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Characterization of Adeno-Associated Virus Capsid Proteins Using Hydrophilic Interaction Chromatography Coupled with Mass Spectrometry

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ABSTRACT

To support adeno-associated virus (AAV)-based gene therapy development, characterization of the three capsid viral proteins (VP; VP1/VP2/VP3) from recombinant AAV can offer insights on capsid identity, heterogeneity, and product and process consistency. Intact protein mass analysis is a rapid, reliable, and sensitive method to confirm AAV serotypes based on accurate mass measurement of the constituent capsid proteins. Compared to commonly applied reversed-phase liquid chromatography (RPLC) methods, we demonstrated that, using a wide-pore amide-bonded column, hydrophilic interaction chromatography (HILIC) could achieve improved separation of VPs from a variety of AAV serotypes using a generic method prior to MS detection. Moreover, HILIC-based separation was shown to be particularly sensitive in detecting capsid protein variants resulting from different post-translational modifications (PTMs) (e.g. phosphorylation and oxidation) and protein backbone clippings, making it ideally suited for capsid heterogeneity characterization. To overcome the challenges associated with low protein concentrations of AAV samples, as well as the trifluoroacetic acid (TFA)-induced ion suppression during HILIC-MS analysis, different strategies were implemented to improve method sensitivity, including increasing the HILIC column loading and the application of a desolvation gas modification device. Finally, we demonstrated that this integrated HILIC-FLR-MS method can be generically applied to characterize a variety of AAV serotype samples at low concentrations without any sample treatment to achieve unambiguous serotype identification, stoichiometry assessment, and PTM characterization.

1. Introduction

Recombinant adeno-associated virus (AAV) vectors have become increasingly popular as gene delivery vehicles to target various human diseases due to their low immunogenicity and cytotoxicity, as well as their effectiveness in transducing a wide range of cell types [1]. As of December 2019, two AAV-based gene therapies have been approved by the US Food and Drug Administration (FDA), and 250 AAV-based clinical trials are currently ongoing or have been completed for numerous disease indications [2–4]. Depending on the targeted diseases, the AAV serotype needs to be carefully evaluated to achieve desired viral infectivity, as each serotype displays different tissue and cellular tropism profiles [5]. Besides naturally occurring AAV serotypes, there are also a rapidly increasing number of engineered serotypes, where the differences in their capsid amino acid sequences and structures contribute to the preferential interactions with various host cell receptors. Despite serotype diversity, all AAV capsids are comprised of 60 copies of three capsid viral proteins (VP; VP1/VP2/VP3) assembled into an icosahedron at a molar ratio of approximately 1:1:10 (VP1:VP2:VP3). While VP1, VP2, and VP3 share the same C-terminal region, the entire VP3 sequence is nested in that of VP2, and the entire VP2 sequence is nested in that of VP1 [6]. It has been reported that the relative expression levels of VP1, VP2, and VP3 can be affected by production methods and can deviate from the 1:1:10 ratio, which can potentially result in decreased infectivity in some cases [7]. Capsid proteins can also inherit post-translational modifications (PTMs), such as phosphorylation and deamidation, during production or under storage conditions, which have also been reported to impact transduction efficiency [8–10]. Therefore, comprehensive characterization of the capsid proteins, with a goal to confirm serotype identity, evaluate capsid protein stoichiome-

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try and integrity, is of great importance to not only expand product knowledge but also ensure product and process consistency.

AAV serotype identity analysis is often achieved by an enzyme-linked immunosorbent assay (ELISA) or western blot methods. However, these methods are limited by the availability of serotype-specific antibodies and might prove insufficient for differentiation of serotypes with high sequence homology [11]. This issue will inevitably be compounded by the rapidly growing number of engineered AAV serotypes. Recently, Bennett et al. reported the application of differential scanning fluorimetry (DSF) for AAV serotype differentiation based on the specific melting temperatures of AAV1-9 and AAVrh.10 [12]. Alternatively, mass spectrometry (MS) based methods have seen increasing applications in AAV serotype identification, which provides highly confident confirmation of capsid identity at the amino acid sequence level. For example, LC-MS based peptide mapping analysis, performed after either in-gel digestion [13] or in-solution digestion [14], not only provides identification of each capsid protein but can also retrieve information on site-specific PTMs. In contrast, intact protein mass analysis, which is often performed on a high-resolution accurate-mass (HRAM) MS system after chromatographic or electrophoretic separation, is a direct and fast technique to identify AAV serotype-specific capsid proteins based on accurate mass measurement with minimal sample treatment. Reversed-phase liquid chromatography (RPLC) coupled to MS has been used by Jin et al. for AAV2 capsid protein identification [14]. Although all three capsid proteins could be readily identified in this study, baseline separation of the three VPs could not be achieved, making this method unsuitable for stoichiometry analysis. We also found that RPLC-based separation of capsid proteins from other AAV serotypes has proved difficult, particularly in separating VP1 from VP2, presumably due to their similarity in hydrophobicity. Compared to chromatographic methods, electrophoresis-based techniques, such as SDS-PAGE and CE-SDS, have been more routinely applied for stoichiometry analysis, due to their excellent resolving power in separating the three VPs. Taking advantage of CE-based separation, Zhang et al. have reported a microfluidic-based CE-MS method for identification of AAV2 capsid proteins [15], in which the three VPs were nearly baseline-resolved prior to MS detection. Finally, intact protein mass analysis can also potentially identify capsid protein variants resulting from PTM formation. Identification of these low-abundance variants could benefit from separation prior to MS detection, which not only alleviates ion suppression from the main species, but also allows accurate mass measurements of variants exhibiting small mass differences (e.g. 16 Da from oxidation). Unfortunately, none of the previously reported intact mass methods have shown the capability of separating capsid protein variants arising from different PTMs.

