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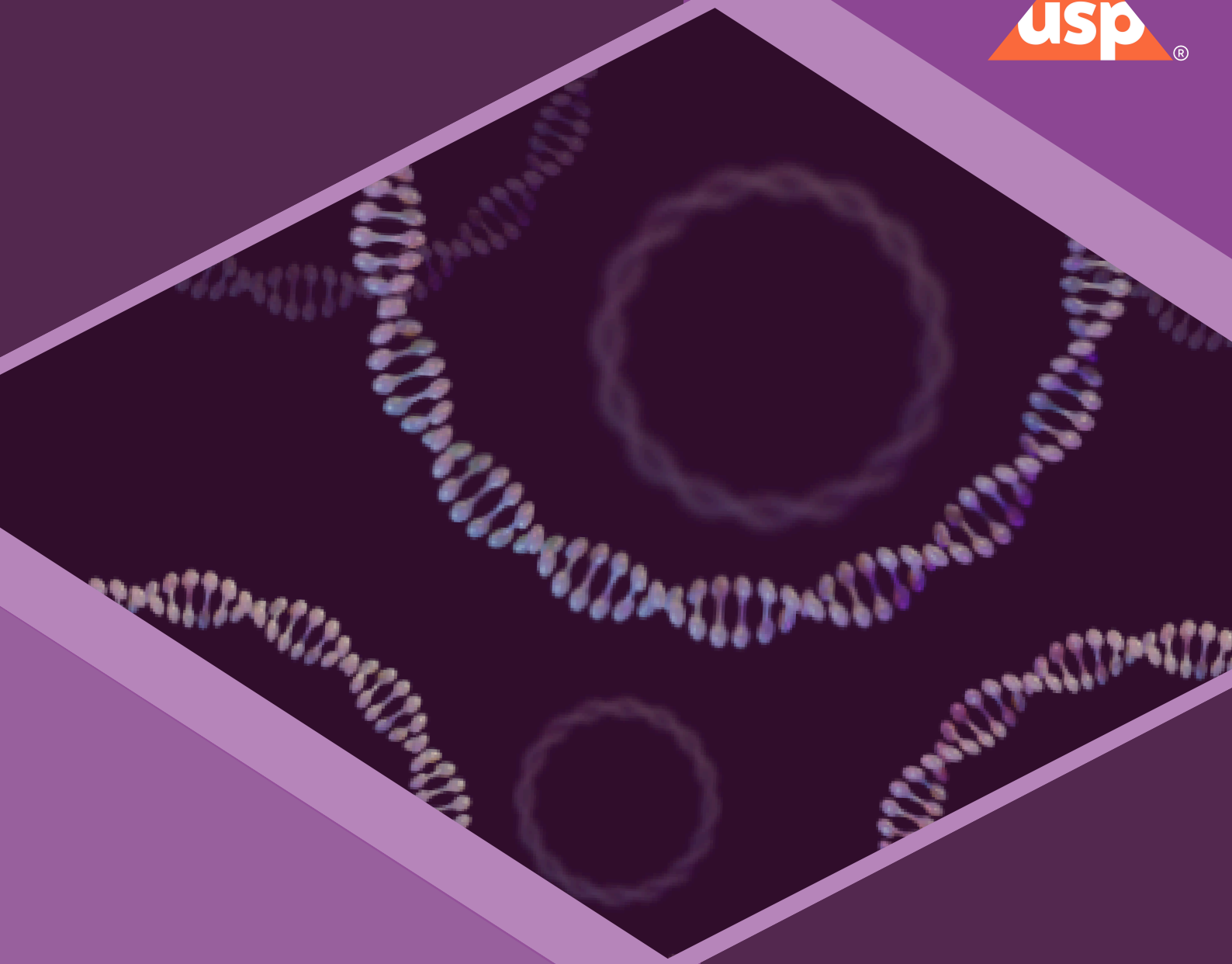

Gene therapy analytical testing

Taylor & Francis

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ABOUT REGMEDNET

RegMedNet is a community site that unites the diverse regenerative medicine community. Through partnership with our sister journal, *Regenerative Medicine*, we seek to educate and inspire to help this exciting field move forward at an even faster rate.

On RegMedNet, you can find free educational webinars, expert opinion and insight, and exclusive peer-reviewed journal articles, as well as the latest news and advances. You can watch, listen and read about every step in the regenerative medicine and cell therapy pipeline, from development, clinical trial and manufacture to regulation and commercialization, all in one place.

FOREWORD

We are pleased to present you with this eBook to conclude our Spotlight feature on gene therapy analytical testing.

The use of plasmid DNA is evolving in the field of advanced therapies and medicinal products, serving as a starting material, the foundation for many biopharmaceutical drug products. Its applications span from cell and gene therapy to vaccine development, offering unprecedented approaches to address rare diseases and debilitating conditions.

As with any component of drug development, it is essential to maintain high standards of plasmid quality to ensure the safety, efficacy and reduction of adverse effects of the final product.

With the increasing demand for these therapies, there is a corresponding need for high-purity standards and purification processes during the production and manufacture of plasmid DNA.

However, there is currently limited guidance and standardization regarding control strategies, production procedures, analytical testing and plasmid DNA specifications among suppliers.

This eBook will explore the necessary quality standards for plasmid DNA and the strategies and resources available to improve the standardization of plasmid DNA as a starting material in advanced therapies, particularly in the development of gene therapies and adeno-associated virus-based gene therapies.

We hope you enjoy reading these expert insights with us.

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The roles, rising demand and purification optimization of plasmid DNA

Christie Childers is a Senior Scientist at Astrea Bioseparations (Cambridge, UK), leading the next-generation development of nucleotide purification solutions based on novel nanofiber technology. Her journey began in the field of molecular biology at Carleton University (Ottawa, Canada), where she received a PhD investigating enzyme purification and post-translational modification for metabolic regulation.

Christie now has four years of industry experience in downstream process development with a past focus on customer-based research and development (R&D), process scale-up and technical transfer of workflows at CPI (Darlington, UK). Christie now leads and develops nanofiber-based pDNA and mRNA processes at Astrea Bioseparations.

In this interview, Christie Childers dives into the evolving roles of plasmid DNA (pDNA) in gene therapy, the growing demand for high-purity material and the purification standards shaping the field. She also shares insights into her work with novel nanofiber adsorbents designed to optimize pDNA purification.

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1 What roles do pDNA play in gene therapy product development and manufacturing?

pDNA plays a key role in nearly all gene therapy products. It serves as the starting template in raw materials for *in vitro* transcription (IVT) manufacturing, viral vectors and other gene therapies. Additionally, pDNA is being developed as a drug product in DNA-based therapies, such as vaccines.

2 How has the increasing demand for gene therapy products impacted the need for high-quality pDNA?

In 2020, the urgent need for high-capacity, high-quality pDNA production became very clear, as hundreds of biopharmaceutical companies transitioned from clinical development to commercial-scale manufacturing in response to COVID-19 [2]. The integrity of this starting material has been shown to be critical to ensuring the safety and efficacy of final therapies, and as such, its industry, efforts are underway to reduce variability in pDNA (whether that template is E. coli generated or synthetic) while increasing analytical stringency wherever possible. As more companies turn to gene therapies for therapeutical applications, the demand for high-quality pDNA continues to grow.

3 What are the key challenges in pDNA manufacturing and how can they be addressed?

Many challenges in plasmid production can be addressed through strict upstream control, either through controlled fermentation processes or synthetic DNA production reactions. These approaches enable the production of high-quality, covalently closed circular plasmid (supercoiled plasmid) in an increasingly high titer, even before downstream clarification processes begin. This production control reduces target-related impurities, minimizing the need for very high-resolution capabilities.

Resolving product-related impurities can lead

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to low yields, particularly when separating target impurities with similar chemistries. Therefore, reducing this processing requirement supports yield maintenance. The rapid success in generating high-quality pDNA at high starting titers, however, has emphasized new challenges: downstream binding capacity, processing flow rates and pressure thresholds are increasingly becoming the rate-limiting factors in scaling production.

There is also a growing need for ultra-scaled-down methods to support screening of new plasmid targets and processes. Some groups require hundreds of R&D-scale plasmid purifications, which can be time-consuming and labor-intensive. Providing small-scale screening methods that can then be directly scaled up to manufacturing capacity will be an advantage moving forward.

4 What standard methods are used to assess pDNA purification throughout the manufacturing workflow?

Many methods are used to ensure pDNA products meet the critical quality attributes required for their intended application. While some small molecule methods that are well established under Good Manufacturing Practice conditions are appropriate, assessing larger biomolecules presents different challenges. For example, certain plasmids will become too large for standard HPLC columns, requiring larger flow paths to accommodate their size.

Restriction mapping and agarose gels are still considered the standard for confirming plasmid identity and purity. Other techniques such as capillary electrophoresis, HPLC and ELISA are used for isoform, host cell RNA, and host cell protein characterization. GMP-grade pDNA must also meet standard endotoxin, bioburden, and mycoplasma requirements as outlined by the US Pharmacopeia (MD, USA) standards [5].

5 Can you share insights on optimizing scalable pDNA purification using novel nanofiber adsorbents?

Nanofiber adsorbents such as pDNA**HERO**® provide a more open flow path for pDNA production. The benefits of this design are two-fold: there are no dead ends in the membrane — often a source of fouling that limits yields — and nanofiber allows for larger plasmids as companies expand the size of their target sequences. Additionally, the reduced running pressures allow nanofiber-based products to operate at higher flow rates, even when fully loaded with plasmid, due to their high binding capacities. This low-pressure processing is critical for accelerating plasmid purification workflows and reducing costly time in GMP manufacturing suites.

AstreAdept® nanofiber is also a robust solution, enabling scaled-down methods to be cleaned in place and reused repeatedly for extensive R&D screening. This durability is especially valuable in smaller-scale production cycles and for companies that choose simulated moving beds for continuous processing as their means to increase production yields as needed. These strategies are typically explored for smaller production batches, such as those required for personalized medicine.

6 How do you see the industry progressing toward standardized quality control for plasmids used in cell and gene therapies?

There is growing momentum toward standardized and automated lysis methods, with the ultimate goal of reducing hands-on

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time for plasmid production. This is already becoming a viable approach with the introduction of technologies such as the alkalizer and other in-line mixing methods [3, 4], which can help reduce variability in the downstream starting materials prior to clarification and capture. There is also increasing interest in more stringent at-line monitoring and *in silico* process monitoring to improve downstream quality.

As the industry grows, a hurdle for manufacturers will be identifying robust, cyclable manufacturing methods. Pump flow rates are likely to become a primary limiting factor, making it essential to maximize capacity through rapid clean-in-place protocols and the reuse of large-scale devices.

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The opinions expressed in this interview are those of the interviewee and do not necessarily reflect the views of RegMedNet, Taylor & Francis or US Pharmacopeia.

RegMedNet Spotlight

Addressing quality standards of plasmid DNA

Ensuring the quality of plasmid-based cell and gene therapy products

Plasmids are composed of DNA sequences that encode key proteins required for the production of cell and gene therapy products. As a critical starting component, plasmids must meet quality standards to ensure product safety and efficacy. There is, however, a lack of commercially available plasmid standards, both in physical reference materials and in documentary guidelines, leading to challenges in standardization such as:

- Limited guidance on control strategies
- Unharmonized production procedures
- Unstandardized analytical testing methods
- Variations of plasmid DNA specification among suppliers

Establishing clear guidelines is crucial for:

- Improving plasmid-based gene therapy products
- Assisting manufacturers in aligning quality standards of starting materials
- Ensuring the safety of gene therapy products

Ensuring quality: plasmid purification and testing

The quality of plasmid DNA has been shown to have a direct impact on product quality.

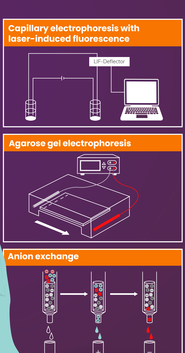
The conformation of the plasmid DNA is one of the most important quality attributes, with supercoiled plasmid being the most desired form.

Nicked **Supercoiled** **Linear**

Prior to production, manufacturers are required to assess the topology of their plasmid starting material to ensure it meets their quality standards.

The three most common methods for assessing plasmid topology are:

- Capillary electrophoresis with laser-induced fluorescence (CE-LIF)
- Agarose gel electrophoresis
- Anion exchange chromatography



Residual plasmid DNA as a process-related impurity

While plasmids are essential in early manufacturing, residual plasmid DNA is an unwanted impurity in the final therapeutic product. Residual plasmid DNA is known and shown to:

- Pose a safety risk
- Trigger unintended immune responses
- Transfer antibiotic resistance genes
- Integrate into a patient's genome

To minimize plasmid-related impurities, downstream purification processes, such as endonuclease treatment and chromatography, are commonly used to remove unwanted DNA fragments. However, to ensure the safety of the product, tests are required to analyze the amount of residual DNA in drug products. Typically, either quantitative PCR (qPCR) or digital droplet PCR (d(d)PCR) are used for this assessment.

Enhancing quality standards for gene therapy

To aid gene therapy manufacturers, USP has developed physical reference materials and a documentary reference standard. The physical reference materials will serve as standards for residual plasmid DNA quantification, qualification and plasmid topology assessment.

USP Standards for plasmid DNA

USP has developed the *General Chapter <1040> Quality Considerations of Plasmid DNA as a Starting Material for Cell and Gene Therapies*. This chapter was collectively drafted by group of experts to provide best practices on sourcing, qualifying and testing plasmid DNA.

Plasmid for Qualification (topology assessment)

- Six plasmids ranging in size from 4.2 kb to 12.3 kb, ensuring all customers have a plasmid that matches the size of the plasmids they use
- These reference materials can serve as controls to support manufacturers in the qualification and validation of different DNA topology methods, as each plasmid will be assessed by CE-LIF, Agarose Gel Electrophoresis and Anion Exchange Chromatography

Plasmid for Residual DNA Quantification

- Consists of linearized plasmid DNA with common target regions for residual plasmid PCR methods
- Serves as a positive control for dPCR and qPCR methods when quantifying residual plasmid DNA
- Functions as a standard curve calibrant in qPCR assays
- Well-characterized using multiple analytical methods, including dPCR, to ensure accuracy and reliability

Learn more about USP gene therapy solutions:

Gene therapy analytical guide
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RegMedNet Spotlight

Perspective
SPECIAL FOCUS ISSUE: The Exciting World of Oligonucleotides

Bioanalysis

Bioanalysis of adeno-associated virus gene therapy therapeutics: regulatory expectations

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The number of gene therapy (GTx) modality therapies in development has grown significantly in the last few years. Adeno-associated virus (AAV)-based delivery approach has become most prevalent among other virus-based GTx vectors. Several regulatory guidelines provide the industry with general considerations related to AAV GTx development including discussion and recommendations related to highly diverse bioanalytical support of the AAV-based therapeutics. This includes assessment of pre- and post-treatment immunity, evaluation of post-treatment viral shedding and infectivity, as well as detection of transgene protein expression. An overview of the current regulatory recommendations as found in currently active and published draft US FDA and EMA guidance or guideline documents is presented herein.

First draft submitted: 31 May 2019; Accepted for publication: 6 September 2019; Published online: 25 October 2019

Keywords: gene therapy • genome integration • immune response • infectivity • neutralizing antibody • total antibody • transgene protein • viral vector shedding

In recent years a variety of gene therapy (GTx) approaches using viral vectors to treat a variety of conditions have been quickly gaining momentum. Many of these gene therapies are aimed at treating rare genetic conditions by introducing a functional copy of a defective gene. Application of these approaches presents an opportunity for a transformative, potentially curative treatment able to correct the root condition caused by a genetic deletion or mutation in a critical gene. In addition to this increase in development of GTx for the treatment of various rare disease conditions, other applications have been increasingly explored, including treatment of cardiovascular diseases [1], Alzheimer, Parkinson's and other chronic life threatening conditions [2,3].

Multiple viral vectors have been investigated for GTx delivery, including adenovirus, lentivirus and many serotypes of adeno-associated virus (AAV) [4]. Due to multiple factors AAV-based delivery has taken the leading position in the field, with more than 130 individual clinical trials currently registered on the ClinicalTrial.gov. AAVs are nonenveloped, single stranded DNA viruses that belong to the parvovirus family. Serotypes of AAV viruses package a relatively small single stranded DNA genome ranging from 4.7 to 5 kb [5] with a relatively simple genome that encodes three capsid proteins: VP1, VP2 and VP3. Wild-type AAV is nonpathogenic and requires a helper virus, for example, adenovirus, for efficient replication to occur. Without a helper virus, AAV vectors will infect a cell, causing a latent state infection with an episomally maintained genome, but viral replication will not occur. Many natural serotypes of AAV have been identified including human and nonhuman primate as well as avian species specific [6]. Several subtypes have been particularly attractive for GTx development, including AAV1 through 9 and other *in vitro* genetically modified serotypes. AAV serotypes can infect dividing as well as quiescent postmitotic cells, which presents an opportunity for long-term expression of transgenes encoded in the viral DNA, without the requirement for target cells to be actively dividing. Currently, two GTx-based therapeutics have been approved in the EU: an AAV-based treatment of lipoprotein lipase deficiency (UniQure: Glybera, taken off market in 2016) [7] and lentiviral based *ex vivo* GTx for treatment of adenosine deaminase severe combined immunodeficiency (ADA-SCID, GlaxoSmithKline, Strimvelis) [8]. In addition, LUXTURNA®, an AAV-2 vector-

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based therapy was approved in the USA for treatment of vision loss due to balletic RPE65 mutation-associated retinal dystrophy [9]. In the coming years, there is every likelihood that multiple other AAV-based treatments will file for approvals.

