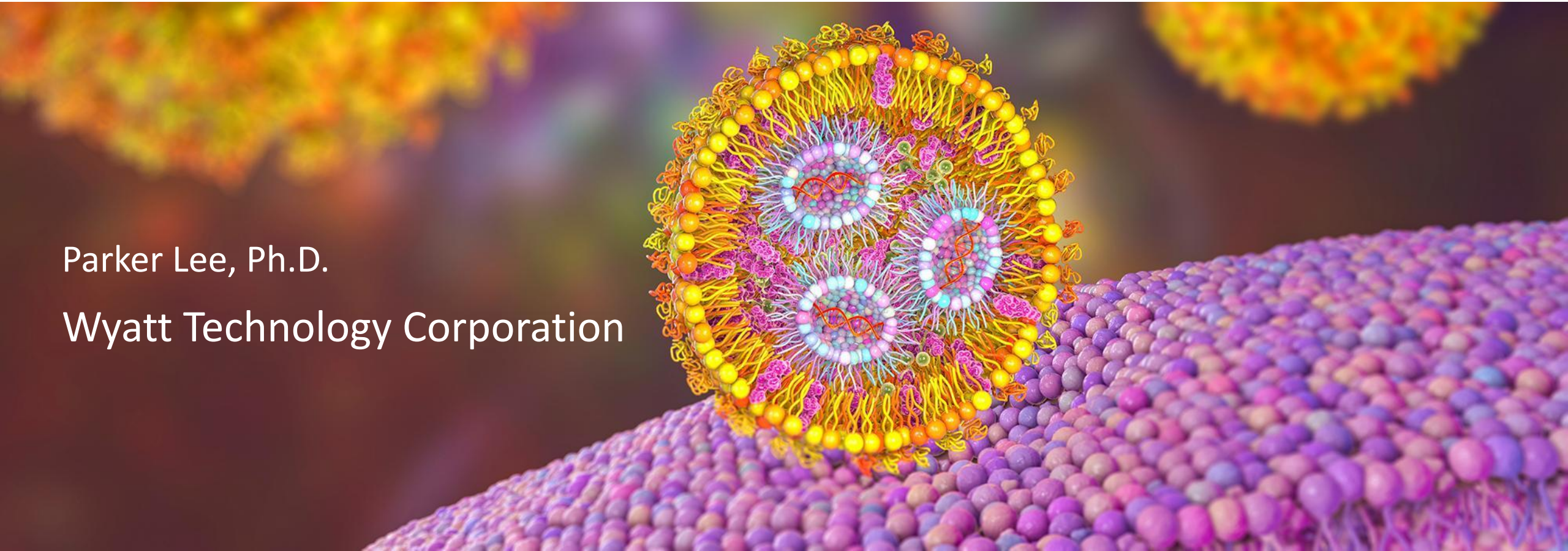
From Proteins to AAVs and LNPs:

Multi-attribute Quantification (MAQ) by Light Scattering



Parker Lee, Ph.D.

Wyatt Technology Corporation

About Waters | Wyatt Technology

- ✔ Founded in 1982 by Dr. Philip J. Wyatt to commercialize multi-angle light scattering (MALS)
- ✔ Award-winning, robust, low maintenance, easy to use instruments that have been validated by thousands of peer-reviewed publications
- ✔ Leading provider of light scattering instruments for solution-based characterization of macromolecules and nanoparticles:

molar mass, size, charge, & interactions

- ✔ Pioneer of SEC-MALS and FFF-MALS, now standard analytical tools in protein, biopharma, biopolymer, synthetic polymer labs and more
- ✔ Pioneer of plate-based dynamic light scattering (DLS), an essential technology for high-throughput protein and nanoparticle formulation
- ✔ Acquired by Waters Corporation in May 2023

SCIENTISTS' CHOICE AWARDS®

Waters™ | WYATT TECHNOLOGY

What can light scattering measure?

Static Light Scattering (a.k.a. multi-angle light scattering, MALS)

- ✔ Molar mass (MM, MW); particle concentration
- ✔ RMS radius (R_g) and spherical radius

DAWN®

ultraDAWN®

Dynamic Light Scattering (DLS, QELS)

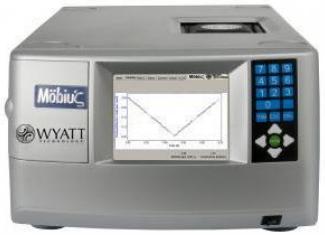
- ✔ Translational diffusion coefficient, D_t
  - Hydrodynamic radius, R_h
  - Particle concentration

DynaPro® Plate Reader

Phase Analysis Light Scattering (PALS)

- ✔ Electrophoretic mobility
  - Zeta Potential, ζ
  - Effective Molecular Charge, Q_{eff}

Mobius™



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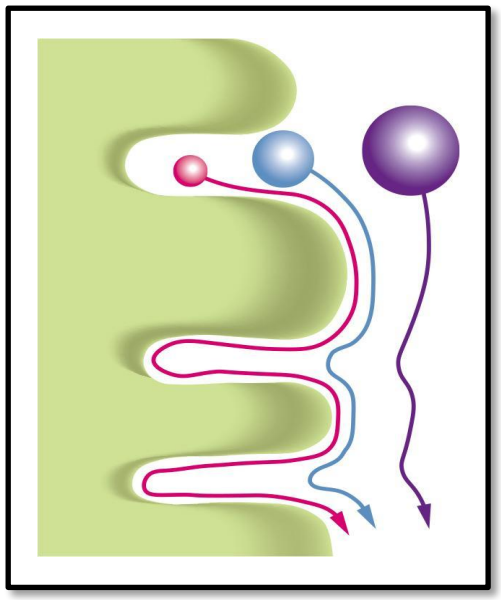
MALS-DLS-UV-dRI following SEC or FFF

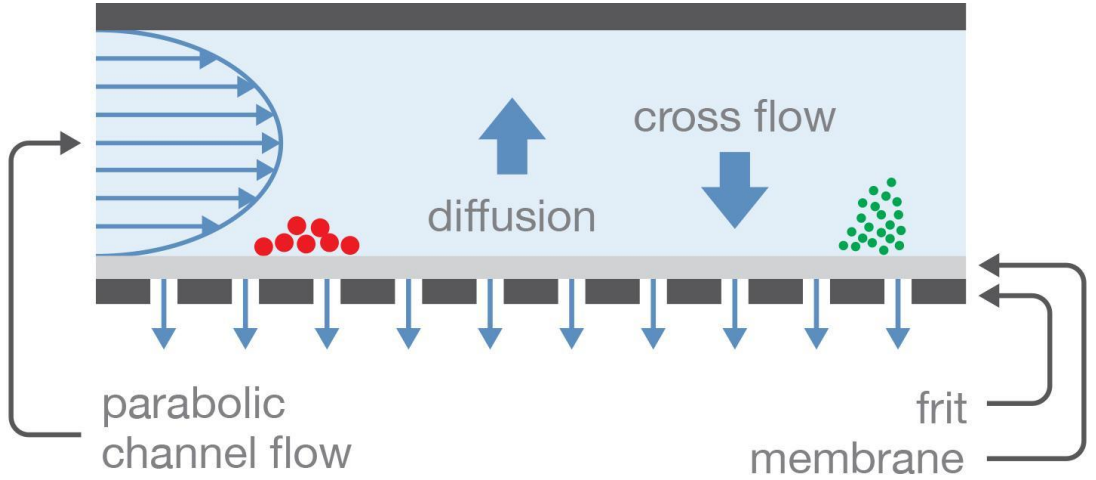
SEC or FFF provides size-based separation

SEC



SEC: elution order by hydrodynamic volume

FFF (AF4)



FFF: the separation tool of choice for NPs and samples that stick to columns and are sensitive to shearing.

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Wyatt solutions for protein and gene therapy products

Screening by DLS

Screening for size distribution, particle concentration, stability, formulation, and turbidity

Charge analysis

Mobius: Automated zeta potential measurement with an autosampler

Inline analysis

ultraDAWN: Real-time MW, size and concentration for process development and PAT

SEC – MALS

First separation-based system to adopt as a platform method for routine analysis of quality attributes

FFF – MALS

Ideal tool for large aggregates, large viral vectors, lipid nanoparticles, and other drug delivery vehicles

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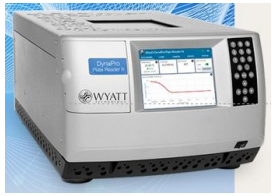
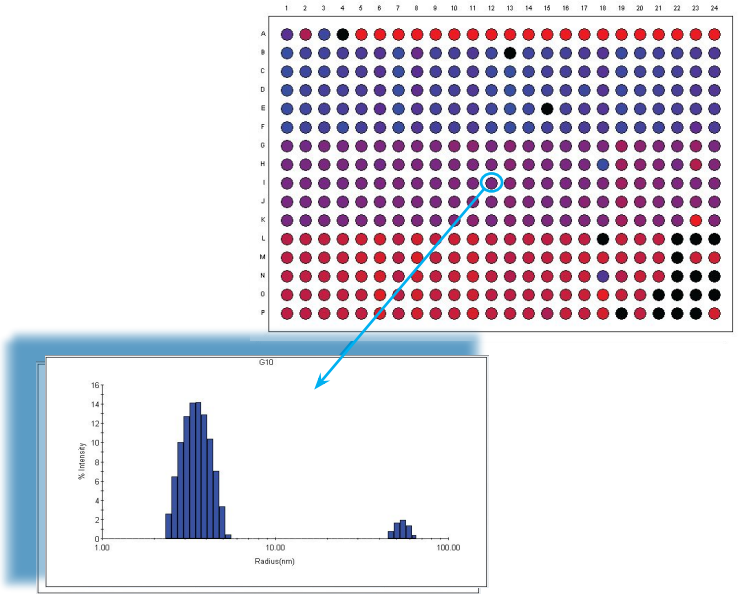
Proteins: Selected DLS applications



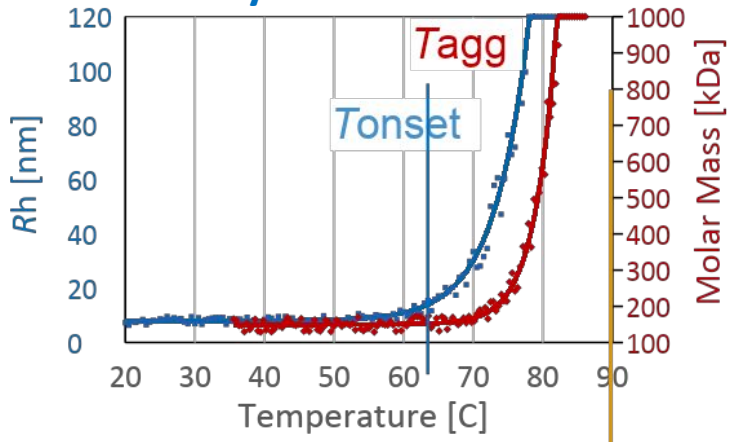
HT screening of size and polydispersity

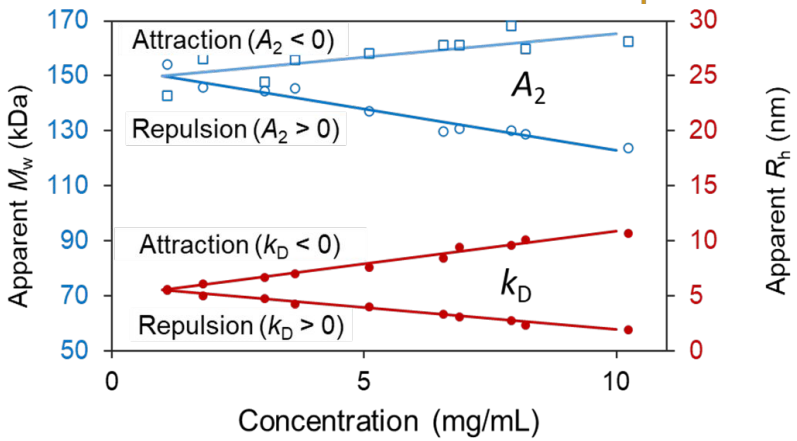


384 wells:

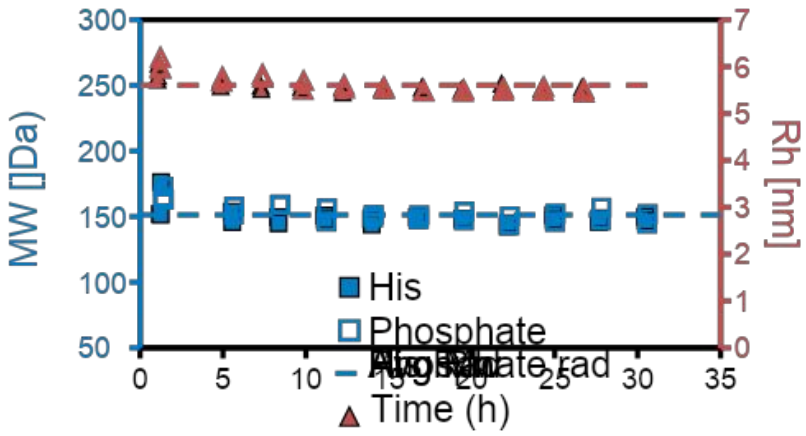
- 5 candidates; 5 pHs; 5 ionic strengths; 3 replicates; 9 controls
- <1.5 hr

