A POWERFUL CAPILLARY ELECTROPHORESIS PLATFORM FOR THE CHARACTERIZATION OF VIRAL CAPSID PROTEINS OF VECTORS USED IN CELL AND GENE THERAPIES

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INTRODUCTION

Viral vectors like adeno-associated viruses (AAV) and lentiviral viruses (LVV) have come to the forefront of biotherapeutic development in recent years in support of cell and gene therapies. Like conventional biologics such as monoclonal antibodies, these viruses require careful manufacturing and testing to ensure product quality, consistency, safety, and efficacy. A series of critical qualities and attributes (together referred to as CQAs) are assigned to control for these. The physiochemical properties of these vectors will determine viral vector identity, purity, stability, and potency. A key challenge in the industry today is that many analytical tools are required for this testing. This requires significant capital investment, training, and space, which means more of the precious materials need to be put aside for characterization and less is available for release. Capillary electrophoresis (CE) serves as a potent technique for characterizing and monitoring several of these CQAs. Among CE instruments, the Maurice™ CE system stands out for the comprehensive characterization of viral capsids. In this poster, we show how Maurice provides both CE-SDS and icIEF analyses for AAVs and LVV.

MATERIALS AND METHODS

MATERIALS

Instrument: MauriceFlex™ (ProteinSimple), cIEF, Turbo CE-SDS, and Flex cartridges (**Figure 1**).
Samples: AAV9 particles (Virovek) at 2 X 10^{13} VP/mL, LVV particles (Takara) at 1.1 X 10^{10} TU/mL.

Additional Materials: Amicon™ Ultra-0.5 Centrifugal Filter Units, 10K MWCO (Milipore UFC500396), and method-specific reagents listed below in **Table 1**.

Table1. Additional reagents used in this study.

Reagent	Vendor	Part Number
Urea powder	SIGMA	33247
Triton X-100 reduced		X100RS
CHAPS hydrate		C9426
Dithiothreitol (DTT)		D0632
Formamide (>99.5%)		47671
Dimethyl sulfoxide (DMSO)		D2650
2-Mercaptoethanol		M6250
ASB -14		A1346
Pharmalyte 3-10	Cytiva	17-0456-01
Pharmalyte 5-8		17-0453-01
pI marker 5.85	ProteinSimple	102225
pI marker 8.40		102229
pI marker 4.65		102223
pI marker 9.50		101996
1% methycellulose		101876
Maurice CE-SDS PLUS Application Kit		PS-MAK03-S
Maurice Turbo CE-SDS Application kit		PS-MAK01-TS

METHODS

Imaged cIEF Analysis of AAV capsid proteins
To 5 µL AAV (2 x 10^{13} GC/mL), 1 µL of 160 mM DTT and 7 µL DMSO are added are mixed. The mixture is then heated at 70ºC for 10 minutes to denature the sample. The denatured sample is then cooled down to room temperature and kept at room temperature prior to analysis. The final IEF sample solution contains 50% formamide, 0.35% methylcellulose, 2% Pharmalyte 3-10, 2% Pharmalyte 5-8, and pI markers 5.85 and 8.40. Denatured AAVs were focused at 1.5 kV for 1 minute, then at 3 kV for 12 minutes and imaged using native fluorescence (20 – 80 second exposures).

Imaged cIEF Analysis of LVV capsid proteins
LVV was inactivated in 0.5% Triton X-100 reduced at room temperature for 20 min. The inactivated LVV is denatured at 95C for 10 min in 16 mM DTT, 1% CHAPS and 0.5% ASB-14. The denatured LVV is diluted by cIEF master mix by 15 times to prepare the final sample solution. The final sample solution contains 9 M urea, 10% CHAPS, 4 mM DTT, 0.25% Triton X-100 reduced , 0.01% ASB-14, 0.35% methylcellulose, 4% Pharmalyte 3-10 and pI markers 4.65 and 9.50. The focusing time is 1.5 kV for 1 min, then 3 kV for 8 min. The samples were imaged using native fluorescence (10 – 80 second exposure time).

Turbo CE-SDS Analysis of AAV capsid proteins
Cold acetone (4X the sample volume) was added to 20 µL of the AAV sample and briefly vortexed. The sample was kept at -20 °C for an hour, followed by centrifugation at room temperature for 10 minutes at 15000 xg to pellet the proteins. The supernatant was removed carefully, and the precipitate was allowed to dry for 5 minutes. The precipitate was dissolved in the same volume of CE-SDS PLUS buffer as the AAV sample (20 µL) and vortexed. For denaturation, 0.7 M β-mercaptoethanol (β-ME) was added to the buffer first, and then incubated at 70 °C for 10 minutes. The sample was then cooled on ice for 5 minutes and spun down with a microcentrifuge before addition of distilled water up to 100 µL final for Turbo CE-SDS. For analysis with Turbo CE-SDS, samples were injected for 8 seconds at 3500 V and separated for 8 minutes at 4200 V. All data were analyzed with Compass for iCE software.

