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Chromatographic strategies for the analytical characterization of adeno-associated virus vector-based gene therapy products

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ABSTRACT

In recent years, the biopharmaceutical industry's interest in gene therapy modalities has increased dramatically. To warrant their quality during manufacturing and upstream/downstream process, fit-for-purpose analytical methods play a crucial role in the overall control strategy. However, characterization of gene therapy products remains challenging due to their large size, structural complexity, heterogeneity, potential instability, and limited sample availability. In addressing some of these challenges with innovative approaches, liquid chromatography (LC) based methods have become an integral part of the currently used analytical toolbox. This review focuses on both established methods and emerging trends in the LC analysis of adeno-associated virus (AVV) vector-based gene therapy products. Each method is discussed to highlight their advantages, drawbacks, and unique capabilities in the analysis of AAV gene transfer vehicles and their corresponding impurities. Taken together, this review provides guidance on the selection of LC-based methods for routine testing and extended characterization of gene therapy products.

1. Introduction

Gene therapy has revolutionized the treatment of previously incurable genetic diseases [1]. As of December 2022, 11 commercial gene therapy drug products have been approved by drug regulatory authorities (see Fig. 1) [2]. In addition, more than 3500 gene therapy clinical trials are currently underway, promising life-altering and one-time treatment options for patients [1,3]. That said, the costs for these therapies can be exorbitant (up to several million USD), which has fueled discussions on equitable access and their affordability [4]. One reason for the high costs are the limited productivity and scalability of current viral-vector manufacturing processes and that a significant portion of this low production yield is required for activity and analytical testing. In this regard, it is hoped that more sensitive, more robust and higher throughput analytical methods might help to reduce the overall costs for gene therapy production.

According to the Food and Drug Administration (FDA), human gene therapies seeks to modify or manipulate the expression of a gene or to alter the biological properties of living cells for therapeutic use. This can be done by replacing, inactivating or transferring genes into cells that takes place either inside the body (*in vivo*) or outside the body before returning them to the patient (*ex vivo*) [5]. Some of these *ex vivo* therapies, such as chimeric antigen receptor T-cells (CAR T) [6], are considered to be at the crossroads between gene therapy and cell therapy [7]. This article focuses on therapies that involve *in vivo* or *ex vivo* genetic modifications and, more specifically, those that require a viral vector to enable the expression of a therapeutic gene (Fig. 1). To date, a variety of viral vectors including adenovirus, adeno-associated virus (AAV), lentivirus and herpes simplex virus, have demonstrated their potential to deliver gene therapies safely and efficiently [8]. These vectors differ mainly in terms of their immune response,

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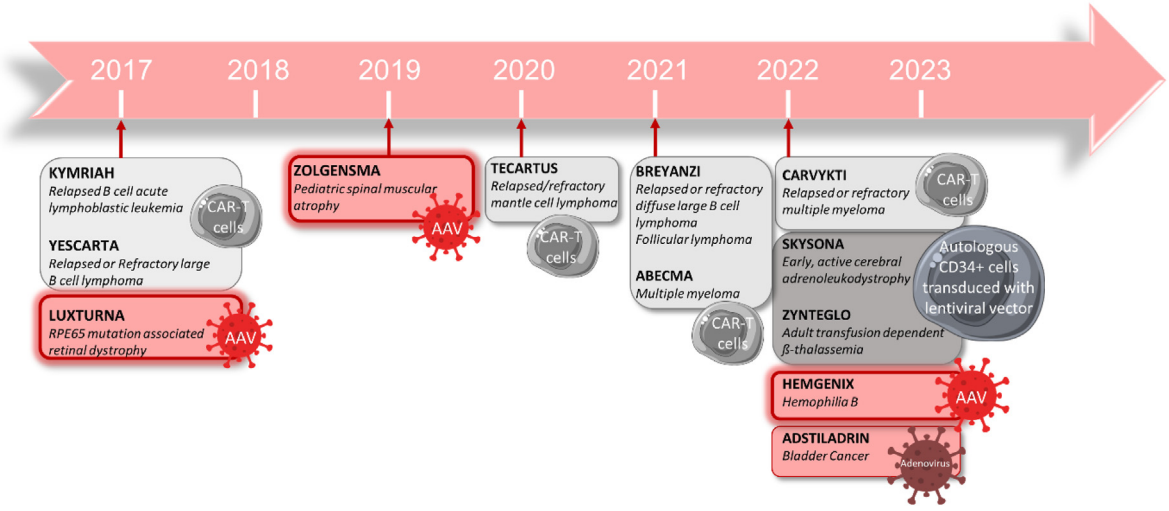

Fig. 1. Timeline of the FDA-approved gene therapies with *in vivo* and *ex vivo* strategies (CAR-T cells) reported in red and grey, respectively.

onset and duration of gene expression, genome integration or extrachromosomal delivery, as well as packaging capacity. Currently, four *in vivo* gene therapy products are approved by the FDA (Fig. 1), targeting various types of genetic diseases [9]. The potential of gene therapy also extends to other diseases that are not strictly genetic, such as HIV or diabetes [10,11].

To be effective, the transgene (genetic payload) must be properly delivered to the target cells [12]. AAVs have many advantages as vectors which has led to their widespread use as a delivery vehicle [13]. Each of the twelve wild-type serotypes has a specific tropism for certain tissues [14–16]. AAVs also have a lower immunogenicity than other viral vectors and allow long-term gene expression [17,18]. Finally, the biological structure of AAVs is relatively simple, meaning that they are comprised of a non-enveloped capsid and a relatively simple protein composition. The capsid consists of approximately 60 copies of three viral proteins called VP1, VP2, VP3 (in a 1:1:10 M ratio, respectively), which are assembled into an icosahedral structure that is 26 nm in diameter. The three capsids are N-terminal splice variants that share a common C-terminus. Their native genome consists of a single-stranded DNA of approximately 4.8 kb in size, containing three genes flanked by two inverted terminal repeats (ITRs). The first gene is the *rep* gene, which encodes four proteins (Rep78, Rep68, Rep52 and Rep40). The second gene is the *cap* gene which encodes the three viral capsid proteins (VPs) (VP1, VP2, and VP3). The third gene encodes for the assembler activation protein (AAP) and is located within the *cap* coding region. This simple structure makes it easy to produce a recombinant AAV (rAAV) in which the viral DNA is replaced by the desired transgene [19].

