Review

Production, processing and characterization of synthetic AAV gene therapy vectors

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Abstract

Over the last two decades, gene therapy vectors based on wild-type Adeno-associated viruses (AAV) are safe and efficacious in numerous clinical trials and have been translated into three approved gene therapy products. Concomitantly, a large body of preclinical work has illustrated the power and potential of engineered synthetic AAV capsids that often excel in terms of an organ or cell specificity, the efficiency of *in vitro* or *in vivo* gene transfer, and/or reactivity with anti-AAV immune responses. In turn, this has created a demand for new,

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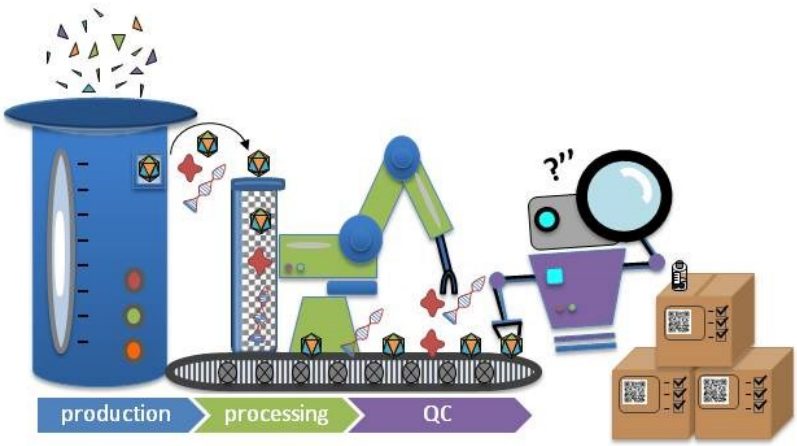
scalable, easy-to-implement and plug-and-play platform processes that are compatible with the rapidly increasing range of AAV capsid variants. Here, we particularly focus on recent advances in methodologies for downstream processing and characterization of natural or synthetic AAV vectors, comprising different chromatography techniques and thermostability measurements. To illustrate the breadth of this portfolio, we use two chimeric capsids as representative examples that have been derived through forward- or backwards-directed molecular evolution, namely, AAV-DJ and Anc80. Collectively, this ever-expanding arsenal of technologies promises to facilitate the development of the next AAV vector generation derived from synthetic capsids and to accelerate their manufacturing, and to thus boost the field of human gene therapy.



El Andari Grimm graphical_abstract

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Abbreviations: AAV, Adeno-associated virus; AEX, anion exchange chromatography; CsCl, cesium chloride; DSF, differential scanning fluorimetry; IAC, immunoaffinity chromatography; IEC, ion-exchange chromatography; LPS, lipopolysaccharide; MS, mass spectrometry; PEG, polyethylene glycol: TOF, time-of-flight; TPP, three-phase partitioning

1 Introduction

Since the dawn of the era of human gene therapy research in the 1970s, hardly any vehicle for therapeutic gene transfer has attracted more attention than the Adeno-associated virus (AAV). As a non-pathogenic member of the *Parvoviridae* family, AAV is composed of a single-stranded DNA genome encapsidated in a 23-28 nm, T=1, non-enveloped capsid. Arguably, the greatest and perhaps also a unique asset of AAV is its extreme amenability to genetic engineering and repurposing of the viral genome and capsid. This flexibility enables not only the construction of recombinant vectors encoding an assortment of therapeutic cargos, but it also facilitates the rational design or molecular evolution of novel capsids with enhanced organ or cell specificity, transduction efficiency and/or lower reactivity with anti-AAV immunity [1-3]. To this end, a vast collection of technologies for capsid engineering have been invented and applied over the years, ranging from site-directed mutagenesis of individual amino acids in the capsid, or insertion of retargeting peptides or larger moieties, to the creation of chimeric capsids composed of multiple parental subunits derived from natural viruses or designed *in silico*.

As these techniques have been reviewed extensively by others and us before [2-7], and as a more detailed discussion is outside our current scope, we will merely highlight two specific strategies and prototypes of synthetic AAV capsids that assume an exemplary role in this article. The first strategy harnesses the principle of DNA family shuffling to create chimeric AAV capsids that are built from blocks derived from a set of two or more input viruses with different properties. Therefore, the parental *cap*(sid) genes of ~2.2 kilobases (kb) are first fragmented into ~100 to 800-base pair (bp) long pieces using a controlled DNaseI digest. Next, these are forced to recombine in a primer-less PCR reaction, in which the individual fragments self-prime enabled by the typically greater than 70% DNA homology of most AAV isolates. A subsequent second PCR is then used to amplify the pool of chimeric *cap* genes for subcloning into a replication- and packaging-competent AAV plasmid. The latter also contains the AAV *rep* gene and inverted terminal repeats (ITRs), which are

required for encapsidation of the chimeric sequences. The ensuing plasmid library of usually more than a million different capsid variants is used to produce a corresponding viral library, which is finally interrogated in cultured cells or animal tissues *in vivo* through two to five iterative cycles of infection, the rescue of *cap* sequences from the on-target cells, re-cloning and production of a secondary library for the next round.

In the 2008 study from the Kay lab that pioneered the use of this technique for AAV capsid evolution [8] and that was quickly succeeded by similar work from the Samulski [9] and Schaffer [10] labs, a chimeric AAV capsid called AAV-DJ was molecularly evolved in cultured human liver cells in the presence of neutralizing anti-AAV antibodies. From the eight distinct AAV serotypes that formed the initial capsid library, five were eliminated during the iterative selection, leaving fragments of AAV2, 8 and 9 that had recombined to yield AAV-DJ (Fig. 1, right). As shown in this work and many follow-up studies [11-13], AAV-DJ mediates highly efficient and specific gene transfer to the mouse liver following peripheral administration, as initially hoped for. Interestingly, it also turned out to be an excellent candidate for gene transfer into other cell types *in vitro* and *in vivo*, explaining why AAV-DJ has been widely used to date for numerous applications and in various models of human gene therapy [11, 14-17]. Moreover, its structure was resolved by cryo-electron microscopy at 4.5Å resolution [18], or, more recently, even at 2.8Å resolution in complex with a heparan sulfate analog [19]. Because of its interesting properties, its broad use and the advanced knowledge of its structure and biology, we will use AAV-DJ as the prototype of a synthetic AAV capsid in this article that was created by forward-directed molecular evolution.

Complementing this strategy is another powerful and original technology that was pioneered by Luk Vandenberghe and his team in 2015 and that is called "ancestral reconstruction" [20]. As the name implies, in contrast to AAV DNA family shuffling, this methodology goes back in natural evolution by computationally predicting and then experimentally recreating ancestors of contemporary AAV capsids that may or may not have existed, but when developed as vectors can display unique and interesting properties. The best-known example in the field is Anc80L65, which is a specific variant of a node called Anc80 that represents the ancestor of a variety of present AAV serotypes including AAV1, 2, 8 and 9 (Fig. 1, left). Akin to AAV-DJ, Anc80L65 also excels in many clinically relevant cells and tissues in comparison to widely used other capsids such as AAV8, comprising the liver of mice and monkeys, or hair cells in the murine cochlea [5, 20-23]. Thus, for the same rationale that applies to AAV-DJ, Anc80L65 and some of its closely related siblings that were

also derived by backwards-directed molecular evolution will be used as a second class of prototypes of synthetic AAV capsids in the following.

