation was able to capture this trend, with the assumption that only live cells could be transfected (Eq. 1)—changes to the membrane of dead cells compromise the ability to bind to cationic complexes.[90] Although the total uptake of plasmid increased at later phases (after 48 h) for $N > 1$, model simulations (Figure 3-6) showed that the uptake of plasmid per live cell decreases, which could be attributed to increased live cell density (Figure 3-2A).

Multi-stage transfections ($N > 1$) maintained a regulated level of Rep proteins compared to one-stage transfection ($N = 1$) (Figure 3-4A). As Rep proteins are expressed from the pRC5 plasmid with the assistance of Helper proteins expressed from the genes on pHelper, the initial (0 h) addition of plasmid was more impactful on Rep production than subsequent (24 h, 48 h) additions of plasmids. Thus $N > 1$ produced consistently less Rep proteins (Figure 3-4A) than $N = 1$. In all cases, synthesis of Rep proteins was maximized at 48 hours after the last addition of plasmids and was then subject to rapid degradation. Plasmid availability was unlikely the contributing factor to the decline of protein synthesis in later time points, for the quantity of plasmids in the cells and the nucleus at 120 h remains at 70%–80% the maximum amount in all N (Figures 3-3 and 3-6).

The reduced capability of the cells to produce more protein later during the

culture could be attributable to ammonia accumulation, an inhibition factor that influences the cellular metabolic stages that govern protein transcription and translation activities [91, 92]. Thus, by incorporating the second-order inhibition effect of elevated ammonia concentration on recombinant protein synthesis, the model captured the rapid decline in Rep protein dynamics (section 3.2.9, Equation 3.10).

The dynamics of capsid titer at early phases (before 72 h) followed a similar trend to that of Rep protein, as the viral proteins that capsids are built upon also require helper and packaging plasmids (Figures 3-4A and 3-5A). In addition, the *cap* gene promoter is also known to be upregulated by the Rep protein [39]. Unlike the Rep protein, capsid titer did not decline rapidly after hitting a peak (Figure 3-5A), as assembled capsids are not prone to rapid degradation compared to individual VP [50, 93]. However, a slight decline was still visible after the capsid titer reached a maximum, suggesting capsids do degrade during culture—previous studies on rAAV transduction efficiency have reported degradation of AAV capsid by proteasome in the cytosol via a ubiquitin-dependent pathway [94].

The rAAV transgene requires the activity of the Rep protein with the assistance of Helper proteins to replicate (Figure 3-1B) [76, 95]. However, the dynamics of these interactions are poorly understood. Model simulations showed that, for every transfection stage added, the concentration of Rep protein per cell increased by 100%, but replicated viral DNA per cell only by 20% (Figure 3-6). However, this effect was negated by lower population of viable cells, making the total replicated viral DNA comparable for all N (Figure 3-3B). The observation is consistent with previous reports that the Rep protein isotype Rep78 has been reported to introduce cell cytotoxicity, and disrupt the cell cycle [96], the modulation of which is critical for viral DNA replication activity. Therefore, these results suggest that the viral DNA replication rate does not increase significantly with the concentration of Rep protein, but rather Rep serves as a catalyst, a threshold quantity of which triggers the replication of viral DNA. Dynamics of genome titer also showed that its degradation was minimal throughout the process, as the portion of DNA encapsidated in capsids was likely protected from cellular and media components that are destructive.

Production of rAAV via triple transfection is known to produce a low ratio (1–30%) of full capsids [8, 81]. In Chapter 2, two limiting factors that caused this low ratio were identified: low nuclear plasmid availability and poorly coordinated timeline between capsid and viral DNA synthesis [27]. In the current experimental design, the media exchange step following transfection was removed to overcome the barrier of low plasmid availability. In the previous study, capsid titer reached a peak at 24 h with media exchange [27], while for $N = 1$ of this study, at the same plasmid dosage and without media exchange, capsid titer reached a peak at 48 h (Figure 3-5A). In addition, multistage transfection methods ($N > 1$) prolonged capsid production compared to $N = 1$ (Figure 3-5A). Although capsid titer decreased with increasing N , the genome titer (Figure 3-5B) was comparable for all N . This suggests that the encapsidation of DNA to produce full capsid requires continued capsid production but at a reduced rate. Once capsid production is sustained, viral DNA replication becomes the limiting factor to genome titer (Figure 3-5B) — towards the end of the cultures, 60%–80% of the replicated viral DNA was encapsidated inside capsids (Figure 3-5C). It was shown in Chapter 1 and the published manuscript [27] that synthesis of viral DNA is a limiting factor. The results of a previous study are consistent as they observed improved full to total ratio by increasing viral DNA synthesis through a cell-cycle modulation mechanism.[97] Overall, here multi-stage transfections ($N > 1$) increased the ratio of full capsids by maintaining genome titer and avoiding the abundance of empty capsids, while using the same quantity of plasmids compared to $N = 1$ (Figure 3-5D).

The improved ratio of full to total rAAV capsids achieved here points to future directions for further optimization of the rAAV production process. First, the dynamics of Rep protein production in relation to viral DNA replication and capsid production across different numbers of transfection stages provide understanding for designing a rational plasmid dosing strategy. As the genome titer remained comparable across different numbers of transfection stages, and did not correlate with the concentration of Rep protein, it is plausible to deliver the RepCap plasmid in a smaller dose at an early phase to initiate replication of viral DNA, and then in a larger dose

at later phase to concentrate capsid production resources. In addition, the ammonia inhibition effect on protein synthesis suggests that a process design which reduces the level of ammonia in the supernatant will also increase capsid titer. Although the fed-batch strategy employed in this study ensured that cell proliferation was not limited by nutrients, the accumulation of byproducts, specifically ammonia (Figure B-2), could inhibit protein synthesis. Other studies have reported that changing the media and harvesting continuously increased titer [22], as the act of media exchange not only supplies the cells with fresh nutrients, but also removes waste accumulation, a feature not possible in batch and fed-batch operations. To this end, a perfusion process can vastly enhance the viral vector production capability by maintaining ammonia byproduct at a non-limiting level to extend the protein synthesis timeline. A continuous platform also has the ability to control plasmid residence time and uptake dynamics, allowing fine-tuning of intermediate process dynamics to optimize product quantity and quality [98].

Chapter 4

Perfusion bioreactor for continuous rAAV manufacturing via transient transfection

4.1 Introduction

The biomanufacturing industry has been shifting from the traditional batch and fedbatch bioprocessing to continuous operations for both small-molecule drugs and biotechnology products. The batch-to-batch variability in the traditional modes of operations is one major issue affecting product quality and causing biologics drug shortage. Continuous processes are advantageous for the ability to sustain operation, maintain consistent product quality, improve speed and reduce cost [98].

Production of rAAV viral vector via transient transfection has been performed in batch processes. The result of fed-batch shake flask experiments in chapter 3 indicates the possibility of extending the viral production process through waste removal and continued, discrete plasmid feeding. The strategy of re-transfection has been explored in a few previous studies, with applications for recombinant protein production and virus-like-particle production [99]. However, the proposed protocols have not demonstrated increased recovery of transfected cells percentage or leveraged the

potentials of the high cell density that a perfusion system can maintain, and have not been applied to more complex system that involves multiple plasmids system such as rAAV production using triple transfection. This last and final chapter will focus on a perfusion bioreactor platform with protocols developed for rAAV production via transfection.

4.2 Materials and Methods

4.2.1 Cell line, plasmids, media and plasmids

FreeStyle™ 293-F cells from Thermo Fisher Scientific (Waltham, MA) were used as host cells. The cells were maintained as described in section 2.2. The 293 Expression Media from Thermo Fisher Scientific (Waltham, MA) was used to culture cells. The plasmids for triple transfection: pAAV-GFP (Part No. AAV-400), pRC5 (Part No. VPK-425), and pHelper (Part No. 340202), were purchased from Cell Biolabs (San Diego, CA) and amplified in-house according to the procedures described in section 3.2.1.

4.2.2 Bioreactor system for cultivation of HEK293 cells

In the present study, the 500mL Applikon stirred-tank bioreactor (Gething, Rochester, NY) used for viral vector production was equipped with a three-blade marine impeller and a micro-sparger. The micro-sparger was used to adjust air and pure O_2 flow rates to maintain dO reading at or above the set point. The pH was maintained at the set point via CO_2 sparging and 0.5M $NaHCO_3$ addition. Antifoam C from Millipore-Sigma (Burlington, MA) was used to control foaming when foam was detected by a foam level controller. The temperature of the bioreactor vessel content was heated through a heating jacket and cooled via a condenser. The PID controllers were automated through the myControls console that was purchased along with the Applikon bioreactor. A summary of the bioreactor control loops are summarized in Table 4-1. The bioreactor was inoculated with a total density of 3×10^5 cells/mL and operated

at 37 °C, dO $\geq$ 40%, pH 7, and 125 rpm. Cells were grown to 1×10^6 cells/mL before the first transfection.