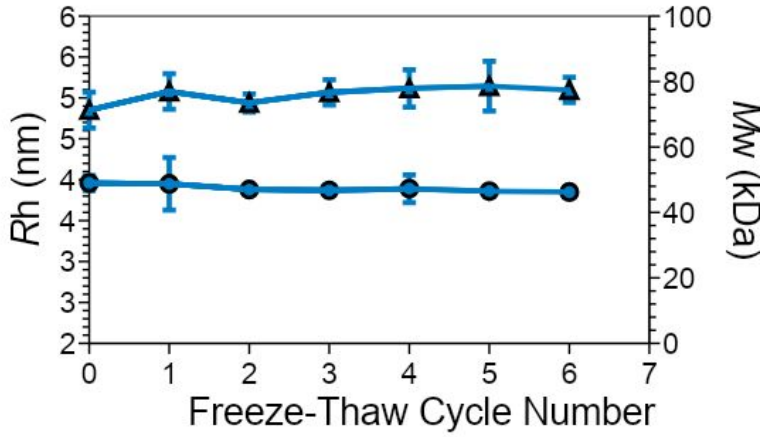
Thermal and colloidal stability



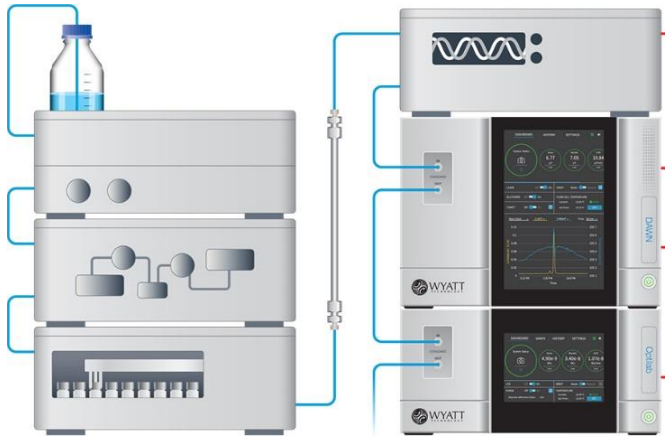


Time course analysis: DLS and SLS





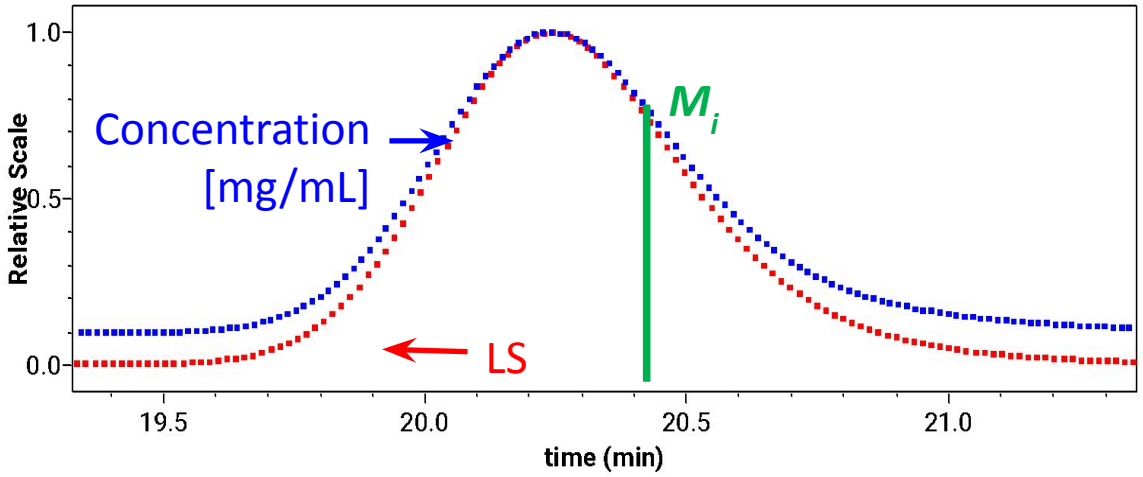
SEC-MALS-UV-dRI for protein therapeutics



UV

DAWN (MALS)

Optilab (dRI)



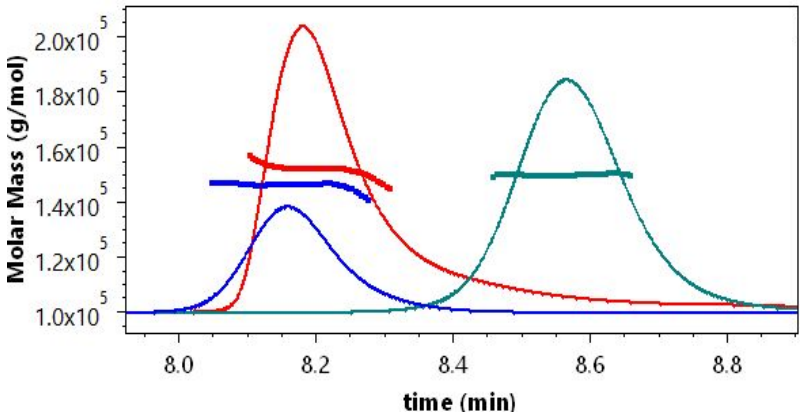
$$I_s \propto c \times M \times \left(\frac{dn}{dc}\right)^2$$

Typical protein applications

- ✔ **Absolute MW of each SEC peak/data slice**
- ✔ **Aggregation**
- ✔ **Protein conjugate analysis: MW + composition**
- ✔ Oligomeric state
- ✔ Heterogeneity of the peak
- ✔ Reversible association
- ✔ UV extinction coefficient
- ✔ Structural information with online DLS
- ✔ SEC method development

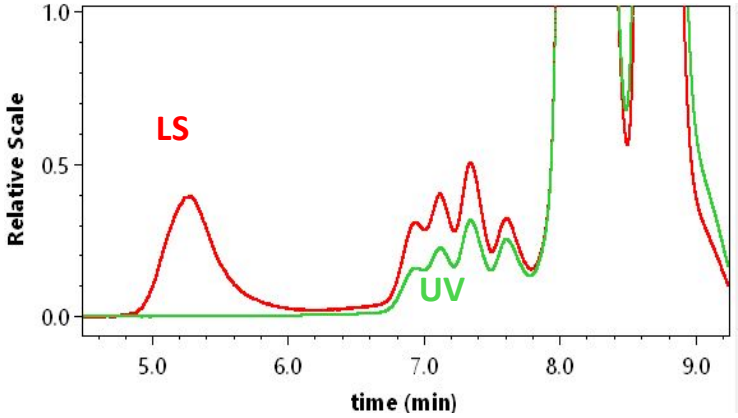
Selected SEC-MALS applications for proteins

Biophysical characterization of proteins



- Protein identity based on absolute MW
- Independent of shape and column interactions
- Key application: lot and process comparability

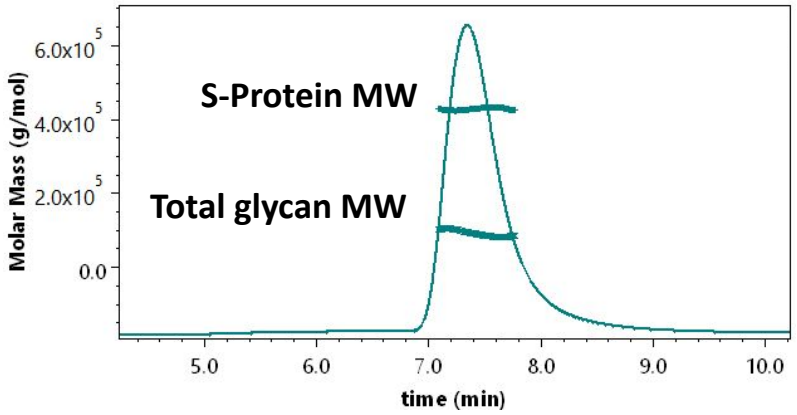
CQA– protein aggregation



- High sensitivity to aggregates
- Key application: FDA requires analysis of aggregates throughout the product life cycle

Composition of protein conjugates

Cell 2019, 176(5):1026-1039



- Key application: PEGylated and glycosylated proteins
- Preferable as a release assay over SEC-UV

Adeno-associated virus (AAV)

S (NanoStar or Plate Reader)

Screening tool for all viral vectors

SEC - MALS

AAV platform method for routine analysis
Applicable in RD, PD, AD and QC
Multi-attribute quantification, MAQ, in one run

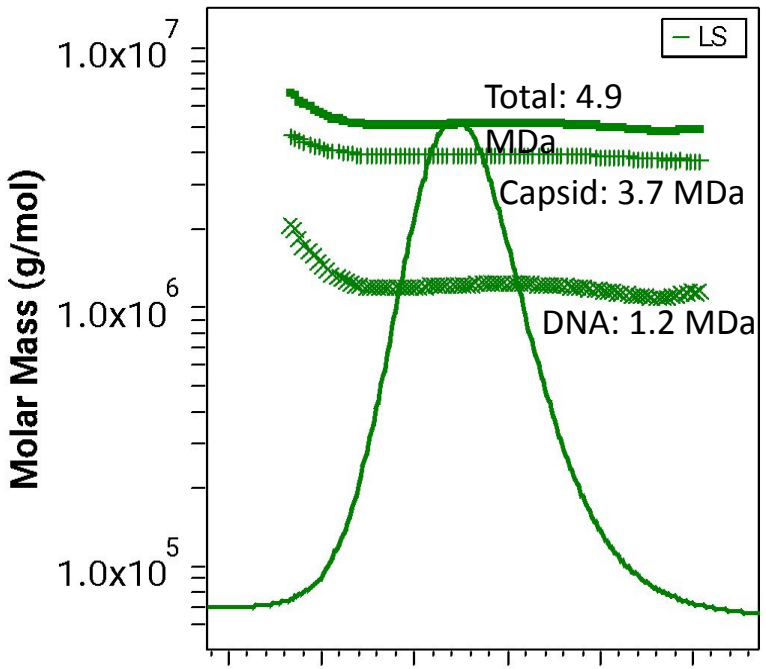
FFF - MALS

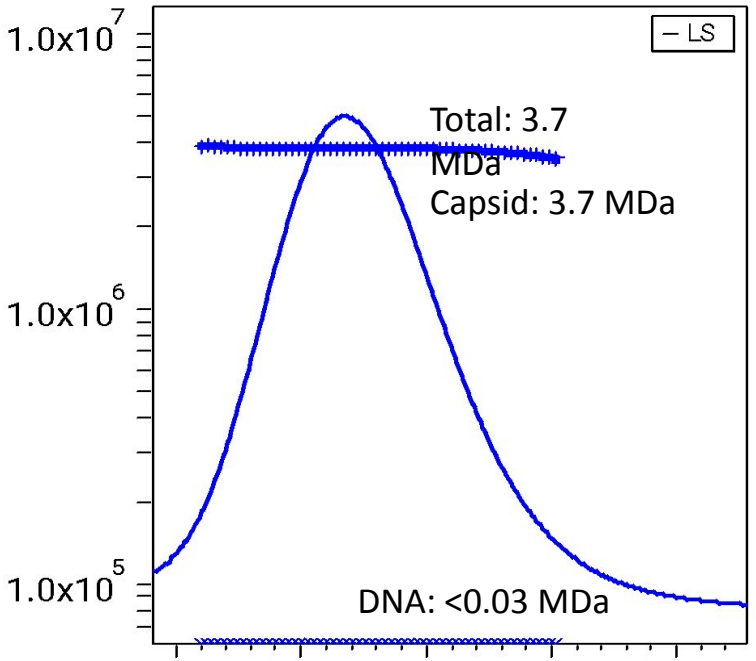
Characterization and quantification tool for large AAV aggregates, large viral vectors and other delivery vehicles

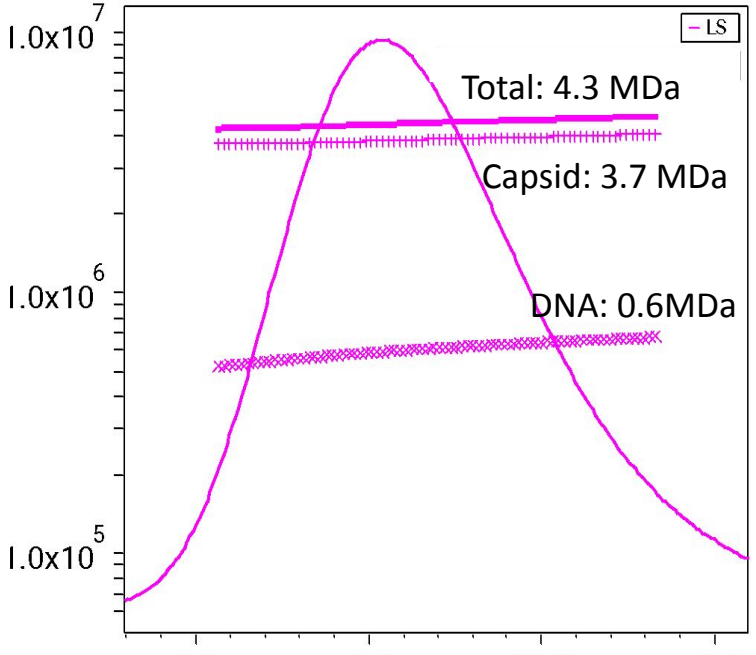
Real-time MALS

Inline AAV monitoring for quick feedback to process development

AAV analysis using Viral Vector Analysis module

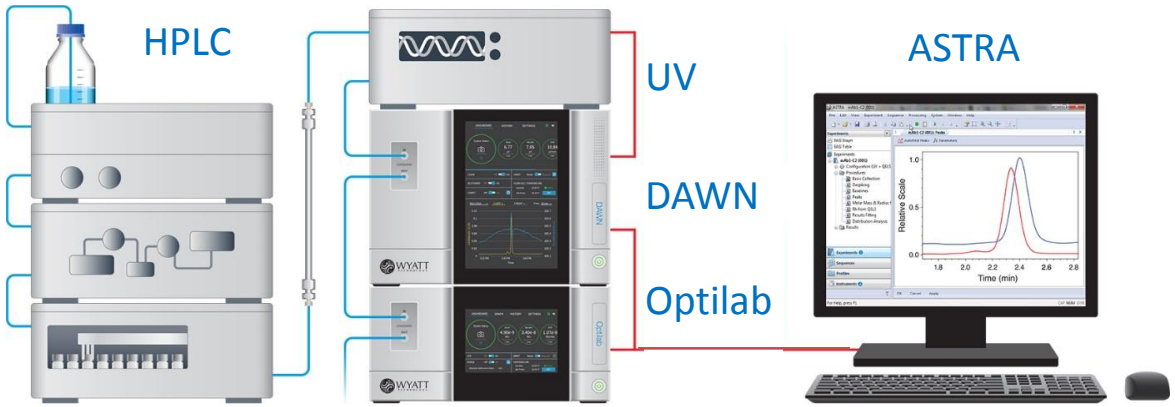






- Determine MW and concentration of capsid and encapsidated DNA
- Equivalent to ELISA/microBCA + ddPCR/qPCR
- ASTRA software calculates AAV-specific critical quality attributes: Cp, Vg, Vg/Cp
- To learn more: wyatt.com/AppNotes, AN1617 (U.S. patent pending)

