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Review

The engineering, use, and mass production of adeno-associated virus as a vector for gene therapy

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ABSTRACT

In recent years, building on a new understanding of cellular biology and human genomics, gene therapy has emerged as a new potential treatment option for both monogenic and complex disorders. Currently, the most successful and researched gene therapeutic vector is the adeno-associated virus (AAV) due to many of its characteristics, which make it a safe and effective gene delivery system. However, one issue impeding its widespread clinical translation has been the extremely large dosages required for an effective therapeutic benefit. A consequence of the need for such high doses is the necessity for large production yields. If this obstacle can be overcome, the use of AAV for gene therapy will likely proceed at an unprecedented rate. This review discusses the use of AAV as a viral vector, including its clinical successes to date, its biology, vector design strategies, the costs and benefits of the most popular production techniques, and a brief discussion of purification strategies.

KEYWORDS: adeno-associated virus, AAV, gene therapy, bioprocessing, viral vector, cell therapy.

INTRODUCTION

Human disease is a multifaceted topic often requiring the input of a variety of disciplines. Some diseases are caused solely by genetic factors; others are due to interplay between one's genes and one's environment; and still others may be completely independent of one's genomic sequence. Regardless of the cause, virtually all diseases can be treated on a cellular level through the introduction of foreign genetic material to a cell to carry out a specific function. This form of treatment has been given the term gene therapy.

There are 7000 known monogenic diseases characterized by mutations in a single gene. These disorders affect millions of people worldwide with life-altering ramifications [1]. However, thanks to the genomic sequencing revolution, the causal gene of 50% of monogenic diseases has been identified allowing for their possible treatment using gene therapy. Contrary to popular belief, gene therapy also shows promise for many complex disorders such as cancer, heart disease, Parkinson's disease, Alzheimer's disease, stroke, diabetes, etc. [1-3] even though some of these disorders are often only partly due to, or even independent of, one's genomic structure.

It is no surprise that due to the tremendous potential of gene therapy for both Mendelian and complex diseases, that over 2200 clinical trials have been conducted since 1989, 65% of which have been in the USA. From an investment stand point, $600 million has been raised for this field since 2013 and funds are expected to exceed $10 billion by 2025 [4].

To comprehend the potential of gene therapy, it is important to discuss it in further detail. For gene therapy to be a viable treatment option, a defect must be identified at the molecular level with a corresponding gene to fix the disease-causing

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defect. Furthermore, there must be a delivery vehicle to introduce the gene into the cellular environment; this is known as a vector [5]. A suitable vector should deliver a gene to a specific cell type, maintain transgenic expression for sufficient time, and have as little toxicity and immunogenicity as possible [6]. Vectors are divided into two classes: viral and non-viral.

Viruses have naturally evolved the capability to deliver genetic material to a targeted cell type with little immune detection and as such have been selected as promising candidates for gene therapeutic vectors. Several viruses have been identified to act as gene delivery vectors. However, due to the safety concerns involving viral infections, vectors are engineered to be replication incompetent [6]. Therefore, viral vector gene deliveries can be considered dead-end infections.

There are two types of viral vectors: integrating and non-integrating. Integrating vectors include retroviruses and lentiviruses, and they are characterized by their ability to randomly insert their DNA into the host cell's genome [7]. Due to this random integration, the use of these vectors is restricted to *ex-vivo* gene therapies to avoid the risk of disease development such as oncogenesis. Non-integrating vectors such as the adenovirus and adeno-associated virus (although it has the potential for site-specific integration) express their vector genome through a nuclear episome without stable integration. As such, transgenic expression is transient and is slowly lost with cell division [6]. Irrespective of the vector type, the product needs to be safe, scalable to industrial yields, and cost-effective [8]. The issue of scalability is vital, because large-quantities of high quality vector are required for clinical translation of the product. As an example, treating muscular dystrophy would require whole-body muscular gene therapy, which would necessitate very high doses [9]. One vector, which has demonstrated substantial promise to fulfill all the aforementioned criteria, is the adeno-associated virus (AAV).

AAV as a therapeutic vector

AAV was first examined as a vector for gene therapy in the 1980's [10]. It is non-pathogenic (replication incompetent), demonstrates very low immunogenicity and has the potential to target non-proliferating cells such as the retina, brain, skeletal muscle, and liver [6, 11]. Even though it is a non-integrating viral vector, it demonstrates persistent gene expression lasting for years [5, 12]. Importantly, AAV has also shown stability in long-term storage over 24 months [13]. It is precisely these attributes that make it such an attractive option for gene therapy [14, 15]. For instance, of all the viral and non-viral vector systems evaluated for retinal gene therapy, AAV was shown to be the safest, most effective, and had the most enduring expression [16, 17].

Admittedly, there are several drawbacks to using AAV as a vector. Due to its small size, there is a limited carrying capacity to genes of approximately 4.8 kb. Further, unless using self-complimentary vectors (scAAV), the onset of expression takes on average several weeks [10]. However, using scAAV reduces the carrying capacity of the vector by half. Additionally, some cell-types are difficult to transduce, or require high multiplicity of infection. This last obstacle of transduction efficiency is largely overcome by vector engineering through rational design or directed evolution [1]. Undoubtedly, the current greatest impediment to widespread clinical translation of AAV-mediated gene therapy is the production process, which is still rather labor-intensive and produces titers below industrial requirements [18]. This last point will be the focus of this review.

The very first AAV clinical trial took place in the 1990's for cystic fibrosis [19] and there are over 70 clinical trials approved today [14]. Due to its success as a vector, AAV has been extensively studied in clinical trials for diseases such as hemophelia B [13], heart disease [20], muscular dystrophy [21], congenital blindness [22], Leber's congenital amaurosis [23, 24], cystic fibrosis [19], Canavan disease [25], α-1 antitrypsin deficiency [26], Parkinson's disease [27], and other diseases [17]. These trials have shown that low toxicity can be attributed to AAV itself; the only toxicity was the inflammatory response involving cytotoxic T cells due to MHC class I presentation of capsid proteins on the cell surface [5].

