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REVIEW

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Critical challenges and advances in recombinant adeno-associated virus (rAAV) biomanufacturing

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Abstract

Gene therapy is a promising therapeutic approach for genetic and acquired diseases nowadays. Among DNA delivery vectors, recombinant adeno-associated virus (rAAV) is one of the most effective and safest vectors used in commercial drugs and clinical trials. However, the current yield of rAAV biomanufacturing lags behind the necessary dosages for clinical and commercial use, which embodies a concentrated reflection of low productivity of rAAV from host cells, difficult scalability of the rAAV-producing bioprocess, and high levels of impurities materialized during production. Those issues directly impact the price of gene therapy medicine in the market, limiting most patients' access to gene therapy. In this context, the current practices and several critical challenges associated with rAAV gene therapy bioprocesses are reviewed, followed by a discussion of recent advances in rAAV-mediated gene therapy and other therapeutic biological fields that could improve biomanufacturing if these advances are integrated effectively into the current systems. This review aims to provide the current state-of-the-art technology and perspectives to enhance the productivity of rAAV while reducing impurities during production of rAAV.

KEYWORDS

capsid recovery, continuous manufacturing, gene therapy manufacturing, in silico modeling, inducible system, multiomics, recombinant AAV (rAAV)

1 | INTRODUCTION

In recent decades, the human genome has been investigated intensively, allowing for the discovery of significant amounts of genes responsible for human genetic diseases. As a result, a new therapy modality has evolved with great potential to cure some of the most prevalent and fatal diseases. Gene therapy is a form of therapy in which genetic material (e.g., DNA or RNA) is introduced into target cells (e.g., patient's cells) to repair or restore related gene expression and, in turn, reverse the effects of the genetic or acquired disease. This therapy serves as a great substitute for traditional medicine and surgery as it is a preventative and proactive approach to correcting medical issues sourced from a patient's genes. Once fully realized, gene therapies will be able to change the life expectancy and quality of life for millions of people around the world.

Gene therapeutics can be classified into two categories based on their use and function: viral or nonviral vectors (Merten, 2016). The efficiency of gene transduction in nonviral systems is much less than that in viral systems, especially in vivo (Nayerossadat et al., 2012). For this reason, viral vectors are currently the more effective form of

gene delivery. Adeno-associated virus (AAV) and lentivirus (LV) are the most widely used vectors for developing and manufacturing gene therapy drugs. LV with efficient transduction of hematopoietic cells (like T cells) is more favored for *ex vivo* applications, like what is used in Chimeric Antigen Receptor T-cell (CAR-T) therapies (Poorebrahim et al., 2019). However, AAV-delivered products have become a more attractive option for in vivo gene delivery due to their non-pathogenicity, low immunogenicity, broad tropisms, and prospective long-lasting gene expression (Athanasopoulos et al., 2000; Ferrari et al., 1997; Patel et al., 2019).

AAV is a non-enveloped, single-stranded DNA virus that belongs to the family of Parvoviridae. The wild-type AAV genome is approximately 4.7 kb in size, containing a *rep* and cap gene flanked by two inverted terminal repeats (ITRs) (Hastie & Samulski, 2015; Wu et al., 2006). Detailed wildtype AAV genome is shown in the upper panel of Figure 1. The *rep* gene encodes four proteins (Rep78, Rep68, Rep52, and Rep40), and the two larger Rep proteins, Rep78 and Rep68, mainly take part in the replication of the genome (Ni et al., 1994), while two smaller Rep proteins, Rep52 and Rep40, play important roles in encapsidation of virion (King 2001). Those four proteins are produced by alternative RNA splicing of transcripts originating from two different promoters: Rep78/68 is regulated by p5 promoter, while Rep52/40 is under the control of p19 promoter (Daya & Berns, 2008). The *cap* gene, under the control of p40

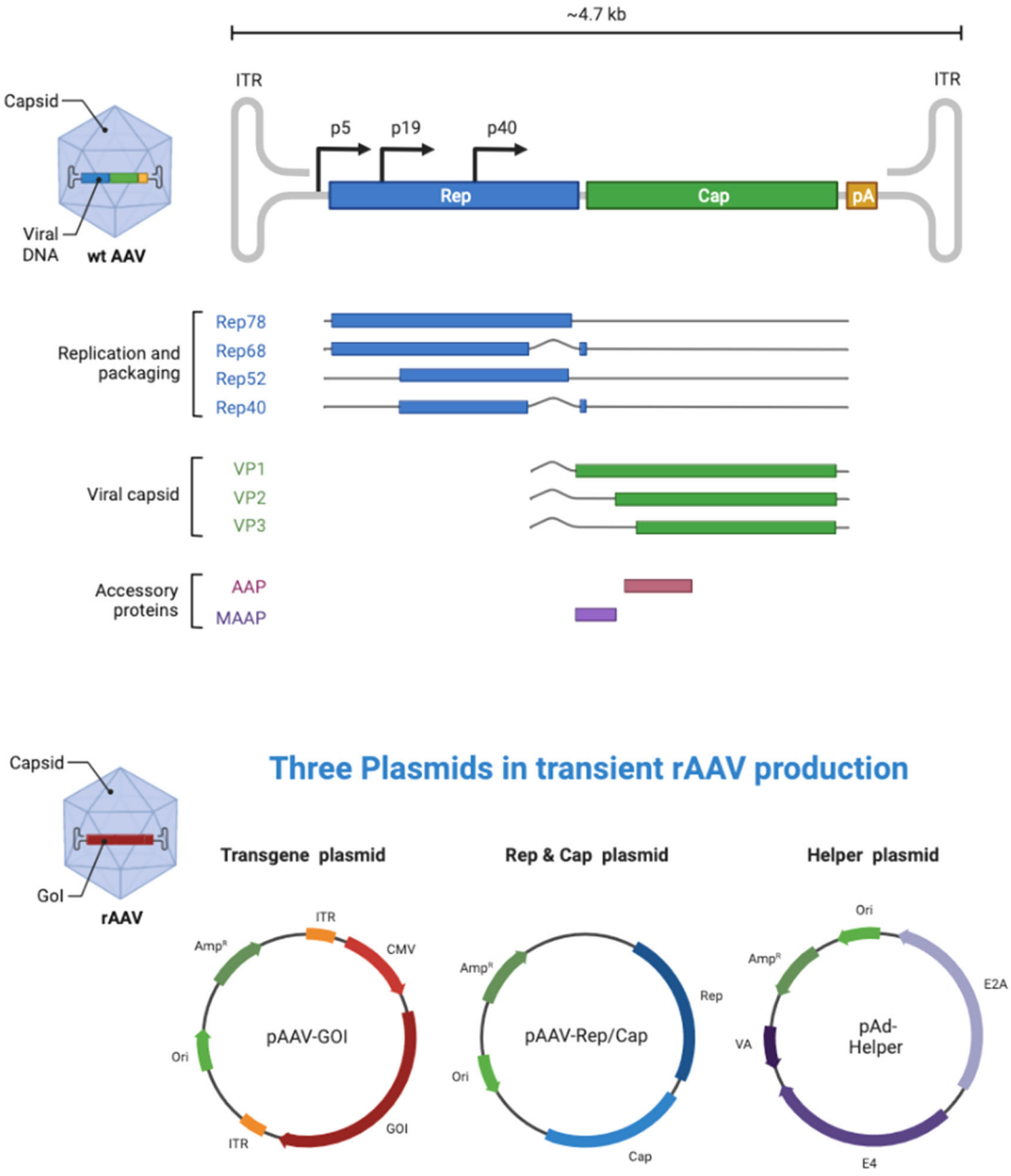

FIGURE 1 The wild-type AAV genome and the structure information of three plasmids for transient rAAV production. rAAV, recombinant adeno-associated virus.

promoter, can be transcribed into three capsid proteins (VP1, VP2, and VP3), assembly-activating protein (AAP) and membrane-associated accessory protein (MAAP). Three capsid proteins are produced from alternative RNA splicing of two transcripts: the larger capsid protein, VP1, is produced from the unspliced transcript, while the spliced transcript produces two smaller capsid proteins, VP2 and VP3 (Daya & Berns, 2008). VP2 translation is initiated by the weak ACG start codon and VP1&3 are expressed more efficiently with the conventional AUG start codon (Sha et al., 2021). With the abundance of VP1/2/3 as 1:1:10 and the help of AAP, viral structural proteins will then assemble together and form capsids. AAV is a Dependoparvovirus, which means the replication of AAV in tissue culture depends on the help of other viruses, such as adenovirus and herpes simplex virus (Aponte-Ubillus et al., 2018). Recombinant AAV (rAAV) is a type of gene therapy vector where the natural coding (such as *rep* gene and *cap* gene), and noncoding (promoters) DNA sequence between the ITR region is replaced with a transgene cassette. As a result of this alteration, rAAV lacks the rep and cap genes in its genome, rendering it incapable of propagating, and can only infect target cells by delivering the gene of interest directly into their nuclei. Detailed information regarding plasmids required for rAAV production is shown in the lower panel of Figure 1. Briefly, three main components are required to produce rAAV: a transgene cassette containing GOI flanked by ITR, a *rep* and *cap* gene, and a helper virus/gene. In rAAV production, the single-strand DNA of rAAV will complete the replication in the host cell nucleus with the regulation of Rep proteins, helper proteins, and host cellular proteins/machinery. The rAAV genome will be packaged into the capsid with the help of Rep proteins. Further details biology of AAV and rAAV production have been reviewed by a few researchers (Meier et al., 2020; Sha et al., 2021; Weitzman & Linden, 2012). Four main production approaches are currently used to manufacture the rAAV, which are: 1) transient transfection of HEK293 cells with two or three rAAV production-related plasmids, 2) stable rAAV-packaging and/or producer cell lines, 3) direct infection of mammalian cells (HEK293 cells or BHK cells) with one or two recombinant Herpes Simplex Viruses (rHSVs), or 4) infection of insect cells (sf9 cells) with two or three recombinant Baculoviruses (rBVs).