Control loop	Setpoint	Actuator (-)	Actuator (+)
pH	7 ± 0.1	CO ₂	0.5 NaHCO ₃
Agitation rate	125 rpm		
Temperature	37	Condenser	Heating jacket
dO ₂	40% saturated air		Air, O ₂

Table 4.1: Bioreactor inner controllers settings

4.2.3 Perfusion system

The perfusion bioreactor system schematic is shown in Figure 4-1, which includes a tangential flow system, the harvest pump (F_h) and the bleed pump (F_b). The tangential flow filtration system consists of a 0.2 μ m hollow membrane filter (Artemis Biosystems, Quincy, MA) driven by a magnetic pump (Levitronix[®], Framingham, MA). The cell-free harvest line is connected to the filter and driven by a controllable peristaltic pump (Masterflex, Radnor, PA). The cell-containing bleed is drawn through a dip tube directly from inside the vessel via a controllable peristaltic pump (Masterflex, Radnor, PA). The volume level inside the bioreactor is controlled via a level sensor to maintain a constant working volume. The working volume, which included the volume inside the vessel and the recirculation tubing, was 370mL.

The process parameters were the concentration of discrete plasmid input [μ g/mL], harvest flow rate [mL/h] and bleeding flow rate [mL/h]. The harvest flow rate F_h [mL/h] and bleed flow rate F_b [mL/h] were manually adjusted periodically to achieve desired cellular density and maintain steady and non-limiting nutrients level. A transfection-free cell cultivation was run to determine a reasonable perfusion rate as a function of total cell density (Figure C-1 and Table C-1). For perfusion with transfection, the total perfusion rate $F_t = F_h + F_b$ was maintained at slightly higher than the transfection-free operation with consideration of the cytotoxicity introduced by the transfection reagent. The discrete plasmid input C_p was adjusted depending

on the cell density at the time of each transfection.

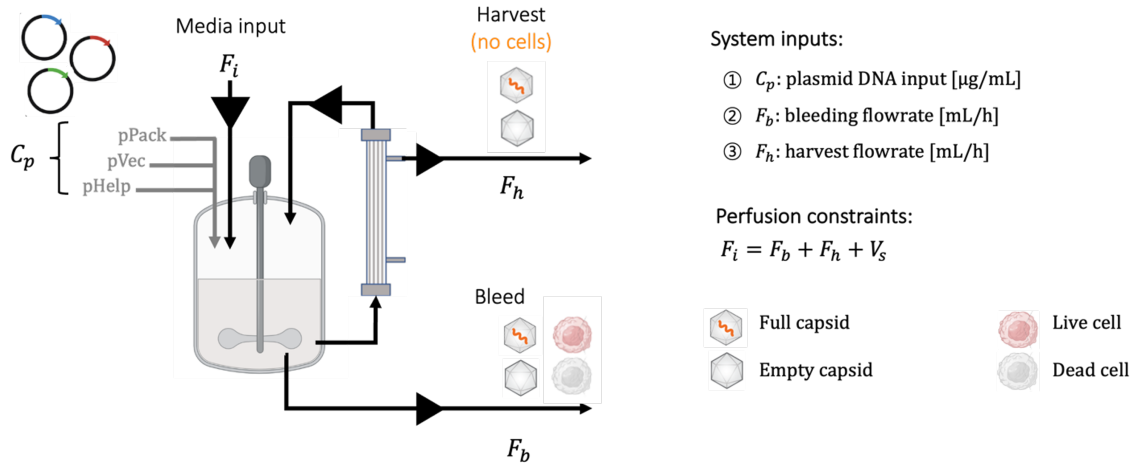


Figure 4-1: Perfusion bioreactor schematic.

4.2.4 Triple transfection of HEK293 cells

Cells in bioreactor were grown in batch mode until 1 million cells/mL. Each plasmid solution was quantified using a NanoDrop Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). The three plasmids were mixed at 1:1:1 molar ratio, and the total plasmid dosage was adjusted depending on the cell density at the time of transfection. The transfection cocktail was prepared by the sequential addition of the plasmid mix, culture medium, and the PEI Max solution. The amount of PEI Max added was calculated so that the PEI:DNA mass ratio came out to 2:1.

4.2.5 Analytical methods

A volume of 7 mL sample was drawn daily for analyses. Culture conditions and measurements include total cell density, viability, metabolite concentrations, and amino acid concentrations. The sample titers were analyzed for total capsids and full capsids via ELISA and ddPCR. The protocols for sampling, storing and assays were as described in the Materials and Methods section 3.2 of Chapter 3. The incubation time between cells and plasmids was determined based on the cell density and availability of nutrients at the time of transfection.

4.2.6 Continuous bioreactor mathematical modeling

Bioreactors have been traditionally modeled as stirred-tank chemical reactors. The material balance equation is

$$\frac{dy}{dt} = \frac{F_{\text{in}}}{V_L}(y_{\text{in}} - y) - \frac{F_{\text{out}}}{V_L}(y - \delta y) + r_y, \quad (4.1)$$

where y is the concentration in the reactor, V_L is the liquid medium volume, F is the media flow rate, the subscripts in and out referring to inlet and outlet streams, δ is the separation factor at the outlet, and r_y is the volumetric reaction rate, which in this case will be calculated from the viral vector production kinetic model previous developed in Chapter 3.

In this perfusion system with bleeding and harvest streams, the equation is expanded to

$$\frac{dy}{dt} = \frac{F_t}{V_L}(y_{\text{in}} - y) - \frac{F_h}{V_L}(y - \delta_h y) - \frac{F_b}{V_L}y + r_y, \quad (4.2)$$

where $F_t = F_h + F_b$ is the total perfusion rate and δ_h is the separation factor at the harvest line.

4.3 Results and Discussion

4.3.1 Logic-based re-transfection scheme

Transfection was performed on days 0, 5, 7, 9 as outlined in Table 4-2. The initial transfection design at 0 h followed the conventional design at 2 $\mu\text{g}/\text{mL}$ of cell culture at 1 million cells/mL. In subsequent transfections, transfections at higher densities were performed. At each of the transfection time, the cells were diluted via bleeding and sampling line to reach the desired transfection density. The first transfection was performed at 0 h, and the perfusion line was stopped for 24 hours. In the subsequent transfections, the perfusion incubation period was experimented at 0 h, 12 h, and 24 h. Finding the right incubation time period is critical – prolonged incubation

Time (day)	Cell density (10^6 /mL)	Plasmid concentration (μ g/mL)	Plasmid concentration (μ g/ 10^6 cells)	Plasmid ratio (pGFP pHelper:pRC)	Plasmid incubation period (h)
0	1.12	2	2	1:1:1	24
5	4.7	2	0.43	1:1:1	0
7	5.1	5.1	1	1:1:1	12
9	7.62	7.62	1	1:1:1	24

Table 4.2: Transfection design.

without perfusion increases the exposure of cells to plasmids, but also risks cell health and viability as nutrients are not replenished. Re-transfection was determined via a model-based calculation on the extracellular PEI/plasmid complexes concentration, using the mechanistic model developed in Chapter 2 and Chapter 3.

The first four transfections were performed at equal plasmid ratio (1:1:1). The first transfection was performed at 2 μ g plasmid DNA/mL and incubated for 24 h before turning on perfusion at 1×10^6 cells/mL, similar to the conventional dosage and condition, to establish a baseline for transfection in perfusion bioreactor. The second transfection was performed at 2 μ g plasmid DNA/mL and 5×10^6 cells/mL without incubation. The third and fourth transfections were performed back to back to recreate the multistage-transfection in fed-batch performed in chapter 3. The plasmid dosage for each transfection was scaled by 1 μ g/million cells.

Model-based calculation was performed to estimate the concentration of plasmids in the extracellular space and in the cell nucleus (Figure 4-2). The concentration estimation was then used to guide the dosing schedule, which is when the concentration dipped to zeros or close to zeros. The second peak, corresponding to the second transfection in which there was no incubation and the plasmid dosage was not scaled with cell density, showed negligible improvement in nuclear plasmid concentration. This result implies that the plasmid dosage should be scaled with cell density and that certain incubation period, when perfusion is turned off, is necessary for effective transfection.

