: A novel fragmentation-based multiple-attribute methodology (MAM) for AAV8 capsid protein analysis

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ABSTRACT

Adeno-associated virus (AAV) is one of the most commonly used delivery vehicles (vectors) in gene therapy development. It is a combination of proteins that form a closed shell (capsid) encompassing a single-stranded DNA called a transgene. The capsid consists of 3 viral proteins (VPs). The post-translational modifications (PTMs) of VPs can directly impact the viral infectivity and vector potency and are therefore considered critical quality attributes (CQAs) for gene therapy development. Here, an accurate mass LC-MS workflow is reported for analyzing AAV8 capsid proteins. A novel fragmentation type based on ExD was investigated using a comprehensive peptide mapping with multiple attribute methodology (MAM) focused on the relative quantification of PTMs. Three VPs were fully characterized with close to 100% sequence coverage. Multiple PTMs were identified with MS/MS confirmation. Some of them were found obtaining different levels in empty and full capsids.

INTRODUCTION

Recombinant AVV vectors are the most widely used vectors in gene therapy due to their low toxicity and longterm expression ability. AAV is a closed shell of proteins, called a capsid that encompasses a single stranded DNA of about 4.8 kD in size. The viral capsid of AAV8 is composed of 3 main proteins, which are 81 kD (VP1), 66 kD (VP2) and 59 kD (VP3) in size. VPs are critical for viral infectivity and vector potency. Thus, complete characterization of the constituent viral capsid proteins of AAV vectors, including their sequences and PTMs, is highly recommended to ensure AAV product quality and consistency. Herein, an accurate mass LC-MS-based peptide mapping with MAM workflow is reported for analyzing AAV8 empty and full capsids.

MATERIALS AND METHODS

Sample preparation:

Multiple lots of AAV8 samples were purchased from Vigene Biosciences. The titers were 5.10×10^{12} GC/mL– 1.10 × 10¹³ GC/mL. Samples were concentrated with an Amicon Ultra filter (0.5 mL, 10 kDa MWCO) and then buffer exchanged to 25 mM of Tris, pH 8.0. The samples were denatured, reduced, alkylated and digested with trypsin, Lys-C and Asp-N with incubation at 37°C overnight. The digest was subjected to LC-MS analysis (Figure 1).

HPLC conditions:

An ExionLC system (SCIEX) with a reversed-phase C18 column was used. The column oven temperature was set to 50°C. The mobile phases were 0.1% formic acid in water (mobile phase A) and 0.1% formic acid in acetonitrile (mobile phase B). 60-min linear gradient ramping from 1% to 40% B was applied for separation. The flow rate was 0.3 mL/min.

MS and MS/MS conditions:

A ZenoTOF 7600 system (SCIEX) was used for peptide mapping analysis with data-dependent acquisition mode, in which electron activated dissociation (EAD), a type of ExD, was used for MS/MS acquisition. Data were processed with Biologics Explorer software from SCIEX (Figure 2) for peptide mapping analysis followed by SCIEX OS software (SCIEX) for MAM processing.

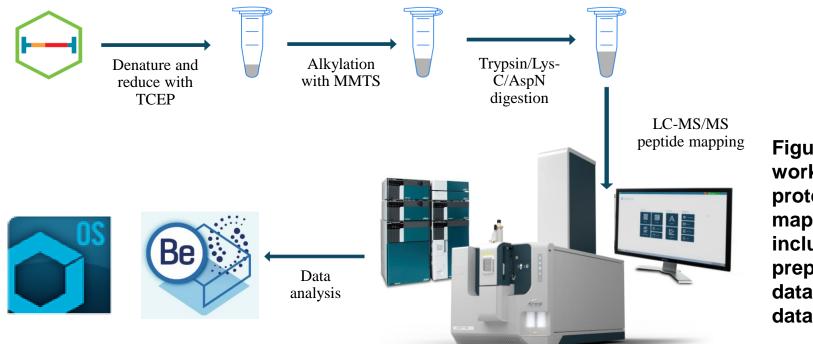


Figure 1. The general workflow for capsid protein peptide mapping analysis, including sample preparation, LC-MS data acquisition and data processing.

RESULTS

Three VPs—VP1, VP2 and VP3—were fully characterized with 100% sequence coverage at MS level and >95% sequence coverage at MS/MS level (Figure 3).

