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Review

Synthetic Biology Design as a Paradigm Shift toward Manufacturing Affordable Adeno-Associated Virus Gene Therapies

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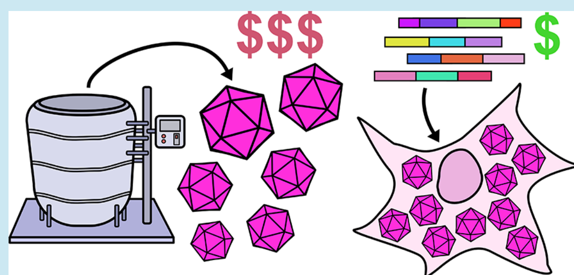
ABSTRACT: Gene therapy has demonstrated enormous potential for changing how we combat disease. By directly engineering the genetic composition of cells, it provides a broad range of options for improving human health. Adeno-associated viruses (AAVs) represent a leading gene therapy vector and are expected to address a wide range of conditions in the coming decade. Three AAV therapies have already been approved by the FDA to treat Leber's congenital amaurosis, spinal muscular atrophy, and hemophilia B. Yet these therapies cost around $850,000, $2,100,000, and $3,500,000, respectively. Such prices limit the broad applicability of AAV gene therapy and make it inaccessible to most patients. Much of this problem arises from the high manufacturing costs of AAVs. At the same time, the field of synthetic biology has grown rapidly and has displayed a special aptitude for addressing biomanufacturing problems. Here, we discuss emerging efforts to apply synthetic biology design to decrease the price of AAV production, and we propose that such efforts could play a major role in making gene therapy much more widely accessible.



KEYWORDS: *gene therapy, adeno-associated virus, biomanufacturing, nanotechnology, viral vectors*

INTRODUCTION

As an emerging technology, gene therapy has promised numerous exciting possibilities: curing genetic ailments,[1,2] dissolving tumors,[2,3] treating neurodegenerative diseases,[4,5] increasing healthy longevity,[6,7] and even helping better equip astronauts for long-term survival on Mars.[8,9] AAV vectors represent one of the most prominent delivery technologies for gene therapy due to their minimal immunogenicity, tissue-selective serotypes, amenability to capsid modifications, and clinical successes.[10−12] In recent years, the FDA has approved three AAV gene therapies: Luxturna for Leber's congenital amaurosis, Zolgensma for spinal muscular atrophy, and Hemgenix for hemophilia B.[13] Yet these so-called miracle treatments represent some of the most expensive therapies in the world, carrying price tags of around $850,000, $2,100,000, and $3,500,000 respectively.[14−16] The titanic prices stem in large part from challenges involved in manufacturing recombinant AAVs.[17,18] As such, new manufacturing innovations are desperately needed so that gene therapy can fulfill its potential. Synthetic biology, a rapidly growing field that takes a rational design approach to biology, has just begun to address the difficulties of AAV manufacturing. In this review, we discuss progress in applying synthetic biology design strategies to develop affordable AAV manufacturing pipelines, and we explore how such approaches may eventually grow to make gene therapy much more accessible to people across the world.

Established AAV Production Methods. AAV manufacturing can be broadly divided into upstream processing and downstream processing. Upstream processing typically involves design and preparation of plasmids, expansion of producer cells, transfection of the cells, and synthesis of AAVs within the cells.[18] Downstream processing typically involves AAV purification, quality control measures, and taking steps to prepare the final clinical formulation. Due to the complexity of this process and the complexity of the involved biology as well as the need for clinical-grade protocols, AAV manufacturing remains very costly.

At the small laboratory scale, a popular workflow for producing recombinant AAVs is triple transfection of mammalian cells followed by ultracentrifugation-based purification (Figure 1A). Exploring this small-scale pipeline will serve as a useful starting point which can be compared and contrasted with industrial-scale approaches. For the triple transfection method, three plasmids are transfected into the mammalian cells: a transfer plasmid, a Rep-Cap plasmid, and a helper plasmid.[19] The transfer plasmid includes the DNA that

