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Review

Analytical methods for process and product characterization of recombinant adeno-associated virus-based gene therapies

Andreas L. Gimpel,[1,2] Georgios Katsikis,[3] Sha Sha,[4,5] Andrew John Maloney,[1] Moo Sun Hong,[1] Tam N.T. Nguyen,[1] Jacqueline Wolfrum,[5] Stacy L. Springs,[5] Anthony J. Sinskey,[4,5] Scott R. Manalis,[3,6,7] Paul W. Barone,[5] and Richard D. Braatz[1,5]

[1]Department of Chemical Engineering, Massachusetts Institute of Technology, Cambridge, MA 02139, USA; [2]Department of Chemistry and Applied Biosciences, ETH Zurich, Zurich, Switzerland; [3]Koch Institute for Integrative Cancer Research, Massachusetts Institute of Technology, Cambridge, MA, USA; [4]Department of Biology, Massachusetts Institute of Technology, Cambridge, MA, USA; [5]Center for Biomedical Innovation, Massachusetts Institute of Technology, Cambridge, MA, USA; [6]Department of Mechanical Engineering, Massachusetts Institute of Technology, Cambridge, MA, USA; [7]Department of Biological Engineering, Massachusetts Institute of Technology, Cambridge, MA, USA

The optimization of upstream and downstream processes for production of recombinant adeno-associated virus (rAAV) with consistent quality depends on the ability to rapidly characterize critical quality attributes (CQAs). In the context of rAAV production, the virus titer, capsid content, and aggregation are identified as potential CQAs, affecting the potency, purity, and safety of rAAV-mediated gene therapy products. Analytical methods to measure these attributes commonly suffer from long turnaround times or low throughput for process development, although rapid, high-throughput methods are beginning to be developed and commercialized. These methods are not yet well established in academic or industrial practice, and supportive data are scarce. Here, we review both established and upcoming analytical methods for the quantification of rAAV quality attributes. In assessing each method, we highlight the progress toward rapid, at-line characterization of rAAV. Furthermore, we identify that a key challenge for transitioning from traditional to newer methods is the scarcity of academic and industrial experience with the latter. This literature review serves as a guide for the selection of analytical methods targeting quality attributes for rapid, high-throughput process characterization during process development of rAAV-mediated gene therapies.

Recombinant adeno-associated viruses (rAAVs) are widely used vectors for *in vivo* gene therapy, primarily because of their non-pathogenicity to humans, low immunogenicity, and long-term gene expression.[1] The US Food and Drug Administration (FDA) recently approved rAAV-based gene therapies for treatment of two rare monogenic diseases: spinal muscular atrophy (Zolgensma)® and Leber congenital amaurosis (Luxturna)®.[2,3] In addition, rAAV-based gene therapies are currently being investigated in over 200 clinical trials for a range of diseases from cancer to neurological disorders.[4] As the clinical potential of rAAV is being recognized, challenges in the scale-up of its biomanufacturing process without compromising potency, purity, and safety are becoming increasingly relevant.[5–7]

The development of manufacturing platforms for biopharmaceuticals requires identification of the critical quality attributes (CQAs) of the product and characterization of the functional relationship between CQAs and process parameters. Defined as "a physical, chemical, biological, or microbiological property or characteristic that should be within an appropriate limit, range, or distribution to ensure the desired product quality,"[8] a CQA is an attribute—broadly corresponding to the product's identity, potency, purity, or safety—with severe impacts on product quality and efficacy if outside its target range. The characterization of the impact of changes to process parameters on these CQAs, such as by using design of experiments,[9] improves understanding of the process, enables its optimization, and ultimately assures product quality by design (Table 1).[10–12] In the case of biopharmaceuticals, such as rAAV, attributes of the drug substance (e.g., identity) and knowledge of impurities—either product related (e.g., inactive product variants and aggregates) or process related (e.g., host cell proteins or DNA)—help identify CQAs early in development.[11,13] Although process-related attributes are not unique to rAAV manufacturing,[11,14] CQAs relating to the rAAV product or product-related impurities are just starting to be clearly identified.[15] In this review, we focus on productivity of rAAV production, the virus titer, and the fraction of capsids with a complete genome (content ratio) and the amount of aggregated capsids as potential CQAs based on their impact on product safety and efficacy.

Quality attributes of rAAV are routinely analyzed using time-intensive quality control assays for batch release testing of the final product to ensure they fulfill regulatory specifications for safe use.[16–19] However, short timelines of product development require economical

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Correspondence: Richard D. Braatz, Department of Chemical Engineering, Massachusetts Institute of Technology, 77 Massachusetts Avenue, Room E19-551, Cambridge, MA 02139, USA.
E-mail: braatz@mit.edu

Table 1. Definition of important terms relating to process development used throughout this work

Critical quality attribute	quality attribute that must be within an appropriate limit, range, or distribution to ensure the desired product quality
Process parameter	variable of the manufacturing process
Process understanding	ability to explain and manage all sources of variability in a process and to reliably predict product quality attributes
Process development	the establishment of a manufacturing process that produces product with the intended product quality attributes
Process monitoring	the monitoring of process parameters or critical quality attributes in, or close to, real time to facilitate the control of an established manufacturing process
Quality attribute	Physical, chemical, biological, or microbiological property or characteristic

Terms are defined and interpreted as by the FDA.[8,10]

analytical methods with rapid turnaround and high throughput. For example, a fractional factorial design used for early screening of a rAAV production platform—with only five process parameters at three levels—required rapid, highly parallelized analysis of 32 experiments.[20] Such throughput inevitably leads to a bottleneck in the analytical capacity of most established quality control assays. Alternatively, rapid, high-throughput analytical methods yield actionable results swiftly, fostering process understanding and accelerating development decisions. However, limited sample quantities and matrix effects (e.g., buffer identity, residual DNA or protein, or ionic strength) pose challenges to such methods in process development.[21–23] Commonly, laboratory-scale production yields only few milliliters (mL) of purified rAAV—equivalent to 10^{12}–10^{14} vector genomes (vgs)—such that the required sample volumes for analysis can cause significant loss of product.[6,7,20,23,24] At the same time, virus concentration in samples covers many orders of magnitude, from 10^{10} to 10^{11} vg/mL at raw culture harvest to 10^{13} to 10^{14} vg/mL in the purified product,[6,7,18–20,24–27] and the sample matrix varies significantly, with the mass concentration of residual byproducts, host cell proteins, and DNA exceeding that of rAAV by 10- to 100-fold in initial downstream steps.[28] This excess of residual byproducts limits the suitability of methods with insufficient specificity, unless extensive sample purification is performed.[21,22]

In this paper, we first introduce the three quality attributes of genome and capsid titers, content ratio, and aggregate content for characterizing the main types of capsids generated in rAAV (Figure 1). To establish these quality attributes as CQAs, we outline both their variability in common production processes and their general impact on safety and efficacy of rAAV products. We then discuss their advantages and disadvantages as CQAs in the context of process development. Finally, we present the current state-of-the-art analytical methods for characterization of CQAs for rAAV and conclude with suggestions for further method development. A list of abbreviations is given in Table 2.