Multiple PTMs were identified with MS/MS confirmation. Representative MS/MS spectra of peptide with PTMs including N-terminal acetylation, phosphorylation, deamidation and oxidation are shown in Figure 4. It is worth noting the identification and localization of the low-level PTMs (~0.1%) were achieved with solid MS/MS verification.

Selected PTMs went through the downstream MAM processing for full and empty AAV8 samples for proof-ofconcept purposes. A batch processing in SCIEX OS software was executed to directly report the level of the PTMs (Figure 5). The levels were calculated as the peak area ratios between modified peptide and the sum of modified and unmodified peptides, based on the peak integrations of MS-level exacted ion chromatograms (XICs). Some PTMs were found obtaining different levels in empty and full capsids (Table 1).



Figure 3. Sequence coverage map for VP1, VP2 and VP3. Green labeling represents the sequences that were verified with both MS and MS/MS, and blue labeling represents the sequences with MS verification.

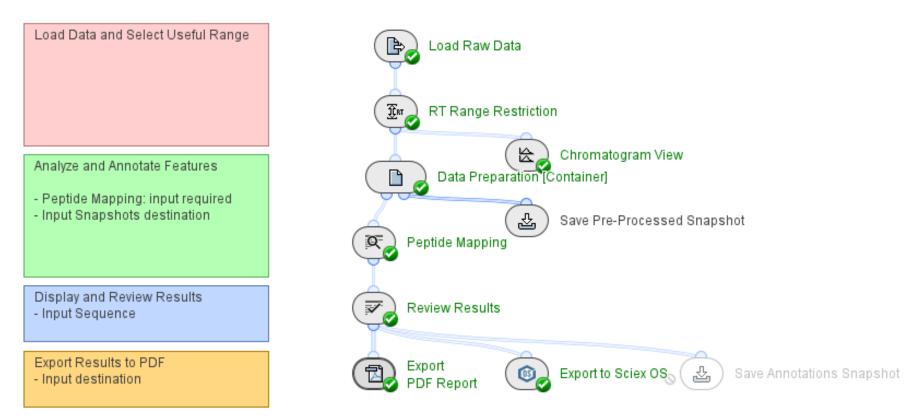


Figure 2. Overview of the peptide mapping workflow using Biologics Explorer software.