Optimal system configuration for AAV-MAQ



- DAWN (MALS), Optilab® (dRI)
- HPLC with UV (DAD/MWD or PDA)
  - w/Agilent HPLC: HPLC CONNECT™ software
  - w/other HPLC: two analog UV outputs required
- ASTRA® 8.1+ VVA Module & SOP Guidance Manual
- 21 CFR Part 11-compliant SW and service options

Comprehensive ***SOP*** Guidance Manual, now embedded in ASTRA

Determination of AAV identity, purity, particle concentration, and capsid content by SEC-MALS

AAV TRAINING & METHOD TRANSFER

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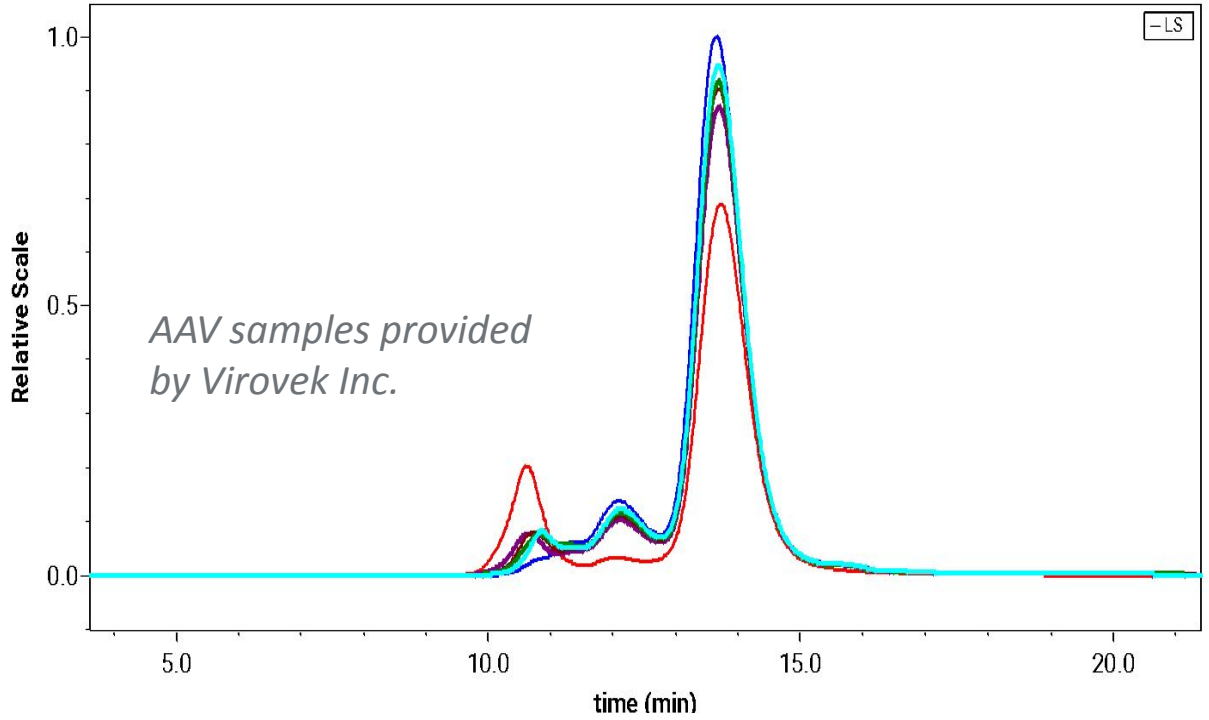
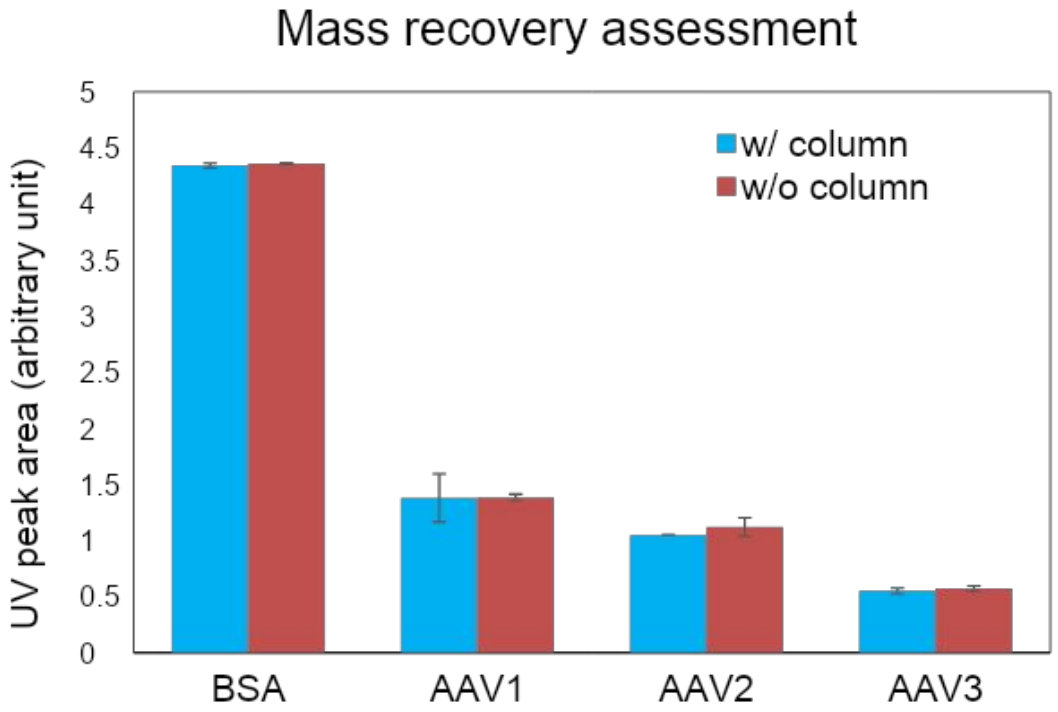
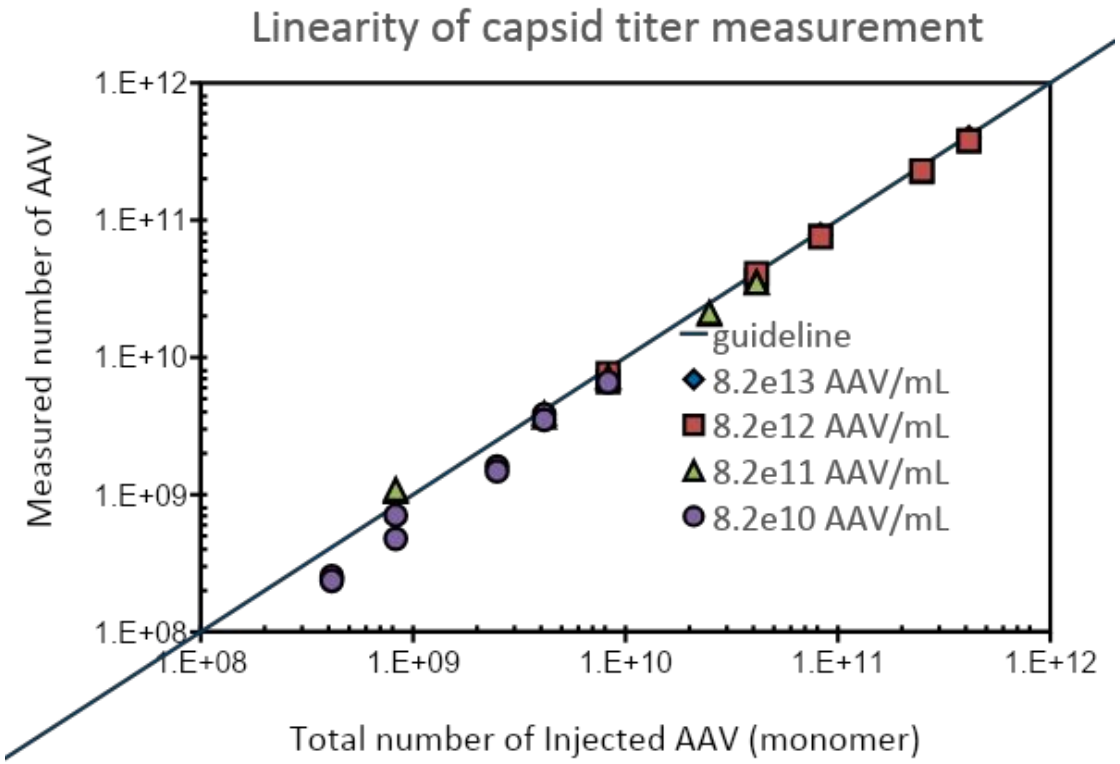
Wyatt SEC-MALS method: AAV-MAQ case study

- The **most accurate and precise quantitation method** for AAV capsid titer.
- **Routine analysis** for capsid ratio (E/F), especially for PD.
- MALS is the most sensitive and quantitative detector for AAV **aggregates**.
- **Platform method** for AAV 1, 2, 3, 5, 6, 8, 9, 10, and engineered serotypes.



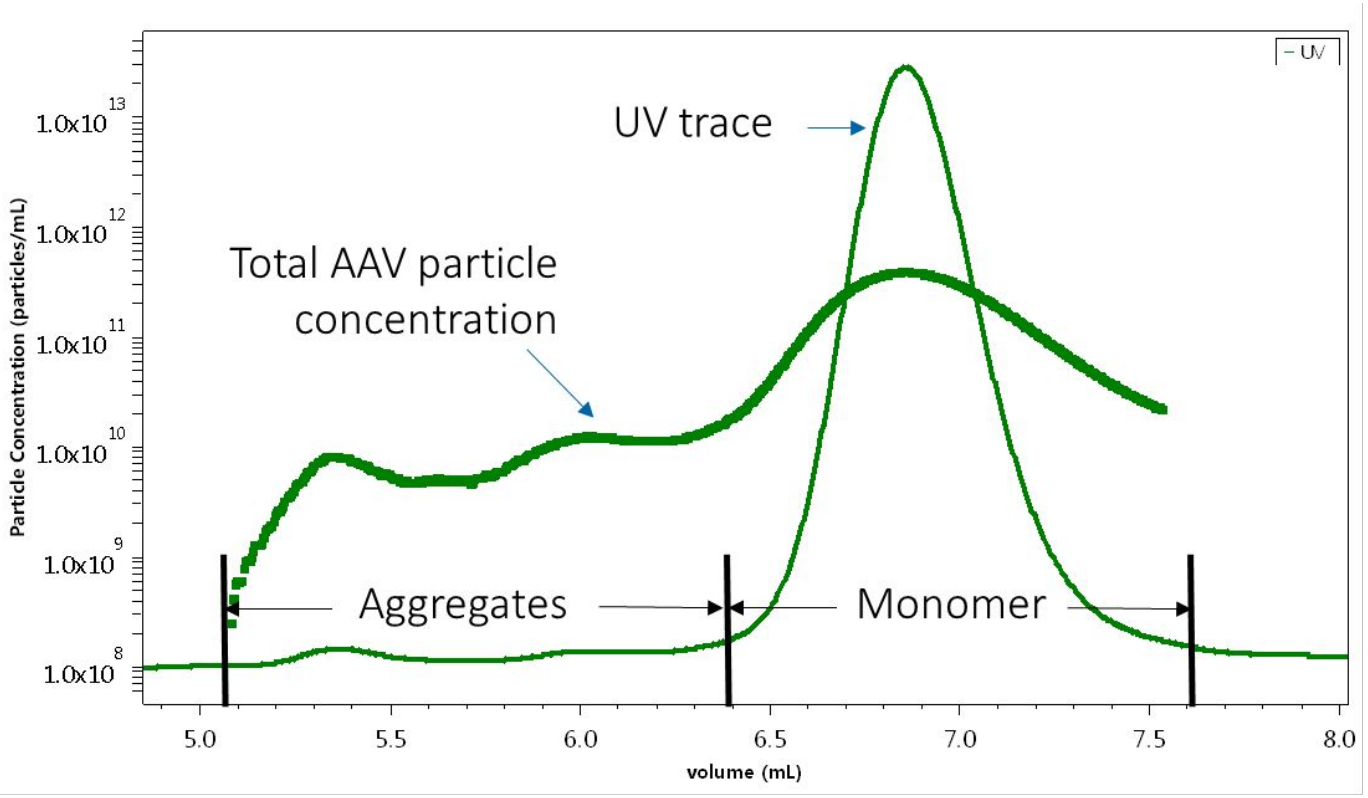
CQA 1: Capsid titer (sensitive, linear, consistent results)





- The most accurate and precise method for capsid titer (the only assumption is 100% mass recovery).
- High sensitivity: LOQ - 5 x 10^{10} AAV/mL with UV 260 and 280 nm as the concentration source.
- Consistent with other protein quantitation methods (ELISA and microBCA).

