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Research review paper

The state of technological advancement to address challenges in the manufacture of rAAV gene therapies

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A B S T R A C T

Current processes for the production of recombinant adeno-associated virus (rAAV) are inadequate to meet the surging demand for rAAV-based gene therapies. This article reviews recent advances that hold the potential to address current limitations in rAAV manufacturing. A multidisciplinary perspective on technological progress in rAAV production is presented, underscoring the necessity to move beyond incremental refinements and adopt a holistic strategy to address existing challenges. Since several recent reviews have thoroughly covered advancements in upstream technology, this article provides only a concise overview of these developments before moving to pivotal areas of rAAV manufacturing not well covered in other reviews, including analytical technologies for rapid and high-throughput measurement of rAAV quality attributes, mathematical modeling for platform and process optimization, and downstream approaches to maximize efficiency and rAAV yield. Novel technologies that have the potential to address the current gaps in rAAV manufacturing are highlighted. Implementation challenges and future research directions are critically discussed.

1. Introduction

Gene therapy has the potential to revolutionize healthcare by offering durable treatments for severe diseases through the delivery of therapeutic genes (Wang et al., 2019). Viral delivery has become a pivotal route in commercial gene therapies, due to its high gene transfer efficiency compared to non-viral methods (Bulcha et al., 2021). Among various viral vectors, recombinant adeno-associated virus (rAAV) has emerged as a natural choice to treat a wide array of diseases, including rare genetic disorders. The rAAV viral vector consists of a single-stranded genome packaged in a non-enveloped capsid, composed by 60 monomeric subunits of three viral proteins (VPs) – VP1, VP2, and

Abbreviations: AC, affinity chromatography; ATPS, aqueous two-phase systems; AEX, anion-exchange chromatography; AFM, atomic force microscopy; AUC, analytical ultracentrifugation; BEVS, baculovirus expression vector system; CEX, cation-exchange chromatography; CBER, Center for Biologics Evaluation and Research; cGMP, current good manufacturing practice; CQA, critical quality attribute; DHM, digital holographic microscopy; ELISA, enzyme-linked immunosorbent assay; EMA, European Medicines Agency; FDA, United States Food & Drug Administration; GOI, gene of interest; HEK293, human embryonic kidney 293; HIC, hydrophobic interaction chromatography; IR, infrared; ITR, inverted terminal repeats; EM, electron microscopy; MP, mass photometry; PCR, polymerase chain reaction; PEG, polyethylene glycol; QTPP, quality target product profile; rAAV, recombinant adeno-associated virus; SEC MALS, size-exclusion chromatography multi-angle light scattering; SMR, suspended microchannel resonator; SNR, suspended nanomechanical resonator; SXC, steric exclusion chromatography; TCD, total cell density; TFF, tangential flow filtration; VCD, viable cell density; VPs, viral proteins; vg, vector genomes.

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VP3 – assembled at an average stoichiometric ratio of 1:1:10 (Wörner et al., 2021). The production of rAAV from cells requires concomitant expression of several structural and accessory proteins encoded in the genes known as *rep* and *cap*. The encapsidated single-stranded transgene cargo codes for the therapeutic gene of interest (GOI) under the control of a tissue-specific promoter.

The widespread clinical interest in rAAV-based therapeutics is due to its low immunogenicity, non-infectivity to humans, and long-lasting gene expression (Wang et al., 2019). Unlike wild-type AAV (wtAAV), rAAV is replication-deficient which makes it suitable for gene therapy (Song et al., 2020). There are over 13 serotypes and 150 variants of AAVs that have varying functional characteristics – transduction efficiency, cell tropism, and immunogenicity – which offer opportunities to engineer rAAV properties for gene therapy applications (Han et al., 2022; Issa et al., 2023). More than 200 clinical trials are underway with rAAV-based vectors for a broad range of diseases. As of this writing (July 2024), eight rAAV-based gene therapies (Table 1) have been approved by the United States Food & Drug Administration (FDA) and/or by the European Medicines Agency (EMA). The ability to supply enough rAAV to meet growing demand for rAAV-based therapies is constrained by current manufacturing practices. For most approved rAAV-based gene therapies, more than 10^{18}–10^{20} vector genomes (vg) would be needed just for treating the U.S. patient population (Table 1). However, current rAAV manufacturing processes are not compatible with such large production, since they yield titers of only approximately 5×10^{14} vg/L, with downstream recovery around 20% (Sha et al., 2021). At the same time, the extremely high manufacturing cost for rAAV, which can exceed $300k per dose (Lyle et al., 2023), contributes to the multi-million dollar price of approved therapies (Table 1). Innovation is needed in large-scale rAAV manufacturing processes that meet product purity, potency, and safety requirements (Clément and Grieger, 2016; Escandell et al., 2022). The need for improving rAAV manufacturing aligns with the quality-by-design (QbD) initiative of regulators, which promotes the adoption of a scientific and risk-based approach to enhance (bio)pharmaceutical manufacturing (Destro and Barolo, 2022; FDA, 2023; Grangeia et al., 2020; International Council for Harmonisation, 2009).

Industrial-scale rAAV vector production in a current good manufacturing practice (cGMP) environment is carried out through either mammalian cells (typically, human embryonic kidney 293 cells, HEK293) or insect cells from the Sf9/Sf21 lines (Fig. 1). In the current industrial practice, the genes for rAAV production are usually delivered to mammalian cells through transient transfection and to insect cells through recombinant baculovirus infection (Sha et al., 2021). Alternative processes that carry out gene delivery to mammalian cells through direct infection with recombinant herpes simplex virus or adenovirus have also been developed (Fu et al., 2023b; Ou et al., 2024). Stably transfected mammalian and insect cell lines have recently emerged as promising alternative platforms for rAAV production (Collins et al., 2023; De Carluccio et al., 2024; Galibert et al., 2021; Mietzsch et al., 2014). Stable cell lines can lead to lower costs and higher rAAV titers, but are currently hindered by long development times and by the toxic effect of leaky expression of the integrated genes (Fu et al., 2023b; Yuan et al., 2011).

A primary challenge in rAAV manufacturing arises from the production in upstream cultures of a substantial proportion of non-functional empty capsids. Increasing the proportion of full capsids in the upstream culture is crucial for improving the productivity and cost-efficiency of the overall process, since additional downstream steps are needed to enrich the full particles before clinical administration. Processes based on transient transfection of HEK293 cells typically yield only up to 10,000 full capsids per cell and a lower (5–30%) percentage of full capsids (Sha et al., 2021). In contrast, the baculovirus expression vector system (BEVS) can produce a high percentage of full capsids (30–50%) at high titers of up to 100,000–400,000 full capsids per cell (Cecchini et al., 2011; Kurasawa et al., 2020). Another critical issue involves the infectivity of rAAV vectors, as it has been found that only a fraction of all the produced full rAAV particles is infectious (Giles et al., 2023; Liu et al., 2023). There have been conflicting results regarding the potency of vectors manufactured from different platforms. While recent studies found that the rAAV vector produced in insect cells was less potent than the capsids derived from mammalian cells (Giles et al., 2023), other works documented higher potency for rAAV produced in the BEVS compared to rAAV from HEK293, instead (Liu et al., 2024). Advances in platform biology and cell culture technology for addressing challenges in upstream rAAV production are not further discussed in the remainder of this article, since several reviews recently focused on this topic (Dobrowsky et al., 2021; Fu et al., 2023; Joshi et al., 2021b; Liu et al., 2024; Ou et al., 2024; Zhao et al., 2020).

A multidisciplinary effort that goes beyond incremental refinement of upstream rAAV production is needed for addressing the current challenges in the manufacture of rAAV-based gene therapies. Rapid and high-throughput technologies that allow measurement of rAAV biophysical and biochemical quality attributes are required for the optimization of upstream and downstream rAAV manufacturing (Gimpel et al., 2021). Specifically, the establishment of analytics for accurate and rapid quantification of the full-to-empty capsid ratio at a cost sustainable in large scale production remains a primary goal (Sripada et al., 2024). Further, the application of model-based process development in

Table 1
rAAV-based gene therapies approved by FDA and/or EMA as of July 2024. vg = vector genome.