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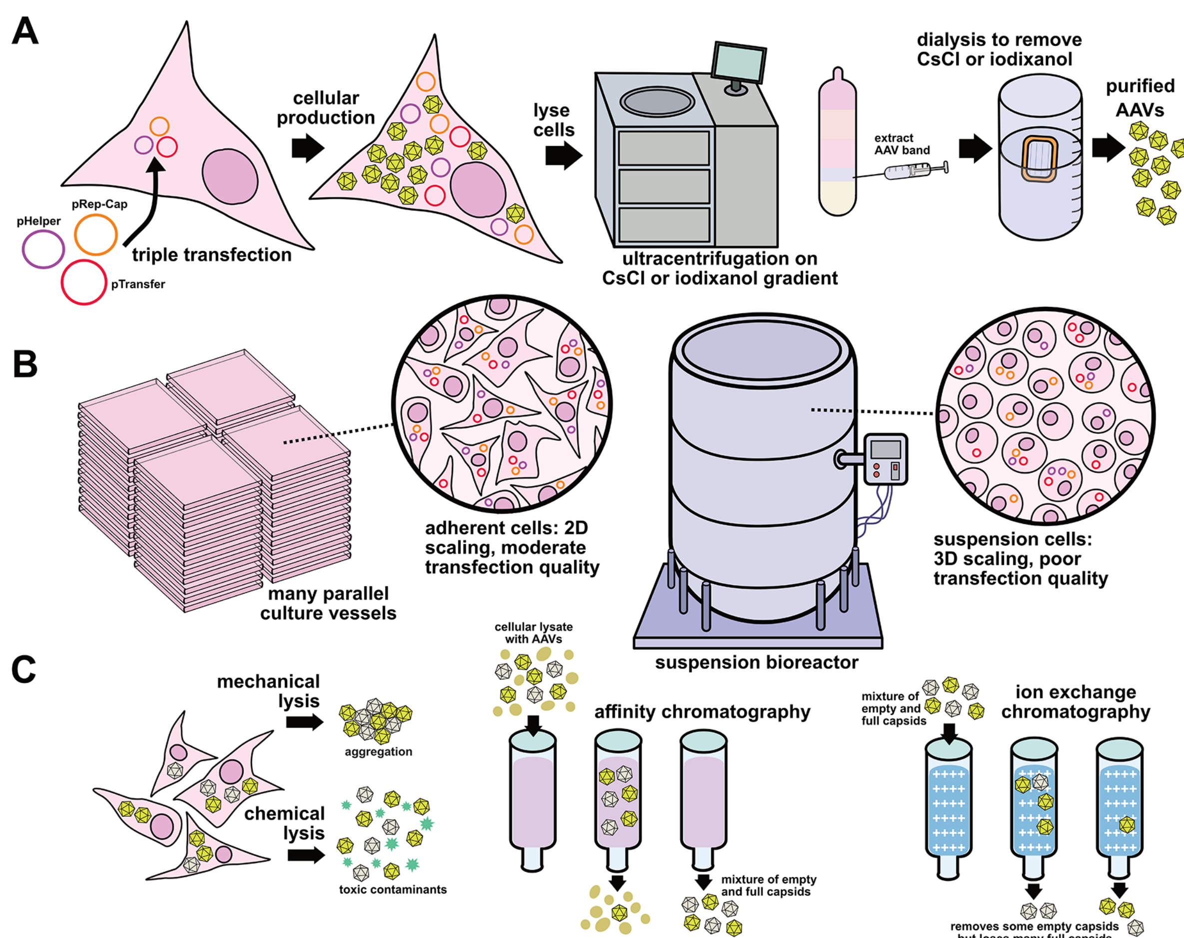

Figure 1. Challenges in AAV manufacturing. (A) Workflow of AAV production at the small-laboratory scale.[19] (B) Some of the difficulties in upstream processing for AAV production at the clinical scale. Adherent cells are not scalable due to their growth on surfaces.[18] By contrast, suspension cells grow in 3D and so represent a more scalable approach, but they are challenging to transfect properly and developing efficacious suspension cell lines is not a trivial task.[27] (C) Some of the difficulties in downstream processing for AAV production at the clinical scale. Mechanical cell lysis can cause losses via AAV aggregation while chemical cell lysis introduces toxic contaminants. Chromatography-based purification methods are much more scalable than ultracentrifugation, but these approaches come with certain drawbacks.[18,32,33] Affinity chromatography cannot separate empty capsids from full capsids. Ion-exchange chromatography can remove some of the empty capsids,[34,35] but also loses many of the full capsids due to poor specificity and the need for extreme pH conditions.[18]

will be packaged into the AAV capsids as well as flanking sequences that form inverted terminal repeats (ITRs). When the DNA is in its single-stranded form, these ITRs fold into secondary structures which help facilitate AAV replication and genomic packaging. The Rep-Cap plasmid encodes the replication proteins Rep78, Rep68, Rep52, Rep40 as well as the capsid proteins VP1, VP2, and VP3 and the accessory proteins MAAP and AAP.[19,20] The helper plasmid encodes the adenoviral genes E4, E2a, and VA. Two more adenoviral helper genes, E1a and E1b, are typically expressed from sites in the mammalian cell genome.[21] It should be noted that some investigators have combined the Rep-Cap and helper plasmids into a single construct to enable a two-plasmid transfection process, though this approach is less common.[22,23] A few days after transfection, recombinant AAVs can be released by lysing the mammalian cells and pelleting the cell debris. Next, the lysate is ultracentrifuged through a CsCl density gradient or an iodixanol discontinuous gradient.[24,25] The fraction containing purified AAVs can then be collected. To remove CsCl or iodixanol, a buffer exchange dialysis process is performed. In preclinical laboratories, these steps are usually sufficient to obtain the desired recombinant AAVs.