Regulatory guidelines have been provided to the industry by both US FDA and EMA to describe general principles of GTx development, including questions related to nonclinical and clinical evaluation, long-term monitoring for serious adverse events, follow-up studies and product quality-related topics [10–15]. Additionally, several indication specific guidelines have been published by the FDA, including GTx development for treatment of retinal [16], hemophilia [17], and rare diseases [18]. However, many questions related to the bioanalytical assessments conducted during development of viral delivery of GTx modalities remain. These and related questions pertinent to the development of AAV-based GTx are reviewed herein with the focus on presenting the current regulatory recommendations based on information available from active or draft FDA and EMA guidance or guideline documents. As many regulatory guidance documents exist to describe development of genetically modified cells and other than AAV viral vector-based treatments, these will remain outside of the scope for the present review manuscript.

Detection of anti-AAV therapeutic immunity

Immunogenicity responses to the components of AAV therapeutic

A significant body of information has been accumulated regarding immunogenicity risks associated with viral vector-based GTx modalities. Immunogenicity concerns include those related to the anticapsid protein and antitransgene protein responses. Both may play a significant role in the success or failure of a GTx-based treatment. For both antivector and antitransgene protein immune responses a potential induction of humoral or cellular-based responses are possible. Relatively high prevalence of antivector antibodies has been described in general population, including healthy individuals. The prevalence of these antibodies, both neutralizing and non-neutralizing, has been reported to be as high as 50% or more in the individual donors tested [19,20]. Antibodies with no clear ability to inhibit viral transduction are typically referred as total antibodies (TAb, also referred to as binding antibodies) while inhibiting antibodies are described as neutralizing (NAb). In addition to neutralizing antibodies, existence of other matrix factors able to negatively impact cellular uptake and/or inhibit transgene protein expression have been proposed [21]. Up until now, limited correlation between TAb and NAb titers have been reported in the literature, although it is reasonable to expect that a higher TAb titer sample should contain some degree of neutralizing activity against viral transduction [22]. Recently, it has been shown that antibodies that bind AAV capsid, but do not have neutralizing activity may enhance transduction of the liver cells [23]. The study highlights the need for a greater understanding of the type of pre-existing anti-AAV capsid antibodies and their impact on the treatment and importance in selecting patients.

The degree of reported pre-existing TAb and NAb immunoglobulins in human subjects prior to GTx treatment depends on several parameters, including the AAV serotype in question, populations tested and specifications of the assay used to detect antibody presence. A variety of reasons for the diversity of anti-AAV immune response have been proposed, including living conditions, population density, hygienic conditions and quality of the health care. A highly diverse geographical distribution of the prevalence of anti-AAV immunity, including neutralizing antibodies, has been presented and discussed [19,20,24,25]. The viral serotype-based variation in pre-existing response has been reviewed elsewhere and is often used as the basis for GTx treatment serotype selection [19,25,26]. Whether measured as TAb or NAb, pre-existing antibody-based immunity against the administered GTx viral serotype may have a negative impact on treatment efficacy [13–15,17,18,27–29]. Regulatory guidelines state that the presence of GTx AAV serotype specific antibodies may prevent delivery of the transgene into the target cells (e.g., liver) therefore limiting treatment efficiency and efficacy [17]. As a consequence, regulatory guidelines recommend that a consideration should be given to the possible impact in subjects that are found to be positive for the pre-existing antibodies to the serotype of AAV used for the GTx [13]. An impact on both safety and efficacy may be anticipated and is recommended to be evaluated and discussed with the regulatory agencies. Importantly, it is recommended that patients that have pre-existing antibody titers above a predetermined and potentially GTx therapeutic specific value may need to be excluded from the treatment [17,18]. Specific recommendations on pre-existing titer values that should be used as a patient exclusion criterion and how they may be defined are not discussed in the currently available regulatory guidelines which may be a reflection of highly diverse nature of diseases intended to be treated by the GTx modality as well as the diversity of the assay types applied to detect NAb and TAb activities.

Therefore, a specific maximum NAb titer value may be assigned as a patient inclusion criterion. The specific cut-off may depend on the NAb assay characteristics, including type of cells used in the assay, multiplicity of infection value or use of a helper virus to improve assay sensitivity. It may be challenging to assign one universal NAb assay-based cut-point value without appropriate degree assay harmonization or standardization. Currently, no nonclinical model exists that would allow for an accurate prediction of pre-existing NAb or TAb impact in human subjects and direct translation of immunogenicity information from animal models to human subjects may not be linear [13]. It may still be possible to apply conclusions derived during preclinical development of the GTx therapeutic, particularly when the same or highly similar assays are used to evaluate presence of NAb or TAb activity in samples collected prior to the GTx application in animals, for example in nonhuman primate (NHP) studies. In the absence of other information, it might be possible to propose how low level NAb or TAb may be expected to inhibit or completely block virus transduction, particularly when applying NHP study data. For example, a broadly referenced NAb titer value of 1:5 was substantiated in the report by Wang *et al.* where a significant suppression of viral transduction and subsequent reduction in the production of the transgene protein (Factor IX) was described in cynomolgus monkeys with higher than the 1:5 threshold NAb titers [27]. Significantly higher NAb threshold values of 1:500 were reported elsewhere demonstrating high dependency of this critical parameter on the assay characteristics [30,31]. The importance of evaluating of anti-AAV NAb activity and its impact on the AAV treatment in large animals other than monkey models had been specifically noted [32].

In addition to pre-existing antibodies, the possible presence of pre-existing cellular immunity against the viral serotype of the GTx vector has been reported [33–35]. Although the exact degree of impact of pre-existing antiviral cellular immunity is not entirely clear at this point and requires further investigation, regulatory guidelines note a potential connection with the selection of GTx treatment route of administration and dosing regimen [28,29].

The host immune system is expected to produce a robust immune response to the nonhuman protein components of the AAV GTx vector. It has been broadly demonstrated that innate and adaptive immunity against GTx modality components, including antivector and antitransgene protein responses, may present a significant challenge for successful development of an AAV-based treatment [34,36]. Regulatory guidelines list both antivector and antitransgene protein responses as potential risk factors impacting GTx treatment safety and efficacy outcome [18]. Generally, early access to methods designed to measure immune response against GTx components early in the GTx development life cycle is recommended [18]. If assays are not available or fully validated at the start of study sample collection, the pretreatment (baseline) and post-treatment material should be stored at appropriate conditions, for example, frozen, before testing [12]. As the assays become available, a fit-for-purpose subject monitoring for the presence of neutralizing and non-neutralizing anti-GTx component antibodies throughout the course of a trial is suggested [18].

A prolonged exposure to transgene protein is expected postadministration of a GTx therapeutic. This is particularly important for patients lacking the endogenous protein (CRIM negative) as their immune system may recognize the transgene protein as foreign. It has been shown that immune responses in CRIM negative patients treated with a protein-based biotherapeutic can present a significant risk and needs to be strongly considered [37]. An immune response against transgene proteins may result in a highly undesirable autoimmune phenomenon and; therefore, a consideration for an assessment of antitransgene protein immune response is requested by the regulators [14]. The exposure to the transgene protein may continue for several years or potentially for the life of the patient. As a result, testing for antitransgene protein immune response may become an important post-treatment monitoring tool [38,39]. Both neutralizing and non-neutralizing antitransgene protein antibodies may impact the efficacy of the treatment either by blocking the specific protein activity or impacting circulating concentration of the protein by accelerating its clearance from the blood compartment. Regulatory guidelines highlight the need to understand both neutralizing and non-neutralizing antitransgene protein immunity as an important element when determining potential impact on treatment safety and efficacy [18]. At the same time, it has also been proposed that testing may be avoided when it is demonstrated that the antitransgene protein immune response is non-neutralizing, not targeting epitopes linked to the specific protein activity and; therefore, may not be impacting product efficacy [11]. Guidelines propose that, where available, neutralizing antibody response to transgene protein should be assessed based on the specific transgene protein activity evaluation [17]. For example, coagulation Factor VIII and IX activity assays can be used to determine impact of antitransgene protein antibody response in the case of GTx therapeutics design to treat hemophilia A and B conditions [40].

In contrast to the transgene protein, circulation of the free viral vector is not expected to exceed days or weeks although viral genetic material can be detected for up to several months postadministration [41]. Because a robust

postdose immunity against viral vector should be expected the value of detection of both TAb and/or NAb response may be questioned. The antiviral response information may still be valuable to understand potential hypersensitivity reactions observed immediately after the treatment or during later observation period and is indirectly proposed by the regulators [18]. As the current GTx treatment paradigm is commonly based on a single administration of the therapeutic, a possibility of repeat dosing remains a highly attractive option and it has been already applied to treat biallelic RPE65 mutation-associated retinal dystrophy [42]. Bilateral subretinal injections of AAV2-hRPE65v2 (LUXTURNA), an AAV2 vector-based therapy carrying gene of retinal pigment epithelial 65 kDa protein, were given to patients during two sequential separate procedures separated by 6–18 days [43]. As such an assessment of post-treatment immune response to both viral vector and transgene protein can be a significant element of the repeat treatment decision process as was pointed out in the Retinal Disorders Indication specific regulatory guidance [16].

Understanding of the potential of cellular antivector and antitransgene protein immune response to impact safety and efficacy of GTx treatment is viewed by the Regulators as a critical element of postdose immunity assessment of GTx products [13]. AAV viral protein processing by the transduced cells followed by presentation within the major histocompatibility class I complex was proposed as a mechanism of induction of a $CD8^+$ cytotoxic T-cell response which has a potential to eliminate virus carrying cells and result in a decline in the transgene protein expression [33–35]. It is recognized that the exact degree of impact of anti-GTx immune response on the safety or efficacy of the GTx treatment may vary from a transient response without any clinical significance to a severe life-threatening condition [11]. Therefore, periodic monitoring for cellular immune response is recommended with the frequency determination based on the anticipated impact and observed clinical signal [17].

Due to the potential of the anti-GTx immune response to impact both treatment safety and efficacy, regulatory guidelines recommend an extended follow-up and monitoring as part of the prolonged observation period that is sufficient to ensure that appropriate clinical signals are detected [11]. The monitoring may include evaluation of both antibody and cellular immunity against the transgene protein, particularly when clinical relevance between specific response type and clinical impact has to be established [11,12]. The exact duration of follow-up monitoring may greatly depend on the serotype of the virus vector, anticipated safety risks and treatment indication. In the example of GTx products designed to treat hemophilia conditions, a short-term monitoring of up to 2 years following GTx product administration is proposed to include antibody and cellular-based immunity to the vector, as well for the presence of neutralizing antitransgene protein specific antibodies (inhibitors). A long-term monitoring scheme (>2 years) should include presence of antitransgene protein neutralizing antibodies [17].

Looking beyond the clinical development phase, a post-BLA approval availability of an appropriate and specific companion diagnostic (CDx) assay to detect antivector immunity in potential human subjects is requested in the regulatory guideline documents [17,18,44]. Development of a therapeutic specific CDx should be conducted in parallel with the pre-approval clinical investigation of the GTx therapeutic allowing for a coordinated submission to regulatory agencies [17]. Industry continues to debate the appropriateness of selecting TAb versus a NAb method as part of the patient inclusion criteria. For both method types, an alignment is still needed to determine whether the regulatory guidelines describing development and validation of assays for detection of immunogenicity against therapeutic protein products apply when working on GTx supporting methods [45].

Detection of anti-AAV therapeutic immune response

The pharmaceutical industry has significant experience developing fit-for-purpose assays designed to detect unwanted immune responses to protein-based biotherapeutic compounds. Information can be found in several regulatory guidelines and industry White Papers [45–48]. Industry and regulatory agencies are generally aligned on the expectations for the requested analytical specifications, including assay sensitivity, methods used to calculate assay cut-point parameter, assay minimal dilution – among others [49]. Typically, a tier-based approach to detect antibody-based immune response is applied [45]. Using this approach, initial screening if conducted for putative total binding antibody is followed by antibody drug-binding specificity test. If the presence of therapeutic specific antibodies is detected and specificity of binding is confirmed, additional characterization of antibody specificity, including analysis for neutralizing antibody activity, follows. Specific details regarding validation of assays designed to detect antibody-based immune responses against biotherapeutics have been discussed in the industry White Papers as well as regulatory guidance documents [45,46,48].

Current GTx-focused guideline documents provide a limited level of detail regarding modality specific TAb and NAb assays while referencing protein biotherapeutics immunogenicity guidance documents mentioned above [18].

Development of appropriate assays designed to detect antivector as well as antitransgene protein antibody response may not require additional regulatory clarification although several assay specific questions remain, mainly whether the methodologies applied to assess unwanted immunogenicity against protein-based biologics are applicable in full when developing similar assays for a GTx therapeutic. For example, sponsors will need to determine whether a tier-based approach to assess immune response is appropriate for a GTx therapeutic, including assessment of antivector and antitransgene protein immune response or should the evaluation be focused on the detection of neutralizing activity only. Sponsors will also need to determine whether a statistically based assay cut-point calculation, which is required for protein biotherapeutics is applicable for a GTx modality, including anti-GTx NAb assay protocols. Other details to be discussed and agreed on include cell lines to be used in anti-GTx NAb protocols, acceptable NAb and TAb assay sensitivity and the nature of positive and negative suitability controls used in the assays. Overall, additional effort will be needed to standardize protocols applied to detect anti-GTx specific immune responses.

Current regulatory guidelines do not provide an in-depth comprehensive discussion of methods designed to evaluate innate immune responses. The innate pathway plays a critical role in the early non-specific response to a viral infection. Various cell types and cellular receptors, such as Toll-like receptors, may recognize and respond to the presence of highly structured viral particles [50,51]. The AAV type viruses have limited potential to interact with toll-like receptors although other mechanisms of innate anti-AAV virus response have been described and reviewed [52].

The Enzyme-Linked ImmunoSpot (ELISPOT) analytical platform was proposed for the purposes of screening peripheral blood mononuclear cells ability to produce INF-γ in the presence of viral and/or transgene protein generated peptides [17]. The ELISPOT methodology has been referenced as the main approach to evaluate cellular immunity against GTx components. Although currently, there are no regulatory guidance documents that provide agencies position on the ELISPOT method development and validation, industry guidelines are available [53,54]. Flow cytometric protocols designed to detect antigen specific T cells have also been developed and may be applied as an alternative approach to the ELISPOT platform [55,56].

Detection of AAV genome material

Exposure & biodistribution in clinical subjects

Biodistribution studies are often a requirement for preclinical studies in AAV GTx development; however, translating preclinical data into the clinic are recognized as being challenging [13]. Regulators require that sponsors of GTx trials understand the kinetics and load of AAV transgene particles shed from patients after dosing. These shedding assay requirements are discussed below. In comparison, a true biodistribution study is not commonly asked for or possible in clinical studies but is discussed in guidance documents [10,13,15]. Standard absorption, distribution and metabolism studies are not directly relevant for AAV GTx; however, either blood or other matrix samples can be used to assess the systemic persistence of the vector, along with potential environmental exposure to caregivers and the general population to the AAV transgene construct [10].

Assessment of shedding in clinical studies

In comparison to typical small and large molecule treatment modalities, traditional pharmacokinetic (PK) measurements using MS approaches are not appropriate for measuring viral GTx kinetics in patients. Instead a quantitative polymerase chain reaction (qPCR)-based assay needs to be developed to measure AAV GTx in patient blood samples. In addition, regulatory agencies require measurement of shed AAV particles from patients in a variety of matrix types, including whole blood, or plasma/PBMCs, saliva, urine, semen and stool depending on route of delivery. These are often required for part of an environmental risk assessment of the AAV GTx. In addition to providing information on the rate of detectable shed AAV, the data from these assays are informative on the kinetics of AAV clearance from these biological matrices. Whole blood samples, or blood components such as plasma and PBMCs, should be assessed to build a profile of clearance from circulation for each AAV GTx in lieu of a more traditional PK measurement. In several countries and regions AAV GTx fall under genetically modified organism regulations and as such the environmental risk of exposure to both caregivers, healthcare workers and the general population will need to be defined through an understanding of the levels of shed viral particles from study participants.

There are limited published guidance's specifically addressing regulatory agency expectations for shedding assays. In general, the validation of these assays should be carried out as per the general biomarker and analytical assay guidance's that are available for the regulatory agencies that the sponsor proposes to seek approval from [57,58]. The serious long-term event monitoring guidance specifies a detection limit of 50 copies per microgram of genomic

DNA [14]. While that number can be calculated for tissue and cells such as PBMCs, it becomes a more difficult number to quantify for whole blood or urine samples. The sponsor is responsible for determining the lower limit of quantification (LLOQ) for the qPCR assays in each matrix to be assessed. Given the high sensitivity of qPCR assays, coupled with the increased specificity of a probe-based assay, it is reasonable to expect that most assays will approach or surpass this recommendation. Care should be taken during the development of the assay(s) to ensure that the extraction efficiency of the AAV GTx in each matrix is reasonable and well understood by the testing laboratory. Additionally, the assessment of PCR inhibitory effects of the matrix, buffers, and storage kit(s) should be investigated as part of assay development and be part of the validation package. A primer target that covers at least a portion of the inserted transgene should be used, in order to minimize potential false-positive numbers from wild-type AAV infections. While AAVs have been shown to be stable [59], short and long-term stability assessments in each matrix, as well as the impact on freeze–thaws should be established. It is reasonable to expect freeze–thaw assessments to be done early in assay development, whereas longer-term stability assessments can be carried out in parallel to assay development. Given the prevalence of AAVs in the general population, special care should be taken during assay development to build in multiple levels of negative controls throughout the extraction and qPCR assay. To avoid differences in manufacturing of preclinical versus clinical material, the clinical lots of AAV material should be used as the standards for the assay, as well as for the initial development and validation of the qPCR assay.