As more AAV clinical applications are emerging, there is a need of understanding the impact of quality attributes on product safety and efficacy. There are many critical quality attributes (CQAs), such as aggregation, full/empty capsid ratio, viral protein ratio and other process-related impurities that need to be monitor to ensure consistent AAV product quality [20]. Various analytical techniques are currently used to characterize this modality, including transmission electron microscopy (TEM) [21], quantitative polymerase chain reaction (qPCR) [22], enzyme-linked immunosorbent assay (ELISA) [23], UV absorbance spectrophotometry, analytical ultracentrifugation (AUC), charge detection mass spectrometry (CDMS) [20,24], and capillary electrophoresis (CE) [25].

This review focuses on chromatographic methods, including ion exchange chromatography (IEX), size exclusion chromatography (SEC), reversed phase liquid chromatography (RPLC), and hydrophilic interaction chromatography (HILIC), as they have increasingly been applied for the characterization of gene therapy products [26]. The article also describes some of the pros and cons of these methods and discusses their suitability for covering various quality attributes of importance [27].

2. Analysis of gene therapy products at the intact level

Detailed characterization of AAV vector particles at the intact level is crucial to ensure the safety and efficacy of gene therapy products. The precarious nature of AAVs needs to be taken into account during analysis; for example, due to its relatively high potential for aggregation and adsorption to surfaces, an AAV drug product is normally formulated in the presence of a non-ionic surfactant (*e.g.* poloxamer 188), and it must be kept at low temperature to increase its stability [28]. The addition of cryoprotectants (*e.g.* glycerol) helps to preserve infectivity upon freeze/thaw cycles [29], suggesting that future formulations may become even more complex [30]. Despite their relative instability, adequate safety measures also need to be considered when the capsid's DNA cargo codes for a tumorigenic/toxic gene product, or when the vector particles are produced with a helper virus (infection potential) [31]. Many AAV samples are now classified as low risk (RG1) and can therefore be safely manipulated in standard biosafety level 1 (BSL-1) laboratories.

Obviously, the functional potency assay is still the most critical assay to address these points, but as it is a rather complex multimodal assay, the chromatographic methods can also be used to answer questions related to the quality of AAVs, such as the number of 'defective particles' [32] and their level of aggregation [33] under non-denaturing chromatographic conditions [34]. Denaturing LC techniques, especially in combination with mass spectrometry (see section 3) furthermore serve the purpose to elucidate important compositional information, such as VP protein phosphorylation and deamidation. Last but not least, fluorescence-based detection can be used to enhance sensitivity and thus facilitate work on low titer samples [35,36].

2.1. Ion exchange chromatography

Biotechnological production of AAVs results in viral particles that contain either the full genome (full), no genome (empty),

fragments of nucleic acid (partial) or overloaded/overfilled capsids [37]. Note that full particles are also referred to as "heavy" capsids, while empty or partial particles can be referred to as "light" capsids [38]. Empty particles that lack the gene of interest (GOI) are a major side-product of recombinant AAV production. As such, they can potentially lead to an unwanted immune response due to the increased total capsid load and reduce the overall transduction efficiency [39,40]. However, other data have shown that empty AAV capsids may enhance gene transfer by evading the preexisting humoral immunity [41]. Whether beneficial or detrimental, the full/empty capsid ratio (F/E ratio) needs to be monitored as an important CQA of AAV products.

Among the available analytical strategies, anion exchange (AEX) chromatography is a reference technique to assess AAVs with respect to their F/E ratio. The AEX separation of full and empty capsids is driven by differences in their relative surface charges [24]. The calculated net isoelectric point (p*I*) for empty AAV is quite similar for different serotypes (p*I* ~ 6.3), but each serotype may behave differently in AEX. This may be due to overall differences in the amino acid composition, resulting in varying capsid surface charge profiles and differing structural characteristics. Nonetheless, full and empty capsids show slight differences in their surface charge (p*I* shift of ~0.4 units) depending on the presence or absence of negatively charged encapsidated genomic DNA [42]. Owing to the charge difference between empty and full AAV capsids, different capsid subpopulations can be sequentially eluted from an AEX column. In AEX separations, the elution order is empty-, partial- and full capsids.

The most commonly used analytical scale packed bed AEX columns are based on polystyrene-divinylbenzene (PS-DVB) beads with large pores ranging from 500 to 10000 Å, or on non-porous polymethacrylate particles. Monolithic anion exchangers are also available and they are based on polymethacrylate substrate containing interconnected, convective channels with a diameter of 1.3 μm [43]. Strong anion-exchangers (quaternary amines) are preferred in most AAV applications, due to the presence of negatively charged DNA payload. Regarding column dimensions, both very short disk-like formats (*i.e.* 4.95 mm) and conventional 50–100 mm long columns are used.

The mobile phase is often buffered with 20–250 mM, 1,3-bis(tris(hydroxymethyl)-methylamino)propane (bis-tris propane or BTP) or with tris(hydroxymethyl)aminomethane (tris) at pH = 7.5–9.

The on-column stability of AAV capsids may be improved by the addition of divalent cations to the mobile phase; it has been shown that 1–2 mM $MgCl_2$ was indeed helpful. In addition to improve sample stability, the presence of $MgCl_2$ slightly shifts the retention times and changes the resolution between empty and full AAV particles [43]. Using higher concentrations of $MgCl_2$ does not provide any further improvement. Other agents, such as glycerol and Poloxamer (which are also commonly used), showed less stabilizing effects.