Although much progress has been made regarding the methods of biological production, the gene therapy field still suffers incredible setbacks when meeting clinical demands while remaining affordable. Since the release of the first gene therapy product, Glybera, in 2012 for the treatment of hereditary lipoprotein lipase deficiency (LPLD), gene therapy has since gained worldwide attention (Dunbar et al., 2018). Hemgenix, the AAV-based gene therapy drug approved by FDA in 2022 to treat hemophilia B, costs $3.5 million (USD) per dose and has become the most expensive drug in the world. The high price of gene therapy drugs is not only due to the fact that they are currently only approved treatment options for several rare disease treatments, but also because of the expensive manufacturing process (D. Wang, Mulagapati, et al., 2019). The manufacturing cost is primarily driven by the low product yield and the high doses of AAV required for treatment. For instance, a maximal dose of alipogene tiparvovec is 6.5×10^{13} vector genomes (vg) for a human that weighs 65 kg (Robert et al., 2017). In another example, clinical doses proposed for muscle dystrophies, such as Duchenne muscular dystrophy (DMD), will exceed the 1×10^{16} vg/patient range (Clément, 2019). Currently, the reported productivity achieved from current bioprocess methods is approximately 1×10^{14} vg/L (Bingnan Gu et al., 2018; Clément, 2019; Grieger et al., 2016), so via brief calculation of the productivity of a pilot-scale (200 L) batch could only meet the demand of two patients, and one manufacturing scale (2000 L–5000 L) batch could support 20–50 patients. High levels of product-related impurities (e.g., empty/partially full viral capsids) during production also make the manufacturing capability hard to meet the market demands. During the rAAV vector manufacturing, product-related impurities include AAV empty capsids, encapsidated host cell nucleic acids/helper DNA, noninfectious AAV capsids, and any other aggregated, degraded capsids (Wright, 2014). Among these impurities, empty capsids most severely truncate the productivity and cause the lot-to-lot variability of products (Grieger et al., 2016; Joshi et al., 2019; Schnödt & Büning, 2017). Purification process that cannot substantially remove this product-related impurity can result in exacerbated immune responses (Manno et al., 2006; Mingozzi et al., 2007) and reduced transduction efficiency (Parker et al., 2003). Additionally, process-related impurities include residual host cell proteins/nucleic acids, residual plasmid DNAs, and residual cell culture medium components/supplements. Substantial efforts have been aimed to reducing these impurities, detailed in the later section. These examples reflect current challenges in rAAV biomanufacturing: high manufacturing costs associated with poor product yields from host cells, high levels of impurities materialized during production, and issues with scale-up of the current bioprocess. Proper capsid design and capsid engineering can effectively improve the rAAV transduction efficiency, thus lowering the dose required for disease treatment (Büning & Srivastava, 2019; Li & Samulski, 2020). More effective use of valuable viral vectors further reduces the market price. These challenges above seem to be the major hurdles limiting the accessibility for the patients. In the following sections, we present the current methods used for the development and manufacturing of rAAV and the critical bottlenecks they face, along with promising technologies which will continue to improve the yield of rAAV over the coming years.

2 | Current rAAV PRODUCTION METHODS AND LIMITATIONS

2.1 | Transient transfection limitations and bioprocess scale-up challenges

Transient transfection is the most utilized method to generate rAAV due to the relatively high productivity of the cells, along with the high infectivity of the harvested rAAV. In addition, transient transfection allows the production of different serotypes of rAAV easily by replacing related plasmids. For these reasons, most gene therapy

products used for clinical trials today have been based on this plasmid transfection approach (Clément & Grieger, 2016). During a typical rAAV-yielding transient transfection procedure, three plasmids (GOI plasmid, RepCap plasmid, and helper plasmid) are cotransfected into mammalian cells (primarily HEK 293), which subsequently produce rAAV in a limited time without integrating viral DNA into the host cell genome (Samulski & Muzyczka, 2014). Current manufacturing approaches and their resulting yields have been reviewed (Clément & Grieger, 2016; Clément, 2019; Robert et al., 2017). Briefly, the current transient transfection methods used to manufacture rAAV-based gene therapy products achieve rAAV titers between 1×10^{13} and 3×10^{14} vg/L in the crude harvests in suspension cell culture (Bingnan Gu et al., 2018; Grieger et al., 2016; Zhao et al., 2020a) and comparable results in adherent culture (Zhao et al., 2020a). However, the adherent cell-based transfection process has its disadvantages, especially in its scalability issue (Srivastava et al., 2021) due to limited vessel surface area, extra procedures (e.g. trypsin treatment) and required operator manipulation (Lock et al., 2010). Here, we focus on the suspension cell-based transfection process and its scale-up challenges.

2.1.1 | Transient transfection issue

There are several hurdles in the transient transfection process that include 1) cytotoxicity in post-transfected HEK293 cells due to either the transfection reagents, such as lipofectamine and polyethyleneimine (PEI), or virus production itself, 2) many different transfection parameters/procedures resulting in variable transfection efficiencies when multiple plasmids are used, 3) issues in reproducibility when scaling up processes for manufacturing purposes, and 4) inherent limitations in rAAV production. The transient transfection is a very complex process which involves temporary introduction of plasmids into the host cell. The foreign plasmids form a complex with transfection reagent, then bind to the host cell membrane, and the complex enters the cells via endocytosis and translocates into the nucleus for the transgene expression (Gutiérrez-Granados, Cervera, et al., 2018). Except for the traditional cell culture process parameters (e.g., temperature, pH, and stir speed), many other transfection process parameters, such as transfection reagent to DNA ratio, plasmid amounts, different plasmid ratios, transfection reagent and DNA complex media volume, incubation time, and cell density at transient transfection, may also affect the transfection process (Gutiérrez-Granados, Cervera, et al., 2018). Previous studies have found that many factors, including transfection reagent to DNA ratio, incubation time, and transfection reagent and DNA complex media volume, can impact the transfection efficiency and cell health posttransfection (Bingnan Gu et al., 2018; Grieger et al., 2016). Optimal cell culture conditions for transient transfection can also depend on the cell line, media formulation, AAV serotype being produced, or other process variables (Grieger et al., 2016; Zhao et al., 2020a). The respective ratio of plasmids (GOI: rep/cap: helper) during transfection can also impact the production of rAAV and must therefore be optimized and regulated (Grieger et al., 2016; Zhao et al., 2020a). Moreover, a high AAV yield is not always guaranteed with high transfection efficiency when using multiple plasmids for the transfection process (Dash et al., 2022). This implies that it is also challenging for multiple plasmids to enter into the nucleus, not only the cytoplasm, for gene expression (Bai et al., 2017). The insufficient yield and recovery of plasmids from bacteria is another challenge, as it fails to meet the high demand for plasmid consumption in rAAV production (Ohlson, 2020).

In addition, root causes for limited rAAV production in mammalian cells might lead to its overall low titer during vector production process. rAAV genome replication could be one limitation, and understanding the AAV DNA replication mechanism could gain insight into increasing rAAV yield (Ning et al., 2023). AAV is one type of virus that its genome replication and genome encapsidation are highly reliant on the host cells (Ning et al., 2023; Schwartz et al., 2009). To complete AAV genome replication, the AAV single-stranded DNA genome will replicate to double-strand DNA under the regulation of the host cell DNA replication system and helper genes (Meier et al., 2020). However, very limited knowledge of AAV genome replication has been gained so far, especially from the perspective of host cell and its interaction with AAV genome. Furthermore, rAAV genome encapsidation could be another bottleneck. One study indicated that only 20%–30% of the replicated transgenes were packaged into capsids (Joshi et al., 2019). Such low encapsidation ratio will be further discussed in the "Complications in Capsid Biosynthesis and Recovery" chapter. Additionally, several publications reported that the host cellular metabolism would be reprogrammed during viral production and viral infection (Prusinkiewicz & Mymryk, 2019; Thaker et al., 2019). However, no research has been conducted on host cell metabolism reprogramming during rAAV production, so limited insights into essential metabolites for rAAV genome replication and capsid synthesis are available. Thus, identifying metabolic pathways involved in viral production and waste/inhibitory metabolites inhibiting the production can be promising directions for future research to enhance productivity and create the cell environment beneficial for viral vector production.

All of these limiting factors contribute to the poor rAAV production yield, and therefore, researchers have been seeking advanced/alternative methods to produce rAAV in a more effective and scalable manner.

2.1.2 | Scale-up issue

Maintaining the consistent productivity and product quality is challenging during rAAV manufacturing, especially when scaling up from bench to production scale. A challenge comes from the complexity and variability of the bioprocess. Besides the procedure of thawing and expanding the packaging cell line in different sizes of bioreactors for seed train, the precise mixing and incubation of transfection reagent and plasmid DNA require an additional step. At a bench scale, the mixing efficiency is well-controlled via vortexing or

shaking. However, it is not easy to achieve comparable mixing at large scales, thus resulting in large discrepancy for polyplex sizes and overall transfection efficiencies. In a recent study conducted by Gu et al., which compared the rAAV production in a 50 L scale stirred tank reactor (STR) with that in shake flasks via triple plasmid transfection in HEK293 cells, they experienced some productivity loss during the scale-up process, because the transfection mix prepared in WAVE bags for use in STRs was not as efficient as the transfection mix vortexed in centrifuge tubes for use in shake flasks (Bingnan Gu et al., 2018). In addition, several raw materials involved in the transient transfection process might contribute to the variability of productivity and the inconsistency of product quality seen at larger scales (Schwartz et al., 2020). This includes different types of plasmids, transfection reagents, basal/feed medium, and packaging cells (Zhao et al., 2020b). The variability in complex raw materials involved in the process can result in lot-to-lot variability of the rAAV vector and needs to be better controlled.