Large-scale clinical AAV production involves numerous difficulties in upstream processing that do not typically occur at the preclinical stage (Figure 1B). Because the AAV plasmids must first be produced at GMP grade in large *E. coli* fermentations, dealing with batch-to-batch variations in plasmid yield and purity represents a challenge.[18] Expansion of adherent mammalian cells also introduces variability since it can be difficult to ensure sufficient consistency in parameters such as pH and oxygen concentration across parallel culture vessels. Animal serum in the culture media can carry adventitious agents which must later be removed. The risk of contamination of the mammalian cell cultures is also a major issue when handling many vessels containing adherent cells. While special types of bioreactors such as those of the iCELLis family[26] can be used in place of hundreds of smaller adherent culture vessels, these systems still have limited overall AAV production capacity.[27] As a consequence of these factors, scaling up adherent cell culture remains challenging and expensive. Suspension-based cell culture represents a more scalable alternative[18] since volume dimensions increase much more rapidly than surface area. Yet it is difficult to develop cell lines that efficaciously synthesize AAVs in suspension, per

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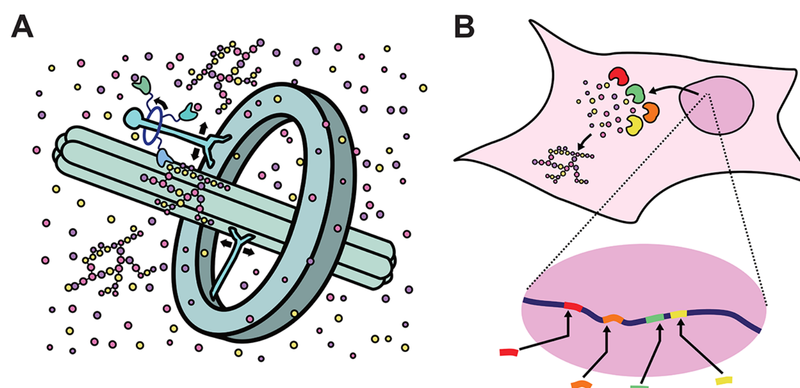

Figure 2. Comparison of nonbiological nanotechnology and synthetic biology as modalities for manufacturing of complex molecules. (A) Hypothetical rendition of a nanotechnology-based molecular assembler which might employ optically programmable "walking" molecules to move catalysts into desired positions and thereby link atoms together in a precise fashion. Though this kind of assembler would represent a broadly revolutionary machine for manufacturing, developing and scaling such a complex piece of technology could take several decades. Existing examples of molecular assemblers[53] are not nearly as broadly applicable as the hypothetical one shown here. (B) Many of the goals associated with nanotechnology can be more easily achieved using synthetic biology, especially when seeking biomolecular products. By reverse engineering the molecular machines already present in cells, it is possible to construct new biological systems which facilitate manufacturing objectives. Another major advantage of synthetic biology is that it employs self-replicating systems, which frequently eases scalable manufacturing of desired products. By contrast, making pieces of nonbiological nanotechnology (without self-replication) often requires challenging synthetic processes, particularly for complex nanodevices.

volume cell densities are typically lower in suspension cultures compared with adherent cultures, and triple transfection of suspended cells often results in the cells taking up suboptimal ratios of the different plasmids. The latter can increase the fraction of empty capsids (which lack DNA) or otherwise defective AAVs produced.[18,27] A potentially helpful strategy in this context involves developing cell lines that express the AAV replication and packaging proteins, though this is complicated by the toxicity of the AAV Rep proteins, leading to the need for gene regulatory strategies to prevent constitutive expression.[28] From a chemical perspective, the triple transfection step involves further difficulties at the clinical scale. Calcium phosphate transfection often results in batch-to-batch variation since its efficacy is sensitive to pH and to impurities.[29] Liposomal transfections show high efficiency and low cytotoxicity, yet they require much more expensive reagents. Polyethylenimine represents another commonly used transfection reagent, yet it is pH sensitive and cytotoxic.[18,30] There exists a clear need for improvements in upstream processing of clinical AAV manufacturing.

Large-scale clinical AAV production also involves challenges in downstream processing which do not typically happen at the preclinical stage (Figure 1C). Cell lysis is necessary to improve AAV yield because producer cells do not release enough of the virus particles. But mechanical lysis methods can trigger AAV aggregation[18] and chemical lysis using Triton X-100 and similar detergents has been shown to exert high toxicity in patients.[18,31] Triton X-100 was labeled as a substance of very high concern by the European Chemicals Agency in 2016 and increasing regulation has hampered its usage for AAV therapeutics. Filtration to remove cell debris after lysis also presents complications since filters often suffer from clogging and it is difficult to optimize filters in a scalable fashion for particular AAV serotypes.[18] For AAV purification, while CsCl or iodixanol ultracentrifugation are useful at the small laboratory scale, ultracentrifugation is difficult to scale up to the much larger volumes of clinical manufacturing.[18,32,33] Column chromatographic purification approaches are thought to represent a more scalable solution, yet many are limited because they cannot distinguish between full and empty capsids. The exception is that certain ion-exchange chromatography methods are capable of removing some of the empty capsids,[34,35] though they employ extreme pH conditions which can damage the AAVs and they often still sacrifice many of the full capsids.[18] Furthermore, chromatography workflows must be optimized depending on the properties of a given AAV serotype.[24] There is a distinct need for better ways of performing downstream processing for clinical AAV production.

