Viral Vector API Characterization of Product-Related Impurities

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FDA's final CMC guidance sets expectations for manufacturing and quality for human gene therapy INDs. This article reviews existing analytical applications, focusing on viral vector characterization of impurities.

In January 2020, FDA issued *Final Guidance for Industry: Chemistry Manufacturing and Control (CMC) Information for Human Gene Therapy Investigational New Drug Applications (INDs)* and other guidance documents for human gene therapies, which set expectations for manufacturing and demonstration of quality (1). These products are applied to deliver genetic material (transgenes) or modified cells that are designed to alleviate or even eliminate a broad range of diseases that are rooted in the genetics of individuals or populations of patients.

This article focuses on one of these guidance documents, reviewing aspects of the chemistry, manufacturing and control (CMC) final guidance (2). The authors' goal is to assess existing and evolving analytical applications and technologies and their associated challenges towards ensuring that viral vectored gene therapy drug substances are well characterized for purity and potency (as outlined in Section V.A.3.b.ii of FDA's CMC guidance).

The analytical methods discussed here are used to identify and quantify the potency and product-related impurities within recombinant adeno-associated virus (AAV) vector gene therapy products, and the potential impact of these impurities on product quality and performance.

This article will highlight the characterization of:

- Empty and partial viral particle contaminants relative to the quantity of full capsids
- Quantitation of the total number of genomes present relative to the viral particles infectivity (i.e., those that are capable of delivering the gene of interest to cells).

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The CMC guidance recommends that multiple product characteristics be evaluated prior to and during early clinical trials in order to identify a product's critical quality attributes (CQAs) and biological properties towards obtaining "relevant and meaningful" information about product potency (1). This approach conforms with that in earlier guidance on potency determination, which acknowledged that "no single test … can adequately measure those product attributes that predict clinical efficacy" and so recommends application of an assay matrix (3).

The new guidance also builds upon an early clinical trial design guidance, which recognizes that cell and gene therapy (CGT) products have unique features relative to other drug products that make the CMC and preclinical data less predictive of safety and potency observed in clinical trials (4):

- Expectation of prolonged biological activity
- Potential for immunogenicity.

The "product-related" impurities to be measured are identified as "defective interfering"; "non-infectious"; "empty capsid particles"; and "recombinant virus contaminants" that are to be reported as ratios (e.g., full:empty or virus particles:infectious units). The brevity of the expected analyses for characterizing and quantitating these impurities belies the complexity of these products, their manufacture, and the challenges of measuring these impurities with the accuracy and precision that have been possible for other drug products, including biotherapeutics.

The presence of partially filled capsids, resulting from encapsidation of fragmented genomes or non-transgene-related DNA contaminants, adds to this complexity.

Analyses applied toward quantifying the viral particles for both genomes present and their efficiency at delivering their genetic payload to cells are important because they are applied in early development to support clinical testing and guide early clinical trials. This review provides a limited overview of product implications that arise from the associated product-related impurities found in AAV vector gene therapy products. It also reviews the range and relative performance of the technical applications used to characterize these impurities.

Analytical ultracentrifugation (AUC) is currently the preferred [analytical] method, but new and modified instrumentation capable of supporting this analysis has the potential to either augment or replace AUC.

Relevance to AAV vectors

The features present in viral-vectored gene therapy products take advantage of the evolved effectiveness of viral agents at transducing cells and delivering genes and genetic elements that can establish long-term expression of transgenes. However, because the human immune system has evolved to identify and respond to viral agents, the vectors themselves, their recombinant nucleic acid contents, and the product-related impurities associated with these vectors have the potential to impact both potency and safety. Observations from clinical trials of vectored gene therapy products demonstrate that the presence of defective or in-

terfering particles in these products can influence the product's efficacy in alleviating the targeted disease or deficiency and can dramatically impact the safety and longevity of the product after administration (5,6).