In summary, due to these challenges encountered at a large scale, transient transfection seems to be more suitable as a provisional production method to evaluate potential gene therapeutic drugs. To meet the current and future demands of gene therapy products, new, highly efficient, scalable, and robust methods need to be established.

2.2 | Obstacles in stable producer cell line development

At first glance, the development of stable producer cell lines through direct infection of cells with the recombinant virus (like Adenovirus, HSV, or Baculovirus) seems to be a more promising approach for the industrializing rAAV production as they present a simplified process procedure compared to transient transfection. Adenovirus and HSV are used as helper viruses to infect mammalian cell lines, while Baculovirus is used to infect insect cell lines. Stable producer cell lines contain the GOI and other genes required for rAAV vector production stably integrated within the cell genome. Normally, stable producer cell lines must be infected with one helper adenovirus containing an essential adenoviral helper gene to start producing rAAV vectors. In early studies, HeLa cells (Jenny et al., 2005; Martin et al., 2013; Thorne et al., 2009) and A549 cells (Farson et al., 2004; Gao et al., 2002) have been chosen as hosts for newly developed producer cell lines. The reported productivity of HeLa-derived producer cell lines ranged from 5×10^4 to 2×10^5 vg/cell (Martin et al., 2013; Thorne et al., 2009). However, the use of both HeLa cells and A549 cells as producer cell lines endure safety issues regarding the proliferation of wild-type helper adenoviruses used to induce rAAV (Farson et al., 2004; Gao et al., 2002; Martin et al., 2013). While the majority of wild-type adenovirus could be removed through purification steps, even small amounts of adenovirus present could ultimately yield undesirable downstream issues related to the vector safety (Monahan et al., 1998). Choosing HEK293 cells and HEK293T cells as a host cell line avoids the use of wild-type adenovirus due to the endogenous expression of Adenovirus-E1A, and as a result, E1-deleted adenovirus itself is used as a helper virus. This highly improves the product safety potential compared to the use of wild-type adenovirus. However, one primary challenge when using HEK293 cell lines as a host cell line is the E1A-mediated activation of AAV promoters p5 and p19 (Chang et al., 1989). The p5 and p19 promoters control the expression of AAV replication proteins (p5 promotes Rep 78/68, p19 promotes Rep 52/40), which are cytostatic and cytotoxic to the host cells when the level of these proteins are high (Yang et al., 1994).

As mentioned previously, Herpes Simplex Viruses (HSVs) can also provide helper virus function (Meier et al., 2020). Normally, direct infection of mammalian cells (HEK293 cells or BHK cells) with two recombinant HSVs is the method applied for rAAV production. Specifically, one HSV contains the *rep/cap* gene, and the other HSV contains the transgene flanked by ITR. So far, some success has been achieved using HSVs to induce rAAV production (Conway et al., 1999; Thomas et al., 2009). It is worthwhile to note that Adamson et al. achieved 3.5–4.7× 10^{14} vg/L yield of AAV in crude harvests after optimizing the variables of production when using EXPI293F cell as a host cell line (Adamson-Small, Potter, Byrne, Clément 2017). However, a downside of using recombinant HSV viruses to produce AAV in mammalian cells is that they present a potential risk of safety issues (Clément, 2019). Although the pathogenic features of HSV have been removed in recombinant HSV (Clément et al., 2009), the undesirable byproduct like replication-competent HSV (rcHSV) could be generated due to recombination of the partial ICP27 gene in rHSV and complementing cells, which might lead to propagation of rcHSV during rAAV production (Ye et al., 2014). In addition, the establishment of GMP-compliant recombinant viral banks is also another great challenge (Clément, 2019).

Infection of insect cells (sf9 cells) with recombinant Baculoviruses (rBV) is another potential alternative approach to produce rAAV. Though BVs mainly infect insect cells and other arthropod cells, rBVs were reported as the vector for protein expression by transducing the mammalian cell (Gupta et al., 2019; Kost et al., 2005), provoking safety concerns related to the exogenous gene. Briefly, based on the original three recombinant BVs proposed by Urabe et al. (2002), many key limitations have been addressed, such as the instability of the rBV (Hacker et al., 2008; Kohlbrenner et al., 2005; Negrete et al., 2007; Smith et al., 2009) and low expression of VP1 which results in partial or complete loss of rAAV infectivity (Chen, 2008; Mietzsch et al., 2014; Urabe et al., 2006). Furthermore, the complicated procedures of each rBV system have been improved with the report of the TwoBac (Smith et al., 2009) and OneBac (Mietzsch et al., 2014; Mietzsch et al., 2015) platforms with the use of an sf9-based packaging/stable cell line. The OneBac platform, for instance, could achieve productivities ranging from 2.5 to 3.5×10^{14} vg/L (Joshi et al., 2019) or >10^5 vg/cell (Wu et al., 2019). So far, the BV-mediated platforms have the potential to achieve comparable titer with the transient transfection platform, but both platforms need further improvements when it comes to meeting the demands of clinical trials and thereafter. Also, research papers showed full/empty AAV capsids ratio produced by insect cells (Joshi et al., 2019;

Joshi et al., 2021) is typically lower than that of mammalian cell production (Grieger et al., 2016; Rumachik et al., 2020) depending on the specific AAV serotype.

No matter which cell lines are chosen as above, the helper/infection virus process will be a potential risk for rAAV production, and the helper/infection virus needs to be eliminated in the downstream process to avoid safety issues related to the final product. In addition, during AAV encapsidation, the AAV capsid might encapsidate the host cell DNA, which will lead to the risk of genotoxicity or immunotoxicity if an oncogene sequence, such as Hela's HPV sequence, HEK293's E1 sequence (Wright, 2014, 2020), HEK293T's SV40 sequence (Ahuja et al., 2005; Carbonell et al., 2019), or nonhuman origin sequence (e.g. BHK cells) exists. However, the mechanism of the encapsidation of non-AAV genomic DNA within AAV particles is yet to be determined. For example, as a previous publication reported, the packaging step is mediated by Rep52/42 proteins, and Rep proteins will bind the ITR Rep binding site and insert the ssDNA genome into a preassembled capsid. If there is a homologous/similar ITR Rep binding sequence in the host cell genome and take this incorrection binding or if there is any other host cell machinery involved in regulation in this process is largely not understood.

Developing a helper/infection virus-free stable producer cell line would be a more promising approach for rAAV production. Helper/infection virus-free stable producer cell lines not only contain the GOI, *Rep* and *Cap* genes, but also include helper genes stably integrated within the cell genome. However, the main hurdle to establishing the helper-virus-free stable producer cell line is to overcome the cellular toxicity from the expression of required vector components (*Rep* gene and helper gene). To date, Rep78 protein was found to activate the caspase-3 and induce cell apoptosis (Schmidt et al., 2000). Also, the Rep78 protein would decrease Cdc25A activity and block the cell cycle in the S phase (Berthet et al., 2005). However, the mechanism of action (MOA) of cytostatic and cytotoxicity caused by Rep protein is yet unclear. Schmidt et al. proposed that the constitutively active ATPase activity of Rep protein leads to the depletion of the cellular ATP pool, thereby triggering apoptosis (Schmidt et al., 2000), although the hypothesis is not confirmed. Moreover, the mechanism of cytotoxicity of helper viral protein was also unclear (Qiao, Li, et al., 2002). E4orf4 was the major E4 production that is responsible for the induction of p53-independent apoptosis while E4orf6 may play a role in this process (Marcellus et al., 1998). E1B55K, one source of helper gene toxicity, inhibited host mRNA export mediated by E4orf6/E1B55K heterodimer (Matsushita et al., 2004). E2A constitutive expression 293 cell line was documented, confirming the tolerance of E2A in host cells (Zhou et al., 1996). Also, host DNA Damage Response (DDR) during viral DNA replication in the host cell might be part of the reason causing cytotoxicity (Ning et al., 2023; Weitzman & Fradet-Turcotte, 2018).

All in all, packaging/stable producer cell line platforms have been in development for more than 10 years. Though they seem to be a more promising alternative choice for rAAV production compared with plasmid transient transfection platforms, many challenges still need to be overcome. Helper/infection virus-related safety issues, low productivity, cytotoxicity from necessary vector components, genotoxicity and immunotoxicity caused by capsid mispackaging and high ratio of empty/partial AAV capsids impurities are the most pressing issues to date. An ideal producer cell line would eliminate the need (and cost) for transfection or viral infection but also be highly efficient at vector production and stable enough for large-scale cell expansion.

2.3 | Complications in capsid biosynthesis and recovery

Often, rAAV yield is negatively impacted during capsid biosynthesis when viral genes are improperly packaged or simply left out. Empty or partially full capsids produced during rAAV production are difficult to reduce and isolate, making them one of the major challenges to overcome when attempting to improve product yield. The presence of empty/partial capsids could reduce transduction efficiency by competing with the fully packaged vectors for receptor uptake of the target cell (Gao et al., 2014), and induce particle aggregation (Qu et al., 2007). Furthermore, empty/partial capsids could serve as potential antigenic material and may activate an undesired innate and adaptive immune responses (Hösel et al., 2012; Mingozzi et al., 2007), thereby posing a threat to the safety and efficacy of products. Some state that capsid impurities behave as decoys for therapeutic AAV to escape from AAV clearance and thereby improve gene transfer efficiency (Flotte, 2017; C. Wang, Mulagapati, et al., 2019; F. Wright, 2014); however, regardless of the magnitude of impact on efficacy and safety, it is essential to fully characterize capsid contents in the final gene therapy product. So far, few studies have reported effective ways to reduce empty/partial capsids levels upstream of manufacturing bioprocessing. Empty/partial capsid formation is mainly caused by improper biosynthesis and assembly of AAV (Sommer et al., 2003), which is further attributed to many factors, such as size and sequence of the vector genome, integrity of the ITRs, cell culture system, and the efficiency of transfection to supply different plasmids (Qu et al., 2007; Schnödt & Büning, 2017; Sommer et al., 2003). A recent mechanistic study for transient transfection production of AAV in HEK293 cells demonstrated that the mismatch timeline of capsid synthesis and viral DNA replication could result in a low ratio of full to empty capsids at harvest (Nguyen et al., 2021). In addition, repressive functions of the Rep protein could hinder capsid further at a later phase (Nguyen et al., 2021). However, further research is needed to facilitate the elucidation of mechanisms and the exploration of remedies.