CQA 2: Quantify aggregation



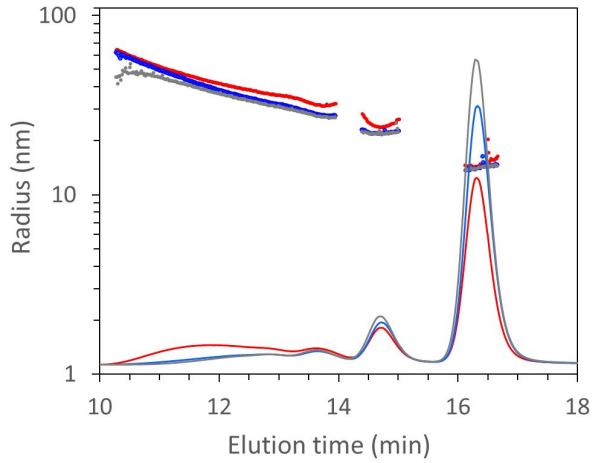
	Concentration [particles/mL]	Content [%]
Monomer	4.22×10^{13}	94.6
Aggregates	0.24×10^{13}	5.4
Total	4.46×10^{13}	100

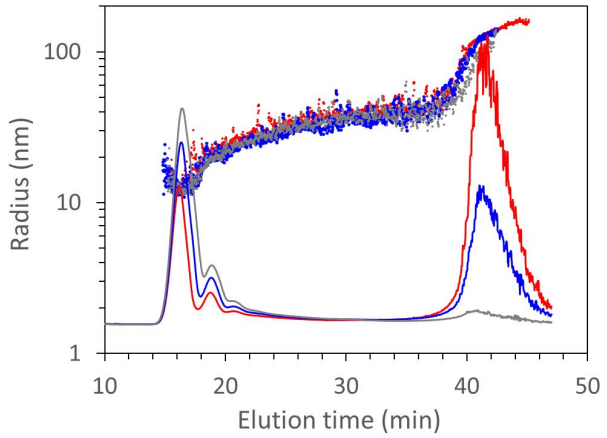
- Particle concentrations are calculated for each data slice to quantify percent monomer and aggregates.
- SEC column may remove large aggregates and FFF is a better alternative. Please read Wyatt AN2004.

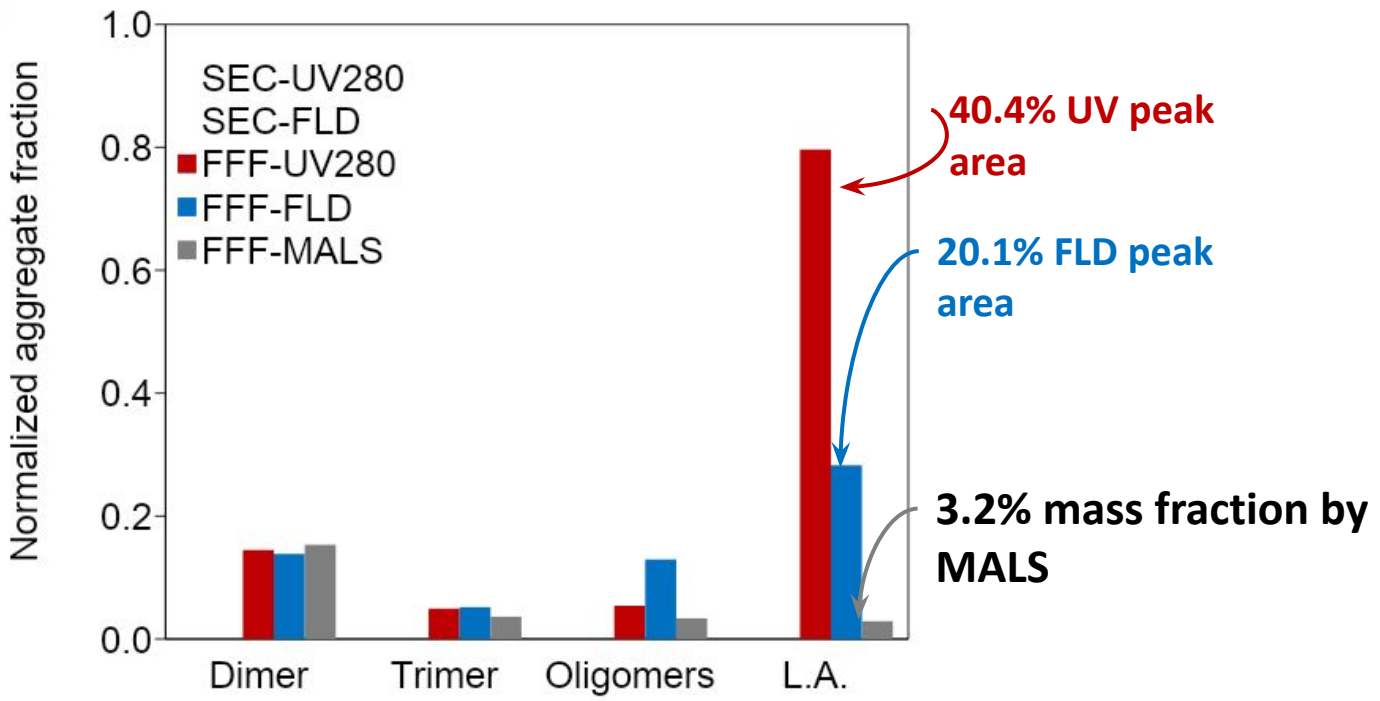
Only FFF-MALS correctly quantifies large aggregates

Large aggregates (L.A.) both absorb *and scatter* light, leading to overestimation of large aggregates.

— Note that large aggregates are only detectable with FFF (filtered out by SEC).

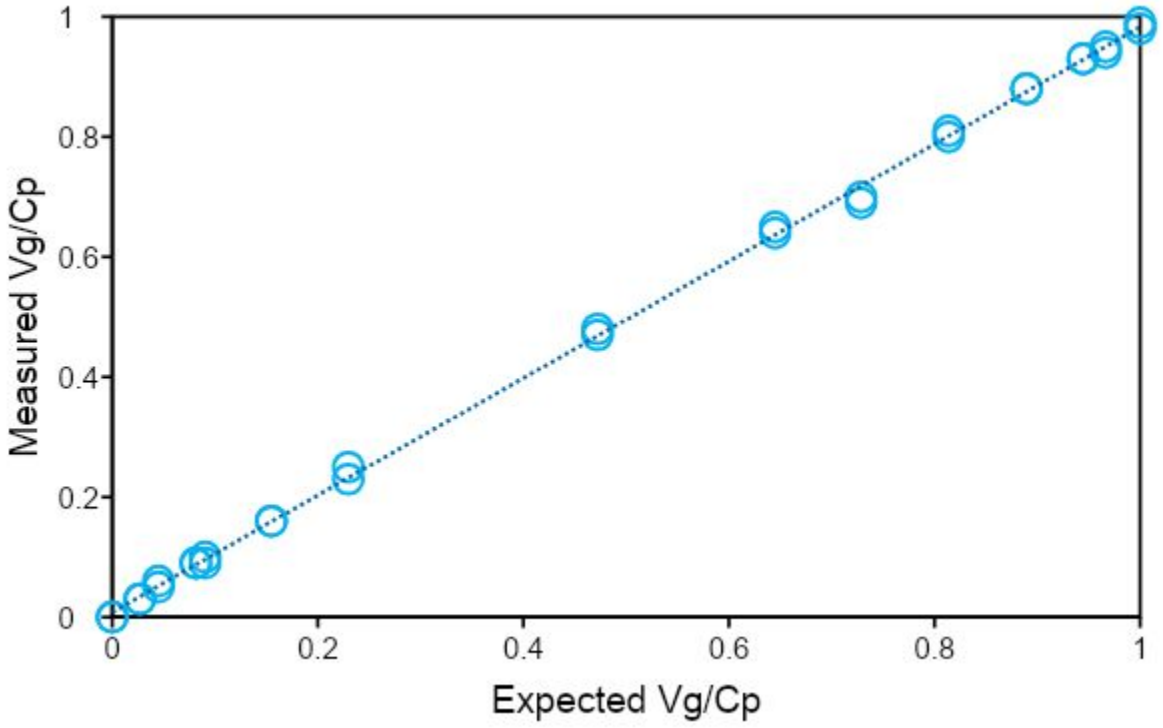






Full details here:
https://www.wyatt.com/library/application-notes/an2004-why-and-how-to-quantify-aav-aggregates-by-fff-mals.html

CQA 3: Genome titer and capsid ratio (V_g/C_p or full/total)



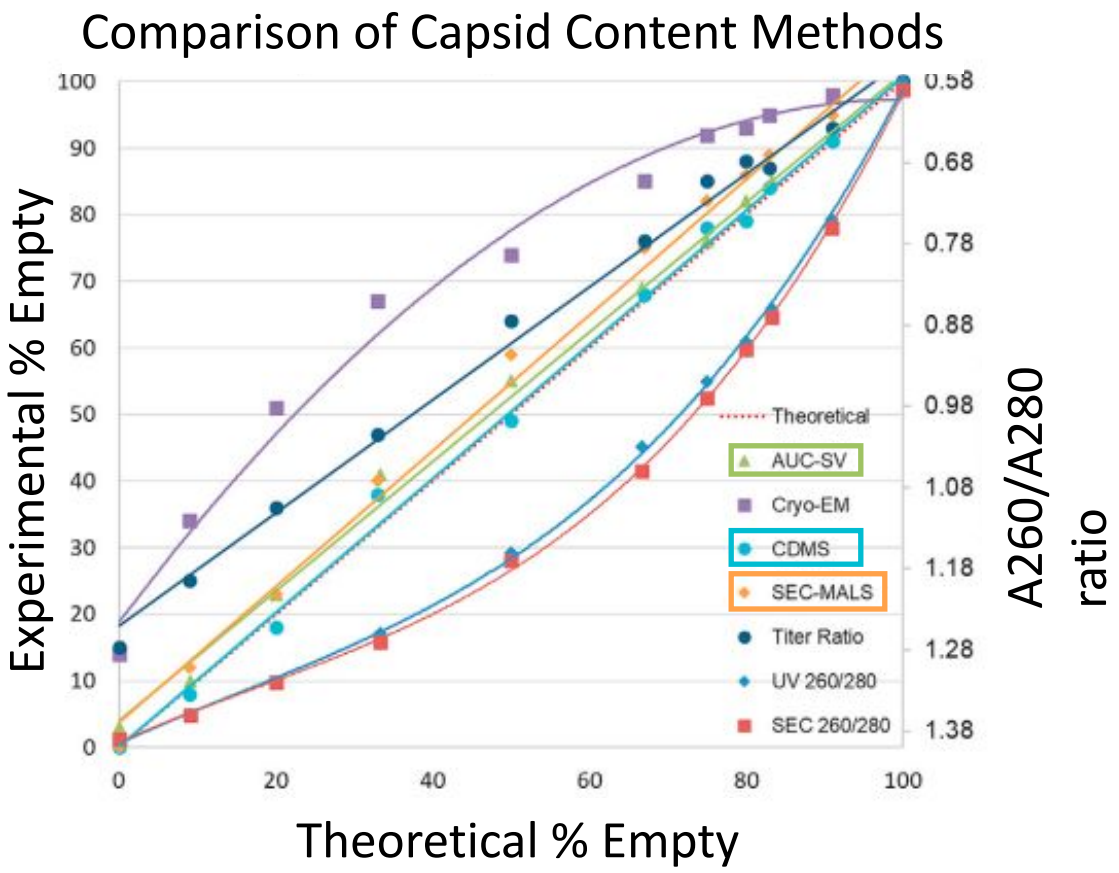
UV1 = UV at 260 nm

UV2 = UV at 280 nm

Empty and full AAV controls were prepared at a concentration of 5×10^{12} AAV/mL, then mixed to create expected V_g/C_p values from 0 to 1.

- Detect and quantify small changes in capsid content arising from empty, partial, or full AAVs.
- Cannot resolve partial AAVs. With low partial content, comparable results with AUC and ddPCR.