					VP1						
		30 L K P G A P K P K A N			60 ENCLORICEDA	70 / N A A D A A A I E H	80	90 			
	LJLGIKLWWA	LKFGAFKFKAN	QQKQDDAKAL	VEFGIKIEGF							K AVIQ
130	140	150	160	170	180	190	200	210	220	230	240
	GAKTAPGKKR	PVEPSPQRSPD	<u></u>	Q P A R K R L N F G	QTGDSESVPD	PQPLGEPPAA	PSGVGPNTMA		NEGADGVGS	5 5 G N W H C D 5 T V	
250	260	270	280	290	300	310	320	330	340	350	360
ALPTYN	NHLYKQISNG	T S G G A T N D N T Y	FGYSTPWGYF	DFNRFHCHFS	PRDWQRLINM	INWGFRP <u>K</u> RLS	FKLFNIQVKE	V T Q N E G T K T I	ANNLTSTIQ	VFTDSEYQLPY	VLGSA
370	380	390	400	410	420	430	440	450	460	470	480
PADVFM	IPQYGYLTLN	N G S Q A V G <u>R</u> S S F	YCLEYFPSQM	IL <mark>R</mark> TGNNFQFT	YTFEDVPFHS	5 Y A H S Q S L D R	LMNPLIDQYL	YYLS <u>R</u> TQTTG	GTANTQTLG	FSQGGPNTMAN	IQA <u>K</u> NW
490	500	510	520	530	540	550	560	570	580	590	600
Q <u>R</u> V S T T	TGQNNNSNFA	W T A G T K Y H L N G	RNSLANPGIA	MATHKDDEER	FFPSNGILIF	G K Q N A A R D N A	DYSDVMLTSE	E E I K T T N P V A	TEEYGIVAD	NLQQQNTAPQI	GTVNS
610	620	630	640	650	660	670	680	690	700	710	720
WQNRDV	YLQGPIWAKI	PHTDGNFHPSP	LMGGFGL <u>K</u> HP	P P Q I L I <mark>K</mark> N T P	V P A D P P T T F N	IQ S K L N S F I T Q	YSTGQVSVEI	EWELQKENSK	RWNPEIQYT	5 N Y Y K S T S V D F	AVNTE
730	740	750	760	770	780	790	800	810	820	830	840
IGTRYL	TRNL										
10	20	30	40	50	VP2	70	80	90	100	110	120
EPSPQR	SPDSSTGIGK	KGQQPARKRLN	FGQTGDSESV		A A P S G V G P N 1			ssss NWHCDs	TWLGDRVIT	TSTR TWALPTY	NNHLY
130	140	150	160	170	180	190	200	210	220	230	240
GGATND	NTYFGYSTPW	GYFDFNRFHCH	FSPRDWQRLI	N N N W G F R P K R	LSFKLFNIQV	KEVTQNEGTK	TIANNLTSTI	QVFTDSEYQL	PYVLGSAHQ	GCLPPFPADVF	MIPQY
250	260	270	280	290	300	310	320	330	340	350	360
5 Q A V G <u>R</u>	SSFYCLEYFP	SQML <u>R</u> TGNNFQ	F T Y T F E D V P F	H 5 5 Y A H 5 Q 5 L	DRLMNPLIDO	<u> </u>	ΤGGTΑΝΤQΤΙ	GFSQGGPNTM	ANQA <u>K</u> NWLP	SPCYRQQRV51	TTGQN
370	380	390	400	410	420	430	440	450	460	470	480
A G T <u>K</u> Y H	LNGRNSLANP	G I A M A T H K D D E	ERFFPSNGIL	IFGKQNAARD	NADYSDVMLI	5 <u>E E E I K</u> T T N P	VATEYGIVA	DNLQQQNTAP	QIGTVNSQG	ALPGMVWQN <u>R</u> E	VYLQG
490	500	510	520	530	540	550	560	570	580	590	600
TDGNFH	PSPLMGGFGL	K H P P P Q I L I K N	T P V P A D P P T T	FNQSKLNSFI	ταγεταανει	EIEWELQKEN	SKRWNPEIQY	' T S N Y Y <u>K</u> S T S V	DFAVNTEGV	Y S E P R P I G T R Y	LTRNL
10	20	20	40	50	VP3	70	80	00	100	110	120
	ADGVGSSGN	WHCDSTWLGDR	VITTSTR TWA		QISNGTSGGA	TNDNTYFGYS	TPWGYFDFNF	FHCHFSPRDW			ΝΙΟΥΚ
120	140	150	160	170	190	100	200	210	220	220	240
K TIANN	LTSTIQVFTD	s <u>ε</u> γ Q L P Y V L G S	AHQGCLPPFP	ADVFMIPQYG	YLTLNNGSQA	VGRSFYCLE	YFPSQMLRTG	NNFQFTYTFE	DVPFHSSYAI	H S Q S L D R L M N F	LIDQY
250	260	270	280	290	300	310	320	330	340	350	360
TTGGTA	NTQTLGFSQG	БРИТМА́ N Q A <u>K</u> N	WLPGPCYRQQ	RVSTTTGQNN	NSNFAWTAGT	KYHLNGRNSL	ANPGIAMATH	KDDEERFFPS	NGILIFGKQ		VMLTS
370	380	390	400	410	420	430	440	450	460	470	480
PVATEE	YGIVADNLQQ	QNTAPQIGTVN	SQGALPGMVW	Q N R D V Y L Q G P	IWAKIPHTDG	NFHPSPLMGG	FGLKHPPPQI	LIKNTPVPAD	PPTTFNQSK	LNSFITQYSTO	QVSVE
490	500	510	520	530	540	550	560	570	580	590	600
NSKRWN	PEIQYTSNYY	<u>K</u> 5 T 5 V <u>D</u> F A V N T	EGVYSEPRPI	GTRYLTRNL							