Since there are structural and qualitative similarities between empty/partial capsids and full capsids, the purification or removal of empty/partial capsids from the crude harvest is also an issue that causes product loss during manufacturing bioprocessing. The current purification approaches to removing the empty/partial capsids are density gradient ultracentrifugation and ion-exchange chromatography. Both cesium chloride (Ayuso et al., 2010) or iodixanol (Strobel,

Miller, et al., 2015) based density gradient ultracentrifugation methods separate capsid byproducts using density difference between different capsids, while iodixanol is more common due to its biocompatibility. However, they are more effective at bench scale, and the technique needs to scale-out instead of scale-up if more products need to be produced (Singh & Heldt, 2022). Ayuso et al. (2010) and Gao et al. (2014) used optimized density gradient ultracentrifugation to reduce the proportion of empty capsids to below 1%. Although high-level purity can be achieved from two or three rounds or coupling with other techniques like diafiltration, the drawbacks of this method are low scalability (Robert et al., 2017; Schnödt & Büning, 2017), the physiochemical effect on the particles, and high time-consuming process (Kramberger et al., 2015; Naso et al., 2017; Qu et al., 2015). The industry has since started the transition into chromatography and membrane-based separation techniques (Moleirinho et al., 2020). Ion exchange chromatography (IEC)-based methods using the differential isoelectric points between empty capsids and full capsids is more common in the industry, with reports of AAV serotypes 1, 2, 4, 5, and 8 showing decreased empty-to-full capsid ratios to 10% and below, using this method (Aebischer et al., 2022; Joshi et al., 2021; Lock et al., 2012; Singh & Heldt, 2022; Tomono et al., 2016). However, due to the very close isoelectric points between empty/partial capsids and full capsids and the limited resolution of chromatography, the ion exchange chromatography used as a purification step will also cause the partial loss of target products. Moreover, some chromatography and resins vary in their specificity and binding capacity depending on the AAV serotypes (Qu et al., 2015). However, this could be counterpoised by the remarkable assets of IEX, including scalability, reproducibility, and compatibility for automation. New columns with different surface chemistry improving separation and resolution are available to meet the new demands (Dickerson et al., 2021); however, further validations on consistency and optimum conditions are required. The challenges associated with the current AAV-mediated viral vector production processes are plentiful and will be difficult to overcome. We believe the current drawbacks to rAAV production can be divided into four themes—transient transfection process, scalability issues, stable cell line development, and biosynthesis and recovery limitations—each having its list of major challenges to be addressed by research labs (Figure 2). Luckily, there have been incredible recent strides in the gene therapy biomanufacturing field, and beyond that will assist in creating the next generation of AAV production platforms with improved production yields. These advances will be discussed in the next section.

3 | ADVANCES IN GENE THERAPY BIOMANUFACTURING

As previously mentioned, different rAAV production platforms pose different challenges, but no matter which platforms are used the rAAV productivity is relatively low compared to the clinical demands. Traditionally, bioprocess improvements are made based on genetic engineering techniques, clonal selection, and bioprocess development (e.g., media formulation and process parameters) (Hacker et al., 2008; Rego et al., 2018; Yu et al., 2021). Such efforts are often labor-intensive and time-consuming because they follow an empirical optimization strategy (Jayapal et al., 2007). Though the design of the experiment (DoE) approach has indeed obtained process improvements for some specific cell lines (Zhao et al., 2020a),

	Critical Challenges in current rAAV manufacturing process	Advanced technology to address the challenges
Transient Transfection Process	Complex Transfection Process Variables	Design of Experiment
	Cytotoxicity caused by transfection reagent	Other promising transfection reagents
	Transfection efficiency of 3 plasmids entering each cell and translocating to the nucleus	Other promising transfection reagents, DTSs and cofactors involved in DNA nuclear transport
	Root cause for productivity limitations: AAV genome replication	Multi-omics
	Root cause for productivity limitations: host cell metabolism and AAV biosynthesis	Multi-omics, In silico model
Scalability Issues (Transfection)	Complexity and Variability of Bioprocess	Continuous culture mode, Multi-omics
	Complex Raw Materials	Multivariate Analysis
Stable Cell Line Development	Helper/Infection Virus Contamination	Develop Helper/infection Free Stable Cell Line
	Genotoxicity and immunotoxicity caused by mispackaging in AAV capsids	Multi-omics
	Cytotoxicity from AAV production required vector components	Multi-omics, Inducible system
Full Capsid Biosynthesis & Recovery	Empty & Partial Capsids biosynthesis	Multi-omics, In silico model
	Product Loss and Impurities During Recovery	MCSGP

FIGURE 2 Summary of challenges faced and advanced technology for improvement in rAAV production. rAAV, recombinant adeno-associated virus.

knowledge of why these changes occur at the molecular or cell biology level is still lacking. In addition, environmental stresses that are placed upon cells during bioprocess improvements, such as selection pressure, changes in growth and production conditions, and media adaptation, can result in various undesired modifications to a cell line, such as chromosomal rearrangements and other epigenetic changes, therefore resulting in phenotypic changes. Undoubtedly, the task of evaluating and comparing the genomic and epigenetic profiles across recombinant cell lines is complex, given that a variety of modifications are possible to occur under a given condition.

Therefore, to increase the rAAV productivity while reducing impurities, more advanced and scientific technologies should be considered and applied, especially the technologies successfully applied in other biologic fields. Figure 2 summarizes the critical challenges and the advanced technologies for improvement in rAAV production.

To address the challenges 1) with cytotoxicity caused by transfection reagents during the transient transfection process and 2) with three plasmids introduced to the cells and entering the nucleus for gene expression without remaining in the cytoplasm, many potential synthetic gene delivery materials have been comprehensively reviewed (Lostalé-Seijo & Montenegro, 2018). DNA nuclear targeting sequences (DTSs) and cofactors involved in DNA nuclear transport in the host cell are also worthwhile for further investigation for nuclear targeting of the plasmids (Bai et al., 2017). As for the issue with complex raw materials in scale-up/manufacturing bioprocess, the multivariate data analysis (Emerson et al., 2020) would help tackle this challenge.

3.1 | Multi-omics as a tool to address productivity and bioprocess scalability issues

Multi-omics technology as an advanced technology may address the hurdles above by applying a system-level approach to understand changes in host cellular physiology and, ultimately, achieve full control over the cell line and/or bioprocess. It refers to the collection and integrated analysis of four main areas, which are genomics, transcriptomics, proteomics, and metabolomics (Lewis et al., 2016). Relevant genes and biological pathways can be identified by comparing cell lines exhibiting different production features both quantitively and qualitatively. Novel biomarkers, such as differentially expressed genes, proteins, and metabolites, will provide insights into production bottlenecks. Gaining this insight would help explain the root cause behind improvements or regressions in cell culture performance and instruct rational modification to achieve the desired phenotypic attributes. Success in utilizing omics technology to unravel production bottlenecks in other therapeutic biological fields provides useful guidance for our rAAV study.

How multi-omics studies can be applied to address rAAV production bottlenecks is summarized in Figure 3. Multi-omics datasets can be used to characterize host cell physiology changes, understand the role of the host cell in rAAV genome replication, capsid assembly, and capsid encapsidation. Rational modifications, either media formulation or cell line engineering strategies, can be utilized to improve the rAAV production bioprocess. With integrated *multi-omics* analysis, the produced datasets can be used for various outcomes and applications, such as improving cellular rAAV production, constructing or improving existing AAV in-silico models, identifying impurities and determining how to reduce them through cell culture, and discovering ways to improve cell performance at higher production scales.

Genomics studies investigate the genetic characteristics of a specific organism by applying bioinformatics to a large set of genomic data. Several studies have employed "Omics analyses" to characterize cell lines (with a major focus on HEK293). To date, genomic information on the HEK293 cell and HeLa cell, including genome sequences, has been published (Adey et al., 2013; Landry et al., 2013; Lin et al., 2014). The genomic information of the HEK293 cell line and its derived cells is available online (http://hek293genome.org/v2/) (Lin et al., 2014). Combined with the reference human genome annotation published by Frankish et al. recently, these resources will greatly facilitate future multi-omics development of human-related cell lines (Frankish et al., 2019). The information on human genome annotation could be found on the website (https://www.gencodegenes.org) (Frankish et al., 2019).

