Forced Degradation Study of AAV Empty Capsid

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ABSTRACT

Stress studies play a vital role in identifying potential degradation pathways which affect the AAV transduction efficiency. Currently, little is known regarding the degradation pathway of various AAV serotypes. The following explorative study focused on two specific serotypes: AAV8 and AAV9, which are widely used in the development of critical gene therapy products.

The forced degradation study was performed to qualitatively and quantitatively compare the effect of freeze-thaw, high temperature, high pH, and oxidative stress on PROGEN's empty AAV8 and AAV9 protein capsids.

Size Exclusion-High Performance Liquid Chromatography (SEC-HPLC), Dynamic Light Scattering (DLS), and Liquid Chromatography-Mass Spectroscopy (LC-MS) were employed to identify the degradation mechanism for the AAV8 and AAV9 serotypes.

Sedimentation Velocity Analytical Ultracentrifugation (SV-AUC) was executed to qualify the developed method for the determination of percent empty capsid for the AAV9 serotype.

EXPERIMENTAL METHODS

Size Exclusion-High Performance Liquid Chromatography (SEC-HPLC)

- Performed on the Waters e2965 HPLC System utilizing the SEPAX SRT-500™ HPLC column and 2x PBS mobile phase to monitor AAV capsid absorbance at 210 nm.

Dynamic Light Scattering (DLS)

- Performed on the Wyatt DynoPro NanoStar preset to derive multimodal hydrodynamic radius and percent polydispersity.

Peptide Mapping (LC-MS)

- Performed on Thermo Vanquish LC System coupled to the Orbitrap 480 Exploris utilizing Thermo Hypersil GOLD™ C18 Selectivity HPLC column and 0.1% FA in water (A) / ACN (B) mobile Phases.

Sedimentation Velocity Analytical Ultracentrifugation (SV-AUC)

- Performed on the Beckman Coulter XLI/A Analytical Ultracentrifuge set to 12000 RPM at 20ºC to monitor empty, full and intermediate capsids sedimentation at 230 nm. BASIS[1] (BioAnalysis SEDFIT Integrated Software) was employed to generate the c(s) distribution from the raw data and final percentages under 21CFR11 and cGMP compliance.