Notably, while these capsids were evolved with two opposed technologies, they can be produced as recombinant gene therapy vectors using the identical protocol. This is due to one of the many strengths of the AAV vector system, which is the fact that the only component that requires adaptation for the production of a new viral capsid variant is the *cap* gene itself. In contrast, all other pivotal factors such as the AAV *rep* gene (typically derived from AAV2) as well as adenoviral helper functions remain unchanged. Consequently, irrespective of the origin—natural or synthetic—of a new capsid type, of its sequence and structure, and of the technology that was employed for its creation, the protocol for AAV vector production only requires a marginal amendment in the form of the cloning of the desired *cap* gene into the AAV helper construct [24]. This is strikingly different, however, for all subsequent steps in the protocol that follow downstream of the initial vector production scheme, *i.e.*, viral particle purification and characterization. One reason is that a significant proportion of the respective technologies that have been developed and reported over the last decades were specifically designed for a subset of AAV isolates. This includes purification methods that are based on virus affinity for a given receptor, such as AAV2 purification via heparin affinity chromatography [25] or protocols for AAV particle characterization that harness serotype-specific monoclonal antibodies, such as capsid ELISAs for selected serotypes [26, 27]. Moreover, the often limited understanding of the biology of new capsids and their biophysical properties hampers the rational selection of a suited protocol for purification and/or characterization from the arsenal of conventional strategies, thus requiring trial-and-error or necessitating the establishment of the novel, capsid-tailored methodologies.

Here, we will use the aforementioned AAV-DJ and Anc family as representative examples to illustrate the flurry of recent activities that aim to expand our options for downstream processing and characterization of synthetic AAV capsid variants, while remaining compatible with (pre-) clinical demands including high yield, purity, quality and consistency. Thereby, this article intends to complement a collection of excellent previous reviews by our colleagues who have comprehensively discussed more traditional technologies for AAV manufacturing. In particular, for further reading, we recommend the overview articles by Ayuso, Mingozzi and Bosch [28], Clement and Grieger [29], or Aponte-Ubillus and co-workers [30], among others [31-33]. Also, we will start by briefly highlighting interesting

recent progress in the upscaling of the upstream AAV production process as it goes hand in hand with downstream optimization. Finally, we will exemplify other technological advances that are related to the progress in AAV downstream processing, comprising latest improvements in AAV characterization through mass spectrometry or thermostability measurement, or in detection and removal of contaminants. An overview of the different methodologies discussed in this article including a selection of pros and cons and representative examples from the literature is given in Table 1.

2 Manufacturing of synthetic AAV vectors

As noted above, AAV production can easily be adapted to newly discovered wild-type isolates or synthetically engineered capsids, by simply replacing the *cap* gene in the preferred AAV helper construct. This is particularly straightforward in the case of protocols based on transient plasmid transfection [34], in which *cap* is encoded on an AAV helper plasmid and typically co-transfected with the AAV vector and another plasmid providing all adenoviral helper functions; the latter can also be combined with the AAV *rep* and *cap* genes on a single construct [24, 35-37]. It is more labor- and time-intensive in the case of stable AAV producer cell lines or scalable systems based on heterologous viruses such as baculoviruses [38], yet the overall principle remains identical. Nonetheless, we note that, to our best knowledge, these alternative systems remain to be harnessed for the production of synthetic AAV capsids. In particular, in the case of the baculovirus system, this may then require additional optimization of the AAV expression cassettes including promoters to maintain a correct stoichiometry and splicing of all capsid mRNAs and proteins as well as other biological AAV properties [28, 39].

Of note, stable lines or helpervirus infection are not the only means of up-scaling AAV production; instead, numerous groups have succeeded at adapting HEK293 cells (a human embryonic kidney cell line that is typically used for AAV manufacturing) to growth in suspension. One example has recently been provided by Blessing and colleagues who cultured an adapted HEK293 suspension cell clone called HEKExpress under serum-free conditions in orbitally shaken bioreactors (OSRs). The latter combine efficient gas transfer with high cell viability and can be scaled up to 1000 liters [40, 41]. Using transient transfection with polyethylenimine (PEI) and POROS CaptureSelect resins for AAV purification via immunoaffinity chromatography, the authors managed to achieve AAV8 and

AAV9 production with a significant recovery (>35%) [40]. While not demonstrated in the original report, this scalable production and purification system can likely be harnessed for synthetic AAV capsids as well.

The latter was shown in a similar study by Grieger and colleagues [42] in which the authors managed to adapt adherent HEK293 cells to grow in suspension under animal component- and antibiotic-free conditions in shaker flasks and WAVE bioreactors. On top, a pipeline was established and optimized that involves triple-transfection of these cells using PEI Max, followed by a combination of discontinuous iodixanol gradient purification and ion-exchange chromatography (IEC). Remarkably, tweaking of the chromatography conditions enabled the removal of frequently observed contamination in parvoviral vectors stocks, namely, ferritin. Further notable is the successful upscaling of the transfection and culturing conditions into WAVE bioreactors of up to 20 liters, frequently resulting in the production of around 1×10^5 vector particles per cell and a total of greater than 1×10^{14} highly pure and >90% full vector particles of various serotypes (AAV1–6, 8, 9). Important to the current article, this method is fully compatible with synthetic AAV capsids, as demonstrated with AAV variants 2i8 [43] and 2.5 [44]. Finally, it was shown that vector yields can be increased through continuous harvest from the medium over 120 hours. As a whole, this technology is very encouraging owing to its universal nature and its ensuing compatibility with synthetic capsids, as well as because virus yields per cell match or surpass those obtained with other HEK293 cell-based systems [42, 45-47] or those using herpes simplex viruses [48] or baculoviruses [49].

Also, we note further interesting studies by, for instance, Emmerling *et al*. who managed to adapt HEK293T cells to growth in fully-controlled, single-use iCELLis™ Nano bioreactors and who obtained substantial AAV2 yields that approximated those achieved in more conventional cell factories [50]. Lastly, we highlight comprehensive recent work by the Mingozzi lab in which Collaud *et al*. harnessed a 10-200 liter suspension cell-based production system to scale up AAV8 vector manufacturing for good laboratory practice toxicology-biodistribution studies [51]. While these and other similar reports used wild-type AAV variants, there is a high chance that these new and optimized AAV production systems are compatible with synthetic capsid variants as well and can thus ideally synergize with large-scale downstream processing.