Transcriptomics utilizes the analysis of gene expression to comprehend changes seen at the transcription level. Previous studies take advantage of microarray and mRNA sequencing (RNA-seq) analysis to identify the specific gene or gene cluster expression (Kang et al., 2014; Sha et al., 2018), thereby identifying changes in cellular pathways associated with disparate process conditions or clonal characteristics. The application of transcriptomics study in enhancing recombinant protein productivity was reviewed previously (Stolfa et al., 2018; Vishwanathan et al., 2014). Furthermore, A transcriptomics study conducted by Rodrigues et al. compared "parental to producer" of two human cell lines producing recombinant retrovirus, and revealed a comprehensive overview of gene expression regulation in producer cell lines (Rodrigues et al., 2013). Subsequent targeted media supplements experiment, based on the identification and regulation of recombinant retrovirus production-related pathways, led to a six-fold productivity enhancement (Rodrigues et al., 2013). Similarly, to address the rAAV productivity limitation, a transcriptomics study can be conducted. Recently, one RNA-seq study revealed that a robust antiviral and inflammatory response was triggered by transient transfection mediated rAAV production (Chung et al., 2023). In addition to comparing producer/non-producer states, different cell lines, clones from the same cell line but with varying productivities can be further investigated using transcriptomics to understand the cellular features for high productivity. Based upon transcriptomic results, media supplementation strategies (Scarrott et al., 2022) and cell engineering targets (Abaandou et al., 2021; Formas-Oliveira et al., 2020) can be proposed and then validated to achieve the desired productivity or quality attributes. According to Scarrott et al.'s (2022) research, adding small molecule chemical additives during production as a media supplementation strategy can

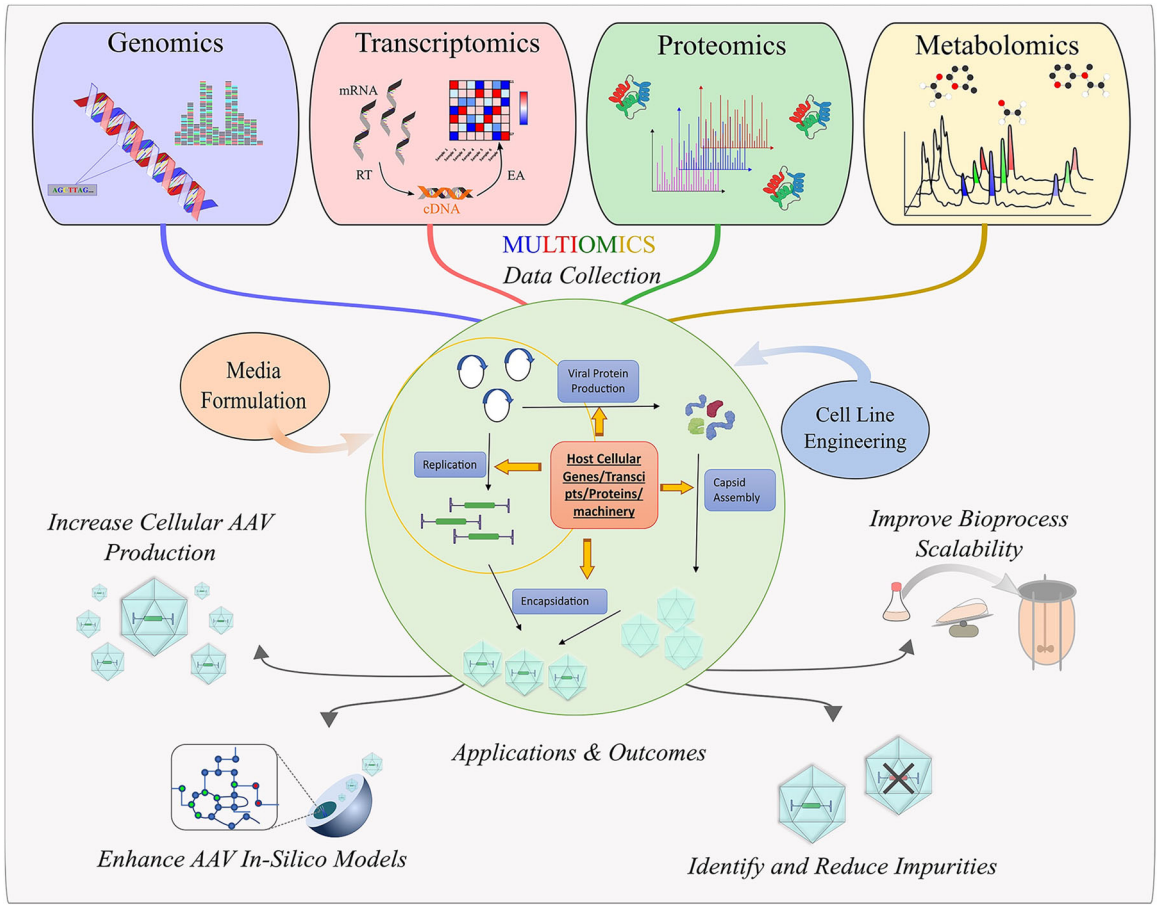

FIGURE 3 An example of how multi-omics datasets can be integrated and analyzed to study host cell physiology changes in adeno-associated virus (AAV) production, and then determine rational modifications (via either media formulation or cell line engineering strategies) which improve the AAV production bioprocess. With integrated *multi-omics* analysis, datasets produced can be used for various outcomes and applications, such as optimizing cellular AAV production, constructing or improving existing AAV in silico models, identifying impurities and how to reduce them through cell culture, and discovering ways to improve cell performance at higher production scales.

enhance rAAV productivity by up to three times in HEK293 cells. In addition, several published resources will be very helpful for the transcriptomics study, which includes human transcriptome databases (Futschik et al., 2018) and transcriptome data for HEK293 cells (Lin et al., 2014) and HeLa cells (Adey et al., 2013; Landry et al., 2013).

Proteomics uncover underlying cellular mechanisms involved in protein expressions throughout the proteome. Proteome has been widely utilized in studying monoclonal antibody (mAb) producing CHO cell lines (Strasser, Farrell, et al., 2021; Walsh, 2018), which currently has been engineered and optimized for higher productivity based on earlier proteome investigations. A study by Lavado et al. compared proteome profiles of transfected and non-transfected HEK293 cells producing Virus-Like Particles (VLP). It showed that decreased transfection efficiency was caused by the overall disruption of cellular homeostasis and downregulation of lipid biosynthesis after transfection (Lavado-García, Jorge, et al., 2020). To reduce the transfection effect, they overexpressed the endosomal sorting complex required for transporting accessory proteins like Nedd4L and CIT (Lavado-García et al., 2021), and achieved 3.3 and 2.9-fold enhancement of VLP production. A recent study (Strasser, Boi, et al., 2021) on host cell proteome of AAV5-producing HEK293 cells revealed significant regulated proteins involved in transfection and viral production process, such as proteins involved in endocytosis and lysosomal degradation, providing valuable insights into underlying cellular mechanisms. Furthermore, DNA packaging into the capsid is another bottleneck during rAAV production, and low amounts of viral particles harvested from cell cultures have gene of interest. Besides Rep52/40 proteins (King 2001) and D-sequence of ITR (Zhang et al., 2021), the host cellular proteins also play important roles in genome packaging, since the reconstitution of genome packaging in cell-free assay requires the addition of cell lysates (Zhou & Muzyczka, 1998). It was reported that wild-type AAV can achieve 95% full capsids (Zeltner et al., 2010), whereas only 5%–30% capsids in crude harvest contain therapeutic elements (Adamson-Small et al., 2016; Joshi et al., 2019). Considering the essential role of host cell factors in encapsidation process, a proteomics study can be applied to identify proteins involved in genome packaging by comparing wild-type AAV and rAAV production. Insights obtained from proteomic studies can be helpful to propose novel control

strategies to overcome productivity and packaging efficiency challenges in the rAAV production process. There are several published resources available that could promote the rAAV production-related proteomics study. The human proteome maps have been drafted over the last decade (Kim et al., 2014), including those based on mass-spectrometry data (Wilhelm et al., 2014). Later, a subcellular map of the human proteome (Thul et al., 2017) was also drafted, which greatly advanced the development and understanding of proteomic networks. Human proteome data could be found on these websites: http://www.humanproteomemap.org/ and https://www.uniprot.org.

Metabolomics allows us to characterize both intracellular and extracellular metabolic requirements more directly and optimize the cellular metabolism for desired cellular phenotypes. The metabolism optimization can mainly be achieved by two approaches: untargeted and targeted metabolomics. The untargeted method aims to identify and semi-quantitate all (measurable) metabolites during a specific period of cell culture. The targeted approach is more hypothesis-driven and mostly used to validate the untargeted outcome. Both untargeted and targeted metabolomics, especially when applied in conjunction, provide input data for in-silico metabolic computational models, such as Metabolic flux analysis (MFA) and Flux balance analysis (FBA) models. Interpretation of these bioinformatic models, along with pathway enrichment and metabolic network analysis, guides bioprocess modification. HEK293 is one of the dominant cell lines used for both recombinant protein production and viral vector production. These therapeutic biologics production induced changes on cellular physiology and metabolic states, thus a metabolism understanding on HEK293 can give a general guide on process optimization (Petiot et al., 2015; Rodrigues et al., 2014). A comprehensive review on HEK293 metabolism optimization can be found in the recent review paper (Petiot et al., 2015). However, production of different virus/protein types has specific metabolic demands and requires particular metabolism optimization. Several metabolism pathways were identified as recruited in the recombinant enveloped virus production, including amino acid and carbohydrate catabolism, lipid, nucleotide and glutathione metabolism, and polyamine biosynthesis (Rodrigues et al., 2013). Medium optimization targeting those major recruited pathways following the metabolomic study led to six-fold increase in productivity in HEK293 cells (Rodrigues et al., 2013). Lack of understanding on host cell metabolism and rAAV biosynthesis hinders production improvement, thus rAAV production-related metabolic biomarkers can be identified via conducting global untargeted metabolomics. Multivariate analysis can be used to identify the cell line-independent metabolites when comparing multiple cell lines. Then, rAAV production-related pathways can be obtained via pathway enrichment analysis and topology analysis. A similar research strategy was reported in other biological field (Monteiro et al., 2014). In addition, there are some useful databases available for metabolomics (as well as other integrative "omics") applications, such as Kyoto Encyclopedia of Genes and Genome (KEGG) (http://www.kegg.jp), Reactome (http://www.reactome.org) and the Human Metabolome Database (HMDB) (http://www.hmdb.ca). The function and explanation of these databases are described by Pereira et al. (2018).