In this study, we developed a novel intact protein mass method for AAV capsid protein characterization, which explores the unique selectivity of hydrophilic interaction chromatography (HILIC), followed by both fluorescence (FLR) and MS detection (HILIC-FLR-MS). We demonstrate that compared to RPLC, this HILIC method is more efficient in separating the three VPs from a variety of AAV serotypes, facilitating stoichiometry analysis. In addition, this method is capable of separating capsid protein variants modified by oxidation and phosphorylation, two commonly occurring PTMs on AAV capsids. To address challenges associated with the low protein concentrations of AAV samples, as well as the TFA-induced ion suppression in HILIC-MS analysis, different strategies have been implemented to improve the sensitivity of this method. In summary, we show that this HILIC-FLR-MS method can be generically applied to a variety of AAV serotypes without any sample treatment. In a single step, this method provides confident AAV serotype confirmation, stoichiometry assessment, and PTM identification, using as little as $\sim$ 30 ng protein on the column. Finally, this method was demonstrated in a case study, where two AAV8 lots produced from two different processes were characterized and compared.

2. Materials and methods

2.1. Materials

AAV serotype samples (AAV1, AAV2, AAV6, AAV7, AAV8, AAV9, AAVhu37 and AAVDJ) were generated by triple transfection of HEK 293Ts at Regeneron Pharmaceuticals, Inc. (Tarrytown, NY, USA). Acetonitrile (ACN), trifluoroacetic acid (TFA), formic acid (FA), Pierce TCEP-HCl (tris (2-carboxyethyl) phosphine hydrochloride), and Invitrogen™ UltraPure™ 1 M Tris-HCl buffer, pH 7.5, were purchased from Thermo Fisher Scientific (Waltham, MA, USA). Propionic acid (PA), acetic acid, urea, and iodoacetamide were purchased from Sigma Aldrich, Co (St. Louis, MO, USA). Isopropanol (IPA) was purchased from VWR International, LLC (Radnor, PA, USA). Sequence grade modified trypsin was purchased from Promega (Madison, WI, USA). All reagents were of analytical grade or higher. Deionized water was provided by a Milli-Q integral water purification system installed with a MilliPak Express 20 filter (Millipore Sigma, Burlington, MA, USA).

2.2. LC-MS methods

LC separation was performed on a Waters I-Class UPLC system (Waters, Milford, MA, USA) equipped with a photodiode array (PDA) detector and a fluorescence (FLR) detector. Both HILIC and RPLC modes utilized mobile phase A as 0.1% (v/v) TFA in water, mobile phase B as 0.1% (v/v) TFA in ACN, a flow rate of 0.2 mL/min and a column temperature of 60 °C for the elution of intact capsid proteins. For HILIC separation, 1 to 3 μL of each AAV sample was injected onto a Waters ACQUITY UPLC Glycoprotein BEH Amide Column (300 Å, 1.7 μm, 2.1 × 150 mm) either directly (1 μL) or using the loading strategy discussed in the Results and discussion (> 1 μL). The HILIC gradient consisted of an initial gradient hold at 85% B for 0.5 minutes, 85% B to 70% B over the next 0.5 minutes, a 2-minute hold at 70% B, then 70% B to 64% B over 16 minutes. The gradient was then set to 0% B over 1 minute and held for 2 minutes for column washing, before ramping to 85% B in 0.5 minutes, and maintained at initial conditions for 7.5 minutes to equilibrate the column for the next run. For RPLC separation, 3 μL of each AAV sample was injected onto a Waters ACQUITY UPLC Protein BEH C4 column (300 Å, 1.7 μm, 2.1 × 150 mm). The RPLC gradient consisted of an initial gradient hold at 20% B for 3 minutes, 20% B to 35% B over the next 2 minutes, a 2-minute hold at 35% B, then 35 to 55% B over 13 minutes, followed by an increase to 95% B over 3 minutes and finally a 5-minute wash step at 95% B before re-equilibrating to initial conditions. For UV detection, the wavelength was set at 280 nm. For FLR detection, excitation (Ex) and emission (Em) wavelengths of 280 and 348 nm, respectively, were employed. Mass spectrometric analysis was performed on a Q-Exactive Plus Orbitrap mass spectrometer (Thermo Fisher Scientific, Bremen, Germany). The resolution was set at 17,500, the spray voltage was set at 3.5 kV, the in-source fragmentation energy was set at 75, the S-lens RF level was set at 60, the source temperature was set at 250 °C, and the capillary temperature was set at 350 °C. Mass spectra were acquired with a m/z range window between 800 and 4000. To counteract TFA-induced ion suppression, a desolvation gas modification device, delivering a dopant gas containing a 3:1 (v/v) ratio of PA and IPA, was implemented on the ion source as previously described [16]. The raw MS data was deconvoluted using Intact Mass™ software from Protein Metrics (Cupertino, CA, USA). The experimental procedure of the peptide mapping analysis of the two AAV8 lots is included in the Supporting Information.