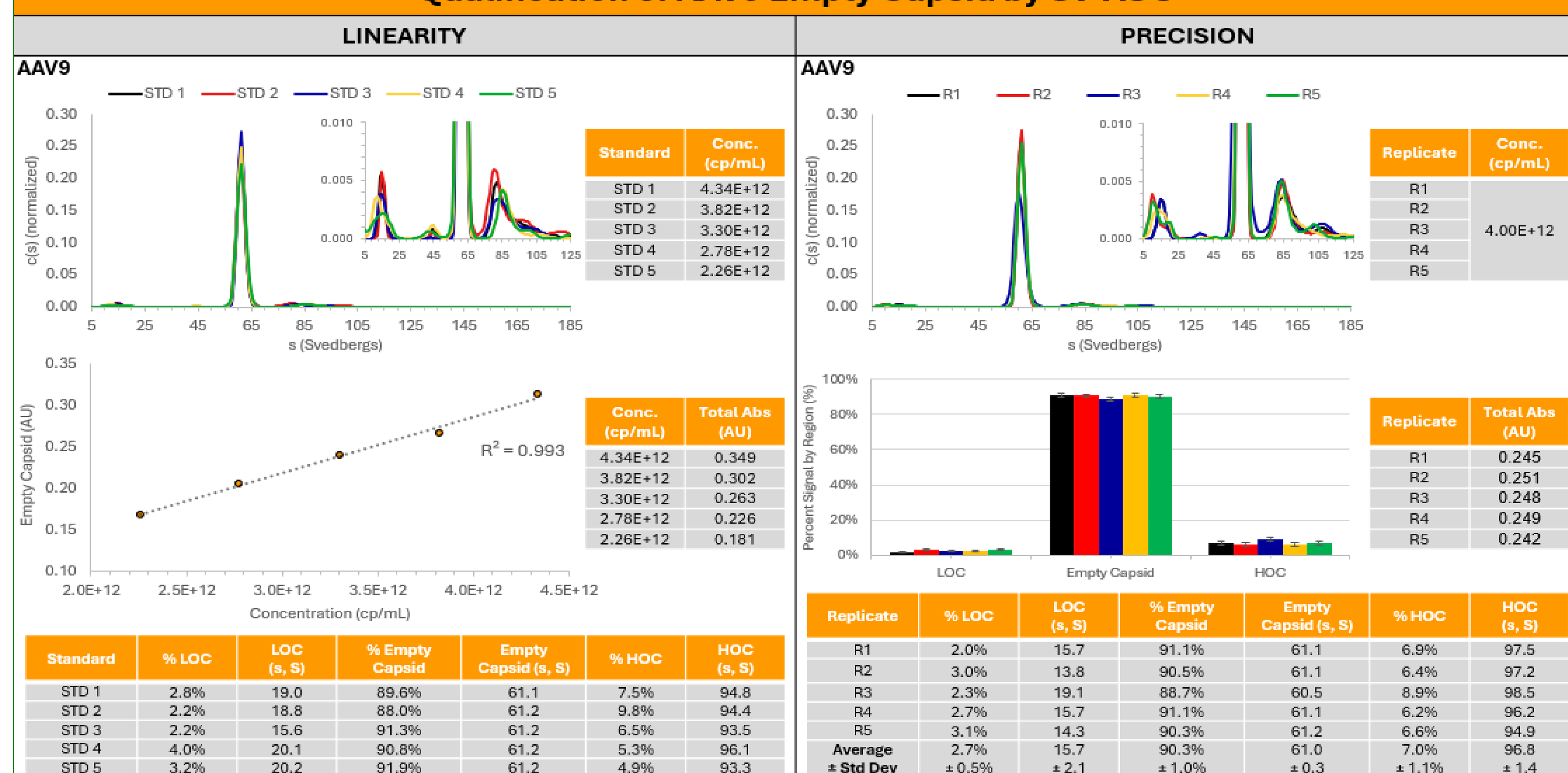
SAMPLE PREPARATION

Size Exclusion-High Performance Liquid Chromatography (SEC-HPLC): For the freeze-thaw (AAV-FT), high temperature (AAV-45°C), and oxidative (AAV-Ox) stressed samples, the PROGEN AAV sample was buffer exchanged into 2x PBS, 0.001% Poloxamer 188. The oxidative (AAV-Ox) stressed samples were further spiked with 0.1% hydrogen peroxide. For the high pH (AAV-pH9) stressed samples, the PROGEN AAV sample was buffer exchanged into 50mM Tris-HCl pH 9, 0.001% P188.

Dynamic Light Scattering (DLS) / Peptide Mapping (LC-MS): AAV samples prepared for SEC-HPLC were aliquoted for DLS and LC-MS testing. LC-MS samples were digested using a combination of Lys-C/Trypsin and Chymotrypsin to maximize peptide coverage.

Sedimentation Velocity Analytical Ultracentrifugation (SV-AUC): The PROGEN AAV sample was buffer exchanged into 2x PBS, 0.001% Poloxamer 188 and diluted to target concentration using 1x PBS.

SV-AUC METHOD QUALIFICATION



Standard	Conc. (cp/mL)
STD 1	4.34E+12
STD 2	3.82E+12
STD 3	3.30E+12
STD 4	2.78E+12
STD 5	2.26E+12

Conc. (cp/mL)	Total Abs (AU)
4.34E+12	0.349
3.82E+12	0.302
3.30E+12	0.263
2.78E+12	0.226
2.26E+12	0.181