The advancement of sequencing, NMR, and MS technologies allows researchers to gather more accurate and cost-effective high throughput omics data. Most studies have utilized single omics approach or at most two omics approaches. However, single "omics" analysis fails to obtain an overall picture of cell physiology in a complicated biological system. For example, transcript profiling provides gene expression changes between groups, whereas metabolite profiling gives a more direct manifestation of phenotype changes. Therefore, a comprehensive understanding on biological systems will require extending single omics further to multi-omics analysis for gene therapy research. Multi-omics studies have been conducted in other biologics research (Ang et al., 2019; Dumaual et al., 2013; Shin et al., 2018; Stolfa et al., 2018). Similarly, multi-omics can be applied to understand the challenges and bottlenecks for rAAV production, such as issues in low productivity, scale-up, and establishing stable producers.

The cytostatic and cytotoxicity of Rep and helper viral proteins are the critical stumbling blocks in establishing helper/infection virus-free stable producer cell line for rAAV production. However, the mechanism of cytotoxicity of Rep and Helper viral protein is still largely not clear. Multi-omics may be a useful tool to investigate the interactions between Rep/helper viral proteins and host cell proteins by overexpressing Rep/helper viral genes in host cells. The changes in host cell gene expression or metabolism reprogramming will offer valuable insights into the underlying cellular mechanisms of cytotoxicity.

Multi-omics approaches can also address the challenges in rAAV manufacturing scale-up. Many papers investigate scale-up issues and identify scale-up dependent biomarkers in biotherapeutic production in CHO cells via the multiomics technology (Gao et al., 2016; Vodopivec et al., 2019). No similar studies have been conducted on cell lines used for rAAV-manufacturing. Multi-omics will be a practical application to evaluate the significant gap in productivity and product quality outcomes across process scales. Furthermore, novel biomarkers discovered by multiomics studies can be used to monitor the scale up processes in rAAV production.

3.2 | In silico modeling to improve yield and reduce impurities

Genome-scale metabolic models (GEMs) is another powerful in silico tool that describes the mechanistic link between genotype and metabolic phenotype via gene–protein–reaction associations (Gu et al., 2019; Hefzi et al., 2016; Huang et al., 2017). The principle and application of genome-scale metabolic models in the biotherapeutic field were reviewed recently (Gu et al., 2019; Huang et al., 2017). It is also worthwhile noting that Hefzi et al. built a genome-scale metabolic model for iCHO1766 and cell-line-specific models for CHO-K1, CHO-S, and CHO-DG44 cells through reconstruction of the metabolic pathways in CHO cells (Hefzi et al., 2016). These models

have been used to analyze the metabolic effects of different bioprocess treatments on productivity. Studies revealed that, compared to those extensively used bioprocess treatments, such as temperature shift and sodium butyrate addition, targeted cell line engineering efforts showed more efficient redirection towards greatly improved productivity (Hefzi et al., 2016). The same type of model is currently being developed for the HEK293 cell by reconstruction of GEMs via integration of various types of data such as multi-omics and kinetic data (Kim et al., 2015), and hopefully can be used to address challenges faced in transfection efficiency, cell line development, and capsid biosynthesis during rAAV production. For example, Quek et al. (2014) developed a genome-scale metabolic model through reduced human genome-scale model Recon 2.0 from >7000 reactions to 357 reactions, and they used the reduced model to investigate the metabolism of HEK cells. Recon 3D is another well-curated and annotated human genome-scale model (Brunk et al., 2018). Together, these models will greatly advance bioprocess performance in gene therapy.

Another application of in silico modeling is to merge and interpret multi-omics datasets (Gu et al., 2019; Ryu et al., 2015). This method allows an interpretation of dynamic intracellular activities and the development of strategies to regulate them. Critical intracellular activities include those that impact not only viral vector titer but also its product quality attributes. For instance, a multi-omics in silico modeling approach would be beneficial to understanding and reducing empty and partial capsids biosynthesis during rAAV vector production. Multi-omics data allows the identification of the key components and optimization of rAAV production in specific cell lines. An in silico model can be generated from the omics data set to evaluate specific genes, metabolites, and pathways related to empty/partial attributes. More potential strategies, such as new media formulations and cell line engineering, can be proposed and aim to improve vector packaging efficiency and reduce empty and partial capsid ratios during AAV preparations. Furthermore, a mechanistic model for rAAV viral vector synthesized by triple plasmid transfection has been developed to uncover the underlying biological processes (Nguyen et al., 2021). Together with omics data, and in silico modeling, the production process bottleneck can be identified and investigated.

3.3 | Synthetic biology/inducible producer cell lines

According to the previous challenges in stable cell lines, developing a helper/infection virus-free stable producer cell line would be a more promising approach for rAAV production. To tackle the cytotoxicity issue induced by continuous expression of viral components, a genetic engineering-mediated inducible system is a very useful and promising technique. Inducible systems in mammalian cells were well-reviewed (Kallunki et al., 2019). Also, Inducible systems have been widely investigated in stable producer cell line development to produce lentivirus vectors (Ferreira et al., 2020). For rAAV vector production, several years of efforts have been focused on the development of inducible systems to regulate *Rep* and helper gene expression in hopes to gain control of cytotoxicity, achieve extended production period and thus increase productivity (Figure 4).

To lower the toxicity caused by Rep proteins, Hölscher et al. (1994) and Yang et al. (1994) utilized glucocorticoid-responsive inducible promoter and metal inducible promoter to regulate the expression of

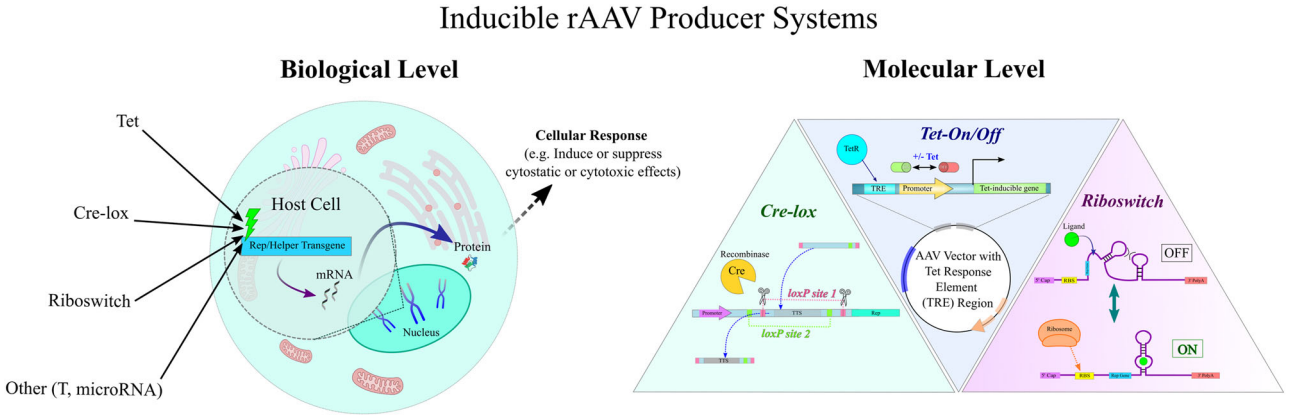

FIGURE 4 (a) Schematic of inducible systems' effect on cells at the biological level. Commonly used inducible systems (Tet, Cre-Lox, Riboswitch, and others) regulate and control expression of *Rep*, helper *genes* and transgenes in hopes to reduce cytotoxicity and/or increase the productivity. (b) Visualization of common inducible mechanisms at the molecular level to regulate cellular function during adeno-associated virus (AAV) production. For Cre-lox, Cre recombinases recognize and catalyze the 34-bp nucleotide sequence named loxP, and then excise or insert a gene sequence between the two loxP sites. For example, excision of a transcriptional termination sequence (TTS) would restore *Rep* expression without removal of the promoter. For Tet-On/Off systems, a Tet Response (TetR) region can be included in an AAV vector upstream of the promoter for the inducible gene of interest. Cells transformed with this vector will only express the gene of interest when Tet is added to the cell culture and TetR interacts with the vector and turns on gene function. In the riboswitch-regulatable expression system, bacteria-derived RNA aptamers combined with aptazymes to induce or knockdown gene expression. In this example, when a ligand is added to the system, the aptamer binds to it and turns on transgene expression by allowing a ribosome to bind to the riboswitch region.

Rep78/68 proteins respectively. However, it is difficult to regulate Rep 52/40 protein expression, since its promoter is embedded in the coding sequence of Rep 78/68. Several research studies described the use of the Cre-lox system to control Rep gene expression in HEK 293 packaging cell lines (Qiao, Wang, et al., 2002; Yuan et al., 2011). Qiao et al. incorporated the transcriptional termination sequence flanked by two loxP sites in the middle of the Rep gene to impede the expression of the Rep protein while maintaining the p5 and p19 promoter activity (Qiao, Wang, et al., 2002). After the infection of the E1-deleted Ad containing the Cre gene, the transcriptional termination sequence flanked by two loxP was excised by Cre recombinases, and the Rep gene was reactivated again (Qiao, Wang, et al., 2002).