Product-related CQAs of rAAV

Virus titer or concentration, as the first potential CQA, refers either to infectious, genome, or capsid titer (Figure 2). Here, we will focus on the genome and capsid titers, because they are practically measurable from a process development perspective. The genome titer represents capsids containing the vector genome (i.e., virions), estimates the potency of a sample, and is primarily used for clinical dosing of rAAV.[25,35] The capsid titer instead represents all capsids, independently of the contained genome. Quantification of total capsids is crucial for the operation and optimization of downstream recovery or purification processes that depend on the load of product rather than its potency, e.g., loading of preparative columns, or pooling of column fractions.[11] Moreover, capsids tend to aggregate depending on their total concentration and environmental conditions, such as the temperature, ionic strength, and pH.[36] Thus, depending on the process unit under consideration, both genome and capsid titers can be indicative of the potency, safety, and efficacy of the product. In contrast, the infectious titer—a measure of biological activity *in vitro*—takes 1–3 days to quantify and exhibits variability of multiple orders of magnitude;[28,34] thus, it is impractical for timely characterization of product potency. Methods for its quantification are based on endpoint dilution assays,[17,19,34,37–39] infectious center assays,[18,34] and transgene expression assays,[17,34,37] and we will not discuss them in this review.

Content ratio, as the second potential CQA, refers to the ratio of viral capsids either missing or having a partial genome, termed empty or partially filled capsids, respectively, and represents the most common product-related impurity in rAAV production (Figure 1). Different factors, such as the length and type of the vector genome (i.e., single stranded or self-complementary), contribute to the variability in the overall ratio of capsids without the intended vector genome.[19,29] In addition, the choice of production platform and the size of the plasmid affect the extent to which reverse packaging of non-transgene sequences leads to the generation of an undesirable subpopulation of capsids partially filled with DNA impurities.[40,41] Empty capsids, as well as capsids with non-transgene sequences, can elicit an unnecessary immune response and compete for vector binding sites, increasing the risk of immunotoxicity and lowering the rate of transduction of full capsids, which are required for product efficacy.[28,30] The ratio of full capsids to all capsids, termed content ratio, can vary from <1% to 30% in the production culture at the time of harvest and is inconsistent between production runs (Figure 1).[6,28–30] Affecting both process efficiency and product purity, the content ratio has been recently added to the regulatory product specifications for virus-based gene therapies and is an important quality attribute in process development.[42,43] Unlike empty capsids, partially filled capsids and full capsids with host cell/plasmid DNA may have additional genotoxic effects, depending on the type of encapsidated genome.[28] Other impurities, besides empty and partially filled capsids, can contribute to immuno- or genotoxic effects, e.g., residual helper viruses (e.g., from upstream transduction steps), residual proteins (e.g., from the host cell or the cell culture medium), or nucleic acids (e.g., host cell DNA/RNA).

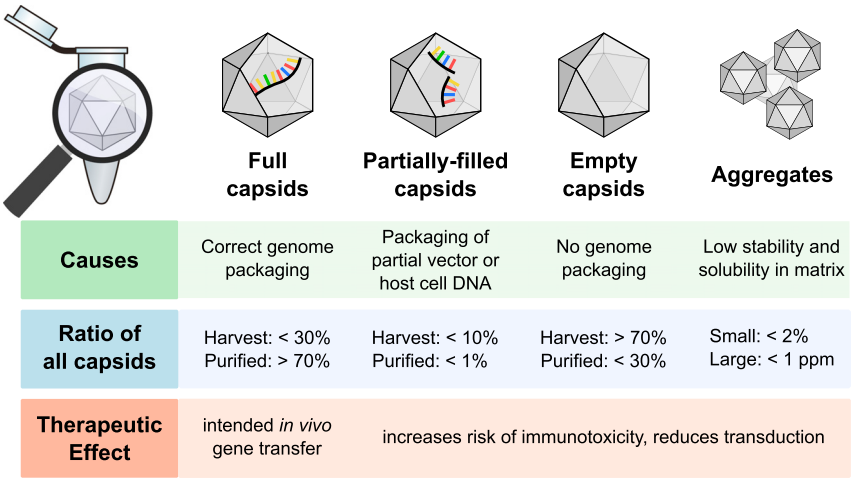

Figure 1. Overview of the main types of capsids generated during rAAV production

The ratio of all capsids is given at the time of harvest of the rAAV production culture ("Harvest"), e.g., in the cell lysate prior to any purification, and in the product after purification ("Purified"), i.e., after the purification of full capsids from the cell lysate but prior to any polishing steps for the near-complete separation of full from empty capsids. The data are based on literature reporting rAAV production on large scales.[6,7,18,19,24,25,28–30] Aggregates are discerned based on size as small (multimers, d < 100 nm) or large (d > 100 nm), with their content as reported for commercial, purified rAAV products.[31–33]

The individual risks associated with these impurities and analytical methods for their quantification have been reviewed in detail,[28,30] so they will not be discussed in this review.

Aggregates of capsids, as the third potential CQA, represent another product-related impurity with detrimental impact on safety and long-term stability of the vector product, as even trace amounts (e.g., <1%) provide nucleation sites for further aggregation.[28,44,45] Both small oligomers (d < 100 nm) and large, subvisible aggregates (d > 100 nm) are present in rAAV preparations,[31–33] and high concentration of capsids, residual nucleic acids, or a low ionic strength in the sample matrix were shown to contribute to aggregation of rAAV2.[36] Thus, aggregation may occur at several points in downstream processing where these conditions are present, e.g., during concentration steps or as result of a buffer exchange. To ensure product safety, minimizing the extent of aggregation and identifying process conditions affecting this product attribute are needed.