CE-SDS PLUS Analysis of LVV capsid proteins
LVV particles (1.1E+10 TU/mL) were heat inactivated at 95°C for 2 minutes. After inactivation, the sample was kept on ice for immediate use or at -80°C for later use. For protein extraction, cold acetone precipitation was used as described above except for dissolving the pellet in 2% SDS containing 200mM bicine (pH 5.5).

Figure 1. MauriceFlex™ instrument and cartridges.

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LVV App Note

AAV App Note

AAV IEF Paper

RESULTS

CHARACTERIZATION OF LENTIVIRAL CAPSID PROTEINS

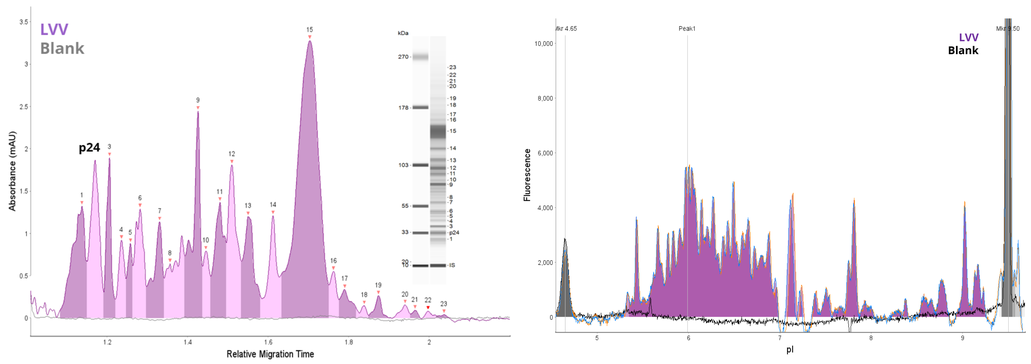


Figure 2. Analysis of LVV by CE-SDS and icIEF. LVV (1 x 10^9 TU/mL) was analyzed as described in the methods and compared to a blank by (A) CE-SDS Plus and (B) icIEF. Both methods offer rapid and unique fingerprints of the LVV capsid proteins.

RESULTS

TURBO CE-SDS CHARACTERIZATION OF AAV CAPSID PROTEINS

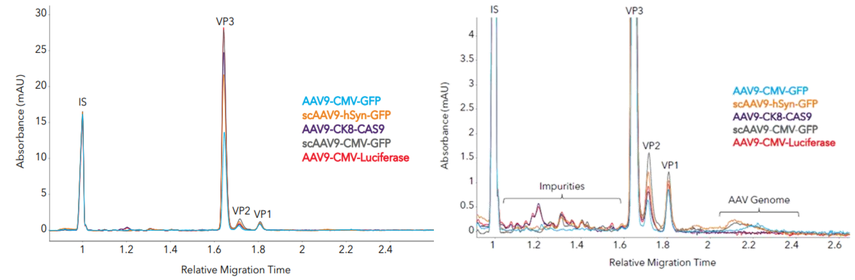


Figure 3. Measuring AAV capsid ratios and impurities in different AAV9 samples with Maurice Turbo CE-SDS. (A) Five AAV samples with different inserts were analyzed with Turbo CE-SDS and analyzed for capsid protein ratio and impurities. (B) Zoom-in to show impurities and AAV genomes.

Table 2

	VP3		VP2	
	PLUS	TURBO	PLUS	TURBO
AAV9-CMV-GFP	16.09	15.10	0.87	0.84
scAAV9-hSyn-GFP	20.73	17.96	1.49	1.34
AAV9-CK8-CAS9	27.31	27.28	1.40	1.36
scAAV9-CMV-GFP	21.40	21.01	1.64	1.80
AAV9-CMV-Luciferase	26.98	26.22	1.40	1.30

Table 2. AAV9 capsid protein ratios from Turbo CE-SDS and CE-SDS Plus (see our other poster!)

Table 3

	Percent Impurities
	Turbo
AAV9-CMV-GFP	6.34
scAAV9-hSyn-GFP	7.29
AAV9-CK8-CAS9	15
scAAV9-CMV-GFP	6.5
AAV9-CMV-Luciferase	16.09

Table 3. Impurity analysis of AAV9 samples.