To control "toxic" helper genes, metal inducible systems and Tetracycline inducible systems were reported in the development of adenoviral producer cells (Kovesdi & Hedley, 2010). An early effort to integrate all necessary components for rAAV production and use the Tet system to control the E1 gene failed to establish the stable cell lines (Qiao, Li, et al., 2002). Other than Tet inducible system, several other promising inducible systems have been reported and might be useful in developing rAAV stable producer cell line. Strobel et al. attenuated transgene cytotoxicity via a riboswitch-mediated system, in which guanine was used as a ligand, when producing rAAV in HEK293 cell lines (Strobel, Klauser, et al., 2015). Furthermore, microRNA (miRNA) was also reported to silence the cytotoxic transgenes during AAV production, which resulted in a 22-fold increase in the AAV vector yield (Reid et al., 2017). Inducible gene expression systems dependent on other cell culture environment factors have also been tested for the development of rAAV producer cell lines. For instance, Emmerling et al. performed a feasibility study where temperature-inducible switches were used to control gene expression of specific genes in the HeLa cells (Emmerling et al., 2013).

Recently, CEVEC Pharmaceuticals reported a helper virus-free producer cell line through stable, sequential transfection of Tet inducible Rep gene, Tet inducible Adenoviral helper genes, Tet inducible Cap gene, and GoI flanked by ITR using CEVEC's Amniocyte Production (CAP) cells as a proof of concept study (Hein et al., 2018; Swiech et al., 2012). Though the technical details related to cell line development are not disclosed, the result is very promising and demonstrates the feasibility of using inducible system/synthetic biology in helper/infection virus-free stable rAAV production. Based on this idea, using multiple inducible switches to regulate the expression of individual genes required for rAAV production is crucial because the demand for these components varies and needs to be controlled separately. However, the coordinated functioning of different inducible switches should be carefully considered. Excessive induction of viral gene expression may cause toxicity to host cells and decrease the vector's production capacity, while insufficient induction of gene expression may not be strong enough to initiate viral replication and production. Furthermore, it is also important to avoid the leakage of gene expression; even the most powerful switch may bring about leakage of gene expression. In this case, two or more switches can be used to prevent leakage of gene expression. Lee et al. proposed to use Destabilizing domains (DD) gene-inducible system and Tet inducible system, and obtained the lowest degree of leakage gene expression of Rep68 by regulating in both transcriptional and posttranslational level (Lee et al., 2022; Lee, 2021). GlaxoSmithKline (GSK) patented a technology combining Cre recombinase with the Tet system in developing AAV producer cell lines, though the Cre recombinase system was used to integrate the AAV production-related components into the host cell in a site-specific manner (Vink, 2020). More inducible switch combinations can be proposed. For example, Tet inducible system and Riboswitch can be used to tightly control toxic gene expression at the transcriptional and translational levels.

Overall, more precise and practical control of inducible switches to control the abundance of different AAV production components is necessary for developing rAAV stable producer cell lines and their regulation of the specific 'toxic' gene expression needs further investigation. Also, two or more different inducible switches can be explored to allow the tight control of the expression of 'toxic' genes like *rep* and helper genes.

3.4 | "Scale-free" continuous manufacturing

As scaling up the AAV bioprocess remains as a tedious hurdle to overcome, using a "scale-free" continuous cell culture environment may offer a solution. There are many obvious advantages to this type of continuous biomanufacturing. First, achieving high cell density is feasible in continuous cultures with the use of cell retention devices and fresh media continuously being added into the culture system. At the same time, harmful by-products or waste inhibitors that impede cell growth are consistently being removed, which should generate high volumetric productivity. Besides, products with unstable structures could be timely removed since the residential time of products in a bioreactor is shorter compared to the fed-batch culture. In continuous cultures, the rate of input and output is equal; therefore, the culture volume could be kept constant, reducing the cost and size of necessary equipment and lab space. In this case, the optimal scale of the production in continuous systems will be determined by the time of bioreactor running rather than by the size of the bioreactor.

Continuous manufacturing has been widely reported as a successful bioprocess for therapeutic protein production, especially in upstream bioproduction(Karst et al., 2017; Patil & Walther, 2017). In the viral vector production field, Gutiérrez-Granados et al. (2018) reviewed the production of viral particles using continuous cultures, and different continuous cultures have been used for the production of different vaccines and viral vectors, including perfusion cultures, two-stage continuous bioreactors, and so on. However, few papers using continuous cultures were reported in rAAV production. Based on the complexity and variability of the transient transfection process, extended gene expression (EGE), which was used widely in other biological fields, might be a promising approach to address the scalability issue in continuous rAAV production. Cervera et al. described using EGE to produce recombinant protein in HEK293 cells by repeated transfection of cell cultures with plasmid DNA and repeated medium exchanges. This method resulted in a 4–12 folds

yield increase in different types of products (Cervera et al., 2015). Fuenmayor et al. further enhanced this approach by combining EGE with continuous culture mode in a bioreactor to produce virus-like particles in HEK293 cells. The results revealed that the titer in the bioreactor was comparable to the shake flask. Still, the bioreactor could achieve a higher cell density, thereby confirming the practicality and potential scalability of this method (Fuenmayor et al., 2019). Lavado et al. further optimized the EGE process parameters by using DoE and achieved 86.7% titer improvement compared with the previously EGE method in HEK293 cells (Lavado-García, Cervera, et al., 2020). Recently, Ladd et al. proposed the proof of concept of continuous transfection for rAAV production in transient adherent HEK293T cell culture systems (Ladd et al., 2022). That is very promising to enhance the rAAV production capacity.

Continuous culture may also be applied and optimized on stable cell lines developed to produce rAAV. Many research papers were reported for other viral vector production in stable cell lines with continuous culture mode. Recently, Alvim et al. used continuous perfusion processes to produce virus-like particles after the generation of stably transfected HEK293 cells. This process improved both viable cell density and the productivity of virus-like particles (Alvim et al., 2019). Broussau et al. constructed a HEK293 stable cell line with the inducible tetracycline and cumate switches (Broussau et al., 2008), and Manceur et al. implemented perfusion strategy with this inducible cell line to produce lentiviral vectors (LV), where higher productivity and scalability were confirmed (Manceur et al., 2017). HeLaS3 cells were also reported to produce an oncolytic adenovirus vector in perfusion mode, and the perfusion strategy could reach a four-fold yield higher than fed-batch cultures (Yuk et al., 2004). As for rAAV production, in a recent study, CEVEC Pharmaceuticals employed a patented Tet-inducible stable producer cell line to produce rAAV using an alternating tangential flow filtration (ATF) perfusion system (Coronel et al., 2021). Compared to batch culture, the productivity in ATF-perfusion culture was improved by 40-fold and achieved 1×10^{15} vg/L, while the full capsid percentage was enhanced by 30%–40% (Coronel et al., 2021). That is a very inspiring and promising proof of concept to produce rAAV via applying continuous culture mode in stable cell lines, though many bottlenecks need to be overcome in the development of helper/infection virus-free stable producer cell line itself. Overall, optimized continuous "scale-free" culture systems could provide a more flexible and cost-saving solution to scaling challenges faced in both transient transfection-based and stable cell line rAAV production by reducing the size of the bioreactor and plant while achieving process intensification (Figure 5).

3.5 | Advanced purification methods to improve capsid recovery and quality

Downstream portions of gene therapy-related manufacturing still need improvements to address product yields, purity, cost and time

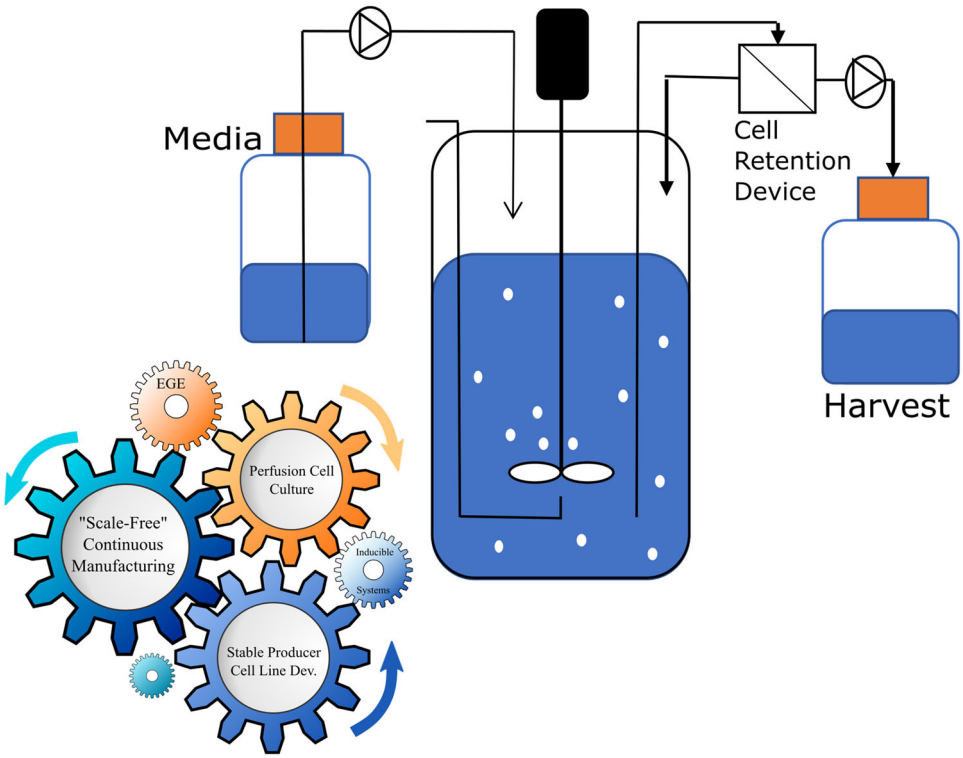

FIGURE 5 A schematic representation of "scale-free" continuous manufacturing. To function ideally, scale-free continuous manufacturing depends on the efficiency of perfusion cell culture methods and cell line development. Perfusion systems using extended gene expression (EGE) would be optimal for transient transfection-based adeno-associated virus (AAV) production. Inducible systems also serve as a catalyst in stable producer cell line development for scale-free systems.

