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Analysis of Adenoviral Vector Proteins by RPLC, Native Fluorescence, and Online MS

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Abstract

Adenovirus (AdV) is being used as a viral vector for vaccines and gene therapies alike. It is comprised of a relatively complex proteome. With this work, we demonstrate the use of difluoroacetic acid (DFA) ion pairing and a 2.7 µm 450 Å phenyl bonded stationary phase to quickly separate the AdV proteins while obtaining high sensitivity mass spectra. This is an RPLC method that facilitates investigating protein copy ratios and measurement of intact protein masses. As such, it should present an excellent starting point for the characterization of current and future AdV based vaccines and gene therapies.

Benefits

- Direct analysis of formulated sample without the need for sample preparation steps

- DFA ion pairing to optimize chromatographic resolution without overly compromising MS sensitivity to support wide dynamic range analyses

- 2.7 µm superficially porous particles for separations that are both UPLC and UHPLC friendly and readily adaptable to the equipment found in different laboratories

Introduction

Dysfunctional genes account for 80% of the 7,136 known human diseases.[1] Gene therapy treatments could be developed to treat many of the monogenic diseases within this list. At the same time, the use of viral vectored vaccines for SARS-CoV-2 is helping to ready the biotechnology industry to make its next step of progress on gene therapy products. A common method of delivery for *in vivo* gene therapy indeed entails the use of a viral vector. When selecting a viral vector and serotype, there are a few key considerations: immune response, insertional mutagenesis, viral tropism, off-target effects, and transgene capacity.[1] These considerations shorten the list of viral vectors dramatically and one candidate remaining is the adenovirus, AdV.

Hundreds of vaccines and cancer trials have used or are using human AdV-based vectors; some trials date back to more than 40 years ago. Currently, there are over 50 AdV studies in development or at a clinical stage.[2] Specific human serotypes, such as AdV5, or non-human serotypes, such as chAdV, are being used to avoid pre-existing humoral immunity.[2]

AdV is a non-enveloped virus whose genomic information is stored as double-stranded linear DNA. It is an efficient tool in gene therapy as it can be prepared to be replication incompetent, packaged with a large transgene, and deliver a non-integrating genetic payload that remains in the form of episomal DNA.[3] Like other viruses, it has a very large molecular weight (150 MDa) and is fairly compact in its size with a diameter of approximately 90 nm. This makes it 6x larger in diameter and 100x greater in mass than a typical mAb. The capsid is comprised of 240 trimers of a so-called hexon protein and it is decorated with protein fibers that extend from the main body of the virus.[3] It has a relatively complex proteome that contains even more proteins than just the hexon and fiber protein. Figure 1 shows a diagram of its proteome and general structure. In addition to the hexon and fiber protein, there is one other major species within the capsid, which is called the penton base protein. On top of these, there are cement proteins that serve a role to hold the capsid components together and yet another set of proteins that can be found in the core of the particle along with the genomic DNA (Table 1).[4–6]