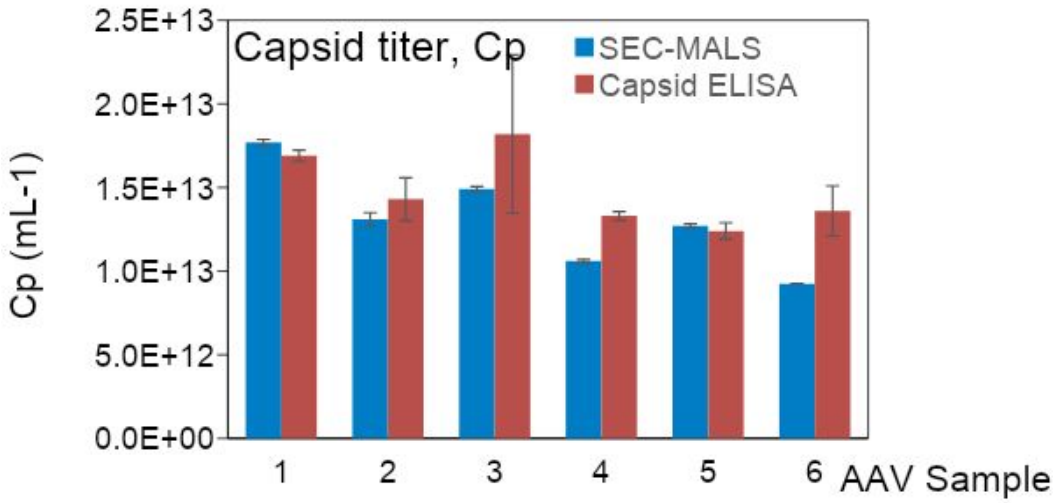
AAV Analysis: Vg/Cp (Full vs Total)

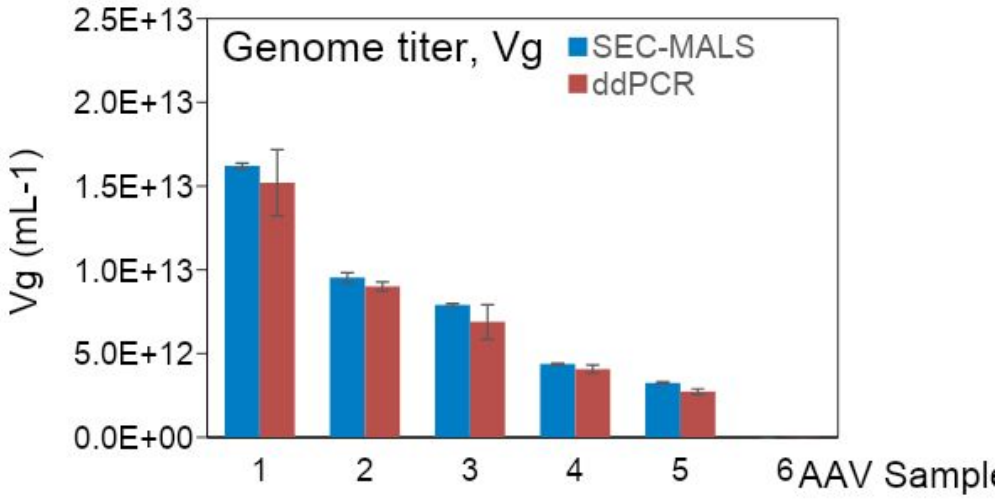


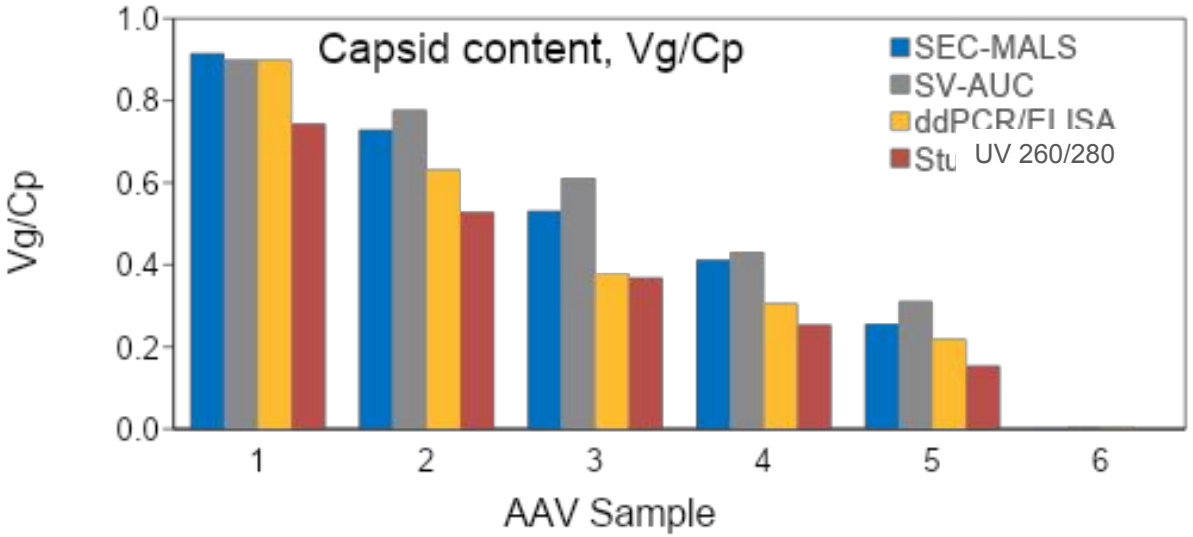
	AAV #1 V_g [mL^{-1}]	AAV #2 V_g [mL^{-1}]
SEC-MALS	(2.3±0.1)x10^{12}	(1.2±0.1)x10^{12}
dd PCR	2.3x10^{12}	1.2x10^{12}
qPCR	1.2x10^{13}	8.5x10^{12}

- Comparison graph is from a paper by **Pfizer** Inc. https://doi.org/10.1016/j.omtm.2021.08.009
- CRO qPCR and in-house ddPCR data as well as AAV samples were kindly provided by Ronald Yeh, **Discovery Biologics Seattle**, Novo Nordisk.

AAV Analysis: Vg/Cp (Full vs Total)

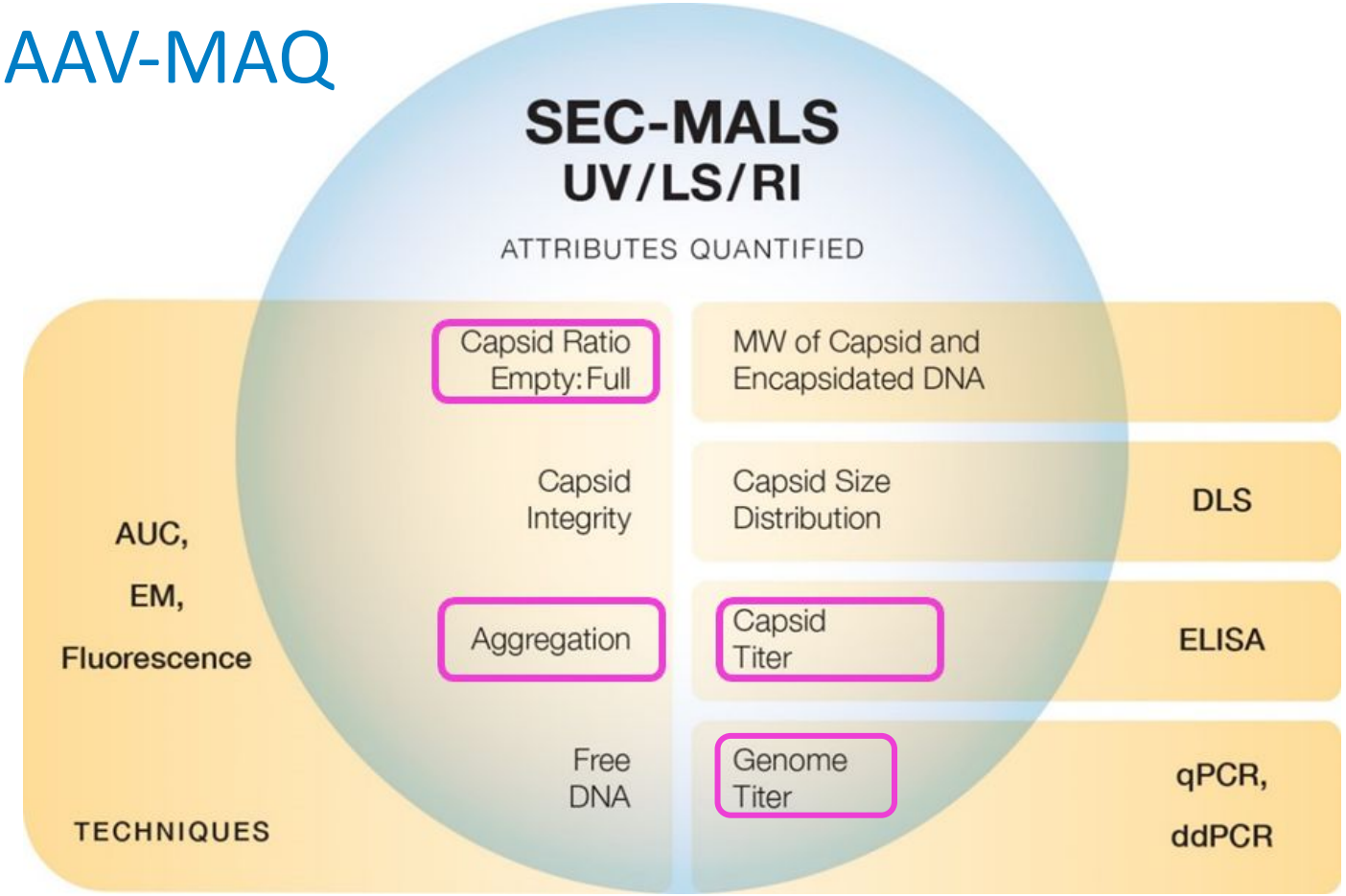






Provided by Brian Troxell, Stride Bio and published in *Human Gene Therapy* (2023). https://www.liebertpub.com/doi/epdf/10.1089/hum.2022.218

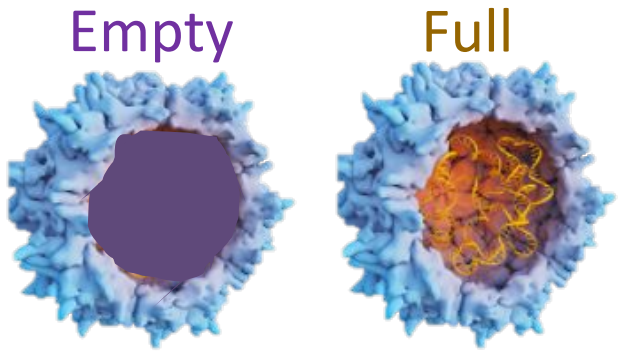
SEC-MALS for AAV-MAQ



(Biomarin) McIntosh, N.L., et al. Comprehensive characterization and quantification of adeno associated vectors by size exclusion chromatography and multi angle light scattering. Sci Rep 11, 3012 (2021). https://doi.org/10.1038/s41598-021-82599-1

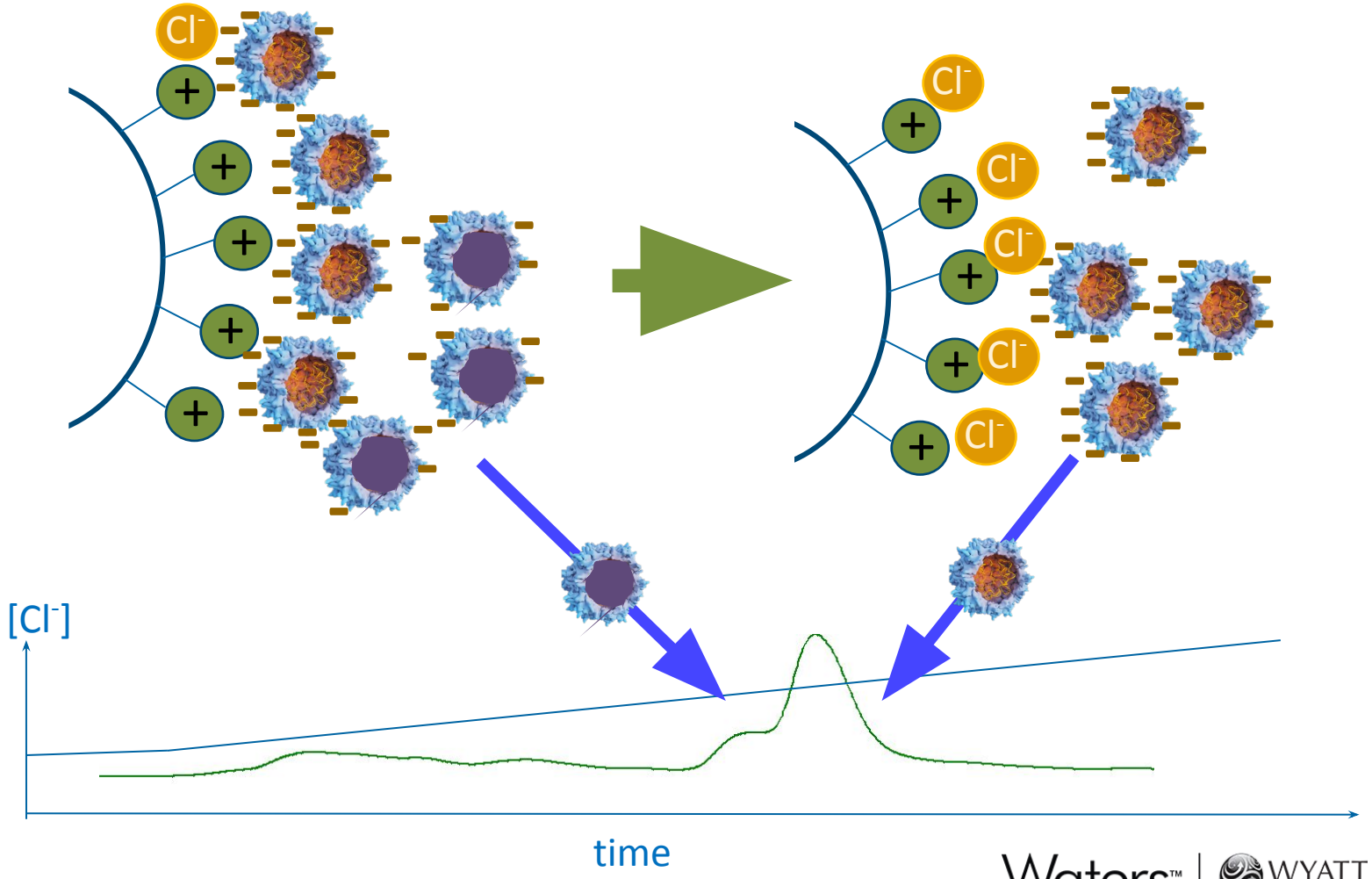
(Pfizer) Werle, A. K. et al. Comparison of Analytical Techniques to Quantitate the Capsid Content of Adeno-Associated Viral Vectors. Molecular Therapy - Methods & Clinical Development (2021) https://doi.org/10.1016/j.omtm.2021.08.009.