Product	Developer	Indication	Approval date	Serotype/ Platform	Dose	Price ($/dose)	US patient population	Total need for US patient population (vg)
Beqvez	Pfizer	Hemophilia B (congenital Factor IX deficiency)	4/2024 (FDA)	AAVRh74/ HEK293	5×10^{11} vg/kg	3.5M	6000	2×10^{17}
Elevidys	Sarepta Therapeutics	Duchenne muscular dystrophy (DMD)	6/2023 (FDA)	AAVRh74/ HEK293	1.33×10^{14} vg/kg	3.2M	50,000	1×10^{20}
Glybera	UniQure	Familial lipoprotein lipase deficiency (LPLD)	10/2012 (EMA)	AAV1/Sf9	1×10^{12} vg/kg	1.2M	300	2×10^{16}
Hemgenix	CSL Behring LLC	Hemophilia B (congenital Factor IX deficiency)	11/2022 (FDA), 2/2023 (EMA)	AAV5/Sf9	2×10^{13} vg/kg	3.5M	6000	1×10^{19}
Luxturna	Spark Therapeutics	Biallelic *RPE65* mutation-associated retinal dystrophy	12/2017 (FDA)	AAV/ HEK293 11/2018 (EMA)	3×10^{11} vg/patient	0.85M	2000	6×10^{14}
Roctavian	BioMarin Pharmaceutical	Hemophilia A (congenital factor VIII deficiency)	6/2023 (FDA), 8/2022 (EMA)	AAV5/Sf9	6×10^{13} vg/kg	2.9M	24,000	1×10^{20}
Upstaza	PTC Therapeutics	Aromatic L-amino acid decarboxylase deficiency	7/2022 (EMA)	AAV2/ HEK293	1.8×10^{11} vg/patient	3.7M	8000	1×10^{15}
Zolgensma	Novartis Gene Therapies	Spinal muscular atrophy (Type I)	5/2019 (FDA), 5/2020 (EMA)	AAV9/ HEK293	1.1×10^{14} vg/kg	2.1M	1600	4×10^{18}

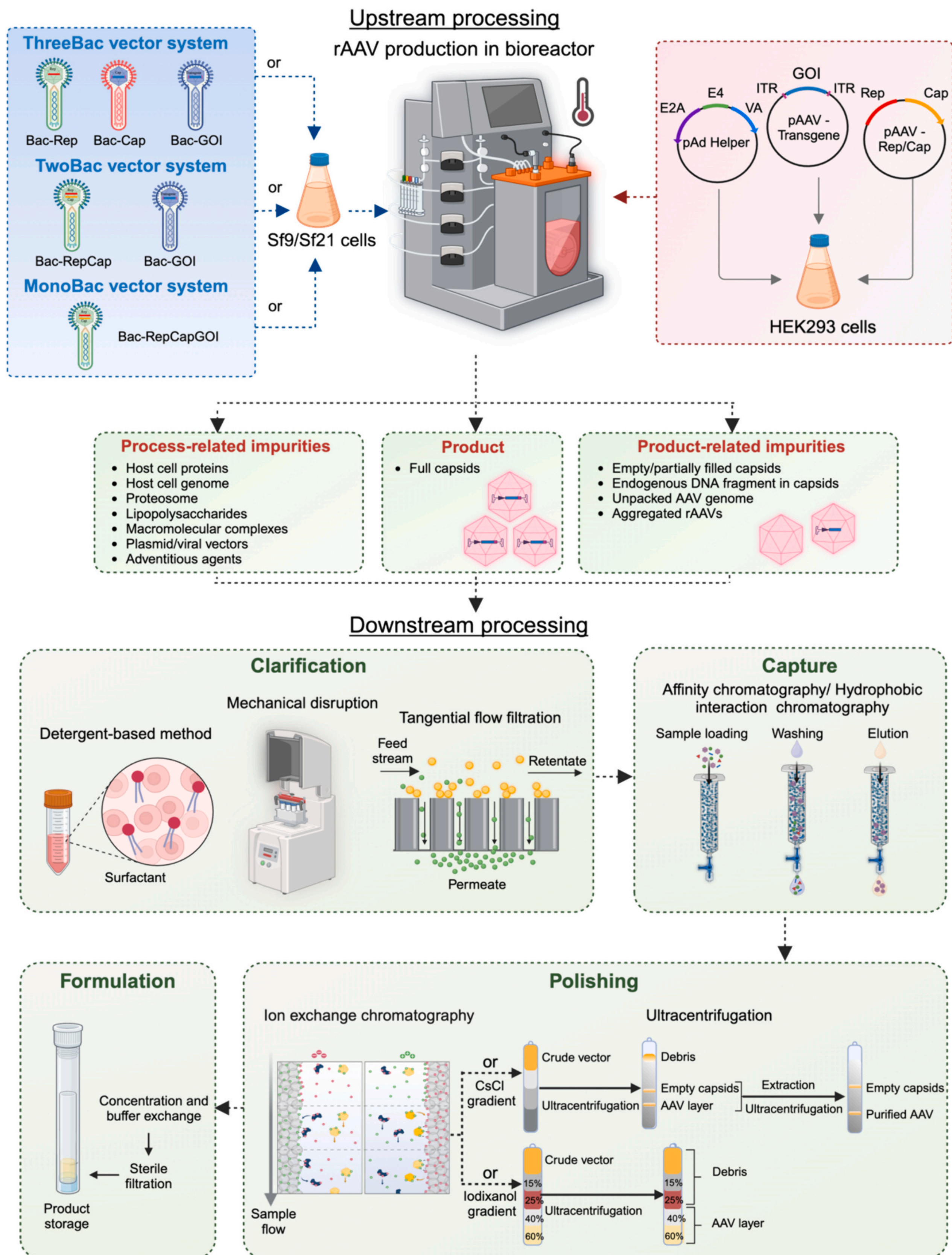


Fig. 1. Overview of rAAV manufacturing workflow. State-of-the-art rAAV platforms for commercial production are typically the baculovirus expression vector system (BEVS) and triple transient transfection in the upstream process. Alternative productive platforms are shown in Fig. 2. Cells cultured in the bioreactor are infected or transfected with appropriate vectors. Product and process impurities are eliminated by the downstream processing phase, which includes clarification, capture, polishing, and formulation, leading to rAAV vectors of high purity ready for delivery.

rAAV manufacturing remains under-explored, although it has been recently shown that mechanistic modeling can provide significant insights into the current bottlenecks in rAAV production (Destro et al., 2023; Nguyen et al., 2021). Irrespective of the technologies used for rAAV production, all current upstream platforms put a significant burden on downstream purification, which is responsible for removing product-related (e.g., empty and partially full capsids and capsids containing host cell DNA) and process-related (e.g., host cell proteins and DNA) impurities (Qu et al., 2015). A significant effort has been dedicated in recent years to the development of a cost-effective and efficient purification process for rAAV, as thoroughly reviewed by Kilgore et al. (2023). Nonetheless, novel technologies are needed to increase the rAAV yield during downstream processing, where currently >80% of the rAAV produced upstream can be lost (Rieser et al., 2021).

This article tackles these gaps by providing a holistic view of the current state and a roadmap for future development in the following pivotal areas related to rAAV manufacturing: (i) in-process analytical technologies, (ii) process modeling, and (iii) downstream rAAV

Table 2
Analytical methods currently used to characterize the critical quality attributes of rAAV.

QTPP category	CQA	Analytical method	Process development	Commercial manufacturing	
			Characterization	QC/ Release	Stability
Identity	Genetic identity	Sequencing	•	•	
		Polymerase chain reaction (PCR)	•	•	
	Viral protein (VP) intact mass	Liquid Chromatography with Mass Spectrometry (LC-MS)	•		
		Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis (SDS-PAGE)	•		
	VP primary structure and PTMs	LC-MS peptide mapping	•		
	VP1:VP2:VP3 stoichiometry	LC with Capillarity Electrophoresis (CE)	•	•	
		LC-MS	•		
	Viral vector size	Charge Detection Mass Spectrometry (CDMS)	•		
		Mass Photometry (MP)	•		
	Thermostability/tertiary structure	Differential Scanning Calorimetry (DSC)	•		•
		Differential Scanning Fluorometry (DSF)	•		
Potency	Infectivity	Tissue Culture Infective Dose 50 (TCID50)		•	•
	Transgene expression	Flow cytometry following in vitro transduction		•	
	Functionality	Cell Based Potency Assay (CBPA)		•	
Content titer	Viral genome titer	Droplet Digital Polymerase Chain Reaction (ddPCR)	•	•	•
		Real-time Polymerase Chain Reaction (qPCR)	•		
		Dye-based binding assay (DyeBA)	•		
	Viral capsid titer	Enzyme-linked Immunosorbent Assay (ELISA)		•	•
		MP	•		
		Size-Exclusion Chromatography with Multi-Angle Light Scattering (SEC-MALS)			
		Static Light Scattering (SLS) / Dynamic Light Scattering (DLS)	•		
		Flow Virometry (FV)	•		
		Optical Density (OD)	•		
		Biolayer Interferometry (BLI)	•		
Purity	Aggregation	Size exclusion-High-Performance Liquid Chromatography (SEC-HPLC)		•	
		SEC-MALS	•		
		Analytical Ultra Centrifugation (AUC)	•		
		MP	•		
		SLS / DLS	•		
		Mini-Transmission Electron Microscopy (Mini-TEM)	•		
		Cryogenic Electron Microscopy (Cryo-EM)	•		
	Content ratio (full:empty capsid)	AUC		•	
		MP	•		
		Mini-TEM	•		
		Cryo-EM	•		
		Anion-exchange chromatography (AEC)	•		
		SEC-MALS	•		
		CDMS	•		
		OD	•		
	Residual host cell DNA [e.g., HEK293, SF9]	qPCR		•	
	Residual helper DNA [e.g., plasmid, rBac, rHSV]	qPCR		•	
	Residual host cell protein	ELISA		•	
	Residual reagents [e.g., transfection reagents, BSA]	ELISA		•	
	Residual DNA fragment sizing	ddPCR	•		
Safety	Replication competent AAV	Serial Passage		•	
	Bioburden	Membrane Filtration (MF)		•	•
	Mycoplasma	PCR		•	
	Viral contaminants	PCR		•	
	Endotoxin	Lumulus Amebocyte Lysate (LAL) Test		•	
	Subvisible particles	Light Obscuration Liquid Particle Counter		•	
Physico-chemical	Appearance	Visual Inspection		•	•
	pH	Potentiometry		•	•
	Osmolality	Osmometry		•	•

purification.