Most AEX AAV separations are performed in salt gradient mode. Salts potentially modulate non-covalent interactions between capsids and interactions between the AAV and the stationary phase. In this regard, Farrah and co-workers observed increased hydrophobic adsorption when anti-chaotropic salts were used [44]. Another study reported improved separation of empty and full AAV capsids by using anti-chaotropes, such as ammonium and tetramethyl ammonium salts, as compared to NaCl [45,46]. Wang et al. evaluated different quaternary alkyl ammonium salts including methyl-, ethyl-, propyl-, and butyl ammonium chloride [43]. The separations were better with quaternary ammonium salts than with NaCl, and longer alkyl chains resulted in greater separation. Method repeatability and robustness of the measurements were also improved with ammonium salts versus NaCl. These results are consistent with the hypothesis that anti-chaotropes enhance hydrophobic interactions between viral capsid proteins, thus increasing the stability of AAV samples.Other anti-chaotropes, including ammonium sulfate and sodium citrate, were also tested, but the separation was inferior to that obtained with quaternary alkyl ammonium salts. When comparing selectivity, peak shapes, the background absorbance of the salts and baseline drifts, tetramethyl ammonium chloride (TMAC) appears to be a good choice [43]. Yang et al. compared NaCl, KCl, ammonium acetate, sodium acetate and TMAC and concluded that TMAC resulted in the best separation, though its moderate acute toxicity can be of potential concern [46] Pranav and co-workers found that trivalent sodium phosphate yielded higher resolution between empty and full capsids than divalent salts (*i.e.* sodium sulfate) [47]. Salt gradients are typically performed with up to 200–350 mM salt concentration, depending on the type of the salt.

Besides salt gradient separation, it is possible to achieve elution with a pH gradient. Wagner and co-workers applied a linear gradient of pH 7 to 10 on WAX and SAX columns, using a mobile phase buffer consisting of 20 mM Tris and 10 mM $MgCl_2$ [48].

The type of buffer species and salts are often selected on the basis of preliminary screening runs [43]. Once the most appropriate combination of buffer type, concentration, mobile phase pH and salt has been found, the gradient program needs to be optimized. Linear salt gradients are often used. However, various multi segmented gradients are also applied as they inherently provide higher selectivity for large solutes. These gradients are often referred to as "modular discontinuous gradient", "step gradient", "two-step gradient" or "multi-isocratic elution mode" methods [49,50]. Fig. 2 shows an example of a step gradient separation. Dickerson et al. recently showed that isocratic elution gradients can also work for the separation of empty and full capsids of AAV2 particles [51]. Hejmowski et al. illustrated an interesting approach based on a step gradient with small conductivity increases of about 1 mS/cm which provided a high separation efficiency for empty and full AAV5 vectors [37].

In terms of method development, it has been shown that to some extent it is possible to develop generic (platform) methods that can work for various AAV serotypes [46,52]. However, further optimization may be required for different serotypes to achieve baseline peak separation while applying the same core principles for method development (mobile phase selection, column selection, and application of multi segmented gradients).

Dual wavelength UV detection is most commonly used for AEX separations of AAVs. Very often 260 nm (specific for DNA) is set and combined with 280 or 230 nm detection (specific for capsid proteins) [50]. The ratio of the two signals is used to determine response factors to quantify empty and full particles. Fluorescence detection alone or in combination with UV is also commonly employed and provides much higher sensitivity than UV. Usually, 280 nm is used for excitation and 336, 340, 350 or 360 nm as the emission monitoring wavelength (depending on the background signal of the mobile phase) [43,46,47,51].

Wagner et al. coupled AEX to multi-angle light scattering (MALS) detection and developed a rapid and simple method for assessing multiple CQAs in a single measurement [48]. This novel and robust AEX-MALS method allowed comprehensive characterization of empty and filled AAVs with regards to the capsid titer, F/E ratio, absolute molar mass of the protein and nucleic acid, and the size and polydispersity of the particles. The method was applicable to different serotypes and can be used as an orthogonal method to other established analytical techniques.

Some limitations include the fact that AEX is not fully suitable for measuring partially filled or overfilled capsids. For this purpose,

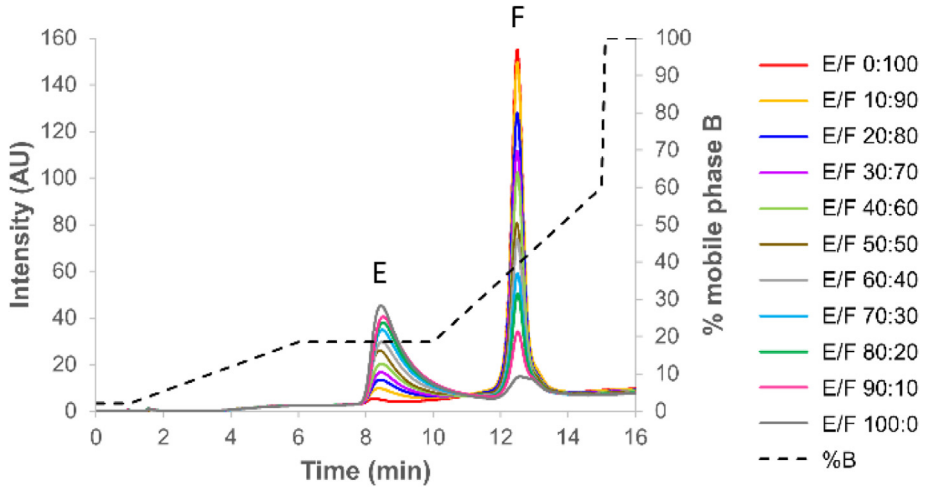

Fig. 2. A multi-segmented gradient AEX separation of empty (E) and full capsid (F) peaks (With permission from Ref. [49]).

analytical ultra centrifugation (AUC) remains the method of choice.

As for future developments and perspectives, to the best of our knowledge, pH-mediated salt gradients have not yet been tested for AAV AEX separations. Such an elution mode could lead to improved separations. In addition, investigating the possibilities of volatile buffers and coupling AEX to mass spectrometry are also areas of potential interest.