3 Methods for AAV vector purification

Today, the AAV field has a plethora of methodologies at its disposal for the harvesting of vector particles from producer cells and/or the culture media and for their subsequent purification, whose description would be beyond the scope of this article; rather, we again refer the reader to seminal previous reviews for details [28-33]. Here, we will briefly recapitulate two of the oldest and most conventional technologies for AAV purification, *i.e.*, by cesium chloride (CsCl) or iodixanol density gradient centrifugation [52]. These protocols are used abundantly because they allow, at least to some extent, for the separation of full (*i.e.*, vector DNA-containing) and empty particles, and because they are largely independent of the capsid. In direct comparison, CsCl-based purification requires more time and work [53-55], but a major benefit is a significantly lower proportion of remaining empty particles (<2%) in contrast to iodixanol (~20%) [33, 53]. *Vice versa*, CsCl is more toxic than iodixanol and can induce adverse effects in animals, thus raising a need for dialysis with physiological buffers before use in *in vivo* studies. Toxicity is a lesser concern with iodixanol owing to its inert and non-ionic nature, permitting the immediate use of iodixanol-purified AAV vectors *in vivo* [56, 57], although the safety in patients with compromised kidney functions is unclear [58]. Either way, rebuffering of iodixanol-purified AAV particles for *in vivo* studies may be indicated because of the relatively high viscosity of the 40% iodixanol phase in which the full particles accumulate and which may hamper vector injection. To this end, a variety of techniques have been tried, including ultrafiltration/concentration with Amicon Ultra-15, 100 kDa MWCO centrifugation tubes, size-exclusion chromatography with desalting columns, hollow-fiber tangential flow ultrafiltration, or PEG-8000 precipitation and concentration. Based on data by Strobel *et al*. [53], ultrafiltration may be most efficient and least time-consuming, and it also yielded highest particle recovery and was most effective at removing iodixanol traces [53]. Still, it remains debated controversially whether CsCl or iodixanol purification results in higher AAV vector bioactivity and product quality. For example, while Zolotukhin *et al*. reported higher transduction with vectors purified with iodixanol [52], Strobel *et al*. found slightly higher potency of AAV vectors that were purified with CsCl [53]. In general, multiple factors may govern transduction efficiency comprising cellular impurities such as ferritin [42, 55, 59, 60], prolonged exposure to CsCl that can reduce infectivity [61], the proportion of full and empty particles [62], repetitive freezing and thawing of vector stocks [63], or precipitation methods [53], which all require further characterization and standardization.

Next to density gradient centrifugation, column-based chromatography has become a popular technology for downstream AAV purification, as it can effectively discriminate AAV particles from impurities including host cell proteins, nucleases, detergents and residual DNA, and can concurrently be used to rebuffer AAV vector samples. Importantly, while AAV purification via chromatography offers less flexibility than the more universal gradient centrifugation technology, it is easily tailored for large-scale production and is compatible with other purification methods, explaining the wide use and frequent commercialization of these techniques [28-32, 64-70].

A first important category is IEC, in which AAVs interact with the column matrix by electrostatic interactions of net surface charges on the viral particle and ion resins in the IEC column. As this interaction depends on the isoelectric point (pI) of the capsids and the pH of the buffers, increasing ion strength via higher salt concentrations in elution buffers enables the efficient recovery of column-bound vector particles. A pivotal asset of IEC is its capacity to remove empty capsids at large scale and under optimized elution conditions, based on the small but significant differences in pI between empty (6.3) and full (5.9) capsids [71-73]. To date, a variety of different anion- or cation-exchange chromatography resins have been developed, many of which are already commercially available. For instance, anion and cation exchangers such as POROS HQ and SP sepharose, respectively, were used for the purification of multiple wild-type AAV serotypes [32, 71, 74, 75]. Additional benefits of using IEC technology for AAV purification comprise its reproducibility and compatibility with automation and upscaling, the independence from chemical reagents (that could represent unwanted impurities in the final product), and the lack of leaching (*i.e.*, detaching of capturing reagents such as anti-AAV antibodies, which is often observed in affinity-based AAV purification methods). Notably, though, IEC cannot discriminate assembled capsids from protein impurities that show a similar pI and thus usually requires a subsequent step using an alternative matrix with distinct pH and salt conditions [31]. Furthermore, the effectiveness of IEC is readily influenced by the characteristics of the input material, and the method cannot be standardized for all AAV capsids including synthetic variants due to their different physicochemical properties.

A second category is affinity chromatography, which exploits either specific AAV substrates that mimic cellular receptors, or which—in immunoaffinity chromatography (IAC)—harnesses monoclonal antibodies or nanobodies that recognize AAV outer shell proteins. Either way, this technique is typically capable of differentiating assembled capsids

from free viral proteins and impurities [35]. One of the earliest examples in this category is heparin-based affinity column chromatography, which was established soon after the AAV2 membrane-associated heparan sulfate proteoglycan receptor had been identified [25, 76]. Although this method is, in principle, also compatible with AAV6 or AAV1 (after a single amino acid change) [77], it is mainly used to purify the AAV2 serotype [29]. Moreover, it is feasible to engineer heparin-binding domains into other AAV capsid variants, but this may interfere with their transduction potency or titers as found with multiple capsids and in various cells [8, 78]. Similarly, AAV2 affinity chromatography purification based on the monoclonal antibody A20 has been reported [26], which specifically recognizes assembled AAV2 capsids and can thus be used for concurrent capsid purification and depletion of free capsid proteins. However, due to the restriction to AAV2 (and AAV3, which is also recognized by A20) and the dependency on a purified monoclonal antibody that is rate-limiting, this technology was not developed further.

Instead, another notable example of an affinity chromatography medium that has already been used extensively for over a decade and that is commercially available is AVB Sepharose High Performance. The ligands in this resin are highly stable, single-domain antibody fragments from the family *Camelidae*, which were isolated from llamas that were naturally infected with AAVs and which are fused to N-hydroxysuccinimide (NHS)-activated Sepharose High Performance [28]. This approach has gained popularity and is relevant for this article since it enables the purification of multiple AAV variants including synthetic capsids (*e.g.*, AAV1-8, AAVrh8R, AAVrh10, AAV12 and AAV6.2) and since it is characterized by a large vector binding capacity, acceptable levels of concentration and recovery, as well as a moderate linear flow-rate [51, 62, 69, 79, 80]. At least one AVB-binding epitope (amino acid sequence SPAKFA) was identified in the AAV capsid, and its transfer from AAV3B (where it was discovered initially) into other serotypes such as AAV8, rh.64R1, AAV9 [81] or into the synthetic AAV-DJ or AAV-DJ/8 [80] improved binding of the ensuing modified capsids to the AVB resin without compromising transduction efficiency. Interestingly, the O'Riordan lab reported preliminary evidence that the AAV-DJ-SPAFKA mutant not only binds to AVB but also tends to produce more genome-containing particles and to yield better transduction than AAV-DJ in cultured Huh7 cells [80].

The discovery of this AVB-binding epitope and the conservation of its function after transfer into multiple AAV capsid variants is critical for at least two reasons. Firstly, it may allow us to predict the suitability of the AVB resin for purification of other AAV capsid

variants, natural or synthetic, purely based on the primary amino acid sequence. This would greatly facilitate the selection of capsid variants in cases where the ease and/or scale of purification are crucial factors. Secondly, capsids lacking this epitope may be engineered and transformed into AVB binders upon grafting of the epitope, as demonstrated by Wang *et al*. [81] or Nass *et al*. [80], highlighting the potential of AVB technology for purification of increasingly diverse AAV capsid variants.

Conversely, a disadvantage of AVB that may hamper its wider use especially for clinical-grade AAV vector purification is the immense costs of the resin material that can rapidly become rate-limiting, in particular when contemplating the purification of multi-liter, bioreactor-derived vector stocks. Moreover, as noted, the AVB resin fails to capture some of the widely used AAV variants such as wild-type AAV9 or the synthetic AAV-DJ capsid (in its unmodified form, see above) [80, 81]. Another general drawback of affinity chromatography over IEC [75, 82, 83] is its inability to separate full and empty capsids, which is not surprising considering their identical amino acid composition [28, 29, 80]. Importantly, Qu *et al*. were able to discriminate between the two populations of AAV2 capsids based on slight differences in charge using IEC [71]. Similarly, the O'Riordan lab has recently shown that combining affinity chromatography and subsequent IEC can result in a significant reduction in empty capsids, enabling the enrichment of up to 80% full, genome-containing capsids depending on the AAV serotype [80].