Performance of analytical methods in characterization of quality attributes

Analytical methods must possess a series of characteristics to demonstrate suitability for their intended purpose (i.e., method qualification and validation), as well as to satisfy specific needs in process development (Table 3). For the latter, these characteristics may also include the suitability to at- or in-line application. Compared to offline analysis, which requires samples to be moved from the point of sampling into a laboratory environment, methods capable of at- or in-line application facilitate the monitoring of a running process with analytical instrumentation either close to the point of sampling (at-line) or directly integrated into the process itself (in-line). The qualification and validation of analytical methods, based on characteristics defined in the regulatory guidance on the validation of analytical procedures,[46] are not required for methods used in process development. Still, most of those characteristics, e.g., accuracy, precision, and specificity, still apply as assays are usually qualified using these characteristics in the early phases of development. Full assay validation based on the regulatory guidance, rather than qualification, is required for assays used in later development phases and for certified production.[11] Table 4 summarizes the characteristics alluded to above for the analytical methods discussed in this review, with key conclusions listed in Table 5.

qPCR and ddPCR

Quantitative polymerase chain reaction (qPCR) and digital droplet PCR (ddPCR) quantify the DNA vector via fluorescence (FS) detection during or after amplification in a thermocycler. They are currently the most widely used methods for the quantification of the genome titer because of their simplicity, specificity, and robustness.[21,35,71,79] For accuracy, these methods require chemical or enzymatic treatment of the sample to digest non-encapsidated DNA and denature capsid proteins to expose the encapsidated DNA.[18,25,35] These treatments render them tolerant to protein or DNA impurities and suitable for unpurified, in-process samples.

qPCR is the standard procedure for genome titration of rAAV reference standard material (RSM), but large multi-laboratory studies highlighted its low precision, with repeatability, indicated by the coefficient of variation (CV), as poor as >30% CV and reproducibility at 70%–100% CV.[37–39] Stochastic and systematic sources of this variability, such as low replication efficiency or choice of the primer's target sequence, have been identified and alleviated with additional DNA pre-treatments and optimized primers,[35,57,70,72,80–82] improving repeatability to 5%–20% CV and intermediate precision to 15%–50% CV.[21,24,25,35] Compared to qPCR, ddPCR does not require a standard curve and measures the endpoint of PCR cycles, exhibiting less sensitivity to variable replication efficiency.[82] Thus, ddPCR is significantly more precise than qPCR—at repeatability of 2%–10% CV and intermediate precision of 3%–8% CV—and more tolerant to matrix effects and inhibitors.[21,35,57,58] Both qPCR and ddPCR exhibit a dynamic range of 2–4 orders of magnitude. When an oversized transgene in AAV is truncated from the terminals, neither qPCR nor ddPCR, using a selected amplicon toward the end of the transgene, can accurately determine the titer of complete genomes, as the PCR replication cycle only considers the presence of the amplicon itself, i.e., the DNA flanked by the primer sequences.[25,83] Moreover, genome titers determined by qPCR and

Table 2. Meaning of the important abbreviations used throughout this work

AEC	anion-exchange chromatography
AUC	analytical ultracentrifugation
BLI	biolayer interferometry
CDMS	charge-detection mass spectroscopy
CQA	critical quality attribute
CV	coefficient of variation
ddPCR	digital droplet polymerase chain reaction
DLS	dynamic light scattering
dRI	differential refractive index
DyeBA	dye-based binding assay
ELISA	enzyme-linked immunosorbent assay
FS	fluorescence spectroscopy
FV	flow virometry
MALS	multi-angle light scattering
MassP	mass photometry
OD	optical density
Ppm	parts per million
qPCR	quantitative polymerase chain reaction
rAAV	recombinant adeno-associated virus
RSM	reference standard material
SD	standard deviation
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SEC	size-exclusion chromatography
SLS	static light scattering
TEM	transmission electron microscopy

ddPCR may differ,[21] complicating the switch from qPCR to ddPCR during development or manufacturing.

ELISA

An enzyme-linked immunosorbent assay (ELISA) quantifies the capsid titer via the photometric activity of a substrate reaction catalyzed by an enzyme linked to monoclonal antibodies specific to a conformational epitope of assembled rAAV capsids. In its most common and specific variant, sandwich ELISA, a capture antibody is used to immobilize rAAV capsids on a solid support, and a detection antibody facilitates quantification with a biotin-conjugated antibody and streptavidin peroxidase catalyzing the biotin/streptavidin peroxidase color reaction.[84] The assay is typically performed after a purification step but is sufficiently robust and specific to be used on samples with moderate matrix effects and free capsid proteins, like cell lysates.[26,37,61,85] ELISA involves multiple lengthy incubation and washing steps prior to analysis, for a total turnaround time of 4–5 h, or up to a full working day.[26,61–63]

Sandwich ELISA was used for the characterization of AAV2 and AAV8 RSM, demonstrating acceptable precision in the multi-laboratory study, with repeatability of 10%–15% CV and reproducibility of

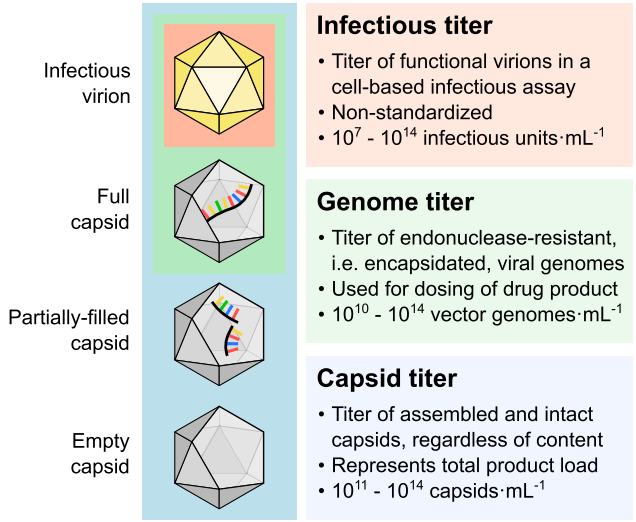

Figure 2. Differences among infectious, genome, and capsid titers of a rAAV sample

Common ranges for the titers during the production process are given in their commonly used units and based on literature reporting rAAV production on large scales.[6,7,18,19,24,25,28,30,34]

around 40% CV.[37–39] Specificity is high, with little cross-reactivity between serotypes, and monoclonal antibodies specific to the conformational epitope of serotypes rAAV1, -2, -3, -5, -6, -8, -9, and rh.10 are commercially available.[61,62] The quantification limit is around 10^8 capsid particles (cps) per milliliter, depending on serotype, and the linear range is limited to 1–2 orders of magnitude, so that pre-titration is recommended for samples of unknown titer.[61,62]

Specificity and robustness to matrix effects are major advantages of ELISA, but in considering the long turnaround time, moderate throughput of only 10 samples per 96-well plate, and labor-intensive sample preparation, it is challenging to implement for routine quantification of capsid titer in a process development setting without automated liquid handling. Recently, optimization of reagent concentrations reduced incubation times to a total of 2.5 h for a single assay, at comparable accuracy and precision, for serotypes 2, 8, and 9.[63] Moreover, ELISA-based rAAV assays were successfully transferred to the well-established Gyrolab systems, a miniaturized ELISA based on an automated, microfluidic platform that promises to reduce turnaround time and improve throughput to 96 samples per hour, while increasing linear range by one order of magnitude.[86] These developments aim to alleviate some of the concerns for ELISA-based assays in process development, but further method development will be required to establish accuracy and reliability compared to traditional ELISA.

