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Review

Challenges in scaling up AAV-based gene therapy manufacturing

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Accelerating the scale up of adeno-associated virus (AAV) manufacture is highly desirable to meet the increased demand for gene therapies. However, the development of bioprocesses for AAV gene therapies remains time-consuming and challenging. The quality by design (QbD) approach ensures bioprocess designs that meet the desired product quality and safety profile. Rapid stress tests, developability screens, and scale-down technologies have the potential to streamline AAV product and manufacturing bioprocess development within the QbD framework. Here we review how their successful use for antibody manufacture development is translating to AAV, but also how this will depend critically on improved analytical methods and adaptation of the tools as more understanding is gained on the critical attributes of AAV required for successful therapy.

Highlights

Adeno-associated virus (AAV) is a safe gene delivery vehicle that has been extensively exploited in clinical gene therapy.

AAV-based gene therapy products are facing manufacturing scale-up challenges due to a limited understanding of their biology and less well-established analytical tools and production processes.

Stress studies and ultra-scale down are two key tools with the potential to accelerate the manufacturing process development of new AAV-based gene therapy products.

Challenges with current analytical techniques for AAV characterisation include long waiting times and then insufficient throughput, resolution, sensitivity, accuracy, or reproducibility. These challenges need to be tackled to enable a standardised product and process development platform.

The rapid emergence of AAV-based gene therapies

The rapid development of advanced therapies presents new possibilities for the treatment of cancers and rare diseases. Of these, gene therapy shows particular promise with recent successes in the clinic. The therapeutic effects of gene therapy are achieved via the manipulation of a genetic sequence or the delivery of genetic materials and have so far been developed towards a wide range of diseases, including autoimmune diseases, neurological disorders, retinal diseases, and various types of cancers [1–3]. Several have now been approved by regulatory bodies, representing major new milestones in gene therapy development. Figure 1 shows a timeline that summarises the development of these approved therapies.

The delivery of genetic materials often requires their incorporation into viral or nonviral gene transfer vectors such as lipid nanoparticles, adenovirus, or AAV. This review focusses on AAV (Figure 2A), a member of the parvovirus family and a viral vehicle widely used in gene therapies and vaccines for its broad cell tropism and low immunogenicity [4]. The genome of wild-type AAV comprises three genes, *rep*, *cap*, and *aap* (Figure 2B), encoding, respectively, key viral replication proteins, capsid proteins (VP1, VP2, and VP3), and the assembly-activating protein [5].

To date, over 150 AAV genotypes and 13 serotypes have been discovered in humans (AAV1, AAV2, AAV3, AAV5, AAV6, and AAV9) and nonhuman primates [4,6], while more have been identified in other species. They vary in cell tropism and transduction efficiency, leading to different functionalities.

AAV genomes must be modified to make them suitable for clinical applications. Wild-type AAV can integrate into the host genome, creating permanent and potentially inheritable genetic modifications that would present significant risks to patients. These risks have been removed in recombinant AAV (rAAV) for clinical use by replacing one or both of the *rep* and *cap* genes with a therapeutic gene of interest and placing the genes removed onto a separate plasmid used only during AAV manufacture. Thus, new AAV particles remain infective but are unable to insert an

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intact AAV genome into human host chromosomes, and are unable to replicate in the wild or after injection into humans.

This review provides an overview of how various tools such as **QbD** (see Glossary), scale down, ultra-scale down (USD), and stress tests can be used to accelerate the development of AAV bioprocesses. We also summarise the current challenges in the analytical tools available for AAV characterisation and bioprocess quality control.

Tools to accelerate bioprocess development

The demand for AAV is growing rapidly as clinical pipelines expand, making the need for a platform large-scale AAV production process more urgent than ever. Their manufacture is complex, requiring cell expansion, transfection, cell culture, virus harvest, and multiple purification and buffer exchange steps. The development and scale up of manufacturing processes for AAV is highly time-consuming and risky due to their inherent instability and complex biology and the relatively few long-term clinical data that can inform the ideal product profile to achieve. Their development also builds on relatively few precedents and lacks the benefits of technical maturity in AAV-specific bioprocesses, cell lines, and analytical instrumentation that are found with more established platforms such as antibodies. These challenges will continue to evolve for many years as future developments increase the process intensity of individual AAV manufacture steps.

Several experimental tools are anticipated to be able to accelerate AAV product formulation and early-stage process development. These build on their well-established success in the development of monoclonal antibodies (mAbs) and include, in approximate order of implementation: (i) developability screening of product candidates; (ii) scale-down and USD tools for rapid bioprocess development; and (iii) accelerated (forced) degradation screening for rapid formulation.