Synthetic Biology Takes a Rational Design Approach. Synthetic biology is a discipline that makes use of design thinking to create new biological systems. Drawing inspiration from the block diagrams of electrical engineering, synthetic biologists conceptually disassemble biological systems into roughly modular parts (genes, regulatory sequences, promoters, protein domains, genetic circuits, riboswitches, etc.) and install these parts into cellular chassis such as *E. coli*, yeast, and mammalian cells.[36] The parts are then tested to see if they can operate in such orthogonal background contexts where they may achieve a reproducible functional outcome. Biological parts are frequently optimized through directed evolution and computational approaches.[36−39] In addition, synthetic biology often leverages contemporary tools like artificial DNA synthesis, Gibson assembly, CRISPR, computer-aided design software, machine learning, and laboratory automation. Finally, the field is characterized by an emphasis on rapid design-test-build-learn cycles. These strategies illustrate synthetic biology's approach to designing new biological systems.

During the past decade, synthetic biology has grown from a nascent field into a thriving industry.[40,41] It has introduced advances such as engineered T cells which seek out and destroy tumors,[41−44] genetically encoded light-inducible ion channels which control neuronal activity,[45,46] and designer bacteria which act as biological fertilizers to increase crop yields.[41,47] Biomanufacturing represents an area in which synthetic biology has demonstrated a particular level of aptitude.[41,48−50] As mentioned, a key strategy in synthetic biology is to transfer useful molecular machinery across species

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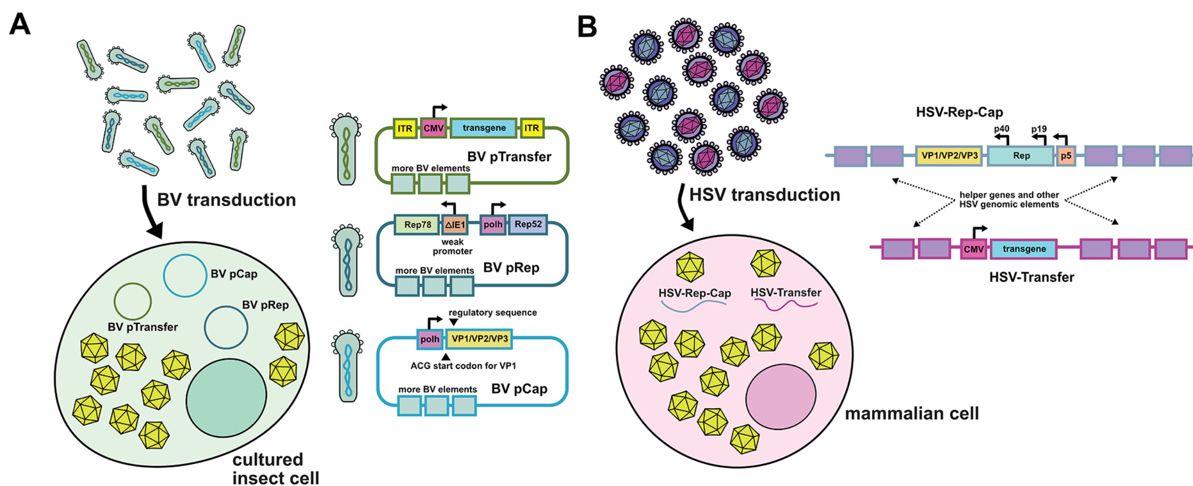

Figure 3. Alternative established methods for AAV production. These methods employ some synthetic biology principles but could stand to benefit from further design improvements to decrease costs. (A) Transduction of insect cells by engineered BVs (carrying AAV components) represents one alternative strategy for making AAVs. A representative BV system design that uses three vectors is displayed here,[63] but similar systems employing one or two vectors have also been developed.[74] The weak ΔIE-1 promoter lowers Rep78 expression level to achieve better yields and the ACG start codon for VP1 facilitates capsid protein production at a ratio closer to that found in AAVs made using mammalian cells (though the ratio is still not completely optimal). (B) Transduction of mammalian cells with engineered HSVs (carrying AAV components) is another alternative method which has been applied to AAV manufacturing. A representative HSV system design which utilizes two vectors is shown here,[72] but there is also a similar HSV approach involving just one vector.[75]

in order to accomplish reproducible outcomes within orthogonal host cells that have beneficial properties for the process in question. Numerous successful synthetic biology companies (e.g., Ginkgo Bioworks, Zymergen, Amyris, Impossible Foods, etc.) specialize in utilizing such strategies for the design of living organisms which synthesize products like dyes, fabrics, foods, materials for electronics, pharmaceuticals, and cosmetics in a sustainable fashion.[41,50,51] These commercial successes illustrate the power of synthetic biology as a manufacturing approach.