ELISA, in conjunction with qPCR, estimates the content ratio of a given rAAV sample by quantifying capsid and genome titers with ELISA and qPCR, respectively (qPCR + ELISA). Accuracy and precision of this indirect method are generally low, as they depend on the cumulative variability of the two methods.[22] Although repeatability of

Table 3. Characteristics of an analytical method relevant to method qualification and validation and needs in process development

Relevance	Characteristic	Description	Importance
Qualification and validation	accuracy	closeness of result to true value	medium[a]
	repeatability	precision under identical conditions, intra-assay precision	High
	intermediate precision	precision within a laboratory, inter-assay precision	High
	reproducibility	precision between laboratories, inter-laboratory precision	medium[b]
	specificity	ability to distinguish between analyte and other components	High
	detection limit	the lowest amount of analyte that can be detected	low[c]
	quantification limit	the lowest amount of analyte that can be quantified	low[c]
	linearity	results directly proportional to the amount of analyte	High
	range	interval of analyte conditions for which the method is linear, accurate, and precise	medium[d]
Needs in process development	sample volume	volume of sample required for routine analysis	High
	robustness	tolerance of method to matrix effects	medium[e]
	turnaround time	time required from sampling to result	high
	throughput	number of samples being processed in parallel	high

The importance of each characteristic during selection of analytical methods during process development is based on guidance for validation of analytical methods[46] and qualification plans during early-stage process development.[11]
[a]Accuracy can be inferred from precision, linearity, and specificity.[11] It may be difficult to establish due to a lack of adequate standards.
[b]Reproducibility only gains importance for lab-to-lab transfer and method standardization.[11,46]
[c]The detection and quantification limits are relevant mainly to assays quantifying low levels of impurities.[46]
[d]Range strongly overlaps with linearity, precision, and accuracy.[46]
[e]Robustness is considered in later stages of assay development.[11,46]

4%–24% CV has been reported,[22] careful consideration of error propagation (qPCR: 5%–30% CV, ELISA: 10%–15% CV, combined: 11%–36% CV) and data accuracy is required for routine estimation of content ratio by this approach.

TEM

Transmission electron microscopy (TEM) creates an image with nanometer resolution using an accelerated electron beam that passes through a thin sample, with transmission dependent on the local electron density. It is commonly used with negative staining, in which a heavy metal stain, commonly uranyl salts, increases contrast. For rAAV (d of around 25 nm), stained, partially stained, and non-stained capsids within images of samples represent empty, partially filled, and full capsids, respectively, and are quantified by manual visual identification.[21,23,87] It is the *de facto* reference method for determination of the content ratio in a laboratory setting but has also been used for the identification of aggregates, deformed capsids, and non-capsid particles, such as host-cell debris.[21,76,77] Although it has been reported that TEM can be used on non-purified samples, it is commonly used only with purified samples, as proteins and cell debris present in the cell lysate can interfere with the accurate identification of rAAV capsids in TEM images.[77,88] Sample preparation consists simply of sample deposition on grids and staining with a heavy metal stain.

The precision of TEM has not been assessed comprehensively. Multiple thousands of capsids must be imaged and analyzed for statistical significance, with the capsid content of individual images varying by around 2%.[23,88] However, unreliable results are common, and poor agreement with orthogonal methods, such as analytical ultracentrifugation (AUC), has been reported.[25,78] Low accuracy is often caused by dimly stained, partially filled particles and uneven staining, leading to low contrast and ambiguity in identification of capsids.[23,88] To this extent, the recent commercialization of low-voltage TEM is promising, as automated image analysis reduced turnaround time to around 6 h and standardized the analysis of large quantities of capsids.[76,77] The versatility of TEM as an assay for content ratio, aggregation, and purity; its simple sample preparation; and its direct visual inspection of the sample is advantageous in a process development setting. However, low throughput and long turnaround limit its application as a routine analytical method, and its systematic disagreement with orthogonal methods must be investigated.

In addition to TEM, cryogenic electron microscopy (cryo-EM) is used for quantifying the content ratio and validated as a release testing assay.[77,88] Although one study found no significant difference in the measured content ratio between TEM and cryo-EM,[89] the latter has been reported to be more robust than the former, owing to the commonly observed interference of cell debris and uneven staining of capsids in TEM.[88]

OD

Optical density (OD; also UV absorption) measures the absorbance of nucleic acids and proteins at 260 nm and 280 nm to quantify capsid titer and content ratio of rAAV samples based on pre-determined extinction coefficients specific to the capsid serotype and molecular weight of its genome.[17,22,25] Denaturation of the capsid by detergents and heat to expose the vector genome is recommended but not performed in some studies.[90] The assay is not specific to rAAV; thus, removal of all other absorbing impurities from the sample, such as residual nucleic acids and proteins, is strictly required for accuracy.[22,85]

The precision of OD is comparable to the combined qPCR/ELISA workflow and repeatability reported as poor as 15% CV for content ratio and 22% CV for capsid titer.[22,70] The method is limited to pure, concentrated samples above 5×10^{11} vg/mL, requiring purification and likely, concentration for in-process samples, but is rapid (about 15 min) and suitable for purified rAAV.[22] The high limit of

Table 4. Data on the most important performance criteria of the analytical methods discussed in this work

Method	Target	Repeatability[a]	Turnaround[b]	Purification	Preparation steps	Sample volume	Range[c]	Key references
AEC	content ratio	<1%–4%	30 min	no	none	5–20 μL	>10^{11} vg/mL	23,47,48
AUC	content ratio	2%	6 h	yes	titration into linear range	400 μL	2 × 10^{12}–5 × 10^{12} cp/mL	23,44,49–52
	aggregation	±1% SD[d]						
BLI	capsid titer	10%	30 min–1 h	no	none	unavailable	10^{8}–10^{10} vg/mL	53–55
CDMS	content ratio	<2%	2 h	yes	buffer exchange	unavailable	nanomolar	56
ddPCR	genome titer	2%–10%	1–2 h	no	removal of non-encapsidated DNA, protein denaturation	2–5 μL	10^{2}–10^{7} vg/mL	21,35,57,58
DyeBA	genome titer	4%−16%	30 min–3 h	yes	removal of non-encapsidated DNA, capsid lysis	1–10 μL	10^{10}–10^{13} vg/mL	59,60
ELISA	capsid titer	10%–20%[e]	2–5 h	no	serial dilution	100 μL	10^{8}–10^{10} cp/mL	37–39,61–63
FV	capsid titer	5%–31.5%[d]	30 min	no	dyeing	195 μL	10^{6}–10^{8} cp/mL	64–66
MassP	content ratio	not available	2–5 min	no	none	0.5–1 μL	10^{12}–10^{13} cp/mL	67–69
OD	capsid titer	2%–22%	15 min	yes	protein denaturation	2 μL–1 mL	5 × 10^{11}–10^{13} vg/mL	22,70
	content ratio	2%–15%						
qPCR	genome titer	5%–30%[e]	1–2 h	no	removal of non-encapsidated DNA, protein denaturation	1–10 μL	10^{5}–10^{10} vg/mL	21,24,35,37–39,57,71,72
SEC-FS	aggregation	<5%[e]	30 min	yes	none	3 μL	>10^{12} cp/mL	33,73
SEC-MALS	capsid titer	not available	30 min	yes	none	30 μL	>4 × 10^{13} cp/mL	74,75
	content ratio	not available						
	aggregation	not available						
SLS-DLS	capsid titer	5%–45%	2–5 min	yes	centrifugation	1–30 μL	6 × 10^{10}–10^{15} cp/mL	31,32
	aggregation	up to >50%						
TEM	content ratio	±15% SD	3–6 h	yes	staining	3–20 μL	not available	23,25,56,71,76–78