Each of these tools is designed to be used at their respective development stages to accelerate the selection of manufacturable product candidates, to optimise the manufacturing process, or for the rapid development of stable dosage formulations, although learning in one can also be used to inform other stages of development. Therefore, efficiencies can be created, for example, by designing the developability screens to generate biophysical data that better inform both the final product formulation and the bioprocess parameter operating windows that retain a stable

Glossary

Fill/finish: the final step in the drug/biological product manufacturing process, where products are stored in suitable formulations and filled into vials.
Permeate flux: the quantification of permeate per unit of time in a membrane separation process.
Quality by design (QbD): a systematic approach that incorporates predefined objectives, risk management, the establishment of process control, and design space for process development.
Quality target product profile (QTPP): a product profile defined in the first step of the QbD paradigm that includes all of the characteristics that the product should possess to achieve the target qualities.
Shear stress in bioprocesses: type of stress generated from fluid flow as a result of the presence of a velocity gradient.
Ultrafiltration/diafiltration (UF/DF): unit operation in a bioprocess used to concentrate and exchange the buffer of the product using a filtration membrane.

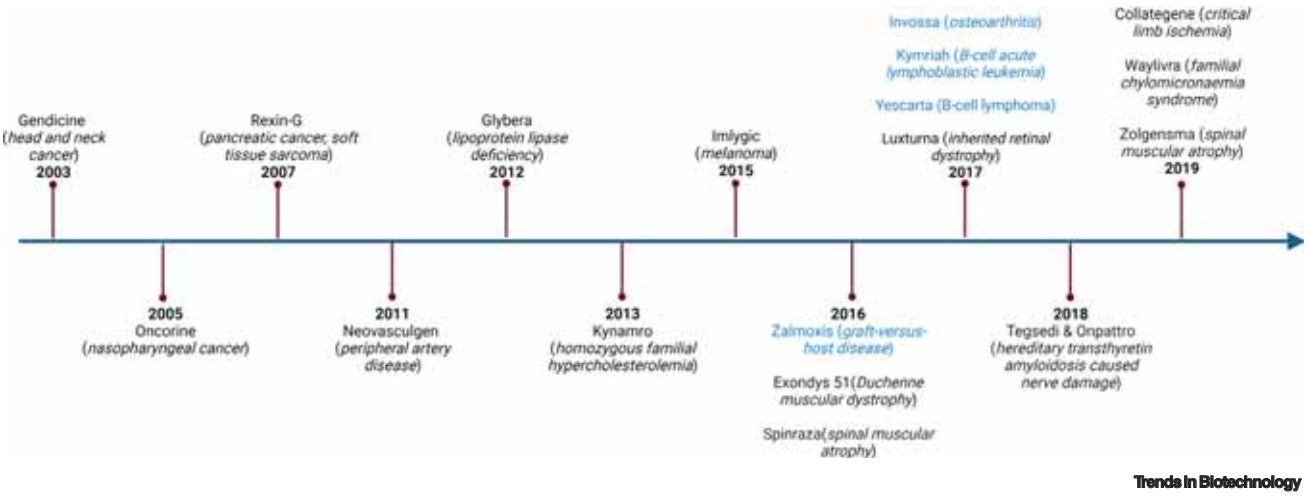

Figure 1. A timeline of cell and gene therapy approvals to date and their target indications (gene therapies, black; cell-based gene therapy products, blue). Glybera and Zalmoxis are withdrawn from use.

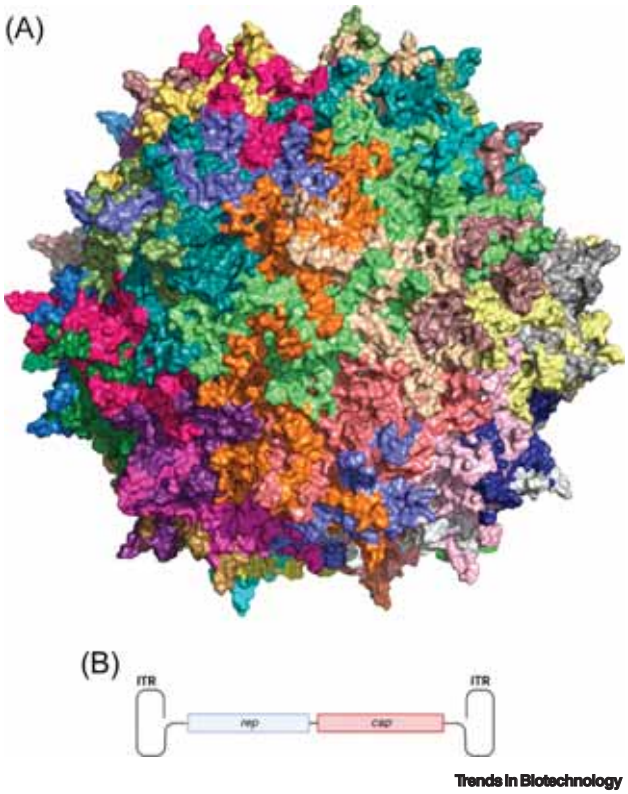

Figure 2. Structure and genome of adeno-associated virus (AAV) serotype 2. (A) Structure of AAV serotype 2 at 2.8 Å with subunits highlighted in different colours. Image generated in PyMol (Delano reference) using PDBID: 1LP3. (B) Schematic for the wild-type AAV genome. The *rep* gene encodes four non-structural proteins. The *cap* gene encodes three capsid proteins obtained by two alternative splices of the mRNA and readthrough translation of the weak ACG start codon used for VP2 expression. The *aap* gene is encoded within the *cap* gene and expressed from an alternative start codon. Abbreviation: ITR, inverted terminal repeat.

product. The design, manufacture, and formulation of mAbs gradually converged on a few robust platform approaches. This further constrained the search space of conditions and led to even greater overlap in many of the experimental conditions used for the three tools. By comparison, the platform manufacturing approaches for AAV have not yet had sufficient time to fully develop and so the conditions tested using the three tools are also evolving.