Unique properties of individual vectors have influenced the choice of vectors applied to various disease/deficiency targets. AAV vectors have been chosen for gene delivery because they are able to transduce multiple cell types and tissues.

In addition, the genes they introduce do not integrate into the host's genomic DNA; gene expression is durable, and wild type AAVs are associated with only weak, transient innate and adaptive immune responses. However, pre-existing immunity in patients is present to many AAV capsid serotypes, and the presence of neutralizing antibodies to the capsid serotype, the route of administration, and the dosage applied can influence product effectiveness, durability, and safety (5).

Because cellular immunity against the viral capsid has also been observed in some clinical trials, immunosuppression is becoming standard practice (6). Questions remain regarding how serotype, vector design, manufacturing parameters, and even target gene overexpression might influence clinical responses.

These clinical observations and the complexity of rAAV products underscore the importance of having well-defined and robust manufacturing processes and analytical methods in place that define their physical, chemical, molecular, and biological properties. Analytical methods applied to establishing those parameters vary widely, as do their sensitivity and limitations. Method-specific variation observed in the data generated further confounds the presence of these impurities with clinical observations (6). Improving the accuracy and precision of methods applied to characterize these products is particularly critical to improving the understanding of their safety and effectiveness prior to and resulting from clinical trials.

Characterizing product impurities such as empty or partially filled capsids, or defective particles that contain the transgene but fail to deliver it to a cell, are therefore increasingly important in understanding and establishing a product's CQAs.

The brevity of the expected analyses for characterizing and quantitating these impurities belies the complexity of these products, their manufacture, and the challenges of measuring these impurities with the accuracy and precision that have been possible for other drug products.

Capsid characterization

The production of AAV vectors can result in capsid generation in which 90% contain only some parts of the transgene, or completely lack the transgene. These partial and empty capsids lack therapeutic benefit and may elicit unwanted immune responses (7,8). Therefore, determining the ratio of full, partial, and empty capsids is considered a CQA for AAV-vectored products. This ratio is expected to correlate with efficacious dosage and safety and must be closely monitored to ensure lot-

to-lot consistency. Various techniques have been developed and applied to characterize AAV vector capsid content (**Table I**).

Initial methods indirectly determined the full and non-full (both partial and empty) AAV capsid ratio using quantitative polymerase chain reaction (qPCR) to quantitate viral genomes present and the total capsid quantified by capsid specific enzyme-linked immunosorbent assay (ELISA). Because the ELISA also quantitates capsid-free proteins, this approach has the potential to misrepresent the actual ratio of full: non-full capsid (7,9,1**0).**

Transmission electron microscopy (TEM). TEM allows direct visualization of full, empty and partial capsids. However, the method is highly laborious and subjective (7), making it less suitable for current good manufacturing practice (cGMP) testing.

Spectrophotometry. Spectrophotometry can estimate viral capsid content, based on characterizing the expected absorbance of the viral capsid and genome to derive their extinction coefficients, by applying the 260/280 ratio to quantify the DNA and proteins in a solution (8). The number of empty capsids in purified vector preparations reduces the A260/A280 ratio in a predictable manner.

The method is quick and easy to perform and utilizes common laboratory equipment. However, it requires highly purified and concentrated (> 5 x 1011 vg/mL) vector, and accuracy can be reduced by impurities associated with more complex compositions (8). As a result, this method is generally only applied for analytical testing during early product development (11).

Anion-exchange high-performance liquid chromatography. Because full capsids have lower isoelectric point (pI) values than the empty capsids due to the negative charge of DNA present in the capsid, anion-exchange high-performance liquid chromatography (AEX–HPLC) and capillary isoelectric focusing (cIEF) allow for characterization of vector capsids. The AEX–HPLC method benefits from the native fluorescence of aromatic amino acids and avoids interference from UV absorption of light at 280nm by packaged viral DNA, improving the accuracy of quantification over other meth-

Table I. Summary, capsid content methods.