The analysis of the AdV proteins is important to the characterization of an AdV vectored advanced therapy medicinal product. With this work, we demonstrate the use of DFA ion pairing and a 2.7 µm 450 Å phenyl bonded stationary phase to quickly separate the AdV proteins while obtaining high sensitivity mass spectra. This is an RPLC method that facilitates investigating protein copy ratios and measurement of intact protein masses. As such, it should present an excellent starting point for the characterization of current and future AdV based vaccines and gene therapies.
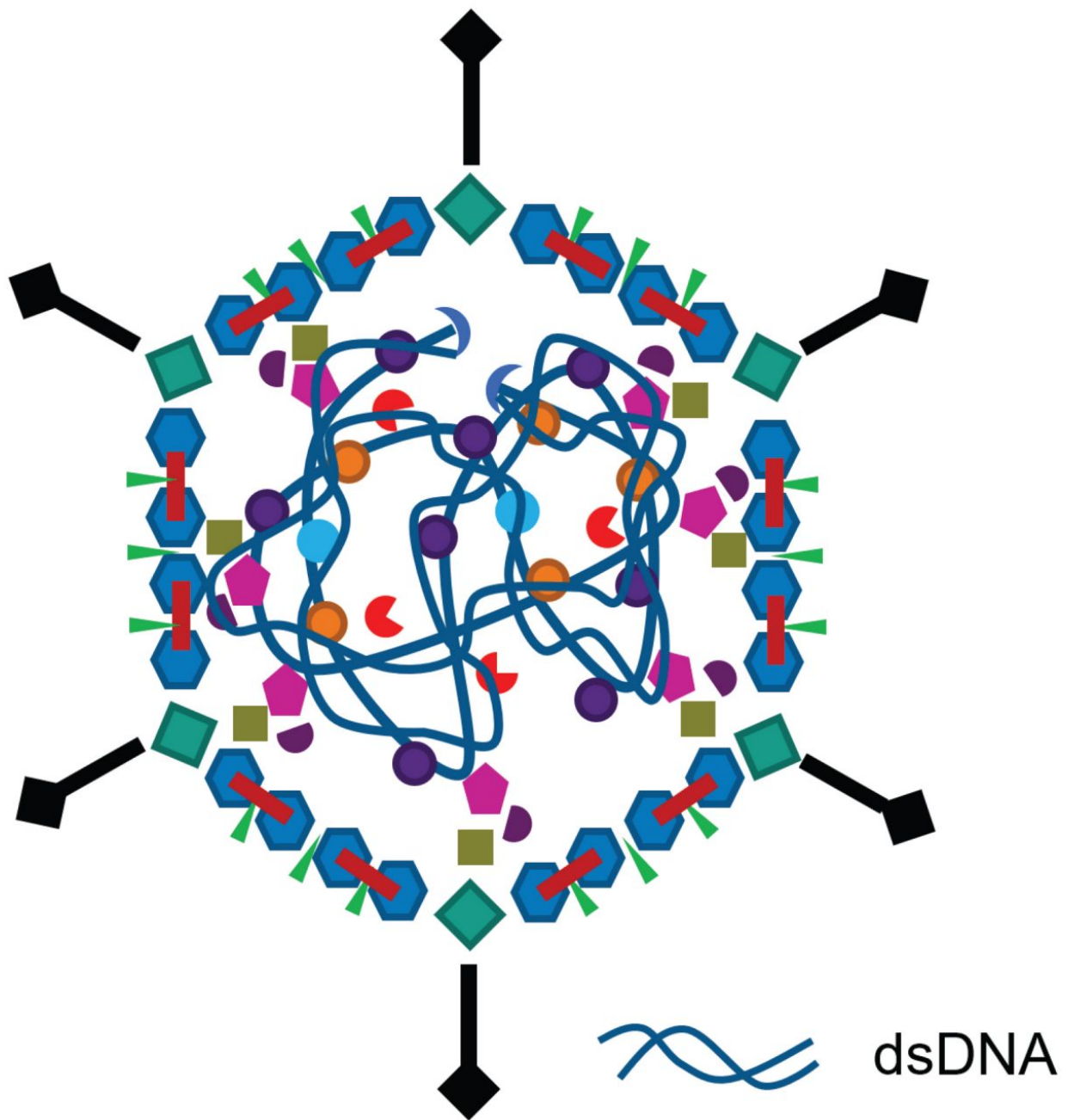
Figure 1. Illustrative depiction of human adenovirus 5 (HuAdV5) based on recent structural understandings outlined by Reddy and co-workers.[4,]

Major proteins		Functional name	Copy number	UniProt
⬡	II	Hexon protein	720	PO4133
◆	III	Penton base protein	60	P12538
➤	IV	Fiber protein	36	P11818
Cement proteins		Functional name		
■	VI	Endosome lysis protein	342	P24937
◤	IX	Hexon interlacing protein	247	PO3281
●	VIII	Hexon linking protein VIII	128	P24936
◢	IIIa	Hexon linking protein IIIa	68	P12537
Core proteins		Functional name		
●	VII	Histone like nucleoprotein	527	P68951
●	X	Protein Mu	290	Q2KS10
⬟	V	Core capsid bridging protein	157	P24938
◖	AVP	Adenovirus protease	15	P03253
●	IVa2	Packaging protein 1	7	P03271
◗	TP	Terminal protein	2	P04499

Table 1. Adenovirus proteins for mature capsids categorized according to their function. Estimates for copy number and each protein UniProt entry is provided. Copy numbers are reported according to values in a review by Ahi and Mittal.[6]

Experimental

Human Adenovirus 5 (HuAdV5) CMV GFP

The sample of human adenovirus type 5 analyzed in this work was obtained from Applied Biological Materials (Richmond, BC, Canada). It is a -E1/-E3 deletion mutant and is thereby replication incompetent. It was engineered with a cytomegalovirus (CMV) promoter and green fluorescent protein (GFP) transgene and prepared to a titer concentration of 1×10^{12} plaque forming units (pfu) per mL. This sample was directly injected onto RPLC chromatography without any additional sample preparation steps.