In addition to AVB affinity columns, other commercially available immunoaffinity resins have recently gained increasing attention and are capable of purifying synthetic AAV capsids, such as POROS AAV9 and AAV8 CaptureSelect Affinity matrices. Here, the ligand is covalently bound to Polystyrene-Divinylbenzene (POROS) beads that have a high capacity of AAV particle binding ($\sim 10^{14}$ vector genomes per ml of resin) [31]. Moreover, POROS resins can effectively reduce impurities within a single run and can resist mechanical pressure of up to 10 Mpa, which in combination with AKTA devices allows for the processing of large volumes of AAV vectors [40]. Their potential was comprehensively demonstrated by Nass *et al*., who reported that AAV8 (80%) and AAV9 (73%) were efficiently recovered from their respective specific resins [80]. Interestingly, sequence similarity may not be a strong predictor of compatibility with a particular POROS resin, as demonstrated with AAVrh8R that was successfully purified with POROS AAV8 but not POROS AAV9, despite its higher similarity to AAV9. Similarly, albeit AAV-DJ is largely homologous to AAV8, it could not be purified via the POROS AAV8 resin (and also not AVB,

see above). Accordingly, until the specific epitopes that are recognized by the POROS systems have been identified, the compatibility of a given AAV variant has to be tested and rational epitope grafting strategies such as reported for the AVB resin remain impossible. Nonetheless, the experience with isolates such as AAVrh8R implies that the increasing collection of off-the-shelf serotype-specific affinity resins may offer sufficient variety to enable the purification of a wealth of AAV capsid variants. Along these lines, the recent release of yet another commercially available resin, POROS AAVX CaptureSelect, is encouraging as it is supposed to display a broad affinity towards numerous AAV serotypes and synthetic capsids [84].

Noteworthy, chromatography technologies are also useful for quality control (QC) of vector stocks as they allow to determine the ratio of full and empty (F:E) capsids [62], partially packaged capsids, and other protein contaminants [42, 85]. Knowledge of this ratio is critical given the accumulating and controversially discussed [86-88] evidence that, on the one hand, the presence of empty capsids may impair transduction efficiencies and exacerbate adverse side effects [89]. On the other hand, it was proposed that empty capsids may be beneficial by acting as decoys for anti-AAV antibodies and by thus helping to overcome preexisting humoral immunity to AAV [90]. For these reasons, a large body of techniques has been devised in the past to measure the F:E ratio, comprising analytical ultracentrifugation (AUC), cryo-electron microscopy, UV spectrophotometry, anion-exchange high-performance liquid chromatography (AEX), or charge detection mass spectrometry (CDMS). We also note other technologies such ELISA combined with PCR, or transmission electron microscopy (TEM), but point out that use of TEM alone is insufficient to determine F:E ratios, and that both, ELISA and qPCR, possess an inherent degree of variability which may be exacerbated during their combination and result in a significant over- or underrepresentation of packaging efficiencies. Particularly notable in the context of the present review is recent work by Wang *et al*., who demonstrated the power of AEX using POROS 50 HQ and CIMac columns to separate full and empty capsids of the synthetic variant AAV6.2 [62]. Impressively, following the optimization of various experimental parameters such as salts and buffering agents, the final method was shown to be accurate, linear, reproducible and sensitive, permitting the detection of low proportions (2.9%) of empty AAV6.2 capsids using relative UV measurements at 260/280 nm. Compared to AUC, one of the most commonly used approaches to determine F:E ratios based on sedimentation velocity, AEX gave a slightly lower resolution and failed to detect a peak of partially

packaged AAV capsids. Still, this may be outweighed by the benefits of AEX, especially its compatibility with automization and its ease-of-use [62].

Finally, we note a recent study by Arden and Metzger, who reported a simple, cost-effective and serotype-independent AAV purification protocol that is merely based on PEG precipitation and two centrifugation steps [91]. Proof-of-concept comprised its application for purification of the AAV variant AAVM41, *i.e.*, a myocardium-tropic, synthetic AAV created by DNA family shuffling of serotypes 1, 6, 7 and 8 [92]. Albeit raw data were only shown for another AAV serotype, AAV6, AAVM41 yields and purity obtained with this streamlined protocol were reported to be high, and *in vivo* bioactivity in mice was preserved. This implies that this inexpensive methodology is well suited to complement existing AAV purification technology based on gradient centrifugation or affinity column purification, albeit it may not be easily scalable.

4 Purification of AAV-DJ

A good example of a synthetic AAV capsid that has been successfully purified using a number of the technologies highlighted above and others is AAV-DJ, the prototype of a capsid generated by DNA family shuffling, *i.e.*, the forced recombination of capsid DNA fragments from multiple parental AAV isolates (here, AAV2, 8 and 9) [8]. Therefore, in this chapter, we will harness this specific capsid to exemplify the diversity of technologies that exists and that has already been exploited for purification of synthetic AAV capsids. Importantly, in Table 1, this will be complemented by an overview of the use of the same technologies for Anc capsids, *i.e.*, the family of synthetic capsids created through ancestral reconstruction including Anc80L65.

In the initial report of AAV-DJ [8], CsCl density gradient centrifugation was used for purification, followed by later studies that used the same protocol [14, 16, 19, 93] or iodixanol density gradient centrifugation [11, 15, 94-97]. Notably, AAV-DJ contains the heparin-binding domain from one of its parents, AAV2, which tempted Liu and Moon to try and purify AAV-DJ via heparin affinity column chromatography [13]. Indeed, a relatively small, 5 ml heparin column in combination with FPLC was sufficient to enable the purification of 3×10^{13} particles from 150 15 cm dishes of triple-transfected HEK293 cells. However, this method was incompatible with the purification of vector particles that had been secreted into the media during production due to column contamination with proteins in the cell culture medium,

resulting in a loss of ~40% of produced particles. The utility of heparin affinity columns for AAV-DJ purification was also independently verified by Xie *et al*.[19], who combined this with three rounds of CsCl density gradient centrifugation in order to purify empty virus-like particles of AAV-DJ and to resolve their structure bound by a heparinoid pentasaccharide. Also, Candelas *et al*. used heparin column-purified AAV-DJ to investigate the role of the T-type calcium channel Cav3.2 in lamina II neurons of the spinal cord in mice [98].

In another study [99], Hashimoto and colleagues compared all four combinations of two plasmid transfection (calcium phosphate co-precipitation and lipofectamine) and two purification methodologies (CsCl and iodixanol density gradient centrifugation), to produce a derivative of AAV-DJ called AAV-DJ/8, in which the aforementioned heparin-binding domain from AAV2 was replaced with the corresponding sequence of AAV8 [8]. This showed that the combination of lipofectamine and iodixanol purification may be best suited to obtain high titers of AAV-DJ/8, albeit questions about the scalability of lipofectamine transfection remained. In line with work by Strobel and colleagues who independently also compared the two purification technologies [53], the choice of method did not affect *in vitro* or *in vivo* vector bioactivity.

Both synthetic AAV capsids, AAV-DJ and its derivative AAV-DJ/8, were also studied by Kimura *et al*. who implemented an original purification protocol based on PEG precipitation, aqueous two-phase partitioning (previously also reported by Guo *et al*. [100]) and iodixanol density gradient centrifugation [101]. To this end, vector particles were produced using a likewise optimized triple-transfection protocol whose hallmarks were a reduction in medium glucose and a pH stabilization, resulting in overall titers of up 1×10^{14} highly pure AAV vector particles per ml. As in the work of Hashimoto *et al*. [99], *in vivo* mouse studies served to confirm that the bioactivity of vectors purified with this improved, rapid and economical protocol was maintained.