3. Results and discussion

3.1. Comparison between HILIC and RPLC methods in separating AAV capsid proteins

The retention mechanism of HILIC is complex and primarily based on the partitioning of analytes between the highly organic mobile phase and the water layer enriched on the stationary phase [17,18]. In biopharma laboratories, HILIC-based separation has often been applied to study glycans or glycopeptides that are released from glycosylated protein biopharmaceuticals. Recently, a growing number of reports have shown that HILIC separation, using a wide-pore amide-based column, could also be successfully applied to intact proteins, including recombinant Fc fusion proteins and highly glycosylated lipases [18–21]. In particular, HILIC-based intact protein mass methods have been used to characterize the glycosylation profile and product-related low molecular weight impurities (LMW) in therapeutic monoclonal antibodies (mAbs) [22,23]. Encouraged by these applications, we investigated if the HILIC method could provide improved separation of AAV capsid proteins when compared to the commonly applied RPLC method. For comparison, the capsid proteins from three AAV serotypes (AAV6, AAV7, and AAV8) were separated by RPLC using a Protein BEH C4 column (300 Å, 1.7 μm, 2.1 mm X 150 mm) or by HILIC using a Glycoprotein BEH amide column (300 Å, 1.7 μm, 2.1 mm X 150 mm), prior to FLR and MS detection. Although formic acid (FA) is more frequently used in RPLC-based intact mass methods due to better MS sensitivity, TFA at 0.1% (v/v) was selected as the ion-pairing reagent in both separation modes for optimal chromatographic separation. A comparable gradient steepness was applied in both methods for a fair comparison. FLR detection, which utilizes the intrinsic fluorescence of tryptophan, was employed to replace UV detection for improved sensitivity (Fig. S1). Consistent with previous reports, only two chromatographic peaks could be resolved from the C4 separation for all three serotypes, even with extensive gradient optimization. Subsequent MS analysis confirmed the co-elution of VP1 and VP2 in the first peak, followed by VP3. In contrast, the HILIC separation of the same AAV serotypes resulted in several resolved or partially resolved peaks for each serotype, which were all subsequently identified by MS analysis (Table S1). For all three serotypes, VP3 eluted first, followed by VP2, and then VP1. Interestingly, although VP1 contains the entire sequence of VP2, it consistently eluted earlier than VP2 on the HILIC column, implying the relatively high hydrophobicity of its unique N-terminus. In addition, HILIC-FLR-MS analysis also revealed several variant peaks that were attributed to oxidation and phosphorylation occurring on individual capsid proteins. Because of the increased hydrophilicity, these variant species exhibited enhanced retention on the HILIC column and eluted slightly later than their unmodified counterparts. Although it is unclear how capsid protein oxidation might impact the potency of AAV-based drug products, it is still of importance to monitor this attribute from a process control perspective. Moreover, a previous report has shown that tyrosine phosphorylation can negatively affect the transduction efficiency of the AAV2 vector [10], suggesting that phosphorylation might be a critical quality attribute for AAV-based drug development. Finally, HILIC-FLR-MS analysis of AAV6 also revealed a partially resolved peak eluting slightly before VP3, which was subsequently identified as an N-terminal fragment of VP3, due to protein backbone clipping between Pro210 and Met211 with subsequent N-terminal Met loss and acetylation of the following alanine (top left panel, Fig. 1 & Table S1). Therefore, it is apparent that the HILIC method is superior to the RPLC method in separating AAV capsid proteins and their variant forms.

3.2. Strategies to increase the HILIC column loading

Compared to therapeutic proteins, AAV samples are typically associated with much lower protein concentrations (0.01-0.1 mg/mL) [24]. Preconcentration of AAV samples by centrifugal filtration can be challenging, as significant sample loss can occur due to the adsorption of AAV on the membrane [25]. Therefore, direct analysis of the AAV sample in its stock solution is highly desirable, not only because it is simple, but it can also eliminate concerns about artifact formation and poor sample recovery that may arise from the pretreatment steps. However, this presents a significant challenge for assay sensitivity. Increasing the column loading amount is the most straightforward way to improve downstream detection. Unfortunately, HILIC analysis of aqueous samples is notorious for its limitation on injection volume, as HILIC separation is very sensitive to the mismatch between the sample solvent (water) and the mobile phase (highly organic). Under our HILIC conditions, the injection volume of AAV samples was kept at 1 μL to ensure optimal chromatographic performance. To increase the column loading without compromising the LC performance, two strategies were applied. First, a larger aliquot of AAV stock solution (e.g. > 1 μL) was adjusted with organic solvent to match the initial mobile phase conditions, and then injected in its entirety onto the HILIC column. For example, straight injection of 3 μL of an aqueous AAV sample led to significant peak distortion, while injection of 20 μL of solvent-matched sample (comprised of 3 μL of AAV stock solution and 17 μL of mobile phase B: 0.1% TFA in ACN) led to highly comparable chromatography as achieved by a 1 μL injection (Fig. S2). However, this strategy needs to be tested on a case-by-case basis, as many AAV samples could precipitate in high percentages of organic solvent after extended storage (> 1 hour) in the autosampler (5 °C). Alternatively, multiple sample loading steps could be performed by repeated 1 μL injections of the AAV samples onto the HILIC column before initiating the gradient for AAV capsid protein elution and separation. The success of this strategy was demonstrated in Fig. S3, where repeated loading steps led to significant improvement in FLR signal without compromising the chromatographic performance (Fig. S3). Both strategies allow for flexibility of direct analysis of diluted AAV samples without preconcentration.