2.2. Size exclusion chromatography

SEC is uniquely positioned for the analysis of intact AAVs as it can sort the sample components by their size in solution, effectively revealing the fraction of the desired monomeric AAV capsids, typically around 23–28 nm in diameter [34], versus larger aggregates and smaller impurities. This can be used for robust quantification of the capsid titer in a rapid analysis using short guard columns [53] (even directly in cell culture media when used in conjunction with AEX) or for more accurate determination of the dimer and higher-order multimer content [33], via the use of longer columns. SEC columns packed with particles having average pore diameters between 400 and 1000 Å have been successfully applied to these sorts of analyses. Such columns are well suited for this type of analysis as monomeric AAV particles fall in the middle of the fractionation range ($K_D = 0.5$). Typically, columns of rather large dimensions (7.8 × 300 mm) containing relatively large particles (≥3 μm; often ≥5 μm) are applied with relatively low flow rates (≤1 mL/min) out of concern for potential sieving and frictional effects. A detailed study has not yet been published to understand the boundaries of these potential effects. Nevertheless, it is believed that heavily aggregated AAV material may not be accurately analyzed by SEC due to filtration effects, de-aggregation, and associated shear forces, in which case asymmetric flow field fractionation (A4F liquid phase separations) has been employed [54,55]. On the other hand, theoretical calculations suggest that connecting SEC columns of different pore sizes in series, although practically difficult to implement, may provide increased resolution of differently sized sub-populations of both aggregates and fragments [56]. Typically, cross-validation of SEC results with orthogonal techniques (such as light scattering and AUC) is required to demonstrate its suitability for detecting various types of aggregates.

Because they possess essentially the same diameter, SEC cannot resolve empty from full capsids. As such, the application of dual-wavelength UV detection allows for the estimation of this important CQA as illustrated in Fig. 3, and this type of content ratio determination method has recently been improved by the use of a less-conventional 260/230 nm wavelength ratio, which extends the linearity and reproducibility of the assay [57]. Notably, results from such an approach have been found to correlate well with those measured by other techniques such as AUC or cryo-electron microscopy, while being more accurate and faster to obtain [58,59].

Coupling SEC to additional detectors offers the opportunity to characterize additional physical properties of AAV capsids, as reported in Fig. 3. In particular, the application of MALS enables absolute estimation of aggregates characteristics (size, dispersity, particle counts, and molecular weights) with an improved sensitivity (signal is proportional to molar mass) [38]. Importantly, by measuring additional optical properties (differential refractive index, light scattering, UV absorption), it is possible to perform compositional analysis and orthogonally determine the F/E ratio without the need for prior calibration [60]. To obtain reliable results using this method, full recovery of the injected particles from the SEC column is required – to maximize the validity of this assumption, it is recommended to use hydrophilic packing materials [61] that are packed into low adsorption column hardware (*e.g.* h-HST or SRT types which are already available from certain SEC column manufacturers) [62–64]. Avoiding non-specific interactions is particularly important for an unbiased analysis of the less stable, but commonly used AAV2 serotype [65]. Alternatively, potential secondary interactions can be limited by an appropriate choice of the mobile phase – of a sufficient ionic strength (typically 2X PBS) to suppress unwanted electrostatic interactions, and in some cases with an organic solvent (*e.g.* 10% EtOH) [38] or non-ionic surfactant additive (*e.g.* 0.005% poloxamer 188) [66].

One of the challenging limitations of SEC is its inability to distinguish partially filled capsids, which usually requires the use of non-chromatographic methods [67]. The other disadvantage, the need for a relatively high sample concentration, can be circumvented by using smaller volume columns (lower sample dilution) with more sensitive detectors, such as a fluorescence detector. Further research into method development using new types of columns is desirable. However, with the improved availability of inert columns that are coupled to multifunctional detectors and used in an increasing number of validated methods, SEC is on its way to become a leading standardized platform for viral vector characterization, driven by the fact that it offers measurements of multiple CQAs and thus reduces the analytical workload.

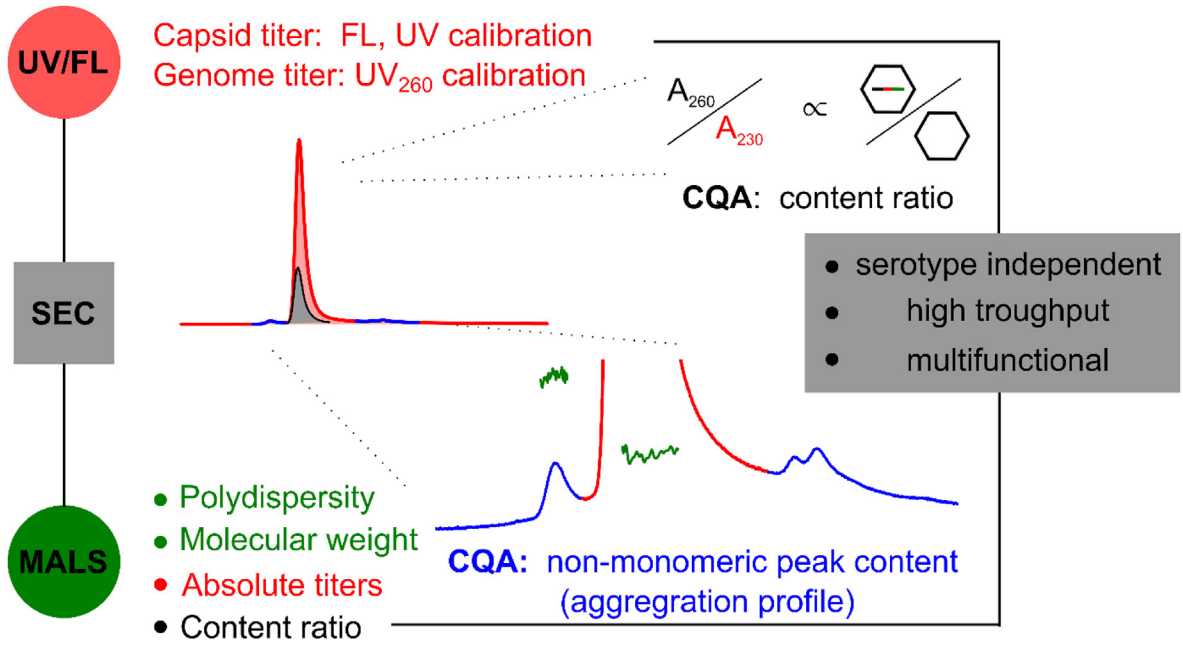

Fig. 3. Type of CQA information that can be obtained for AAVs with SEC *via* specific measurements based on the detector employed. Capsid titers and aggregation profile (blue trace) can be directly obtained *via* optical detectors (Fluorescence or UV), but MALS detector is more sensitive for large oligomers. Similarly, full/empty content ratio can be determined *via* determination of UV absorption ratio at capsid characteristic (e.g. 230 nm, red trace) and genome specific wavelength (260 nm, black trace), or based on molecular weight measurements (green trace) with MALS. The latter detector also offers capability for further biophysical characterization (e.g. polydispersity, absolute capsid titers).