2. In-process sensors for rAAV manufacturing

With the intricacies of triple transfection and BEVS infection, large-scale rAAV production processes have traditionally been unpredictable, leading to fluctuations in quality and yield (Carbonell et al., 2019; Srivastava et al., 2021). Current rAAV manufacturing practices are being transformed by the adoption of emerging sensors specifically designed for rAAV production, as well as sensor technologies with proven effectiveness in the production of recombinant proteins. These technologies can be categorized as (i) analytical methods for monitoring rAAV quality attributes and (ii) sensors for monitoring cell culture process parameters. Real-time characterization of rAAV critical quality attributes (CQAs), such as the viral titer and the full-to-empty capsid ratio, would provide critical insights on the production; however, current technologies are not capable of providing such rapid measurements. Monitoring the cultivation process, such as tracking viable cell density and dissolved oxygen level, provides insights into the process dynamics and allows for immediate controls to optimize yield and quality. However, existing process controls are insufficient to maintain low levels of process variability for rAAV as they did for recombinant protein production. There is a need to better understand how currently measurable process parameters affect production, and search for additional parameters that will enhance the control on rAAV CQAs. Therefore, the improvement of sensor technologies in rAAV production is a necessary step towards greater reliability and control, lowering the cost of gene therapy manufacturing and ensuring more products make it into the clinic faster.

2.1. Current and emerging sensors for monitoring rAAV production

2.1.1. Analytical methods for monitoring rAAV quality attributes

Current industry practices for analyzing rAAV attributes involve a large suite of analytical methods, each with their own challenges. These include analytical ultracentrifugation (AUC) and electron microscopy (EM) for distinguishing and quantifying full, partial, and/or empty capsid subpopulations, polymerase chain reaction (PCR)-based methods for determining the genome titer, and enzyme-linked immunosorbent assay (ELISA)-based approaches for quantifying the capsid titer. However, these methods come with limitations, such as the need for substantial material quantities (AUC), manual inspection and counting of particles (EM), low-throughput (AUC and EM), and high error rates (ELISA and PCR) that can lead to low overall accuracy on calculating full-to-empty ratio. A list of other analytical methods currently used to evaluate attributes in the rAAV quality target product profile (QTPP) is summarized in Table 2. The table is categorized by metrics such as identity, potency, purity, and safety, as well as the stage in product development cycle each technique is suitable for. A comprehensive overview of these techniques and of their performance have recently been presented elsewhere (Gimpel et al., 2021; Werle et al., 2021).

While a long-term goal for improving rAAV manufacturing is to achieve real-time product characterization and release testing, there are a number of technological gaps that need to be addressed. The next generation of techniques need to (i) shorten the measurement turnaround times from hours- to minutes-timescales, (ii) lower the amount of sample material required, and (iii) provide deeper structural understanding of the rAAV product. There is a suite of emerging technologies that are filling these gaps. For instance, size-exclusion chromatography multi-angle light scattering (SEC MALS) offers an alternative solution for simultaneously measuring capsid titer, content ratio and aggregation by detecting the average molecular weight and concentration of specific species separated by SEC (McIntosh et al., 2021). While SEC MALS cannot resolve the masses of intact (full) and partially full capsids, it will likely become highly effective in early process development because of its ability to measure multiple CQAs with high throughput and low material requirement. Similarly, the recently developed mass photometry (MP) technique measures the mass distributions of single rAAV particles on a minute timescale and with sub-picomole sample consumption (Wu et al., 2022). A comparative advantage of MP over SEC MALS is its enhanced mass resolution in quantifying heterogeneous impurities, such as partially full capsids, in addition to empty and full-genome-containing capsids. Additionally, a number of recent academic developments have provided alternative methods to characterize rAAV content. The suspended nanomechanical resonator (SNR) has been used for quantifying aggregation and distinguishing between empty and full capsids by directly measuring rAAV mass (Katsikis et al., 2022). SNR achieves a precision corresponding to 1% of the genome-holding capacity of the rAAV capsid and can obtain results from a few microliters of sample within minutes. Microfluidic electrophoresis systems can also rapidly measure content ratio by independently obtaining capsid protein and single-stranded DNA profiles (Coll De Peña et al., 2024). This method achieves an average turnaround time < 5 min/sample, but has detection limits of 3.4×10^{11} VP/mL and 1.6×10^{11} genome copies/mL, which are far less sensitive than ELISA and ddPCR. Atomic force microscopy (AFM) has also been used to distinguish between full and empty rAAV capsids due to their differing particle heights (Nam et al., 2023; Zoratto et al., 2021). However, a challenge for an AFM-based technique is the low sampling throughput. In addition to content ratio, the characterization of VPs is critical for controlling viral infectivity and vector potency, given that a growing number of rAAV capsids are now engineered from the wild type to improve transduction efficiency while lowering adverse immune response (Li and Samulski, 2020; Shirley et al., 2020). Peptide mapping analysis enabled by mass spectrometry has been utilized for serotype identification and primary structure evaluation of specific rAAV capsid VPs (Lam et al., 2022; Serrano et al., 2023; Toole et al., 2021). Despite these advances, there is still a substantial gap in the ability to perform real-time measurements on rAAV attributes directly from the crude culture.

2.1.2. Sensors for monitoring cell culture process parameters

Cell culture monitoring is crucial for maintaining precise cultivation processes, ultimately ensuring consistent product quality and yield. Factors such as pH, dissolved oxygen, cell growth, and viability are known to affect production of many biologics. While these process parameters have been translated to monitoring rAAV production, they are insufficient for reducing process variability and enabling feed-forward model-based control. There is a need for deeper biological understanding of the rAAV production process and identification of specific biological programs that can be monitored and controlled. Investment in process monitoring will facilitate more efficient process development, thereby reducing time-to-market.

Physicochemical parameters such as temperature, pH, pO_2, pCO_2, osmolality, and metabolites are known components of cell culture processes that influence protein production, but there is overall limited literature on optimization of the parameters to improve rAAV titers (Coplan et al., 2024). For instance, CO_2 plays a vital role in stabilizing the pH of the medium, but dissolved CO_2 can become a detrimental and undesirable by-product of cell culture processes. High levels of dissolved CO_2 can lead to unwanted metabolic shifts, inhibit growth, and ultimately decrease productivity (Román et al., 2018). In addition, while media osmolality is usually set between 260 and 450 mOsm/kg, recent reports showed that osmolarity can have serotype-dependent impacts on rAAV yield (Rego et al., 2018; Shen and Kamen, 2012). Furthermore, the real-time monitoring and optimization of key metabolic parameters, such as substrates and metabolites, will also likely enable higher titer and more reproducible processes. For example, high glucose levels in cell culture are known to negatively impact rAAV production by promoting acidification, presumably through excessive glycolysis (Kimura et al., 2019). Glucose monitoring has traditionally been done with at-line or off-line glucose meters, which measure glucose levels electrochemically. Emerging technologies offer continuous online and in-situ glucose monitoring, by measuring the hydrogen peroxide production

or the oxygen consumption due to glucose oxidase (GOx)-catalyzed reaction of glucose oxidation via amperometric or optical transducers (Lederle et al., 2020; Pontius et al., 2020). In-line Raman-based spectrometry has also been used to monitor glucose and lactose levels in HEK293 processes (Brown et al., 2024).

Two critical physiological parameters in rAAV upstream process are viable cell density (VCD) and viability, i.e. the percentage of living cells relative to the total cell density (TCD). Accurate determination of cell density (number of cells per unit volume) forms the basis of all stages of cell cultivation, from establishing a rudimentary subcultivation routine in process development to defining a process control strategy in commercial production. With the increasing application of perfusion processes, there is a growing demand for continuous biomass/cell density determination. This control is vital in regulating the cell-specific perfusion rate. In fed-batch processes, adjusting feeding rates based on continuous cell density measurement aids in determining optimal feed rates, thus reducing the cost of goods.

VCD has traditionally been determined by manual counting of viable cells with viability labeling like the trypan-blue. Automated cell counters such as Vi-CELL BLU and Nova Flex2 are image-based cell analyzers that are improved from traditional manual viability assays by automating sample preparation and reducing measurement variability. Image analysis algorithms allow these systems to more consistently classify detected objects into categories such as debris, viable and dead cells. However, analytical approaches based on morphological features can be cell line-dependent and susceptible to changes in cell morphology during scale-up. Alternatively, capacitance probes allow for in-line monitoring of VCD, facilitating feedback control loops for VCD-based feeding, bleeding, and osmolality control, which enable successful qualification in an industrial environment (Schwamb et al., 2021). Since cell cluster formation occurs frequently in HEK cell culture, capacitance measurement may be more suitable than image-based counting. However, capacitance measurements of VCD are dependent on cell volume, which can change over time and requires re-calibration during a production run.