In October 2015, Spark therapeutics reported positive results from their phase III trials for Leber's congenital amaurosis 2, which may result in the first commercial gene therapy to be approved in the USA [4]. Sub-retinal administration of rAAV2 vectors was reported to be well tolerated and

led to significant improvement in the patients' vision [10, 28]. However, while this would be the first gene therapy to be approved in the USA, clinical trials for lipoprotein lipase (LPL) deficiency using a rAAV has already led to the first EU-licensed gene therapy product [1]. The product, Glybera, is the first and only gene therapy to be approved in the western world [17].

Glybera, also known as alipogene tiparvovec, uses a rAAV1 platform, which carries a vector genome encoding the gene *LPL* [29]. Patients with a defective copy of this gene experience elevated levels of serum triglycerides causing life-threatening pancreatitis. Familial LPL deficiency is an autosomal recessive disorder and, therefore, rescue from this condition requires one copy of the wild-type gene. The LPL protein is produced in skeletal muscle and adipose tissue, but only skeletal muscles are targeted for vector transduction. The transgene used in the Glybera product is a 1.9 kb truncated variant of the LPL protein and can, therefore, fit within the AAV1 viral capsid. While other AAV serotypes also transduce skeletal muscle cells, AAV1 showed the highest efficiency and most humans possess less neutralizing antibodies against it. The first clinical trial used $1x10^{11}$ vg/kg (vector genomes/kilogram) and $3x10^{11}$ vg/kg as the low and high dose cohorts, respectively. The results corroborated the need for higher doses to boost transgene expression. Therefore, the second trial changed its production platform to increase the yield, although this new method decreased the potency of the vector. Nevertheless, this time around the low and high doses were $3x10^{11}$ vg/kg and $1x10^{12}$ vg/kg for low and high dose cohorts, respectively. The higher dose was considered the minimally effective dose for positive results. However, this was still considered too low of a dosage, because patients returned to baseline after 6 months. This is important to note, because it demonstrates the reality that for the average human weighing 80.7 kg, over $1x10^{14}$ vg will be required for a single treatment; and many argue that doses as high as $1x10^{17}$ vg will be required for systemic delivery and widespread implementation of rAAV gene therapy for several diseases. In their study [29], the authors claim that changes in the manufacturing process should be expected due to the infancy of the gene therapy field. It is also important to note that while Glybera does not cure the disease, it represents a preferred alternative to the current method of treatment of LPL deficiency, which is the goal for new and innovative products. Moreover, despite the uncertainty of its clinical efficacy, it represents the coming of a new era in medical treatment and sets a precedent for future AAV-based gene therapy products.

AAV biology

In order to appreciate the potential of AAV as a vector, it is fundamental to have an understanding of its basic biology. AAV has never been associated with any human disease, which explains its popularity and success as a vector for gene therapy [18]. In fact, it can infect a variety of tissues without apparent toxicity. Moreover, unlike retroviral vectors, AAV has the ability to infect quiescent cells, in addition to proliferating cells, which increases its value as a therapeutic vector [9, 10, 30].

AAV was initially discovered in 1965 as a contaminant of an adenovirus preparation [5]. AAV belongs to the family *Parvoviridae* and the genus *Dependovirus* meaning a productive infection requires a helper virus [31]. AAV is a 25 nm, non-enveloped virus with a 4.7 kb single stranded genome [1]. The single stranded DNA (ssDNA) genome occurs in both the sense and antisense orientation at equal frequencies. 12 human serotypes and more than 100 serotypes from non-human primates have been discovered to date.

The interaction of the AAV with its host has adopted two alternate styles containing lytic and lysogenic stages [32]. As such, AAV is only capable of replication in the presence of a helper virus, namely adenovirus or herpes simplex virus (HSV) [33, 34]. It is AAV's inability to replicate on its own, which makes it a non-pathogenic virus. Therefore, it is only upon co-infection of a cell that has been previously infected by AAV that AAV genome replication, viral gene expression and capsid production become possible. More specifically, it is only a subset of adenoviral genes that are necessary for helper function (*E1A*, *E1B*, *E2A*, *E4*, and *VA* RNA) [5]. This is known as the lytic life cycle, because AAV production will result in cell lysis. Conversely, in the absence of a helper virus, AAV genome replication is limited, gene expression is repressed and the AAV genome can set up latency by site-specifically integrating into chromosome 19q13.4 *via* non-homologous recombination [32].

This ability makes AAV the only mammalian DNA virus known to be capable of site-specific integration [5]. The 4-kb region of chromosome 19, now termed AAV Site 1 (AAVS1), is the locus of the gene *MBS85* (myosin binding subunit 85) also known as *PPP1R12C* (protein phosphatase 1, regulatory inhibitor subunit 12C) whose product has been shown to be involved in actin organization [35]. Furthermore, the AAVS1 locus is near several muscle-specific genes, *TNNT1 and TNNI3* [5].

The AAV genome contains 3 open reading frames (ORFs). The two main ORFs are entitled *Rep* and *Cap*, while the third ORF is a nested alternative reading frame within the *Cap* gene. Flanking *Rep* and *Cap* on either side of the genome are the inverted terminal repeats (ITRs) [10].

The left ORF codes for the *Rep* gene, which produces four non-structural proteins denoted Rep78, Rep68, Rep52 and Rep40 [1]. These four different proteins are produced from the same ORF using two different promoters and alternative splicing. Rep78 and Rep68 are produced using the P5 promoter from unspliced and spliced transcripts, respectively. Similarly, Rep52 and Rep40 are respectively produced using the P19 promoter from unspliced and spliced transcripts [5]. Rep proteins are responsible for genome replication (hence the name), but they also play important roles in site-specific genome integration, genome encapsidation, and transcriptional regulation [15]. More specifically, Rep78 and Rep68 possess site-specific endonuclease activity, which are vital functions in genome integration [36, 37]. Additionally, they can positively or negatively regulate AAV gene expression in the presence or absence of a helper virus, respectively. Rep52 and Rep40 function by assembling ssDNA for packaging within the AAV capsid. Furthermore, all four proteins contain ATPase and helicase activity [5, 38].