Figure 4-3 shows cell density and viability during the course of continuous viral production. Most times, the viability was maintained at $\geq 90\%$. Between day 11–12, there was a malfunction in the pump of a cell counting device, leading to low

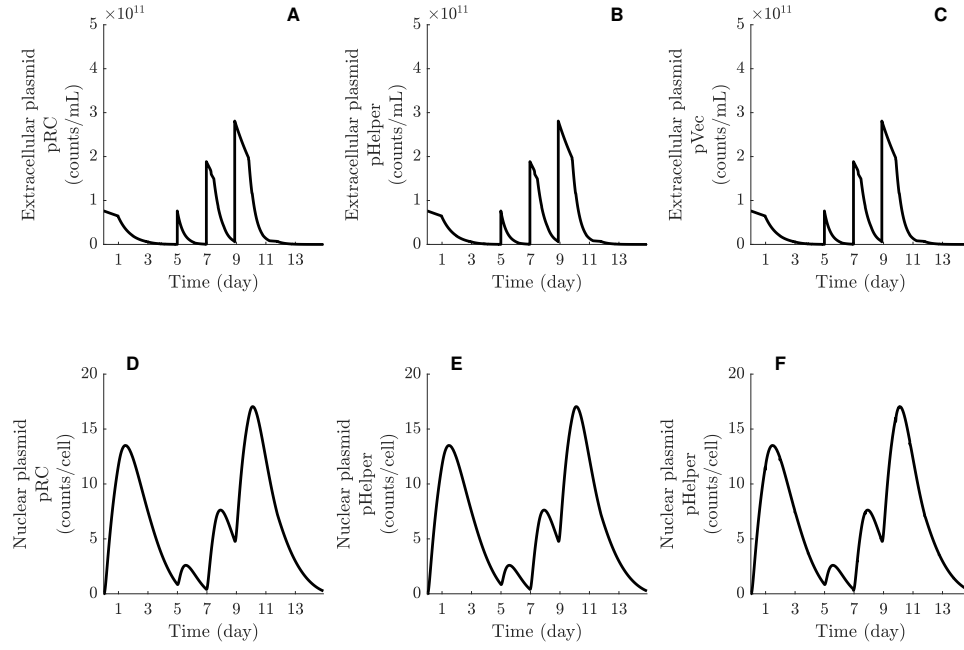


Figure 4-2: Model simulation of plasmids concentration in the extracellular space and in the cell nucleus.

growth and viability. After disconnecting the device and running harvest and bleeding in combination, the viability was recovered. In Figure 4-3, the model predicted accurately the dynamics of cell growth and death influenced by transfection in the perfusion system, using the same cell growth and plasmid delivery kinetic parameters as reported in Chapter 3.

The pump flow rates, including harvest rate F_h and bleeding rate F_b , are recorded in Table C.2 along with the total perfusion rate $F_t = F_h + F_b$. The bleed pump was turned on periodically to adjust cell density to the desired density for transfection. The total perfusion rate was maintained around 1-3 vvd with a working volume of 370 mL. The demonstrated perfusion rate is only slightly higher than the reported perfusion rate reported in another study for growing pure HEK293 culture without transfection [62] and in the preliminary study (Appendix C). The pump operation value was reasonable as in the case for transfection, it is not only important to maintain stable metabolite levels, but also flush out cytotoxic transfection reagent.

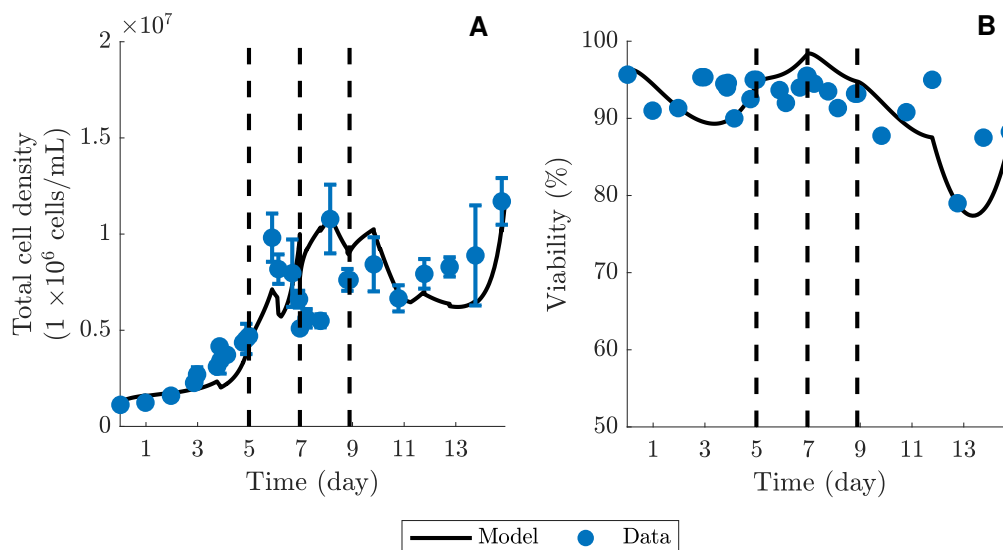


Figure 4-3: Model vs. data of cell density and viability. (A) Total cell density. (B) Cell viability. Dotted line represents time of transfection.

In Chapter 3 of this thesis, a multi-stage transfection protocol in a fed-batch operation was demonstrated. A fed-batch supplements nutrients so the metabolites are non-limiting. However, a fed-batch process does not provide the flexibility to remove by-products and manipulate the materials and cell density. Perfusion processing has the ability of exchanging the spent medium with fresh medium thereby reducing the concentration of byproducts inside the bioreactor. In this study, culture metabolites were closely monitored at least every 24 hours. The metabolite profiles: glucose, glutamine, lactate and ammonia were maintained at reasonable levels (Figure 4-4). The glucose level dropped below 0.5 g/L on day 10 and day 12. Day 10 was a day after transfection on day 9 and after the perfusion had been turned off for 24 hours. The viability (Figure 4-3B) dropped to slightly below 90% but increased right after the perfusion system was turned on. It has been demonstrated in transfection-free cell

growth that low but non-depleted glucose is not detrimental to cell growth (Appendix C). During the course of continuous production, lactate was maintained at a steady level between 2–3 g/L and ammonia was maintained at approximately 1 mM.

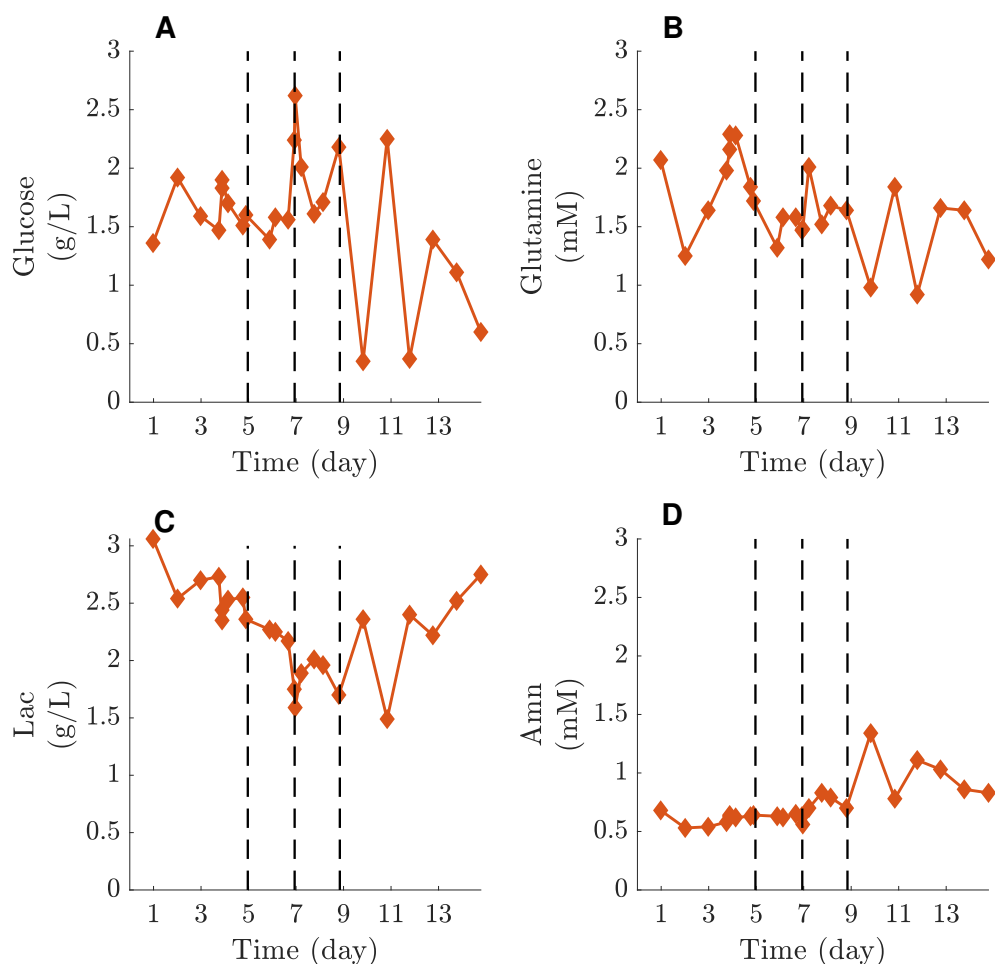


Figure 4-4: Metabolites concentration.