3.3. Increasing HILIC-MS sensitivity using a desolvation gas modification strategy

In this study, the protein concentrations of the tested AAV samples ranged from 0.01 to 0.09 μg/μL. For highly diluted AAV samples, up to 3 μL of the stock solution was injected onto the HILIC column using the aforementioned strategies, leading to a column loading amount of as little as ~ 30 ng. Although this amount is sufficient for FLR detection, thanks to the presence of multiple Trp residues in the three VPs from all serotypes, it still presents a significant challenge for MS analysis, particularly for the detection of minor variant forms. This challenge is further compounded by the use of TFA as an ion-pairing reagent in the HILIC method, which is notorious for its ion suppression effect [26]. To mitigate TFA-induced MS sensitivity loss, Gargano et al reported a capillary HILIC-MS method which applies a dopant gas (ACN + 1% PA) to a Bruker CaptiveSpray ESI interface to reduce TFA ion suppression [27]. Adopting a similar strategy, we applied a previously reported device that could readily modify the desolvation gas on a Q-Exactive MS system for regular flow LC-MS analysis [16]. By modifying the desolvation gas with acid vapor from a mixture of PA and IPA (3:1, v/v), we have previously shown that the MS responses of tryptic peptides from a TFA-based LC-MS analysis could be boosted by approximately 5-fold. To evaluate if this strategy could also be applied to improve the characterization of AAV capsid proteins,

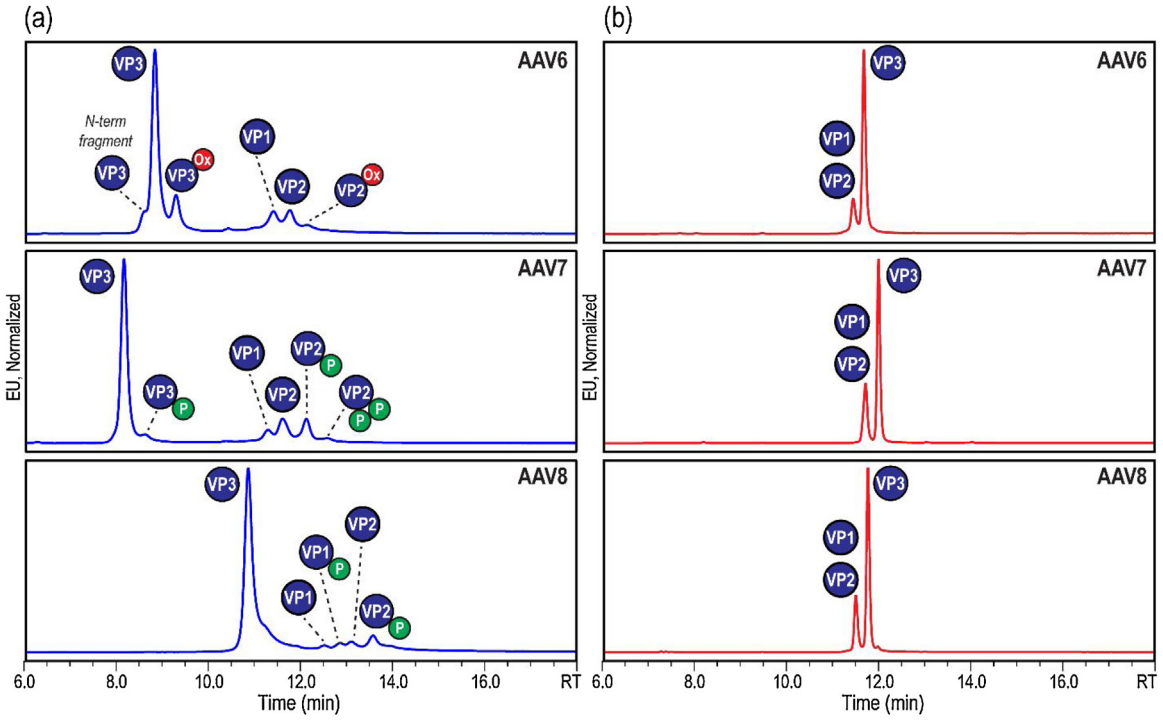

Fig. 1. Comparison of LC separation profiles of AAV6 (top), AAV7 (middle) and AAV8 (bottom) capsid viral proteins (VPs) using: (a) Waters ACQUITY UPLC Glycoprotein Amide column (300 Å, 1.7 μm, 2.1 mm × 150 mm) and (b) Waters ACQUITY BEH C4 Column (300 Å, 1.7 μm, 2.1 mm × 150 mm). The FLR traces were monitored using λ_{em} = 280 nm and λ_{ex} = 348 nm. The FLR peak identities were confirmed by accurate mass measurement and annotated. Ox: oxidized; P: phosphorylated.