3. Analysis of gene therapy products at the viral proteins level

The AAV capsid consists of approximately sixty copies of three viral proteins (VPs) which are arranged in an icosahedral shape with a molar ratio of 1:1:10 (VP1:VP2:VP3). Transcribed by the *cap* gene *via* alternative splicing, the three VPs share a very high degree of sequence similarity, with the VP3 (~60 kDa) sequence completely integrated into the VP2 (~65 kDa), and the entire VP2 sequence embedded in VP1 (~80 kDa). In addition, VPs from different AAV serotypes typically share a high degree of sequence homology (51–99%) although changes in the primary sequence confer specific binding affinity for different host cell receptors to the AAV capsids. In addition, VP variants (containing *e.g.* deamidation, oxidation, isomerization, acetylation, phosphorylation, glycosylation, SUMOylation, and ubiquitination) have been reported as a result of degradation processes or post-translational modifications (PTMs), not including engineered VPs containing specific point mutations [68,69]. Considering the complex mechanism of AAV capsid assembly, the VP1-VP3 ratio is a key parameter for monitoring capsid integrity. Changes in this ratio among different batches may indicate process inconsistency. In addition, the characterization of VP variants modified by degradation or PTMs is necessary as they may affect the efficacy of AAV products, for example by reducing the transduction efficiency. Capillary electrophoresis (CE)-based methods are routinely used to obtain baseline separation of the VPs, as it is necessary to determine their stoichiometry. However, orthogonal LC-MS methods are also required, especially for obtaining information at the amino acid sequence level to determine the primary sequence for serotype confirmation and to perform characterization of PTMs. In this context, RPLC is the most commonly used technique, although HILIC is a very promising option too. For an adequate release of intact VPs, a simple denaturation protocol can be used, for example by incubating intact viral capsids with 10% acetic acid for 15 min at room temperature. Canonical enzymatic digestion procedures can be used to prepare samples for peptide mapping, although further optimizations are desired in the face of limited sample availability [68–74].

3.1. Reversed phase liquid chromatography

In depth characterization of VPs is essential to meet health authority expectations on the quality aspects of gene therapy medicinal products [75]. RPLC is a robust analytical method with the potential to support this task as it allows confirming AAV serotype and capsid identity by analyzing VPs at both intact protein and bottom-up level. Direct RPLC-MS analysis for the comprehensive characterization of VPs derived from different rAAVs was first reported by Jin et al. [76] The method development involved the analysis of AAV2 capsid VPs at the intact protein level using a bridged ethyl hybrid (BEH) C8 column (2.1 mm × 100 mm) and 0.1% formic acid (FA) as a mobile phase additive. Although the VPs were not well separated (with VP1 and VP2 coeluting in the same peak), the direct hyphenation to MS allowed the identification of all 3 VPs as well as some additional variants with modifications: acetylation (+42 Da mass shift) of both VP1 and VP3. In addition, full sequence confirmation of the AAV2 VPs was achieved by LC-MS/MS analysis using peptide mapping. The authors were able to apply the method to confirm the sequence of both the N-terminal and C-terminal fragments of six different AAV serotypes, thus monitoring the capsid protein heterogeneity by LC-MS. As an extension of this work, an RPLC-FLD-MS method was developed with the aim of achieving chromatographic resolution of at least the 3 major VPs forms [73]. A systematic comparison of the separation of AAV8 capsid proteins using different mobile phase additives (FA or difluoroacetic acid (DFA)) and stationary phases (C4, C8, C18 with different pore sizes) led to the selection of a BEH™ C4 Column with 300 Å pore size and DFA as a mobile phase modifier as optimal chromatographic conditions. As shown in Fig. 4a, this RPLC-FD-MS workflow was applied to the analysis of six different rAAV serotypes and allowed the separation of the three VPs in most of the

cases. The improved chromatographic separation facilitated mass analysis of the different VP variants. As shown in Fig. 4b for the deconvoluted MS spectra of AAV8 VPs, the accurate mass measurements allowed the identification of VP1 and VP3 as N-terminal methionine truncated and acetylated variants; VP1 and VP2 as additional phosphorylated proteoforms (+80 Da mass shift); and VP3 as an additional resolved fragment corresponding to the truncation of the VP3 sequence in the C-terminal region (hydrolysis of the acid-labile D_{659}-P_{660} bond). With the exception of phosphorylation, which was identified at intact protein level, additional PTMs (deamidation, oxidation, and methylation) were identified (and quantified) exclusively *via* bottom-up level of analysis (peptide mapping). It should be noted that Oyama et al. questioned the validity of evaluating the stoichiometry of VPs using only an LC-MS approach [74]. The authors applied a RPLC-UV-MS workflow to the analysis of VPs from 3 different serotypes and compared the results obtained by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and capillary gel electrophoresis (CGE). Interestingly, VP ratios derived from the LC-MS analysis differed from those obtained by SDS-PAGE and CGE. Indeed, the harsh LC-MS conditions (low pH + high temperature) induced truncation at the C-terminus of VP3. The authors therefore suggested to combine LC-MS identification of viral proteins together with the peak assignment obtained in CGE for an accurate and quantitative evaluation of the VP stoichiometry [74]. With these insights, it is clear that VP protein RPLC methods can be further optimized. With the fact that VP proteins are frequently phosphorylated, it would seem prudent for research to be performed on metal versus low adsorption column hardware (such as h-HST or PEEK lined). Secondly, further efforts should be made to optimize milder elution conditions and/or quicken run times to minimize on-column hydrolysis [77].