4.3.2 Capsid titer and full capsid titer dynamics in bioreactor vessel

The genome titer and capsid titer inside and outside the cells (Figure 4-5) at different time points are characterized via ddPCR and ELISA, respectively. The media used in perfusion bioreactor study was different from the multistage transfection study in

chapter 3, therefore the kinetic model parameters were updated (Table 4.3). With only adjustments of viral production parameters, the model was able to predict the trend of capsid and vector genome production (Figure 4-5). Subsequent transfections at high densities yielded over one magnitude more product than the conventional design (Figure 4-5A, day 3 vs. day 11).

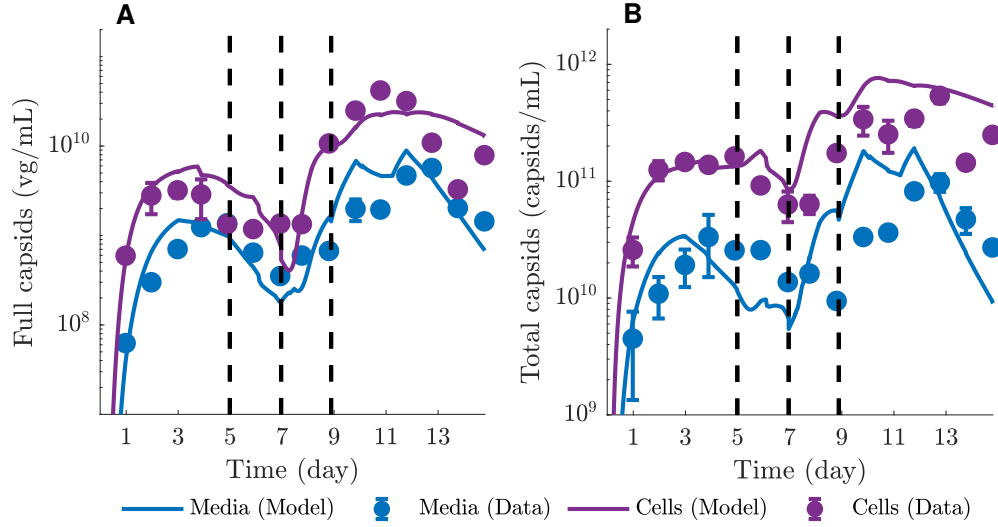


Figure 4-5: Model v. data of capsid titer and genome titer inside the bioreactor vessel.

Parameter	$k_{\text{Rep syn}}$	$k_{\text{Cap syn}}$	$k_{\text{secrete,cyto}}$	$k_{\text{secrete,media}}$	$k_{\text{replication,DNA}}$	$k_{\text{pack,DNA}}$
Value	5.20E+04	1.23E+02	3.6	2E-02	5.72E+02	1.24E-01

Table 4.3: Viral production kinetic parameters in perfusion experiment.

4.3.3 Accumulation of genome titer

The total amount of viral capsid and genome at a certain time in vessel can be calculated as follows

$$\text{fCap}_{\text{vessel}} = ([\text{fCap}_{\text{media}}] + [\text{fCap}_{\text{cells}}])V_L \quad (4.3)$$

$$\text{tCap}_{\text{vessel}} = ([\text{tCap}_{\text{media}}] + [\text{tCap}_{\text{cells}}])V_L, \quad (4.4)$$

assuming that the tangential flow filter allowed 100% of the viral particles to pass through to the harvest stream. In a study where the same membrane was utilized in a continuous process to produce influenza A virus, the efficiency of the membrane was evaluated and 100% of the viral particles were able to pass through the membrane [100].

The accumulation of total genome titer obtained from the continuous cultivation can be calculated as follows

$$\text{fCap}_{\text{harvest}} = \int_0^T [\text{fCap}_{\text{media}}] F_h dt \quad (4.5)$$

$$\text{fCap}_{\text{bleed}} = \int_0^T ([\text{fCap}_{\text{media}}] + [\text{fCap}_{\text{cells}}]) F_b dt \quad (4.6)$$

The calculations are similar for total capsid titer:

$$\text{tCap}_{\text{harvest}} = \int_0^T [\text{tCap}_{\text{media}}] F_h dt \quad (4.7)$$

$$\text{tCap}_{\text{bleed}} = \int_0^T ([\text{tCap}_{\text{media}}] + [\text{tCap}_{\text{cells}}]) F_b dt. \quad (4.8)$$

Since the viral capsid and genome capsid measurements are discrete, sparse and did not align with changes in pump operations, the viral capsid and genome quantity in between measurements were extrapolated and trapezoidal rule was applied to calculate the interval titer accumulation. Therefore, equations 4.5 and 4.6 become

$$\text{fCap}_{\text{harvest}} \approx \sum_i \frac{\text{fCap}_{\text{media}}^i + \text{fCap}_{\text{media}}^{i+1}}{2} F_h^i \quad (4.9)$$

$$\text{fCap}_{\text{bleed}} \approx \sum_i \frac{\text{fCap}_{\text{media}}^i + \text{fCap}_{\text{cells}}^i + \text{fCap}_{\text{media}}^{i+1} + \text{fCap}_{\text{cells}}^{i+1}}{2} F_b^i \quad (4.10)$$

and equations 4.7 and 4.8 become

$$\text{tCap}_{\text{harvest}} \approx \sum_i \frac{\text{tCap}_{\text{media}}^i + \text{tCap}_{\text{media}}^{i+1}}{2} F_h^i \quad (4.11)$$

$$\text{tCap}_{\text{bleed}} \approx \sum_i \frac{\text{tCap}_{\text{media}}^i + \text{tCap}_{\text{cells}}^i + \text{tCap}_{\text{media}}^{i+1} + \text{tCap}_{\text{cells}}^{i+1}}{2} F_b^i \quad (4.12)$$

The total titer, including vessel, harvest stream and bleed stream is then calculated as follows

$$\text{fCap}_{\text{total}} = \text{fCap}_{\text{harvest}} + \text{fCap}_{\text{bleed}} + \text{fCap}_{\text{vessel}} \quad (4.13)$$

$$\text{tCap}_{\text{total}} = \text{tCap}_{\text{harvest}} + \text{tCap}_{\text{bleed}} + \text{tCap}_{\text{vessel}}. \quad (4.14)$$

The accumulated capsid titer and genome titer are shown in Figure 4-6. It is notable that for all transfections, especially vector genomes (panel A), most products are retained in the cells for at least 2 days after transfection. It is also important to note that most vector genomes were produced during the first 2–3 days after transfection, after which the productivity was plateaued.

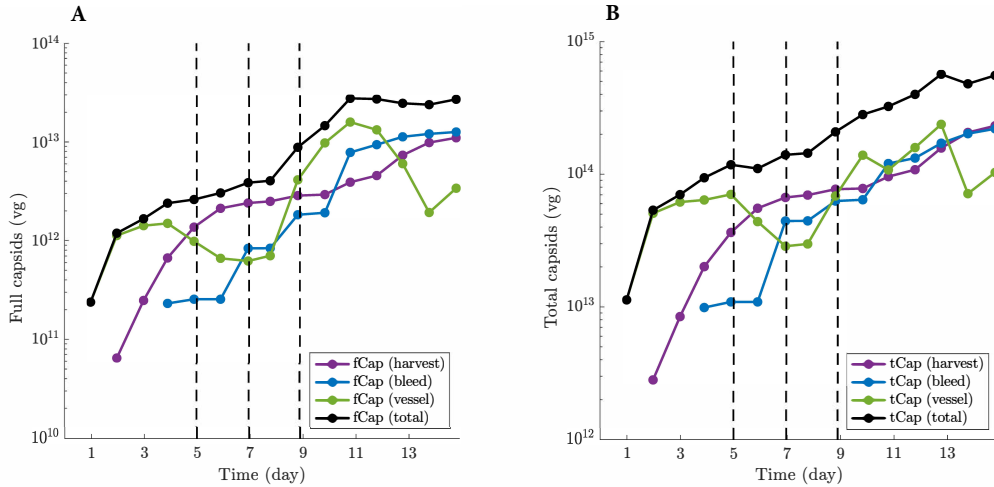


Figure 4-6: Cumulative capsid and genome titer over time. (A) Genome titer in bioreactor, cumulative genome titer in bleed stream, harvest stream, and total genome titer. (B) Capsid titer in bioreactor, cumulative capsid titer in bleed stream, harvest stream, and total capsid titer.