AEC to resolve empty and full AAV



pI 6.3 5.9

Eluent: 20 mM BTP, 2 mM MgCl2, 200 mM NaCl, pH 9.0



Wang, Chunlei, et al. "Developing an anion exchange chromatography assay for determining empty and full capsid contents in AAV6.2." *Molecular Therapy-Methods & Clinical Development* 15 (2019): 257-263.

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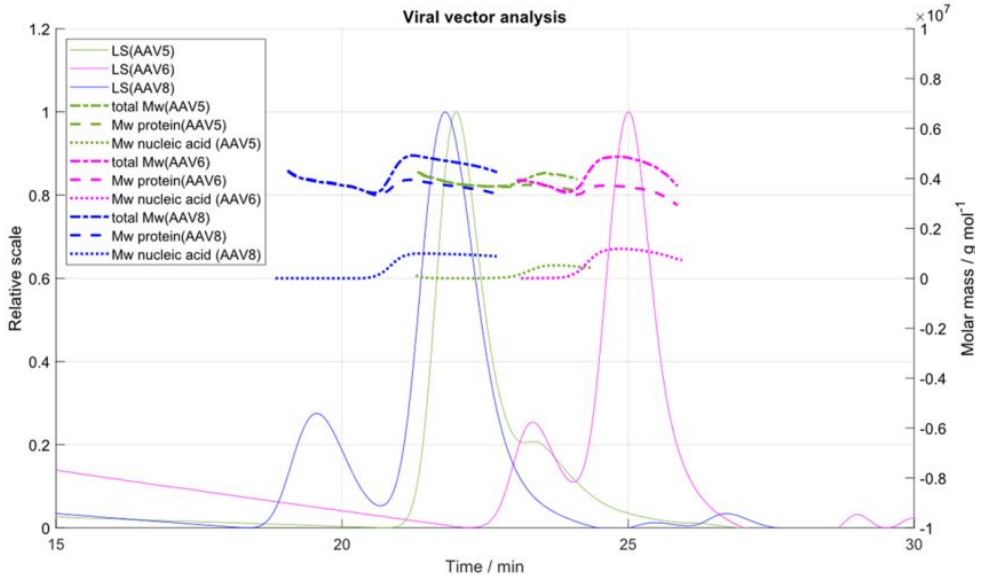
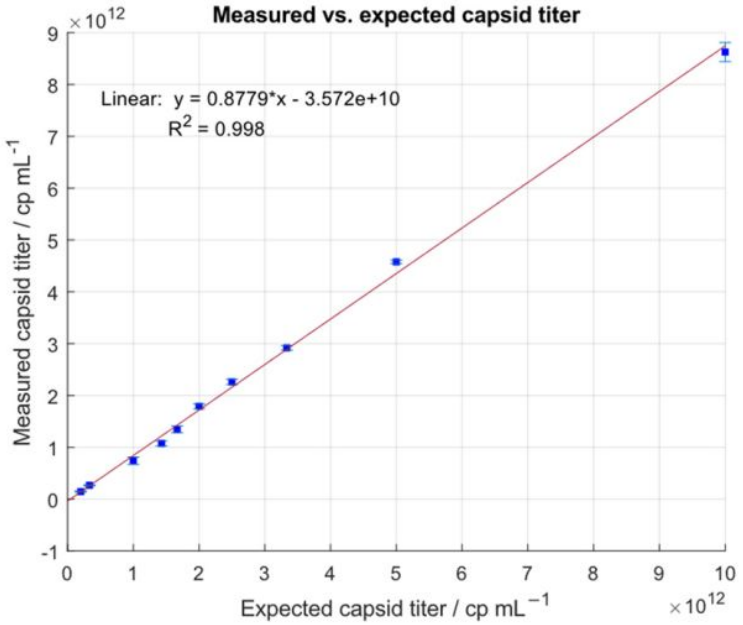
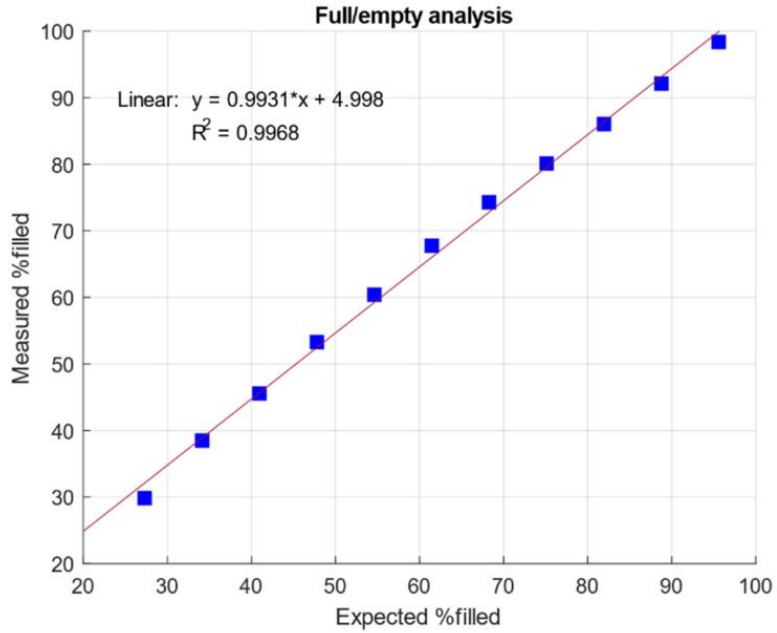
AEC to resolve empty and full AAV

AAV8







- IEX-MALS can be applied to varied serotypes of AAV to separate empty and full capsids.
- Method development is required for different serotypes and careful consideration needs to be given to gradient type and processing.

Wagner, Christina, et al. "Biophysical-Characterization of Adeno-Associated Virus Vectors Using Ion-Exchange Chromatography Couple to Light Scattering Detectors." *International Journal of Molecular Sciences* 23 (2022): 12715.

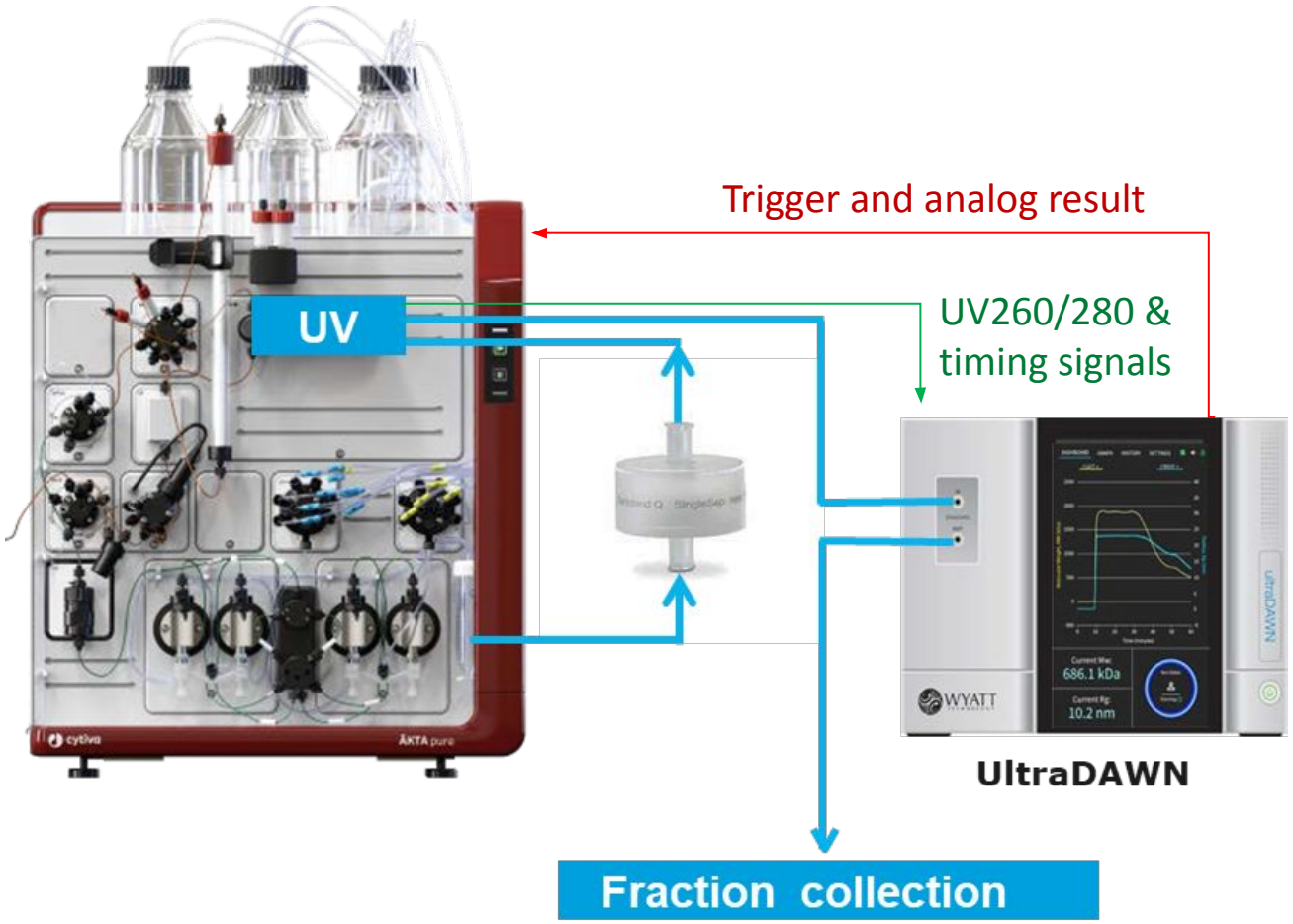
Real-time analysis of AAV for DSP development

RT-MALS with ultraDAWN and OBSERVER software

- In-line to 150 mL/min
- On-line for higher flow rates
- Chromatography, UF/DF, fill-finish
- Feedback of AAV attributes for process control

Attributes measured:

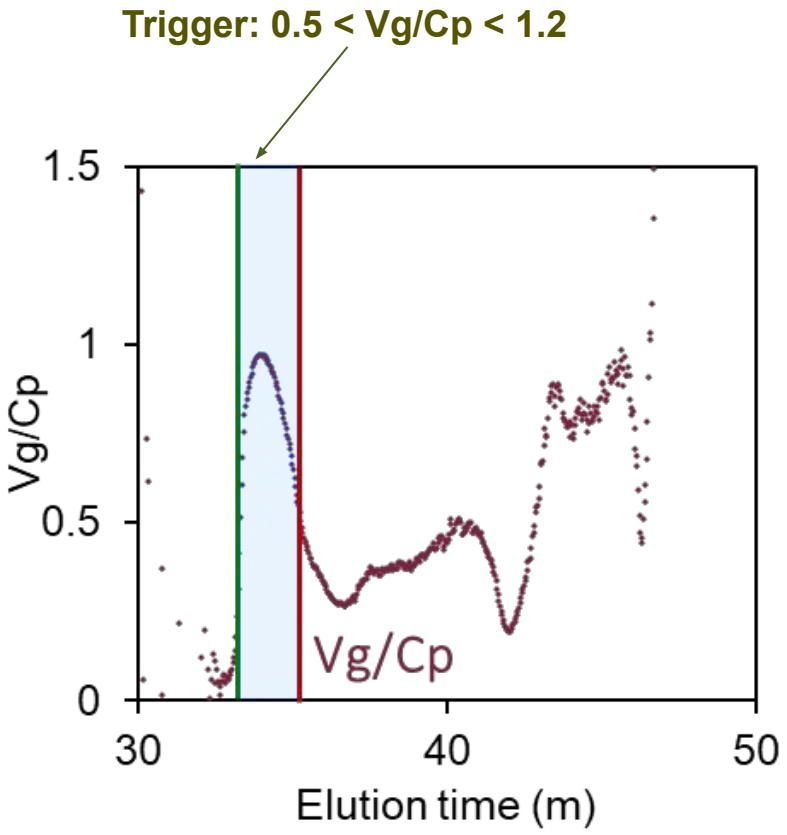
- Vg/Cp
- N_{capsid}
- N_{genome}
- MW_{capsid}
- MW_{genome}
- R_g