3.2. Hydrophilic interaction chromatography

With a complementary retention mechanism to RPLC by discriminating analytes based on their hydrophilicity, HILIC has been reported as an alternative technique for separating VPs proteoforms. The most relevant contribution in this context, comes from Liu et al., who demonstrated that HILIC can improve the separation of VPs from a variety of AAV serotypes using a generic method prior to Fluorescence (FD) and MS detection [78]. As shown in Fig. 5, the separation achieved by the reported HILIC method outperformed the RPLC-based approach. More specifically, capsid proteins of three different AAV serotypes – AAV6, AAV7, and AAV8 – were separated by HILIC using a wide-pore BEH Amide stationary phase and by RPLC using a wide-pore BEH C4 Column. Both columns had the same dimensions and were packed with the same types of particles (300 Å, 1.7 μm, 2.1 mm × 150 mm), only the chemistry was different. Separations were performed with the same ion-pairing reagent (0.1% TFA v/v) and change in B per time unit (%B/min). In addition, to mitigate ion-suppression during mass analysis, the MS desolvation gas was modified with propionic acid and isopropanol. Although the RPLC method was not sufficiently optimized to separate VP1 and VP2, the comparison clearly showed that HILIC offered a better separation of the VP variants. In fact, this is currently the only published method being capable of separating both oxidized and phosphorylated proteoforms. All these variants exhibited higher retention times compared to their unmodified counterpart due to their increased hydrophilicity. In addition, their chromatographic separation promoted MS analysis of minor species such as the oxidized forms which might otherwise go unnoticed in an intact level RPLC analysis. The developed method was further applied for batch-to-batch comparison, indicating different levels of oxidation and phosphorylation between two batches of AAV8. The results were then confirmed by peptide mapping

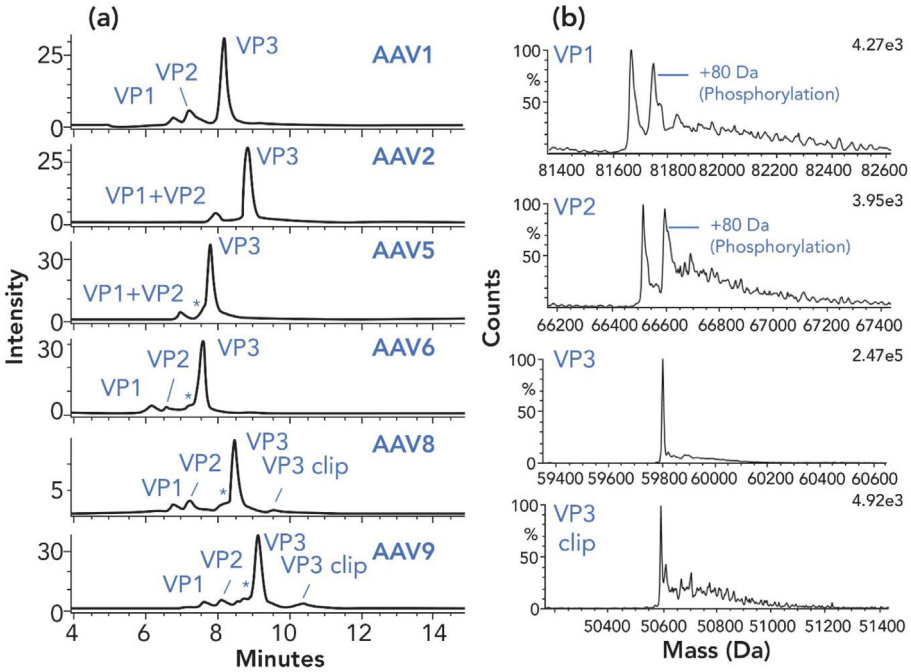

Fig. 4. The separation of six AAV serotypes (a) with fluorescence detection and (b) deconvoluted MS spectra of VP1, VP2, VP3, and VP3 clip in AAV8 are shown. Peaks annotated with * are observed variants with the same mass (isomer) of VP3. Separation conditions included an ACQUITY™ BEH C4, 2.1 × 100 mm, 1.7 μm, 300 Å column. The mobile phases were 0.1% DFA in water (A) and 0.1% DFA in acetonitrile (B) and the separation was performed with a gradient from 32% to 36% B in 16 min at a flow rate of 0.2 mL/min. The column temperature was 80 °C and the injection volumes were 1.0 μL for fluorescence detection and 10 μL for MS detection (With permission from Ref. [73]).