Given that a qPCR assay has the potential to detect fragments of DNA as well as the full AAV/transgene complex, most regulatory bodies will additionally expect a DNAse treatment of each sample type. This will allow for the differentiation between AAV protected transgene DNA versus fragmented single strand DNA in samples which could artificially increase AAV/transgene quantification.

Assessment of infectivity in clinical studies

As a component of the environmental risk assessment, regulatory agencies may request further characterization of the shed material for infectivity and growth. The EMA guidance [58] specifically requests that sponsors develop an infectivity type assay but indicate that if the LLOQ of that assay once fully characterized is found to be significantly higher than results found from shedding assays, that a sponsor may not need to run the infectivity assay. Since the assay may utilize the same end point qPCR as the shedding assays, it therefore may be recommended to develop both assays in parallel. Currently, no regulatory guidance exists requiring these cell-based infectivity assays to be validated to a specific standard. There are clear expectations for infectivity cell-based assays for other delivery modalities based on replication competent viral vectors [57].

The development of an infectivity assay is difficult, as AAV alone does not induce cytopathic effect in cell culture and while AAV may be internalized in cells, the virus may not be infectious. To assess infectivity, a susceptible cell line is treated with the shed material, in the presence or absence of a helper virus, incubated and then assessed for viral internalization using a qPCR-based assay, ideally using the same assay used to assess shedding. During the development of the infectivity assay, the cell line of choice, seeding density, and incubation time must be optimized, a limit of detection (LOD) and LLOQ established, the reproducibility of these results determined, and the stability of the infectious material assessed. Using a qPCR-based readout, PCR inhibitors should also be assessed and minimized.

Assessment of genome integration in clinical studies

The genomic payload of recombinant AAV vectors does not need to integrate into the host genome in order to be biologically active and the DNA remains primarily episomal. AAV are considered to be nonintegrating vectors in regulatory guidelines [10,13,15]. However, random integration events can be observed in AAV with a low frequency of between 0.1 and 1% of transduction events [60]. There are some studies that have shown increased liver tumors when neonatal mice were injected with AAV in association with integration into specific sites in the mouse genome that do not have a human homolog [61]. However, others have not found tumor induction when adult mice are treated with AAV [62–64]. Long-term follow-up of 135 hemophiliac dogs for greater than 10 years [65–67], and NHP for greater than 5 years [68] that were treated with AAV vectors replacing factor IX have not found any evidence of tumor induction. Additionally, there have been no reported incidences of AAV vector-induced neoplasia in humans in over two decades of AAV use in clinical trials. However, developers of AAV gene therapeutics should plan to discuss AAV genomic integration with regulatory agencies early in the development process to ensure there is no concern regarding the specific AAV construct or intended disease population that the vector will be used in.

Regulatory expectations for qPCR-based assays

To accurately quantify the amount of shed and/or infectious material as required by multiple regulatory guidance documents, a validated, quantitative qPCR assay is necessary. The Minimum Information for the Publication of Quantitative Real-Time PCR Experiments (MIQE) document provides methodology considerations for the design of qPCR assays and experiments [69,70]. When designing qPCR-based assays, nonspecific dyes or sequence-specific probes are available as reporters. Nonspecific dyes (such as SYBR Green, SYTO-13 and SYTO-82, etc.) intercalate into dsDNA, but could detect nonspecific amplification leading to concentration overestimation or false-positive signals. The use of fluorogenic probes leads to specific binding of the probe to the targeted sequence, providing greater specificity in qPCR amplification [71]. When designing qPCR assays, special consideration in primer and probe design should be given with regards to amplicon size, GC content and location [72]. When considering GTx constructs, the qPCR amplicon should include a portion of the transgene to verify the delivery of the genetic payload and avoid an over estimation based on the potential presence of empty capsids. After initial screening of potential qPCR assays, primer and probe concentrations should be optimized. Several approaches are available for optimization of qPCR assays, one of which is the use of full factorial design. This approach estimates the effect of each PCR factor on assay performance and may detect interactions between PCR factors while determining optimal reagent concentrations [73]. The optimal assay should be selected based on a combination of amplification efficiency, highest fluorescence values and lowest cycle threshold (Ct) counts. Amplification efficiency is determined based on the slope of the standard curve using the formula $E = 100 \times (10\text{-}1/\text{slope} - 1)$ [74]. The ideal slope for 100% amplification efficiency is approximately -3.3, but the acceptable range for a quantitative PCR assay is between -3.1 and -3.6 (90–110%) [70,74]. Sensitivity is critical to the quantification of shed material. Sensitivity should be assessed across a linear dynamic range ($R^2 > 0.98$) of ideally 5–6 $\log_{10}$ and determine the LOD and LLOQ and to verify the assay is sensitive enough to reach the 50 copies per µg of genomic DNA, as referenced above [69,75]. Assessing the linear range of the assay requires the development and characterization of an appropriate standard curve.

Developing a standard curve for GTx constructs, with respects to shed material, requires optimizing the standard curve in all sample type matrices. This will ensure accurate quantification in each sample type and help identify any matrix-induced effects during DNA extraction [76]. For initial primer/probe evaluation, linearized plasmid can be used; however, the use of plasmid for quantification of AAV material can lead to an overestimation of the titer [77]. For AAV constructs, standard curve development and final assay validation should be performed using encapsulated DNA, preferably using the same serotype capsid used in clinical dosing. For quantification of shed material, internal spike-in controls should be used to characterize extraction efficiency and evaluate PCR amplification quality and inhibition [71]. Multiplexing assays in a single well should be considered to evaluate the target amplicon as well as the internal control as this provides greater power to the qPCR analysis [69]. Validating a multiplexed assay requires evidence demonstrating that the presence and amplification of multiple targets in a single well is not impaired by the other assays and that the efficiency and LOD of the assays are the same as when the assays are performed on their own [69].

Specificity, reproducibility and robustness must be determined in order to validate qPCR assays. Specificity should be assessed using tools such as NCBI BLAST or equivalent technologies and should be assessed during optimization by testing target sequence in the presence of human genomic DNA [69,78]. Reproducibility experiments should be performed to verify the precision of results for the same method using the same samples performed by different operators and multiple instruments. If the assay is to be performed at multiple laboratories, reproducibility should be evaluated across all locations. A well-designed qPCR assay should demonstrate a percent CV less than 25% for these reproducibility experiments [74]. As a component of validation, robustness must also be assessed by testing different master mixes, different qPCR instruments, accounting for variation in assay run parameters (i.e., annealing/extension temperatures and times, etc.) and performing guard-banding experiments [79].

An alternate approach to standard qPCR assays is digital droplet PCR, which has become a more established and mature technology over the past several years. During a droplet digital PCR (ddPCR) assay, DNA and the target assay are dispersed into thousands of individual oil droplets where individual PCR reactions can occur [70,80]. This reduces the amount of background DNA in each reaction enabling greater detection of low copy amplicons. ddPCR has advantages over qPCR in that the technology provides an absolute quantification of copies/ml without the use of a standard curve, is less affected by sample inhibitors, and is considered more precise [81]. However, ddPCR is generally more expensive, has a smaller dynamic range, and has limited multiplexing capacity [81]. As

ddPCR is a relatively new technology, most of regulatory guidance's reference the use of qPCR for quantification of genomic material. We anticipate that as the use of ddPCR becomes more prevalent that this will change. However, if ddPCR is selected as the end point assay of choice we would recommend that, bridging studies be carried out to verify the assay of choice performs as well, if not better, when using ddPCR as compared with conventional qPCR.

Detection of transgene protein

As a growing number of GTx products enter clinical development, particularly in rare diseases [82], the need for transgene protein detection has become increasingly pressing as an important contribution to GTx development. While some regulatory guidance documents specifically mention the use of the transgene protein measurement in the context of preclinical toxicology or preclinical and clinical pharmacodynamics [10,15]; detailed guidance does not yet exist for clinical samples. Depending on the disease pathophysiology, some GTx program may use suitable functional end points or efficacy biomarkers instead of, or in addition to, transgene protein expression. Examples of such biomarkers are factor activity as primary end point in hemophilia GTx clinical trials [17], or serum phenylalanine levels in a mouse model of human phenylketonuria [83]. However, a more detailed discussion of considerations for biomarkers in GTx is beyond the scope of this article.

The objective of most AAV GTx approaches is gene replacement to achieve long-term stable transgene protein expression at levels that are therapeutic [39]. While scientific advancements are expected to be made also in diseases with multigene defects, current GTx approaches focus mainly on treating monogenic diseases, in other words, with a single-gene defect. Therefore, the aim is to achieve transgene expression of a single protein at an expression level resulting in meaningful clinical benefit.

Measuring the transgene protein can be a critical aspect of GTx development, both preclinically and clinically. Preclinically, this assessment contributes to the selection of drug constructs for further development, is recommended to help with setting a suitable dose for preclinical studies and to determine an initial clinical dose in a subsequent first in-patient trial [10]. Assessment of preclinical transgene protein expression profile is also recommended to identify the potential for induced toxicity if expression is too high or if aberrantly expressed in nontarget tissues [10]. In early clinical studies, understanding of the transgene protein expression contributes to dose selection and importantly enables correlation with effectiveness of the treatment and clinical outcome, as shown in GTx clinical trials for hemophilia B [84]. In a rare disease guidance document, the FDA lays out issues for evaluation and validation of surrogate biomarkers, including transgene protein [85]. In some GTx strategies, transgene protein expression may be pursued as a surrogate end point and if it is considered reasonable likely to predict clinical benefit, it may be used as a basis for accelerated approval.

Across this evolving field of GTx, there is a broad range of classes of transgene proteins requiring their detection including soluble proteins, enzymes, structural and membrane proteins as well as intracellular proteins located in specific subcellular compartments. Many of these proteins or even protein classes have not been encountered in bioanalytical laboratories. Furthermore, significant technical challenges can exist when the transgene protein and the endogenous counterpart are not fully identical but need to be analytically differentiated. Reasons for this difference could be mutations or other modifications in the endogenous protein leading to its absence or reduction in function. In addition, in some preclinical investigation, the human transgene protein derived from the clinical drug product needs to be differentiated from the respective endogenous protein in a preclinical species [86]. Furthermore, AAV packing size limitations of the plasmid-encoded vectors may result in the design of shortened, truncated transgene proteins compared with the endogenous form [87]. At last, depending on the etiology of the disease, these proteins need to be measured in a range of tissues and biofluids. For tissue-based transgene protein assays, like for any other tissue protein assays, optimized extraction conditions need to enable high and reproducible recovery [88], while being compatible with the downstream analytical method. A variety of technologies are required for transgene protein detection accommodating the above considerations. These range from ligand binding assays, western blots, tissue staining techniques, to more recently protein MS. The latter technique is particularly suitable for the quantification of tissue proteins [89,90]. For example, it has been shown to be able to quantifiably measure dystrophin protein in skeletal muscle tissue where previously only western blot methods were available [91,92]. However, it has become clear that to address the emerging, significant bioanalytical needs for transgene protein expression and associated regulatory expectations, improved and advanced bioanalytical methods are needed.

The expression of transgene protein in patients can be compared with a control, normal population and depending on the disease and sample availability also to the patient's own baseline. Furthermore, depending on what is known about the course a disease takes in the absence of intervention (natural history) and the associated

Table 1. Analytical tests requested by the current guidance documents in support of GTx modality therapeutics.

Phase of GTx treatment or therapeutic development	Test type	Additional details	Request level	Ref.
Pre-administration	Antivector immunity	Total antibody response	Recommended	[13–15,17,18,27–29]
		Neutralizing antibody response	Recommended	
		Cellular response	Discussed	
Post-administration	Antivector	Total antibody response	Discussed	[12,18]
		Neutralizing antibody response	Discussed	
		Cellular response	Discussed, potentially critical	
	Antitransgene protein	Total antibody response	Discussed	[11,13,14,17,18]
		Neutralizing antibody response	Discussed, potentially critical	
		Cellular response	Discussed, potentially critical	
	Vector shedding	Vector shedding	Required until subject negative	[57,58]
	Infectivity	Infectivity	Dependent on shedding data	
	Transgene protein	Transgene protein	Discussed, possible biomarker	[10,15]
Follow-up	Antitransgene protein	Total antibody response	Discussed	[11,12,17]
		Neutralizing antibody response	Discussed, proposed	
		Cellular response	Discussed, proposed	
	Vector shedding	Vector shedding	Required if subject not negative during trial	[57,58]
	Infectivity	Infectivity	Dependent on shedding data	
Post-approval	Antivector CDx	Total antibody response or neutralizing antibody response	Required	[17,18,44]

CDx: Companion diagnostic assay; GTx: Gene therapy.

expression of the endogenous protein that is replaced by the GTx, a prospective natural history study can be conducted [18,93]. As outlined in the regulatory guidance documents, natural history studies can help design and conduct clinical trials. Target protein expression can be monitored through different phases or clinical stages and considers demographic, genetic, environmental and other variables that correlate with disease and outcomes in the absence of GTx treatment [93]. Target protein data can then contribute to building a correlation between endogenous protein expression and function that will help with establishing a correlation of transgene protein expression and outcome in GTx clinical trials.

Regulatory guidance documents dedicated solely to the bioanalysis of transgene protein expression do not exist. The FDA document provides some guidance for analytical validation of surrogate biomarkers that include the transgene protein [85]. It states that analytical validation should be confirmed before starting the clinical trial and should evaluate several factors including assay sensitivity, specificity, range of results that can be measured, standardized methods of sample collection, shipment and preparation as well as reproducibility of the results. However, for more specific analytical guidance the FDA bioanalytical method validation and EMA bioanalytical guidance documents are applied where possible and appropriate to the protein class, detection method and importantly the use of the data (Table 1) [94,95].

Conclusion

Immune response potential against viral vector-based GTx modality therapeutics is highly unique and impactful. While pretreatment immunity against protein-based biotherapeutics is not commonly expected, it has been broadly shown that antiviral antibody and cellular immunity is highly prevalent and potentially detrimental for the GTx treatment. It is therefore imperative to understand and assess potential impact and ability to measure antivector immune response before treatment is initiated. Although antibody-based pretreatment immunity has been viewed as the main risk element, cellular immunity can also be considered during patient assessment prior to the treatment. Development of specific companion diagnostic tests, hence becomes an expectation for a successful approval and launch of a GTx treatment.

Due to highly foreign nature of the virus and potentially transgene product protein as well, post-treatment immunity can be expected. Hence, subject monitoring becomes an important element of the treatment protocol. Regulatory guidelines describe monitoring for the specific immune response to the virus and transgene protein.

Guideline documents and industry publications also focus on the need to assess potential cellular response and potential patient treatment implications.

Guidelines list immune response assessment is a part of the follow-up monitoring, particularly when there is evidence of clinical relevance between antiviral or antitransgene protein immunity and clinical impact.

Multiple countries and regions classify GTx therapies under genetically modified organism regulations. As such understanding the potential environmental risk of exposure to caregivers and to the general population by an AAV GTx is a requirement. Additionally, understanding the PK of systemically delivered AAV GTx requires additional whole blood, or blood component, assay development and modeling.

At last, transgene protein expression can be an important measurement for some GTx programs, both preclinically and clinically. Guideline documents and literature focus primarily on its use as a biomarker.

Future perspective

With an increasing number of GTx therapeutics in development, we believe that further alignment around regulatory expectations and requirements will be needed in the future. Questions related to the relevance of certain tests, the degree of analytical method fit-for-purpose validation, analytical data relevance to the patient selection and requirements for post-treatment monitoring will likely to be further discussed and refined. Although several GTx-related guidance documents are already available, additional GTx bioanalytical guidance documents may be needed.

Executive summary

- Pretreatment immunity to gene therapy (GTx) therapeutic can be expected and should be evaluated due to high potential to impact treatment outcome.
- Antibody based and cellular anti-GTx immunity have been detected pre- and post-treatment.
- Post-treatment immune responses to viral vector proteins as well as to the transgene protein can be expected.
- Shedding assays are required by health agencies for multiple matrices, as well as whole blood or blood components to assess environmental risk and pharmacokinetics.
- Detailed analysis of post-treatment antitransgene protein immune response can be critical to understand GTx treatment impact.
- Companion diagnostic assay-based assessment of pretreatment antiviral vector immunity is likely to be required after the regulatory approval.
- Transgene protein expression can be an important biomarker measurement for some GTx programs.

Financial & competing interests disclosure

All the authors are employees of Pfizer, Inc., and may own respective stock or other form of equity. The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

No writing assistance was utilized in the production of this manuscript.