Method	Capsid detection*	Run time	Throughput	Comments
ELISA/qPCR	F,E	Hours	High	Two separate methods, Indirect calculation
TEM	F,E	~ 1 day	Low	Subjective results requiring human interpretation
UV Spec	F,E	Min	High	Requires very pure preparations along with knowledge of extinction coefficient of capsid
AEX-HPLC	F,E	30 min	High	Reproducible and robust, HPLC commonly used in regulatory environment
cIEF	F,E,P α	<1 hour	High	Requires very pure preparations due to UV detection
SEC-MALS	F,E	30 min	High	Provides capsid concentration and degree of aggregation
AUC	F,E,P	~6 hours	Low	Fully characterizes partial capsids. Large samples size requirement
CDMS	F,E,P	~2 hours [15]	Low	Fully characterizes full, partial, empty capsids. Currently not commercially available

* F=Full, E=Empty, P=Partial, α=Partial, not fully characterized. ELISA/qPCR is Enzyme-Linked Immunoassay/quantitative polymerase chain reaction; TEM is transmission electron microscopy; UV spec is ultraviolet spectroscopy; AEX-HPLC is Anion-exchange-high-performance liquid chromatography; cIEF is Capillary isoelectronic focusing; SEC-MALS is size-exclusion chromatography with multiple angle light scattering; AUC is analytical ultrcentrifugation;CDMS is charge detection mass spectrometry.

ods. AEX–HPLC analyses are fast and allow high throughput, making them a good fit for QC laboratories (12), but doesn't resolve partially filled capsids. cIEF has higher resolving power than AEX–HPLC for distinguishing full and partial capsids but does utilize UV detection at 280nm, which can be problematic with impure samples (13).

Size-exclusion chromatography with multi-angle light scattering (SEC–MALS). SEC–MALS also resolves full and empty capsids by hydrodynamic volume and size separation and determination of the mass and molar mass of the capsid and DNA. This allows calculation of the capsid content, provides total capsid concentration and aggregation estimates. Although also limited by its inability to resolve partial capsids, it is otherwise well suited to QC laboratories (12).

Analytical ultracentrifugation (AUC). AUC separates the AAV capsids by their sedimentation properties and allows characterization of full, empty, and partially full viral capsids. Analysis is performed with *in-situ* monitoring and can quantify and characterize capsid preparations independent of the serotype, DNA size, or form present. Disadvantages include the lack of 21 *Code of Federal Regulations* Part 11 compliant software, the need for large sample size, low throughput, and a long run time (12).

Charge detection mass spectrometry (CDMS). CDMS is a single ion technique that differs from conventional MS because it simultaneously detects the mass-to-charge ratio (m/z) by measuring the velocity of an ion with known electrostatic energy, and charge (z) by utilizing a sensitive amplifier, allowing the mass of each ion to be determined (14). CDMS has shown the ability to resolve capsids that contain the entire vector genome from those that contain partial and empty capsids (15). While no commercial systems specific for CDMS are currently available, there are commercial instruments whose use can be modified (16). In the authors' experience, AUC is currently the preferred method for performing these analyses, but the application of multiple orthogonal methods is generally acceptable for early phase clinical trials. The apparent utility of CDMS and new/modified instrumentation that is capable of supporting this analysis has the potential to either augment or replace AUC.

Clinical observations and the complexity of recombinant adeno-associated virus (rAAV) products underscore the importance of having well-defined and robust manufacturing processes and analytical methods in place.

Potency determination

Early approaches to quantitate vector genome (VG) included dot blot DNA assays, southern blot, ultraviolet (UV) spectrometry, and fluorometry. More recently, real-time quantitative PCR (RT-qPCR) and digital droplet PCR (ddPCR) have emerged as the industry standards. The precision of each approach can be influenced to varying degrees by several AAV product-related factors, including secondary structures of the AVV genome, process impurities (e.g., residual DNA or protein), and buffer matrix.