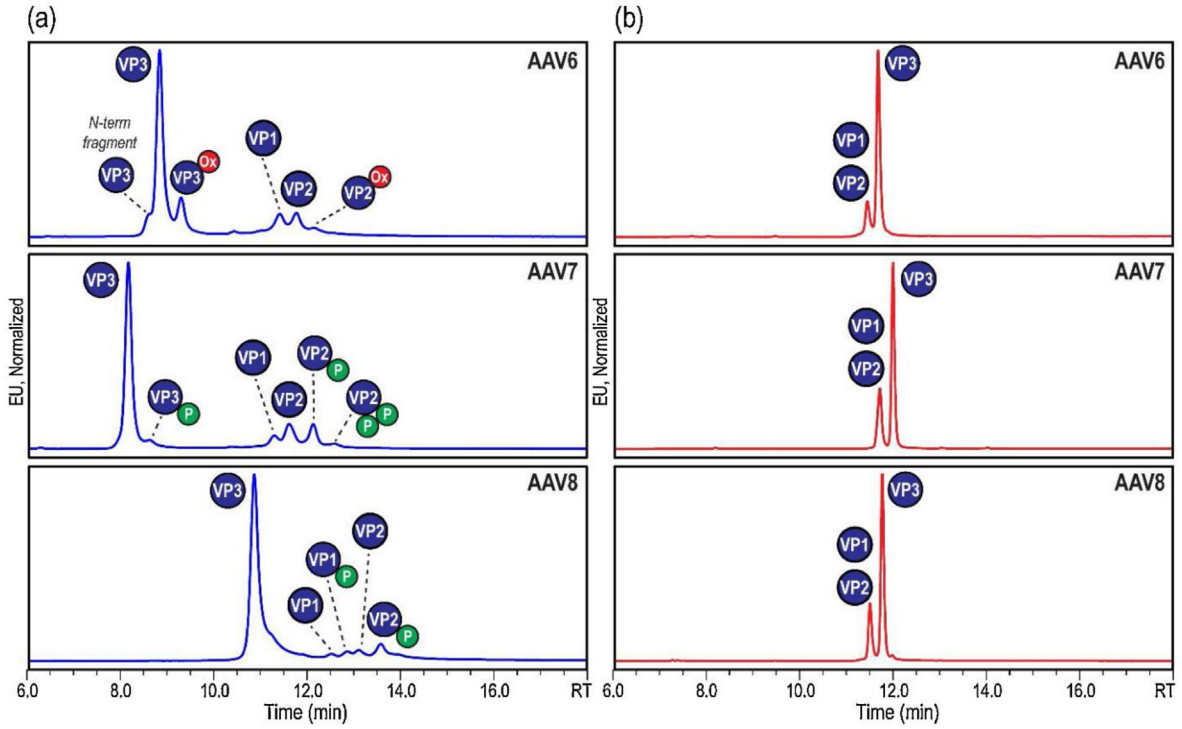

Fig. 5. Comparison of LC separation profiles of AAV6, AAV7 and AAV8 capsid viral proteins (VPs) using: (a) Waters ACQUITY UPLC™ Glycoprotein Amide column (300 Å, 1.7 μm, 2.1 mm × 150 mm) and (b) Waters ACQUITY BEH C4 Column (300 Å, 1.7 μm, 2.1 mm × 150 mm). Fluorescence intensity (EU) was monitored using λem = 280 nm and λex = 348 nm and normalized across all samples. The FD peak identities were confirmed by accurate mass measurement and annotated as follows: Ox: oxidized; P: phosphorylated (With permission from Ref. [78]).

analysis, corroborating the potential of HILIC in supporting process development of rAAVs.

4. Perspectives

In addition to the widely implemented chromatographic modes described in the previous sections, there are several more advanced analytical strategies that can be used to further improve the analytical characterisation of gene therapy products. The most relevant are outlined in this section.

First of all, it is important to note that the limited availability of samples requires the adaptation of existing analytical methods developed for other biopharmaceutical products. Indeed, due to the difficulties in large-scale production and purification of AAVs, resulting in low sample yields, the analyst often has only micrograms of rAAVs to cover a range of required assays. In this context, miniaturization of LC (microLC, capLC and nanoLC) is attracting increasing attention, especially for bottom-up analysis (*i.e.* peptide mapping, host cell protein analysis). Some authors have recently reported a 1 h enzymatic digestion method using only 1.25 μg of AAV VPs, providing >98% protein sequence coverage and reproducible relative quantification of different PTMs of the VPs [73].

In addition to rAAV vector analysis, chromatographic methods may also be used to examine the integrity of the single stranded DNA (ssDNA) payload incorporated into viral vectors, as an alternative to established methods (qPCR, next generation sequencing or CGE methods). As already reported for long RNA constructs up to 8000 nt, at least two chromatographic methods can be used for this purpose, namely ion-pairing reversed phase liquid chromatography (IP-RPLC), which has the potential to be coupled to MS, and AEX, both of which are being developed as useful techniques for the characterization of genomic material [79,80]. These approaches have not yet been used to assess the integrity of the rAAV genome, but this is expected to happen very soon [81].

A highly promising on-line two-dimensional (2D) LC-MS method has been recently reported. This approach is capable of determining the F/E ratio and characterizing viral proteins at the intact level in a single experiment [82]. The first dimension utilizes AEX to separate empty and full capsids by applying a salt gradient with TMAC, as shown in Fig. 6A. Then, for the selected peaks of interest, on-line trapping was performed using a multiple heart-cutting approach to remove MS-incompatible salts, as shown in Fig. 6B. Capsid denaturation was performed on-column by injecting the intact AAV capsids into a trapping column, followed by washing with the initial RPLC mobile phase composition for 10 min. Due to the high column temperature and the acidic mobile phase an increase of aspartic acid-proline bond-clipping species was observed. In the second dimension, RPLC using DFA as ion pairing reagent was coupled to MS for intact mass analysis. Fig. 6C shows the RP analysis of the VPs (2nd dimension). Finally, Fig. 6D shows the potential of the 2D-LC approach for the comprehensive characterization of an AAV8 product. As illustrated, the three peaks separated by AEX were fractionated separately by multiple heart cutting and the corresponding fractions were analyzed by RPLC-MS, highlighting the differences in terms of phosphorylation between the three AEX peaks. The 2D-LC-MS method was successfully applied for different AAV serotypes (AAV8, AAV5 and AAV1) and allows high-throughput and multi-attribute characterization of AAV in a single experiment with minimal sample handling.

There are several ways to extend the use of 2D-LC for AAV characterization. First, other chromatographic modes can also be considered, such as SEC in the first dimension to study HMWs in AAV samples, or HILIC to obtain alternative selectivity for viral proteins in the second dimension. Alternatively, IP-RPLC in the

