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 are 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, scalable, easy-to-implement, and plug-and-play platform processes that are compatible with the rapidly increasing range of AAV capsid variants. Here, the focus is 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, two chimeric capsids are used as representative examples that are 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.
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 (Figure 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 4.5 Å resolution,\[18\] or, more recently, even at 2.8 Å 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 temporary 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 (Figure 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, 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, that is, 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 Griege,\[29\] or Aponte-Ubillus et al.,\[30\] 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.\[39,40\]

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,
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                                | • difficult to scale up                                               | 8      | 20    |
|            |                                                 | • possible in each lab                                                | • vast plasmid DNA amounts needed for larger vector batches          |        |       |
|            |                                                 | • high particle yields                                                | • hard to customize                                                  |        |       |
|            | Stable cell lines                               | • reproducible                                                        | • potential cellular instability over time                            | Not reported | Not reported |
|            |                                                 | • scalable up to clinical manufacturing                               | • may require helper virus                                           |        |       |
|            | Helper viruses                                  | • scalable (especially with baculoviruses and suspension insect cells)| • contaminating virus                                                | Not reported | Not reported |
|            |                                                 | • high particle yields                                                | • instability of helper virus                                         |        |       |
|            |                                                 |                                                                      | • biological AAV properties may be altered                           |        |       |
| Purification| Crude lysates                                   | • quick, cheap and easy to generate                                   | • various contaminants                                               | 103    | 20    |
|            |                                                 | • compatible with any AAV capsid variant                              | • stocks usually not titrated                                         |        |       |
|            |                                                 | • high particle yields                                                | • unclear ratios of full:empty capsids                               |        |       |
|            | CsCl (cesium chloride) density gradients        | • good separation of full:empty capsids                              | • cumbersome                                                          | 8      | Not yet reported |
|            |                                                 | • high purity                                                         | • hard to scale up                                                    |        |       |
|            |                                                 | • versatile                                                           | • open system prone to contaminations                                |        |       |
|            |                                                 |                                                                      | • time-consuming                                                      |        |       |
|            |                                                 |                                                                      | • toxicity                                                            |        |       |
|            | Iodixanol density gradients                     | • versatile                                                           | • difficult to scale up                                               | 11     | 84    |
|            |                                                 | • decent purity                                                       | • open system prone to contaminations                                |        |       |
|            |                                                 | • enrichment of full capsids                                          | • time-consuming                                                      |        |       |
|            | IEC (ion-exchange chromatography)               | • ability to separate full and empty capsids in large scale           | • different physicochemical AAV properties require adaptation         | 80     | Not yet reported |
|            |                                                 | • easily scalable                                                     |                                                                      |        |       |
|            |                                                 | • versatile                                                           |                                                                      |        |       |
|            | IAC (immunoaffinity chromatography)             | • very specific                                                       | • serotype-selective (lesser concern with AAVX)                      | 13     | 84    |
|            |                                                 | • easily scalable                                                     | • antibody leaching                                                   |        |       |
|            |                                                 | • high yields and purity                                              | • no removal of empty capsids                                        |        |       |
| Characterization | MS                                         | • enables detection of post-translational capsid modifications       | • requires special, expensive equipment as well as experience         | Not reported | Not reported |
|            |                                                 | • useful for lot-to-lot control                                       | • often demands large sample volumes                                 |        |       |
|            |                                                 | • can distinguish AAV serotypes and genomes                           | • yields no data on capsid structure                                 |        |       |
|            | Thermostability                                 | • simple, cheap, robust and fast                                     | • not all serotypes can be segregated                                 | Not yet reported | 20    |
|            |                                                 | • can distinguish serotypes                                           | • sensitive to changes in buffer and pH                              |        |       |

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.

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 up-scaling of the transfection and culturing conditions into WAVE bioreactors of up to 20 liters, frequently resulting in the production of around 1×10^8 vector particles per cell and a total of greater than 1×10^16 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 or those using herpes simplex viruses or baculoviruses.

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 iCellLis Nano bioreactors and who obtained substantial AAV2 yields that approximated those achieved in more conventional cell factories. Finally, we highlight comprehensive recent work by the Mingozzi lab in which Collaud et al. harnessed a 10–200 L suspension cell-based production system to scale up AAV8 vector manufacturing in which—in immunoaffinity chromatography (IAC)—harnesses specific AAV substrates that mimic cellular receptors, or 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.