One other feature that has helped to shape the tools used to accelerate mAb development is QbD. This approach is encouraged to de-risk the development and scale up of manufacturing processes for biologics, by creating an understanding of how the process parameters impact product properties and ultimately its clinical performance [7–9]. As shown in Figure 3, this approach defines critical quality attributes (CQAs) as readily measurable biophysical and physicochemical product properties that ensure the required clinical performance as defined in the **quality target product profile (QTPP)**; critical material attributes (CMAs) and critical process parameters (CPPs) are identified as factors that could significantly impact the CQAs if they are varied [8]. Therefore, the three experimental tools defined previously have the potential to identify CMAs and CPPs and even begin to quantify their boundaries.

The platform manufacturing approach for mAbs and extensive clinical data from many products means that their CQAs can now be defined relatively quickly. By comparison, the CQAs for AAV

Figure 3. Workflow of the quality by design (QbD) framework. Abbreviations: CQA, critical quality attribute; CMA, critical material attribute; CPP, critical process parameter; QTPP, quality target product profile.

may continue to expand as more products generate clinical data. This is also likely to lead to changes in the range of conditions tested and measurements made using the three tools described here.

Scale-down and USD tools for AAV manufacturing bioprocess development

Scaled-down platforms use the same process unit operations as pilot- to large-scale manufacture and with sufficiently tight control to achieve high reproducibility, but with much smaller operating volumes that can often be run in parallel to rapidly explore and optimise process variables. Early pilot-scale optimisation of manufacturing processes strongly informs the acceptable design space for CMAs and CPPs as well as potential control strategies to maintain them in the design space [10]. Therefore, scaled-down platforms can extend this to a broader exploration of bioprocess operating parameters, giving a deeper understanding.

This approach is now well established for mAb bioprocess development, and some aspects can be readily transferred to AAV despite the significant difference between these products. For example, miniaturised bioreactors such as ambr™ (developed by Sartorius) can give comparable cell culture performance, environmental control performance, and antibody productivity observed in large bioreactors, making them suitable as predictive tools for large-scale cell culture/fermentation [11–14]. A scaled-down approach using 24 deep-square well plates has already been used to optimise the production of lentiviral vectors in suspension culture and so has good potential to succeed similarly for AAV production [15].

Other powerful scaled-down systems, such as microscale chromatography columns and microlitre batch incubation, have also been widely used as high-throughput screening methods for chromatographic conditions and resin selection [16–18]. These scaled-down platforms are well established for antibodies and are already being adapted to accelerate AAV bioprocess development. Of course, the specific screening conditions will differ for AAV, and perhaps even between serotypes, accommodating the known CPPs of elution conductivity and pH for AAV anion exchange chromatography, which impact the empty–full capsid separation performance [19].

In contrast to scale down, USD tools do not replicate a process operating at a small scale but are instead designed to precisely mimic the critical conditions and exposures encountered in a large-scale process unit of operation using only millilitres of samples [20]. They can be used to optimise products, solution conditions, or bioprocess parameters and define the CPP design space for each unit of operation. In particular, they can provide a cause-and-effect understanding of the impacts of CPPs on the product CQAs, which is essential for the QbD framework.

Fill/finish and **ultrafiltration/diafiltration (UF/DF)** steps are often found to be major causes of functional AAV titre loss, and so USD could potentially be used to identify the causes of these

losses. Such losses are proposed to derive from shear-induced virus aggregation during the concentration steps [21,22]. However, while UF/DF and fill/finish are two major sources of **shear stress in bioprocesses** alongside microcavitation in pumps and valves [23], further investigation is needed to confirm that shear stress is the main cause of AAV losses. This was the case for therapeutic proteins, where aggregation was attributed to shear stress not alone, but in combination with exposure to air–liquid interfaces or adsorption onto solid surfaces during the chromatography purification step, or through the occurrence of cavitation due to high shear rates in pumps and valves [24–29]. In addition, the pH of the solution and the solid surface roughness in the solid–liquid interface were found to affect the extent of protein aggregation during shear stress [27].

A recent USD investigation describes the effects of protein concentration, shear rate, and transmembrane pressure on the **permeate flux** and mAb aggregation during UF/DF [30]. Comparison with pilot-scale filtration experiments showed similar correlations between the tested conditions and the permeate flux. Increased filtration times and product concentrations were found to correlate with increased turbidity in both the USD and the pilot-scale UF/DF model, indicating a mechanism in which an increased protein concentration led to more frequent protein collisions, a higher propensity to aggregate, and so greater turbidity. Despite the good accordance between the permeate flux in the two models, differences in the extent of shear exposure between the two models led to a minor disagreement in the turbidity detected. Thus, further modifications are required to make a more accurate USD mimic of the impact of shear stress in UF/DF. In another study, the impact of shear stress on plasmid DNA during centrifugation was accurately predicted by a USD model [31].