The table does not include the methods discussed in Other methods, due to a lack of sufficient available data. The method abbreviations used are listed in Table 2.
[a]Repeatability given as coefficient of variation (CV), unless otherwise noted. SD, standard deviation.
[b]Turnaround time includes sample preparation, but not sample purification, if applicable.
[c]vg, vector genome; cp, capsid particle.
[d]Repeatability was not determined specifically for rAAV.
[e]Includes median repeatability estimated from the intra-laboratory results of the characterization studies for AAV8 and AAV2 RSM.[37–39]

quantitation and small linear range of OD, commonly two orders of magnitude in size, could be improved by spectrophotometers with shorter or variable path lengths.[11,91] With its simple instrumentation, OD is automatable and in theory, adaptable to online process measurement, but in practice, the purification and denaturation steps limit its application in process development. Instead, OD is often used intrinsically as a detector in methods based on physical separation of capsids, such as anion-exchange chromatography (AEC) or AUC.

AUC

Sedimentation-velocity AUC relates the sedimentation profiles of a sample subjected to centrifugal forces to the distributions of molecular weight and size of particles. In a single experiment, AUC assesses the presence of aggregates and quantifies the relative content of empty, partially filled, and full capsids.[23,49,50] AUC is distinct from density-gradient ultracentrifugation, a common process used for purification and enrichment of full capsids during downstream processing of rAAV, as AUC monitors particle sedimentation during centrifugation using either UV absorption or Raleigh interference. Sensitivity of the detectors and the risk of induced density gradients limit AUC to purified samples without residual impurities or excipients, and prior titration into the small linear range of the detector, 2 × 10^{12}–5 × 10^{12} cp/mL, is needed.[49,51,52,92]

AUC has two advantages for the quantification of the content ratio: it is highly repeatable, at only 2% CV, and distinguishes partially filled from empty and full capsids.[49] AUC is generally not quantitative for the quantification of aggregates if their content is below 3%–5%,[50] rendering analysis of rAAV samples, with typical aggregate contents below 1%, challenging (Figure 1). This high quantification limit is due to poor accuracy and low reproducibility, with standard deviation up to around ± 1% in aggregate content, caused by improper alignment of hardware components.[44,50,52,93] In general, AUC requires large sample volumes, 400–500 μL, at high concentrations around 2 × 10^{12}–5 × 10^{12} vg/mL, representing a significant loss of unrecoverable material during early production stages.[23,49,51] With throughput limited to 7 samples in around 6 h, routine use of AUC in process development is challenging. Instead, it is well suited as an orthogonal method to validate more rapid methods with poorer resolution.[23,44]

Table 5. Main advantages and disadvantages identified for each analytical method in this work

Method	Key advantage	Key disadvantage
AEC	robust method with high reproducibility and potential for online characterization of capsid content and titer	high limit of quantitation, resolution of empty and full capsids is poor, and method development is required for each serotype
AUC	*de facto* standard method for quantifying partially filled capsids; useful for validation of aggregates	long turnaround, low throughput, requires large amounts of purified sample
BLI	fast, at-line method with high specificity and increased throughput compared to ELISA	requires serotype-specific antibodies and further method development; no published literature on rAAV
CDMS	capable of quantifying partially filled capsids	still in experimental stage, no significant advantages over AUC
ddPCR	more accurate and precise than qPCR, less sensitive to replication efficiency and matrix effects	not as commonly used industrially yet, less mature than qPCR
DyeBA	simple, fast, and scalable alternative to PCR-based methods; not genome dependent	not suitable for non-purified cell lysate; possible matrix effects
ELISA	most common method for capsid titer quantification, high specificity for intact capsids	long turnaround times and low throughput for most serotypes, labor intensive
EM	most common method for quantifying content ratio, characterization of aggregation possible, allows direct imaging of sample	low throughput and long turnaround times; image analysis is challenging
FV	rapid, simple assay for process samples with high specificity	only rAAV2 and -3 supported commercially, bias in capsid titer results compared to ELISA; no published literature on rAAV
MassP	rapid quantification of the content ratio in small sample volumes	accuracy, precision, and robustness for rAAV5 quantification unknown
OD	rapid, simple, and automatable quantification method for sufficiently pure samples	low precision, samples must be completely pure from protein and DNA impurities
qPCR	most common method for quantification of genome titer, specific and relatively fast	requires standard to be run in parallel, sensitive to variability in replication efficiency and matrix effects
SEC	common, rapid method to assess aggregates; can be combined with MALS to determine capsid content	issues with filtration, non-specific interactions, and deaggregation of large aggregates; no published literature on rAAV
SLS/DLS	rapid and non-destructive method with high throughput capable of online operation	accuracy and precision are poor, and sensitivity to optical properties of sample is significant; cannot resolve small aggregates
Soft sensors	yields real-time data on important process variables during operation within the production vessel	robustness unknown, challenging to validate

The table does not include the methods discussed in the Other methods, due to a lack of sufficient available data.