All existing methods for determining cell viability require validation against trypan blue staining, which examines cell membrane integrity and is considered the gold standard (Schwamb et al., 2021). However, this binary classification of cell states does not consider cells that are dying but still have functional membranes. These dying cells are likely changing their specific productivity but current VCD measurements consider them the same as truly viable cells. For apoptotic cell death, cells undergo a long series of physical and biochemical events during the 12–24 h after a cytotoxic event, and the loss of plasma membrane integrity occurs only at the end of the process. The transient changes in cell fitness are not detectable with existing VCD measurements, but are known to closely associate with metabolic and transcriptomic shifts in apoptotic cells. The relationship between cell fitness level and rAAV productivity has yet to be examined. Since rAAV production with both HEK293 and Sf9 systems induces a high degree of cytotoxicity, emerging techniques that offer a more detailed view of cell state changes will enhance our understanding and control over the cultivation process.

Techniques such as next-generation sequencing have enabled temporal characterization of cell states which provides deeper insight into host cell inflammatory response during rAAV production and potential genetic targets to improve production (Chung et al., 2023; Pistek et al., 2023). While offering insights on both genomic and transcriptomic levels, they often require multi-step manual preparations, limiting their use to off-line analysis rather than real-time monitoring. In contrast, emerging imaging-based technologies, such as digital holographic microscopy (DHM), can detect morphological features indicative of changing cell state in real time. DHM has recently been established as a promising online monitoring tool for Sf9 VCD and infection efficiency during rAAV production (Pais et al., 2020). Another promising approach for monitoring cell state involves measuring biophysical features such as cell size, density, and stiffness. The suspended microchannel resonator (SMR) has been used to simultaneously monitor multiple physical attributes of single cells with a throughput of 30,000 cells per hour (Kang et al., 2019; Wu et al., 2024) and is suitable for real-time process monitoring. In the field of functional precision medicine in oncology, the SMR has been used in clinical laboratories (Kimmerling et al., 2022) for rapid ex vivo drug sensitivity testing because the buoyant mass of primary cancer cells following drug treatment can predict patient response (Stevens et al., 2024). A critical aspect of cellular attributes such as buoyant mass and density is that they reflect changes in cell state much earlier than viability and viable cell count (Byun et al., 2015; Grover et al., 2011; Hecht et al., 2016). This may be particularly helpful in monitoring rAAV production as cells experience cytotoxicity from exogenous agents, such as Rep78 and helper proteins E2a and E4, that instigate cell cycle arrest and apoptosis while making the rAAV products (Berthet et al., 2005; Jing et al., 2001; Marcellus et al., 1998; Schmidt et al., 2000).

2.1.3. Soft sensors for real-time monitoring of rAAV manufacturing

During process development, interconnections between process measurements and crucial variables that cannot be measured in real-time, such as certain product quality attributes, can be harnessed to develop soft sensors, namely in silico sensors based on mathematical models. From the available process measurements, mathematical models are trained to provide estimations of critical process variables and product quality attributes that cannot be directly measured in real-time (Randek and Mandenius, 2017; Togkalidou et al., 2001). Recently, soft sensors have been implemented to estimate the cell and metabolite concentrations in mammalian cell cultures from fluorescence and Raman spectroscopy (Abu-Absi et al., 2011; Berry et al., 2015; Faassen and Hitzmann, 2015; Maruthamuthu et al., 2020). In rAAV manufacturing, the ability to estimate rAAV concentration through soft sensors would significantly improve process development efficiency and process control, as, to date, there are no analytical methods capable of directly measuring rAAV titer from the crude culture (Iglesias et al., 2023). Recent studies have shown that real-time data collected from Raman spectroscopy, automated cell counter, and holographic imaging can be potentially harnessed to predict lentiviral and rAAV capsid and genome titers when trained with machine learning models (Morder et al., 2022; Pais et al., 2020). Despite the potential to enhance process monitoring, a considerable challenge for soft sensors is their robustness during process scale-up, where process parameters could vary and reduce model performance. Although soft sensors have not yet been extensively applied to rAAV manufacturing in current platform development or in cGMP production, they hold considerable promise for the future of rAAV manufacturing.

2.2. Integration of novel analytical technologies into commercial-scale manufacturing

2.2.1. Challenges associated with adopting novel technologies in cGMP production

The implementation of new sensors involves financial investment, time, and risk. During process development, a wide array of new technologies and parameters are evaluated to aid process optimization and product characterization. As processes move into scale-up and validation, the range of potential monitoring methods is typically narrowed down to those deemed critically important to the process. For instance, a pCO2 probe may provide significant benefits during process scale-up by providing critical data to fine-tune process parameters. Once the process moves to large-scale production under cGMP, the added value of continuously monitoring pCO2 may not justify the cost and risk of operating the probe system. Hence, large-scale operations tend to favor the use of simple and extremely robust technologies that have been thoroughly validated in the past. This is partly due to the risk of mechanical breakdown and a lack of technical expertise on the manufacturing floor to operate newly introduced, complex sensor

technologies.

Although complex sensors have potential benefits, adoption within a GMP context can be slowed down by the extensive validation process necessitated by regulatory requirements. To ensure the integrity and reliability of the data produced, validation often involves the use of parallel orthogonal methods, i.e. those that measure the same attribute but through different mechanisms. In keeping with the current FDA guidelines, when changes to an assay are proposed at any stage of the product lifecycle, companies are required to conduct a comprehensive risk assessment. This risk assessment must evaluate the potential impact such a change could have on product quality. For example, if a company wants to transition from a manual ELISA assay to an automated method, they are required to conduct side-by-side comparability testing from the two methods on the same material for the single attribute that the assay is measuring. This added complexity and the associated costs often limit the number of new technologies that can be feasibly tested and potentially incorporated into the manufacturing process. A critical practice is to preemptively store enough retains (samples, reference standards, and reference materials) from analytical methods used at all stages of the product development cycle. This will greatly reduce the difficulties of implementing comparability tests required for new methods to be incorporated into production at a later stage.

2.2.2. *Path to large-scale adoption*

Since January 2020, the FDA has released a series of updated regulatory guidelines specific to cell and gene therapies. These revised guidelines encourage improvement to the analytical procedures for measuring impurities, replication, titer, and infectivity (FDA, 2024). These guidelines suggest that manufacturers include more detailed characterizations in their regulatory filings, a trend that may intensify as the field continues to mature and develop. This changing landscape offers a promising opportunity for novel techniques that can provide greater accuracy and precision in measuring rAAV attributes than the current gold-standards. In a commercial manufacturing environment operating with an approved process, the introduction of any new process-analytical technology largely depends on its ability to demonstrate a preferable risk-to-benefit ratio. Therefore, for new technologies to be successfully integrated, they not only need to offer significant advantages but also meet the highest standards of reliability, risk management, and cost-effectiveness. To facilitate the de-risking of introducing novel technologies, FDA's Center for Biologics Evaluation and Research (CBER) has created the CBER Advanced Technologies Team (CATT) to offer an interactive platform for communication between CBER and prospective developers of novel manufacturing and test technologies. This mechanism provides early feedback to developers about what would be needed to ensure that a submission containing the technology could be approved (FDA, 2021).

3. The use of mathematical modeling to enhance rAAV production

Mathematical models are useful resources for enhancing the efficiency of biopharmaceutical manufacturing and to reduce the time for process development and scale-up (Destro and Barolo, 2022; Hong et al., 2018; Narayanan et al., 2020). This section discusses strategies for improving rAAV production through mathematical modeling.

3.1. *Developing models for rAAV manufacturing*

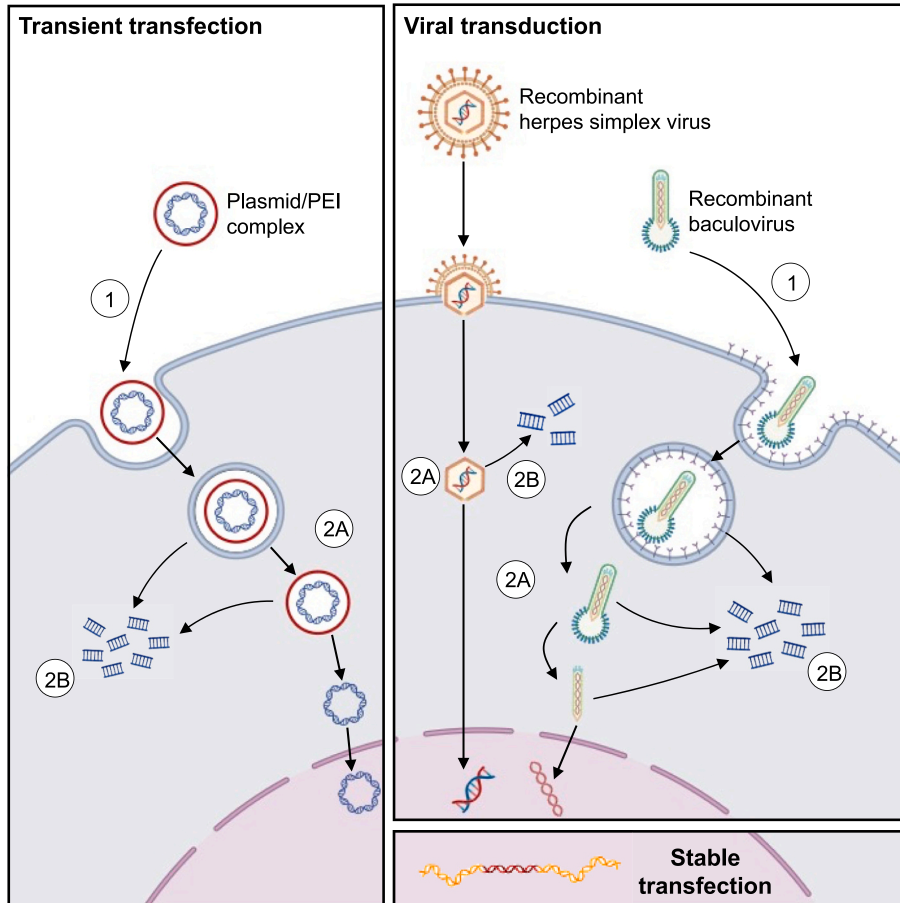
3.1.1. *Mathematical modeling of biopharmaceutical processes*

A model is a mathematical representation of a physical process, consisting of equations that connect a set of inputs to a set of outputs. Models with different degrees of detail and different input/output combinations can be developed for a given process, based on the intended application. For rAAV production, modeling efforts can span from the scale of the intracellular reactions inside of cells producing rAAV up to flowsheet models, encompassing both cell culture and purification unit operations.