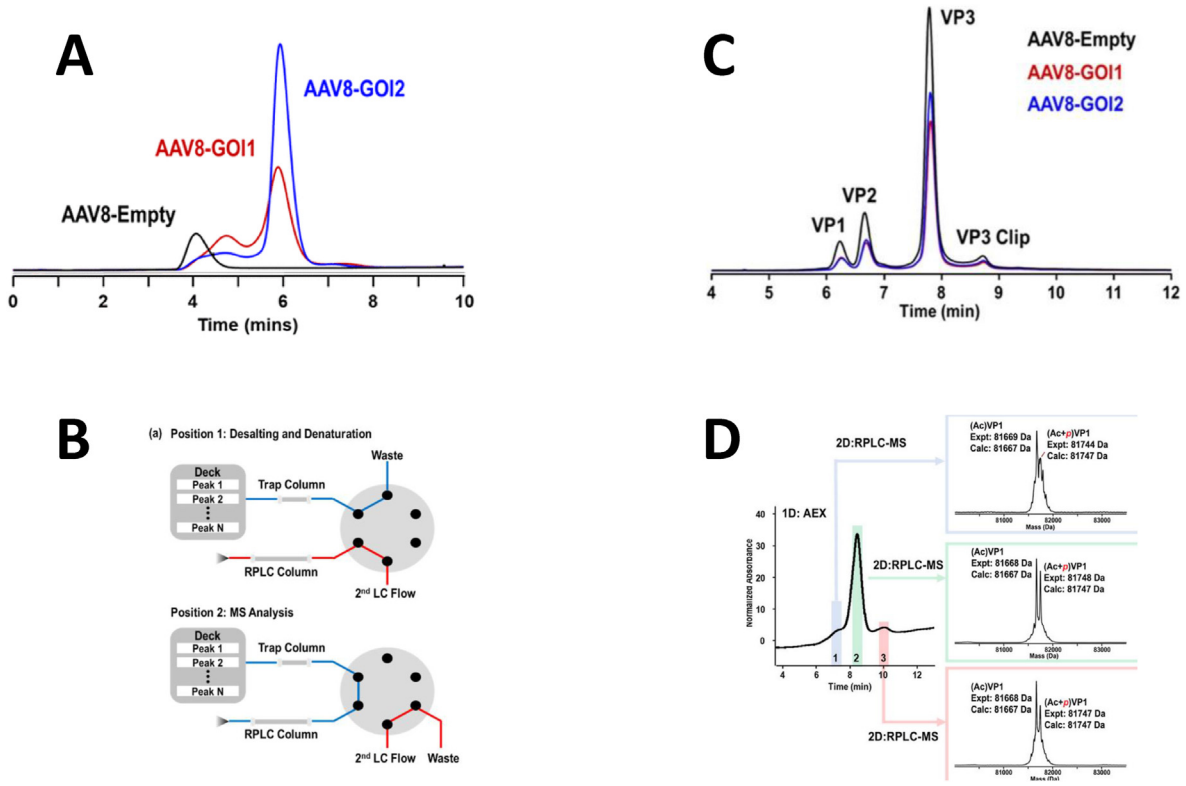

Fig. 6. 2D-LC-MS platform for the simultaneous multi-attribute characterization of AAV8 product. (A) Optimized AEX separation of empty and full AAV8 capsids, using TMAC as the salt gradient component. (B) Scheme of the heart-cutting 2D-LC (AEX-RPLC) setup. (C) Optimized RPLC analysis of AAV8 viral proteins using DFA as the ion-pairing reagent. (D) Chromatogram and mass spectra obtained when using the 2D-LC-MS for the characterization of AAV8 product. (With permission from Ref. [82]).

second dimension could be considered to assess genome integrity, following an AEX separation in the first dimension to isolate empty and full virus particles. Finally, a 4D-LC-MS platform has recently been developed for the detailed characterization of PTMs of monoclonal antibodies and related compounds [83,84]. Such a platform has the potential to be used for rAAV characterization using RPLC in the first dimension for separation of VPs, followed by on-line reduction, trypsin digestion and peptide mapping by RPLC-MS of each peak observed in the first dimension. This multidimensional strategy would allow not only the identification, but also the localization of all structural modifications (*e.g.* clipping, oxidation, phosphorylation) within each VP. However, there are also numerous technical problems to overcome *e.g.* material constraints, stability of AAVs, adsorption phenomena.

MS is undoubtedly an important tool for the analysis of gene therapy products as it is recognized to be one of the most powerful analytical techniques in protein characterization [85]. In our opinion, there are two innovative MS-based strategies that deserve attention in the coming years. The first is ion mobility spectrometry-mass spectrometry (IM-MS), which adds a gas phase separation dimension based on shape [86]. To the best of our knowledge, there are no reports on the application of LC-IM-MS to gene therapy products, but the approach may be highly informative, due to the complementarity of the three separation dimensions. CDMS is another relevant strategy that has demonstrated its applicability to the resolution of heterogeneous mixtures of very large masses [87]. It simultaneously measures the charge and mass-to-charge ratio of individual ions, allowing direct determination of their mass. It has been successfully used to characterize gene therapy vectors [88]. However, coupling CDMS with a chromatographic dimension is still a challenge and has not yet been reported.

Last but not least, there is a clear need for analytical methods with short turnaround times and simple sample preparation to facilitate the monitoring of an ongoing process with analytical instrumentation close to the point of sampling (at-line characterization) [20]. In this context, ultra-short chromatographic columns of only a few millimeters length can be considered, as their utility for protein analysis in RPLC and IEX modes has already been successfully demonstrated [89,90]. Due to the particular retention mechanism of large molecules (*e.g.* intact rAAVs or viral proteins), the column length has less influence on the control of their retention. Comparable performance can therefore be achieved for both long and ultra-short columns, but ultrashort columns will inherently provide significantly reduced analysis times. To our knowledge, this approach has not yet been applied to the characterization of gene therapy products.

5. Conclusions

As highlighted throughout this review, gene therapy products are difficult to characterize due to their large size, structural complexity, and heterogeneity. Nevertheless, several chromatographic workflows have been developed to measure the ratio of full/empty capsids, to study the presence of HMWs in AAV samples, to confirm the identity of viral proteins and to assess PTMs. Still, there is a need to better understand how these impurities affect product quality. Although some of these chromatographic methods are already well established in industrial and academic practice, several research groups are working on the next generation of chromatographic methods, such as the incorporation of multi-step salt gradients in AEX, the use of low adsorption column hardware

and inert packing materials in SEC, the addition of more informative detectors (MALS) in IEX and SEC, the optimization of mobile phase composition and stationary phase chemistries in all chromatographic modes, and the development of multidimensional LC.

Despite all these improvements, it is important to note that generic (platform) LC conditions are not necessarily applicable to the analysis of all AAV serotypes, and some of them remain more difficult to analyze due to their varying chemical stability, solubility, adsorption behavior, and physico-chemical properties. To date, AAV8 appears to be one of the less challenging serotypes for LC analysis and it has been widely utilized as benchmark throughout the scientific literature. On the other hand, occasional reports on the analysis of AAV2 suggest that this serotype is more difficult to analyze. Another crucial aspect in the development of a new chromatographic method is the need to systematically validate the analytical results with reference methods (*i.e.* AUC, dynamic light scattering, CGE, bio-layer interferometry), as some artefacts may be generated during the analysis.

Given the complexity of AAV-based products, we see an increasing importance of multidimensional LC in the future, as it is expected to provide multi-attribute characterisation of AAV products in a single run. Coupling of orthogonal LC techniques with MS is also expected to contribute fundamentally to a better understanding of gene therapy products at the molecular level, but technical advances in MS instruments and software are desirable.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:Tobias GRAF and Raphael RUPPERT are working for Roche (Penzberg, Germany) who are producing some gene therapy products as new drugs. BEH, ACQUITY and UPLC are trademarks of Waters Technologies Corporation.

Data availability

No data was used for the research described in the article.

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