To date, USD models have successfully simulated large-scale bioprocess conditions for numerous biologics, including plasmid DNA [31], mAbs [32], adenovirus [33], and cells [34]. However, no literature has yet reported USD models to predict the behaviour of AAV under shear stress.

Developability and forced degradation screening

Stress tests play an important role in determining product stability under process, storage, shipping, and in-use conditions and are well established for platform biologics such as mAbs. They are implemented during product formulation optimisation, but also in early product candidate screening as part of an overall developability platform, which also includes several biophysical characterisation measurements.

For developability screening, stress tests aim to determine whether a product will remain stable and soluble under the range of conditions and timescales (hours to days) typically used during manufacture. This also provides an initial scoping of control measures and potential conditions for product formulations, with biophysical data that can inform the design space for CPPs and CMAs during bioprocess development [35].

Developability screens have increasingly included more extreme (accelerated or forced degradation) conditions designed to weed out product candidates that are likely to be challenging to formulate into products that remain stable over months to years, using typical buffers and ideal protein concentrations. This therefore overlaps with the accelerated degradation stress conditions used in formulation development and optimisation.

Accelerated degradation uses elevated temperature, agitation, or light exposure to allow degradation pathways in product formulations and final dosage forms to be studied on much shorter timescales than the intended product shelf life. This makes them highly suited to rapid formulation

development by screening for the most suitable excipients to stabilise products and then for their optimisation. For example, the stabilising effect of trehalose, now a commonly used excipient for therapeutic proteins, was discovered through a heat shock (thermal stress) test [36].

Stress tests apply moderate (for manufacture developability) or extreme (for formulation development) conditions to biologics that can be grouped into physical stress (e.g., temperature, light, mechanical shear, surface adsorption) and chemical stress (e.g., pH, denaturants, oxidation, reduction). While accelerated stability and light exposure conditions used for regulatory filings are highly regulated by International Council for Harmonisation (ICH) guidelines such as ICH Q1A and ICH Q1B [37,38], forced degradation conditions used during development are often more variable [39], especially for biological products with properties distinct from general drug products. Stress conditions for biological products 'should be selected on a case-by-case basis' (ICH Q5C [38]). This allows different forced degradation conditions to be selected based on whether the purpose is for formulation development, product developability screening, or simulation of bioprocess stress.

Comparison between stress tests for mAbs and AAV

Through years of experimentation, stress tests used for the development of mAbs have become relatively standardised through industry consensus around established product and manufacturing platforms. However, mAb stress conditions do not translate simply to AAV products and so these still need to be investigated and refined. Reported AAV stress conditions vary significantly, leading to different outcomes, but also depend on a wide variety of AAV serotypes with different biophysical characteristics [40]. A standardised set of stress tests, perhaps tuned to certain serotypes, would facilitate platform-based process and formulation development for AAV products.

It is worth considering whether the development of stress tests for AAV can learn from those developed previously for mAbs. Examples of previously reported stress tests for AAV and mAbs are shown in Table 1 and reveal wide variability in the conditions explored for AAV and mAbs, as well as some similarities.

Storage at 4°C for a number of weeks provides the control conditions for most thermal stress tests with both of these biologics. This fits with the long-term storage aims for both AAV and mAbs of stability at 4°C for up to 1–2 years given the availability of a 4–8°C cold chain throughout global transport and storage facilities.

Elevated thermal stress is widely established for accelerated degradation of mAbs in formulation development and regulatory filings. Thermal melts of most mAbs reveal that their first thermal transition is typically above 45–50°C [75]. Therefore, to focus on the kinetics of degradation (primarily aggregation) from native proteins, accelerated degradations tend not to exceed 40–50°C to avoid global unfolding of the protein. The ICH guidelines also do not exceed this temperature. However, some studies have used higher temperatures, presumably to differentiate particularly stable formulations.

By contrast, the thermal melting points for AAV vary widely by serotype from 66.5°C to 89.5°C [6]. However, reported accelerated thermal degradation tests for AAV are often biased towards rapid high-temperature heat shocks, such as 65–80°C for 15–30 min. These would seem to be focused on causing partial capsid denaturation and DNA release and are often used to compare the thermostability of AAV variants. However, such extreme conditions are not representative of accelerated stability testing for formulation or bioprocessing conditions. Additionally, such extreme stresses may not be a good predictor of longer-term stability at lower storage

Table 1. Example stress tests reported in the literature for AAV and mAbs and comparisons with some ICH guideline accelerated stability study conditions