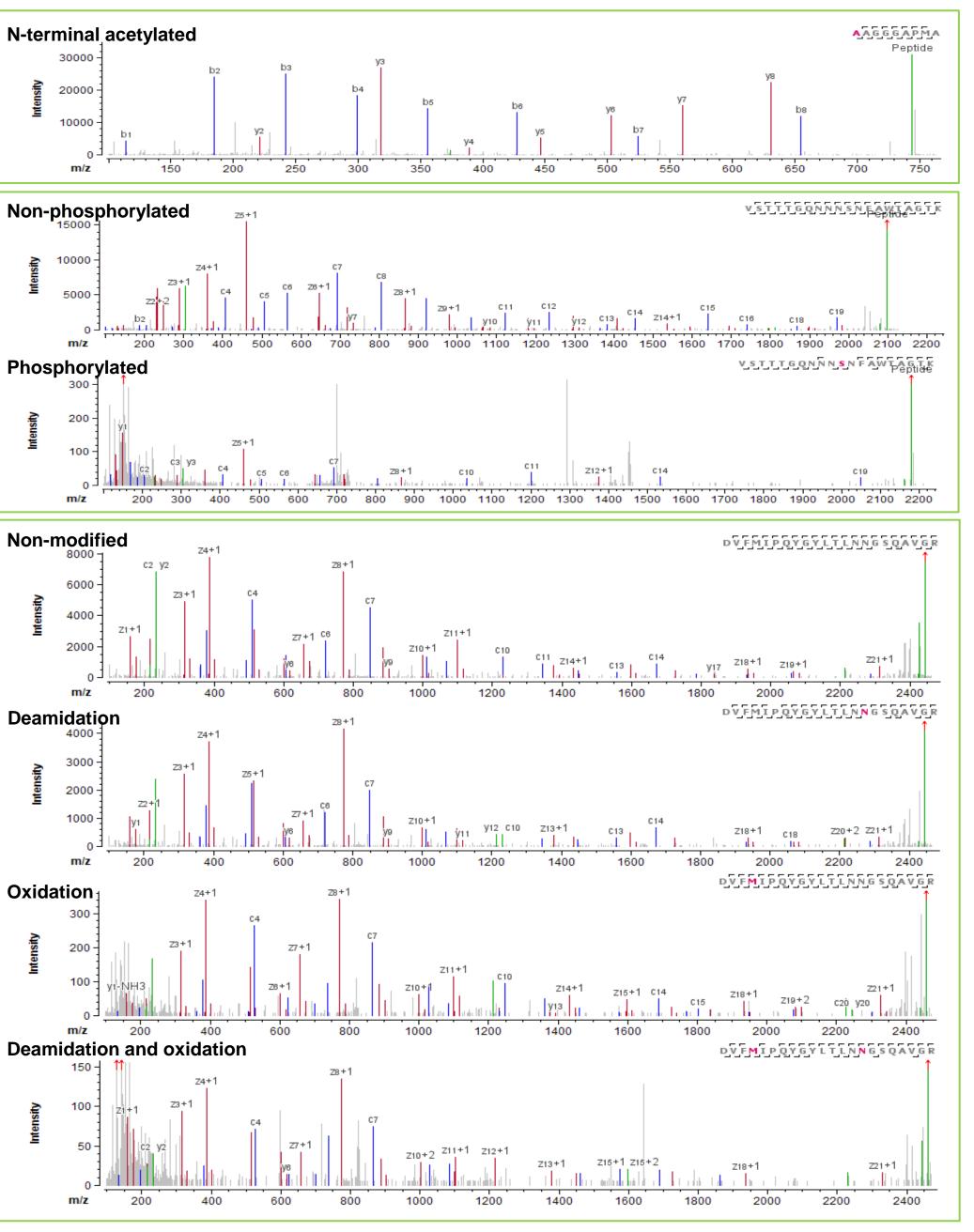


Figure 4. Representative EAD MS/MS spectra for AAV peptides. From top to bottom: (acetyl)AAGGGAPMA with N-terminal acetylation from VP3; VSTTTGQNNNS(phos)NFAWTAGTK from all VPs (including native and phosphorylated forms); DVFM(oxi)IPQYGYLTLNN(dea)GSQAVGR from all VPs (including native, deamidation, oxidation, and deamidation + oxidation forms).