The strength of synthetic biology as a methodology for biomanufacturing arises from its ability to creatively organize systems which exert reproducible control over processes occurring at the nanoscale. Eric Drexler's foundational vision for nanotechnology centers on the idea of creating synthetic molecular machines which could assemble any desired structure atom by atom (Figure 2A).[52] Yet similarly powerful molecular machines already exist in the biological world, converting the problem of molecular manufacturing into an exercise in reverse engineering (Figure 2B). The design-oriented thinking of synthetic biology has arguably made significant progress toward fulfilling some of Drexler's proposals for nanotechnology. We suggest that synthetic biology design represents a promising strategy for assembling the complex macromolecular structures of AAVs.

Synthetic Biology Strategies in AAV Manufacturing. Though synthetic biology for AAV manufacturing is still in its early days, a handful of promising technologies in the area have started to emerge. Certain established methods that use some synthetic biology principles have existed for many years and may serve as the foundation for more systematic gains in production capacity. These include baculovirus (BV) expression vectors which synthesize AAVs inside of insect cells[54,55] as well as herpes simplex virus (HSV) platforms for transfecting mammalian cells and providing helper functions.[56] On the more contemporary side, newer synthetic biology technologies have shown promise for upending current paradigms of AAV production. Yeast-based AAV production systems are under development, though these are still limited in their production capacity and may require extensive further engineering to achieve their full potential.[57−59] Synthetic biology has recently been used to create a self-attenuating helper adenovirus system as a novel approach for AAV production.[60] To address challenges in AAV plasmid production, an *in vitro* technology utilizing enzymatic machinery has been developed for synthesis of large quantities of GMP-grade linear AAV DNA constructs called doggybone DNA (dbDNA).[61,62] These designs represent some examples of synthetic biology for AAV manufacturing and may help inspire and inform future research in the field.

Production via recombinant baculovirus (BV) represents an alternative AAV manufacturing strategy which employs synthetic biology by installing biological parts into an insect cell chassis and using engineered BV for delivery (Figure 3A).[54,55] This process works through infection of cultured *Spodoptera frugiperda* Sf9 cells or similar to a BV carrying the transgene and a BV carrying the replication genes and capsid genes.[63,64] Some investigators have instead put the replication genes and the capsid genes in two separate BVs. These BV and insect cell platforms support AAV replication, though the mechanism by which they facilitate the helper functions is poorly understood.[65] A key drawback of insect cell techniques is that they typically lead to altered capsid compositions (abnormal VP1, VP2, VP3 ratios) and therefore the resulting AAVs exhibit lower therapeutic potencies.[66−68] Since the Sf9 system uses the *polh* promoter instead of the p40 promoter of the wild type, it cannot employ the usual splicing regulation to generate appropriate VP1:VP2:VP3 ratios. As such, leaky ribosome scanning instead is utilized to adjust the capsid protein ratios. This involves the ribosome occasionally transitioning past the first start codon as a consequence of the specific DNA sequence near the translation initiation site.[69] Some benefits of insect cell systems include their capacity to circumvent transfection issues through the use of BV infection, their ability to support growth of insect cells in suspension at higher densities than mammalian cells, their usage of serum-