The right ORF encodes the *Cap* gene, which, like *Rep*, produces numerous proteins from a single transcript [1]. Using the P40 promoter, *Cap* produces three viral capsid proteins (VP1, VP2, and VP3) in a 1:1:10 molar ratio [5]. Alternative splicing produces a spliced and unspliced version of the P40 transcript. The unspliced transcript produces VP1 (87 kDa), while the spliced transcript produces both VP2 (72 kDa) and VP3 (62 kDa). The same spliced transcript can produce two proteins, because VP2 is translated using an unconventional ACG start codon and VP3 is translated from a downstream AUG codon [5]. Additionally, nested within the *Cap* ORF is an alternative reading frame coding for the Assembly-Activated Protein (AAP), which localizes AAV capsid proteins to the nucleolus and assembles the three Cap proteins into the 60-mer icosahedral viral capsid [3, 15, 39].

There are generally two ways in which viruses package their genomes; either through binding of the viral genome by the capsid proteins, or by insertion of the genome into the preassembled capsid. It has been demonstrated that AAV uses the second mechanism [40]. The precise method is not fully understood, but it has been proposed that Rep40 and Rep52 play a key role in the shuttling of the ssDNA genome into the preassembled capsid. This interaction between the structural proteins, the Rep proteins and the genome involves specific amino acids and, therefore, a single mutation can lead to a deficiency in packaging [30]. Furthermore, even though the viral genome is single stranded, it appears that both sense and antisense DNA strands are packaged with equal frequency [5].

As aforementioned, flanking the *Rep* and *Cap* ORFs are the ITRs, which are 145 base pair (bp) palindromic sequences [16]. The first 125 nucleotides (nt) of the ITR is the palindromic portion, which folds upon itself to form a T-shaped hairpin-loop secondary structure [5]. The ITR plays a vital role in all aspects of the AAV life cycle including DNA replication, transcription, genome packaging, regulation under non-permissive conditions, and site-specific integration. One consequence of the hairpin structure is the creation of double stranded DNA (dsDNA) with a free 3' end. This free 3' end functions as a primer for second strand synthesis and is thus an origin of replication [41]. The creation of dsDNA is a vital and rate-limiting step in the expression of transgenes in AAV-mediated gene therapy. It has also been proposed that another function of the hairpin structure is to prevent recognition of the ssDNA genome by the immune system of infected or transduced cells [16]. Located within the ITR are adjacent motifs known as the Rep-binding site (RBS) and the terminal resolution site (TRS). These two elements are critical to the AAV lifecycle by interacting directly with Rep proteins [32].

Interestingly, AAVS1 shares many sequence similarities with the ITR, specifically a 33 bp region, which contains a RBS adjacent to a TRS [41]. The site-specific integration into AAVS1 [42] requires Rep68/78 binding site (RBS) and a nicking site, that present similarities with the TRS. The two proteins enable this site-specific integration by specifically and simultaneously binding to both the viral and AAVS1 RBS and creating nicks at both the viral and AAVS1 TRS. Several studies have even reported that Rep78 alone is sufficient for integration [38, 43, 44]. Also, necessary for integration are 16 bp of a 138 bp region of the P5 promoter called the integration efficiency element (IEE) [5]. Efficiency of integration into AAVS1 has been reported to be 68-82% [43]. However, two other integration sites have been identified as well in regions that contain RBS homologs such as 5p13.3 (AAVS2) and 3p24.3 (AAVS3), albeit at much lower frequencies [45].

In addition to their direct helper functions, it is also believed that helper viruses are crucial for efficient packaging of the viral genome into the capsid. It has been proposed that they may induce the host cell into entry of S-phase, which leads to the cellular changes required for AAV assembly [30]. For example, proteomics analysis identified 10 cellular proteins involved in activities such as binding and shuttling the AAV capsids between the cytoplasm and nucleus, which are instrumental to proper AAV assembly [46].

Vector design

Even though viruses are often used as gene delivery vectors, it is important to recall that they did not evolve for medical applications. As such, several challenges arise in their use as therapeutic vectors [1]. These challenges manifest themselves as barriers to transduction, namely lack of cellular receptors for vector binding, intracellular trafficking obstacles such as endosomal and proteosomal escape, onset of gene expression, genomic capacity, immune evasion, etc. [47] For example, the majority of the population has been exposed to and has subsequently developed antibodies against AAV. In fact, 72% of the population has neutralizing antibodies against AAV2 alone [1]. This can obviously impact gene delivery, and one method to circumvent this (higher clinical doses) has already been discussed at length. However, many of the challenges can also be addressed by engineering the viral capsids to release them from their evolutionary constraints and confer unto them novel phenotypes [1, 47]. The capsid is responsible for viral tropism, infection kinetics, and immunogenicity and, therefore, the sequence of exposed amino acids can greatly influence the efficacy of gene therapy [13]. Rational design of AAV capsids, directed evolution, self-complementary vectors, and trans-splicing are just some of the methods bioengineers are utilizing to expand the potential of AAV as gene therapy vectors [9, 48].

Rational design refers to the engineering of a vector against a specific obstacle for which there exists prior knowledge of the viral or cellular mechanisms. For example, if the amino acid sequence that acts as an epitope for antibody binding has been identified, one can mutate this region of the capsid to evade immune detection [1]. Previous claimed methods for eluding antibody detection relied on co-administering animals with empty and full particles and having the empty ones act as decoys by binding to the antibodies. However, as was already seen in a previous section, empty particles can also bind to cell surface receptors and decrease transduction efficiency. Rational design is also used for conferring alternative viral tropisms when the transduction of a specific cell-type needs to be increased or for the transduction of previously non-permissive cell-types. For example, a recent study inserted ankyrin repeat motifs specific to the human epidermal growth factor receptor 2 (HER2 or ERBB2) at the N-terminus of VP2 resulting in an active targeting of tumor cells that overexpress that receptor [1]. Another prime example for rational design was implemented to circumvent the issue of innate cellular immunity against AAV. Specific tyrosine residues of the AAV capsid can be phosphorylated upon cell entry resulting in ubiquitylation and proteosomal degradation of AAV virions. It was demonstrated that mutation of these tyrosine residues to phenylalanine, which differs from tyrosine only in its inability to be phosphorylated, enabled vectors to avoid degradation by this pathway. This results in higher transduction efficiencies and greater transgene expression [10, 16]. This also leads to a reduced risk of a cytotoxic T lymphocyte response, because it is believed that the presentation of AAV capsid epitopes triggers this

reaction against AAV. Therefore, with less virion degradation, there is less MHC class I presentation of the capsids [1].