It is important to evaluate the yield and plasmid efficiency of each transfection. To this end, the net yield of full capsids and total capsids from each transfection was

calculated and presented in Figure 4-7 and Figure 4-8. The first transfection yielded in total 2.66×10^{12} vg. A comparable batch production of rAAV in a suspension bioreactor typically yield 1×10^9 – 1×10^{10} vg/mL [20, 27, 101], which results in a total of 3.7×10^{11} – 3.7×10^{12} when scaled up to a 370 mL production. This result suggested that the first transfection in the study is comparable to the yield of a conventional batch production. In the second transfection, the total yield obtained was half the yield of the first transfection. Low yield in second transfection was the result of lower dosage per cell and no plasmid/cell incubation, leading to low plasmid uptake, as shown in the model simulation in Figure 4-2B–D. For the third and fourth transfections, plasmid input is scaled along with cell density and spread over two doses, following the multi-stage experiment strategy in Chapter 3. The third transfection yielded more than two times and the fourth transfection yielded six times more vector genomes than the first transfection (Figure 4-7A). The third and fourth transfection together produced more than eight times the amount of the first transfection, which is equivalent to a batch production. This result means that the perfusion system and protocols allowed for successful transfection and viral vector production at high cell density in the same amount of time. Conventional production scale-up for viral vector in suspension bioreactor needs hundreds of liters bioreactors [2] — this study established a platform that produces rAAV at high cell density, which allows for scale-up without needing a larger bioreactor or conventional batch processes operated in parallel, reducing footprint and allowing higher flexibility [102]. In addition, continuous production allows much faster production compared to a conventional batch process that takes ~ 3 weeks to start up [101], enabling faster clinical trials and much higher speed to market.

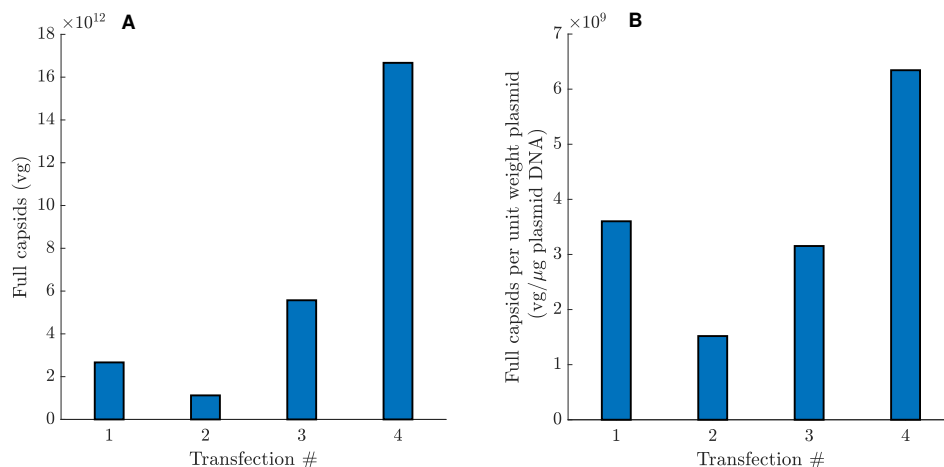


Figure 4-7: Net genome titer for each transfection. (A) Total genome titer (vg). (B) Genome titer per unit weight plasmid (vg/ μ g plasmid DNA).

In transient transfection, plasmids cost accounts for 40% of the raw materials cost [101]. As shown in Figure 4-7B, the fourth transfection had almost two times more productivity than the first transfection. Together with the third transfection, the back-to-back transfections at high cell density resulted in 32% higher plasmid usage efficiency, lowering the development cost and therapeutic cost, increasing patient accessibility.

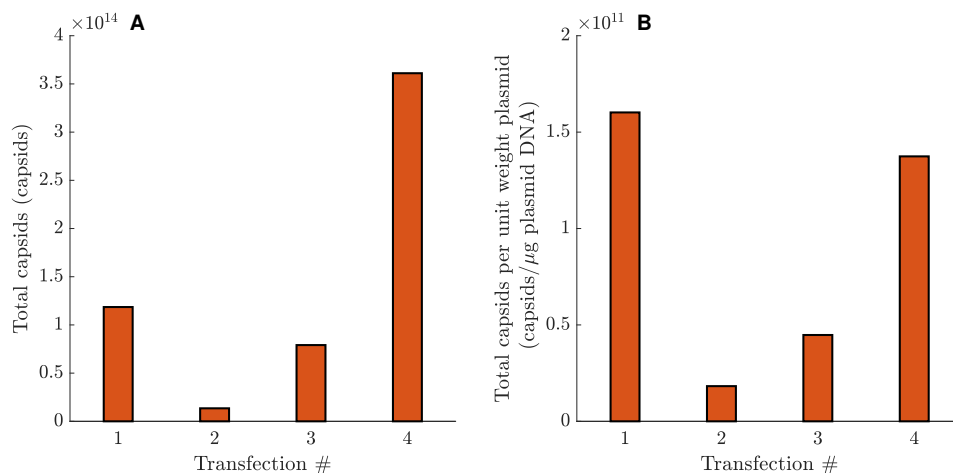


Figure 4-8: Net genome titer for each transfection. (A) Total genome titer (capsids). (B) Genome titer per unit weight plasmid (capsids/ μ g plasmid DNA).

The net production of total capsid obtained for each transfection is shown in Figure 4-8. The third transfection produced slightly lower total capsids than the first transfection, while the fourth transfection produced three times as many as the first transfection. However, this increase in total capsid production was not as high as the increase in vector genome production (Figure 4-7A). The calculation of total product purity shows that the third and fourth transfection product at high cell density transfection had three times as high of full capsid in harvest than the first transfection, which is comparable to a conventional batch transfection (Figure 4-9).

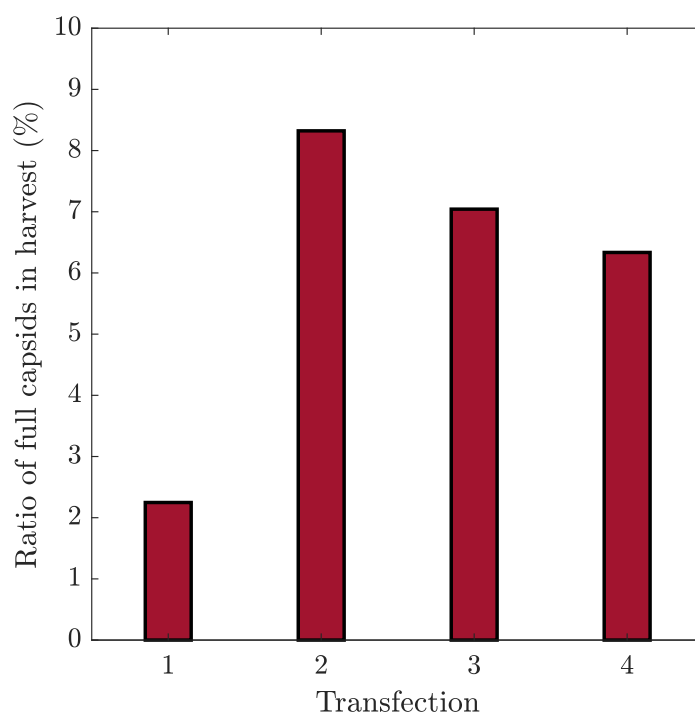


Figure 4-9: Percentage of full capsid in harvest in product from each transfection.

4.3.4 Conclusion

In this thesis chapter, a continuous process in perfusion bioreactor for rAAV manufacturing via transient transfection was successfully demonstrated. Model-based calculation was performed to guide the plasmid dosage and dosing schedule. The system consists of an automated bioreactor connected to a perfusion system enabled

by a viral harvesting unit, a tubular membrane that retains the cells while allowing viral particles to pass through, as well as a bleeding line. The harvest rate and bleeding rate, which also determine the total perfusion rate, were adjusted based on a previous perfusion experiment without transfection. A protocol for continuous production using transient transfection was developed. For each transfection, the cells were transfected followed by a period of cell-plasmid incubation of approximately 24 h, when the perfusion is turned off.

The perfusion system was able to achieve healthy cell growth and viability even after transfection, maintain steady levels of nutrients and avoid spiking of byproducts such as lactate and ammonia. Continuous production after transient transfection at high density produced multiple batches at once without needed to scale up the bioreactor or running many batches in parallel. In addition, the high density production demonstrated increased vector genomes per unit plasmid weight by more than 30%. The model developed was able to predict cell growth and death influenced by transfection in perfusion cultivation with the same plasmid delivery kinetic parameters reported in Chapter 3. With slight adjustment in viral production kinetic parameters, the model was able to capture the trends in vector genomes and total capsids.

Chapter 5

Conclusion

In this thesis, systems engineering methodologies are employed to develop a continuous platform for manufacturing via a model-based approach.