	Stress condition (AAV)	Stress condition (mAb)
Thermal stress ICH Q1A guideline [41] General case: 40°C ± 2°C for 6 months Refrigerated drug products: 25°C ± 2°C for 6 months		
Other studies	15 min at 21–80°C [42] 30 min at 37, 55, and 65°C [43] 24 h at 20°C or 20 min at 75°C [44]	30 days at 45°C [45] 10 days at 40°C [46] 7–60 days at 25 and 40°C [47] 14 days at 4, 40, and 50°C [48] 10 min at 74°C [49] 1–3 days at 60°C [50]
pH stress	Phosphate-citrate buffers, pH 4.0, 5.5, 6.0, 7.5 [51] Citrate or phosphate buffers, pH 5.6, 6.2, 7.4 with 155 mM NaCl [52] Phosphate-citrate buffers, pH 7.4, 5.5, 6.0, and 4.0 with 150 mM NaCl, 5 min at 75–86°C [53]	100 mM citrate, pH 8.6, at 4 or 37°C [54] 100 mM citrate, pH 3.5 100 mM Tris-HCl, pH 8.5 [55] 14 days at 30°C in 0.1 M citrate and 0.2 M Na_2HPO_4 at pH 3 [56] 1–7 days at 25°C in 0.5 N NaOH, pH 10 [57]
Freeze–thaw stress	Ten freeze–thaw cycles (–80°C 5 min, 21°C 5 min) [58] Nine freeze–thaw cycles (–80°C, 21°C 1 h; store –80°C for 24 h) [59] Five freeze–thaw cycles (–60°C 1 h, 25°C 1 h) [60] Ten freeze–thaw cycles (–80°C 1 h, 21°C 30 min) [61]	–80°C 15 min, 25°C 20 min [49] 30 freeze–thaw cycles (liquid nitrogen 5 min, 25°C 5 min) [62] Five freeze–thaw cycles (–20°C 24 h, 25°C 5 min) [63] 12 freeze–thaw cycles (–80°C, 37°C 5 min) [64]
Oxidation	None found	1 mM peracetic acid at 30°C for 2 h [65] 3% H_2O_2 and 10% AAPH at 25°C for 1, 3, and 7 days [55] 0.05% H_2O_2 at 4°C and 37°C for 0–72 h [54] 0.2% H_2O_2 at room temperature for 24 h [66]
Reduction	None found	500 mM DTT, 37°C, 30 min [67]
Mechanical/shear stress	UF through various membranes; normalised feed rate of 350 l/h/m^2 and transmembrane pressure of 5–20 pounds per square inch gauge (psig) [68]	Exposure to shear rates 250 000 s^{-1} and 150 000 s^{-1} for 30–51 ms [28] Shear stress in rotating disk device at 50, 100, 125, 150, 200, and 250 rps for 2 h at 3°C [69] Sonication on ice at 30% amplitude for 1 and 3 min [70] Shaking device agitation for 1–3 days at 300 rpm [50] Vortexing at 3200 rpm for 5 min to 15 h [71]
Light exposure ICH Q1B guideline [37] Exposure to near UV at ≥200 Wh/m^2 for ≥ 2 × 10^6 lxh		
Other studies	UV light, 10 min [44] 126 mW/cm^2 UV light, 40 min [72]	5000 klxh, 25°C [48] 0.24–1.2 × 10^6 lxh white light + 40–200 Wh/m^2 UV light at 25°C [57] 302 nm light, 30 min or 1 h [73] 8000 lx UV light for 20–525 h at 4°C [74]

temperatures, as has been found to be a problem when comparing high- and low-temperature aggregation kinetics for mAbs [76].

Extreme-pH incubations are also well established for mAb developability screening, often combined with thermal stress. Incubations at pH 3–4 for mAbs evaluate their tendency to denature and aggregate more rapidly below pH 5, but also their survival at the low pH used for affinity chromatography elution and viral inactivation steps. Incubations at pH 8.5–10 also commonly evaluate susceptibility to deamidation and the propensity of mAbs to aggregate under high-pH

conditions used in ion-exchange chromatography. By contrast, for AAV, the reported screens evaluate a range of pH 4–7.5 [6,43,44,58,77]. This reflects the current focus on AAV losses that most likely result from aggregate formation and surface adsorption under standard bioprocessing or formulation pH conditions, and so an early assessment of stability in these conditions is useful in designing and optimising the bioprocess.

Currently, deamidation is not widely considered a CQA for AAV, which explains the lack of high-pH tests, although deamidation of AAV has been reported to alter vector function [78]. Meanwhile, the purification of AAV often uses affinity capture chromatography with elution at low pH. It remains to be seen whether this low pH will lead to emerging challenges in manufacture, potentially when process intensities increase to give higher viral titres.

Freezing–thawing represents a manufacturing challenge common to both AAV and mAbs. Drug substance materials are often frozen after primary manufacture to avoid long hold times prior to fill-and-finish operations, especially when this requires transport to a different site. Process scheduling challenges can also lead to wide hold-time variability and so it is best avoided by freezing the materials instead. Therefore, a freeze–thaw stress test is useful to evaluate the developability of AAV or mAbs in terms of tolerance to freezing–thawing. The reported freeze–thaw tests for mAbs and AAV both use freezing and thawing temperatures of –80°C and 25°C and between five and 12 cycles, reflecting a fairly standardised freeze–thaw process used in manufacturing.

Oxidising or reducing agents such as H_2O_2 and DTT have been used to accelerate oxidation or reduction reactions for antibodies. However, no examples could be found for the screening of AAV, possibly reflecting the current absence of these reactions from CQA lists, justified by a lack of clinical evidence to date. There is wide variability in the conditions reported for oxidation and reduction screening of mAbs, with oxidations in 0.05–3% H_2O_2 or peracetic acid and reductions with up to 500 mM DTT. Time will tell whether oxidations or reductions eventually emerge as CQAs for AAV products, requiring an appropriate stress test.