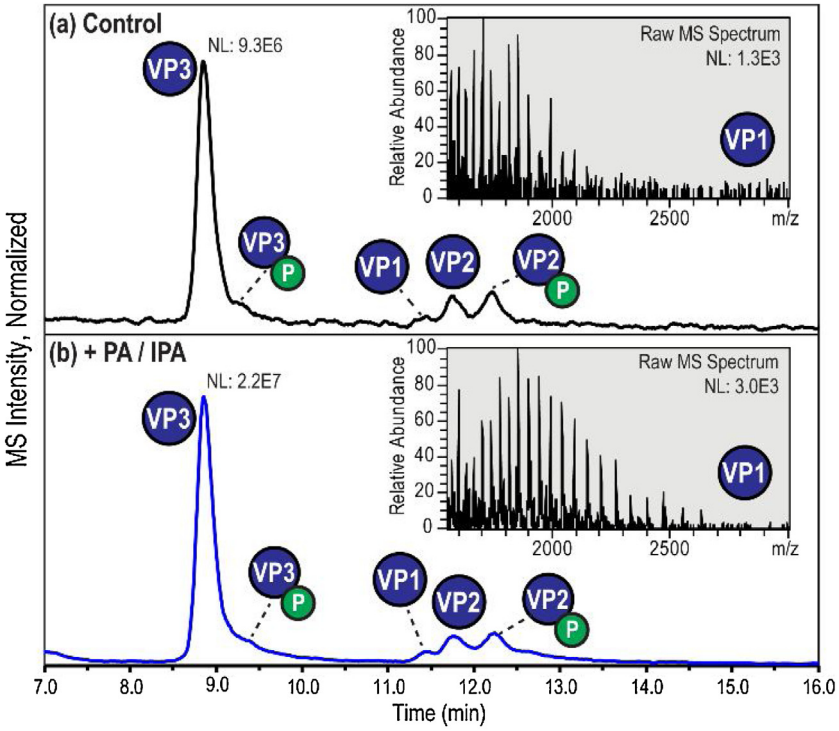

Fig. 2. Total ion chromatograms (TICs) from the HILIC-FLR-MS analysis of an AAV7 sample (a) without and (b) with modified desolvation gas using a mixture of PA and IPA (3:1, v/v). The raw mass spectrum of VP1 from each sample is displayed in the corresponding inset.

HILIC-FLR-MS analysis of an AAV7 sample was performed both with and without the use of PA/IPA modified desolvation gas (Fig. 2). By comparing the total ion chromatograms (TICs) and the raw MS spectra, it is apparent that application of PA/IPA modified desolvation gas led to a significant improvement in MS sensitivity (~2-3 fold) as well as improved spectrum quality. In addition, this modification also did not lead to an obvious change in protein charge state distribution. Compared to peptides, the gain in MS sensitivity by this strategy is less prominent for capsid proteins, presumably due to a larger number of TFA anions residing on the protein sur-