Similarly, Yu *et al*. reported a protocol for AAV purification based on three-phase partitioning (TPP) combined with CsCl density gradient centrifugation [102], which is capable of separating full from empty particles and of removing 90% of cellular proteins, while concurrently increasing the capacity of ultracentrifugation by up to 10-fold, depending on the AAV serotype. TPP is a non-chromatographic methodology that is based on the separation of tertiary butanol into two phases upon mixing with ammonium sulfate, and the additional separation of target proteins such as AAV particles into a third phase between the other two. The impressive proof-of-concept data included purification of synthetic AAV-DJ particles

from a 25 liter production run, resulting in a total of ~5×10^{15} vector genomes and an ~80% recovery rate. The fact that next to AAV-DJ, the method was also validated with numerous wild-type AAV suggests that it is widely applicable and, based on the authors' calculation, useful for stocks in the 10^{17} vector genome-scale.

A special variation of a protocol for AAV-DJ purification was reported by Kukisi and colleagues [103], who used this vector to selectively and artificially activate specific neural circuits in the medial amygdala of mice, to study the role of olfactory signals for sexual behaviour. To this end, cells transfected with the AAV helper and vector plasmids were collected, suspended in artificial cerebrospinal fluid and subjected to four freeze-thaw cycles. Subsequently, the cell lysate was treated with benzonase and centrifuged twice at 16.000×g for 10 min, yielding a supernatant that was directly used for the *in vivo* experiments.

Finally, we note a study by Lakhan and colleagues who used AAV-DJ vectors to deliver the *COX2* gene to fracture sites in a mouse femoral fracture model, where it effectively transduced mesenchymal stem cells [17]. For vector purification, the authors harnessed the AAV Purification Kit from Cell Biolabs Inc., which is based on an affinity matrix that is not further specified but suitable for AAV2 and AAV-DJ, and which claims to yield >60% recovery and >95% purity following a three-hour protocol (https://www.cellbiolabs.com/aav-purification-standard-kit). The same kit was also used by Yoo and colleagues in a study where they harnessed AAV-DJ to express a cocktail of heart reprogramming and regeneration factors *in vitro* and *in vivo* [104].

5 Methods for AAV vector characterization

Akin to the arsenal of technologies for AAV vector purification, there is a wealth of reported strategies for the subsequent characterization of vector particles, whose comprehensive discussion would be beyond our present scope. Therefore, in the following, we will restrict ourselves to a selection of methodologies that we deem particularly interesting and promising for their (future) application to synthetic AAV capsids, and that, to the best of our knowledge, have not been reviewed extensively before. We apologize to all other authors who have developed an alternative, sensitive and powerful techniques that we cannot review in great detail here for space reasons, such as SDS capillary gel electrophoresis [105].

5.1 Mass spectrometry (MS)

Over the last 1.5 decades, MS has been used to characterize AAV capsid protein integrity and post-translational modifications, as well as to identify contaminating cellular proteins in AAV vector preparations (see next chapter). Concurrently, it has also been recognized as a versatile and powerful technology to confirm AAV serotype identity and as an assay for lot release testing. Among the first to apply MS for the study of AAV capsid biology were Salganik *et al*., who harnessed MS to identify proteolytic cleavage sites in the AAV1 capsid [106]. Capsid modifications were also studied by Murray and colleagues, who specifically assessed the glycosylation of AAV2 capsids and reported evidence that this particular post-translational modification may not occur [107]; a conclusion that was later confirmed by Jin *et al*. [85].

However, the general view of AAV as a non-glycosylated virus changed with more recent studies, *e.g.*, a 2018 report by the Xiao group who used MALDI-TOF and high-resolution LC/MS to identify N-glycosylation on amino acid N499 of the AAV8 capsid, at least in a fraction of the stock they investigated [108]. Interestingly, the same amino acid was also among the numerous asparagine residues on the AAV8 capsid that Giles *et al*. discovered to be deaminated by LC tandem-mass MS, and it was the one showing the greatest stock-to-stock variation in terms of deamidation level, for reasons unknown [109]. As shown in the same study, the phenomenon of deamidation is not unique to AAV8 but was also observed for seven other isolates, *i.e.*, AAV1, AAV3 to 5, AAV7, AAVrh32.33 and AAV9, and may negatively affect capsid assembly, transduction efficiency, tissue tropism and immunoreactivity. An interesting concept with relevance for AAV manufacturing that emerged from this work is that of a "deamidation clock", according to which progressing deamidation correlates with losses in vector activity, thus providing an advantage to freshly translated virions for transduction [109]. Hence, MS-based analysis of capsid deamidation may become an important part of future workflows to quantify consistency and lot-to-lot reproducibility during AAV vector manufacturing.

Likewise, Mary *et al*. used MALDI-TOF/TOF analysis as well as a combination of nanoUPLC and Triple-TOF MS analysis to identify post-translational modifications in ten AAV serotypes, AAV1 through 9 and AAVrh10, and detected a variety of such events including glycosylation, phosphorylation, acetylation, ubiquitination and SUMOylation [110]. Interestingly, while some of these occurred at evolutionarily largely conserved residues in multiple serotypes, others were specific for one or more viral isolates, implying their potential for identification or confirmation of particular capsids during vector manufacturing. The fact

that LC/MS but not MALDI-TOF analysis revealed N- and O-glycosylation in AAV2 adds to the controversy about this specific post-translational modification in AAV2 and concurrently highlights the importance of using utmost sensitive detection technology. Similar to the deamidation events reported by the Wilson group [109], the broad spectrum of modifications detected here could affect immune recognition of the capsid as well as its stability, cellular entry and trafficking, or other aspects of its intracellular fate [110]. Of note, using LC-MS, Jin *et al*. also independently detected acetylation on the VP1 and VP3 N-termini of six different AAV serotypes, *i.e.*, AAV1, 2, 5, 7, 9 and rh10, and speculated on a link to capsid protein degradation or ubiquitination [85]. The second conclusion of this work with relevance for the topic of the present article was that the unanimous determination of VP1, VP2 and VP3 masses could serve as a generic but highly specific and rapid method for capsid serotype identity testing and to ensure product consistency during AAV manufacturing, even though its usefulness for synthetic AAV variants remains to be demonstrated to date.

Until then, the great potential of MS technology for confirmation of AAV serotype identity and batch release testing has already been exemplified in 2009 in a comprehensive study by Van Vliet *et al*., who developed a protocol involving VP protein separation by gel electrophoresis, band excision, trypsin digestion and LC/MS/MS [111]. As demonstrated in various proof-of-concept experiments, this workflow was capable of separating closely related serotypes AAV1, 2 and 8 from each other as well as from the more distinct AAV4 and 5. Moreover, it also enabled the recognition of a point mutation in the AAV4 capsid at VP1 position 544 (K544E) that had previously been detected by conventional Sanger sequencing, even though the two AAV4 variants were indistinguishable *in vivo* in the mouse retina.

The usefulness of MS techniques for identification and quantification of AAV capsids and their components was further validated in three later studies, including work by Snijder *et al*. in 2014 who illustrated the power of high-resolution Orbitrap MS for the study of VP protein stoichiometry in assembled AAV1 capsids [112]. Intriguingly, their data suggest that AAV1 exists in a total of eight stoichiometries, composed of 0-2 copies of VP1, 8-11 of VP2, and 48-51 of the major capsid protein VP3. In turn, this implies that AAV(1) capsid assembly is a stochastic rather than a predefined process whose outcome depends on the expression levels of each capsid protein. This idea of inherent variability between individual particles is an appealing hypothesis that could readily explain the inability of even atomic-level crystallography approaches to precisely resolve the unique regions of VP1 and VP2.