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Editorial

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Bioanalysis

Complexity and diversity of bioanalytical support for gene therapy modalities

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First draft submitted: 18 August 2020; Accepted for publication: 29 October 2020; Published online: 26 January 2021

Keywords: AAV • adeno-associated virus • bioanalysis • gene therapy • GTx

Ex vivo and *in vivo* gene therapy (GTx)-based methodologies for treatment of various human conditions are experiencing a clear and dynamic renaissance, with several therapeutic modalities and platforms evaluated preclinically or already in patients. A significant number of gene therapies are developed to treat various cancers or rare genetic diseases. These revolutionary and potentially disease-modifying approaches to medical intervention are intended to work through recruitment of elements of the immune system to attack cancer cells or to correct the root of the underlying condition caused by a single gene defect with a single or limited number of treatments.

Although development of the viral vector delivery *in vivo* GTx modality was initiated almost a generation ago, only recently, and mainly due to the advent of adeno-associated virus [AAV]-based technology, has it shown a strong and real potential for practical application. AAVs are ssDNA containing, relatively small, nonenveloped parvoviruses that can be constructed to carry up to 5 kb transgene payloads [1]. AAVs are commonly viewed as nonpathogenic and can infect dividing and quiescent postmitotic cells in the absence of a helper virus to form an episomally maintained genome. A number of natural serotypes of AAVs that can infect human cells have been identified, with additional designer serotypes actively in development [2]. AAVs therefore offer a significant opportunity for an effective *in vivo* GTx intervention; an opportunity that has been very actively and broadly pursued by academia and industry investigators.

Chimeric antigen receptor expressing T-cells (CAR-T) CTx modality is an example of *ex-vivo* GTx that represents highly successful and promising approach to treat various types of oncological conditions [3,4]. Two CAR-T-based products have received required regulatory approvals (YESCART, KYMRIAH) [5,6]. Genetically modified T-cells are designed to carry a CAR protein that binds to a tumor cell expressed target [3,4]. Additional intracellular domains are added as part of the CAR construct in order to increase CAR-T cytotoxic potential. Such complexity is achieved by employing various *ex vivo* GTx techniques, which modify patient or donor T-cells with either lentiviral, onco-retroviral systems or by applying nonviral gene transfer methodologies [3,7–9].

As one might expect, the high complexity of GTx modalities aligns with a significant increase in expectations and requirements for the bioanalytical evaluations requested in support of nonclinical and clinical studies. The diversity and broad nature of questions, methods and reported data are noteworthy. In the case of GTx modality, therapeutic and bioanalytical support may include detection of the viral genome in circulation or in tissues (particularly during nonclinical evaluation), expression of transgene and transgene protein and/or its activity, evaluation of binding and neutralizing antibody-based as well as cellular immune responses against viral capsid and transgene protein [10–14]. Safety monitoring may require evaluation of GTx genome integration, shedding and infectivity of the virus post dose [15,16].

To enable adequate bioanalytical support, there is a clear expectation for access to a broad range of analytical platforms and methodologies. Notably, a range of bioanalytical methods may be applied to address a given objective. For example, expression of transgene may be evaluated by detection of mRNA, transgene protein by either ligand binding assay (LBA) or LC–MS protocol or by detecting corresponding biologic activity. This diversity is in stark contrast with bioanalytical expectations for a typical protein-based biotherapeutic or a small molecular weight chemical compound.

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The diversity of analytical platforms applied in support of GTx and CTx modalities cuts across many platforms and often includes PCR and LC–MS based protocols, LBA and various cell-based methodologies [10,17]. Access to laboratories with a broad range of bioanalytical capabilities is therefore highly desirable to ensure successful support of a GTx or CTx modality therapeutic.

Complexity of questions and methodologies is paralleled by the complexity of the regulatory landscape that is clearly in development and still forming. Guidelines have been published by the US and European agencies to reflect on the general principles of GTx and CTx development. These cover topics related to nonclinical, clinical and post-approval phases of development [12–14,18–20]. Separate regulatory documents have been made available to present regulatory agencies' position on treatment of specific indications, including retinal, hemophilia and rare diseases [21–23]. In some cases, researchers may be able to apply agency recommendations made for protein-based biotherapeutics. For example, evaluation of anti-transgene protein and possibly anti-viral capsid immunogenicity responses may be conducted based on the recommendations described in previously issued US FDA and European Medicines Agency guidelines [24,25]. Evaluation of anti-GTx immunity also has a strong connection with a likely needed companion diagnostic or similar test designed to detect pre-existing anti-viral capsid antibodies, which can be requested in order to determine whether a patient can be admitted for the treatment. Such connection between pre- and post-approval phases of therapeutic development is quite unique to the GTx modality. The regulatory landscape may be incomplete and lacking some of the guidelines at this point. Such perceived gaps include lack of guidelines that would describe regulatory expectations for bioanalysis of transgene protein expression by either LC–MS or other methods and guidelines on the value and methodologies to conduct cellular immune response assessment. Industry white papers are available to describe methods for detection of cellular immunity [26]. A general position of regulatory agencies may be available although with a limited description of bioanalysis specific questions, for example in the guidance documents describing shedding and infectivity evaluations [15,16].

Focusing on the viral vector delivery GTx and to understand current clinical development landscape of the modality, information available on the ClinicalTrials.gov website was searched in the early 2019 based on the following criteria [27]:

- Keywords applied: 'gene therapy' AND 'virus' + 'AAV' OR 'adeno' OR 'HSV' OR 'lentivirus';
- Items excluded: cell-based therapy studies, antiviral treatment-focused studies;
- To report on the indications and viral vector type, only studies with unique therapeutic were retained.

The search produced 173 unique items (studies) with a significant majority (~72%) registered as early Phase (I/II) clinical investigations. Several studies were identified as Phase III or II/III (total ~10%) and a very limited number were listed as Phase IV (~1%). The majority (>75%) of the individual sponsors were found to conduct between one and three studies each with only a handful of sponsors supporting greater than six studies at the time data were collected. In the vast majority of cases (~75%), GTx therapeutics were AAV based, including AAV2, 5, 8 and 9 or a recombinantly produced AAV serotypes. Other viral vectors, including adenovirus and herpes simplex virus, were fond to be investigated for treatment of oncology conditions.

Approximately a half of the ongoing studies were designed to investigate GTx-based treatment of rare diseases, including hemophilia A and B, muscular dystrophies, mucopolysaccharidosis and Leber hereditary optic neuropathy. Oncological conditions were listed as the primary indication in approximately 20% of protocols. Importantly, nonhereditary conditions such as Alzheimer's disease, Parkinson's disease, heart failure, cardiomyopathy, myocardial ischemia or rheumatoid arthritis also appear on the list of potential GTx modality-based indications [28–30].

It is reasonable to expect that the number of GTx and CTx-based therapeutics will continue to grow quickly in the future. Significant complexity and diversity of bioanalytical support expectations related to *in vivo* and *ex vivo* GTx modalities require concerted and expedited effort to provide an aligned position accepted by the regulatory, industrial and academic participants. Many considerations related to the bioanalytical support of GTx and CTx modalities remain in development and will require additional clarification. Better understanding of required end points, methods and analytical platforms to use and expectations for the generated data will be key in ensuring successful advancement of the modalities.

Financial & competing interests disclosure

Boris Gorovits is employed by Pfizer, Inc., which is involved in development of gene therapy products. The author has no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

No writing assistance was utilized in the production of this manuscript.

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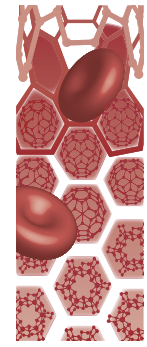

Characterization and quantification of adeno-associated virus capsid-loading states by multi-wavelength analytical ultracentrifugation with UltraScan

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Aim: We present multi-wavelength (MW) analytical ultracentrifugation (AUC) methods offering superior accuracy for adeno-associated virus characterization and quantification. **Methods:** Experimental design guidelines are presented for MW sedimentation velocity and analytical buoyant density equilibrium AUC. **Results:** Our results were compared with dual-wavelength AUC, transmission electron microscopy and mass photometry. In contrast to dual-wavelength AUC, MW-AUC correctly quantifies adeno-associated virus capsid ratios and identifies contaminants. In contrast to transmission electron microscopy, partially filled capsids can also be detected and quantified. In contrast to mass photometry, first-principle results are obtained. **Conclusion:** Our study demonstrates the improved information provided by MW-AUC, highlighting the utility of several recently integrated UltraScan programs, and reinforces AUC as the gold-standard analysis for viral vectors.

First draft submitted: 30 May 2023; Accepted for publication: 4 September 2023; Published online: 25 October 2023

Keywords: adeno-associated virus • analytical buoyant density gradient equilibrium • gene therapy • multi-wavelength analytical ultracentrifugation • sedimentation velocity • UltraScan • viral capsid quantification • viral vector

Gene therapy is gaining increased attention for its potential to treat or cure a broad range of genetic diseases. Over the past few years, several gene therapy products have been approved by the EMA and the US FDA, while thousands more are candidates in clinical trials [1–3], demonstrating their importance in today's therapeutic arsenal. One of the most established vector systems used is the adeno-associated virus (AAV) [4] due to its minimal pathogenicity and broad clinical applicability [5], resulting in three FDA-approved AAV gene therapies to date [2]. The AAV capsid consists of about 60 viral protein subunits made up of VP1, VP2 and VP3, typically in a 1:1:10 ratio [6]. After the capsid is formed, the recombinant AAV genome containing the gene of interest is packaged as ssDNA into the capsids, resulting in full AAVs. Notably, this method relies on the efficient transfection of three or more unique plasmids into a single cell, resulting in the production of full capsids as well as unwanted by-products, including empty and partially filled capsids.

Product-related impurities such as empty capsids, a critical quality attribute, can induce antigen presentation in the cell, leading to increased immune responses and proteasomal degradation of viral capsids, potentially leading to therapeutic failure [7], while also competing with full capsids for cell receptor entry. Therefore, it is essential to accurately characterize AAV-loading states, and methodology must be developed to: efficiently purify full capsids from empty, aggregated and partially filled capsids; quantify gene therapy drug formulations for the precise amount

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of full capsids compared with the other loading states; and identify contaminants. Previously developed analytical methods are limited in their ability to characterize or quantify product-related impurities [8]. Challenges encountered in the characterization of AAV formulations relate to the accuracy and resolution with which this quantification can be made, the throughput and cost of the analysis, and the sensitivity with which the measurement can be made [9]. Other AAV characterization techniques that focus on the characterization of the capsid-loading states include transmission electron microscopy (TEM), size-exclusion and anion exchange chromatography and mass photometry (MP) [10]. PCR and ELISA are used for the quantification of capsid titers [11]. Each method provides satisfactory results, but with various limitations [10,12,13]. The ability of analytical ultracentrifugation (AUC) to characterize and accurately quantify the composition of an AAV sample, in a single experiment with minimal sample preparation, is a significant advantage of AUC [14]. AUC can detect empty, partially filled and filled capsids, and contaminants such as free nucleic acids, DNA length heterogeneity in partially filled capsids, aggregates and full capsids with nucleotide loads fractionally greater than one genome [15]. In addition, the UltraScan software is designed to adhere to Title 21 of the Code of Federal Regulations Part 11 (21 CFR Part 11) requirements, allowing AUC to be integrated into a current GMP environment [16].

Current AUC methods primarily employ sedimentation velocity (SV) experiments for AAV characterization [14,17,18]. SV measures the transport of macromolecules dissolved in solution; derives the sedimentation and diffusion coefficients of all species in a mixture; and reports their partial concentrations, buoyant molar masses and shape factors. Despite the popularity of AUC in characterizing empty/full capsid ratios, several shortcomings exist. First, the most often cited SV design only measures between one and three wavelengths (generally 230, 260 and 280 nm) and at times is matched with Rayleigh interference experiments [17]. These methods overestimate filled capsids due to protein and nucleic acid spectral overlap [19]. Therefore quantification is always distorted when the DNA and protein extinction coefficients are not separated [20], which is only possible when measuring in multi-wavelength (MW) mode [21]. Secondly, SV is a low-throughput technique, and analysis is considered difficult, requiring significant training and time investment. Thirdly, SV experiments require relatively large amounts of sample (~0.5 ml/experiment, 0.3–0.9 optical density [OD] at 260–280 nm). In an effort to reduce sample requirements, analytical band centrifugation has recently been explored as an alternative to characterize AAV composition [22,23].

In this study, we report improvements to AUC methods used for the characterization and quantification of AAVs. Specifically, we applied MW analysis to SV and analytical buoyant density equilibrium (ABDE; sometimes also called density gradient equilibrium) experiments with AAVs. With the availability of new and improved AUC instruments, MW analysis has been demonstrated to be a powerful new tool for the characterization of the interactions between two or more molecules with distinct absorbance profiles [19,24–28]. AAV consists of a protein coat and encapsulated DNA, two molecules with unique but overlapping chromophores. Hence MW-AUC is ideally suited to characterize and quantify the composition of AAV samples. MW-AUC methods take advantage of the optical system in the latest Beckman Optima™ analytical ultracentrifuge and new software modules developed for UltraScan [29]. The value of SV analysis performed at multiple wavelengths, and manually analyzed, in a labor-intensive approach was previously demonstrated for the analysis of AAVs by Maruno *et al.* [22,24]. Richter *et al.* also demonstrated the ability of MW-AUC to obtain DNA insert length and derived wavelength-specific correction factors for the respective insert size, and compared their results with those from several characterization methods [18]. Recently developed UltraScan software modules now largely automate the MW-AUC analysis workflow, reducing the requirement for training and time spent analyzing AUC data [16,21,30–32].

MW-AUC offers a second spectral dimension for the separation of macromolecules in solution, in addition to the traditional hydrodynamic separation, which can resolve and identify loading states, distinguish full capsids from contaminants and resolve unique species with high precision. SV experiments provide separation based on mass, size, anisotropy and density. SV offers access to a large particle size range and excels by detecting even small amounts of contaminants. ABDE experiments separate AAV-loading states in a CsCl gradient based only on their density. ABDE experiments offer high resolution, sensitivity and throughput, with minimal sample requirements and rapid data analysis [31,33,34]. Below, we apply MW capabilities to SV and ABDE experiments for the analysis of AAV capsid-loading states and compare our results with TEM and MP measurements. We show that both MW-AUC methods achieve excellent agreement with TEM and MP, although AUC offers improved statistics due to bulk observation and enhanced resolution while reducing sample requirements.

Materials & methods

AAV production

AAV8 were prepared by the Gene Vector Core at Baylor College of Medicine (TX, USA) using a published method [35]. HEK293T cells were maintained in DMEM (cat. no. CM002-050, GenDEPOT, TX, USA) supplemented with 10% fetal bovine serum (Avantor Seradigm, PA, USA) and antibiotic–antimycotic (cat. no. CA002-100, GenDEPOT), in 15-cm dishes at 37°C, 5% CO_2 in a humidified CO_2 incubator. AAV was packaged by a three-plasmid transient transfection with iMFectin Poly DNA Transfection Reagent (cat. no. I7200, GenDEPOT). A total of 8 µg of DNA was combined with iMFectin, and 1 ml of the DNA cocktail was overlaid per dish. Cell-associated and media-secreted AAV were collected separately 72 h after transfection. Cell-associated AAV was recovered by cell lysis using 5% deoxycholate and subsequently treated with DNase I and RNase. Media-secreted AAV was precipitated with 40% PEG8000 supplemented with 2.5 M NaCl to a final concentration of 8% PEG. Both AAV fractions were combined and purified by CsCl ultracentrifugation as described by Ayuso *et al.* [36].

AAV9:CAG-GFP was generated at the CLOVER Center, Caltech (CA, USA), by triple transfection of HEK293T/17 cells (American Type Culture Collection, VA, USA; cat. no. CRL-11268) cultured in 15-cm dishes [37,38] and purified using a precipitation-based method. Briefly, 3 days after transfection, the producer cells were lifted by adding 10 mM EDTA to the media. After being spun down at 2000 × g for 10 min, viral particles in the cell pellets were purified with the AAVPro purification kit (TaKaRa Bio, CA, USA, cat. no. 6675) according to the manufacturer's instructions. AAV9:CAG-mNeonGreen samples were produced in HEK293T/17 cells cultured in 15-cm dishes with the triple transient transfection method and then purified by iodixanol gradient ultracentrifugation as previously described [37]. Virus titers were measured using qPCR targeting the WPRE region of the viral genomes [37].

Empty AAV5 was generated by Pharmaron (Liverpool, UK) using the HEK293 cell line and triple transfection. Following harvest, the feedstock was clarified and subsequently purified. The purification process consists of three chromatography steps: affinity chromatography, cation exchange chromatography and anion exchange chromatography.

Electron microscopy

TEM analysis for AAV8 BCM #1 was performed by the Baylor College of Medicine CryoEM Core. Samples were stained on Quantifoil 2/2 200Cu^+ 8-nm ThinC grids (Quantifoil Micro Tools GmbH, Jena, Germany) using a 2% uranyl acetate solution (Sigma Aldrich, MO, USA). The images were taken using a 200-kV JEOL 2200FS electron microscope (JEOL Ltd, Tokyo, Japan) using a Direct Electron DE-20 camera (Direct Electron, CA, USA). The images were then analyzed by FIJI ImageJ software (NIH, MD, USA).

AAV9:CAG-mNeonGreen 1 TEM experiments were performed in the Caltech CryoEM center. Samples were stained on Carbon Type-B 200 Cu grids (Ted Pella, CA, USA) using 3% uranyl acetate solution (Sigma Aldrich). The images were taken by a Tecnai T12 120 kV electron microscope (FEI, OR, USA) using a Gatan 2k × 2k CCD (Gatan, CA, USA).