AEC

AEC has been used to separate empty and full rAAV capsids of several serotypes (1, 2, 5, 6, 8, 9) on a positively charged stationary phase based on the difference in their isoelectric points caused by the encapsulated, negatively charged genome.[47,94,95] AEC is one of the most widely used purification processes for the separation of empty and full capsids during downstream processing of rAAV and can be used on validation-friendly high-performance liquid chromatography (HPLC) systems equipped with OD or FS detectors to quantify the content ratio in the eluate.[23,48] Sufficient separation between empty and full capsids requires development and optimization of mobile phase conditions for each serotype, but baseline separation of full and empty particles, as well as resolution of partially filled capsids, has not been achieved so far.[23,47,48,95] The method is applicable to samples from downstream purification processes without the need for additional sample purification or preparation.[23,48] Sensitivity is too low for quantification of raw harvest, however, with the lower end of the UV detector's linear range at around 10^{11} vg/mL.[48]

Accuracy of AEC was verified by comparison to TEM and AUC, and high repeatability (<1%–4% CV) and intermediate precision (2%–5% CV) using UV detectors have been reported.[23,48] The method's concentration range is limited by sensitivity of the detector, with a lower limit of around 10^{11} vg/mL similarly to OD, but the limit of detection is reduced by an order of magnitude using FS detectors.[23,47,95] If sample concentration is not a limiting factor, then the high throughput and reproducibility of this method are advantageous for routine use in process development. AEC is also attractive as an online instrument, given the automatability of HPLC systems and nondestructive nature of the method.

DLS

In dynamic light scattering (DLS), the average particle size of a sample is quantified by measuring the time-unsteady intensity of light scattered by its solutes over time due to Brownian motion. DLS quantifies aggregate content and estimates the capsid titer of purified rAAV samples without prior sample preparation, within <5 min.[31,92]

The estimated capsid titer is generally inaccurate but was improved compared to ELISA using multi-angle DLS.[31] The particle size distribution obtained from DLS is only semiquantitative compared to

microscopy and of low resolution.[50,96–98] Overall precision of the method is low, with repeatability reported as poor as 45% CV for capsid titer and 85% CV for aggregate content using multi-angle DLS.[31] Operated in batch mode, DLS is a non-destructive method with high throughput and commonly used in conjunction with static light scattering (SLS).[32]

SLS

In SLS, commonly implemented as multi-angle light scattering (MALS), the average particle molecular weight and size of a sample are quantified by measuring the time-steady intensity of light scattered by its solutes due to Rayleigh scattering. Batch SLS has been used exclusively combined with DLS, improving accuracy of the capsid titer estimate for rAAV samples compared to DLS on its own. Although the combined workflow of SLS/DLS can determine the average molecular weight, the presence of aggregates prevents accurate estimation of capsid content.[32] SLS/DLS requires no sample preparation, yields results in about 2 to 5 min, and is amenable to 384-well plates.[31,32,92]

A major challenge for accuracy and precision of SLS/DLS remains high sensitivity to the refractive index of the sample that must be known *a priori*.[31,32,92] Reliability is especially poor due to the difference in refractive index between samples of empty and full capsids, leading to errors of up to 33% if the refractive index is incorrectly assigned. SLS/DLS is a rapid, non-destructive method with immense throughput and significant potential as a low-volume screening tool for capsid titer and aggregates in process development. Its short analysis times and large dynamic range, covering six orders of magnitude,[32] render it attractive for routine process monitoring as well.

MassP

In mass photometry (MassP), the mass of particles is quantified at the single-particle level by measuring the light scattered by the individual particles as they are bound on a glass surface. MassP works by attenuating the reflected light from an illumination source using a partial reflector, while collecting the scattered light from the particles of interest largely unaffected.[67] The interferometric contrast between scattered and reflected light can be related to the mass of the particles through a calibration procedure applied both to proteins[68] and nucleic acids.[69] MassP has been recently used to characterize the content ratio of rAAV5, using down to 1 μL of sample at 10^{12}–10^{13} cp/mL with a measurement time of only few minutes.[99] The short turnaround time and low sample demand of MassP render it a promising tool for routine, real-time process monitoring of the rAAV content ratio. Its robustness, especially regarding protein/DNA contaminants and other sources of noise, and performance characteristics, such as its accuracy and precision, will still need to be established for characterization of rAAV in the future.

SEC

Size-exclusion chromatography (SEC) separates solutes of below subvisible size (d < 100 nm) based on their hydrodynamic volume. Used on HPLC systems with UV, FS, or differential refractive index (dRI) detectors to quantify the eluting species, it is the primary method to assess aggregates in the biopharmaceutical industry due to its simplicity, throughput, and speed.[92,100] For rAAV, SEC-FS has successfully quantified the content of small aggregates and fragments in purified rAAV samples for multiple serotypes.[33]

Automated, SEC-based methods are suited for at-line application and offer potential as rapid screening tools for aggregates in process development. They are generally more accurate and precise than orthogonal methods, e.g., AUC or DLS, but accuracy for large aggregates is limited by the SEC separation mechanism: filtration of aggregates by the stationary phase, nonspecific interactions between aggregates and the column frit, and dissociation of reversible aggregates due to dilution in the mobile phase are common.[44,50,92,100,101] These limitations must be assessed with orthogonal methods, foremost AUC, to validate SEC-based methods, but no comprehensive comparison study has been published to support their use for rAAV aggregation.

MALS detectors are now commonly used in-line with UV, FS, or dRI to determine and confirm the molecular weight for individual species being separated by SEC.[92,101] With this configuration, quantification of capsid titer and content ratio is possible for purified samples using SEC-UV-dRI-MALS, based on the monomer peak's average molecular weight and total concentration.[74] Including the quantification of aggregate content, this combined workflow thus targets all product attributes discussed in this review. Although promising, this technology must overcome the challenges related to SEC, e.g., accuracy for large aggregates, and SLS/DLS, e.g., sensitivity on optical properties of the sample. Also, its high-reported quantification limit, at around 10^{13} cp/mL in purified samples, renders the technology unsuited for in-process samples in which the capsid titer is commonly at 10^{11}–10^{14} cp/mL (Figure 2).

FV

Flow virometry (FV) quantifies intact vector capsids and vector genomes, each labeled with a specific fluorescent antibody, in a microfluidic flow cell. Akin to flow cytometry, a well-established analytical method for cell counting,[102] FV is commonly used for virus detection and quantification in virology.[103] Sample preparation includes a short, 30-min labeling step, and the method has been successfully applied to non-purified samples from different downstream purification steps.[104,105] Application to rAAV is currently limited to quantification of capsid titer for serotypes 2 and 3, as FV depends on the availability of a serotype-specific antibody, and simultaneous identification of empty and full capsids with this method has only been achieved for large, enveloped viruses (i.e., lentivirus).[105]

FV was shown to yield capsid titers differing by two orders of magnitude compared to ELISA for the rAAV2 reference material despite similarities in labeling methodology, questioning the method's accuracy.[64,105] Repeatability for rAAV2 and -3 is reported around 5% CV but commonly 10%–30% CV in independent studies on other viruses.[64–66] The method's high throughput and simple sample preparation are beneficial for use in process development and especially

in-process monitoring, but no data to support its accuracy and precision for quantifying rAAV are available in the peer-reviewed literature.