Mechanical stress tests based on agitation are well established in the development of stable mAb formulations, and simulate mechanical shear stresses during the transport of products in vials or prefilled syringes [79,80]. Similarly, the use of USD technologies is established for mapping the shear sensitivity of mAbs in the conditions found in centrifugation, pumps, and filtration processes [32]. The challenges of AAV manufacture are revealing particular steps in which AAV losses are particularly difficult to minimise, but little has yet been done to pick apart the relative roles of surface adsorption, self-interactions, and mechanical shear in these product losses. Thus, USD approaches and accelerated degradation studies, similar to those used for mAbs, have significant potential for understanding these mechanisms in more detail, and ultimately defining a suitable developability screen.

Finally, light stress has a standard test that is applied to product formulations according to the ICH Q1B guidelines [37]. This test is performed to determine whether the drug product will experience changes when exposed to UV light during transport. The light-stress test is an extreme test given the low exposures likely during manufacture and storage in packaging materials. However, it may yet emerge that light at lower levels of exposure is an important feature impacting product quality. This is an area in which more details continue to emerge for mAbs due to the more widespread adoption of mass spectrometry (MS). Similar analysis of AAV may eventually reveal degradation processes of concern, but currently there is no obvious need for a light-based developability screen in addition to the existing final product testing according to ICH Q1B guidelines.

In general, the relative lack of stress conditions developed for AAV compared with antibodies reflects the much greater maturity of the mAb manufacturing industry, where CQAs have been developed for a significantly greater number of mAb products and candidates. However, the list of CQAs for mAbs increased gradually over time and a similar trend may be expected for AAV as pipelines expand and as bioprocesses evolve towards higher process intensities. As mAbs have been extensively developed to higher intensity over nearly 50 years, there are potentially many standards and techniques that could be adapted for AAV. The current manufacturing process for AAV products already uses unit operations that were largely adapted from mAb bioprocessing (Figure 4). As these processes evolve further for AAV, more consideration will be needed to make the developability and USD stress conditions more suitable to mimic the process stresses experienced by AAV.

Even with extensive knowledge and experience with mAbs as a reference, relatively little has yet been done to establish a consensus or standard set of stress conditions for AAV or to test the transferability of mAb stress conditions to AAV. This is partly hampered by the lack of suitable analytical techniques to monitor many AAV degradation processes, an incomplete understanding of likely AAV CQAs for future products, and even insufficient access to standard AAV sample materials.