Contrary to the problems solved through rational design, sometimes the challenges are so mechanistically complex or no prior knowledge of the system exists that conscious engineering cannot be employed [10]. In these circumstances, directed evolution is employed. Directed evolution exploits genetic diversity and subsequent selection to improve the function of a vector [1, 3]. It uses the wild-type virus as a template for the creation of vectors with modified or enhanced phenotypes often by combining serotypes [49]. More specifically, *Cap* is diversified to create huge genetic libraries on the order of 10^7 and a selective pressure representing an obstacle encountered in gene therapy is applied to isolate the most successful variant [1, 10]. The genetic diversity is created either through error-prone polymerase chain reaction (PCR), DNA shuffling (fragmentation of AAV *Cap* genes followed by reassembly based on homology), random insertions in a defined genomic location (usually an exposed area of the capsid), or defined insertions in a random genomic location [1, 49]. These methods can also be combined. The viruses are then packaged so that each capsid contains the genome of the *Cap* variant that encodes it. Following this, selection pressures are applied to the millions of capsid variants *in vitro* or *in vivo*. Selection pressures can be positive such as cell receptor binding, or negative such as neutralizing antibodies [10, 49]. Successful variants can subsequently undergo iterative selection where their mutated *Cap* ORF acts as a template for a new round of selection [1]. Directed evolution has been applied to neutralizing antibodies, transduction of non-permissive cell types, crossing biological barriers, etc. and has led to transduction increases upwards of 100-fold [1]. It is quite clear that this engineering technique has tremendous potential. However, it is currently quite labor intensive [16].

In addition to vector improvements that rely on capsid engineering, there is often the necessity to engineer other aspects of the vector genome for improved transgene expression. This is especially seen for transgenic expression. Because the vector genome is composed of ssDNA, the onset of gene expression, while influenced by several factors, is limited primarily by the time required for the synthesis of the complementary DNA strand [10]. RNA polymerase recognizes only double stranded DNA (dsDNA) for transcription, and it has been proposed that FKBP52 binds to the ITR and inhibits second strand synthesis [5]. Furthermore, although both sense and antisense strands of the vector genome are packaged with equal frequency, it is unlikely that any given cell will be transduced with both allowing for their complimentary hybridization [16]. As a result, several weeks are often required for optimal transgene expression; and for many diseases, there is a specific window of time required for gene expression. One innovative solution to increase the rate of transgene expression is the use of a self-complimentary vector (scAAV) which encodes for both a sense and antisense copy of the transgene in the vector genome separated by linker DNA [10]. Therefore, the genome folds upon itself to form dsDNA independent of the cell's replication machinery [5, 16]. However, because of encoding two copies of the transgene, the carrying capacity of the vector is cut in half.

This issue of carrying capacity has also been tackled through clever means. Due to the small size of the virion, rAAV have been shown to be capable of packaging genomes up to 5 kb in size, after which 5' truncations begin to occur [1]. However, trans-splicing seems to overcome this barrier [50]. It takes advantage of AAV's natural proclivity to form head to tail concatemers through recombination of their ITRs. Therefore, two rAAV are produced: one contains the 5' portion of the transgene, and the other encodes the 3' end. Strategically placed on each of these vector genomes are splice sites. Upon transduction of the same cell, recombination of the ITRs occurs resulting in one long vector genome. After transcription of this recombined vector genome, splicing occurs, which removes the recombined ITR and brings the 5' and 3' of the transgene together resulting in a functional protein [5]. The issue of limited cargo space can also be overcome by using small fragments of cDNA together with a site-specific endonuclease for precise genome editing. This has been proposed as a solution for autosomal dominant diseases, which can also be treated through RNA interference [1].

It should also be noted that another technique to ensure transgene expression restricted to a specific

cell type is the use of cell-specific promoters. For example, vectors targeted to cone cells of the retina were achieved using the cone arrestin, blue opsin, and red/green opsin promoters [51-53]. Conversely, if high levels of expression are required, ubiquitous promoters such as CMV can be used [16].

rAAV production

To produce or manufacture recombinant AAV (rAAV), several elements are required in *cis* and in *trans*. The only components needed in *cis* are the ITRs flanking the transgene. As aforementioned, the ITRs play direct roles in genome replication and genome encapsidation. Therefore, it is imperative that they be present for the vector genome to replicate and be encapsidated within the gene therapy rAAV vector. More specifically, it is believed that only the RBS is required as it is the RBS that directly interacts with the Rep proteins that carry out these functions [11]. Interestingly, most rAAV constructs use ITRs isolated from the AAV2 genome [16].

The elements required in *trans* are the *Rep* and *Cap* genes, as well as the helper virus functions [54]. However, studies have shown that not all four Rep proteins are necessary for rAAV production; one P5 and one P19 protein may suffice [31]. In terms of the helper virus, while the natural AAV lifecycle requires an adenoviral or HSV infection, this creates issues in the purification of rAAV for clinical use. When infecting producer cells with adenovirus to generate rAAV, adenovirus particles themselves are also generated and co-purified with rAAV [55]. This might have some serious safety concerns when injected into patients. To avoid this, the production process uses plasmids containing the necessary adenoviral helper genes, rather than the virus itself. As a result, the transfected cells will only produce rAAV vector. In addition to the safety reasons for eliminating adenoviral production, one would also want to limit adenoviral replication to prolong the cellular viability of the producer cells thus maximizing rAAV yields. Furthermore, it has been shown that only low copy number of helper genes is necessary for rAAV production making virus-free helper plasmids a viable option, which would achieve much lower copy numbers than a replicating adenovirus [15]. As noted above, the adenoviral helper genes needed for induction of AAV production are *E1A*, *E1B*, *E2A*, *E4* (specifically *E4ORF6*), and *VA* RNA. However, most production techniques, such as those using HEK 293 cells, omit the necessity for including *E1A* and *E1B* on the helper plasmid. Because HEK 293 cells were transformed using the *E1* region of adenovirus, those genes are constitutively expressed in these cells and would, therefore, be redundant on the helper plasmid [14]. In terms of the role the *E1* region plays, it has been reported that E1A enhances the gene expression of cellular genes integral to AAV production such as *YY1*, which regulates the P5 and P19 promoters [31].