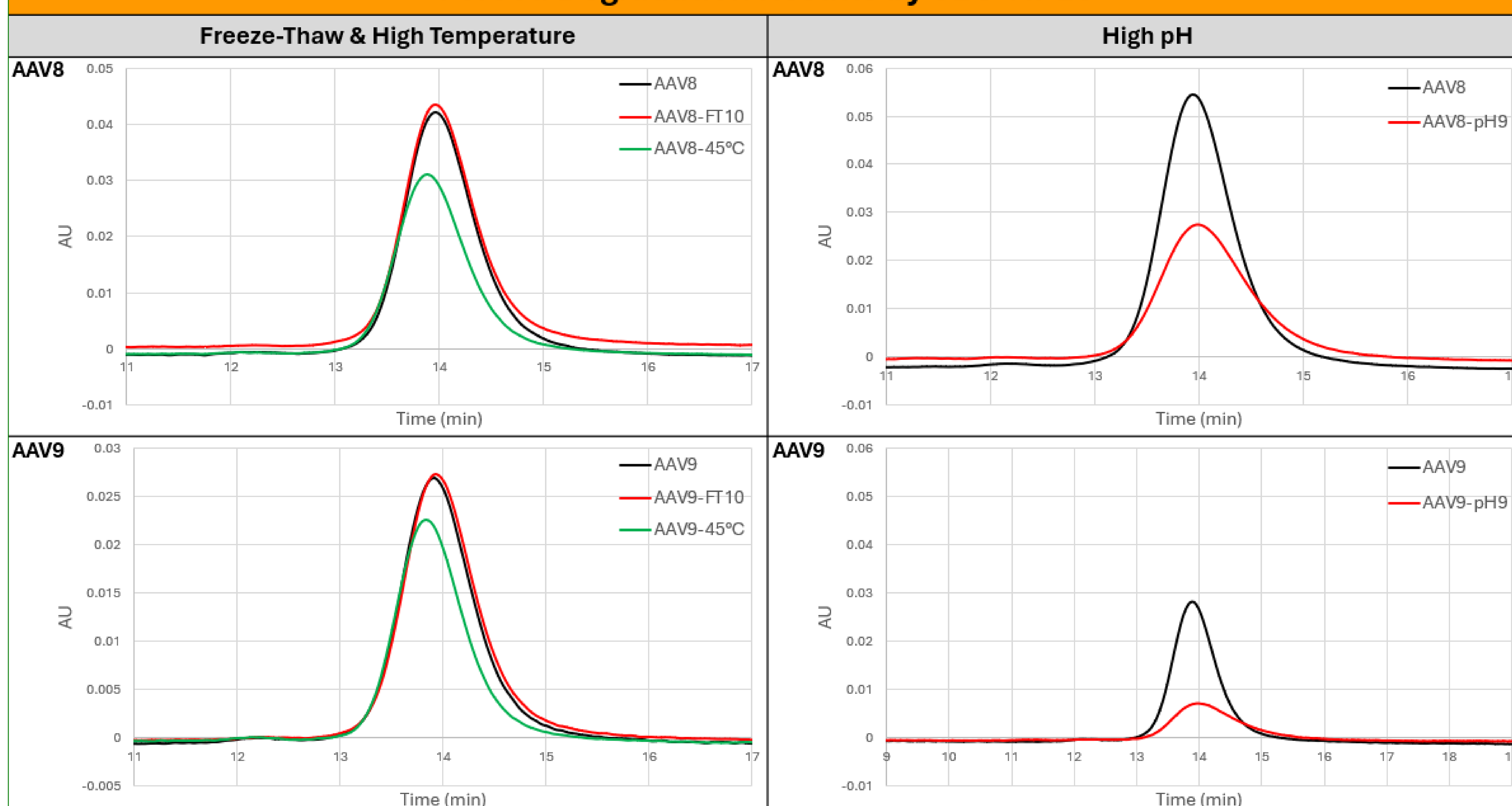
Standard	% LOC	LOC (s, S)	% Empty Capsid	Empty Capsid (s, S)	% HOC	HOC (s, S)
STD 1	2.8%	19.0	89.6%	61.1	7.5%	94.8
STD 2	2.2%	18.8	88.0%	61.2	9.8%	94.4
STD 3	2.2%	15.6	91.3%	61.2	6.5%	93.5
STD 4	4.0%	20.1	90.8%	61.2	5.3%	96.1
STD 5	3.2%	20.2	91.9%	61.2	4.9%	93.3