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. 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. Although this

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. Here, we will briefly recapitulate two of the oldest and most conventional technologies for AAV purification, that is, by cesium chloride (CsCl) or iodixanol density gradient centrifugation. 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 but a major benefit is a significantly lower proportion of remaining empty particles (<2%) in contrast to iodixanol (~20%). Vice versa, CsCl is more toxic than iodixanol and can induce adverse effects in animals, thus raising a need for diafiltration with physiological buffers before use 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, although the safety in patients with compromised kidney functions is unclear. 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 diafiltration/concentration with Amicon Ultra-15, 100 kDa MWCO centrifugation tubes, size-exclusion chromatography with desalting columns, hollow-fiber tangential flow ultrafiltration, or polyethylene glycol (PEG)-8000 precipitation and concentration. Based on data by Strobel et al., ultrafiltration may be most efficient and least time-consuming, and it also yielded highest particle recovery and was most effective at removing iodixanol traces. 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, Strobel et al. found slightly higher potency of AAV vectors that were purified with CsCl. In general, multiple factors may govern transduction efficiency comprising cellular impurities such as ferritin, prolonged exposure to CsCl that can reduce infectivity, the proportion of full and empty particles, repetitive freezing and thawing of vector stocks or precipitation methods which all require further characterization and standardization.
method is, in principle, also compatible with AAV6 or AAV1 (after a single amino acid change). It is mainly used to purify the AAV2 serotype. 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. Similarly, AAV2 affinity chromatography purification based on the monoclonal antibody A20 has been reported, 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. 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. 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 or into the synthetic AAV-DJ or AAV-DJ/8 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-SPAKFA 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.

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. First, 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. Second, capsids lacking this epitope may be engineered and transformed into AVB binders upon grafting of the epitope, as demonstrated by Wang et al. or Nass et al. 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). Another general drawback of affinity chromatography over IEC is its inability to separate full and empty capsids, which is not surpris-
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. 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 automation and its ease-of-use.

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. Proof-of-concept comprised its application for purification of the AAV variant AAVM41, that is, a myocardium-tropic, synthetic AAV created by DNA family shuffling of serotypes 1, 6, 7, and 8. 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, that is, the forced recombination of capsid DNA fragments from multiple parental AAV isolates (here, AAV2, 8, and 9). 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, that is, the family of synthetic capsids created through ancestral reconstruction including Anc80L65.

In the initial report of AAV-DJ, CsCl density gradient centrifugation was used for purification, followed by later studies that used the same protocol or iodixanol density gradient centrifugation. 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. 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., 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.

In another study, 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. 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, 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.) and iodixanol density gradient centrifugation. To this end, vector particles were produced using a likewise optimized triple-transfusion 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., 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, 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 L 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 AAVs 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, 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 behavior. 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 3 h protocol (https://www.cellbiolabs.com/aav-purification-standard-kit). The same kit was also used by Yoo et al. 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

Over the last 1.5 decades, mass spectrometry (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, for example, a 2018 report by the Xiao group who used MALDI-time-of-flight (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 deamination level, for reasons unknown.[109] As shown in the same study, the phenomenon of deamination is not unique to AAV8 but was also observed for seven other isolates, that is, 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 “deamination clock”, according to which progressing deamination correlates with losses in vector activity, thus providing an advantage to freshly translated virions for transduction.[109]

Hence, MS-based analysis of capsid deamination 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 deamination 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, that is, 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. 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
5.2. Capsid Thermostability

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 have limited 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. and then extensively and independently validated in two publications by the groups of Luk Vandenberghe, Eduard Ayuso, and Mavis Agbandje-McKenna in 2017. The hallmark of this powerful biochemical technique, called differential scanning fluorimetry (DSF) or AAV-ID, 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 unambiguously identify divergent serotypes, as exemplified using AAV1, 2, 5, and 8. 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 ($\approx 70 ^\circ$C) and the highest for AAV5 ($\approx 90 ^\circ$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. 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. In line with the data from Rayaprolu et al., $T_m$ 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., 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., 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 $5 \times 10^5$ purified AAV5 (the most stable serotype) particles in 25 µL. Extending the previous studies, 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., 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 AAV6, 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. Specifically, Zinn et al. determined the $T_m$ of their lead AAV candidate, Anc80L65, to be $\approx 92 ^\circ$C and thus $15–30 ^\circ$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 (Figure 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 also to 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 $2 \times 10^{11}$ 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, that is, 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 or 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] mL$^{-1}$, which complies well with the FDA-recommended upper limit of 5 EU kg$^{-1}$ 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[83] 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 <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. First, 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 (Pakhiri 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.[153] 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, that is, 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.[159] 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]

6. 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.[122] 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.[28,53] 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 Paulik 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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