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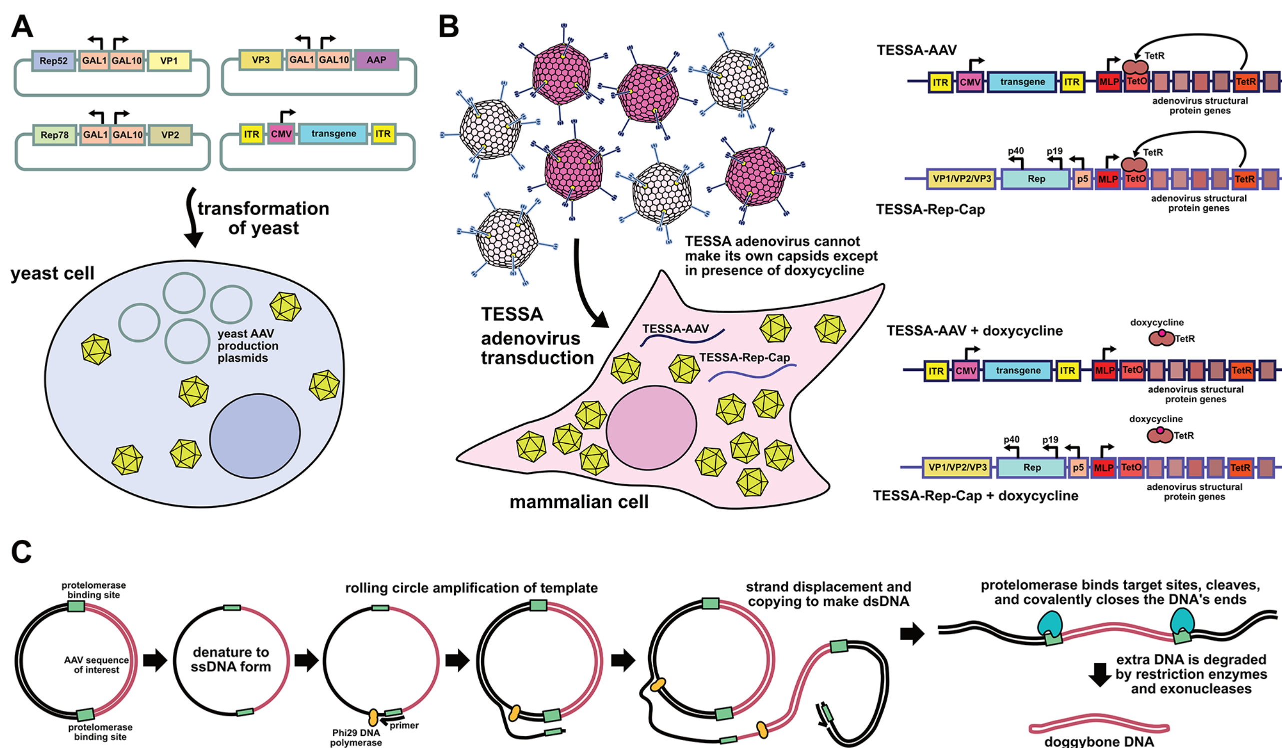

Figure 4. Emerging synthetic biology approaches for AAV production. (A) Yeast platforms for AAV production have been explored, though they suffer from very low yields and poor AAV potencies.[57,76] Synthetic biology engineering of yeast has the potential to ameliorate these issues and pave the way for establishing yeast as a low-cost production system for AAV manufacturing. Nonetheless, there remain significant challenges for yeast systems to overcome. (B) TESSA utilizes helper adenoviruses (TESSA-AAV and TESSA-Rep-Cap) which contain designed genetic circuits.[60] These engineered adenoviruses repress production of their own capsid proteins except in the presence of doxycycline. TESSA achieved 30-fold higher yield than triple transfection without generating infectious adenovirus or replication-competent AAV contamination. (C) *In vitro* production of dbDNA may act as a more easily scalable alternative to AAV plasmids. The dbDNA synthesis process leverages Phi29 DNA polymerase for rolling circle replication and protelomerase for covalently closing the ends of target sequences flanking AAV DNA. Since the final dbDNA can denature into a circle of linear ssDNA with terminal protelomerase binding sites, it acts as the substrate for further amplification, enabling easy production of large amounts of the product.

free media which decreases the risk of adventitious agents, the inability of most human pathogens to replicate in cultured insect cells, and the inability of BVs themselves to replicate in humans.[64,70] Despite the intriguing advantages of BV-based AAV production methods, the manufacturing of AAVs remains generally challenging. Though BV methods utilize some synthetic biology strategies, we argue that they have the potential to act as a foundation for more advanced biological engineering efforts that could yield higher gains in AAV production.

Herpes simplex virus (HSV) complementation platforms are another alternative for AAV manufacturing that use some approaches from synthetic biology in the engineering of HSV vectors (Figure 3B).[56] Much like adenovirus, HSV can act as a helper virus and provide essential genes for AAV replication in mammalian cells.[55,71] In this case, the minimum helper genes include U_L5, U_L8, U_L52, and U_L29.[71] The platforms often employ two replication-deficient HSVs: one carrying the desired AAV genomic DNA and another carrying the AAV Rep-Cap genes.[72] Because they can infect cells directly, HSV platforms circumvent the difficulties of large-scale transfections in a similar fashion to BV systems. HSVs furthermore have been shown to facilitate production of AAVs after infecting mammalian cells adapted for growth in suspension, making the platform more scalable than methods which rely on adherent cells.[73] A major drawback of HSV methods is that they require production of two distinct HSV vectors to high titer prior to using them for making the AAVs, adding to the overall complexity of the process. While HSV systems have promise, they have not yet led to substantially lowered costs overall. Much like BV platforms, HSV systems could still benefit from more complex synthetic biology design improvements to enhance their cost-effectiveness.