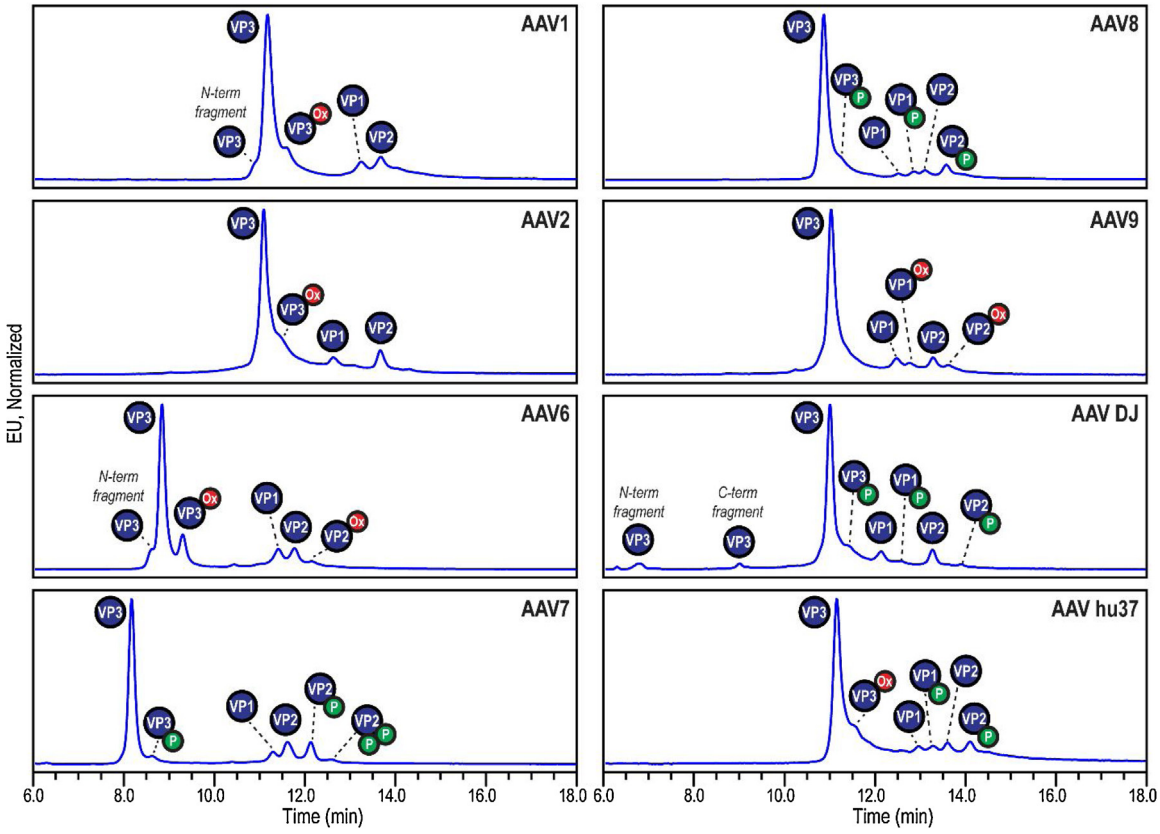

Fig. 3. HILIC-FLR-MS analysis of AAV serotypes (AAV1, AAV2, AAV6, AAV7, AAV8, AAV9, AAV DJ, and AAV hu37), monitored using λ_{em} = 280 nm and λ_{ex} = 348 nm. The fluorescence intensity (EU) was normalized across all samples. The FLR peak identities were confirmed by accurate mass measurement and annotated. Ox : oxidized; P : phosphorylated.

face that cannot be effectively replaced by PA during the ESI process [28]. Nevertheless, the apparent improvement in MS sensitivity, as achieved by this method, is beneficial for the identification of low-abundance proteoforms, such as VP1, which was expressed at much lower levels.

3.4. Generic application of HILIC-FLR-MS for a variety of AAV serotypes

To test generic applicability, a variety of AAV serotypes (AAV1, AAV2, AAV6, AAV7, AAV8, AAV9, AAVDJ, and AAVhu37) were subjected to HILIC-FLR-MS analysis for capsid protein characterization (Fig. 3). The injection volume and column loading strategy used for each serotype sample was specified in Table S1. Using a generic 30-minute method, the three VPs and their variant forms for each of the eight AAV serotypes were readily separated. The subsequent MS analysis, enhanced by PA/IPA modified desolvation gas, allowed for sensitive and accurate mass measurements of each FLR peak, including those present at low levels. By comparing the observed and predicted masses, the identity of each AAV serotype was unambiguously confirmed based on three of its unique capsid proteins with mass errors under 2 Da (Table S1). Moreover, the capsid protein variants, as a result of PTM formation or protein backbone clipping, were also confidently identified with mass errors under 4 Da (Table S1). Interestingly, for all the tested serotypes, including the three previously discussed, the same elution order of VP3, VP1, and VP2 was observed. Consistent with findings from previous reports [8,14,15], MS analysis confirmed the removal of the N-terminal methionine and the subsequent acetylation of the following alanine in VP1 and VP3, as well as the removal of the N-terminal threonine in VP2 from all serotypes. In addition, oxidized and phosphorylated variants were also identified in a majority of the tested AAV serotype samples. Because of the increased hydrophilicity, the variants exhibited enhanced retention on the HILIC column and were effectively separated from the unmodified capsid proteins. It is worth noting that MS detection of these low-abundance variants has benefited from the chromatographic separation step, which not only reduces ion suppression from the co-eluting species but also allows accurate mass measurement of variants bearing small mass changes. For example, without chromatographic separation, low levels of oxidation variant (+16 Da) are unlikely to be detected by MS at intact capsid protein levels (60-80 kDa), as it is challenging to resolve its mass from that of the main species. Furthermore, as previously discussed in the example of AAV6, this HILIC method is also capable of separating capsid protein fragments. Analysis of AAVDJ also revealed two low-abundance, early-eluting FLR peaks, which were identified as the N-terminal and C-terminal fragments of VP3, due to protein backbone clipping between Gly454 and Gly455 or Asn458 and Thr459, respectively (Table S1). Because of the high chromatographic resolution, this HILIC method could potentially be used for capsid protein stoichiometry analysis. By combining the FLR peaks of all proteoforms from each VP, an estimated stoichiometric ratio was calculated for each AAV serotype sample. As shown in Table S2, a majority of AAV serotype samples exhibited ratios close to the expected value of 1:1:10 (VP1: VP2: VP3), although several samples showed values that deviated from it significantly. It is understood that different production methods or processes could lead to different expression levels of the three VPs [29]. However, it is also important to note that two issues might compromise the accuracy of the stoichiometry measurement by this HILIC method. First, in contrast to UV-based detection, the fluorescence response from each VP may