Based on the type of equations, models are classified as mechanistic, data-driven, or hybrid. Mechanistic models describe in mathematical terms the physical, chemical, and biological phenomena occurring within a process (Pantelides and Renfro, 2013). In contrast, data-driven models are made of equations that do not have an easily interpretable physical meaning and, instead, rely on large amounts of training data for building a connection between inputs and outputs (Venkatasubramanian, 2019). Machine-learning and artificial intelligence models fall within this category. Hybrid models include both mechanistic and data-driven components (Narayanan et al., 2019; von Stosch et al., 2014). Automated tools exist for rapid construction of data-driven models (Cozad et al., 2014; Sun and Braatz, 2021). In contrast, time-consuming work from experts in both computational and physical science is required for developing mechanistic models. Although harder to develop, mechanistic modeling has a lower data requirement, offers a deeper understanding of the process, and can have predictive capabilities even beyond the training conditions. In general, a trade-off needs to be considered when selecting a modeling approach. A proper balance must be found between the desired accuracy for the application of interest, the time available for model development, the computational resources at hand, the amount of available data, and the budget allocated for conducting new experiments. Therefore, mechanistic models have emerged as more promising tools in the context of rAAV manufacturing, for which data are often limited and expensive to obtain.

3.1.2. *Mathematical models for rAAV manufacturing*

Existing models for rAAV production focus on upstream manufacturing (Fig. 2 and Table 3), where the overall process can be enhanced by increasing the titer of full capsids and by decreasing the production of empty capsids. Notably, a lower production of empty capsids reduces (and potentially removes) the need to enrich the full capsid concentration downstream. Mechanistic modeling of viral particle production at the intracellular and bioreactor scales has recently been discussed by Canova et al. (2023). Joiner et al. (2022) recently reviewed process modeling of rAAV production via transient transfection of HEK293 cells, including models focusing on the metabolism of HEK293 cells and models describing rAAV production. The mechanistic model recently proposed by Nguyen et al. (2021) represents the state-of-the-art in modeling rAAV manufacturing in mammalian cells. The model describes rAAV production via triple transfection of HEK293 cells, through equations accounting for the main steps of the intracellular reaction-transport network (Fig. 2). Validation with literature and in-house-collected datasets showed that the model accurately predicts the dynamics of polyethylenimine-mediated plasmid delivery and of production of full and empty rAAV capsids in different experimental conditions. Although the intracellular reaction-transport networks for rAAV production in mammalian and insect cells have some steps in common, intrinsic differences between the two systems must be considered for process modeling (Fig. 2 and Table 3). rAAV manufacturing in insect cells involves gene transduction through baculovirus infection, rather than transfection. Hence, rAAV virion formation in insect cells occurs in parallel with the cascade of reactions initiated by baculovirus infection (Rohrmann, 2019; Virgolini et al., 2023). Destro et al. (2023) recently developed the first mechanistic model for rAAV production in insect cells. The model can predict with high accuracy the dynamics of full and empty rAAV capsid formation, along with the dynamics of baculovirus infection, for different productive systems, such as TwoBac and ThreeBac (Fig. 1). The level of several intermediates involved in rAAV production, including replicative and non-replicative proteins, as well as non-encapsidated vector genomes, is also estimated with high accuracy by the model. The model by Destro et al. (2023) was developed for batch rAAV production in insect cells and can simulate two waves of baculovirus infection, aligning with common industrial processes. In these conditions, a high baculovirus titer is



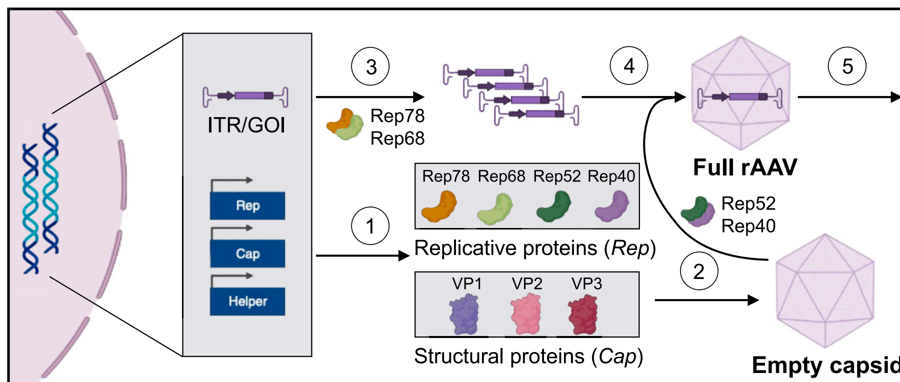


Fig. 2. rAAV manufacturing: intracellular reaction-transport network. For mammalian cells, the delivery of the genes for rAAV production is carried out through transient transfection or through transduction with recombinant herpes virus or adenovirus (not shown). For insect cells, gene delivery for rAAV production is performed through recombinant baculovirus. Alternatively, mammalian or insect cell lines stably transfected with the genes for rAAV production can be developed. Independently from the gene delivery route, rAAV production occurs through analogous intracellular steps across the different platforms. Replicative proteins and viral proteins are first expressed. Viral proteins assemble into the viral capsid, while Rep78 and Rep68 intervene in the amplification of the inverted terminal repeat/ gene of interest (ITR/GOI) cassette. Rep52 and Rep40 mediate the final step of viral encapsidation. Legend and reference kinetics for the steps of the network are reported in Table 3.

inoculated in the cell culture to ensure that all cells are infected either immediately after inoculation (first wave) or after the first wave of infected cells releases baculovirus progeny, initiating a second wave of infection. Recently, (Destro and Braatz, 2024) presented vitraPro, a software toolkit for simulating transduction-based biomanufacturing processes characterized by several waves of viral transduction and propagation, such as batch processes with low viral titer at inoculation, as well as continuous processes. The software is a useful tool to support the development of innovative platform and process designs for rAAV production in insect cells, since it can estimate the dynamic distribution of viral genome copy numbers and infection age in a cell population. Additionally, vitraPro can account for the presence of defective interfering particles, which can impact productivity and process efficiency (Frensing et al., 2013).

Table 3
Kinetics of rAAV manufacturing: triple transient transfection of HEK293 cells vs. baculovirus/Sf9 system (TwoBac platform). The parameters are reference values, approximated and converted to comparable units of measurement from the estimations reported in Nguyen et al. (2021) and Destro et al. (2023). The parameters may vary significantly for other HEK293-based or Sf9-based production platforms not considered in this table. The listed steps refer to the schematics of Fig. 2. The reported characteristic times correspond to the duration necessary for approximately 60% of a relevant species to undergo a specific biochemical or biophysical step. For instance, approximately 60% of the extracellular plasmid/baculovirus is internalized by cells during the reported characteristic time for cellular uptake.