Subsequently, Pierson and colleagues harnessed charge detection (CD)MS to dissect the DNA content of AAV8 vectors containing single- or double-stranded genomes, permitting

them to demonstrate its power to quantitatively and rapidly characterize various subpopulations comprising particles with a full or partial genome, empty capsids as well as impurities [113]. With this resolution and these features, CDMS seems well suited to complement the other technologies, especially since it allows to monitor not only capsid but also genome integrity.

Finally, another useful addition has more recently been reported by Zhang *et al*. who applied a novel, automated, microfluidic ZipChip CE/MS methodology to identify AAV2 capsid proteins within a very short period (four minutes) and from a minimal sample volume of 5 nl [114]. To illustrate the resolution of this technology, the authors successfully distinguished wild-type AAV2 from a triple point mutant of the same serotype, reminiscent of the data by Van Vliet *et al*. [111] but achieved in much shorter processing time. Notably, this study also again confirmed the single N-terminal acetylation on VP1 and VP3 that has been reported previously [85, 109].

5.2 Capsid thermostability (AAV-ID)

The MS-based technologies highlighted above allow to distinguish capsid serotypes including variants with minimal sequence divergence, and are thus, in principle, useful to validate proper labeling and batch-to-batch consistency of AAV vector stocks for clinical use. However, most of the reported protocols may be limited in the applicability for a variety of reasons, including the requirement for large substrate volumes or for special instrumentation, and their inability to yield higher-level structural information beyond primary capsid protein sequence.

These and other gaps can potentially be filled by another technology that was introduced into the AAV field in 2013 by Rayaprolu *et al*. [115] and then extensively and independently validated in two publications by the groups of Luk Vandenberghe, Eduard Ayuso and Mavis Agbandje-McKenna in 2017 [116, 117]. The hallmark of this powerful biophysical technique, called differential scanning fluorimetry (DSF) or AAV-ID [117], is its ability to rapidly, easily and accurately measure AAV capsid thermostability (melting temperature, T_m), by monitoring capsid unfolding in real-time in response to temperature gradients in the presence of SYPRO Orange. The latter is a hydrophobic dye whose fluorescence at 570 nm is quenched by solvent molecules but which starts to fluoresce once it binds to hydrophobic pockets, such as those that are normally located inside the AAV capsid and become exposed during heat-induced denaturing. Already in 2013, the McKenna and Bothner labs recognized the potential of DSF to determine AAV capsid T_m and to then use these values to unanimously

identify divergent serotypes, as exemplified using AAV1, 2, 5 and 8 [115]. Intriguingly, recording of thermal denaturation curves using DSF revealed distinct and narrow transition temperatures that were characteristic for each serotype, with the lowest observed for AAV2 (~70°C) and the highest for AAV5 (~90°C). Further analysis showed that these T_m are neither determined by capsid protein VP1 nor by the presence of an AAV genome inside the capsid, which was later confirmed in the aforementioned follow-up studies [116, 117]. Importantly, the authors also performed a first mixing experiment in which AAV2 and AAV5, the two serotypes with the greatest difference in T_m, were pooled and analyzed by DSF. This yielded two distinct and serotype-specific peaks, and thus illustrated the power of this technique to concurrently identify and resolve at least two different capsid variants.

The latter was subsequently extended and much more profoundly demonstrated by Pacouret *et al*., who used DSF to analyze a collection of 67 AAV stocks and who convincingly showed its ability to discriminate six different serotypes by their T_m fingerprint, namely, AAV1, 2, 5, 6.2, 8 and 9 [117]. In line with the data from Rayaprolu *et al*. [115], T_m were largely independent of the presence or size of an encapsidated genome, as well as of production (mammalian versus insect cells) or purification protocols (iodixanol versus POROS). Moreover, the methodology—called AAV-ID here—is highly robust, as illustrated via its reproducible application in two different labs. Also interesting is that the signal amplitude at the apex of the T_m peak correlated with vector dose in a linear fashion over at least one order of magnitude, illustrating the ability of AAV-ID to provide information on particle concentration. Furthermore, Pacouret and colleagues showed that the thermostability measure is strongly affected by the pH of the AAV vector formulation buffer in a serotype-dependent manner, providing clues about a link between capsid stability and cellular mechanisms affecting AAV genome release, including acidification in the endosome. However, the finding that in some cases, fluorescence transition was obscured by contaminants, implied that AAV-ID may be limited to AAV stocks above a certain purity threshold. Finally, reminiscent of the MS data by Van Vliet *et al*. [111], AAV-ID was shown to be able to distinguish various pairs of AAVs that differ in a few residues, such as AAV1 and 6.2.

These conclusions were essentially mirrored in the work by Bennett *et al*. [116], who studied ten different AAV serotypes (AAV1 through 9, AAVrh.10) and who showed that most of them can be distinguished by their unique T_m, except AAV7, 9 and rh.10 whose T_m was in a very similar range around 77°C. Also in this work, melting temperatures were affected by buffer formulation and pH in a serotype-specific manner, but not by the presence or absence

of a genome inside the particles. Notably, the method proved to be very sensitive and capable of detecting as little as 5×10^{11} purified AAV5 (the most stable serotype) particles in 25 µl. Extending the previous studies [115, 117], Bennett and colleagues demonstrated that the VP3 capsid protein is the sole determinant of thermostability (and sufficient to assemble capsids). Finally, akin to Pacouret *et al*. [117], it was shown that DSF can distinguish a pair of AAV serotypes differing in single amino acid, by swapping residue E/K531 between AAV1 and 6, respectively.

Of interest for this article, the same lab has applied various techniques including DSF to also assess the thermostability of synthetic AAV capsids, especially variants that were designed *in silico* through ancestral reconstruction [20]. Specifically, Zinn *et al*. determined the T_m of their lead AAV candidate, Anc80L65, to be ~92°C and thus 15 to 30°C higher than that of AAV2 or AAV8. This came as a surprise considering the relatively poor vector yields that were obtained with Anc80L65 as compared to AAV8, but it may imply that higher activation energy is required not only to disassemble but also to assemble Anc80L65 capsids, albeit this needs to be validated. An even further increase was noted for another reconstructed capsid from the AAV1-3 lineage, Anc127, whereas two others, Anc81 and Anc82 (Fig. 1), had a lower T_m than Anc80. The fact that the latter two are evolutionary intermediates between Anc80 and extant serotypes illustrates the power of DSF to not only distinguish synthetic AAV variants based on T_m, but to also gain insights into natural virus evolution.

Last but not least, we highlight a recently reported alternative technology dubbed intrinsic (i)DSF, which provides remarkable precision and sensitivity (down to 2×10^{11} AAV particles) in the absence of external dyes such as SYPRO Orange [118]. Briefly, this approach relies on the inherent red-shift of tryptophan residues under UV light when exposed to a hydrophilic rather than a hydrophobic environment, as is the case during AAV capsid disassembly. Similar to the preceding studies using SYPRO Orange-dependent DSF, also iDSF was shown to enable the discrimination of various natural serotypes (albeit not all; *e.g.*, the AAV3/AAV8 pair could not be distinguished) and to yield similar T_m values. Notably, iDSF is relatively simple, fast and inexpensive, making it attractive for high-throughput screening and sample monitoring during the purification process. Finally, albeit not demonstrated in the pilot study by Rieser and colleagues, the fact that tryptophan residues within AAV capsids are largely conserved across serotypes implies great usefulness of this method also for synthetic AAV capsid variants in the future.