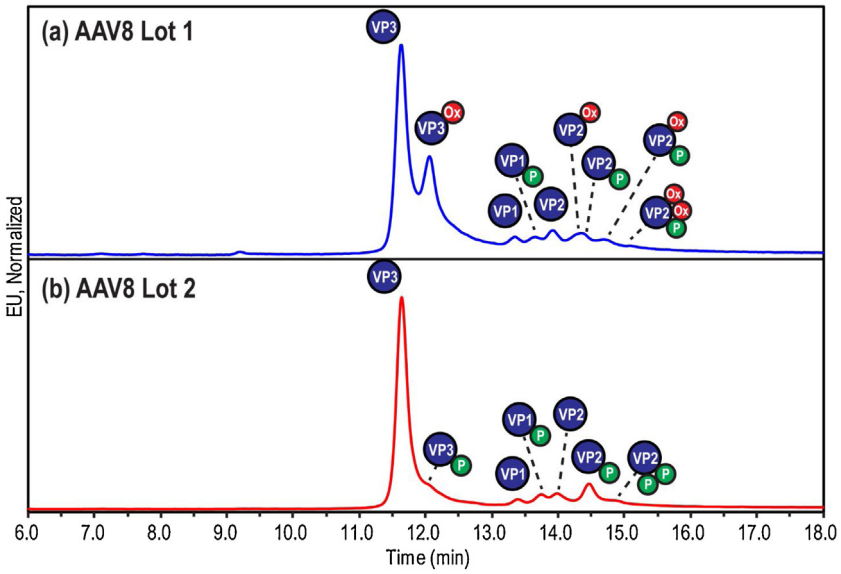

Fig. 4. Comparison of HILIC-FLR chromatograms of AAV8 from (A) Lot 1 and (B) Lot 2, monitored using λ_{em} = 280 nm and λ_{ex} = 348 nm. The fluorescence intensity (EU) was normalized across both samples. The FLR peak identities were confirmed by accurate mass measurement and annotated. Ox : oxidized; P : phosphorylated.

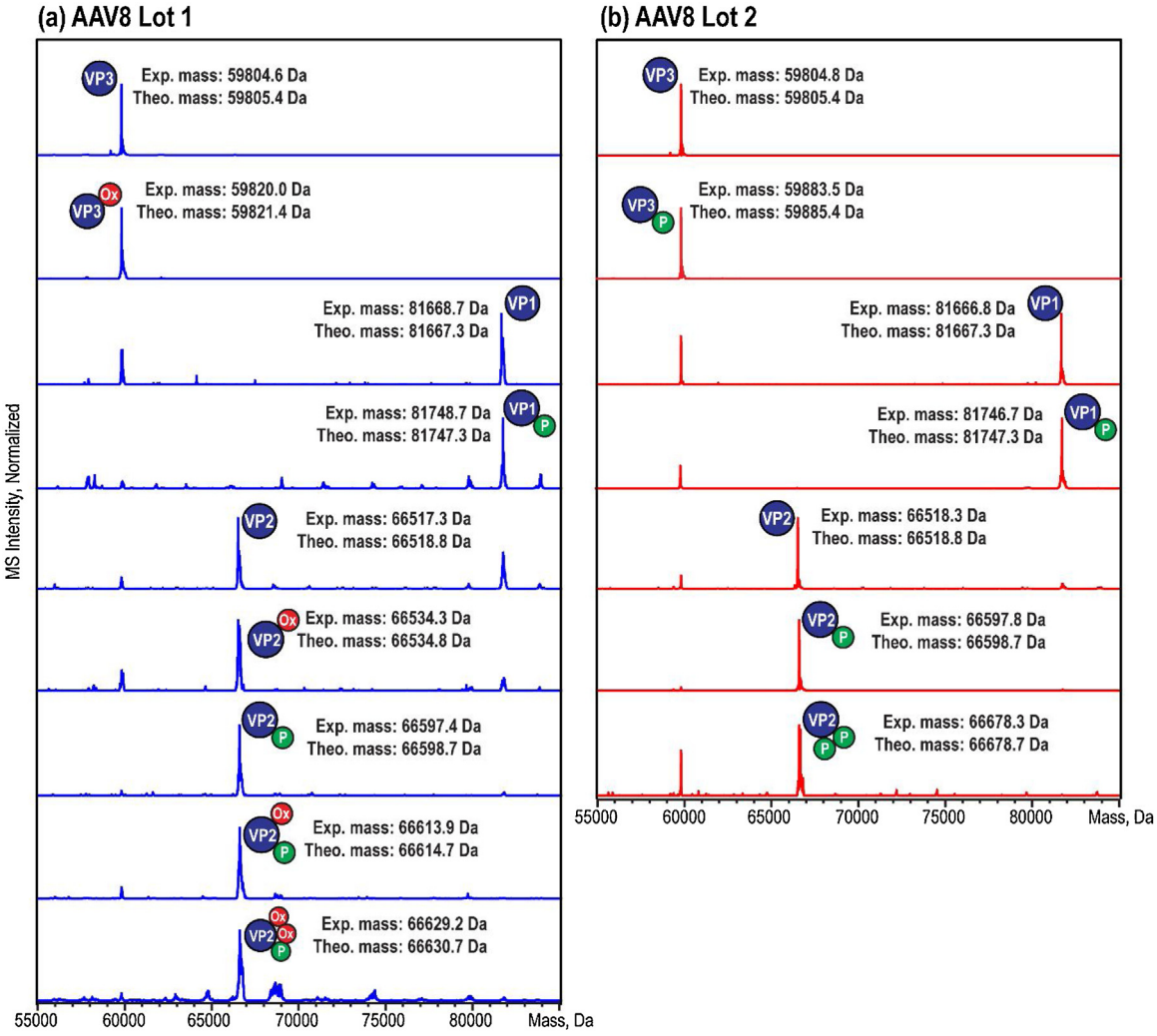

Fig. 5. Deconvoluted mass spectra from the HILIC-FLR-MS analysis of (a) AAV8 Lot 1 and (b) AAV8 Lot 2. Ox : oxidized; P : phosphorylated.