Step	HEK293 (triple transient transfection)		Baculovirus/Sf9 (TwoBac platform)	
	Parameter	Reference value	Parameter	Reference value
Delivery of genes for rAAV production				
1. Uptake	Characteristic time (plasmids)	100–1000 h	Characteristic time (baculovirus)	2 h
2. Trafficking				
2 A. Transport to nucleus	Characteristic time (plasmids)	200 h	Characteristic time (baculovirus)	2 h
2B. Degradation	Plasmids reaching nucleus	1–5%	Baculovirus reaching nucleus	40–60%
3. Replication	–	–	Baculovirus replication	Up to 10^4–10^5 copies/cell
rAAV production				
1. Transcription and translation	Transcription	Unknown	Transcription from polh/p10	500–600 nucleotide $cell^{-1}$ h^{-1}
	VP and Rep synthesis	10^{-3} molecule $cell^{-1}$ h^{-1}	Transcription from ΔIE1	50–60 nucleotide $cell^{-1}$ h^{-1}
			Max translation rate for VP and Rep transcripts	3×10^9 nucleotide $cell^{-1}$ h^{-1}
2. Capsid assembly	Characteristic time	$<< 1$ h	Characteristic time	$<< 1$ h
3. Vector genome amplification	Amplification rate	50 vg $cell^{-1}$ h^{-1}	Amplification rate	5000 vg $cell^{-1}$ h^{-1}
4. Encapsidation	Encapsidation rate	50 vg $cell^{-1}$ h^{-1}	Encapsidation rate	5000 vg $cell^{-1}$ h^{-1}
5. Secretion	Characteristic time	Serotype dependent	Characteristic time	$> >$ 24 h

3.2. *Using mathematical models for enhancing rAAV manufacturing*

Mathematical models can enhance rAAV manufacturing in all stages of the pipeline, with applications in both the process engineering and molecular biology domains (Fig. 3). From a process engineering perspective, mathematical modeling can be used to optimize the design and the operation of a process. From a molecular biology perspective, mathematical modeling can support the engineering of the cell lines and of the transfection and/or transduction agents used in the productive platform. The use of mathematical modeling for supporting pharmaceutical development and manufacturing is specifically encouraged by regulators under the QbD (International Council for Harmonisation,

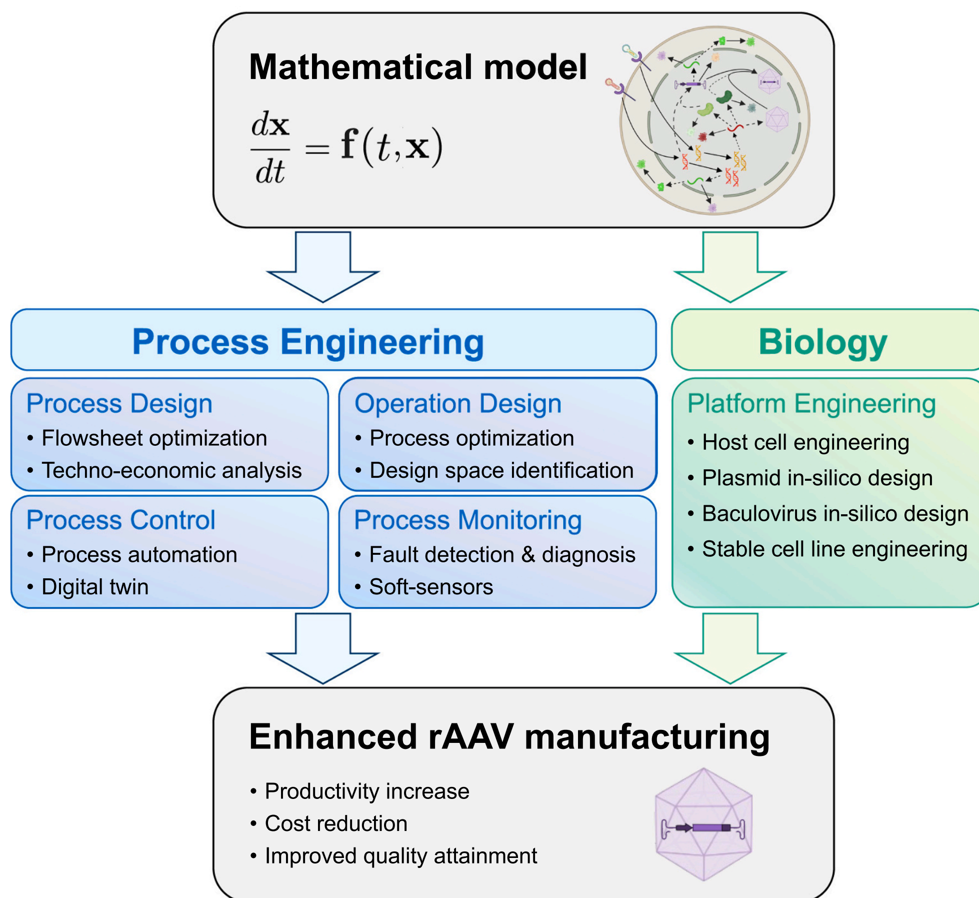


Fig. 3. Mathematical modeling to enhance rAAV manufacturing: model-based strategies in the domains of process engineering and molecular biology. Multiscale models linking macroscopical phenomena with the intracellular reaction-transport network for rAAV production are used to identify and tackle process bottlenecks. The representation of the intracellular reaction-transport network is adapted from Destro et al. (2023).

2011, 2009; Tian et al., 2019).

3.2.1. Process design

Model-based process design is a widespread tool within the process industry for optimizing the flowsheet of a plant (Chen and Grossmann, 2017). In this context, models are used to determine the type and the layout of upstream and downstream unit operations that maximize productivity and minimize costs, with given product quality constraints. Lyle et al. (2023) recently carried out a model-based analysis of the process economics for rAAV manufacturing through transient transfection of HEK293 cells. The authors compared flowsheets employing adherent and suspension cultures for the upstream process, and flowsheets using ultracentrifugation and anion-exchange chromatography for enriching the full capsids concentration downstream. Considering a population of 1000 patients, the most cost-effective flowsheets for high dose therapies (10^{14} vg per dose or more) were based on suspension cultures and anion-exchange chromatography. Adherent cultures and ultracentrifugation are feasible options only for therapies requiring small doses of about 10^{12} vg (e.g., for ophthalmic applications). Model-based process economic evaluations have also been used to compare viral vector production with transient transfection and with stable producer cell lines (Comisel et al., 2021) and to benchmark batch and continuous approaches to rAAV manufacturing (Yang et al., 2023). The analyses demonstrated that, for large-scale production, switching to continuous manufacturing is economically advantageous. Future studies on model-based process design hold potential for further enhancing rAAV manufacturing, especially by comparing different productive platforms with detailed models.

3.2.2. Operation design

Mathematical models can enhance the operation of existing plants (Içten et al., 2020; Wang et al., 2017). Zhao et al. (2020) recently used design of experiments and response surface modeling to identify a combination of plasmid concentrations and cell density that enhanced rAAV production in HEK293T cells across several serotypes and capsid variants. Petiot et al. (2015) discussed how models of the metabolism of HEK293 cells can be leveraged for optimizing feeding strategies, medium design, and other culture conditions for improved productivity of viral vectors. Metabolic flux analysis (MFA) and flux balance analysis (FBA), a simplified class of steady-state mechanistic models, have been used for modeling the metabolism of HEK293 cells and to improve the productivity of several processes (Martínez-Monge et al., 2019), although not yet for rAAV. Similarly, models of the host cell metabolism could be leveraged to improve rAAV manufacturing, by ensuring that producer cells have enough reservoirs of nutrients and metabolites to maximize rAAV production. Metabolic models would also be useful to address the cell density effect, which hinders rAAV production at densities above 5 million cells per mL in both Sf9 and HEK293 (Petiot et al., 2015). In downstream purification, recent advancements have demonstrated model-based process development for the separation of full and empty rAAV capsids through ion-exchange chromatography (Gomis-Fons et al., 2024; Keller et al., 2024). Enhanced operating conditions for the removal of empty particles were identified through in silico experiments on mechanistic models, significantly reducing the experimental effort necessary for process development, as further discussed in Section 4.2.3.

Advanced approaches to model-based operation design are employed in several manufacturing sectors (e.g., by the chemical industry) to determine the operating conditions that optimize productivity under a set of quality constraint (Biegler et al., 2002; Fujiwara et al., 2005). These approaches have the potential to boost rAAV productivity and significantly lower the manufacturing cost. Mathematical modeling is especially encouraged within the QbD framework to support the identification of the design space, namely the multidimensional space of operating and feed conditions that have been demonstrated to provide quality assurance (Destro and Barolo, 2022; García-Muñoz et al., 2015; International Council for Harmonisation, 2009).

3.2.3. Process monitoring and control

In process monitoring, models are used in fault detection and diagnosis for detecting deviations from normal operating conditions that might compromise the product quality (Venkatasubramanian et al., 2003). As discussed in Section 2.1.3, mathematical models play an additional crucial role for process monitoring within soft-sensing frameworks. In process control, models are used to determine the optimal control actions to maximize productivity and minimize costs, while guaranteeing the product quality (Hong et al., 2018; Rawlings et al., 2017; Seborg et al., 2016). In rAAV manufacturing, potential applications of models for process control include optimization of the initial concentration of plasmids/recombinant baculoviruses to supply to the host cells and real-time estimation of culture conditions and harvest time. Ultimately, model-based digital twins can be developed as a digital replica of a process, communicating in real-time with their physical counterparts to support the monitoring, control, and optimization of the process operation in real time. FDA recently highlighted the benefits of the use of digital twins and artificial intelligence to support pharmaceutical manufacturing (FDA, 2023, 2022).