CDMS

Charge detection mass spectrometry (CDMS) measures the charge and mass-to-charge ratio of individual ions simultaneously, allowing direct determination of their mass. Pierson and colleagues[56] report successful resolution of empty, partially filled, and full capsids and quantification of capsid content using CDMS. The method is accurate and repeatable (<2% CV). Compared to AUC—the only other method capable of resolving partially filled capsids—the turnaround time of CDMS is lower, at 2 h, but the instrumentation is less mature and still in development. Although CDMS is unlikely to rival AUC for identification of capsid subpopulations with partial and fragmented genomes in the short term, the technology has recently been commercialized by Megadalton Solutions, with a focus on improving the application to rAAV.[106]

DyeBAs

Direct quantification of the genome titer from at least crudely purified samples is possible using FS signals from dye-based binding assays (DyeBAs). The approach is simple and fast, requiring neither special instrumentation nor genome-dependent probes to yield results within 30 min–3 h.[59,60] The method is precise, with repeatability and intermediate precision reported within 4%–17% CV and 7%–16% CV, respectively.[59,60] Similar to PCR-based methods, DyeBA requires the removal of non-encapsidated DNA and capsid lysis during sample preparation. However, the genome titer found with this method for an rAAV reference material differed significantly from results obtained with qPCR and instead agreed with capsid titers obtained by ELISA.[59]

With a low required sample volume of only 1–10 μL and a linear range of 10^{10}–10^{13} vg/mL,[59,60] covering the scope of titers expected during manufacturing (Figure 2), DyeBA is an attractive method for process development. However, the accuracy and robustness of this method must still be established, especially regarding common matrix components that have been shown to interfere with the assay.[59]

BLI

Bio-layer interferometry (BLI) quantifies biomolecular interactions and analyte concentration by measuring changes to a submerged probe's optical thickness caused by the analyte binding to specific antibodies immobilized on the probe's surface.[11,107] This high-throughput, label-free method is specifically targeted for at-line use, provides acceptable precision, requires neither sample purification nor preparation, and has been successfully used in process development for biopharmaceuticals and vaccines.[11,53,108] It was recently commercialized for quantification of the rAAV capsid titer on the well-established Octet system, with support for 96- and 384-well plates.[54,109] BLI is promising for use in rAAV process development, but literature supporting its accuracy and precision for this application is currently not available to assess accuracy and robustness.

Soft sensors

Recently, the monitoring of rAAV production culture and prediction of the intra- and extracellular rAAV titer, among other process-related quality attributes, with *in situ* FS spectroscopy[110] and online digital holographic microscopy,[111] were reported. These methods employ soft sensors—estimators in the form of regression models or neural networks trained on existing process data—to process sensor data and predict process variables in real time. These methods are the only report of real-time process analytical technology for rAAV production processes in the literature at this point, but considerable validation will be needed for the routine use of such soft sensor-based technologies.

Other methods

In this section, we present characterization methods for which application to rAAV or related biologics has been reported, but because of insufficient data on their assay performance, we will not discuss them thoroughly. For this reason, they are not included in Tables 4 and 5.

Capillary isoelectric focusing, based on the difference in the isoelectric points of capsids, has been developed to determine the content of full, partially filled, and empty capsids.[55] On the validated system, resolution was superior to AEC, but limited data on accuracy and precision for rAAV are available.

Nanoparticle tracking analysis characterizes particle movement due to Brownian motion, like DLS, but directly visualizes particles via an ultramicroscope. The method is intended to quantify rAAV capsid titer and aggregation, but sensitivity is insufficient unless gold nanoparticles are used to improve the scattering signal, raising concerns about accuracy and precision.[98,112]

Gas-phase electrophoretic mobility molecular analysis, also called electrospray-differential mobility analysis, relates the electrophoretic mobility of singly charged particles to their size and molecular weight. The method rapidly quantifies various small, non-enveloped viruses and characterizes their integrity at reasonable accuracy and precision.[113–115] No use in rAAV characterization has been reported yet, but quantification of capsid titer and content ratio is a foreseeable potential application.

Asymmetric flow field-flow fractionation (AF4), a technology separating particles in two perpendicular flows based on their hydrodynamic volume, has been used instead of SEC for separating rAAV aggregates for analysis with MALS. AF4-MALS can resolve and quantify aggregates up to micrometers in size, thus including the large aggregates commonly removed by SEC-based methods.[116,117]

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) is a widely used method to assess rAAV purity by visualizing the capsid proteins and potentially remaining protein impurities, with

protein stains after separation based on molecular weight. A semi-quantitative estimate of the capsid titer can be directly measured from SDS-PAGE stains, by comparison to a reference standard for silver-stained gels[118] and using infrared-FS scanning for gels stained with Coomassie brilliant blue.[119]

Other methods have not been used specifically for rAAV but nevertheless show promise as analytical tools for rAAV due to existing, comparable applications. First, surface plasmon resonance, like BLI, quantifies the concentration of an analyte by measuring changes to the optical properties of a sensor chip, in this case, a shift in the resonance angle of free electrons exited by polarized light, caused by analyte binding to immobilized antibodies on its surface. It successfully quantifies adenoviruses in downstream samples,[120] with low throughput being its main drawback in comparison to BLI.[121] Second, mass measurements of particles using suspended micro- and nanochannel resonators are already being used to characterize and quantify aggregates in recombinant proteins.[122] Recent improvements in the latter technology—attaining attogram precision at the level of individual nanoparticles[123]—suggest the potential for characterizing rAAV by their mass in suspended nanochannel resonators.

State-of-the-art and remaining challenges

The number of analytical methods to characterize rAAV has increased dramatically in the last few years, and multiple methods are now available to characterize the quality attributes presented here. In general, established methods for the characterization of rAAV are commonly based on standard techniques in molecular biology.[6,17,25,71,85] Even though these methods are mature and in widespread use throughout the biopharmaceutical industry, their use for rAAV characterization in process development has limitations, most commonly insufficient throughput or precision. These needs have led to the commercialization of several novel methods, promising lower turnaround times without compromising precision.

For the quantification of the genome titer, PCR-based methods are the industry standard and remain unrivaled but have important limitations. First, although standardization of these methods is theoretically possible by defining pre-treatments and primer selection, while using established reference materials, this has proven challenging in practice. As a result, the current use of different assay protocols renders titer results incomparable, jeopardizing accuracy and reproducibility of rAAV dosing.[21,57,72] Second, PCR-based methods are indifferent toward the integrity of the vector genome, as both complete (i.e., functional) and truncated (i.e., non-functional) genomes with intact amplicon contribute to the genome titer. As the impact on potency and safety of the product differs significantly between these encapsidated genomes, accurate quantification of the genome titer as CQA for estimating potency should take genome integrity into account. Thus, we expect that complementary technologies, like qPCR/ddPCR, with multiple primers[57] or next-generation sequencing, which is capable of characterizing encapsulated DNA at full genome resolution,[124,125] will contribute to quantifying the genome composition of capsids comprehensively in the future.