be biased towards the ones containing more tryptophan residues. In addition, as each VP species can be further isolated into different proteoforms by HILIC separation, co-elution of proteoforms from different VPs may occur, making peak integration inaccurate. Finally, it is still unclear whether the recovery of different VPs might differ as a result of varying adsorption on the HILIC column. Nevertheless, this HILIC method could still be applied semi-quantitatively to compare the relative expression levels of the three VPs from different lots or processes.

3.5. Application of HILIC-FLR-MS for batch-to-batch comparison

The utility of this HILIC-FLR-MS method was demonstrated in a comparability case study, where two AAV8 lots produced from two different processes were compared at capsid protein levels. By comparing the FLR profiles, it was apparent that although the main FLR peaks (VP3) from the two lots showed identical retention time, notable differences were observed in the overall profiles (Fig. 4). Subsequently, the mass spectrum of each FLR peak was averaged and deconvoluted, and the resulting spectra were shown in Fig. 5. The identity of each FLR peak was then assigned by comparing the observed and predicted masses, after taking the common PTMs into consideration (Table S3). After annotating each FLR peak, it became clear that the AAV8 lot 1 exhibited much higher levels of oxidation, while lot 2 showed significantly higher levels of phosphorylation. Because both oxidation and phosphorylation increase the hydrophilicity of the capsid proteins, those variants eluted later than their unmodified forms on the HILIC column. Interestingly, as VP2 with 1 × oxidation and VP2 with 1 × phosphorylation were both observed in lot 1, their relative retention times indicated that phosphorylation had a slightly greater effect on increasing HILIC retention compared to oxidation. Because of the enhanced MS sensitivity, low-abundance variants, including those that were unique for each lot, were all confidently identified. For example, VP2 with 1 × oxidation and 1 × phosphorylation was only observed in lot 1. VP2 with 2 × phosphorylation was only observed in lot 2, which suggested the presence of two phosphorylation sites in VP2. Combining the results from both lots, a total of 11 different proteoforms from the three VPs were identified with mass errors under 2 Da. It is also worth noting that this analysis did not reveal any N-glycosylated nor O-glycosylated capsid proteins, despite that HILIC-based separation should be highly efficient in isolating and detecting those species. Hence, the findings indicate that N-glycosylation and O-glycosylation, if present, should be at very low levels in both AAV8 lots. This observation is also consistent with a recent study, where very low levels of N-glycosylation on AAV8 were only detected after HILIC-enrichment and PNGase F treatment in ^{18}O water [30]. Subsequently, peptide mapping analysis after tryptic digestion confirmed these findings, in which higher levels of oxidation were found on several methionine residues (Met212, Met374, Met473 and Met561) in lot 1 and a higher level of phosphorylation was found on Ser149 in lot 2. N-glycosylated peptides were not detected in either lot (Table S4). Finally, by combining different proteoforms from each VP, the capsid protein stoichiometry of each lot was estimated and found comparable between the two (Table S5).

4. Conclusions

With the rapid growth of AAV-based gene therapies in the pharmaceutical market, there is an increased demand for improved analytical methods to support product and process development. Here, we developed a HILIC-FLR-MS method that can be generically applied to characterize capsid proteins from a variety of AAV serotypes to achieve unambiguous serotype identification, stoichiometry assessment, and PTM characterization. To circumvent issues with the low protein concentrations of AAV samples, as well as the TFA-induced ion suppression in HILIC-MS analysis, we developed strategies to increase the HILIC column loading and boost MS response using a desolvation gas modification device. As a result, this method can be directly applied to low protein concentration AAV samples without any sample treatment. Compared to peptide mapping analysis, although this method cannot reveal site-specific PTM information, it prevails in higher throughput and reduced sample processing, and might prove particularly valuable in supporting process development, where a large number of samples might need to be evaluated in a timely fashion. Because of the high chromatographic resolution from HILIC separation, this method may be applied with FLR detection alone in certain scenarios, where a well-characterized standard is included as a comparator. This should further expand the applicability of this method in laboratories with limited mass spectrometry capability.

Disclosures

Anita P. Liu, Shailin K. Patel, Tao Xing, Yuetian Yan, Shunhai Wang, and Ning Li are full-time employees and shareholders of Regeneron Pharmaceuticals Inc.

CRediT authorship contribution statement

Anita P. Liu: Methodology, Investigation, Formal analysis, Writing - original draft. **Shailin K. Patel:** Investigation. **Tao Xing:** Investigation. **Yuetian Yan:** Investigation. **Shunhai Wang:** Conceptualization, Methodology, Writing - review & editing. **Ning Li:** Supervision, Writing - review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jpba.2020.113481.

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