RT-qPCR and ddPCR have inherent distinctions. While qPCR functions over a wider dynamic range and is more economical, ddPCR of-

fers key advantages. One important benefit is that ddPCR provides absolute quantification of vector genomes, whereas qPCR uses indirect quantitation relative to a reference standard, which may or may not be available or properly represent the rAAV product.

Additional ddPCR advantages result from the droplet compartmentalization of individual DNA molecules that reduces the potential for inhibitory effects of sample matrix or impurities, and droplets concentrate the target sequence. When compared, ddPCR has shown increased accuracy and precision (16).

It should be noted that VG titers derived from these methods have been observed to differ significantly when applied to individual vector lots, and that "viral structure, aggregates, and impurities" and results obtained can "be altered by sample preparation" (17).

While determination of vector particle quantity can be derived by the physicochemical and molecular methods already described, another important parameter for estimation of potency is the ability of the particle preparation to deliver the transgene to cells; referred to as the product's "infectious titer." The combination of VG and infectious titer are applied to derive the virus particles:infectious units ratio referenced in the CMC guidance.

Determination of the infectious titer applied to QC testing of AAV drug substance and drug product generally follows the TCID50 method described by Zhen *et al.* (18). Although this method was developed using qPCR, ddPCR is also potentially an option.

Importantly, the accuracy of the TCID50 method can be influenced by the presence of aggregates and impurities, increasing assay variability (18,19). For this reason, other methods have been developed and evaluated with varying success at discriminating between infectious particles and non-infectious controls. These include infectious center assays that add complexity by use of membrane transferred nucleic acid and probe hybridization, and a method that applies permissive cell lines to eliminate the necessity of using helper virus (20).

Digital droplet polymerase chain reaction (ddPCR) provides absolute quantification of vector genomes, whereas quantitative polymerase chain reaction (qPCR) uses indirect quantitation relative to a reference standard, which may or may not be available or properly represent the recombinant adeno associated virus (rAAV) product.

More recently, a relative infectivity method has been proposed as an alternative to the TCID50 method for use in screening early AAV vector candidates that also eliminates the need for a helper virus (19). The variability associated with infectivity methods may be further exacerbated when measures of potency progress to expression or functional activity assays, as these measure transgene activity downstream from the transduction events quantitated by PCR methods. As a result, it is unlikely that infectious titer mea-

surement by some method will be replaced, especially early in product development and clinical material assessment. However, having multiple orthogonal methods applied to determine AAV product potency would support acceptance of modifications, improving the reliability of infectious titer quantitation (4).

Summary

Viral vector products, and rAAV in particular, may be a heterogeneous mixture of empty and partial capsids, noninfectious particles (containing DNA, but do not result in detectable *in-vitro* DNA amplification), and infectious particles (complete vectors that enter the cell and *in-vitro* DNA amplification and transgene expression is detectable).

Particles that do not result in expression/amplification are considered product-related impurities that can impact product efficacy and immunogenicity, and thus must be quantified (3). Production conditions and purification processes can dramatically impact the levels of these impurities.

While there is some debate around the impact of these impurities on product performance (4), the regulatory guidance identifying these particles as contaminants suggests attempts should be made to at least reduce, if not eliminate, non-transgene expressing particles. To do that effectively, accurate and precise measurements of these particles must be available.

As described previously, extensive efforts are being made to apply a variety of instruments and applications toward achieving that objective, but it will take additional effort to identify and refine standardized methods that can be universally applied across the range of AAV vectors being developed, as has been done for other biopharmaceutical products. Because of their complexity and heterogeneity, it is likely that an analytical matrix still may be required even when acceptable standardized methods are available.

As directed by regulatory guidance, product developers should consider the development and application of orthogonal methods to characterize their AAV-vectored gene therapies. This approach will ensure the proper selection of and specifications for CQAs.

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