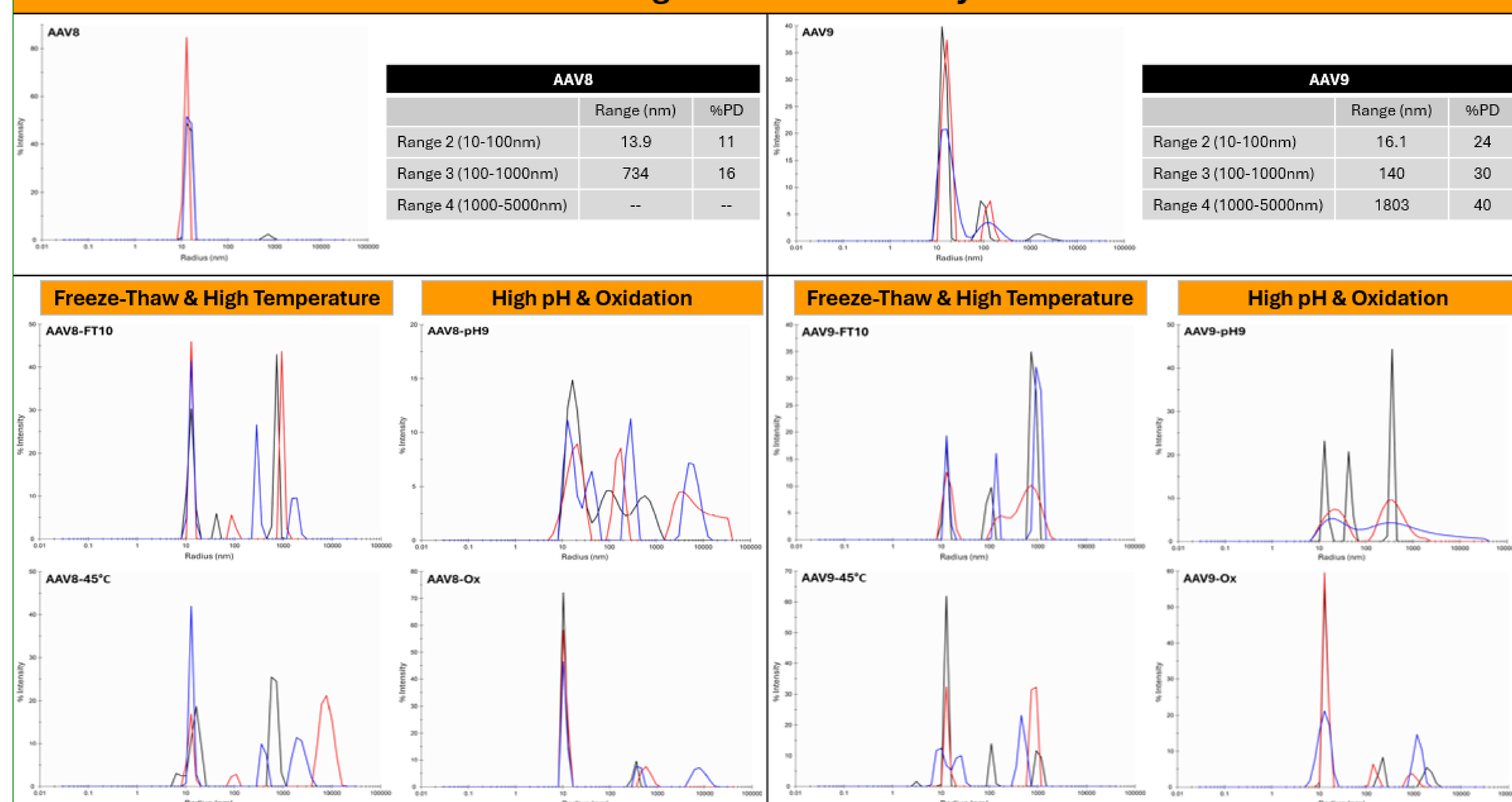
Replicate	Conc. (cp/mL)
R1	4.00E+12
R2	
R3	
R4	
R5	