5.3 Bacterial and cellular contaminants

Frequent and usually adverse contamination of AAV vector stocks is endotoxin, *i.e.*, a lipopolysaccharide (LPS) found in the outer membrane of Gram-negative bacteria. Particularly concerning is that even minute amounts of LPS can trigger severe adaptive immune responses in intravenously injected mammals including humans and potentially lead to an inflammatory response, sepsis, multi-organ failure and, ultimately, death. Exacerbating this concern is that bacteria are found almost everywhere in a laboratory setting, including in buffers, on lab-ware and surfaces as well as in the plasmid DNA that is produced and used for AAV vector production. Moreover, proteins such as the AAV capsid shell tend to readily interact with LPS over a broad range of isoelectric points, implying that AAV serotypes and natural or engineered capsid variants may all display at least some degree of affinity toward endotoxin.

Therefore, we consider a recent study by Kondratova and colleagues from the Zolotukhin lab that tackled this particular AAV contaminant as important, timely and worth highlighting [119]. In this work, the authors succeeded at establishing a new, rather simple but very effective protocol that permits to reduce the endotoxin content of AAV stocks to virtually undetectable levels of <2.5 endotoxin units [EU] per ml, which complies well with the FDA-recommended upper limit of 5 EU per kg body weight for intravenously injected biopharmaceuticals [119, 120]. Briefly, the hallmarks of this protocol are the initial treatment of AAV stocks with mild detergent, followed by a series of buffer exchange and concentration steps under experimental conditions that prevent adverse AAV particle aggregation. Importantly, a thorough analysis of the final products yielded no evidence for perturbations of transduction efficiency, capsid thermostability, protein composition or capsid morphology. Even more important in the context of the present article is that the authors successfully validated their new technology with eight different AAV capsid variants, including wild-types AAV2, AAV5, AAV8, AAV9 and AAVrh10 as well as synthetic capsids, such as AAV-DJ [8] and AAV-TT [121]. Also very encouraging are the high recovery rates of between 50 and 96% of starting material, which surpass the low yields of typically less than 10% that are often observed with commercial LPS removal kits.

Collectively, these advantageous properties make this new protocol highly attractive especially for academic laboratories who work with different AAV capsid variants and who will thus benefit most from the combination of inexpensive, off-the-shelf reagents, quick turn-around time, high recovery, versatility and effectiveness at LPS removal. However, its

scalability to large(r) volumes has remained unclear to date and should be established in future work.

Of note, bacterial endotoxins are not the only contaminants that have been detected in AAV preparations, but others have likewise reported the presence of cellular proteins. In one notable 2014 study, Dong and colleagues used a combination of two proteomics approaches, gel electrophoresis liquid chromatography-mass spectrometry (GeLC-MS) and two-dimensional gel electrophoresis, to assess the purity of cesium chloride gradient-purified AAV2 vectors and detected 13 co-purifying cellular proteins, including known AAV host factors such as nucleolin and nucleophosmin [87]. One of them, protein SET, was also found in stocks of serotypes AAV5, 6, 8 and 9 that were purified with the same method. Interestingly, though, it was absent from AAV2 stocks that were purified using IEC, and it seemed to specifically co-purify with full, DNA-containing AAV2 capsids, but not with the excess of empty particles, for reasons unclear. This work and related other studies (see below) are intriguing because the data have multiple implications for AAV biology and vector manufacturing. Firstly, such identification of contaminating or co-purifying (under specific conditions) host cell proteins is highly informative as it expands our still limited understanding of natural AAV biology, which may ultimately allow to rationally engineer better vectors and/or to improve manufacturing protocols. There is evidence that cellular contaminants may actually enhance AAV vector transduction [55, 122] (unpublished own observations), and there are also numerous reports of mammalian serum proteins that interact with AAV capsids and affect their properties (Fakhiri *et al*., submitted). Accordingly, it may not necessarily be desirable to comprehensively eliminate all cellular factors from AAV vector preparations during purification, in particular not those that could act as transduction enhancers. Secondly, considering the speed of many proteomics technologies, the rapid and robust detection of contaminating proteins by these methodologies could well be implemented as a routine procedure for AAV quality control.

Similar findings and conclusions were also reached by Strobel *et al*. in a 2015 study where they compared two methodologies for AAV purification by density gradient centrifugation (see also above) and used nanoscale liquid chromatography-tandem mass spectrometry (nanoLC-MS/MS) analysis to investigate the identity of 13 additional protein bands in purified AAV8 vector preparations [53]. Notably, these authors identified several proteins that overlapped with those from the Dong *et al*. study [87], including SET, nucleolin, mitochondrial single-stranded DNA-binding protein and nucleophosmin, and the latter two were independently also detected as proteins that co-purified with AAV8 by Aloor *et al*. [108],

which emphasizes their role in the AAV life cycle. Also noteworthy is that some of the contaminants in the work by Strobel and colleagues [53], such as nucleolin and nucleophosmin, were specifically detected in the CsCl-purified samples but absent from the iodixanol-purified batches, which could inform future purification protocols.

Finally, in another noteworthy example, the Samulski lab utilized mass spectrometry to identify a small contaminating protein of around 20 kDa, *i.e.*, ferritin, which they observed in AAV stocks of multiple serotypes that were purified by iodixanol density gradient chromatography followed by IEC [42]. In negatively stained transmission electron microscopy pictures, this protein formed small, circular structures of up to 10 microns, which were independently detected in an AAV5 stock in a more recent study from the Zolotukhin lab [59]. Ferritin was also among the contaminating proteins in AAV8 stocks that were investigated earlier by the Lamla lab [53]. Besides, our lab has recently observed the presence of ferritin protein and structures in various recombinant AAV and bocaviral vector preparations (Fakhiri and Grimm, manuscript in preparation). As noted above, unlike endotoxins, the presence of ferritin will most likely not interfere with AAV (or bocaviral) functionality and is thus a lesser concern. Moreover, if desired, it can be removed by tweaking the IEC conditions, as illustrated by Grieger *et al*. [42].

Conclusions

It may seem ironic that the smallest of all viruses that are being developed and evaluated as vectors for human gene therapy, AAV, has rapidly become the biggest and undisputed star that continues to enjoy the limelight. The reasons for this astounding success are manifold but are largely based on the ever-expanding portfolio of ingenious, versatile and powerful techniques for AAV vector manufacturing, ranging from methodologies for high-throughput capsid diversification and large-scale vector production, to tailored and innovative concepts for AAV particle purification, characterization and quality control for lot releases. Not surprisingly, most of the technologies for downstream AAV vector processing had originally been developed for and validated with, wild-type AAV serotypes due to their long availability and our advanced understanding of their biology. Importantly, though, their development has also paved the way for the implementation of new manufacturing technology that is compatible with the many rapidly emerging, non-natural viral variants, too. Concurrently, this process is fostered by our steadily increasing knowledge of the function and properties of