Some groups are investigating yeast as potential low-cost production systems for AAV therapeutics (Figure 4A). Yeast represents an attractive chassis since it grows rapidly in suspension, its growth media are cheaper, and it is much easier to transfect properly.[57] As an example, Barajas et al. developed a preliminary demonstration of AAV production in *Saccharomyces cerevisiae*.[55,76] They transformed yeast with four plasmids: a vector expressing Rep52 via the GAL1 promoter and VP1 via the GAL10 promoter, a vector expressing VP3 via the GAL1 promoter and AAP via the GAL10 promoter, a vector expressing Rep78 via the GAL1 promoter and VP2 via the GAL10 promoter, and a vector containing a transgene flanked by ITRs. This setup facilitated synthesis of the necessary proteins at roughly appropriate ratios. In addition, Galli et al. developed and analyzed a similar yeast AAV production system.[57] Unfortunately, the AAVs made using these approaches were less infectious even than those produced via BV systems and the overall yield was around 1000-fold lower than standard mammalian cell methods. Aponte-Ubillus et al. took partial steps toward ameliorating the low yield by leveraging liquid chromatography mass

spectrometry to analyze proteomic differences between yeast producing AAVs and a nonproducing control.[58] Bioinformatic analysis of the results identified several proteins which could each be overexpressed to enhance AAV yields. Though this overexpression strategy only enhanced AAV yield by up to 3-fold, Aponte-Ubillus suggested that more dramatic improvements might arise through the synergistic effects of overexpression of multiple carefully chosen proteins at once. Because of the drawbacks in current yeast platforms, extensive further engineering will be necessary if yeast is to gain footing as a viable AAV manufacturing tool. Despite these daunting barriers, past successes using synthetic biology approaches to radically engineer cellular systems (including yeast cells) for novel biosynthetic processes[77−79] indicate that yeast platforms may still have promise.

Though adenoviral helper systems have not been in common use for some time since they introduce infectious adenoviruses and can enable replication-competent AAV,[55] a recent study by Su et al. employed synthetic biology to design a self-attenuating helper adenovirus platform which circumvented those drawbacks and gave a 30-fold higher yield compared with triple transfection (Figure 4B).[60] For this platform, which was called Tetracycline-Enabled Self-Silencing Adenovirus (TESSA), a tetracycline operator (TetO) was inserted ahead of the adenovirus major late promoter (MLP) and the tetracycline repressor coding sequence was added under control of the MLP. This created a negative feedback loop which precluded expression of adenovirus structural protein genes (which are encoded ahead of the MLP) except in the presence of the doxycycline inducer. By contrast, the adenoviral genome replication and helper genes were unaffected since they are not transcribed from MLP. The AAV genomic sequence was placed in the E1-deleted region of the adenovirus genome, making TESSA-AAV. Su et al. also made an adenovirus that provided the Rep-Cap genes for the AAVs, called TESSA-Rep-Cap. Rather than an AAV genomic sequence, the E1-deleted region of this adenovirus contained the AAV Cap genes expressed from a CMV promoter as well as the AAV Rep genes under the control of a wild-type p5 promoter. Co-infecting mammalian cells with TESSA-AAV and TESSA-Rep-Cap allowed production of AAVs with desired genetic cargos. Su et al. furthermore developed a propagation method in which AAVs carrying the cargo of interest were used to coinfect cells alongside a TESSA-Rep-Cap vector, thereby producing more AAVs of the same type. By starting with an AAV2 capsid and then using a TESSA-Rep-Cap vector encoding a cap gene from a different serotype, this propagation method also facilitated easy packaging of AAV genetic cargos into desired capsids. Finally, Su et al. demonstrated that the AAVs synthesized via TESSA actually showed enhanced potencies compared to those made via triple transfection approaches. As such, AAVs made via TESSA might eventually show clinical efficacy at lower doses. TESSA's very high yields, negligible levels of viral contaminants, easy applicability to a wide range of systems, and remarkable ability to generate AAVs with enhanced potencies position it as a prime synthetic biology innovation which may help make AAV therapies substantially more affordable in the future.

An intriguing innovation in the synthetic biology space known as doggybone DNA or dbDNA could circumvent difficulties in AAV plasmid manufacturing.[61,62] This dbDNA consists of linear double-stranded DNA (dsDNA) that is covalently closed at its ends. When AAV dbDNA sequences are transfected into cells, efficient production of infectious AAV particles occurs. Synthesis of dbDNA involves two enzymes, Phi29 DNA polymerase and protelomerase. Starting DNA must include a pair of protelomerase binding sites flanking the AAV sequence (Figure 4C). After denaturation of the starting DNA into single stranded form and priming, Phi29 DNA polymerase performs rolling circle amplification. Strand displacement from the rolling circle and copying of the displaced strand leads to formation of linear dsDNAs containing the AAV sequence. Next, protelomerase acts on its binding sites to covalently close the dsDNA ends, forming dbDNA. Remaining extraneous DNA is digested and the final dbDNA is used as the substrate for further amplification by the same reaction. This *in vitro* process can produce gram-scale quantities of GMP-grade DNA for AAV manufacturing and has the potential to replace plasmid-based approaches, which are often more variable and difficult. The process employs a novel configuration of biological enzymes as molecular machines to synthesize dbDNAs as a potentially superior alternative to AAV plasmids.