Replicate	Total Abs (AU)
R1	0.245
R2	0.251
R3	0.248
R4	0.249
R5	0.242

Replicate	% LOC	LOC (s, S)	% Empty Capsid	Empty Capsid (s, S)	% HOC	HOC (s, S)
R1	2.0%	15.7	91.1%	61.1	6.9%	97.5
R2	3.0%	13.8	90.5%	61.1	6.4%	97.2
R3	2.3%	19.1	88.7%	60.5	8.9%	98.5
R4	2.7%	15.7	91.1%	61.1	6.2%	96.2
R5	3.1%	14.3	90.3%	61.2	6.6%	94.9
Average ± Std Dev	2.7% ± 0.5%	15.7 ± 2.1	90.3% ± 1.0%	61.0 ± 0.3	7.0% ± 1.1%	96.8 ± 1.4

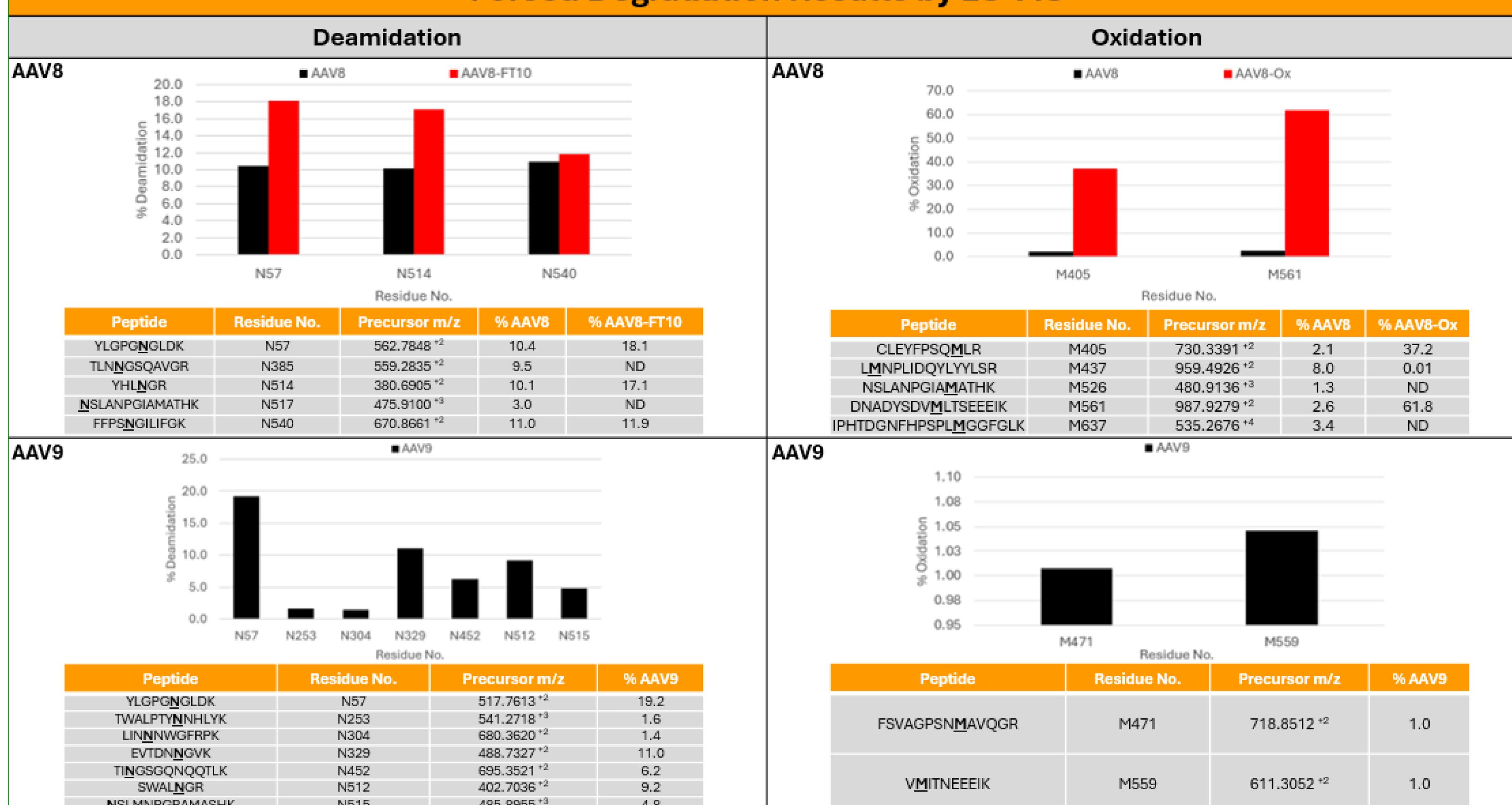
FORCED DEGRADATION STUDY





AAV8	Range (nm)	%PD
Range 2 (10-100nm)	13.9	11
Range 3 (100-1000nm)	734	16
Range 4 (1000-5000nm)	--	--