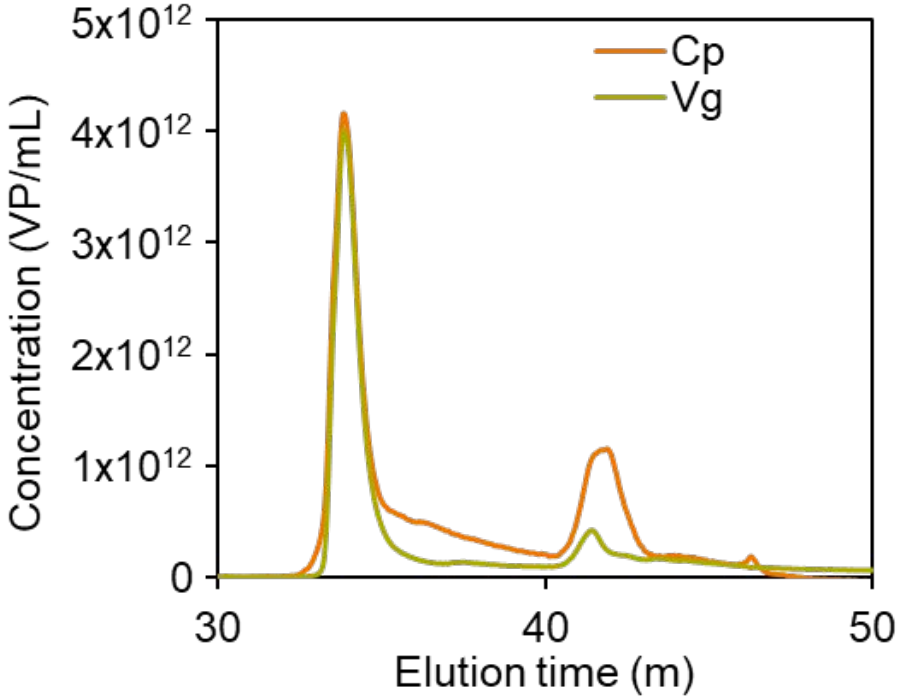


AAV (and other small viral vectors): Vg/Cp, titer, aggregates

Attributes:

- Vg/Cp
- N_{capsid}
- N_{genome}
- MW_{capsid}
- MW_{genome}
- R_g

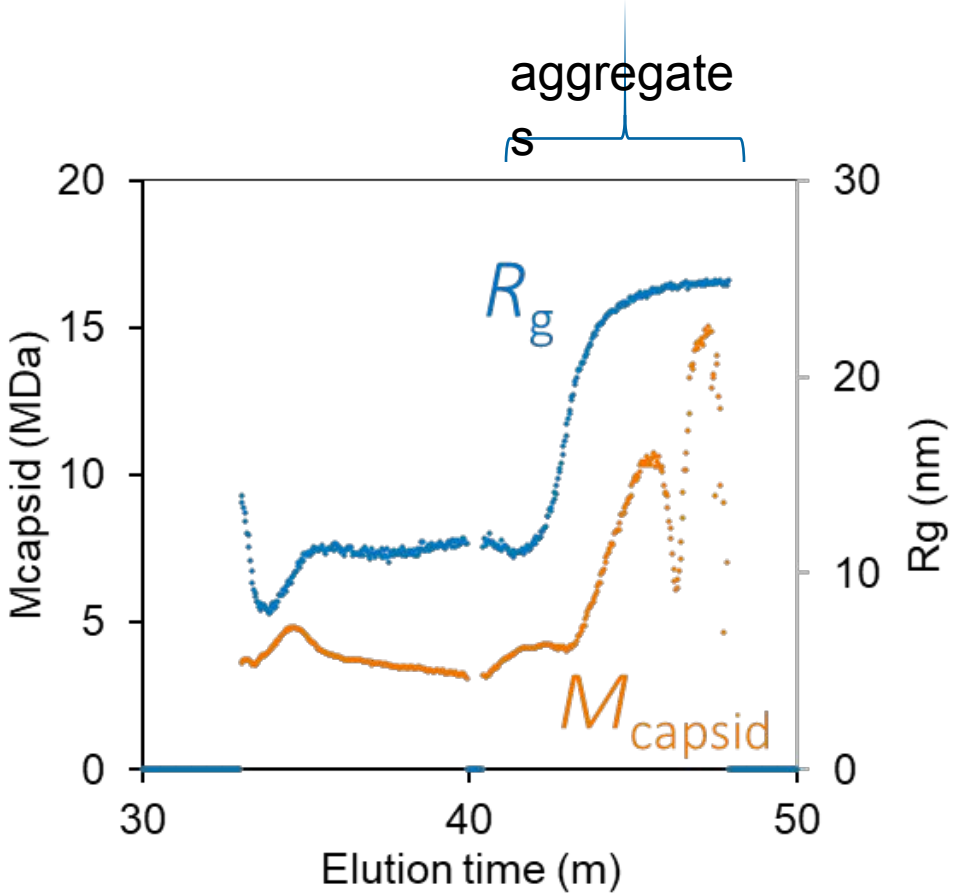


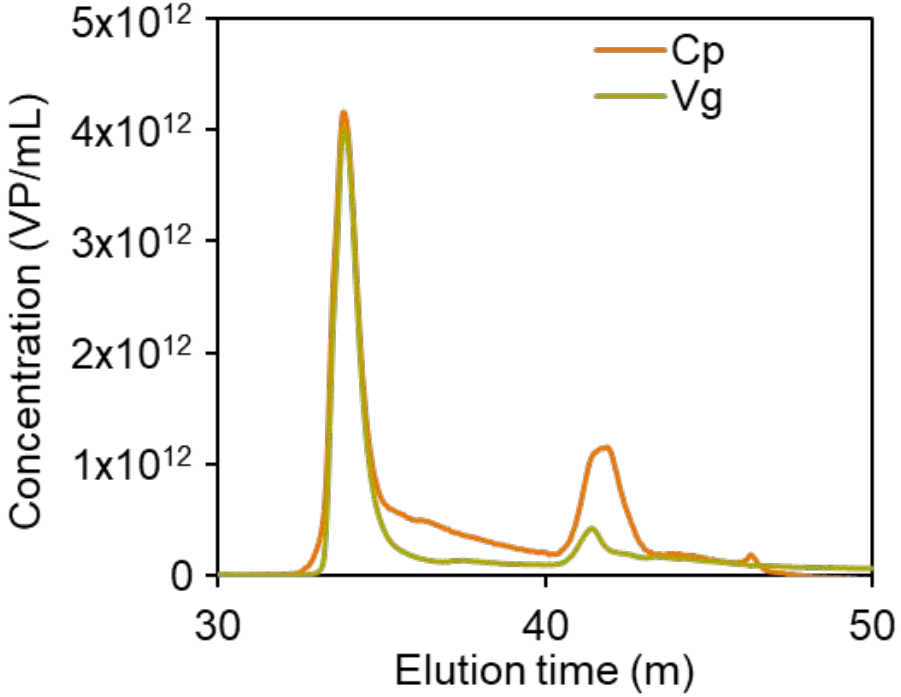


AAV (and other small viral vectors): Vg/Cp, titer, aggregates

Attributes:

- Vg/Cp
- N_{capsid}
- N_{genome}
- MW_{capsid}
- MW_{genome}
- R_g





Lipid Nanoparticles (LNPs)

DLS (NanoStar or Plate Reader)

Screening tool for LNPs

SEC - MALS

LNP method for size, polydispersity, aggregation, and payload in a single run

FFF - MALS

Characterization and MAQ tool for large LNP systems and aggregates

Real-time MALS

Inline LNP monitoring for quick feedback on formulation and DSP

LNP physical attributes and assays

Attribute	Assay	SEC/FFF-MALS-UV-dRI
mRNA integrity	Gel, qPCR	✔
LNP size	DLS	✔
LNP distribution	DLS	✔
Physical stability	DLS	✔
LNP number concentration	NTA	✔
LNP morphology	TEM, Cryo-EM	✔ (R_g/R_h)
LNP charge	PALS	Possibly with EAF4
Encapsulation efficiency	Fluorescence	✔ new
mRNA concentration	Fluorescence	✔ new
Lipid concentration	LC-MS	✔ new

Challenges in the traditional assays for LNP formulations

Unlike proteins and AAVs, LNP-NAs are heterogeneous in size, MW and composition/payload.

- DLS: quick screening tool for LNP size and polydispersity

  Drawback: Lacks resolution

  The presence of minute quantities of large particles (~ 1%) can significantly skew the average size.

- Ribogreen assay: rapidly quantifies average RNA content

  Drawback: Indirect reporter with high error (~ 30%)

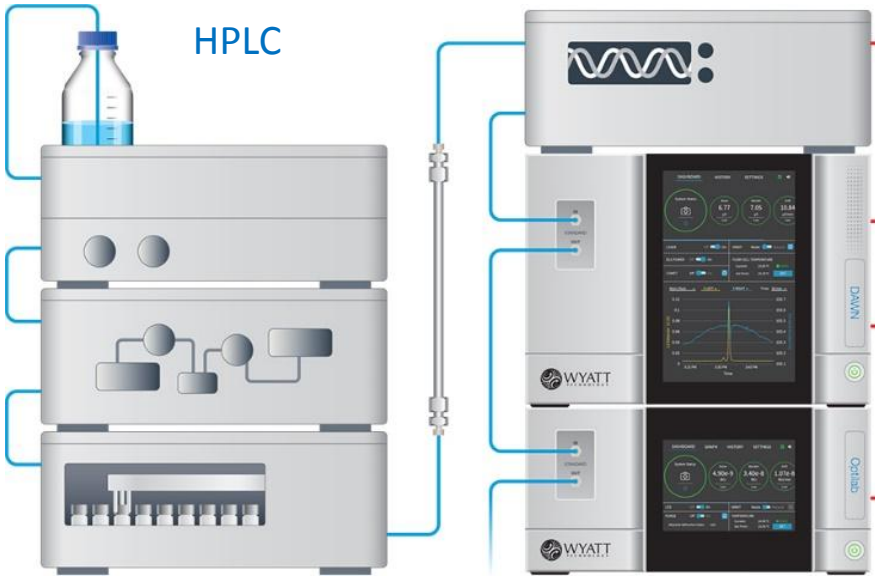
  Does not reveal dosing as a function of LNP size.

SEC/FFF-MALS: a single tool for high-resolution distributions of size and size-based payload

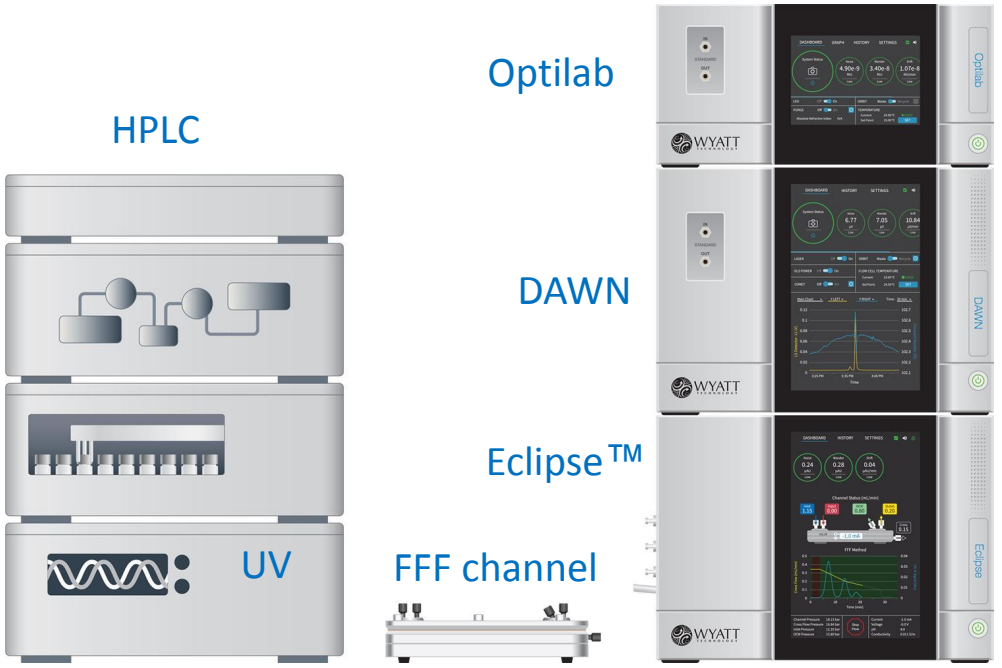
Invaluable in process development and optimization