While it may seem that the production process has been completely elucidated, it remains one of the factors currently impeding AAV's translation to the clinic. Even though AAV demonstrates low immunogenicity, the majority of the population has been exposed to it, which has resulted in a large portion of the population possessing AAV neutralizing antibodies limiting gene delivery [6]. These pre-existing immune responses necessitate higher titer of AAV to ensure gene delivery to the target cells. Furthermore, off-target binding is seen, which can dilute the administered dose resulting in lower number of vectors reaching the desired tissue. Finally, some cell types require a very high multiplicity of infection, which refers to the number of viral particles per cell needed for successful transduction. With all of this in mind, it becomes apparent that high yields of rAAV are required so that patients can receive high doses [30]. Current vector requirements are in the range of $1x10^{15}$-$1x10^{16}$ vector genomes (vg) per patient. However, it has been suggested that yields greater than $1x10^{17}$ vg will be required for large-scale clinical application [14]. On a per cell basis, the current best yields seem to converge to less than 1 log range always bouncing between $1x10^{4}$-$1x10^{5}$ vg/cell across the numerous production platforms [31]. Therefore, the current goal would be to increase the yield per cell.

Ideally, it would be most efficient to progress in large-scale production by improving upon the companies' protocols that are currently producing clinical-grade (quantity and quality) rAAV. However, the methods used in clinical manufacturing are usually not published, which hinders the growth

and advancement of this field [14]. Despite these 'trade secrets' of sorts, significant progress has been made in large-scale production and purification of AAV [30, 56, 57]. There are three main methods currently used for clinical-grade rAAV production: transient transfection, recombinant baculovirus or herpes simplex virus (HSV), and packaging/producer cell-lines [11, 58]. Transient transfection utilizes plasmids to deliver the vector genome, the *Rep*/*Cap* sequences and the helper virus genes to the mammalian producer cells. Recombinant baculovirus/ HSV are viral vectors used to transduce insect or mammalian cells, whereas, packaging or producer cell lines have part or all the genetic elements stably integrated into the host cell's genome [9]. Naturally, each method comes with its own set of advantages and disadvantages [59].

Transient transfection

The most widely used method for AAV production is helper virus-free transient transfection, typically performed using HEK 293 cells and either calcium phosphate, polyethylenimine (PEI), or cationic lipids (Lipofectamine) as the transfection reagent [4, 9, 30]. Normally, triple transient transfection is performed by transfecting the producer cells with three separate plasmids simultaneously. One plasmid encodes the vector genome, another encodes *Rep*/*Cap*, and the third encodes the adenoviral helper genes. Sometimes, however, double transient transfection is used where *Rep*/*Cap* and the helper genes are combined onto a single plasmid [14]. This second method is less common though it presents challenges in terms of scalability [11].

Initially, these transfections were performed using adherent cells, but this too soon became a major challenge in terms of scalability. Using adherent cells, a typical good manufacturing practice (GMP) operation would require more than one hundred cellstacks for yields lower than $1x10^{15}$ vg and more than 500 cellstacks for yields greater than $1x10^{16}$ vg [14]. This is because scaling-up production means increasing the number of producer cells; and adherent cell densities are limited by the surface area of their culture plates or roller bottles since they grow as monolayers [31]. Therefore, scaling-up adherent cell systems requires a very large number of flasks, roller bottles, or cell factories and the manipulation of each of these vessels increases the production time and risk of contamination [4]. Subsequently, it was realized that this issue could be altogether avoided by transitioning to suspension cultures, which allow cell expansion based on volume instead of surface area [31]. Assuming normal cell densities, there is approximately the same number of cells in ~10-50 cm^2 of adherent cell-culture as there is in 1 mL of suspension. This translates into 1 L of suspension culture producing the same amount of AAV as fifty 50 cm^2 plates [31]. It is thus quite apparent that suspension cultures provide scalability to industrial-scale bioreactors that adherent cultures cannot match. Furthermore, they permit much easier collection of cells and supernatant for purification purposes [4]. Under optimal conditions, transfected HEK 293 cells grown in suspension were reported to generate AAV serotypes 1-6, 8 and 9 at yields greater than $1x10^5$ vg/cell, which is greater than $1x10^{14}$ vg/L of cell culture at a concentration of $1x10^6$ cells/mL [14, 55]. In this scenario, the optimized parameters of the transfection reaction included compatible serum-free media optimal for cell growth and transfection, the transfection reagent, transfection conditions, and cell density [4, 55]. PEI was selected as the transfection reagent, because it is non-toxic to the cells, can transfect them efficiently, is inexpensive, and does not require a medium exchange post transfection. Furthermore, the authors optimized the actual transfection protocol reporting optimal yields with a 2:1 PEI Max to DNA ratio using 1 µg DNA/mL of cells. They, additionally, found an ideal plasmid molar ratio of 2:1.5:1 for the helper genes, *Rep*/*Cap* and vector genome plasmids, respectively. Lastly, they found that rAAV vector production was optimal when the transfecting volume was 5% of the final volume of the cell culture at a density of $1x10^6$ cells/mL [55]. Aside from higher vector genome yields, they also reported significantly reduced empty capsid particles upon ion exchange chromatography purification.

In addition to optimizing transfection protocols, one can also attempt to increase rAAV yields by optimizing the plasmid sequences themselves. This was done by Emmerling *et al.* [60] to avoid unnecessary gene products, which would take away from the cells' resources. Specifically, a wild type AAV2 sequence lacking the ITRs was cloned into a pUC backbone. The *Rep* and *Cap* sequences were separated and cloned onto different plasmids, followed by inactivation of both *Rep78* expression

and an artificial RBS in the plasmid backbone. Additionally, dispensable promoters and unnecessary start codons were removed to limit the expression of non-functional or truncated proteins [60]. This was further improved upon recently. A new split packaging plasmid system was re-designed to yield as high as $2.7x10^5$ vg/cell in HEK 293T cells [61].