First, a mechanistic model was developed for triple transfection production platform to link transfection inputs to outputs, and estimated the relevant parameters. Since the biological mechanism of rAAV synthesis will remain largely the same for other transfection methods and even productive infection or stable production cell line, the model can be easily adapted to other production methods and serotypes with small modifications. For instance, a double transfection can be derived by removing one set of gene delivery parameters and species and re-estimating protein synthesis constants. Transfection production data from Chahal et al. (2014) showed that the quantities of genome-containing particles of different serotypes produced in the same platform are comparable [20]; therefore, the proposed mechanistic model can be adapted to the production of various rAAV serotypes with slight parameter adjustments. This mechanistic model can provide valuable insights to experimental data, identify process bottlenecks and guide future research. Leveraging the model in a quality-by-design implementation has great potential to facilitate process development and advance gene therapy manufacturing [70].

Second, a multistage-transfection method to produce rAAV in fed-batch was presented. The rapid saturation of the rate of viral capsid synthesis combined with the late onset of viral genome replication could be responsible for the low ratio of

full capsids at harvest. Additionally, low plasmid availability in later stages of cell culture inhibited capsid production activity [27]. In Chapter 3, a multi-stage transfection strategy was investigated — this protocol extends the viral capsid production timeline to improve the ratio of full to empty capsids. Keeping the total plasmid used constant, the ratio of full capsids in the harvest was improved while quantity of full capsids, or genome titer, was preserved. In parallel, the mechanistic model of rAAV production was further generalized through molecular analysis. Model analysis provided insights into the dynamics of rAAV synthetic pathways, including plasmid uptake, capsid synthesis, viral DNA replication, and genome encapsidation, that allow rational experimental design for further improvements of rAAV production.

Last, a continuous process in perfusion bioreactor for rAAV manufacturing via transient transfection was successfully demonstrated. Model-based calculation was performed to guide the plasmid dosage and dosing schedule. The system consists of an automated bioreactor connected to a perfusion system enabled by a viral harvesting unit, a tubular membrane with 10 μm pore size that retains the cells while allowing viral particles to pass through, as well as a bleeding line. The harvest rate and bleeding rate, which also determine the total perfusion rate, were adjusted based on a previous perfusion experiment without transfection. A protocol for continuous production using transient transfection was developed. For each transfection, the cells were transfected followed by a period of cell-plasmid incubation of approximately 24 h, when the perfusion is turned off. The perfusion system was able to achieve healthy cell growth and viability even after transfection, maintain steady levels of nutrients and avoid spiking of byproducts such as lactate and ammonia. Continuous production after transient transfection at high density produced multiple batches at once without needing to scale up the bioreactor or running many batches in parallel. In addition, the high density production demonstrated increased vector genomes per unit plasmid weight by more than 30%. The model developed was able to predict cell growth and death influenced by transfection in perfusion cultivation with the same plasmid delivery kinetic parameters reported in Chapter 3. With slight adjustment in viral production kinetic parameters, the model was able to capture the trends in vector

genomes and total capsids.

The validation of the mechanistic model and demonstration of continuous platform in this thesis have great implications in further improvements of the rAAV manufacturing process. In recent years, many efforts have been devoted to the model-based optimization of processes in biotechnology and bioengineering. Among these efforts is the dynamic optimization, or open loop optimal control, of bioreactors as reviewed in several articles [103, 104]. The methods imperative to solving these problems have been studied and cases studies have been demonstrated in the literature [105, 106]. Dynamic optimization can compute the optimal operational trajectory that maximizes an objective function — the objective function can be customized and represents process indices such as productivity, product quality/purity, or an economical index derived from the operational profile [105]. More often than not, a multi-objective needs to be considered when a process requires more than a single index for evaluation, in which case a Pareto optimality can be achieved. [107]. Once the optimal trajectory has been reliably computed, it can be implemented using different control strategies, such as adaptive control [108] or model predictive control [109]. Dynamic optimization has been studied mostly on fed-batch operations, with recent extension to continuous system [110, 111]. The method is commonly applied on microbial system, with a few a studies on mammalian cell systems in recent years, notably the production of monoclonal antibodies [112]. A mechanistic model capable of describing the process system dynamics influenced by process inputs is the necessary condition before dynamic optimization can be applied. Therefore, with the developed mechanistic model, the immediate next step to follow up on this result would be to apply a dynamic optimization to determine the best operating policies, i.e. optimal dosing schedule, optimal plasmid dosage as well as optimal plasmid ratio. The optimization should be formulated with the constraints representing the physical operating constraints, such as the upper and lower limits of plasmid/cell incubation periods, the harvesting and bleeding flow rates etc. The design of a continuous operation powered by model-based optimization and control will greatly increase productivity, reduce both operating costs and product time to market [113, 114].

Appendix A

Mechanistic Modeling Supplementary Materials

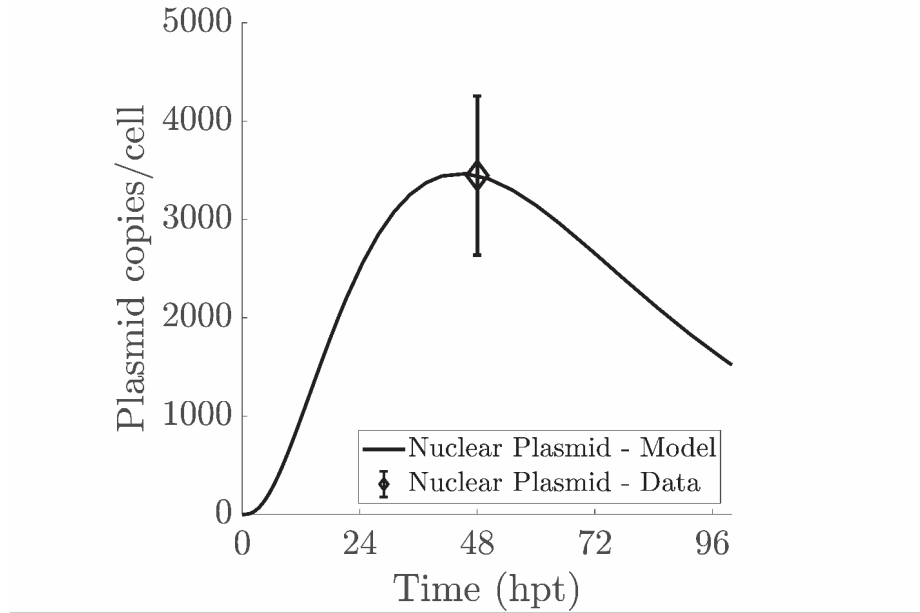


Figure A-1: Dynamics of nuclear plasmid copies per cell in PEI-mediated transfection of HEK923-EBNA1 cells. The model was fitted to experimental data from Carpentier et al. [45] with initial plasmid input of 2.7×10^5 plasmids/cell.

Appendix B

Multistage Transfection Supplementary Materials

Cellular concentration of individual plasmids

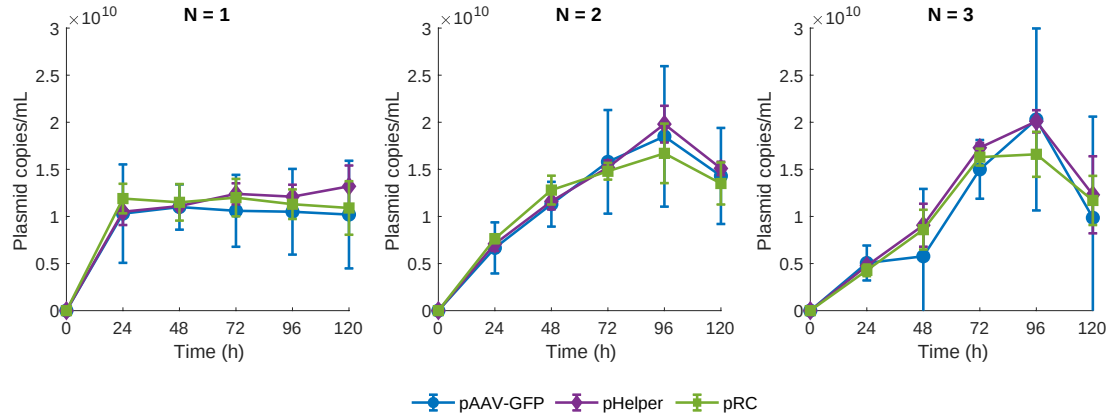


Figure B-1: Quantity of different types of plasmids in cells. Each experiment was performed in duplicate and measured twice; the error was calculated from all four measurements at each time point.

Ammonia concentration buildup in transfection culture

For modeling purposes, the ammonia profile (Figure B-2) was fitted using cubic polynomials as functions of times. The ammonia concentration is then approximated using the polynomial parameter. The polynomial parameters are shown in Table B.1.