For the quantification of the capsid titer, available methods can be broadly categorized as antibody-based or optical methods. Whereas both groups achieve similar levels of repeatability at usually <20% CV, antibody-based methods trade a longer turnaround for high specificity and vice versa. Especially, conventional ELISA, the most established method for capsid quantification, is laborious, requires 3–4 h to complete, but reliably quantifies non-purified in-process samples. Recently developed antibody-based methods, like BLI, FV, or miniaturized ELISAs, promise rapid, high-throughput analysis while requiring less sample preparation, but there is scarcity of published, peer-reviewed literature applying these methods to rAAV or other viral vectors. A key challenge for all antibody-based methods remains the need for serotype-specific antibodies, which are presently only available for the most common serotypes. To this extent, the demonstration of the validity of such methods, for which the FDA encourages the use of reference materials,[126]is also challenging, since standardized reference materials are only available for rAAV2 and rAAV8,[37,38] necessitating development and qualification of in-house reference materials and controls for other serotypes. On the contrary, the simplicity and high throughput of rapid optical methods, usually intended for at- or online use, are attractive for process development. However, these methods lack specificity and robustness, being unable to characterize non-purified samples, and there are insufficient experimental data for rAAV quantification to support the accuracy and compare the precision of all optical methods apart from OD. Overall, each group of methods is already appropriate for different applications, depending on sample purity and demanded analytical capacity, but methods based on miniaturized ELISAs or light scattering show potential to become a universal method for at- or in-line use during process development.

For the quantification of the content ratio, every method is subject to a tradeoff between throughput and resolution. Among high-resolution methods, AUC remains the only viable method capable of quantifying partially filled capsids, despite requiring substantial sample amounts. CDMS and capillary isoelectric focusing may match the resolution of AUC with lower turnaround time but are still in development. Overall, the severely limited throughput afforded by high-resolution methods diminishes the added value of quantifying partially filled capsids in a context of process development. To exclusively distinguish empty and full capsids, however, other rapid methods with shorter turnaround and higher throughput compared to AUC are available. Of those, AEC is the most promising technology, as it is both more robust (i.e., suitable for in-process samples) and precise (i.e., repeatability <4% CV) than other methods. Moreover, quantification of capsid titer with AEC, either directly or via a standard curve, may become feasible with further development to increase its resolution and mature its application to rAAV.[47,48] SLS already provides simultaneous quantification of content ratio, capsid titer, and aggregate content at high throughput, but limited published literature on this method prevents an unequivocal assessment of its capabilities for rAAV. Generally, in quantifying the content ratio by using any method presented here, two caveats must be considered. First, results between orthogonal methods are often not in agreement, and

Box 1 Key issues for analytical methods in process development.

- Well-established methods are incapable of satisfying the short turnaround time, high throughput, and sample diversity encountered in process development.
- Experience with upcoming methods that do satisfy these needs is too scarce to justify their use.
- Comparison of analytical methods and their results is impeded by the absence of standardized protocols and well-characterized standard materials, other than for serotypes 2 and 8, which may act as an established reference between laboratories.

especially, the accuracy of negative-staining TEM is often questioned.[23,25,78] Moreover, the contribution of partially filled capsids to the signals of empty and full capsids remains ambiguous for low-resolution methods. Second, the informative value of the content ratio should not be overstated, as the content ratio oversimplifies the heterogeneous genome composition of capsids, particularly in distinguishing between vectors with a complete, functional genome or a fragmented, non-functional genome of comparable size. This is also the reason why the concentration of full capsids, as estimated from a capsid titer and content ratio, should not be considered equivalent to the genome titer.

For the quantification of aggregates, the lack of primary literature on the application of analytical methods to rAAV impedes straightforward comparisons. Additionally, many methods cover vastly different size ranges, from dimers to large (submicron-sized) aggregates. Generally, SEC-based methods are the long-standing industry standard due to their simplicity and high throughput, but they are not suitable for quantifying large rAAV aggregates due to possible filtration, non-specific interactions, and deaggregation. Both EM and AUC can assess aggregation, but their low throughput and poor accuracy for small aggregate concentrations are limiting factors in process development. Optical methods, such as dynamic light scattering, on the other hand, show potential as at-line methods to quantify large aggregates, but their precision is poor (i.e., up to >50% CV), and accuracy remains challenging if optical properties of the matrix are uncertain. In any case, additional research on the mechanisms and factors of rAAV aggregation is required and will need to rely on multiple orthogonal methods, especially to accurately quantify large aggregates.

Conclusions

In the development—and especially commercialization—of new analytical methods for the characterization of CQAs of rAAV, emphasis is increasingly placed on short turnaround times, high throughput, and simple sample preparation. Notably, chromatography- and light-scattering-based methods have progressed to facilitate rapid, high-throughput characterization of three potential CQAs: capsid titer, content ratio, and aggregate content, directly at- or online. Nonetheless, experience using those methods in academic or industrial practice is scarce, and the goal of real-time bioprocess monitoring remains far beyond the capabilities of any analytical method at present. This is at least in part due to a mismatch between the capabilities of established analytical methods and the analytical needs in process development (Box 1).

Identifying and alleviating bottlenecks in rAAV production will unlock the advantages of process understanding similar to those that have dramatically improved production of other complex biopharmaceuticals. The first step lies in developing analytical methods that can support the short timelines and high throughput of process development while maintaining good reproducibility and specificity. The analytical methods discussed in this review have begun to overcome some of these challenges and show progress toward rapid, at-line characterization of rAAV. Although the three CQAs discussed in this review are the most relevant to development of rAAV currently, other CQAs (e.g., capsids with host cell/plasmid DNA, replication-competent AAV) have been proposed and will also guide process development as the field of rAAV gene therapy matures in the future.[15] We envision that analytical methods for these CQAs will provide the insights on product and process characteristics necessary to address the current challenges of both rAAV biomanufacturing, including upstream (e.g., transfection efficiency) and downstream (e.g., removal of empty capsids) processing, as well as clinical use (e.g., transduction efficiency).

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AUTHOR CONTRIBUTIONS

Conceptualization, P.W.B. and R.D.B.; investigation, A.L.G., G.K., S.S, S.L.S., A.J.M., and M.S.H.; writing – original draft, A.L.G., G.K., S.S., S.L.S., A.J.M., M.S.H., and T.N.T.N.; writing – review & editing, J.W., S.L.S., A.J.S., S.R.M., M.S.H., P.W.B., and R.D.B.; visualization, A.L.G.

DECLARATION OF INTERESTS

S.R.M. is a co-founder of Affinity Biosensors, which develops techniques relevant to nanoparticle characterization. All other authors declare no competing interests.

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