Other attempts have been made to increase production yields by optimizing the transfection conditions. A 2013 study [15] reported significantly higher yields by cultivating the cells at 32 °C compared to the standard 37 °C post transfection. The authors hypothesized that this occurred due to an arrest of cells in G2/M phase at this lower temperature. This, subsequently, resulted in larger cell size and thus elevated protein production. Furthermore, gene expression analysis of the HeLa cells used in this study led to the identification of three genes and 16 miRNAs up-regulated up to 7-fold and 2-fold, respectively.

Additional studies have also found a strong relationship between cellular gene expression and AAV production. Recently, Satkunanathan *et al.* [30] identified up to 44 cellular proteins associated with AAV production. One such protein was Y-Box Protein 1 (YB1), which is a DNA and RNA-binding protein. The authors found that by downregulating YB1 expression through the shRNA sequence Y4, AAV producer cells achieved a 45-fold and 7-fold increase in vector genome titers and infectious genome titers of AAV2, respectively [30]. Furthermore, they found a 12-fold increase in *Rep* expression, a 13-fold increase in vector DNA production, and a 7-fold decrease in *Cap* expression in these knockdown cells. They propose a mechanism where YB1 competes with both adenoviral protein E2A and Cap proteins for binding to the ITR sequences. YB1 has a higher affinity for ssDNA, specifically the motif GGGG(TT), which is present in the AAV2 ITR in the region involved in genome encapsidation [30, 62, 63]. Normally, the N-terminal region of AAV capsid proteins binds to this region of the ITR resulting in genome packaging into the preassembled capsid [64]. Therefore, YB1 may interfere with the natural ability of the capsid protein to bind to this region resulting in a decrease in genome encapsidation efficiency. Furthermore, competition with E2A, which also binds to the ITRs and promotes DNA replication [65], may result in lower number of vector genomes. The authors further explain that even though there was a 7-fold decrease in *Cap* expression, the amount of capsid protein in harvested vector samples was the same regardless of *YB1* expression indicating that vector capsid particle formation is independent of the amount of capsid protein produced by the cells [30]. Therefore, the rAAV yield is likely dependent on the number of vector genome copies. However, the authors note that while unpackaged DNA was 7 times higher in knockdown cells, the packaged DNA was only 4 times higher, signifying that the packaging mechanism is also a limiting factor to higher yields.

Due to the many ways that one can optimize a transfection protocol, it is no surprise that most clinical trials have utilized transfection as their means of production [14]. One can clearly see the numerous benefits of transfection including simple protocols, ease and low cost of producing the raw materials, the flexibility in terms of producing many types of rAAV, and the potential for relatively high yields of $1x10^5$ vg/cell or $1x10^{15}$-$1x10^{16}$ vg for 10-100L production. While this is sufficient for treatments requiring low doses and orphan diseases, it is simply not enough vector for instances involving a larger number of patients or when higher doses are necessary. An estimated increase of one to two orders of magnitude is expected to be required for these cases [4]. Furthermore, despite the relatively high titers and the low cost of reagents when production is small-scale, transfection is not cost-effective for industrial scale-up [9, 14]. This is partly due to the fact that the main variable in transfection scale-up is the number of cells being transfected. Normally, with a very high transfection efficiency, ~80% of cells get transfected. Therefore, because industrial processes will require huge cell numbers, even with 80% transfection efficiency, millions, if not billions, of cells will remain untransfected. Unlike viral infection, the absence of cell-to-cell transmission for transfection processes limits rAAV production to those 80% (at best) initially transfected with plasmid DNA [31]. Moreover, because plasmids are incapable of replication in mammalian cells, the copy numbers of the transfected plasmids are significantly lower than the levels reached by wild type AAV [31]. Subsequently, the transfection of

a large number of cells is considered a bottleneck in clinical manufacturing [4].

Recombinant baculovirus/herpes simplex virus

Baculovirus naturally infects invertebrates and, therefore, the Baculovirus method of production uses Sf9 insect cells [66]. Insect cells are often used for large-scale production of heterologous proteins due to the large cell densities they reach in suspension, their high level of protein expression, and the high similarity of their post-translational modifications to those seen in mammalian cells [14]. As such, the Sf9cells/baculovirus expression system presents all the necessary attributes for large-scale rAAV production. In fact, Glybera is produced using the recombinant baculovirus system [67].

Similar to transient transfection, there are numerous ways of implementing this method of manufacture. In the 3-bac system, three recombinant baculoviruses are used; one virus encodes the rAAV vector genome, the second encodes *Rep*, and the third encodes *Cap*. With this technique, no helper virus genes are required as the baculovirus infection performs this function [68]. The 2-bac system once again has the vector genome on its own, but combines the *Rep* and *Cap* cassettes into a single baculovirus [69]. A 2009 study [70] developed a system whereby the authors stably inserted copies of *Rep* and *Cap* into the Sf9 genome under the control of baculovirus regulatory elements. Upon infection by the rAAV genome-containing baculovirus, the entire system was induced to begin the production of rAAV.

Irrespective of the protocol, once a small population of insect cells is infected, they are added to a bioreactor containing a larger population of Sf9 cells to inoculate it so that the baculoviral infection spreads. This method has been shown to generate yields over $1x10^{16}$ vg at the 200 L scale, which is $2x10^{4}$ vg/cell or $1x10^{14}$ vg/L [4, 14]. Moreover, analysis of the rAAV produced from Sf9 cells indicate that they are physically, biochemically, and biologically equivalent to those produced in HEK 293 cells [31]. However, disadvantages are seen on a molecular level, where there is instability of the genomic sequences within the baculovirus, a failure to assemble the AAV virions with the correct ratio of capsid proteins, and downstream obstacles eliminating baculovirus from the rAAV product [14, 31, 71].

Another method using recombinant viruses utilizes HSV, with the first clinical trial using this production platform having taken place in 2010 for α1-antitrypsin deficiency [14, 26]. Because HSV is a helper virus itself, only two recombinant HSVs are required; one carrying the vector genome, and the other carrying *Rep* and *Cap* [14]. To improve the safety of the system, recombinant HSV are created with inactivated *ICP27* expression making them replication deficient (d27.1 HSV variant) [14]. Because they are replication deficient, to produce the rHSV in the first place, they are produced in V27 cells, which express the necessary genes enabling replication.