Reversed Phase Liquid Chromatography (RPLC)

LC system:	ACQUITY UPLC H-Class PLUS Bio System
Detector:	ACQUITY UPLC FLR
Ex wavelength:	280 nm
Em wavelength:	360 nm
Scan rate:	20 Hz
Column:	BioResolve RP mAb Polyphenyl 450 Å, 2.7 µm, 2.1 x mm (Waters p/n: 186008946)
Column temp:	80 ˚C
Sample temp:	8 ˚C
Injection:	10 µL of 10x10^{12} pfu/mL
Flow rate:	0.4 mL/min
Mobile phase A:	0.1% DFA (v/v) (IonHance DFA, Waters p/n: 18600 in 18.2 MΩ water
Mobile phase B:	0.1% DFA (v/v) in LC-MS grade acetonitrile

Time (min)	mL/min	A	B	Curve
Initial	0.4	90	10	*
20.00	0.4	50	50	6
20.50	0.4	10	90	6
21.00	0.4	90	10	6
30.00	0.4	90	10	6
Run time = 30 minutes, MS Divert for first 2 minutes				

Mass Spectrometry

MS system:	Vion IMS QTof Ion Mobility Quadrupole Time-of-flig
	Mass Spectrometry
Mode:	ESI+
Acquisition window:	1000–4000 *m/z*
Source temperature:	150 °C
Sample cone:	150 V
Offset:	100 V
Collision energy:	15 eV
Scan rate:	2 Hz
Desolvation temperature:	650 °C
Desolvation gas flow rate:	1200 L/hr
Cone gas:	300 L/hr
Instrument control:	UNIFI v1.9.13

Results and Discussion

In 2018, van Tricht and co-workers reported on the robust design of an LC method for the analysis of proteins present in the viral vectors of the Janssen AdVac Technology.[7] Through their method development work, they showed it was possible to improve the resolution of AdV proteins while shortening run times from 130 to just 17 minutes. To do so, a 2.1 x 250 mm column packed with 5 µm 300 Å C_4 bonded silica was replaced with a 2.1 x 150 mm column packed with 1.7 µm BEH 300 Å C_4. AdV type 26 and type 35 vaccine candidates were then studied along with a multifactorial DoE to determine an optimal mobile phase. The ion pairing agent trifluoroacetic acid

(TFA) was employed and found to yield optimal results at a concentration of 0.175%. While a high resolution, high throughput separation was achieved, online MS was not pursued due to concerns over ion suppression. Peak identification was alternatively facilitated by fraction collection and secondary peptide mapping analyses. Here, we have explored the use of RPLC for AdV protein characterization but have prioritized direct hyphenation to MS. Rather than TFA, DFA was employed. Moreover, the use of DFA was paired with a stationary phase based on 2.7 µm superficially porous particles and a structurally rigid, high coverage phenyl bonding. This is a column technology (BioResolve RP mAb Polphenyl) that has previously been found to facilitate higher protein recoveries and to be amenable to milder elution conditions.[8]

With this approach, a sample of human adenovirus 5 (HuAdV5) containing a GFP transgene was subjected to analysis. Serial detection of the eluting proteins was achieved through native fluorescence detection followed by ESI-MS with a time-of-flight mass spectrometer, and the sample was directly injected onto the column without any prior sample preparation. A 20-minute gradient consisting of a change from 10 to 50% acetonitrile was applied along with 0.1% DFA mobile phases. A 2.1 x 150 mm column was operated with a 0.4 mL/min flow rate and maximum system pressure during the run of 6700 psi. Example chromatograms are displayed in Figure 2. Fluorescence detection was sufficiently sensitive to provide a detailed view of the protein sample. Peak areas were seen to spread across a wide dynamic range as would be predicted for an AdV sample and viral particles that are built with protein components varying by up to three orders of magnitude in their copy number. Peak widths in the observed separation ranged from 2.5 seconds at 50% height, as with the peak at 7.46 minutes, or 5.8 seconds, as with the peak at 14.46 minutes. The average peak capacity of this separation was estimated to range from 200 to 480 for selected peaks throughout the separation.