Mass photometry

An AAV5 empty sample was acquired in triplicate on a Refeyn Samux mass photometer (Oxford, UK). A microscope coverslip was cleaned consecutively in Milli-Q® water, isopropanol and Milli-Q water prior to mounting a silicone gasket on the coverslip; then 10 µl of phosphate-buffered saline (PBS) was added to a gasket well and the instrument was focused and locked. Next, 10 µl of AAV5 empty reference sample was added to the PBS solution for a final AAV concentration of 2×10^{11} particles/ml. The sample in the well was aspirated using a pipette prior to acquisition of data. Analysis of the data was performed in DiscoverMP (Oxford, UK), where an AAV mass calibration was applied to convert the measured ratiometric contrast (between scattered and reflected light) to mass. Because the masses for both empty and full particles are known, the empty:full ratios can be determined by this method.

Analytical ultracentrifugation with MW SV detection

MW-AUC SV experiments were performed in a Beckman Coulter Optima analytical ultracentrifuge (Beckman Coulter, IN, USA) at the Canadian Center for Hydrodynamics at the University of Lethbridge. Samples were diluted to 0.3–0.4 OD at 260 nm with dPBS (Thermo Fisher Scientific, 14190144, WI, USA). Standard Beckman 1.2-cm epon-charcoal centerpieces, fitted with quartz windows, were filled with 460 µl of sample. In each experiment, a single cell was measured, and loaded with two samples, utilizing both the sample and reference channel, and

measured in intensity mode. AUC cells were loaded into an AN60Ti rotor and equilibrated at 4°C for 1 h before the rotor speed was increased to 14.5 kr.p.m. and scan collection began. A speed between 14.0 and 14.5 kr.p.m. will maximize the number of scans collected, because the flash lamp rate and rotor speed are synchronized at this speed, reducing the scanning time to ~8 s per channel of each cell. A temperature of 4°C was used to minimize the density variation in response to small, local temperature changes, which nearly eliminates sample convection during the run. Convection can occur in experiments where large analytes are measured at low rotor speeds in dilute buffers when the Optima AUC's temperature control is activated [39]. The reduced temperature also increases the buffer density and viscosity, resulting in slower sedimentation and permitting additional data to be collected in MW mode. Intensity data were collected from both channels of a single cell, from 240 to 290 nm, every second wavelength. All data were analyzed using UltraScan 4.0 [29] as described previously [21,40]. Briefly, each triple (data from a single cell, channel and wavelength) was analyzed using two-dimensional spectrum analysis (2DSA) [41,42], which removes the time and radially invariant noise in the intensity data, fits the boundary conditions (meniscus and bottom of the cell) and refines the final model using an iterative approach. Residuals from the iteratively refined 2DSA models were examined for randomness and used to generate a time-synchronized sedimentation profile for each wavelength, which was used for MW deconvolutions [20,21,32].

Diffusion-corrected sedimentation coefficient profiles were generated using the enhanced van Holde–Weischet analysis [43] implemented in UltraScan [29] from the deconvoluted SV data. Dual-wavelength AUC results were obtained from the same MW-SV experiments, but using only the iterative 2DSA results from the 260- and 280-nm triples. The buffer density and viscosity corrections were calculated with UltraScan using the partial concentration of each buffer component.

Analytical buoyant density equilibrium

ABDE with AAV samples was performed in CsCl density gradients. ABDE experiments provide high resolution information on AAV-loading states, while requiring a significantly reduced amount of AAV sample compared with SV experiments. Like SV experiments, ABDE experiments can also be performed in MW mode to add spectral information to separate and quantify protein and DNA signals. ABDE experiments distinguish solutes based on their buoyant densities in a density gradient and produce sharp peaks for each solute that are easily quantifiable with the ABDE peak fitter in UltraScan, which calculates the density of each peak based on peak position by calculating the density gradient profile [31]. The peaks will occur at the positions where the solutes' densities equal the density of the gradient. Together, MW and density characterization provide an unambiguous, orthogonal AAV capsid characterization method.

ABDE experiments were performed in a Beckman Coulter Optima analytical ultracentrifuge at the University of Lethbridge. Samples were diluted between 0.2 and 0.25 OD at 260 nm (measured in a 1-cm path length), with a final CsCl density of 1.36 g/ml, unless mentioned otherwise, prepared in 10 mM Tris (pH 8.0) buffer and measured at 60 kr.p.m. Titanium 3-mm centerpieces (Nanolytics Instruments, Potsdam, Germany) were assembled in standard Beckman Coulter cell housings, fitted with sapphire windows and one 3-mm spacer above and below the centerpiece to align the centerpiece fill holes with the fill holes of the cell housing. For accurate quantification, the OD needs to remain within the dynamic range of the detector (0.1–0.9 OD for most wavelengths). The 3-mm centerpieces serve two purposes. First, by reducing the path length from the typical 12-mm cell used in SV experiments to 3 mm, only one-quarter of sample volume is required. Second, a reduced path length significantly decreases the radial refraction error generated by the lens-shaped CsCl gradient, which has a high refractive index.

The density difference generated in the gradient between the meniscus and the bottom of the cell depends on the centrifugal force (F), which is given by $F = m\omega^2 r$, where m is the mass of the sedimenting particle, ω is the angular velocity and r is radius. Therefore, faster rotor speeds and longer columns provide the maximum density range and solute separation. In this configuration, eight samples can be measured simultaneously by utilizing both channels of the cells and all four rotor holes. When measuring with the AN50Ti rotor, 16 samples can be measured in a single run, but only up to 50 kr.p.m. It should be noted that as the rotor speed is decreased, the gradient becomes shallower, increasing the CsCl concentration near the meniscus compared with high-speed runs. Therefore when rotor speeds are decreased, the concentration of CsCl required to visualize empty capsids may also need to be decreased, otherwise the empty AAVs peak will not separate from the meniscus.

For ABDE experiments, scans were collected in intensity mode at equilibrium, from 236 to 288 nm every second wavelength for a total of 27 wavelengths. However, because the sample at equilibrium no longer changes its distribution in the gradient, unlimited time exists to collect up to 100 wavelengths per channel, which is possible

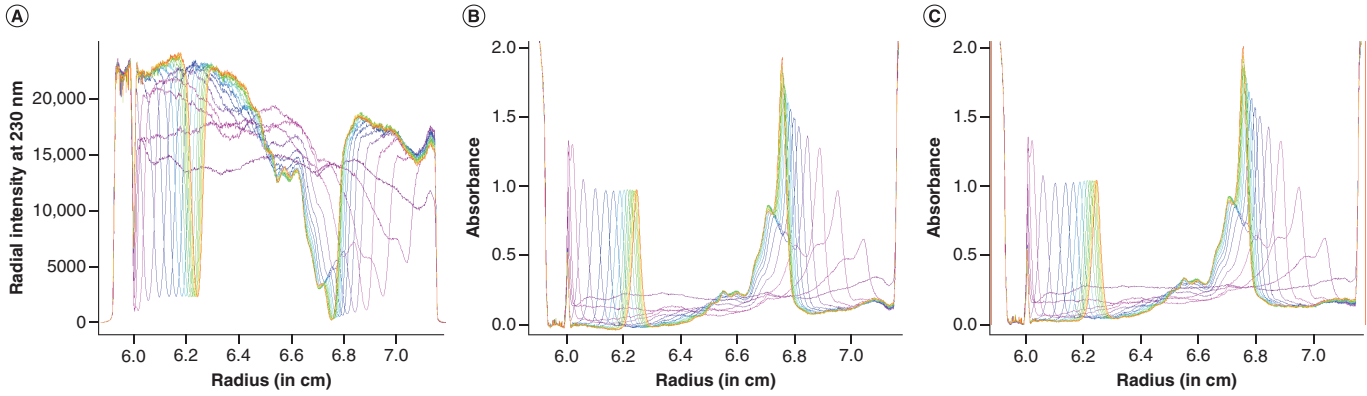

Figure 1. Noise correction of an analytical buoyant density equilibrium adeno-associated virus experiment. (A) Raw analytical buoyant density equilibrium intensity data from an adeno-associated virus sample approaching equilibrium, collected on the Optima™ AUC (only a single wavelength is shown). **(B)** Pseudo-absorbance conversion of data shown in **(A)** without time-invariant noise correction. **(C)** The same data after processing with the UltraScan's noise-correction module (Pseudo-Absorbance). The color gradient indicates time.

with UltraScan's data acquisition software for the Optima AUC. Data collection was performed throughout the experiment to determine when equilibrium was reached, but only the last scan from each wavelength, after the CsCl gradient was established, was used in the analysis. The gradient was deemed established after the solute peaks stopped shifting, approximately after 5 h at 60 kr.p.m. and at 20°C. Viral particles are relatively large compared with typical proteins, resulting in smaller diffusion coefficients and narrower peaks in an ABDE experiment. The width of the peaks formed in an ABDE experiment is directly proportional to the diffusion coefficient of the analyte and the temperature, and inversely proportional to the viscosity of the solvent. Given that AAV capsids have approximately the same size and shape regardless of their cargo load, and the temperature is constant, the width of each capsid peak is only a function of the local viscosity and density at a particular gradient position, which is primarily a function of the local CsCl concentration. Narrow peaks improve baseline resolution between peaks and reduce the amount of sample needed up to 40-fold compared with SV experiments.

For ABDE experiments, all data of interest are time-invariant. Hence, time-invariant/radially-invariant noise corrections as performed in SV experiments [44] cannot be used. Therefore a new noise-processing module was developed in UltraScan, called Pseudo-Absorbance, to process the time- and radially-invariant noise contributions [30]. The Pseudo-Absorbance module in UltraScan offers extensive baseline processing features that allow experimental data to be collected in intensity mode, removing time- and radially-invariant noise, and avoiding the amplification of stochastic noise by a factor of $\sqrt{2}$ [30,40]. The module converts intensity data to pseudo-absorbance data using the time-invariant intensity fingerprint of the photomultiplier tube in the Optima AUC as a function of radius and wavelength. This process extracts the time-invariant background signal from experimental data for each wavelength without the addition of stochastic noise, avoiding the use of a buffer channel for absorbance subtraction. As a result, the signal-to-noise ratio and precision of ABDE data are significantly enhanced. At the same time, the capacity of the instrument is doubled, because the reference channel can be used for another sample [30,40]. The effect of the noise-processing algorithm is illustrated in Figures 1 & 2. Figure 1A shows the samples approaching equilibrium measured in intensity. Figure 1B shows the data after conversion to pseudo-absorbance, but without any noise correction, while Figure 1C shows the data after noise correction, which corrects the baseline of the experiment to zero absorbance. In Figure 2, the effect of time-invariant noise contributions resulting from the photomultiplier tube can be seen clearly in the negative baseline observed near 6.2 cm in the red line. Noise processing in UltraScan restores an accurate baseline and recovers reliable peak amplitudes across the entire radial domain. After pseudo-absorbance conversion, the ABDE experiments are edited by selecting the meniscus and cell bottom positions and are spectrally deconvoluted into the spectral contributors, protein and DNA, as explained below.

The ABDE experiments can be analyzed with a recently added module in UltraScan for peak fitting [31]. This program calculates the integral and buoyant density of each analyte from its peak size and position, and other experimental parameters such as the meniscus position, bottom of the cell, gradient-forming material properties and concentration and the rotor speed. Because the density of each analyte is constant, even when any of these experimental parameters are varied, this calculation provides an unambiguous and reproducible

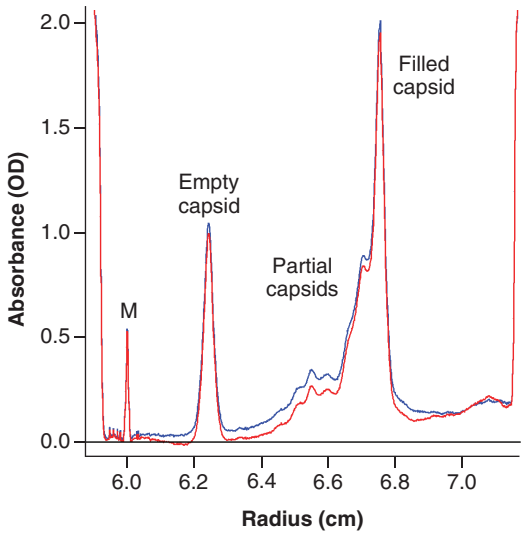

Figure 2. Effect of time-invariant noise correction. Shown is the last scan of the analytical buoyant density equilibrium experiment shown in Figure 1. Red: without time-invariant noise correction. Blue: with time invariant noise correction. Note the negative absorbance for the uncorrected scan near 6.2 cm.
M: Meniscus position; OD: Optical density.

characterization of each analyte. Together with the MW characterization, this approach provides a very high precision for AAV characterization. For accurate quantification of capsid peaks, it is essential to also process ABDE baseline contributions from CsCl refractive artifacts and potential absorbing contaminants in the sample or buffer, as described by Savelyev *et al.* [30], before processing the data with the peak integration module of UltraScan [31].

MW deconvolution

MW-AUC data were deconvoluted into the spectral contributions from each absorbing analyte (protein and DNA) as described previously [20,21]. If the buffer or gradient-forming material absorbs in the measured wavelength region, it must be included in the spectral deconvolution. The amounts of sample required to measure pure AAV protein and DNA extinction spectra are difficult to obtain. Instead, we chose to measure a dilution series from albumin (Bio Basic cat. no. D0024, Markham, Canada), serving as a pure protein standard, and a highly purified DNA plasmid between 240 and 290 nm. A GENESYS™ 10S benchtop spectrophotometer (Thermo Fisher Scientific) was used to collect all extinction profiles. When measuring between 240 and 300 nm, the absorbance spectrum from albumin is sufficiently similar to that of the viral capsids because only tryptophan and tyrosine contribute to the spectrum in this range; hence it can be used instead of the empty capsid spectrum to obtain a pure spectrum. However, when measuring below 240 nm, the capsid absorbance also depends on the peptide backbone absorption, whose molar absorptivity is primarily a function of the number of peptide bonds in the protein. Because the ratio of the number of peptide bonds and the number of aromatic side chains is not constant, measurements that include wavelengths below 240 nm require a purified empty capsid protein spectrum. A sum of Gaussian terms was fit to each dilution series and scaled to 1.0 OD at 280 nm for protein and 260 nm for nucleic acid, using the Spectrum Fitter program in UltraScan [29]. Whenever MW-AUC data are deconvoluted into contributions from individual chromophores, the 3D-residuals viewer from UltraScan should be used to assess the quality of the decomposition to make sure the basis functions are properly representing the original spectrum [32]. An example of our MW deconvolution fits for a single scan can be seen in Supplementary Figure 1, and for a single radial position in Supplementary Figure 2. It is recommended that the user view each scan and radial position to ensure random fits and low root mean square deviations.

Because accurate molar extinction coefficients are also difficult to obtain for single-stranded DNA cargo, packaged inside a protein capsid, we have adopted a different approach for ABDE experiments: we chose to normalize the protein and DNA extinction vectors used for spectral deconvolution such that spectral contributions from protein and DNA observed for the filled capsid species are equal in magnitude. Filled capsids have a known density and are typically the major species in AAV preparations, and hence provide sufficient signal and are readily identified. Comparison of the normalized absorbance facilitates identification of empty capsids (no spectral contribution from DNA), partially filled capsids (spectral contribution from protein exceeds the DNA contribution) and filled capsids (protein and DNA spectral contributions are equal in amplitude), as well as overloaded species (spectral contribution from DNA exceeds the protein spectral contribution). Precise molar extinction coefficients are generally not available

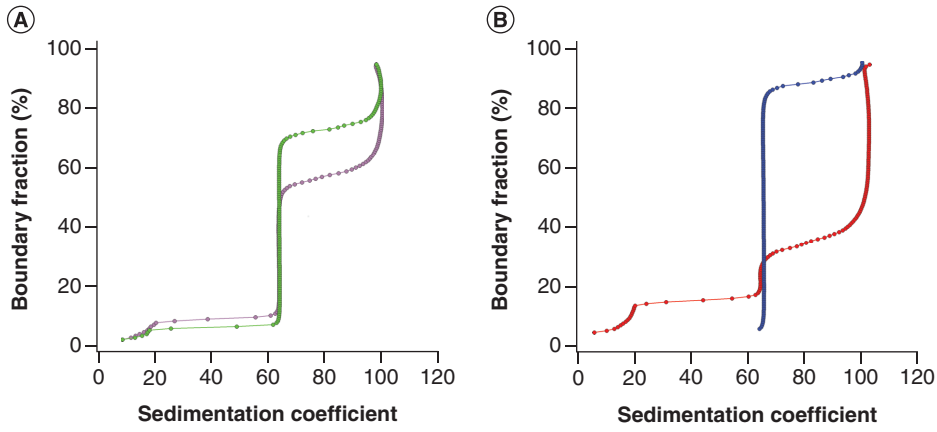

Figure 3. Comparison of the enhanced van Holde–Weischet distributions of the 260/280 nm and multi-wavelength analytical ultracentrifugation results. SV AUC data for AAV9:CAG-GFP analyzed using **(A)** only the 260 (pink) and 280 nm (green) wavelengths and **(B)** the deconvoluted MW SV AUC method (blue: 280 nm; red: 260 nm).
AAV: Adeno-associated virus; AUC: Analytical ultracentrifugation; MW: Multi-wavelength; SV: Sedimentation velocity.

and are not needed to perform the identification of loading states of AAV peaks. Using this approach, it is possible to uniquely determine the relative amounts of protein and DNA of each capsid species present in a mixture by comparison with a filled capsid.