The vectors purified after recombinant HSV production are of high quality with a reported increased potency, increased transgenic expression, and a reduction in the number of empty capsids [14]. Chulay *et al.* [72] generated yields greater than $1x10^{5}$ vg/cell from $1\text{-}2x10^{11}$ cells, which was greater than $1x10^{16}$ vg at the 100 L scale. However, due to issues during purification, their final yield was only $2x10^{4}$ vg/cell.

Stable cell-lines

Due to the relatively high costs, regulatory efforts, and safety concerns of the previously discussed methods, much interest has veered towards the development of stable producer or packaging cell lines for rAAV production [15]. It is hoped that the establishment of these cell lines will improve production in terms of both the quantity and quality of vectors, while allowing for a more rapid production process by decreasing the number of steps involved [30].

There are two types of cell-lines one can establish for rAAV production: packaging cell-lines and producer cell-lines. Packaging cell-lines have stable integration of *Rep* and *Cap* in the host cell's genome [11]. These cells produce rAAV upon transfection with the vector genome in tandem with either an adenoviral infection or transfection with a helper plasmid. Equally, it is also possible to induce rAAV production through an infection with a recombinant adenovirus that codes for the rAAV vector genome, thus eliminating the need for transfecting the transgene [14]. Conversely, a producer cell-line contains all genetic elements required for rAAV production stably integrated in the host cell's genome including *Rep* and *Cap*, the

transgene flanked by the ITRs, and the necessary adenoviral helper genes [15]. However, many producer cell-lines do not contain the helper genes and are induced through either infection or helper plasmid transfection. Therefore, perhaps a better way to distinguish between packaging and producer cell-lines would be the presence of the vector genome [11]. Either way, for producer cell-lines, production is a single step induction, which makes them more straightforward for the cGMP (current good manufacturing practice) process [8].

There are several advantages to using producer cell-lines over the previous methods discussed. Firstly, while transfected cells are limited to generating particles up to ~96 hours post-transfection, stable cell lines can produce rAAV until cell death [73]. Secondly, if using a cell-line with all genetic elements integrated, there are no contaminating plasmids or viruses, which abridges the purification process [4]. Lastly, the commercial scalability of producer cell-lines is far superior to that of the other methods [9, 11]. By culturing them in suspension, producer cell-lines have been applied at the 250 L scale and are expected to reach 2000 L [8]. Yields of $1x10^5$ vg/cell have been reported with 70-90% vector-containing particles, which stands in stark contrast to transfection methods where it can be 15-20% [11, 14].

The current method for physically creating cell-lines relies on random recombination events. Stable transfection is performed with plasmids coding for the vector genome, *Rep*, *Cap*, helper genes and a selection marker (or a variation of these elements depending on the type of cell-line being developed). Cells with successful integrations are selected for by drug resistance and then assessed for production yield [8]. To achieve high yields, it is important that the chromosomal structure at the site of integration allows for high gene expression [11]. The productivity screen is usually performed on a small-scale in 96-well plates by inducing rAAV production. Approximately one dozen of the highest producing cell clones will be expanded to shake flasks and screened for their ability to grow in the proper serum-free growth medium. Importantly, stability through sufficient number of population doublings will be examined, as this is required for cGMP certification and the establishment of master cell-banks [8].

While the development of stable cell-lines appears to be the ideal method of large-scale rAAV production, it comes with a set of issues. Perhaps most obvious, due to the stable integration, there is less flexibility to alter the capsid serotype or the vector genome, as each cell-line will produce only one type of rAAV [11]. Another potential issue, present also in other production methods, is the copy numbers of each genetic component in the cells. In a natural AAV infection, the vector genome is the viral genome and, therefore, *Rep*, *Cap* and the viral genome are present in equal copy numbers [11]. This 1:1:1 ratio is perhaps the optimum for efficient production. However, analysis of producer cell-lines have shown that the vector genome is usually 100-fold more abundant than *Rep* and *Cap*, which may interfere with encapsidation competence. Furthermore, studies have shown that the use of helper plasmids supplied in *trans* to induce packaging cell-lines significantly reduces rAAV titers compared to using live adenovirus. One such study [74] found that upon adenoviral infection of a cell-line with stably integrated *Rep*/*Cap* genes, the integrated genes underwent a dramatic amplification resulting in 100-fold increase in copy number. However, this amplification was not seen with the helper plasmid. Furthermore, the authors found that the localization of some adenoviral proteins was abnormal when using the plasmid as compared to the live virus.

Clearly, the current production of stable cell-lines is quite arduous and needs to be repeated for every transgene-serotype combination [14]. Additionally, because helper genes are difficult to incorporate into cell-lines due to their toxicity, most cell-lines have relied on live virus [9]. The resulting safety concerns can be circumvented to an extent by using viruses with adequate safety features and purification strategies such as heat-inactivation or nano-filtration [8]. Lastly, perhaps the greatest challenge is that some of the gene products required for rAAV production are cytotoxic, specifically Rep proteins [4]. It is believed that Rep's toxicity stems in its inhibition of the oncogenes expressed for cellular transformation, which would inhibit cellular proliferation [43]. Subsequently, methods of regulating *Rep* expression under an inducible system are necessary for the viability of a cell-line, especially due to the recent shift from HeLa cells to HEK 293 cells used for these stable cell-lines.

Until recently, all cell-lines were created in HeLa cells, because it was assumed that the presence of HPV genes *E1*, *E2*, and *E6* might support adenoviral helper functions thus increasing rAAV yield [11]. Several of these cell-lines were produced for clinical trials, all requiring adenoviral infection [14]. However, the presence of the HPV genes, while complementing production, presented a safety risk [11]. Therefore, focus shifted towards HEK 293 as the ideal cell-type in which to create stable cell-lines. The main reason for this is the presence of *E1* gene region in the HEK 293 cells and therefore, *E1*-deleted replication incompetent helper adenoviruses could be used to supply helper functions. However, this is a double-edged sword, as it is E1A that mediates *Rep* expression through the P5 and P19 promoters [9, 75]. Therefore, the issue of Rep cytotoxicity became apparent and the need to regulate its expression became necessary.