The value of performing protein RPLC with DFA ion pairing is in being able to optimize chromatographic resolution without introducing the ion suppression and gas phase adduct formation that can come with the use of TFA.[9] The sensitivity of the online MS detection observed in this analysis speaks to the potential of DFA based LC-MS. Figures 2B and 2C present the total ion count (TIC) and base peak intensity (BPI) chromatograms obtained for the HuAdV5 sample. From these data, it can be seen that total ion counts approaching $2x10^7$ were obtained, so too were raw mass spectra that could be readily deconvoluted into molecular weight information. The first two peaks of the separation exhibited masses of 1.3 and 3.6 kDa. Their charge states, isotopic resolution, and monoisotopic masses confirmed them to be peptide sized analytes. Such species are present in the AdV proteome, so this is a predictable result. A cursory look at the more strongly retained peaks showed there to be protein species ranging in mass from 10 up to approximately 110 kDa. Several of these species and their masses were interrogated in greater detail.
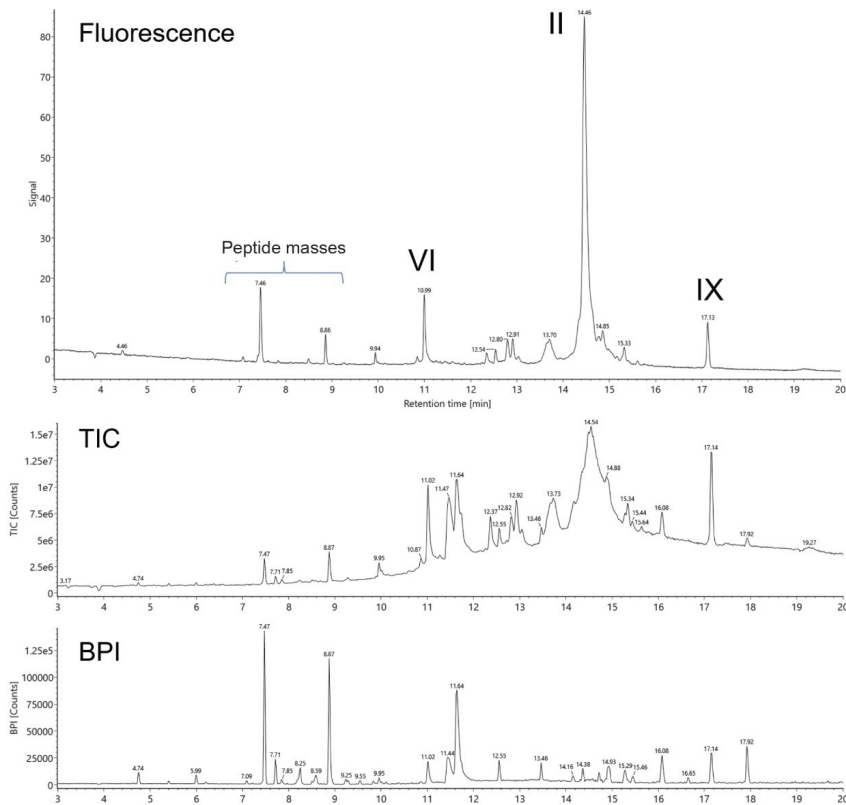
Figure 2. LC-MS chromatograms of adenoviral vector proteins from HuAdV5 GFP as obtained with 2.1 x 150 mm BioResolve RP mAb Polyphenyl 450 Å 2.7 µm Column and difluoroacetic acid (DFA) modified water/acetonitrile mobile phases. Separations were performed with an ACQUITY UPLC H-Class PLUS Bio System, 10 µL injections of 10×10^{12} pfu/mL sample, a flow rate of 0.4 mL/min, and a column temperature of 80 °C. Fluorescence detection was performed along with intact mass analysis by Tof mass spectrometry.

The peak eluting at 10.99-minutes produced the summed mass spectrum shown in Figure 3A. Deconvolution by MaxEnt 1 yielded a molecular weight of 22,100 Da. This is a mass that uniquely matches to protein VI from HuAdV5, and the mass error of the assignment is 0.0 Da. That is, it is an exact match for the sequence annotated as UniProt KB accession number P24937. This protein is also known as the endosome lysis protein and is the highest copy number cement protein present in the AdV particle. Another potential protein identification can be illustrated through the analysis of the LC peak at 17.13-min. The summed mass spectrum for this peak is shown in Figure 3B. The inset shows a corresponding deconvoluted mass spectrum, wherein a mass of 14,369.2 Da was observed. There is only one AdV protein predicted to be in this molecular weight range, and it is protein IX. It too is a cement protein. Protein IX plays a role to interlace between some of the major components of the AdV

capsid structure. The molecular weight observed for Protein IX was not in close agreement with what is predicted from UniProt P0328, which is a mass of 14,327.0 Da. The resulting mass difference is 42.2 Da, which may be attributable to an acetylation or the result of a sequence variant. An alignment of other UniProt submissions shows that AdV protein IX exhibits sequence variability that could likewise explain this mass difference, such as an alanine to leucine or isoleucine substitution.