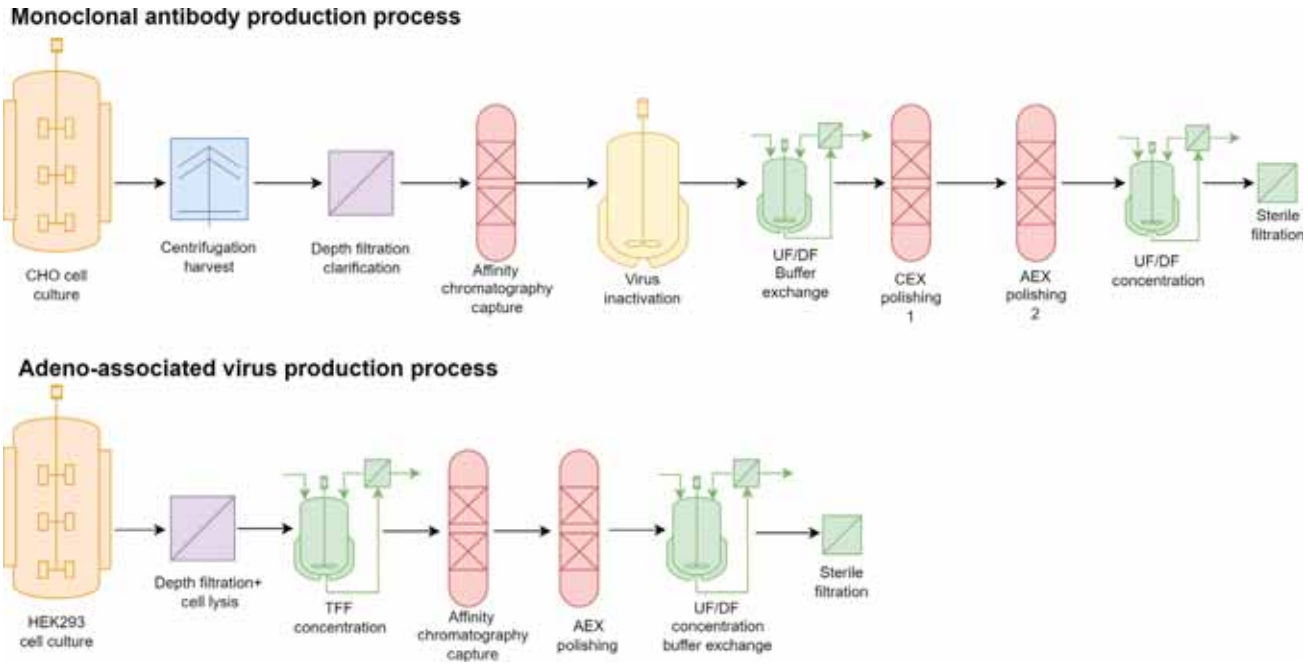

Figure 4. A comparison between the conventional monoclonal antibody (mAb) and adeno-associated virus (AAV) production processes. Top: The mAb production process begins with cell culture. In this step, cells proliferate and start to produce mAbs. CHO cells are used here as an example of the common mammalian cell lines used for full-length mAb production. Using the mammalian cell expression system, the mAbs are produced extracellularly. Cells are removed through the centrifugation step and media containing the secreted mAbs are collected and filtered using a depth filtration technique to further remove insoluble debris. Affinity chromatography then captures mAbs through the affinity ligands on the chromatography resin. The resultant low-pH eluate from the chromatography step is transferred to the virus deactivation tank to deactivate any endogenous virus carried by the mammalian producer cell line. Ultrafiltration/diafiltration (UF/DF) buffer exchange prepares the product for two additional chromatography steps, cation-exchange chromatography (CEX) followed by anion-exchange chromatography (AEX), which remove further impurities such as host cell protein, host cell DNA, and leached affinity ligand. To ensure the transfer of the product into a suitable formulation buffer and condense the product volume, UF/DF is performed. The concentrate of this step then goes through a final step of filtration to ensure the sterility of the product before it is formulated into vials. Bottom: The AAV manufacturing process also begins with cell culture of, for example, the HEK293 producer cell line. Since AAV is produced intracellularly, the cell lysis + depth filtration step is used to release the intracellularly produced AAV particles by lysing the cells and then clarifying the lysate by removing the lysed cells. Tangential flow filtration (TFF) is then used to concentrate the AAV particles prior to their capture by affinity chromatography from the clarified lysate, followed by an AEX step to remove further impurities. Similar to the mAb manufacturing process, UF/DF is then applied to concentrate the product and perform buffer exchange. The concentrated and buffer-exchanged AAV solution is filtered for a final time to ensure the sterility of product before fill/finish.

Current challenges in analytical method development for large-scale AAV manufacture

As a tool for monitoring product CQAs during production, analytics are of great importance. The evolution of analytical capabilities will enable improved developability and accelerated stress testing, and so here we briefly summarise the current analytics and their challenges.

During AAV production, their CQAs, such as the potency, identity, purity, and stability of the viruses, are monitored to ensure product safety and efficacy in clinical applications [81,82]. Each of these qualities is attributed to a different property, such as infectious titre, viral genome titre, genome identity, capsid stoichiometry, or aggregation. Any changes in these properties could undermine the efficacy of the product or lead to serious safety concerns. For instance, empty viral capsids are difficult to remove during the manufacture of AAV. Due to their lack of a therapeutic genome, they would have no direct therapeutic effect and yet may trigger immune responses against the AAV viral capsid [83]. However, empty capsids may still have a role in achieving overall therapeutic efficacy by acting as decoys to neutralise pre-existing immunity towards AAV [84]. Clearly, a consistent empty-to-full-capsid ratio is an important CQA.

Current analytics for AAV characterisation and monitoring were largely designed and optimised for smaller biologics, leading to various insufficiencies with AAV. The adaptation of existing analytics to AAV products is particularly challenging due to their large molecular weight, their oligomeric complexity, the mix of protein and ssDNA, and the low concentrations of viral particles compared with typical mAb concentrations during bioprocessing [81]. In Table 2, a summary of

Table 2. Examples of current analytical methods used in AAV quality control[a]

CQA	Test	Analytical method	Current issue
Potency	Infectious titre [87]	TCID50	Time-consuming and noisy
	Viral genome titre [87,88]	qPCR or ddPCR Combine with ELISA [87]	ELISA is time-consuming and exhibits 10–20% error rate [87] qPCR is less accurate than ddPCR and has lower dynamic range; higher cost for ddPCR
Identity	Genome identity [89]	NGS, ddPCR	High cost for NGS
	Capsid stoichiometry and heterogeneity [89]	LC-MS, CE-IEF, SDS-PAGE	LC-MS and SDS-PAGE are slow; too slow for real-time monitoring; LC-MS is resource intensive
Purity	Full:empty capsid ratio [88]	AUC, TEM, ELISA/dd(q)PCR, AEX HPLC, U/HPLC-MS, CE-IEF, 260:280 UV spectrometry	Differentiating empty and full capsids is challenging [90]; AUC accurate but low throughput; serotype-specific ELISA unavailable for all AAV serotypes [81]
	Partially full capsids	ddPCR, NGS	ddPCR can show a false-positive result if partial D/RNA is present
	Aggregates [89]	SEC, DLS, AUC, nanoparticle tracking	SEC removes the largest particles; DLS is non-quantitative for particles; AUC is low throughput; nanoparticle tracking is good for AAV aggregates but cannot resolve at <30 nm
Process-related impurities	Residual HCP [91]	ELISA	ELISA is time-consuming and exhibits 10–20% error rate
	Residual DNAs (plasmid, host cell DNA) [91]	qPCR or ddPCR	qPCR is less accurate than ddPCR and has lower dynamic range; higher cost for ddPCR
	Residual transfection or lysis reagents [91]	U/HPLC	Results may not be accurate or reproducible at low residue concentrations [90]