Yuan *et al.* [9] came up with an ingenuous method of inducing *Rep* expression. Using HEK 293 cells, they created a stable cell-line by integrating an inactive copy of *Rep* where its ORF was interrupted by an intron flanked by *loxP* sites. In tandem, they created a recombinant *E1A*/*E1B*-defective adenovirus coding for the *Cre Recombinase* gene (Ad-Cre). Upon infection with Ad-Cre, the inactivating intron was spliced out of the *Rep* coding region resulting in *Rep* expression and rAAV production. This cell-line generated yields of $1.3x10^5$ vg/cell ($8x10^{13}$ total vg per Nunc cell factory), with an optimal Ad-Cre multiplicity of infection of 5. The authors note that the cell lines were very stable and portrayed identical growth to the parental cell-line. However, they also report that despite using a replication-deficient adenovirus, inactivation and elimination of the virus from the product is still necessary through heat inactivation, high hydrostatic pressure, or chromatography.

Emmerling *et al.* [60] also came up with a very clever approach for inducing rAAV production. Upon the discovery that cultivation at 32 °C significantly increased production for triple transfection (earlier section), they decided to create a stable cell-line in HeLa cells with *E1A* under the control of a 32 °C temperature-sensitive inducible promoter. Therefore, by using this temperature shift to both induce the system and increase yields, they successfully produced high vector titers. They found dozens of genes regulated up to 11-fold and 16 miRNAs regulated more than 2-fold at this lowered temperature. Furthermore, they discovered that while using live helper virus increases the yield at 37 °C, it makes no difference whether helper genes or live virus is used at 32 °C. Other studies have also found a relationship between gene expression and temperature change [76].

Purification

Regardless of the method of production, rAAV have to be purified from the cell culture. This involves lysing the cells to release the rAAV and then isolating the rAAV from the rest of the cell debris and medium [77]. Because AAV capsids are extremely resistant, the purification process takes advantage of AAV's ability to withstand high temperatures, freeze-thaw cycles, and exposure to acids and organic solvents [31]. As briefly mentioned above, if using live helper virus, one method of viral clearance is through heat inactivation since the AAV capsid can withstand high temperatures. Since the field is relatively new and is still being developed, there is no universal protocol, and most manufacturers develop their own methods for their specific product. Despite this, all methods are variations of the following [14, 59]: The producer cells are lysed either through chemical means (lysis buffer) or through mechanical means. Next the lysate undergoes benzonase treatment to remove nucleic acids. Finally, the rAAV particles will be isolated by chromatography, filtration or ultracentrifugation using an iodixanol or cesium chloride (CsCl) density gradient, or some combination of the three [78]. Satkunanathan *et al.*, [30] describe their purification protocol in some detail where they begin with a freeze-thaw cycle (5x) followed by centrifugation of the cell debris at 2000 g. Following this, the supernatant was filtered through a 0.45 μm filter and was further concentrated by chromatography. Khan *et al.*, [79] describe a serotype-specific method (AAV2) using a heparin affinity column, since AAV2 naturally binds to the heparin sulfate surface receptor.

Irrespective of the exact protocol, it is important to purify the product based on density so that empty virions can be separated from those containing the vector genome [80]. Empty capsids pose a threat to the efficacy of the treatment, as they could

competitively bind to cellular receptors and inhibit the transduction of therapeutic vectors [79]. One way to measure the ratio of empty to full particles is through a 2% uranyl acetate stain, which is taken up by empty particles differently than full ones allowing quantitation using electron microscopy [55]. Seeing how there is no current production technology that does not produce empty particles as a by-product, purification procedures that remove empty particles from full particles are vital. However, while the production of empty particles is inevitable, many purification techniques have been improving upon the ability to limit this number in the final product. Grieger *et al.* [55] designed a purification strategy, which they claim to be universal for all serotypes and results in a 10:1 full to empty ratio. They propose that ion exchange chromatography could be used to purify all serotypes by modifying certain parameters. Their protocol also employed a perfusion technique where vector was purified from the medium every 24 hours and only after 120 hours was it extracted from the cell pellet. This resulted in 6-fold higher yields. Therefore, it is quite clear that purification strategies can play an equally important role in maximizing the final yield. A 2010 study [81] came to similar conclusions when they reported that using iodixanol gradient centrifugation resulted in higher yields, purity, and transduction efficiency when compared to vectors purified by CsCl gradient, while successfully eliminating empty capsids.

Once the vector has been properly purified, it is customary to titer the final concentration. The best ways to do this involve southern blots, dot blots, or quantitative PCR (qPCR) [79]. For example, using qPCR a stock can be quantified by removing any unencapsidated DNA with benzonase treatment after cell lysis. Following the removal of nucleic acids, the AAV capsids are disassembled with proteinase K thus releasing the vector DNA, which can easily be measured with the proper primers [30]. It is also possible to measure the total amount of vector genome replicated by the cell by foregoing the initial benzonase treatment.

It is important to emphasize that vectors destined for the clinic must be manufactured and released under current Good Manufacturing Practices (cGMP) following a series of stringent guidelines issued and updated by the regulatory authorities.

Conclusion and future trends

Many aspects of this review underline the tremendous potential of AAV as a gene delivery system and the very active research and development activities focusing on translation to the clinic. While there are still several obstacles to overcome, as have been noted above, AAV-mediated gene therapy will likely emerge as a modern and exciting treatment option for many previously untreatable or alternatively treated diseases in the very near future. Very promising options for large-scale manufacturing build on advanced development for packaging or stable producer cell-lines amenable to high yield and cost-effective production of functional and safe AAV vectors. It is expected that a number of AAV-mediated gene therapy will get marketing licenses for the treatment of a variety of diseases. This will likely open the doors and increase funding for many other gene and cell therapy using alternative vectors. Therefore, the importance of tackling the problems associated with AAV-vector design, production platforms, process intensification and robustness as well as quantitation and quality control of AAV-products cannot be overstated.

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CONFLICT OF INTEREST STATEMENT

The authors report no conflicts of interest.

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