IMAGED cIEF CHARACTERIZATION OF AAV CAPSID PROTEINS

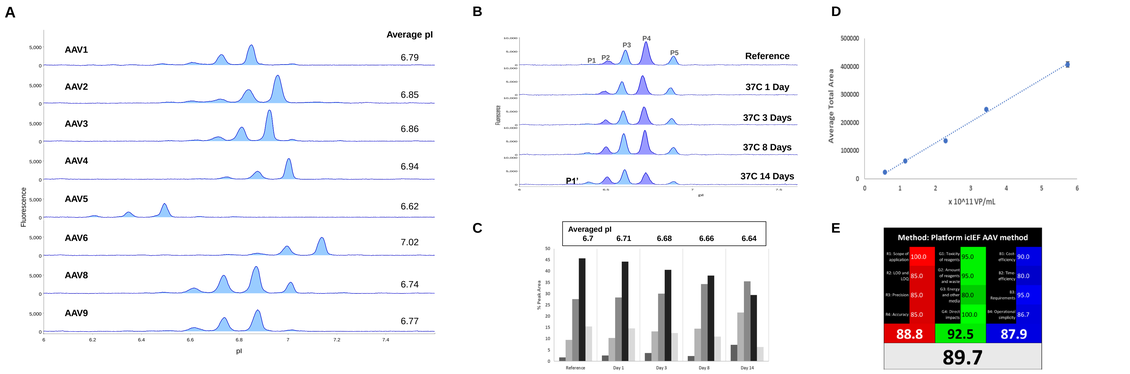
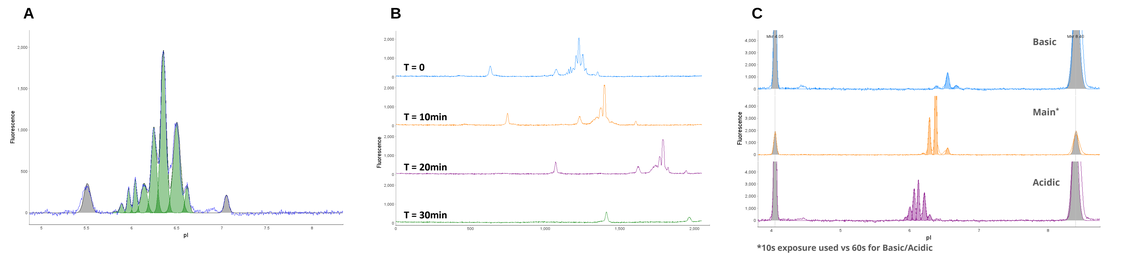


Figure 4. Imaged cIEF AAV Capsid Protein Method. (A) AAVs 1–9 were denatured prior to analysis by icIEF with native fluorescence detection (20s exposure). The averaged pI was determined from 3 injections of each AAV sample. **(B)** AAV8 (4.7 ×10^{11} VP/mL) was incubated at 37 °C prior to analysis. **(C)** Quantitation of AAV8 averaged pI (top panel) and %PA changes over time (bar graph). **(D)** AAV9 was denatured and then serially diluted two-fold from ~ 6 x 10^{11} VP/mL to 6 x 10^{10} VP/mL prior to analysis in triplicate on Maurice. A strong linear relationship was observed, with $R^2 > 0.99$. **(E)** Evaluation of icIEF method for adherence to the principals of green/white chemistry standards, receiving an 89.7% rating. *See Wu and Heger, Green Analytical Chemistry 3 (2022), 100027 for more details.*

MAURICEFLEX™ cIEF FRACTIONATION OF AAV CAPSID PROTEINS



*10s exposure used vs 60s for Basic/Acidic

Figure 5. AAV9 Capsid Protein Fractionation. **(A)** AAV9 was loaded onto a Flex cartridge. **(B)** Capsid proteins mobilized out of the window within 30min. (C) Fractions were checked on an icIEF cartridge to ensure isolation of individual acidic (VP1), main (VP3), and basic (VP2) species.

CONCLUSIONS

- A single platform, MauriceFlex™, can be used to characterize a variety of viral vectors used in cell and gene therapies through its CE-SDS, imaged cIEF, and charge based fractionation capabilities.
- Turbo CE-SDS provides AAV/LVV capsid protein ratio and purity analysis in under 6min per sample.
- Imaged cIEF is a powerful stability-indicating identity assay for viral capsid proteins.
- MaricheFlex™ fractionation allows for downstream characterization of charge variants by mass spectrometry and other techniques.

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