3.2.4. Platform engineering

Mathematical models can support the development of improved production platforms for rAAV. Previous studies used FBA and MFA metabolic models to enhance production of various recombinant proteins in CHO cells (Huang et al., 2017). Recently, Lavado-García et al. (2021) employed design of experiments and response surface modeling to identify genes whose overexpression and underexpression are correlated with improved HIV-1 virus-like particle production in HEK293 cells. Similar approaches can be applied to support genetic engineering of host cells for rAAV production, including producer cell lines. Mathematical models can also guide the enhancement of plasmids (or recombinant baculoviruses) used in rAAV manufacturing with mammalian (or insect) cells. Mechanistic models are particularly useful for understanding and overcoming the current productivity bottlenecks. For instance, a mechanistic model revealed that the large production of empty capsids in mammalian-cell based processes results from delayed vector genome amplification with respect to capsid production during transient transfection of HEK293 cells (Nguyen et al., 2021). Similarly, a mechanistic model indicated that low rAAV genome amplification, controlled by limiting replicative protein levels, is the main factor that limits rAAV production in current insect-cell based processes (Destro et al., 2023). In future studies, mathematical models can support the enhancement of productive platforms through in silico simulation of novel constructs and through the estimation of optimized expression rates for replicative and structural AAV proteins.

3.3. Future opportunities for enhancing rAAV manufacturing through mathematical modeling

Several opportunities exist for boosting rAAV productivity in upstream and downstream manufacturing through model-based techniques conventionally employed in process engineering for process optimization, monitoring, and control. Mathematical models can also support the development of platforms with enhanced rAAV productivity. For instance, in silico experiments can be used to estimate the rAAV productivity that could be achieved with novel plasmid/baculovirus constructs or engineered cell lines. For this purpose, mathematical models explaining in more detail the physical phenomena occurring during rAAV manufacturing are needed. Recently developed models for rAAV production in mammalian (Nguyen et al., 2021) and insect (Destro et al., 2023) cells represent a quantitative and interpretable summary of the currently available mechanistic knowledge on upstream rAAV manufacturing. These models serve as a foundational framework for further studies on rAAV manufacturing platforms. Both models are

readily extendable to other production methods with, respectively, mammalian and insect cells, also including stably transfected producer cell lines. The models are expected to capture the production dynamics for different serotypes of rAAV with minimal changes in the model parameters (Chahal et al., 2014; Destro et al., 2023). To further improve the models, follow-up studies should decouple steps of the reaction-transport network that have previously been lumped together for identifiability issues. The models can also be improved by explicitly modeling additional phenomena of the host metabolism. This is especially important for mammalian cells, in which rAAV production occurs in parallel with the cell cycle, and for which a cellular immune response to rAAV production has recently been discovered (Chung et al., 2023). Data-driven and hybrid modeling are interesting options towards this objective.

4. Purification of rAAV

4.1. The current state of rAAV purification technologies

The goal of downstream purification is to remove process- and product-related impurities to below specified levels, while minimizing losses of functional rAAV vector. Each purification step in the process decreases the total yield of the final product. For example, five purification steps in series that each have an 87% yield will lead to an overall yield of only 50%. Improvement of the yield at each step to 95% would improve the five-step purification yield to 77%. Given the significant cost of upstream rAAV manufacturing (Lyle et al., 2023), the purification process plays an important role in both the scalability and cost of goods of the final rAAV product.

Process-related impurities represent materials that are introduced during the process or that are a byproduct of the process. Examples include transfection reagents, plasmids, baculovirus, host cell DNA, host cell proteins, proteosomes, and other macromolecular complexes. In contrast, product-related impurities relate to the rAAV product itself, and include empty capsids, partially full capsids, capsids containing endogenous DNA fragments, and aggregates of rAAV and its components. Product-related impurities are the most difficult to remove due to their similarity to the final product. For example, removal of empty viral capsids from full capsids is a major challenge in rAAV purification due to the similarity between the two. Both empty and full capsids display the same proteins and epitopes and have nearly identical surface charges. Hence, a robust and high yield purification strategy is needed to retrieve full rAAV capsids with minimal loss. Insufficient removal of product- and process-related impurities reduce the efficacy of a dose and can cause immunotoxicity in patients.

The typical downstream steps involved in rAAV production (Fig. 1) include cell harvesting, cell lysis, lysate clarification, capsid capture using affinity chromatography, polishing using ion exchange chromatography or ultracentrifugation, buffer exchange, and product formulation. The proportion of rAAVs retained intracellularly versus those released to the extracellular media depends on the AAV serotype and on the production cell type (Lock et al., 2010; Vandenberghe et al., 2010). Therefore, it is common practice to harvest rAAV both from inside and outside cells to increase the overall yield. Cell lysis is typically done via freeze-thaw, mechanical disruption, or using non-ionic surfactants. Freeze-thaw is only suitable for sample volumes lower than 10 mL (Marichal-Gallardo et al., 2021). Mechanical disruption and chemical treatment using non-ionic surfactants are the preferred approaches for efficient cell lysis at larger scale (Marichal-Gallardo et al., 2021; Srivastava et al., 2021). Typically, clarification of lysate to separate the soluble fraction from other cellular debris is done using dead-end or tangential flow filtration (TFF) prior to chromatographic separation of capsids (Negrete and Kotin, 2008).

Affinity chromatography (AC) is often the first step towards the capture and enrichment of rAAV capsids downstream. Affinity resins using ligand chemistries (e.g., AVB and CSAL8/9) that target common epitopes across different rAAV serotypes are available (Florea et al., 2023). Most resins can be regenerated efficiently (~65–80% overall efficiency across all rAAV serotypes), without carrying over contamination (Florea et al., 2023). Since AC separates capsids from process-related impurities based on the interaction of the resins with the capsid viral proteins, this approach cannot discriminate between full rAAV capsids and product-related impurities.

Downstream polishing through multiple processing steps is necessary to remove product-related impurities. Selective purification of full capsids from the recovered total capsids can be achieved by exploiting the small charge or density difference between full and empty capsids. Full capsids are more negatively charged compared to the empty capsids and therefore can be separated by cation/anion-exchange (CEX/AEX) chromatography using buffers at a specific pH. Furthermore, full capsids are comparatively denser than empty or partially full capsids and can also be separated by ultracentrifugation. However, ultracentrifugation has low yield (<20%), can only process small volumes, and is time-intensive (Marichal-Gallardo et al., 2021). Therefore, CEX/AEX chromatography is currently the only route for separating full and empty capsids at large production volumes. CEX/AEX chromatography is also a key tool to remove other charged impurities, such as host cell DNA.

A final downstream purification process will be comprised of multiple chromatography operations in series. For example, ion exchange chromatography following affinity chromatography is efficient in removing both the host cell DNA and protein (>90%), but leads to significant loss of capsids (Table 4) and can only improve the percentage of full capsids to ~80%. Other purification approaches that do not use affinity chromatography have also been developed, such as hydrophobic interaction chromatography (HIC) followed by CEX and AEX as well as the use of CEX and AEX alone. A comparison of the total yields after each purification step is shown in Table 4 for different starting materials and purification trains. HIC-CEX-AEX demonstrates performance below that of the AC-AEX process in terms of overall rAAV yield. In contrast to AC-AEX and HIC-CEX-AEX, a combination of CEX-AEX improves rAAV recovery to ~65% after the initial CEX step and ~ 59% of the initial rAAV after the AEX step, using cell pellet as starting material. Additionally, the CEX-AEX process is only capable of enriching full capsids to between 70 and 80%.

While AEX achieves only partial enrichment of full capsids, leading to a content ratio of about 70–80% in the eluate, further improvements in the percentage of full capsids can be made by performing multiple rounds of AEX (Qu et al., 2007; Tomono et al., 2016). However, this leads to further reduction in yield (between 60 and 70% from each additional round of AEX (Qu et al., 2007). As an example, a two-stage process using both cation- and anion-exchange chromatography achieved >99% full capsids at around 50% recovery for rAAV1 and rAAV8 (Okada et al., 2009).

Table 4
Recovery of rAAV viral genome after different purification steps starting from different starting material. Percentages refer to the initial rAAV concentration in the starting material. Data taken from Rieser et al. (2021).

Purification protocol	Purification step	TFF filtrate as a starting material (%)	PEG precipitate as a starting material (%)	Cell pellet lysate as a starting material (%)
AC-AEX	AC	89	69	59
	AEX	32	44	37
HIC-CEX-AEX	HIC	23	3	0.3
	CEX	15	0.3	0.2
	AEX	5	–	–
CEX-AEX	CEX	–	–	65
	AEX	–	–	59

–: Not done.

4.2. Emerging technologies and opportunities to improve rAAV purification

This section presents several emerging technologies in rAAV purification: novel affinity ligands, chromatographic systems with innovative stationary phases, model-based process development, steric exclusion chromatography, and aqueous two-phase separation systems. Research opportunities in these areas and in the broader field of rAAV purification are highlighted.