An analysis of the most abundant species in the chromatogram is noteworthy. This protein marked with a 14.46-minute retention time was detected with the mass spectrum shown in Figure 3C. Deconvolution of this signal produced an observed molecular weight of 107,899.1 Da. The only AdV protein with a predicted molecular weight anywhere near this observed mass is protein II or the hexon protein. Uniprot KB accession number P04133 predicts a molecular weight for this protein that is approximately 23 Da lighter, at a mass of 107,875.9 Da. Despite the difference between observed and theoretical masses, the identification of this protein as the hexon protein is quite credible as it is the most abundant protein in an AdV particle with an estimated 720 copies per viral particle. The analysis of alternative UniProt AdV5 sequences shows there to be a number of common hexon protein sequence variants. One or more of these sequence variants and their corresponding amino acid substitutions might explain the molecular weight that has been observed for this sample's hexon protein. Additional investigation at the peptide level would be needed to confirm the mass deviations observed.
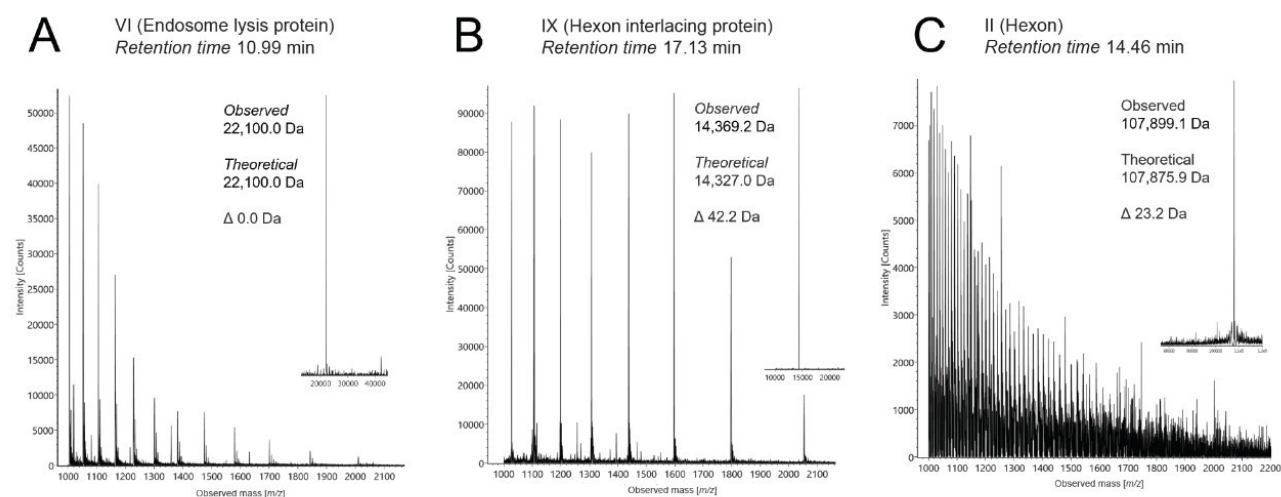

Figure 3. Time of flight mass spectra from ESI-MS obtained for three of the abundant species observed in the HuAdV5 GFP sample. Raw mass spectra and Max Ent 1 deconvoluted masses (inset) from chromatographic peaks observed at (A) 10.99 min, (B) 17.13 min, and (C) 14.46 min. Putative identifications based on the observed MS data have been proposed.

Conclusion

A technique has been proposed for the RPLC analysis of AdV proteins that facilitates both optical and online MS detection such that it is possible to investigate protein copy ratios and simultaneously confirm molecular weight information. This method entails the use of DFA ion pairing and a 2.7 µm 450 Å phenyl bonded stationary phase to effectively separate the AdV proteins while obtaining high sensitivity mass spectra.

This method presents a viable starting point for the characterization of current and future AdV based vaccines and gene therapies. It is expected that this field will grow with demands on both vaccines and gene therapies, and deep analytical understanding will help to overcome barriers for approvals and adoption by the general public. With its ability to produce high titers, efficient transduction, and large packaging capacity, the AdV viral vector is well-positioned as a delivery vehicle for even more advanced therapy medicinal products.

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