constraints, scalability, and automatability. To overcome the challenge of yield loss caused by the resolution limitation of ion-exchange chromatography, continuous chromatography, which has been extensively used in monoclonal antibody field, could be an alternative solution (Angelo et al., 2018; Silva et al., 2015; Steinebach, Ulmer, Decker, et al., 2017; Steinebach, Ulmer, Wolf, et al., 2017) Multi-Column Counter-Current Solvent Gradient Purification (MCSGP) is one of the most recent continuous purification methods involving two columns instead of one to overcome the trade-off between yield and purity, which is a common concern in single-column batch chromatography. During extraction in batch chromatography, the pure product portion is collected while the overlapping portion, a mixture of product and product-related impurity, is discarded to reach certain product purity. However, the discarded fraction may contain a certain amount of the target product. MCSGP internally recycles the overlapping fraction and runs onto a second column, while continuously extracting the pure product (De Luca et al., 2020; Krättli et al., 2013). This ensures the minimum product loss without any accumulation of impurities and may provide benefits, especially in capsid recovery where the sample contains many product-related impurities including empty capsid. To address current challenges in effectively differentiating and separating partial capsids from empty and full capsids, IEX columns can be used in the MCSGP format. Capsids are particularly difficult to characterize due to very little differences among each capsid type. IEX columns would separate full, partial, and empty capsids based on their difference in isoelectric point (pI). Due to negatively charged DNA encapsidated inside the capsid, the full capsid surface is negatively charged compared to the empty capsid, which lacks any DNA, and the partial capsid, which contains a shorter DNA fragment. Due to the charge variation among AAV capsids, different capsid subpopulations are sequentially eluted from the IEX column under a shallow gradient elution. Under AEX (anion exchange chromatography) using the buffers with pH above the empty capsid's pI, the empty capsid will elute first, followed by partial and full capsids. The use of the CEX (cation exchange chromatography) column results in the opposite separation profiles. The countercurrent achieved by periodic inlet/outlet switch allows full utilization of column capacity as well. Figure 6 displays one example of how MCSGP can be applied using AEC followed by CEX to separate full AAV capsids from empty and partial capsids. In the future, the development of validated models would allow for theoretical validation, more efficient process optimization, and applications for an automated process (De Luca et al., 2020).

There has been a paradigm shift in downstream purification for gene therapy products from ultracentrifugation to chromatography, mostly due to scalability, reproducibility, and capability to adapt automated and in-line settings. Multiple detection monitors coupled with chromatography could further improve the sensitivity of AAV detection and the quality of final AAV products. An intrinsic fluorescence detector can be used over UV to provide more objective full vs empty capsid ratios (Gagnon et al., 2021). Full and empty capsid determination using UV detection is based on the comparison between UV absorbances at 280 nm and 260 nm. 260 nm is most strongly absorbed by DNA; however, the full capsid quantity is typically overestimated because both proteins and DNA contribute to the total UV signal at 280 nm. Equipment of fluorescence detector overcomes the issue by monitoring the native fluorescence of aromatic amino acids among capsids, such as tryptophan, tyrosine, and phenylalanine (Fu et al., 2019; Sommer et al., 2003). The signal is mostly generated by tryptophan abundant in VP1 (15 residues) and VP2 and VP3 (12 residues) (Gagnon et al., 2021). Light scattering is another area of emerging interest. This enables relatively easier

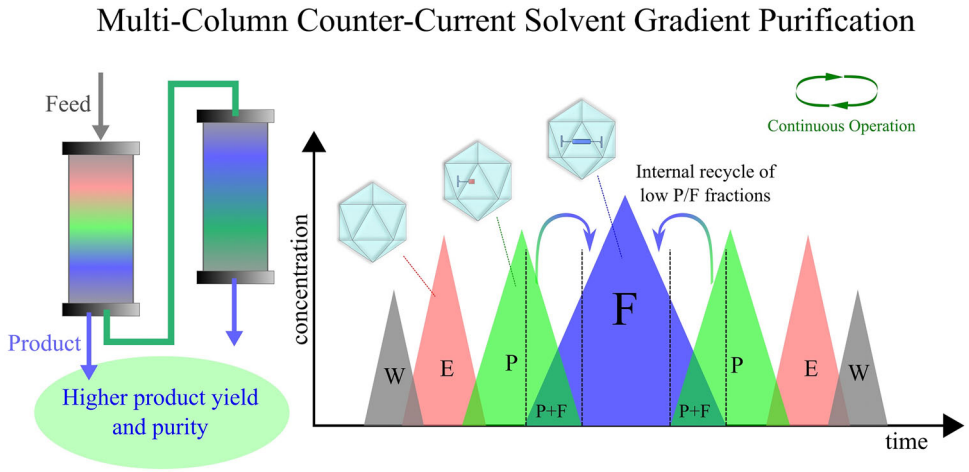

FIGURE 6 Example of a multi-column counter-current solvent gradient purification (MCSGP) process design as a potential method to separate and collect empty (E), partial (P), and full (F) capsids. This process involves cascade of counter current chromatography columns, ideally IEX columns to separate viral capsids based on pI. Under AEX (Anion Exchange Chromatography) using the buffers with pH above the empty capsid's pI, Empty capsid (E) will elute first followed by partial (P), and full (F) capsids. The use of CEX (cation exchange chromatography) column results in the opposite separation profiles. The countercurrent achieved by periodic inlet/outlet switch allows full utilization of column capacity as well. Addition of more columns leads to higher purity and yield, yet higher purification costs.

detection of the complex chromatogram, and when coupled with AEX, it is shown to provide a more reasonable representation of full capsid closer to that of the intrinsic fluorescent detector when compared with UV absorption. Single-angle light scattering is sufficient to provide the capsid ratio in crude and purified samples, while multi-angle scattering (MALS) provides more accurate information on the capsid characterization (Amartely et al., 2018; Amartely et al., 2019). Dynamic light scattering (DLS) monitor will be more suitable for quick assessment of large differences between full and empty capsids during different cell lines and transfection method development (Gagnon et al., 2021).

4 | CONCLUSION AND PERSPECTIVES

With the rise in clinical demand, AAV-mediated gene therapy manufacturing issues related to low AAV yield, high ratio of empty/partial capsids, low scalability, and stable cell line development need to be addressed urgently. A fundamental problem lies with the lack of knowledge in AAV biology over the past 50 years. We still lack sufficient knowledge in the AAV life cycle, such as replication, packaging, and its interaction with helper genes/proteins and host cells, although some related research are in progress (Maurer & Weitzman, 2020; Meier et al., 2020). This knowledge is essential and critical for the improvement of rAAV productivity and the reduction or elimination of empty/partial capsids. In other words, how to improve the yield and packaging efficiency of AAVs, and mechanisms related to replication and packaging need to be further investigated.

Muti-omics provides us with an "inside look" of a cell, systematically and comprehensively. By investigating the genome, transcriptome, proteome, and metabolome of host cells, researchers have been able to clarify the causes behind changes in cell line performance. Combined with another solid and useful tool *in silico* modeling (e.g., Genome-scale metabolic models), rational modifications via cell line engineering and process modification, including media formulation, can be instructed to attain the desired phenotypic attributes (high rate and efficiency of AAV replication and packaging), and thereby improve the rAAV productivity and lower or eliminate the empty/partial capsids. Future research will be committed to integrating multi-omics in different levels with reconstruction/constraint of in silico modeling to improve the accuracy of system analysis and provide rational modifications.

Development of helper/infection virus-free stable producer cell lines might be the next-generation platform to produce rAAV, where AAV *rep* and cap genes, as well as the required Adenoviral helper genes, are incorporated into the host cell line genome. The development of a helper/infection virus-free system would enable an efficient transition to the commercial manufacturing (Adamson-Small et al., 2017). With the help of genetically engineered inducible systems, control and regulation of the toxic proteins expressed by *Rep* and helper genes may be possible, which is one of the critical factors to consider when generating helper-virus-free producer cell lines. Future AAV vector studies focused on more precise control of inducible switches and regulation of the specific gene expression would be worthwhile. For instance, regulation of the cytostatic and cytotoxicity involving AAV Rep/helper proteins would be significant in improving production yields. Also, the interaction between Rep/helper proteins and host cell genome/proteins should be further investigated, so that rational modification can be carried out to attenuate/eliminate the cytotoxicity of those proteins. Multi-omics technology makes this possible, allowing researchers to gain insights and tackle this problem from a mechanistic angle.

"Scale-free" continuous manufacturing is a very useful technology for seed train and process intensification in therapeutic biologics. In gene therapy biomanufacturing settings, it will help tackle scalability issues, especially for the transfection/infection platforms. One feature and advantage of "scale-free" continuous manufacturing is process intensification. For further research, the cell density effect (high cell density leads to low transfection/infection efficiency and low productivity) might pose a challenge to this process. Multi-omics could be useful to investigate the cause behind the cell density effect and promote judicious modifications. In addition, multi-omics technology could be a powerful tool to analyze and study the changes in cell culture performance (i.e., viral titer and other critical quality attributes) in different scales of manufacturing. Advanced downstream purification technology (e.g., MCSGP) could promote more efficient capsid recovery by further reducing the contamination of empty/partial capsids while maintaining good quality full capsids.

Overall, the potential technologies discussed in this review paper will greatly enhance rAAV biomanufacturing with effective incorporation into current systems. These approaches would one day improve rAAV productivity in host cell lines, reduce or even eliminate certain rAAV product-related impurities and promote scalable and robust bioprocesses.

AUTHOR CONTRIBUTIONS

Qiang Fu, Ashli Polanco, and Yong Suk Lee conceived and wrote the review. Ashli Polanco designed figures. All authors critically revised and approved the final manuscript.

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DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available in the references provided. These data were derived from the references available in the public domain.

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