4.2.1. Affinity ligands

The state-of-the-art affinity resins POROS CaptureSelect AAVX (Thermo Fisher Scientific) and AVB Sepharose (Cytiva) have revolutionized rAAV capture, by allowing the separation of diverse rAAV serotypes, without the need to create a new affinity resin for each rAAV product (Florea et al., 2023; Kilgore et al., 2023; Nass et al., 2018). The AAVX resin, with a dynamic binding capacity of 10^{13}–10^{14} viral particles per mL of resins, supports a platform purification process across various AAV serotypes, with recovery rates exceeding 95% for several serotypes (Florea et al., 2023). In comparison, the AVB Sepharose resin (binding capacity $>10^{12}$ vg/mL) provides a wider range of product yields, 50–92% for serotypes AAV1, AAV2, AAV5, and AAV6 (Nass et al., 2018). An issue with state-of-the-art affinity resins is their requirement of strongly acidic elution conditions (pH $\leq$ 3), which can reduce rAAV transduction activity and shorten the resin's lifespan (Kilgore et al., 2023). Recently, in silico design was used to develop serotype-agnostic AAV-targeting peptide ligands that combine high-rAAV-capture properties with the requirement of mild elution conditions (pH $\sim$ 6) (Chu et al., 2023; Shastry et al., 2024). Conjugated to Toyopearl resin, the developed peptide ligands exhibited selectivity, binding capacity, and yield comparable to AAVX and AVB resins. Notably, rAAV2 vectors purified through the novel peptide ligands exhibited up to 80% transduction activity, marking a remarkable 60-fold increase with respect to state-of-the-art AAVX and AVB resins and showing the benefits of elution in mild conditions (Chu et al., 2023; Shastry et al., 2024). Furthermore, peptide ligand-based resins can be manufactured at a much lower cost than conventional affinity resins for rAAV purification, which rely on antibody-derived ligands (Shastry et al., 2024). Following these examples, future advances in the development of serotype-agnostic affinity resins with higher selectivity, dynamic binding capacity, and rAAV yield that also require mild elution conditions would greatly benefit rAAV purification. Enhanced affinity resins hold the potential to streamline the process, reduce production costs, and improve the availability and affordability of rAAV-based therapies.

4.2.2. Chromatographic systems with innovative stationary phases

Chromatographic systems with innovative designs for the stationary phase, such as monoliths and membranes, have recently demonstrated promising performance in rAAV purification (Kilgore et al., 2023; Lavoie et al., 2023; Lothert and Wolff, 2023). Monoliths, which consist of a continuous porous stationary phase, facilitate high convective flowrates of the mobile phase through interconnected channels (González-González et al., 2017). Compared to resins, monoliths have lower void space and more accessible channels for rAAV particles (Kilgore et al., 2023). Monoliths are usually functionalized with ion-exchange ligands, although different types of molecules, including, for instance, affinity ligands, can be utilized. Monolith-based ion-exchange chromatography has achieved a promising performance in full/empty rAAV particles separation, with enrichment >90% of full capsids across different serotypes (Chen et al., 2024; Di et al., 2024; Dickerson et al., 2021; Joshi et al., 2021a). Membrane-based chromatography is also acquiring importance in rAAV purification, especially in ion-exchange chromatography. Fan et al. (2022) recently isolated rAAV2 from a Sf9 lysate with high productivity (2.4×10^{13} capsids mL^{-1} min^{-1}) and selectivity, by using a strong AEX membrane functionalized with triethylamine, forming a quaternary amine ligand. A capsid yield of 65% was achieved, with an infectivity of 76%. Future research on monolith- and membrane-based chromatographic systems holds significant potential for further enhancing productivity, yield, and selectivity. Addressing current scalability challenges and high costs will be critical for advancing these technologies and optimizing rAAV purification processes.

4.2.3. Model-based process development of chromatographic systems

As discussed in Section 3, model-based process development offers significant advantages over heuristic and experiment-intensive approaches. Recent studies on rAAV purification have introduced advanced workflows and applications, highlighting the benefits of model-based design of chromatographic systems. For instance, mechanistic modeling was recently applied for the first time to optimize an anion exchange (AEX) chromatography process for full/empty rAAV6 separation (Gomis-Fons et al., 2024). A reactive-dispersive model was successfully calibrated through data obtained from linear gradient experiments. The model was then utilized to optimize parameters such as mobile phase composition and elution conditions, including single-step versus two-step elution modes. The model led to an optimized design that experimentally achieved a purity of 64.5% and a recovery yield of 88.2%, significantly reducing the experimental effort needed to reach these results. In another study, Keller et al. (2024) presented a workflow for rAAV full/empty capsid separation that combined high-throughput screening for resin and buffer selection with mechanistic modeling for optimizing the operating conditions of the chromatography system. Future research should build on these examples, which demonstrate the potential of model-based methodologies to enhance rAAV purification processes. By exploiting advanced modeling, more efficient and effective purification processes can be achieved, ultimately improving the scalability and cost-effectiveness of rAAV production.

4.2.4. Steric exclusion chromatography

Membrane-based steric exclusion chromatography (SXC) is a promising technology in rAAV downstream purification. It exploits molecular crowding and volume exclusion effects caused by the addition of a crowding agent, such as high molecular weight polyethylene glycol (PEG), to a solution containing the species to be purified. The target, in the presence of a highly concentrated PEG solution, is then captured without a direct chemical interaction on a nonreactive hydrophilic membrane and later eluted by a buffer not containing PEG. Larger molecules with higher hydrodynamic diameters, such as rAAV, are more prone to interact with the stationary phase than smaller ones, such as host cell proteins and DNA. SXC has many advantages including the use of physiological pH and salt concentration; further, SXC is serotype-independent, inexpensive, single use, exhibits a high yield of rAAV particles (>95% recovery), and can remove host cell DNA (>94% clearance) and proteins (>80% clearance) (Marichal-Gallardo et al., 2021). Compared to a binding capacity of $\sim 10^{14}$ vg/mL and $\sim 10^{12}$ vg/mL in commercial affinity resins, the binding capacity of SXC is almost 10 times higher (Marichal-Gallardo et al., 2021). Despite these potential benefits, SXC requires multiple rounds of purification, and its scalability has not been tested yet. Additionally, like affinity chromatography, SXC is only capable of capturing all rAAV particles and additional purification steps to separate full from empty capsids will be needed.

4.2.5. Aqueous two-phase systems

Aqueous two-phase systems (ATPS) offer a scalable and cost-effective method for rAAV purification and are also suitable for continuous processing (Moleirinho et al., 2020; Molino et al., 2013; Teixeira et al., 2018). Separation via ATPS consists of a liquid-liquid extraction, in which molecules partition between two aqueous phases based on their physicochemical properties. The two phases of an ATPS are generated by adding to an aqueous solution a soluble polymer (usually, PEG) and a salt or by using pairs of polymers (e.g., PEG and dextran). ATPS are particularly attractive for the separation of viral vectors from process-related impurities, although downstream polishing

is usually needed for removing product-related impurities, such as empty capsids (Arden and Metzger, 2016; Joshi et al., 2023; Suleman et al., 2022). Guo et al. (2012) first demonstrated the use of ATPS for rAAV purification, obtaining 95% recovery of rAAV8 from an HEK293 lysate. Compared to purification through one single round of CsCl gradient density centrifugation, ATPS separation required shorter processing time and achieved higher purity. More recently, Fu et al. (2023a) reported over 99% recovery of rAAV particles from a crude cell lysate, with >95% impurity removal. A recent economic evaluation showed that ATPS-based purification of viral products can lower capital costs by up to 90% and operating costs by 50% compared to chromatography (Nold et al., 2024). However, ATPS implementation is challenging due to the need to optimize a vast design space – including variables such as pH, polymer and salt types and their concentrations – for each product (Molino et al., 2013; Nold et al., 2024). Poor design can result in inadequate purification, necessitating downstream polishing steps, which has limited ATPS adoption in industry.

4.2.6. *Future opportunities to improve rAAV purification*

The efficiency of the downstream purification process clearly has a significant impact on the yield and cost of the final product. While current approaches have been sufficient to enable the production of the first approved gene therapies, there is significant room for improvement. Technologies that improve rAAV yields and simultaneously eliminate purification steps will have the biggest impact on cost and availability. Technologies that are serotype-agnostic and that can easily be applied across the broad diversity of current rAAV therapies under investigation will also be more attractive. The emerging technologies discussed in this section show promise in overcoming several purification challenges. However, further research and development are necessary to fully address the existing issues in rAAV downstream manufacturing. Exploring out-of-the-box alternative purification processes, such as crystallization, which has shown potential for different serotypes (DiMattia et al., 2005; Lane et al., 2005; Xie et al., 2004), should also be considered.

5. Conclusions

The current paradigm for AAV gene therapy production has been quite successful in supporting the approval and commercial production of AAV therapies to date. However, anticipated demand will tax current production processes beyond their ability to deliver sufficient high-quality AAV vectors at a reasonable cost. This article highlights the current state of technological advancement to address this challenge in the areas of in-process analytical technology, process and mechanistic models, and downstream purification. The continued adoption of advancing process analytical technology to AAV production will increase process reliability and final product quality. Yet, an opportunity exists to develop and adopt tools to effectively measure AAV quality in real time. Such technologies would provide unprecedented control over product quality. While process and mechanistic models have demonstrated marked improvements in product quality and process performance for a variety of manufacturing scenarios, including in pharmaceutical production, they have yet to be applied to AAV production processes. Recently developed models highlight the potential gains and opportunities for models to uncover areas for process improvement and to significantly reduce time and effort in experimental design. Coupling them with advanced sensors enables model predictive process control and will reduce variability and improve quality. Finally, downstream purification of AAV represents an area of need. Current processes result in significant losses of material, largely due to the need to separate empty capsids from full capsids. Improved chromatography resins and new modes of purifying AAV represent steps forward. Yet, significant technological development is still needed. Continued evolution of rAAV manufacturing processes will be needed to meet demand and make these therapies available to those that need them.

Declaration of competing interest

The authors declare no conflict of interest.

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