AAV9	Range (nm)	%PD
Range 2 (10-100nm)	16.1	24
Range 3 (100-1000nm)	140	30
Range 4 (1000-5000nm)	1803	40



Peptide	Residue No.	Precursor m/z	% AAV8	% AAV8-FT10
YLGPGNGLDK	N57	562.7848 $^{+2}$	10.4	18.1
TLNNGSQAVGR	N385	559.2835 $^{+2}$	9.5	ND
YHLNGR	N514	380.6905 $^{+2}$	10.1	17.1
NSLANPGIAMATHK	N517	475.9100 $^{+3}$	3.0	ND
FFPSNGILIFGK	N540	670.8661 $^{+2}$	11.0	11.9

Peptide	Residue No.	Precursor m/z	% AAV8	% AAV8-Ox
CLEYFPSQMLR	M405	730.3391 $^{+2}$	2.1	37.2
LMNPLIDQYLYYLSR	M437	959.4926 $^{+2}$	8.0	0.01
NSLANPGIAMATHK	M526	480.9136 $^{+3}$	1.3	ND
DNADYSDVMLTSEEEIK	M561	987.9279 $^{+2}$	2.6	61.8
IPHTDGNFHPSPLMGGFGLK	M637	535.2676 $^{+4}$	3.4	ND

Peptide	Residue No.	Precursor m/z	% AAV9
YLGPGNGLDK	N57	517.7613 $^{+2}$	19.2
TWALPTYNNHLYK	N253	541.2718 $^{+3}$	1.6
LINNNWGFRPK	N304	680.3620 $^{+2}$	1.4
EVTDNNGVK	N329	488.7327 $^{+2}$	11.0
TINGSGQNQQTLK	N452	695.3521 $^{+2}$	6.2
SWALNGR	N512	402.7036 $^{+2}$	9.2
NSLMNPGPAMASHK	N515	485.8955 $^{+3}$	4.8

Peptide	Residue No.	Precursor m/z	% AAV9
FSVAGPSNMAVQGR	M471	718.8512 $^{+2}$	1.0
VMITNEEEIK	M559	611.3052 $^{+2}$	1.0

CONCLUSIONS

SEC-HPLC / DLS: Forced degradation of AAV8 and AAV9 empty capsid was executed by freeze-thaw, high temperature, high pH, and oxidation via hydrogen peroxide. Aggregation was mainly observed by DLS in high temperature, high pH, and oxidative samples.

Only the loss stressed of the AAV monomer was observed by SEC due to the high molecular weight nature of the aggregates formed. No aggregation was observed in the samples after completion of the 10 freeze-thaw cycles.

LC-MS: Peptide mapping concluded a high percentage of deamidation at surface exposed asparagine N57 for AAV9 (up to 20%), whereas deamidation of asparagine residues for AAV8 were approximately 10%. Low levels of methionine oxidation were identified for the AAV9 at two methionine residues, whereas AAV8 methionine oxidation was observed at five methionine residues at low levels except for M437 with 8%.

The peptide coverage was low in the stressed AAV9 and AAV8 samples possibly due to low starting concentrations and additional sample loss due to aggregation. Only methionine M405 and M561 were detected after oxidative stress with an increase of oxidation up to 37% and 62%, respectively.

SV-AUC: AAV9 empty capsid was used to qualify the SV-AUC method. Empty capsid standards are ideal for spiking studies (i.e., accuracy) associated with cGMP method validation. The results indicate excellent linearity and precision in the concentration range of interest.

References

1. Yarawsky, A.E., Gough, E.S., Zai-Rose, V. et al. BASIS: BioAnalysis SEDFIT integrated software for cGMP analysis of SV-AUC data. *Eur Biophys J* 53, 111–121 (2024).