Synthetic biology innovations have begun to demonstrate their power in lowering the costs of AAV production. Though synthetic biology has matured as a discipline,[40,41] it still represents a relatively young field and so its potential in gene therapy manufacturing has not been thoroughly explored. But the methodologies described in this review point to the burgeoning promise of synthetic biology for creating cost-effective AAV manufacturing processes. There now exists an enormous space of ripe directions by which synthetic biology could help make AAV therapies dramatically more affordable and accessible.

The Future of Synthetic Biology in AAV Manufacturing. Because AAV manufacturing employs cellular chassis as tiny factories to synthesize and assemble biological parts, it makes sense that there are numerous inroads for synthetic biology to redefine how this process is performed. In addition, while the upstream manufacturing process may represent a more obvious target, synthetic biology could also enhance downstream manufacturing. Since existing commercial entities have focused more on process optimization than biological design, we propose that the convergence of synthetic biology with AAV production presents a diverse array of under-exploited commercial opportunities. As an industry, AAV manufacturing is poised for technological disruption by synthetic biology.

Systematic engineering of cellular chassis for AAV production represents an area where synthetic biology could aid in making more efficient and cost-effective nanofactory platforms. While some engineering tweaks have improved existing cellular platforms,[63,68,75] there remains much ground to explore in terms of extensive genomic design.[80] Some commercial entities are beginning to delve into systems-level genome engineering. The startup Asimov reports that they are employing computer-aided design, machine learning, and multiomics characterization to drive synthetic biology efforts toward developing cellular platforms for manufacturing biologics including lentiviruses, monoclonal antibodies, and AAVs.[81] Likewise, the startup called 64x Bio has created a proprietary genetic barcoding method which reportedly allows tracing levels of viral vector productivity to single parent cells and thereby facilitates very high throughput genetic screens of combinatorially engineered cell lines.[82] Though the inner workings of these startups currently remain mysterious, their

emergence indicates that synthetic biology may indeed be poised to modernize the biological engineering processes of viral vector manufacturing.

Rational design represents another key area of synthetic biology which can enhance AAV production. After all, experimental directed evolution and its *in silico* analogues can much more rapidly produce superior results if guided by cleverly chosen core designs.[83,84] That is, AAV production may benefit from rationally designed cellular chassis circuits which give new functions to host cells and systems biology approaches might be applied afterward to optimize the function of said circuits. For example, since cell lysis difficulties can decrease yields,[18,31] we suggest that one might incorporate a gene which overexpresses a lytic protein such as a phospholipase upon chemical induction. As another possible direction, we propose capitalizing upon the Nelson Bay orthoreovirus fusion-associated small transmembrane protein or a similar fusion protein which induces formation of multinucleate syncytia.[85] The design would involve creating a mammalian cell line that inducibly forms such multinucleate syncytia through expression of existing or engineered fusion proteins. We speculate that the syncytia might act as high-volume and high-density AAV nanofactories which could achieve greater titers than traditional cellular platforms. In the context of downstream processing, rational and computationally guided protein engineering strategies could facilitate rapid generation of novel affinity reagents which enable scalable column-based purification of AAVs regardless of serotype. There is a world of possibilities for creative rational engineering toward improved AAV manufacturing.

Since AAV manufacturing is limited by extremely high production costs, contemporary synthetic biology innovations may act as a much-needed salve. As has been promised by startups like Asimov and 64x Bio, the combination of high-throughput directed evolution methodologies with contemporary machine learning may create next-generation producer cells that act as nanofactories for upstream processing. Creative rational design of novel biological functions which enhance AAV production may provide another route toward making more scalable platforms. Rationally designed functions and novel directed evolution methods may furthermore synergize since the latter might be employed to optimize the former. Leveraging synthetic biology may open many doors toward a future of more affordable AAV gene therapies.

OUTLOOK

Gene therapy represents a remarkable technology with the potential to dramatically change the world for the better. But as with any powerful new technology, gene therapy's capacity to positively influence humanity will depend on its accessibility. Some fear that gene therapy could exacerbate inequalities in the case that only the wealthy are able to access its gifts.[86,87] Though these arguments have often been applied to the more ethically challenging questions associated with germline gene editing, they are also relevant to somatic gene therapies. Equitable distribution of gene therapies will be important to create a future where everyone thrives. AAVs are one of the most promising vectors for gene therapy,[10−12] yet they come with the drawback of enormous costliness largely due to manufacturing difficulties.[14,15,17,18] Since synthetic biology has thus far shown impressive successes in numerous kinds of biomanufacturing,[41,48−50] rational biological design may play a central role in solving the problem of AAV production. Indeed, synthetic biology has already shown promise for AAV manufacturing, and we speculate that further strides in the area could make the AAV gene therapies of the future 100-fold or even 1000-fold less costly than those of today. Though achieving such goals will of course require a significant level of coordinated effort, we are optimistic that synthetic biology may put them within reach. We believe that all people deserve to benefit from the fruits of science and technology. We therefore propose capitalizing upon the power of synthetic biology to ensure the accessibility of AAV gene therapy. In our view, this research will have lasting benefits for the future of humanity.

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