Table B.1: Fitted cubic polynomial parameters to ammonia concentration profile (Figure B-2).

	t^3	t^2	t^1	t^0
$[\text{Amn}]_{\text{one-stage transfection}} = f_1(t)$	2.15E-06	-4.40E-04	4.75E-02	1.23E-02
$[\text{Amn}]_{\text{two-stage transfection}} = f_2(t)$	2.44E-06	-4.65E-04	4.79E-02	2.33E-02
$[\text{Amn}]_{\text{three-stage transfection}} = f_3(t)$	3.15E-06	-5.61E-04	5.19E-02	6.67E-03

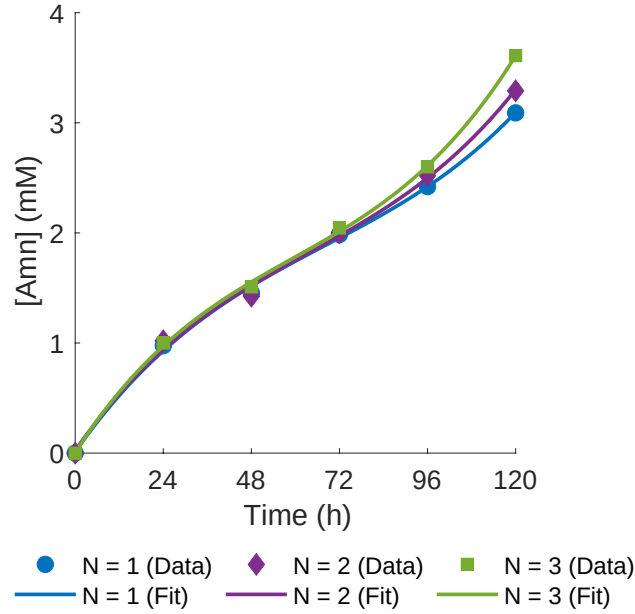


Figure B-2: Ammonia concentration in transfection cultures.

Metabolite profiles

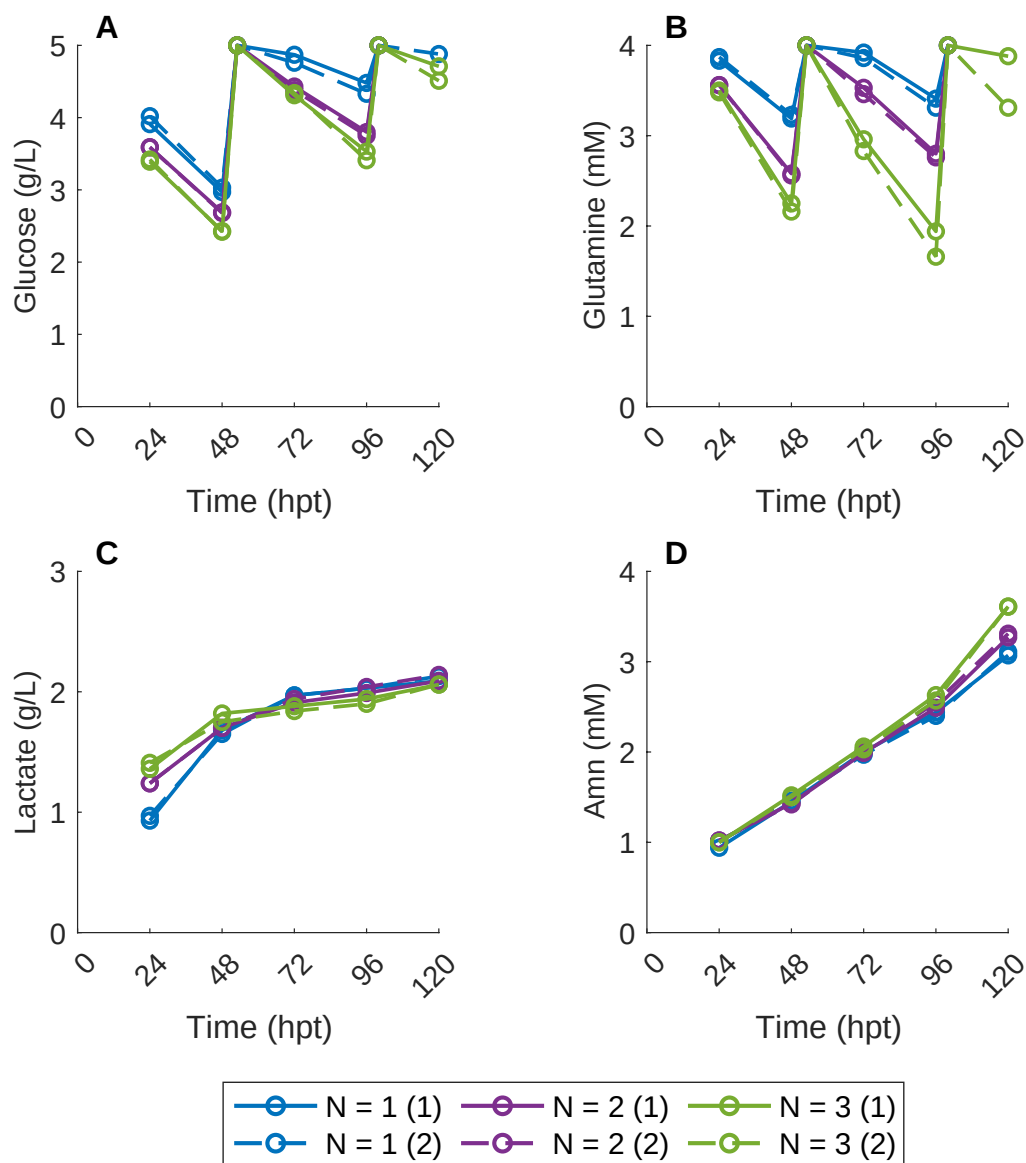


Figure B-3: Metabolite profiles of the cultures. (A) Glucose concentration [g/L]. (B) Glutamine concentration (mM). (C) Lactate concentration [g/L]. (D) Ammonia concentration (mM). Glucose and glutamine were supplemented at 48 h and 96 h to replenish the levels to 5 g/L and 4 mM respectively.

Capsid distribution

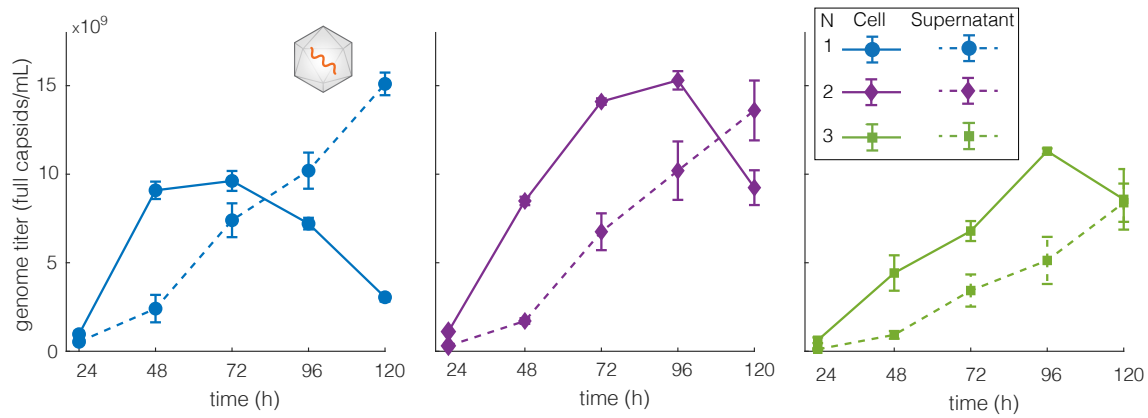


Figure B-4: Distribution of genome capsids in cells and in supernatant per mL of suspension. Error bars are the average of standard errors and propagated errors from for $n = 2$ and duplicate measurements.

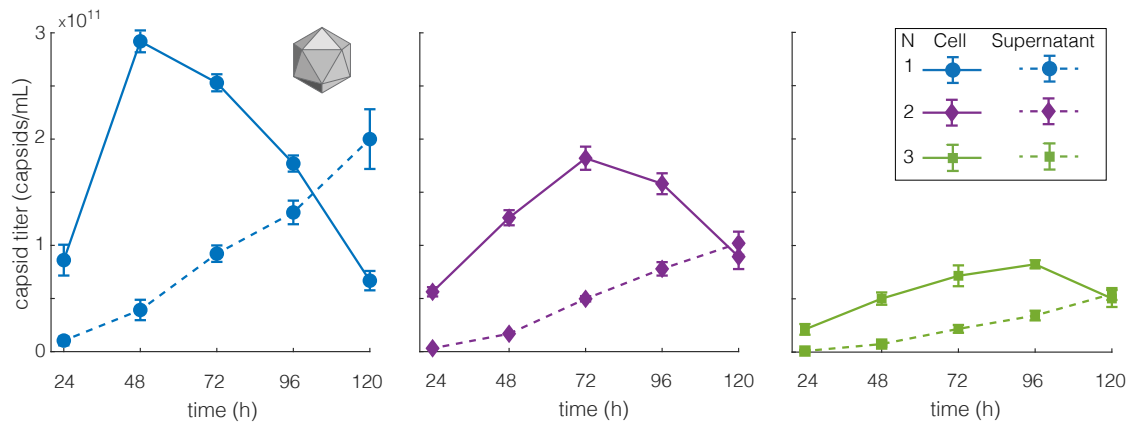


Figure B-5: Distribution of total capsids in cells and in supernatant per mL of suspension. Error bars are the average of standard errors and propagated errors from for $n = 2$ and duplicate measurements.