Results

SV experiments

To determine the composition of AAV formulations, we performed MW-AUC SV experiments, which resolve AAV capsids and contaminants based on their sedimentation coefficient profile and spectral properties, offering excellent resolution. MW-AUC (collected every second wavelength from 240 to 290 nm) separates the results into the spectral contributors of AAV, the protein capsid and DNA transgene, generating individual hydrodynamic distributions for each macromolecule [21]. The empty:full capsid ratio can be directly quantified from the integral sedimentation coefficient distributions generated in the van Holde–Weischet analysis for the MW data [43]. Figure 3 compares the 260- and 280-nm van Holde–Weischet distributions for an AAV9:CAG-GFP sample with the MW-AUC SV results. Figure 3A suggests that 45% of the total concentration sediments as a 100 S species (filled capsids) and 45% as a 65 S species (empty capsids) when measured at 260 nm, while the 280-nm results suggest that 30% of the total concentration sediments as a filled capsid and 65% as an empty capsid. The remainder (10% for the 260-nm measurement and 5% for the 280-nm measurement) sediments at a lower sedimentation coefficient (<20 S), and its identity is unknown. The MW-AUC analysis is shown in Figure 3B. Here, the protein and DNA signals are separated into individual van Holde–Weischet distributions, which reflect the relative contribution of each macromolecule in the hydrodynamically separated data. For filled capsids, both DNA and protein signals contribute, while empty capsids only show protein contribution and partially filled capsids show both protein and DNA signals, with an excess of protein signal. Free nucleic acids are identified by the absence of co-migrating protein signal. Based on the protein signal, the MW-AUC results show that the relative concentration of the 100 S species (filled capsids) contributes only 10% of the total protein signal, while the remaining 90% of the protein signal sediments at 65 S (presumably empty capsids). The DNA signal shows that 65% of the DNA co-migrates with the 100 S species (filled capsids) and 20% co-migrates with the 65 S species, indicating that the 65 S species is not truly an empty capsid but contains a small amount of bound nucleic acid, allowing us to classify the 65 S species as a partially filled capsid. The remaining 15% of the DNA signal sediments slower than 20 S, but without any co-migrating protein contribution, identifying the slower species as free nucleic acid. These MW-AUC results demonstrate several key points. First, because absorbance spectra for protein and DNA have significant overlaps in the range 200–300 nm, single-wavelength analysis will always add the DNA signal to the signal of the protein species and overestimate the amount of full capsids. Second, the presence of a small amount of DNA signal in the 65 S species demonstrates that the S-value alone is insufficient to classify capsids as empty, partially filled or filled capsids, and that quantification of AAV capsid-loading states using single-wavelength AUC is inherently unreliable.

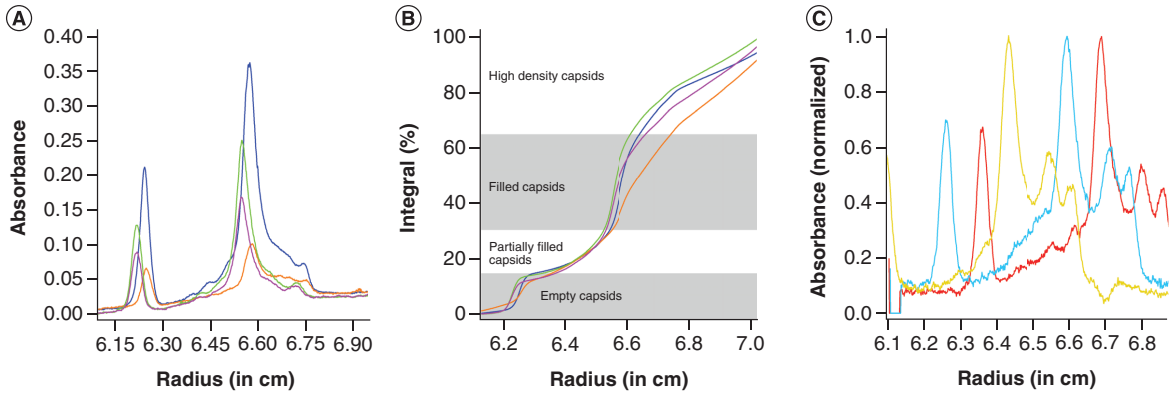

Figure 4. Analytical buoyant density equilibrium optimization. Only the deconvoluted protein absorption pattern is shown for ABDE experiments for ease of reading. **(A)** Deconvoluted protein signal from ABDE experiments of AAV9:CAG-mNeonGreen 1 at four different AAV concentrations (3.64×10^{12} [orange], 6.36×10^{12} [green], 9.55×10^{12} [pink] and 1.36×10^{13} vg/ml [blue]). **(B)** Normalized integral distribution of the data shown in **(A)**, demonstrating that the relative concentrations of each species remain constant. **(C)** Normalized results for AAV9:CAG-mNeonGreen 2 in different CsCl concentrations (1.35 [red], 1.36 [cyan] and 1.38 g/ml [yellow]). ABDE: Analytical buoyant density equilibrium; AAV: Adeno-associated virus.

Third, MW-AUC analysis is required to accurately identify any contaminants as proteins, DNA or protein–DNA complexes. Fourth, in cases where molar extinction coefficients are available, the protein and DNA distributions will be reported in molar units and MW-AUC can report directly the number and ratio of protein and DNA molecules in each capsid species. Because protein and DNA spectra have significant spectral overlap, failure to account for the relative contribution of both biopolymers will always lead to an overestimation of the filled capsids, regardless of wavelength used. Examples of the normalized spectra for AAV5 and DNA are shown in Supplementary Figure 3. The relative error for overestimating the amount of filled capsids varies as a function of wavelength. For the case shown in Supplementary Figure 3, the relative error at 280 nm is 0.91:1, 2.89:1 at 260 nm and 0.124:1 at 230 nm. While the quantification error is reduced at 230 nm, it is not zero, and single-wavelength measurements will always yield incorrect relative quantities when the extinction contributions from DNA are not properly taken into account. MW-AUC is the only reliable approach to mitigate this problem.

Analytical buoyant density equilibrium

ABDE experiments use a density gradient-forming material to separate analytes based on their densities, supporting reduced sample volume requirements and increased throughput. The linearity of the ABDE method was evaluated to test its ability to obtain proportional results for different AAV concentrations (Figure 4A & B). For clarity, only the deconvoluted protein signals are shown in Figure 4. AAV9:CAG-mNeonGreen 1 was measured at four concentrations ranging between 3.64×10^{12} and 1.36×10^{13} vg/ml (0.11–0.40 OD at 260 nm in a 1-cm path length). The deconvoluted protein signals from each concentration are shown in Figure 4A (without normalization), and their normalized integral distributions are shown in Figure 4B, which confirm equal ratios of empty, partially filled and filled capsids. A minor shift in radial position across all peaks is seen in Figure 4A, which results from a slight variation in loading volume. For all concentrations, empty capsid peaks appear at a density between 1.19 and 1.20 g/cm^3 (6.17–6.3 cm) and contribute approximately 15% to the total signal, while the partially filled capsids contribute approximately 15% of the signal and appear between 6.35 and 6.5 cm. The filled capsids appear between 6.5 and 6.65 cm and have peak densities between 1.30 and 1.31 g/cm^3 and contributed approximately 35% of the total signal. Unexpectedly, two discrete higher-density species are identified between 6.65 and 6.8 cm with peak densities between 1.37 and 1.38 g/cm^3 that contribute approximately 35% of the signal; these were identified in all samples that contained filled AAVs and are further discussed below.

To optimize the run time and AAV peak formation, various CsCl concentrations, rotor speeds and CsCl column lengths were tested. The effect of varying CsCl concentrations on peak distributions for sample AAV9:CAG-mNeonGreen 2 is seen in Figure 4C. As the CsCl concentration is increased from 1.35 g/ml (red) to 1.36 g/ml (cyan) to 1.38 g/ml (yellow), all AAV peaks shift to the left. At a CsCl concentration of 1.36 g/ml, an optimal distribution of AAV peaks was observed (at 60 kr.p.m.) because it positions empty, partially filled and filled capsids

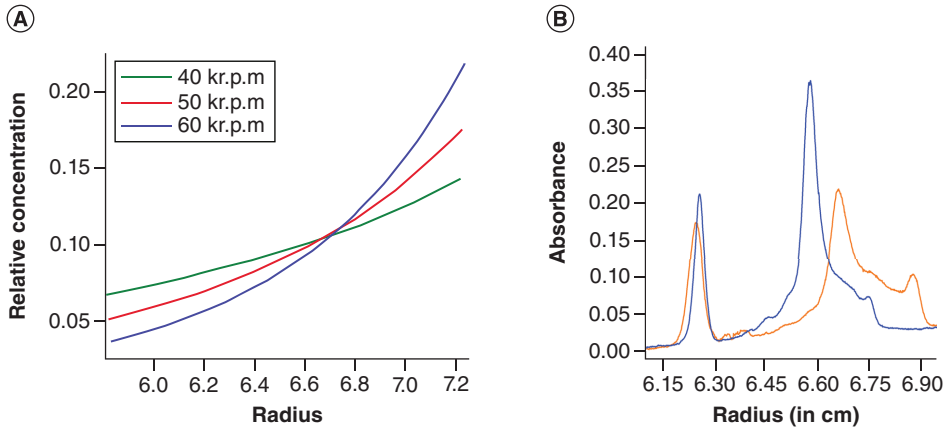

Figure 5. The effect of speed on CsCl gradient formation. (A) Simulated gradient shapes for CsCl at 40,000 (green), 50,000 (red) and 60,000 r.p.m. (blue). **(B)** AAV9:CAG-mNeonGreen1 measured at 60,000 (blue) and 50,000 r.p.m. (red).

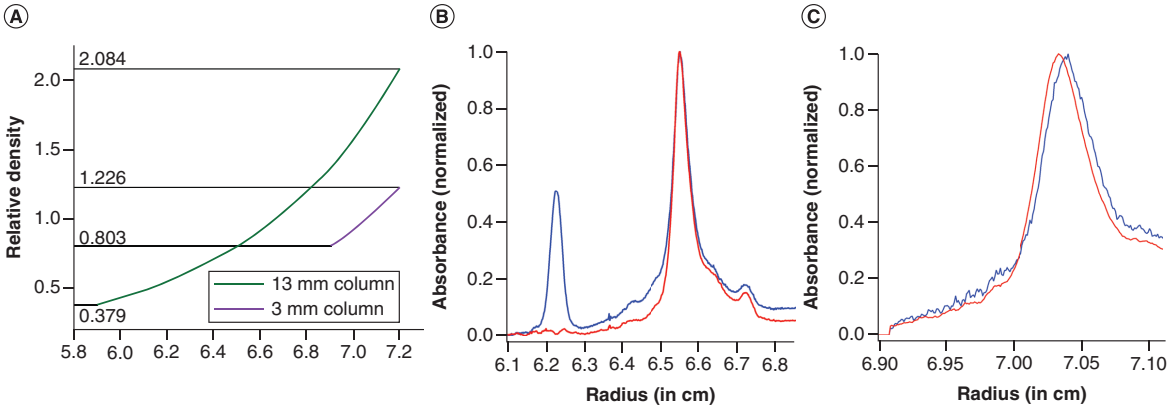

Figure 6. The effect of column length on CsCl gradient. (A) Simulated CsCl gradient formation in a 0.3-cm centerpiece filled with a 13-mm column (green) and 3-mm column (purple). CsCl gradients from long-column experiments (13 mm) provide a 3.6-fold higher dynamic density range than short-column experiments (3 mm). **(B)** 13-mm column experiment at 1.36 g/cm^3 CsCl, **(C)** 3-mm column experiment at 1.35 g/cm^3 CsCl. In **(B)** and **(C)** the DNA signal is shown in red and the protein signal in blue.

in the center of the solution column, providing baseline separation between the empty and full species, as well as the meniscus and bottom of the cell. At the highest CsCl concentration, the peak corresponding to empty capsids was no longer visible and floated to the top of the cell. At all three CsCl concentrations, the apparent density was approximately 1.20 g/cm^3 for empty AAVs and 1.30–1.43 g/cm^3 for the filled AAV capsid and higher-density peaks.

Rotor speed can also be used to modulate the CsCl gradient profile. In Figure 5A, an overlay of the CsCl equilibrium density gradient profile was simulated for three rotor speeds (40, 50, and 60 kr.p.m.), demonstrating the variation in the density profile. Sample AAV9:CAG-mNeonGreen 1 was measured at 50 and 60 kr.p.m. to demonstrate the resulting variations in peak positions (Figure 5B). At 50 kr.p.m., the gradient is shallower and tends to broaden the peaks, especially toward the bottom of the cell. At 50 kr.p.m., the gradient requires 9 h to reach equilibrium (4 h longer than at 60 kr.p.m.). The reduced speed slightly shifts the empty capsids to the left, while the filled AAV peaks are shifted to the right, closer to the bottom of the cell. Although a reduction in rotor speed reduces the density range over which AAV samples can be measured, this approach increases baseline separation and allows the investigator to focus on a particular density range with higher resolution.

The effect of CsCl column length was simulated with the ASTFEM simulation routine in UltraScan [45,46], calculating the CsCl distribution for a long (13-mm) column and a short (3-mm) column (Figure 6A). The variation in column length clearly demonstrates that at 60 kr.p.m., the CsCl gradient distribution in the long

column (13 mm) provides a 3.6-fold higher dynamic density range than the short column (3 mm). Although this approach was advocated by Sternisha *et al.* [33], we do not recommend to use short column centerpieces for ABDE experiments involving AAV, because the entire range of capsid-loading states cannot be distinguished by this approach. The ratio of the maximum density over the minimum density in a 13-mm column is 5.5, but only 1.5 for a 3-mm column. When measuring AAV9:CAG-mNeonGreen 1 in the 3-mm column, at 1.36 g/ml CsCl the sample did not sufficiently separate from the meniscus; therefore the CsCl density was reduced to 1.35 g/ml. At 1.35 g/ml, in contrast to the long-column experiments, only a single peak (likely corresponding to the filled capsid species) was observed in the short-column experiments (Figure 6B & C). Therefore the dynamic range of the 3-mm column experiments is insufficient to simultaneously detect empty, partially filled and filled AAV capsids in a single experiment. Overall, the best experimental design for AAV experiments employed a CsCl concentration of 1.36 g/ml at 60 kr.p.m., 20°C, using a long column (12–13 mm) in a 3-mm centerpiece, and measuring wavelengths 240–290 nm. It is recommend to use optically pure CsCl that does not absorb, such as UltraPure™ CsCl, optical grade (Thermo Fisher Scientific, cat. no. 15507023). In addition to background absorbance from CsCl, the high refractive index of CsCl can also contribute to background signal, which must be subtracted. To correctly account for the baseline contribution from any absorbance or refractive effects, it is advised to also measure the signal from a solution with an identical CsCl concentration in a separate channel. This reference channel absorbance can be used for all other channels in the same run that are performed at the same CsCl concentration. The baseline contribution from CsCl can then be removed from all samples in the experiment using the pseudo-absorbance program in Ultrascan [30]. To further mitigate the effects of refractive artifacts from CsCl gradients, we recommend to shorten the optical path length by employing 3-mm centerpieces. In order to withstand high hydrostatic pressures at high speed and high CsCl densities, we recommend to use titanium centerpieces (Nanolytics Instruments), which are rated up to 60,000 r.p.m.

In our experiments, we have observed the presence of contaminants and refractive artifacts resulting from CsCl gradients that contribute to baseline variations, which are also dependent on wavelength (e.g., Figures 2, 4, 5, & 6). The intensity recordings from a single channel filled with CsCl at the same concentration and approximately the same loading volume as all other channels suffices to provide the necessary baseline correction data for the pseudo-absorbance conversion module in UltraScan [30]. In this context, it is important to understand that the radially invariant noise component of the last scan in an ABDE experiment, which is considered to be at equilibrium, is also time-invariant. Any systematic baseline contributions observed in the equilibrium scan can therefore be considered to be part of the time-invariant noise and handled with the algorithms discussed by Mortezazadeh and Demeler [30].

Comparison of MW SV, ABDE & TEM

The ABDE, SV and TEM results were compared in order to demonstrate their equivalence. SV analysis of sample AAV8 BCM #1 revealed filled capsids sedimenting at 95 S (Figure 7A), along with a nucleic acid signal sedimenting below 20 S. The ABDE results (Figure 7B) show a major peak at 6.54 cm with a density of 1.29 g/cm^3, corresponding to the filled capsids. Once again, two smaller higher-density peaks are found, at 6.66 and 6.74 cm, corresponding to densities of 1.34 and 1.38 g/cm^3, respectively. When the deconvoluted protein and DNA signals are normalized to the amplitude of the filled capsid, it is clear that the two higher-density peaks share an identical protein:DNA ratio with the filled capsid peak. The identity of these higher-density peaks is not known. One possibility is that they represent overfilled capsids containing more than one complement of the DNA genome. This possibility was rejected because the MW analysis of both samples clearly demonstrated identical protein:DNA ratios in the higher-density peaks compared with the filled capsid peaks (Figure 7A). A larger capsid, accommodating more proteins and DNA, is also unlikely because the velocity data of AAV8 BCM #1 do not indicate the presence of any faster-sedimenting species. We speculate that the higher-density species are the result of a leaky capsid, allowing a small amount of CsCl to enter the capsid, binding to the DNA. The second species could be a capsid containing externalized DNA, or DNA or RNA fragments attached to the exterior of the capsids, freely complexed by CsCl, increasing the overall density of each species. For the TEM analysis, 1460 particles were counted (Figure 7C), and only two empty particles were identified.