natural versus synthetic AAV capsids, which will ideally allow the adaptation of existing technology to future capsids once their structural and biophysical characteristics have been unraveled. These aspects were in the center of the present article, but we acknowledge that owing to the sheer complexity of this research field and the flurry of activities by academic and industrial entities, we could only offer a glimpse into the relevant advances, and we, unfortunately, had to omit important and informative work by other colleagues for reasons of space and clarity. Nonetheless, we at least want to mention that the strategies highlighted in the present article synergize with, and are perfectly complemented by, a wealth of other, equally important and informative approaches. Examples of such work that we could not discuss here despite its relevance for synthetic AAV capsids include the investigation of methodologies for cell harvest and lysate clarification, comprising a large variety of chemical or physical approaches such as freeze-thawing and low-speed centrifugation, which is particularly suitable for small-scale preparations and has thus been used preferably in our recent comparison of libraries of synthetic AAV capsid variants in cultured cells [123]. In contrast, other technologies such as microfluidization or lysis with detergents such as Triton X-100 are better suited for large-scale manufacturing of natural or synthetic AAV capsids, albeit the use of chemicals necessitates their subsequent monitoring in the final product as potential impurities [28, 33]. Moreover, we note the emerging use of deep sequencing techniques for analyses of the contents of AAV vector particles (plasmid backbones, cellular DNAs, fragmented genomes) [124]. This comprises non-selective but sensitive single-stranded DNA virus sequencing (SSV-Seq) technology for detection of illegitimate residual cellular or plasmid DNA as reported by the Ayuso lab [125] or Fast-Seq methodology from the Paulk lab [126]. Techniques such as the latter are becoming increasingly important as more and more AAV gene therapies including synthetic capsid-based stratagems are being tested in patients, raising a need for new and robust processes to detect, quantify and eliminate product impurities, as comprehensively reviewed by Penaud-Budloo and colleagues [33] as well as Ayuso, Mingozzi and Bosch [28]. Along the same lines, we note exciting new insights into the role of primary or secondary sequence elements in the vector genome that govern replication and/or encapsidation as well as AAV vector DNA integrity [127]. Combined with the advanced technologies that were summarized here and with further ongoing innovations in the field of AAV capsid evolution, including rational design [128] and machine learning [129], this offers plenty of reason to be optimistic about the future of AAV vector biomanufacturing and its continued contribution to the success story of human gene therapy.

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Conflict of interest

D.G. is a co-founder, shareholder and designated chief scientific officer (CSO) of AaviGen GmbH. J.E.A. declares that he has no conflict of interest.

Data Availability statement

Data sharing not applicable – no new data generated

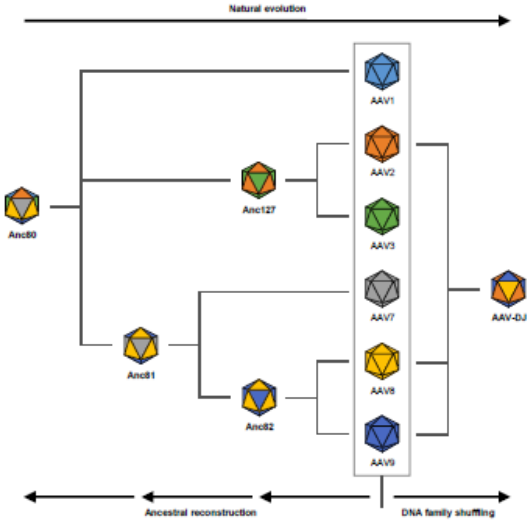

Figure 1. Evolution of synthetic AAV capsids AAV-DJ (right) and Anc (left). Shown in the box are the six AAV wild-types that form the basis for all synthetic capsids depicted in this figure and discussed in the text. AAV-DJ was derived by DNA family shuffling of AAV serotypes 2, 8 and 9, *i.e.*, through forward-directed molecular evolution. The Anc family (Anc80-82, 127) was *in silico* designed using ancestral reconstruction, *i.e.*, through backwards-directed molecular evolution starting with all shown six wild-types. Note that this is not a genuine phylogenetic tree and that evolutionary relationships have been simplified. The actual phylogeny is depicted in Figure 1 in the original work by Zinn and colleagues [20].

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Table 1. Methodologies for production, purification and characterization of synthetic AAV capsids

Step	Technology	Pros	Cons	AAV-DJ	Anc
Production	Transient plasmid DNA transfection	• easy and cheap to adapt to new AAVs • possible in each lab • high particle yields	• difficult to scale up • vast plasmid DNA amounts needed for larger vector batches	8	20
	Stable cell lines	• reproducible • scalable up to clinical manufacturing	• hard to customize • potential cellular instability over time • may require helper virus	Not yet reported	Not yet reported
	Helper viruses	• scalable (especially with baculoviruses and suspension insect cells) • high particle yields	• contaminating virus • instability of helper virus • biological AAV properties may be altered	Not yet reported	Not yet reported
Purification	Crude lysates	• quick, cheap and easy to generate • compatible with any AAV capsid variant	• various contaminants • stocks usually not titrated • unclear ratios of full:empty capsids	103	20
	CsCl density gradients	• good separation of full:empty capsids • high purity • versatile	• cumbersome • hard to scale up • open system prone to contaminations • time-consuming • toxicity	8	Not yet reported
	Iodixanol density gradients	• versatile • decent purity • enrichment of full capsids • less toxic than CsCl	• difficult to scale up • open system prone to contaminations • time-consuming	11	84
	IEC	• ability to separate full and empty capsids in large scale • easily scalable • versatile	• different physicochemical AAV properties require adaptation	80	Not yet reported

	IAC	• very specific • easily scalable • high yields and purity	• serotype-selective (lesser concern with AAVX) • antibody leaching • no removal of empty capsids	13	84
Characterization	MS	• enables detection of post-translational capsid modifications • useful for lot-to-lot control • can distinguish AAV serotypes and genomes	• requires special, expensive equipment as well as experience • often demands large sample volumes • yields no data on capsid structure	Not yet reported	Not yet reported
	Thermostability	• simple, cheap, robust and fast • can distinguish serotypes • independent of specific instrumentation	• not all serotypes can be segregated • sensitive to changes in buffer and pH	Not yet reported	20

Note that this table merely summarizes the subset of technologies that are discussed in this article and that only one representative example (numbers are literature references) is always listed for the use of these techniques in the context of AAV-DJ and/or Anc capsids. For more comprehensive overviews of these and other methodologies including additional pros and cons, we refer the reader to excellent previous reviews and original articles as referenced in the text. The numbers indicated are related to the corresponding references.

Jihad EL Andari has obtained a Ph.D. in Molecular Microbiology from the University of Freiburg (Germany). Following a postdoctoral stint at the Center of Synthetic Microbiology (SYNMIKRO) at the University of Marburg (Germany), he is now a senior postdoctoral fellow in the laboratory of Dirk Grimm at the medical faculty and BioQuant center of the University of Heidelberg (Germany). His current research focuses on AAV capsid engineering with the aim to develop novel synthetic vectors for safe and efficient therapeutic gene delivery to the human musculature and the central nervous system.

Dirk Grimm holds the Professorship for Viral Vector Technologies at the medical faculty of the University of Heidelberg (Germany). For over 25 years including a six-year postdoctoral stint at Stanford University (CA, USA), his research has been dedicated to the engineering of Adeno-associated virus (AAV) capsids and genomes towards novel human gene therapy vectors with superior efficiency, specificity and safety. To this end, his team including Dr. El Andari applies a wide variety of state-of-the-art technologies for AAV diversification, production and high-throughput *in vivo* screening in small or large animals, comprising next-generation sequencing, barcoding, single-cell RNA sequencing and machine learning.