[a]Abbreviations: AEX, anion-exchange chromatography; AUC, analytical ultracentrifugation; CE-IEF, capillary isoelectric focusing; ddPCR, digital droplet PCR; DLS, dynamic light scattering; LC-MS, liquid chromatography–mass spectrometry; NGS, next-generation sequencing; PAGE, polyacrylamide gel electrophoresis; qPCR, quantitative PCR; SEC, size-exclusion chromatography; TCID50, median tissue culture infectious dose; U/HPLC, ultra/high-performance liquid chromatography.

the analytics used for AAV and their respective challenges shows that significant room remains for improvement. Although the current techniques are sufficient to analyse AAV CQAs during production, they are not always highly accurate, are often time-consuming, and are not available for real-time monitoring. Therefore, more robust analytical methods are needed for almost all AAV CQAs, with higher throughput, greater sensitivity and dynamic range for typical AAV titres, and ideally the ability to monitor product CQAs in real time [85].

MS has emerged as a powerful technique for the quantification and identification of host cell proteins (HCPs) in mAb production, as well as many other mAb attributes including glycosylation variability, oxidation, deamidation, chemical adduct formation, fragmentation, and C-terminal clipping. A recent MS analysis of adenovirus heterogeneity has linked a key structural composition change to infectivity, thus demonstrating how some analytical approaches can be usefully transferred from mAbs to gene therapy products [86].

A recent study introduced an approach to aid the development of robust analytics [92] analogous to the QbD approach. However, rather than identifying the desired product qualities, this approach starts with identifying targets for analytics, followed by a series of screening, evaluation, and improvement procedures to produce the most optimal analytics. A scoring system is used to rank the different analytics by taking into account both their constraints in operability and their performance [92]. The analytical techniques with the highest scores proceed to improvements and further assessment. One example in this study validated the feasibility of the approach for the development of adenovirus analytics, indicating its applicability also for AAV.

Concluding remarks

AAV is a safe and promising candidate for gene therapies and has experienced rapid growth over recent years. However, the bioprocess development is currently time-consuming with a high level of risk due to few commercialised examples as precedents and a lack of robust industry standards. As more AAV-mediated gene therapies move from the clinic to commercialisation, the regulatory guidelines will continue to evolve and are likely to increase the analytical burden (see Outstanding questions). Furthermore, technical innovations will increase the intensity and scalability of the AAV manufacturing process, placing new physical demands on analytical methods. Based on precedence from other biologics, this is likely to come through further media optimisation alongside improved process monitoring and control. Downstream processing is also still evolving for AAV and represents a major technical challenge. A widely used laboratory-scale purification technique for AAV is ultracentrifugation on caesium chloride (CsCl) or iodixanol gradients [93], which allows good separation of full and empty viral capsids, but it is not scalable. Chromatography is commercially scalable and so great efforts have been made to produce suitable AAV serotype-specific affinity columns. There is also a strong interest in anion and cation exchange chromatography as a lower-cost option, which also avoids the low-pH conditions used in affinity chromatography.

In this evolving landscape, the application of developability screens and scale-down and accelerated stability tests can inform QbD to ensure the robust scalability of AAV bioprocesses. However, process development challenges are still hindering the establishment of a robust, large-scale AAV production platform. To meet these challenges, it will be necessary to establish validated standards, analytics, product CQAs, and suitable developability screens. Due to their complexity, the standardisation of AAV products could be challenging, but it will be needed to alleviate the increased regulatory burdens anticipated for gene therapy products. To achieve this, attempts should be made to better understand what are the essential product CQAs and to anticipate which of the other potential CQAs are likely to arise as we learn more about products in the

Outstanding questions

What additional CQAs are likely to emerge as AAV-based gene therapy products become more widespread? Research will need to focus on ensuring that appropriate analytical methods are available and validated.

How well do we understand the instabilities of AAV during bioprocesses and for formulated products? Further research should map degradation pathways for AAV in more detail and evaluate the factors that influence them.

As bioprocessing advances increase the process intensity of AAV manufacture, how will this impact degradation pathways and in-process stability for AAV? The development of accelerated degradation tests and USD devices will need to keep pace with any emerging critical instabilities.

clinic. Analytical methods should also be further improved to enable faster analysis on smaller samples, as the gradual shift from 'potential' to 'actual' CQAs will bring a greater analytical burden. In the near future, it is anticipated that AAV production and the analytics developed by vendors will be more customised to AAV biology. Meanwhile, more attention may be drawn to continuous and single-use bioprocesses, following the evolution pattern of mAb bioprocessing.

As the technology becomes more advanced, other strategies emerging to assist the bioprocess development of mAbs may also be adapted for use with AAV. In particular, computer-aided bioprocess design [94], data mining, process control, and bioprocess equipment design [95] are becoming major trends for mAb platforms that also could be adopted early for AAV. Compared with the well-established mAbs, the development of AAV is still taking its first baby steps, but by learning from mAbs where possible, the road to maturity for AAV manufacture should be a much shorter one.

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Declaration of interests

The authors have no interests to declare.

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