SV analysis (Figure 7D) of AAV9:CAG-mNeonGreen 1 revealed a heterogeneous composition consisting of 55% filled capsids (95 S), 15% empty capsids (~60 S) and approximately 20% partially filled capsids (70–93 S) (Figure 7E). ABDE analysis of the same sample revealed a peak containing mostly protein signal, with a density of 1.20 g/cm^3 at 6.25 cm (12.5% of the protein signal), corresponding to empty capsids. Between 6.35 and 6.54 cm, the protein and DNA absorbance gradually increases but never reaches a 1:1 ratio as observed in the filled capsids

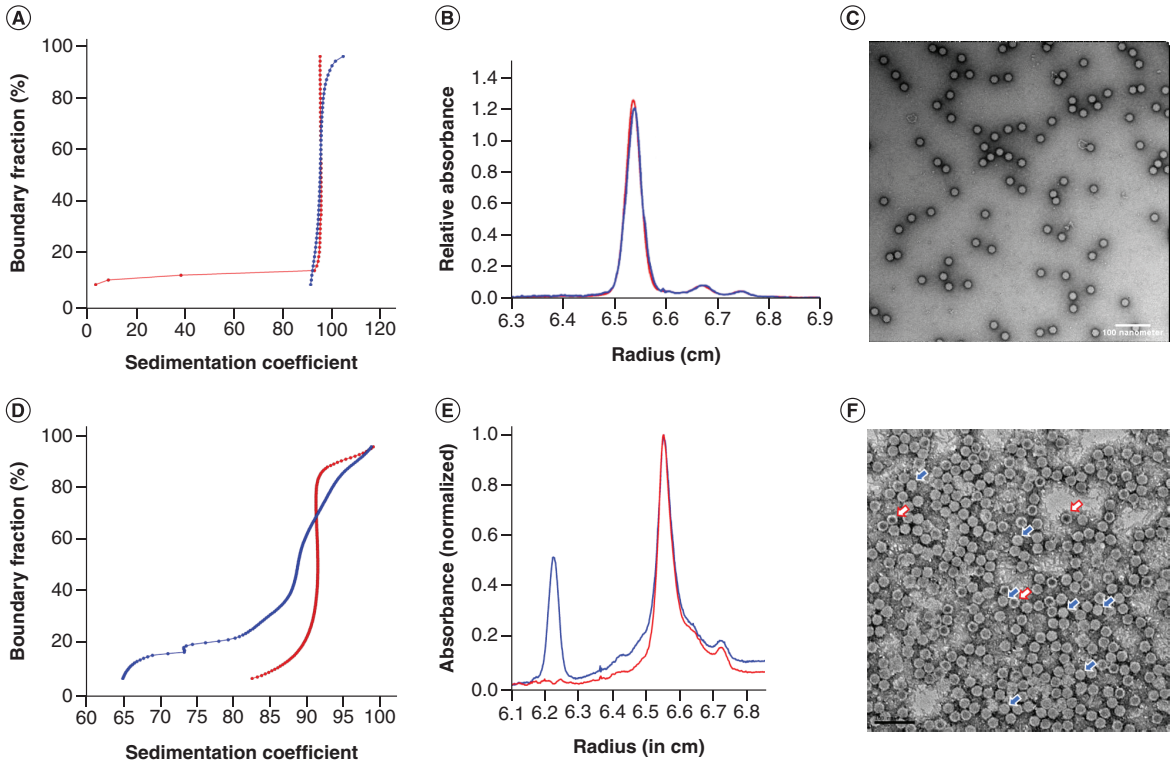

Figure 7. Adeno-associated virus formulations measured using sedimentation velocity and analytical buoyant density equilibrium multi-wavelength analytical ultracentrifugation, deconvoluted into its protein absorbance pattern (blue) and DNA absorbance pattern (red), and by transmission electron microscopy. The top panel shows AAV8 analyzed by **(A)** SV MW-AUC, **(B)** ABDE MW-AUC with protein and DNA deconvolutions normalized to 1.3 Optical density and **(C)** transmission electron microscopy; no empty AAVs are visible. The bottom panel shows AAV9:CAG mNeonGreen 2 analyzed by **(D)** SV MW-AUC, **(E)** ABDE MW-AUC with protein and DNA deconvolutions normalized to 1 OD and **(F)** transmission electron microscopy; the blue arrows show full AAVs and the red arrows empty AAVs.
AAV: Adeno-associated virus; ABDE: Analytical buoyant density equilibrium; MW-AUC: Multi-wavelength analytical ultracentrifugation; OD: Optical density; SV: Sedimentation velocity.

(6.57 cm). This suggests the presence of a heterogeneous mixture of partially filled capsids containing varying lengths of nucleic acids. The remaining three high density peaks, between 6.55 and 6.77 cm (corresponding to a density of 1.30–1.38 g/cm^3, respectively), all exhibited a 1:1 ratio of protein:DNA. Together these peaks contribute approximately 51% of the protein signal, similar to the ratio of filled capsids identified by SV. By TEM, the sample was identified to contain approximately 20% empty capsids and around 80% filled capsids, but the method was unable to distinguish partially filled capsids (Figure 7F), likely counting partially filled capsids as filled capsids. Finally, an AAV5 preparation of empty capsid was compared by SV, ABDE and MP (Figure 8). The SV results showed a sedimentation coefficient of ~65 S, while the ABDE experiment resulted in a single peak at 6.19 cm and a density of 1.20 g/cm^3. MP resulted in a single peak with a mass of 3797 kDa.

Discussion

Currently, the only well-established method with sufficient resolving power to detect empty, partially full and full AAV capsids in a single experiment is AUC [47,48]. However, single-wavelength AUC exhibits deficiencies in quantification and identification of sample species. In this study, we describe two improved AUC methods, SV and ABDE, leveraging MW detection, which can greatly improve accuracy, resolution and throughput and significantly reduce AAV sample requirements. Data acquisition and analysis with UltraScan automates the AUC data acquisition, analysis and reporting workflow and supports 21 CFR part 11 requirements to facilitate GMP integration. In addition, the high-performance computing possible with UltraScan greatly accelerates data processing. The results from these methods were compared with traditional 260/280 nm AUC experiments, cryo-TEM and MP

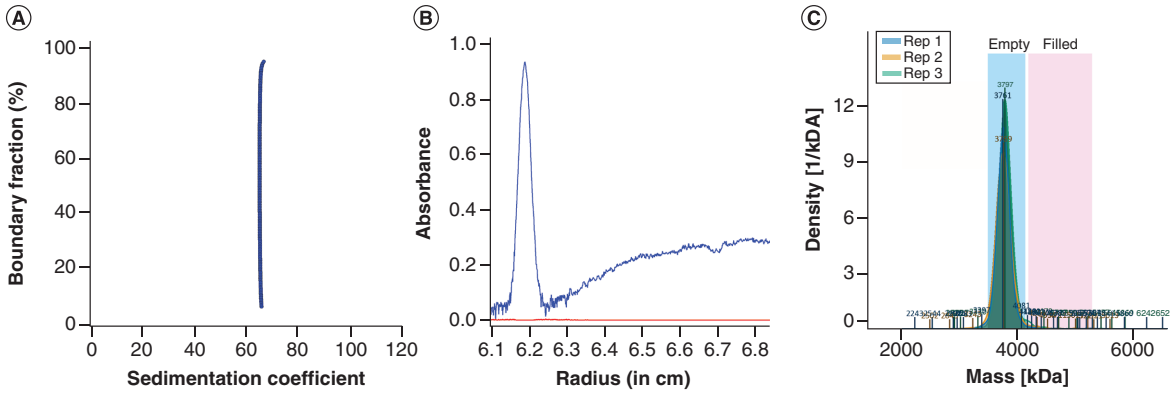

Figure 8. Comparison of AAV5 measured by sedimentation velocity and analytical buoyant density analytical ultracentrifugation and mass photometry. (A) Sedimentation velocity multi-wavelength analytical ultracentrifugation and **(B)** analytical buoyant density equilibrium multi-wavelength analytical ultracentrifugation. For both, after the spectral deconvolution only the protein signal (blue) was present. **(C)** Results from mass photometry measured in triplicate.

measurements, demonstrating significant improvements in accuracy and statistics. Finally, our study demonstrates that MW-AUC methods can be applied to multiple AAV serotypes and transgenes without modification, and potentially – with minor modifications to rotor speed, run length and CsCl concentration – to other viral vector systems. Our improvements addressed multiple limitations that prevent the widespread adoption of AUC methods for AAV characterization. The high AAV sample requirement for SV experiments is the most critical limitation. However, it should be noted that sample recovery after SV experiments is possible, to permit further testing. By switching to ABDE experiments, the required sample amount is significantly reduced by 20- to 40-fold. Two factors contribute to the significant sample reduction. The first factor relates to the differences by which the solutes are distributed throughout the cell in each experiment. In any AUC experiment, the measured signal should ideally match the dynamic range of the detector. The sample requirements to reach this limit are very different for the two experiments. In SV experiments, the loading concentration is uniformly distributed over the entire column length. In ABDE experiments, at equilibrium, the entire signal is concentrated into one or more narrow peaks. This concept is illustrated in Supplementary Figure 4, where the orange shaded regions reflect the sample required to reach an OD of 0.4 in either experiment. Secondly, the lower sample requirements are a consequence of the enhanced signal-to-noise ratio achieved through the improved noise processing possible in UltraScan [30]. MW-AUC provides additional data points through observations at multiple wavelengths, increasing the accuracy and amplifying the signal-to-noise ratio through additional data points by combining all wavelengths in a global fit which separates the protein and DNA signals [20,21]. Because data are collected at equilibrium, a filled rotor with 16 samples can be measured at up to 100 wavelengths in the Optima AUC, greatly enhancing sensitivity and throughput at the same time.

MW-AUC offers an orthogonal verification through spectral deconvolution of protein and DNA signals to unambiguously identify and quantify AAV-loading states and other contaminants with the highest accuracy and reproducibility. This eliminates uncertainty about filled capsid ratios because the protein capsid can be identified without contribution from the DNA signal, providing an unambiguous mass ratio for each species. It also provides the spectral identity of contaminants, which is essential to assure drug purity and patient safety. The ability to simultaneously characterize the presence of contaminating species and quantify capsid ratios is an important benefit of the MW-AUC approach. SV experiments excel at simultaneously addressing a larger range of solute properties, useful for contaminant identification, but are limited by their throughput and higher sample requirements. For ABDE experiments, the relevant information is accessed after equilibrium has been reached, allowing not only more time for data collection at multiple wavelengths, but also for collection of additional cells and channels, significantly improving throughput. Our studies included a comparison with negative-stain TEM experiments, which showed excellent agreement when only full or empty capsids were present, but the presence of partially filled capsids could not be established by TEM.

ABDE experiments revealed a previously unknown feature of AAV capsids: the presence of two additional higher-density peaks with the same protein:DNA ratio as the full capsid (Figures 4A–C, 6B & 7B & E). We hypothesize that the higher-density peaks are a result of Cs ion complexation by DNA in potentially leaky capsids. Capsid instabilities under certain conditions have been reported earlier [49,50], pointing to a possible undesirable species in therapeutic formulations. Because CsCl and other density gradient-forming materials change the chemical environment of AAV capsids in terms of ionic strength compared with the physiological buffers used in SV experiments, and because these chemicals are commonly used to purify AAV capsids, further research is warranted to better understand the identity of these two higher-density species and the role of pH and CsCl in the solvent as factors potentially affecting capsid stability.

Conclusion

Our study demonstrates the relevance and reliability of SV and ABDE MW-AUC for the characterization and quantification of AAV-loading states in AAV serotypes 5, 8 and 9. It highlights the improved information obtained from MW analysis, demonstrating its abilities to detect correct ratios of empty, partially filled and full AAVs. The ABDE approach promises significant sample savings compared with the traditional SV method, and revealed additional species not seen in SV. The UltraScan software observes stringent requirements for 21 CFR Part 11 compliance in its design, facilitating current GMP integration and automation of sample analysis. Together, these advances will help reinforce AUC as the gold standard analysis method for viral vectors.

Summary points

- Multi-wavelength (MW) analytical ultracentrifugation (AUC) methods are presented for the quantification of adeno-associated virus capsid-loading ratios and the identification of product-related impurities and contaminants.
- The presented methods correct errors encountered in dual-wavelength AUC, which overemphasize filled capsids.
- In contrast to transmission electron microscopy, MW-AUC methods can detect and correctly quantify partially filled capsids.
- In contrast to mass photometry, MW analytical buoyant density equilibrium (ABDE) methods provide results that do not require reference standards; the densities of each peak are unique for each capsid-loading state.
- MW-ABDE methods separate protein from DNA signal and allow proteinaceous capsids to be measured separately from the DNA.
- MW-ABDE methods require 20- to 40-fold less sample for comparable sensitivity as sedimentation velocity AUC.
- MW-ABDE methods offer significantly higher throughput by leveraging both channels in a centerpiece for sample measurement.
- MW-ABDE methods identified two new previously unknown high-density capsid species that may result from adeno-associated virus exposure to CsCl. Further research is needed to characterize these species.

Supplementary data

To view the supplementary data that accompany this paper please visit the journal website at: www.futuremedicine.com/doi/suppl/10.2217/nnm-2023-0156

Financial disclosure

This work received funding from the Beckman Institute CLOVER Center at Caltech and the NIH BRAIN Initiative Armamentarium (UF1MH128336), Canada 150 Research Chairs Program (C150-2017-00015), Canada Foundation of Innovation (CFI-37589), Canadian Natural Science and Engineering Research Council (DG-RGPIN-2019-05637), Cancer Prevention and Research Institute of Texas (RP190602), NIH-NIGMS (1R01GM120600), NSF (TG-MCB070039N) and the University of Texas (TG457201). The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed. This includes employment, consultancies, honoraria, stock ownership or options, expert testimony, grants or patents received or pending, or royalties.

Competing interests disclosure

The authors have no competing interests or relevant affiliations with any organization or entity with the subject matter or materials discussed in the manuscript. This includes employment, consultancies, honoraria, stock ownership or options, expert testimony, grants or patents received or pending, or royalties.

Writing disclosure

No writing assistance was utilized in the production of this manuscript.

Data availability statement

The UltraScan software used to analyze the AUC data is open source and freely available from the Github repository (https://github.com/ehb54/ultrascan3). The AUC data are available in openAUC format upon request from the authors and are stored in the UltraScan LIMS server at the Canadian Center for Hydrodynamics.

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EXPERT OPINION ON BIOLOGICAL THERAPY
2022, VOL. 22, NO. 9, 1067–1071
https://doi.org/10.1080/14712598.2022.2060737

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EDITORIAL

Addressing high dose AAV toxicity – 'one and done' or 'slower and lower'?

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ARTICLE HISTORY Received 25 January 2022; Accepted 29 March 2022

KEYWORDS Gene therapy; adeno-associated virus; AAV toxicity; hepatotoxicity; thrombotic microangiopathy; hepatocellular carcinoma; dorsal root ganglia toxicity; AAV capsid engineering; immune tolerance

1. Introduction

While there has been substantial progress and notable achievements in the use of adeno-associated virus (AAV) gene therapy vectors for treatment of rare diseases, setbacks related to vector toxicity and immunogenicity represent major challenges for the field. In many cases, these two issues appear to be inextricably linked[1–3]. Immunogenicity of AAV vectors is thought to cause or exacerbate some of the more serious adverse events associated with AAV gene therapy, such as hepatotoxicity and thrombotic microangiopathy (TMA). Moreover, these adverse events tend to be correlated with vector dose, increasing in both prevalence and severity with higher doses [1,4]. Not surprisingly, high vector doses are also associated with increased immunogenicity, leading to a vicious circle when vector doses of 1E14 vg/kg or higher are required for efficacy in neuromuscular diseases [1,5]. These issues will likely require a multipronged approach to improve vector transduction efficiency and mitigate vector immunogenicity, and a perhaps a shift from the 'one-and-done' paradigm of gene therapy to a 'slower and lower' model of administering multiple lower doses of vector to achieve a similar therapeutic benefit.

2. Existing challenges and learnings from early clinical trials

Immunogenicity of AAV gene therapy is much more complex and nuanced than that for most protein biologic therapies, which are typically affected only by anti-drug antibodies (ADAs) [2,6]. ADAs also limit systemic AAV gene therapies through preexisting anti-AAV neutralizing antibodies that occur through natural exposure to wildtype AAV and which limit patient eligibility for gene therapy, and through de novo formation of neutralizing ADAs that develop after treatment and prevent the ability to re-dose AAV gene therapies. However, many of the inflammatory toxicities of AAV gene therapies appear to be driven by innate immune responses and adaptive CD8 T cell responses [4].