▙	ک ک	88	
Component Name	🗸 Area 🗸	Mass Error ⊽	*1
AAGGGAPMA_Acetyl_[Protein_N-term]_M+H_1	6.510e6	-0.3	2
AAGGGAPMA_Acetyl_[Protein_N-term]_M+2H_2	4.144e4	-0.3	2
AAGGGAPMA_Acetyl_[Protein_N-term]_NotUsed	N/A	N/A	2
AAGGGAPMA_M+H_1	1.403e5	-0.7	2
AAGGGAPMA_M+2H_2	N/A	N/A	2
AAGGGAPMA_M+2H_Notused	N/A	N/A	2
VSTTTGQNNNSNFAWTAGTK_Native_M+2H_2	6.134e6	0.8	0
VSTTTGQNNNSNFAWTAGTK_Native_M+3H_3	7.227e6	0.6	0
VSTTTGQNNNSNFAWTAGTK_Native_M+4H_4	1.627e4	0.1	0
VSTTTGQNNNSNFAWTAGTK_Phospho_[S11]_M+3H_3	1.219e4	-0.5	0
VSTTTGQNNNSNFAWTAGTK_Phospho_[S11]_NotUsed_1	N/A	N/A	0
VSTTTGQNNNSNFAWTAGTK_Phospho_[S11]_NotUsed_2	N/A	N/A	0
DVFMIPQYGYLTLNNGSQAVGR_Native_M+3H_3	1.432e7	2.1	3
DVFMIPQYGYLTLNNGSQAVGR_Native_M+2H_2	4.988e6	1.0	3
DVFMIPQYGYLTLNNGSQAVGR_Native_M+4H_4	2.626e5	0.6	3
DVFMIPQYGYLTLNNGSQAVGR_Deamidated_[N15]_M+3H_3	7.477e6	1.3	3
DVFMIPQYGYLTLNNGSQAVGR_Deamidated_[N15]_M+2H_2	2.022e6	0.3	3
DVFMIPQYGYLTLNNGSQAVGR_Deamidated_[N15]_M+4H_4	1.363e5	-0.2	3
DVFMIPQYGYLTLNNGSQAVGR_Oxidation_[M4]_M+3H_3	1.341e5	-0.3	3
DVFMIPQYGYLTLNNGSQAVGR_Oxidation_[M4]_M+2H_2	9.589e3	0.4	3
DVFMIPQYGYLTLNNGSQAVGR_Oxidation_[M4]_NotUsed_1	N/A	N/A	3
DVFMIPQYGYLTLNNGSQAVGR_Deamidated_[N15]_Oxidation_[M	5.713e4	0.1	3
DVFMIPQYGYLTLNNGSQAVGR_Deamidated_[N15]_Oxidation_[M	6.065e3	-0.6	3
DVFMIPQYGYLTLNNGSQAVGR_Deamidated_[N15]_Oxidation_[M	N/A	N/A	3

Figure 5. MAM processing user interface in SCIEX OS software. Left: result table with peptide and ion information, peak areas and calculated PTM%. Right: peak integration parameters, XICs and MS spectra.

CONCLUSIONS

EAD-based data-dependent analysis serves as an efficient workflow for in-depth peptide mapping combined with MAM in AAV8 capsid protein analysis.

REFERENCES

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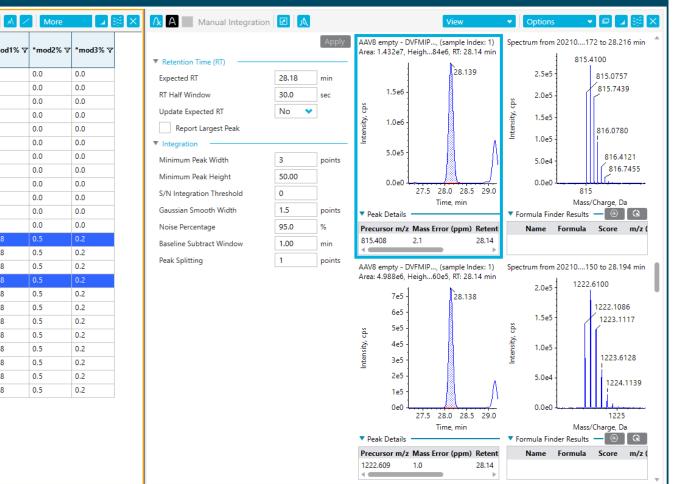


Table 1. Levels of selected PTMs in empty and full capsids, calculated using SCIEX OS software processing

Peptide sequences	Modification %		
	Empty	Full	
(acetyl) A AGGGAPMA	2.1	4.7	
VSTTTGQNNN S (phos)NFAWTAGTK	0.1	0.1	
DVFMIPQYGYLTLN N (dea)GSQAVGR	32.8	26.9	
DVF M (oxi)IPQYGYLTLNNGSQAVGR	0.5	0.6	
DVF M (oxi)IPQYGYLTLN N (dea)GSQAVGR	0.2	0.2	

Xiaoying Jin, Lin Liu, Shelley Nass, Catherine O'Riordan, Eric Pastor and X. Kate Zhang. Direct Liquid Chromatography/Mass Spectrometry Analysis for Complete Characterization of Recombinant Adeno-Associated Virus Capsid Proteins. Human Gene Therapy Methods, 2017, 5, 255-267. 2 A new electron activated dissociation (EAD) approach for comprehensive glycopeptide analysis of therapeutic proteins. SCIEX technical note, RUO-MKT-02-12980-A.