Appendix C

Perfusion Bioreactor Supplementary Materials

Cell growth in perfusion bioreactor without transfection

The first step towards validation of the perfusion system was to grow cells without transfection. The goals of this experiment were (1) to confirm that the perfusion setup and operation parameter was conducive to cell health before conducting any transfection experiments and (2) provide data on cell growth kinetics in perfusion system. The cell density, viability and nutrition profiles are shown in Figure C-1. The operating parameters are summarized in Table C.1. The perfusion rate was adjusted as cell density increased to accommodate the metabolic needs of the cell culture. The results indicated that the environment under this operating condition is conducive to healthy cell growth and waste accumulation is kept at a reasonable level. Although glucose level was quite low at times, cell growth was maintained at an exponential rate, indicate the nutrients levels were non-limiting.

Time [h]	72–96	96–120	120–144	144–168
Perfusion rate [vvd]	0.5	0.7	1.5	2

Table C.1: Perfusion rate at 350 mL working volume inside the bioreactor.

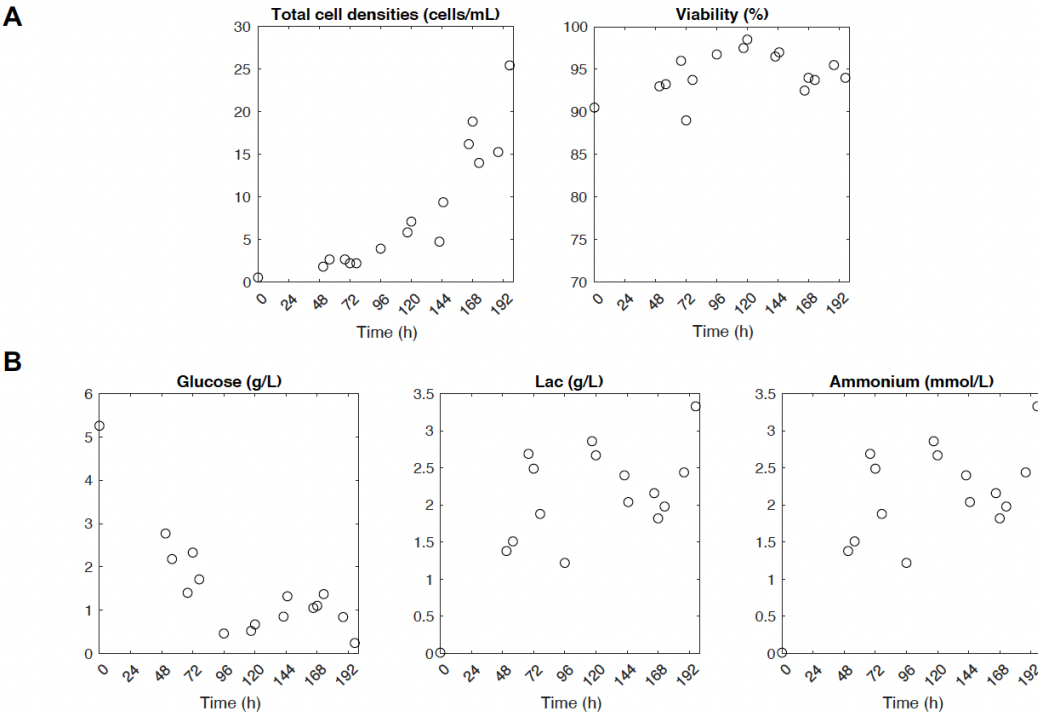


Figure C-1: Transfection-free perfusion cell density and nutrients. (A) Total cell densities and cell viability. (B) Metabolites concentrations.

Pump operation data

Time (h)	F_h (mL/h)	F_b (mL/h)	F_t (mL/h)
0.25	0	0	0
23.5	15.3144	0	15.3144
72.58333333333333	22.9716	0	22.9716

90	7.6464	17.67	25.3164
92.4166666666667	15.3036	17.67	32.9736
93.5833333333333	22.9554	0	22.9554
119.25	50	0	50
119.75	33.2964	0	33.2964
121.25	32.2980952380952	0	32.2980952380952
141.25	22.7045454545455	14.2916666666667	36.9962121212121
146.083333333333	22.7045454545455	61.6666666666667	84.3712121212121
147.083333333333	22.7045454545455	15.4166666666667	38.1212121212121
149.583333333333	23.13	7.71	30.84
161.583333333333	23.13	15.417	38.547
166.25	38.547	0	38.547
166.583333333333	0	0	0
167.083333333333	0	0	0
174.083333333333	3.083	3.083	6.166
174.583333333333	23.125	0	23.125
175.083333333333	0	0	0
179.25	23.125	0	23.125
195.75	23.125	7.71	30.835
212.25	90	0	90
213.25	0	0	0
236.25	23.125	7.708	30.833
241.25	0	0	0
241.833333333333	23.125	7.708	30.833
259.416666666667	23.125	3.083	26.208
269.916666666667	0	0	0
282.75	23.125	3.083	26.208
306.25	27.75	3.08333333333333	30.8333333333333

355.75	154.17	3.08	157.25
358.25	27.75	0	27.75
358.616666666667	154.17	0	154.17
358.95	308.33	0	308.33
359.25	0	0	0
378.516666666667	38.54	0	38.54
378.8025	38.54	7.71	46.25
404.25	23.12	7.71	30.83

Table C.2: Pump data.

ELISA and ddPCR measurements

Table C.3: Total AAV5 capsids from ELISA measurement.

Sampling time (h)	Total capsids (capsids/mL)			
	Cells	Cells error	Supernatant	Supernatant error
23.5	2.58E+10	7.18E+09	4.49E+09	3.15E+09
47.3	1.25E+11	2.35E+10	1.09E+10	4.22E+09
71.5	1.46E+11	8.29E+09	1.92E+10	6.79E+09
93.3	1.38E+11	6.21E+09	3.33E+10	1.82E+10
117.3	1.63E+11	8.64E+08	2.56E+10	2.05E+09
141.3	9.20E+10	3.60E+09	2.57E+10	6.74E+08
166.3	6.31E+10	1.85E+10	1.37E+10	3.44E+08
186.3	6.37E+10	1.14E+10	1.62E+10	1.14E+09
211.3	1.74E+11	5.56E+08	9.44E+09	8.89E+07
235.8	3.39E+11	9.35E+10	3.33E+10	8.72E+08
258.8	2.52E+11	7.72E+10	3.63E+10	1.61E+09

282.8	3.43E+11	4.55E+10	8.19E+10	5.91E+09
306.3	5.38E+11	7.39E+10	9.78E+10	1.68E+10
330.3	1.44E+11	8.58E+09	4.71E+10	1.17E+10
354.8	2.49E+11	2.25E+09	2.71E+10	8.87E+08
378.3	1.46E+11	3.11E+10	4.52E+10	6.89E+09

Table C.4: Full AAV5 capsids from ELISA measurement.

Sampling time (h)	Full capsids (vg/mL)			
	Cells	Cells error	Supernatant	Supernatant error
23.5	5.94E+08	7.38E+07	6.26E+07	3.47E+06
47.3	2.80E+09	1.07E+09	3.00E+08	3.37E+07
71.5	3.20E+09	5.42E+08	7.05E+08	6.12E+07
93.3	2.88E+09	1.36E+09	1.24E+09	1.19E+08
117.3	1.34E+09	1.99E+08	1.37E+09	7.74E+07
141.3	1.18E+09	1.27E+08	6.43E+08	2.85E+07
166.3	1.36E+09	1.92E+08	3.52E+08	2.49E+07
186.3	1.34E+09	1.86E+08	5.97E+08	7.30E+07
211.3	1.07E+10	1.46E+09	6.72E+08	8.69E+07
235.8	2.49E+10	1.89E+09	1.99E+09	5.41E+08
258.8	4.18E+10	2.48E+08	1.95E+09	1.16E+08
282.8	3.18E+10	2.09E+09	4.69E+09	3.70E+08
306.3	1.09E+10	5.86E+08	5.68E+09	2.54E+08
330.3	3.28E+09	2.33E+08	2.03E+09	1.75E+08
354.8	7.91E+09	4.51E+08	1.44E+09	7.06E+07
378.3	3.81E+09	2.87E+08	2.90E+09	2.42E+08

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