Early clinical trials in hemophilia reported delayed elevation in serum levels of liver transaminases, which is indicative of liver inflammation and hepatoxicity. In some patients, the increase in liver enzymes correlated with a decrease in transgene expression and the appearance of circulating AAV-specific CD8 T cells [4]. In many cases, the elevated liver transaminase levels and further loss of transgene expression could be successfully treated with steroids. The induction of an adaptive immune response indicates the activation of innate antigen-presenting cells (APC), such as dendritic cells, which express toll-like receptors (TLR) involved in recognition of pathogens [7]. The DNA payload of AAV vectors may contain CpG motifs that bind and activate TLR9, an innate immune sensor for unmethylated CpG dinucleotides. Similarly, the capsid itself can be recognized by TLR2 resulting in activation of the APCs which can lead to the induction of adaptive immune responses. A curious aspect of the hepatoxicity associated with AAV gene therapy is the delayed onset, typically occuring 4–8 weeks after dosing. This delay suggests that AAV capsid antigens persists in some tissues and that some secondary event, or possibly cumulative hepatic stress, may cause activation of capsid-specific CD8 T cells [8].

Adverse events related to elevated liver transaminases have now been widely reported in AAV gene therapy clinical trials, with increased prevalence at higher vector doses. AAV gene therapy for neuromuscular diseases has typically required doses of 1–3E14 vg/kg. Onasemnogene abeparvovec, an AAV9 therapy for spinal muscular atrophy (SMA) and the first systemic AAV gene therapy approved by the FDA, has been administered to over 1400 patients. Approximately one-third of the patients receiving a dose of 1.1E14 vg/kg have experienced at least one adverse event of hepatotoxicity [1]. Three patients have been reported to experience severe hepatoxicity 7–8 weeks after treatment. Liver biopsies indicated hepatocyte degeneration and inflammatory infiltrates comprised primarily of CD8 T cells. All three patients recovered after treatment with methylprednisolone. However, tragically, three patients with X-linked myotubular myopathy (XLMTM) died 20–40 weeks after receiving a vector dose of 3.5E14 vg/kg. The patients presented with signs of severe hepatotoxicity, with bilirubin levels 35–60 times the upper limit of normal and delayed elevation in AST and ALT, and evidence of cholestasis, periportal and bile ductal reaction, and secondary fibrosis at autopsy. All three patients showed preexisting evidence of hyperbilirubinemia and intrahepatic cholestasis prior to

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treatment. Reports indicated a lack of inflammatory cellular infiltrates and ineffective treatment with steroids and immunosuppressants. However, an early innate immune response to the massive AAV dose could have contributed to hepatic stress resulting in exacerbation of the underlying disease [8]. The clinical trial was halted and eventually restarted at a lower dose of 1.3E14 vg/kg. Unfortunately, a fourth patient died after treatment with the lower dose [9].

High vector doses of AAV have also been associated with TMA, or atypical hemolytic uremic syndrome (aHUS), a syndrome characterized by hemolytic anemia, thrombocytopenia, and acute kidney injury, and associated with complement activation [1,10,11]. Complement is a key component of the innate immune system and is comprised of plasma proteins that undergo a complex cascade of biochemical reactions that can result in direct killing of pathogens or opsonizing them for clearance, as well as chemoattraction and activation of innate immune cells and the release of vasoactive mediators. AAV can activate complement through binding of antibodies that fix complement (classical pathway) or direct binding of complement components (alternative pathway). In SMA, nine cases of TMA have been reported, including one death [12]. The overall incidence is low, with over 1400 patients treated; nonetheless, one of the affected subjects had complications with persistent hypertension while another case took three months to resolve. In Duchenne muscular dystrophy (DMD), two small clinical trials involving two different AAV product candidates reported four cases of severe adverse reactions (SAEs) involving aHUS out of 15 total patients dosed [11,12]. One case occurred at a dose of 5E13 vg/kg, while the other three occurred at vector doses of 1–3E14 vg/kg. In addition, in September, 2021, Pfizer announced an amendment to their Phase 3 clinical trial in DMD to exclude patients with certain mutations as a result of three SAEs, including two cases of myocarditis [13]. More recently, a patient death in the Pfizer Phase 1b trial was reported on 21 December 2021. No further details were available, and the clinical trial was put on hold [14].

In addition to hepatotoxicity and TMA, other potential risks include hepatocellular carcinoma (HCC) and dorsal root ganglia (DRG) toxicity, which have been observed in animal models of AAV gene therapy [1,15–17]. HCC has been primarily associated with vector integration at high AAV doses in neonatal mice, particularly with vectors that employ a strong promoter that may result in transactivation of protooncogenes [15,16]. Pre-neoplastic hepatic lesions have also been reported in long-term canine studies [18]. HCC has been reported in one hemophilia patient, although subsequent investigation indicate that this incidence was unlikely to be related to the AAV gene therapy vector [19]. Despite the uncertain relevance to humans, a clinical program in phenylketonuria remains on clinical hold due to observations of HCC in the preclinical animal model [20]. DRG toxicity has been observed in nonhuman primates (NHP) and other animal models, primarily associated with direct administration of AAV into the cerebral spinal fluid or high intravenous doses [17]. DRG toxicity appears to be associated with high levels of transgene expression in DRG neurons which may cause neuronal stress [21]. The clinical significance of DRG toxicity is uncertain, although ataxia and tremors has been reported in some NHP, and ataxia and proprioceptive deficits have been reported in piglets receiving an intravenous vector dose of 2E14 vg/kg [17,22]. Histological evaluation at autopsy revealed evidence of DRG toxicity following AAV gene therapy in a human patient with giant axonal neuropathy and a patient with familial amyotrophic lateral sclerosis [1]. The latter patient reported tingling sensations and pain in the extremities after an intrathecal AAV dose of 4.2E14 vg.

The different manifestations and relative incidence of adverse reactions suggest that multiple variables may contribute to the overall risk, such as vector design, vector manufacturing and quality attributes, underlying disease, and comorbidities. Retrospective analysis of hemophilia B gene therapy trials suggest that higher CpG content correlated with higher incidence of liver enzyme elevation, stronger CD8 T cell responses and less durable transgene expression [23]. In DMD, a third clinical program, from Sarepta, has yet to announce a serious adverse event related to aHUS, with 77 patients treated as of October 2021 [24]. The basis for a high incidence of aHUS in two programs but not a third is unknown. It is conceivable that different capsids would have different profiles with respect to fixation of complement or binding to preexisting antibodies. One major unknown with all AAV clinical trials is the degree of impurities from the manufacturing process, such as percentage of empty capsids or host cell impurities [1,5]. Since AAV doses are calculated based on determination of vector genomes (or 'full' capsids), the ratio of empty:full capsids could have a huge bearing on the total capsid dose.

3. Moving forward

For all the notable successes and continued promise of the AAV field, there is clearly room to improve the safety and efficacy of recombinant AAV vectors, particularly for indications that currently require high vector doses. A capsid dose of 1E14 vg/kg represents a dose of over a quadrillion viral particles (not including empty capsids) in a young child, which is far greater than the total number of cells in the body (Figure 1). Increasing the ratio of full:empty capsids and reducing CpG content provide an immediately actionable strategy to improve safety. Another potential strategy to increase transduction efficiency and reduce CD8 T cell responses is to introduce capsid mutations that minimize processing by the proteasome [25], which results in capsid degradation and presentation of immunogenic epitopes on MHC class I molecules, or eliminating MHC-binding epitopes in capsids. Longer term strategies include sophisticated capsid engineering approaches to design or evolve capsids that show better targeting of specific tissues, such as muscle [26]. Improvements in promoter and enhancer design could also improve efficacy at lower doses, although the potential risks of HCC associated with strong promoters and DRG toxicity with transgene overexpression must be considered [16,21]. In addition, engineering of more efficient transgenes, as exemplified by the Padua variant used in hemophilia B [27], could allow for lower vector doses. Ultimately, a breakthrough in radically improving transduction efficiency may require a better

understanding of the key rate limiting steps of AAV transduction, including transit through the cell from the endosome to the nucleus, viral uncoating, maintenance as a stable episome, and interaction with host cell factors [26].

Both capsid engineering and pharmacological strategies are being pursued to mitigate the immunogenicity of AAV capsids. Some strategies, such as mutagenesis of capsids to remove antibody epitopes [26] or use of plasmapheresis [28] or IgG protease [29] to eliminate anti-AAV antibodies, only address the preexisting antibody component, without addressing innate immunity or T cell responses. Indeed, the very presence of preexisting anti-AAV antibodies is indicative of prior exposure to AAV and a high likelihood of the presence of AAV-specific memory T and B cells [30]. High vector doses, which can overcome low levels of neutralizing antibodies, has enabled some groups to loosen the threshold antibody titer for inclusion into clinical trials [31], although preexisting memory T cells and complement fixation by preexisting low-titer antibodies could still potentially exacerbate the toxicities observed with high vector doses. Combination immunosuppressive regimens, such as rapamycin and corticosteroids or rapamycin and rituximab are being evaluated clinically for mitigation of immunogenicity [32]. Rapamycin is an inhibitor of the mTOR pathway, which dosed chronically, can inhibit effector T cell activation. Rapamycin encapsulated into nanoparticles (ImmTOR) is also being evaluated for the induction of antigen-specific immune tolerance to AAV vectors. A dose of ImmTOR nanoparticles administered at the time of AAV gene therapy treatment has been shown to inhibit T and B cell responses to AAV and enable vector redosing [33]. Rapamycin nanoparticles have also been shown to have hepatoprotective properties in animal models of liver inflammation [34]. Notably, rapamycin has also been shown to enhance AAV vector transduction through induction of autophagy [35].

4. Expert opinion

The stakes are high for mitigating the toxicity of high vector doses. The benefit of AAV gene therapy has been undeniable in SMA and hemophilia, and early data are highly promising in DMD. Even in XLMTM, those patients that tolerated therapy showed remarkable benefit. Further improvement might be achievable if the therapeutic window could be safely widened. The ultimate goal should be to provide similar efficacy at lower vector doses. This may require a combination of vector engineering and pharmacological strategies to increase vector transduction efficiency and mitigate immunogenicity. AAV gene therapy has been historically viewed as a 'one-and-done' therapy, meaning that a single dose of therapy was expected to provide years, if not a lifetime, of therapeutic benefit. This notion has been challenged by the waning of efficacy observed in some clinical trials and the loss of efficacy observed in patients that experience hepatotoxicity. If the barrier to vector redosing, currently limited by the formation of high titers of neutralizing antibodies after initial exposure, can be overcome, it not only opens the possibility to restore therapeutic benefit in patients that have experienced loss of transgene expression but may also provide a safer path for the treatment of neuromuscular diseases by allowing for multiple smaller doses of gene therapy rather than a single high dose of 1–3E14vg/kg. The potential of providing a therapeutic outcome by delivering multiple lower doses over a finite period of time (e.g. 1–3 months) may herald the next gene therapy paradigm that facilitates the coming wave of approved gene therapy drugs with lower toxicity profiles for unmet rare diseases – 'one-and-done' may be substituted with 'lower and slower.' One potential challenge to this approach is that vector re-dosing is likely to elicit a stronger anamnestic immune response. An immunosuppressive approach may not be sufficient to prevent the formation of memory T and B cells that would drive a secondary immune response. A strong

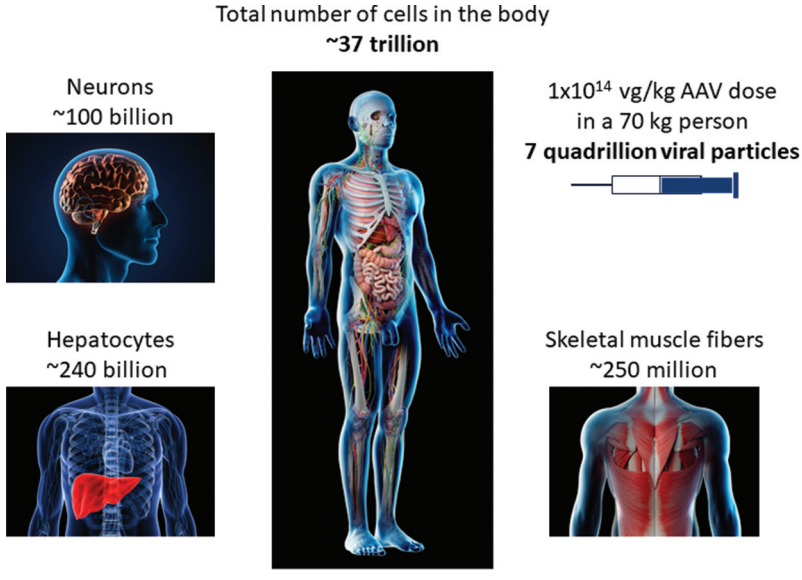

Figure 1. An estimate of the number of cells in the human body compared to the number of full viral particles in a high vector dose of AAV.

Estimated number of total cells, neurons, hepatocytes, and skeletal muscle fibers in the human body [36] and the number of full viral capsids in a 1E14 vg/kg dose of AAV for a 70 kg human. Image credits: Male anatomy and male back muscles by Sebastian Kaulitzki/Science Photo Library via Getty Images; Human brain by Comotion Design via Getty Images; and Human liver by Ingram Publishing via Getty Images.

regulatory T cell response would likely be required to maintain immune tolerance. Combination therapies may need to be employed, particularly with high vector doses.

Funding

This paper was not funded.

Declaration of interest

TK Kishimoto is an employee, shareholder and executive officer of Selecta Biosciences. RJ Samulski is an employee of the University of North Carolina and a consultant of Asklepios BioPharmaceutical (AskBio). The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

Reviewer disclosures

Peer reviewers on this manuscript have no relevant financial or other relationships to disclose.

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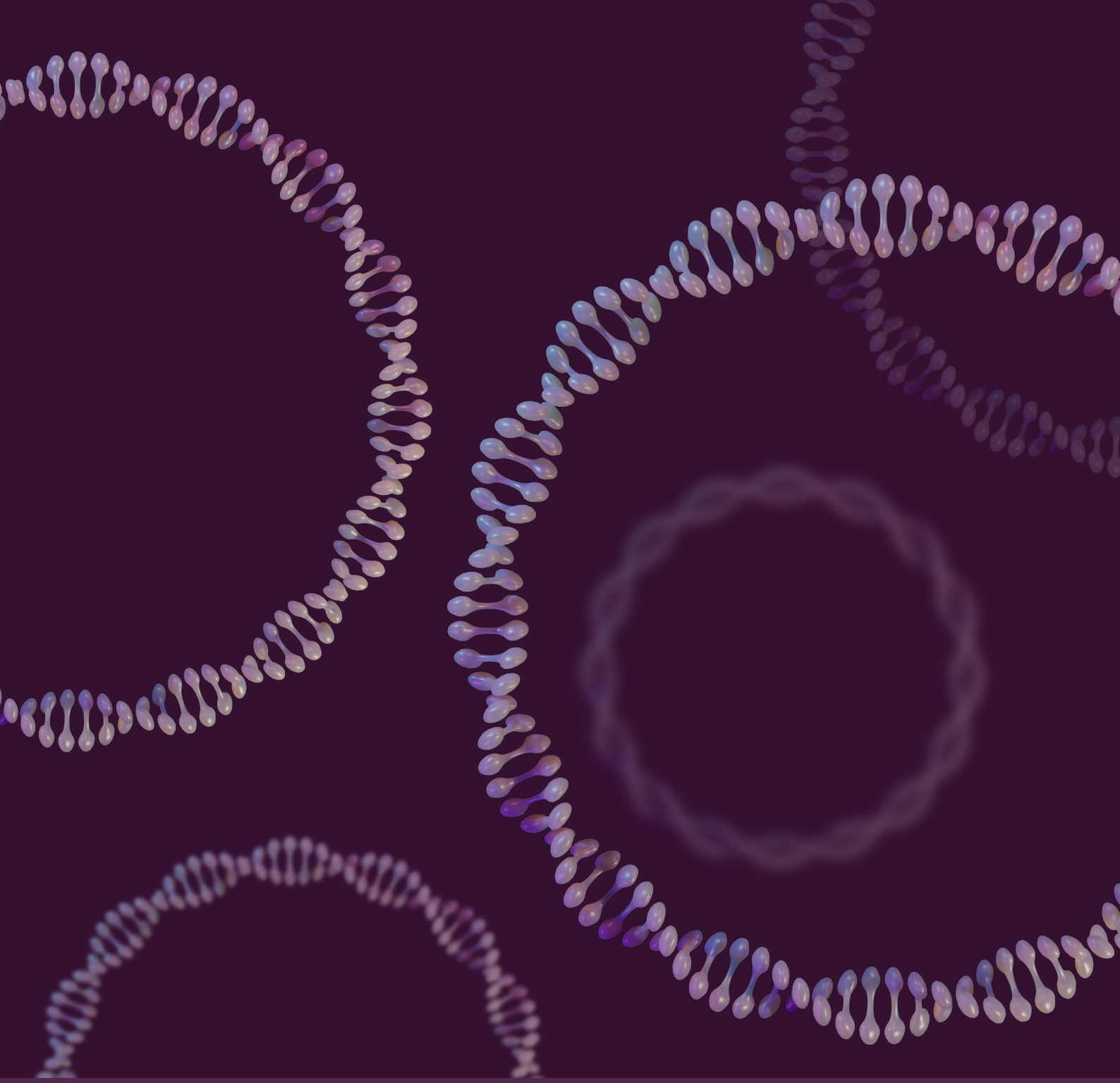

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