MALS-DLS-UV-dRI following SEC or FFF

SEC



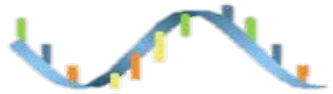
FFF



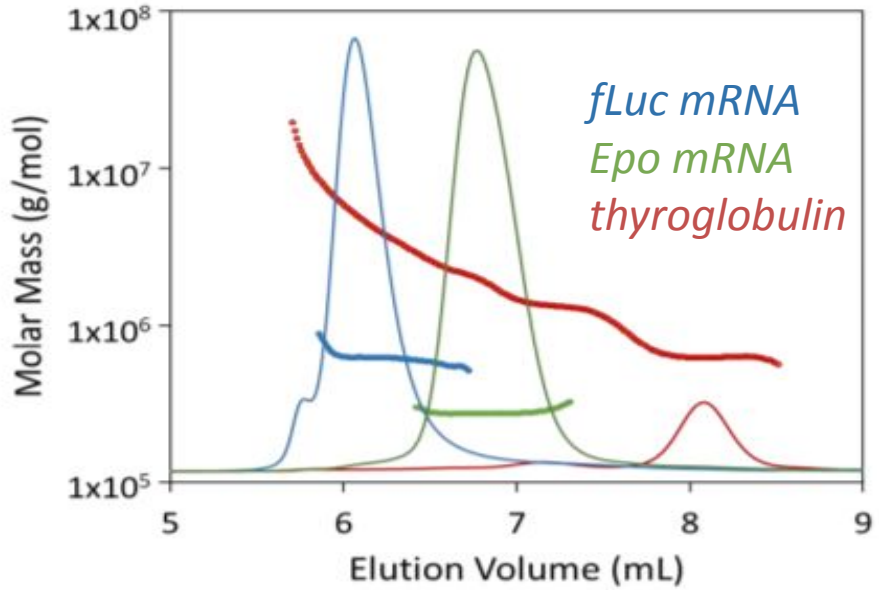
Eclipse with SEC switch option supports both SEC and FFF modes

- Multiple on-line detectors: DAWN (MALS-DLS), UV (260 nm), Optilab (dRI)
- ASTRA and VISION (FFF) software packages are 21 CFR Part 11 compliant

SEC or FFF to separate mRNA



SEC



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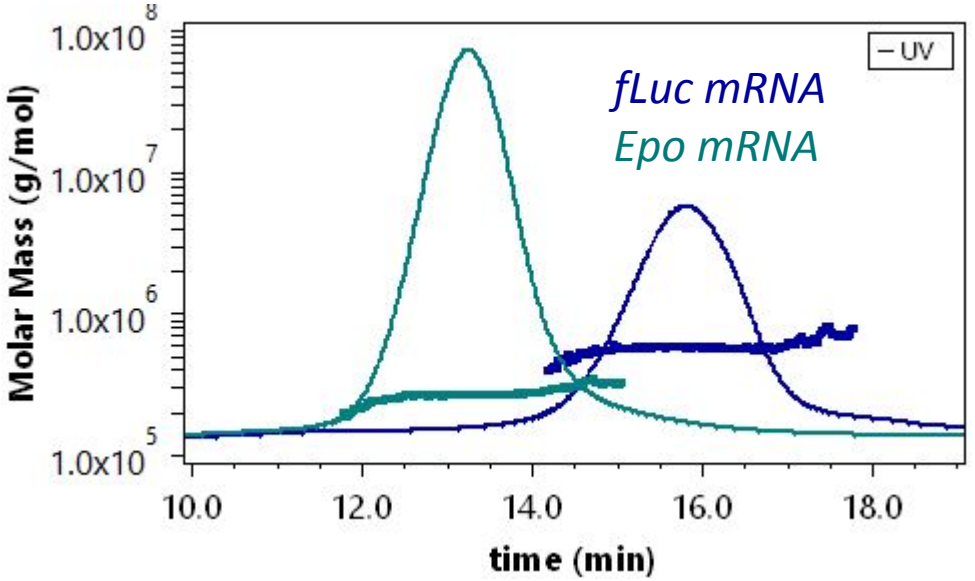
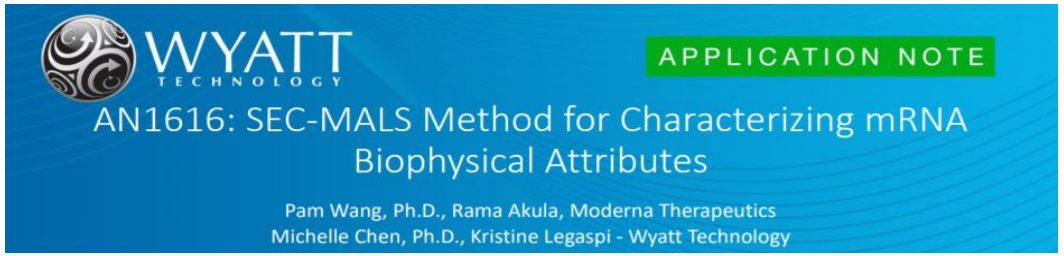
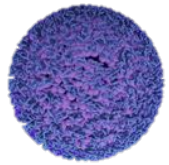


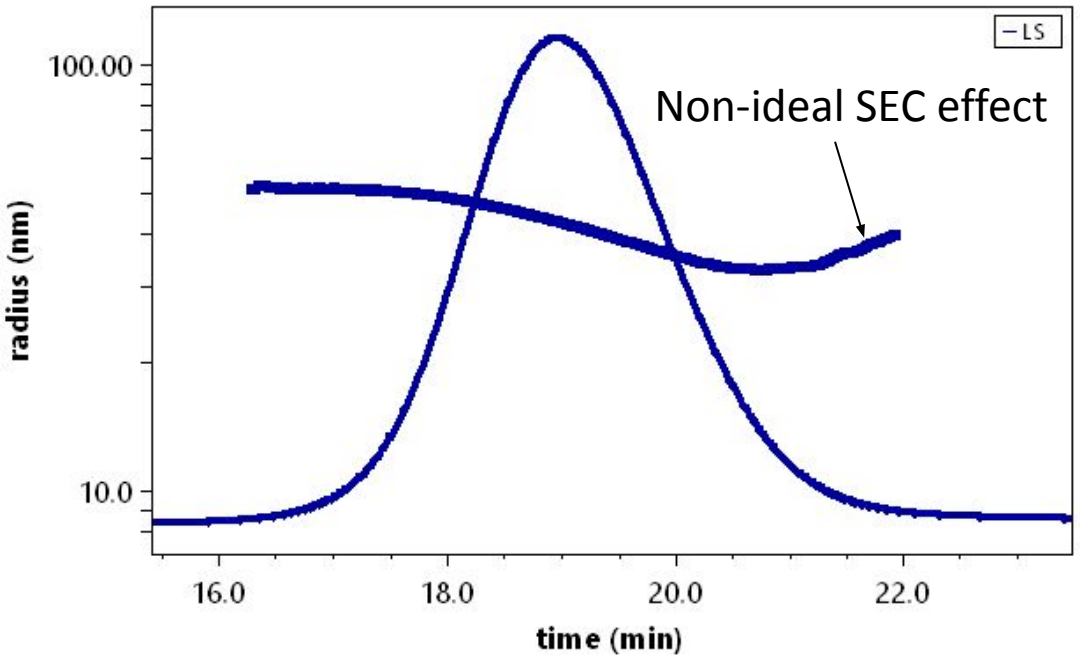


	M_w [kDa]	Agg [%]	R_g [nm]	R_h [nm]	R_g/R_h
EPO	272 ± 1	4.8	15 ± 1	12 ± 1	1.2 ± 0.1
fLuc	622 ± 1	2.6	20 ± 1	17 ± 1	1.2± 0.1

SEC or FFF to separate LNP



SEC



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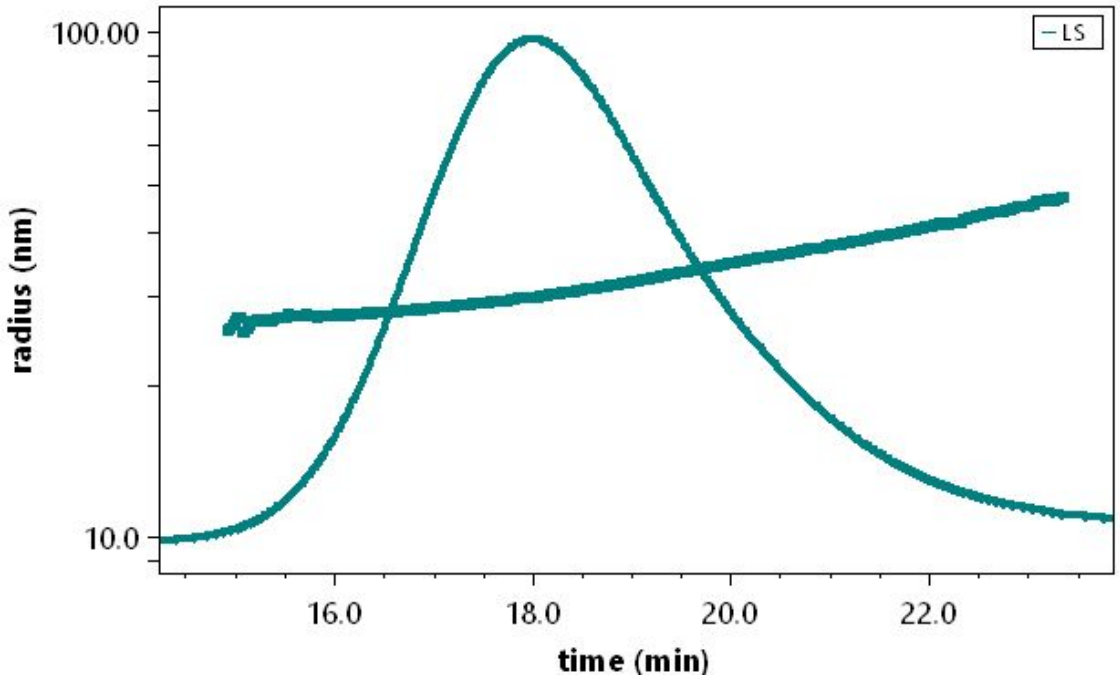
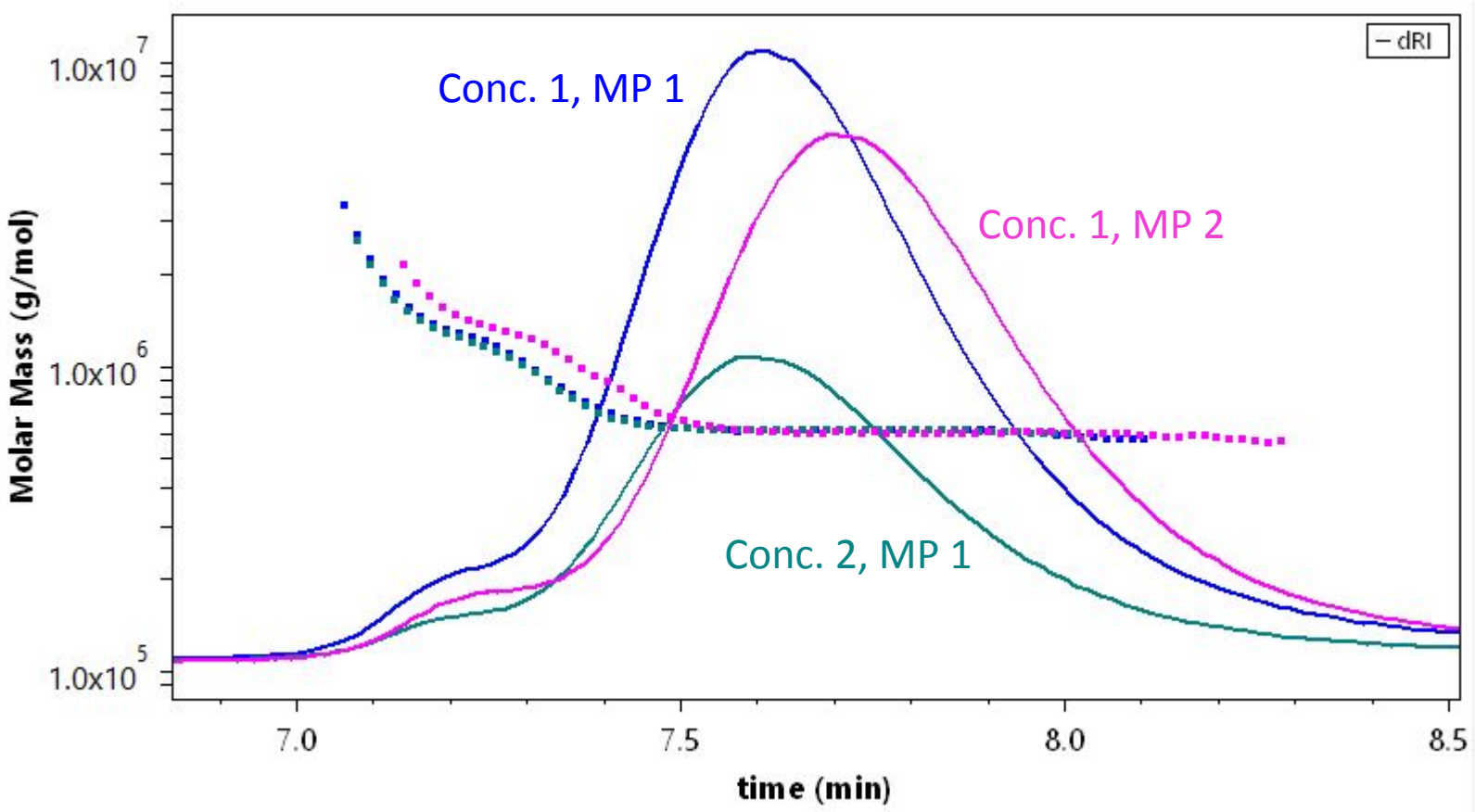


- LNP is polydisperse with continuous size/MW distribution.
- FFF for LNP stability studies, separation between free RNA and LNP-RNA, and sticky LNPs.

Measuring RNA integrity by SEC-MALS

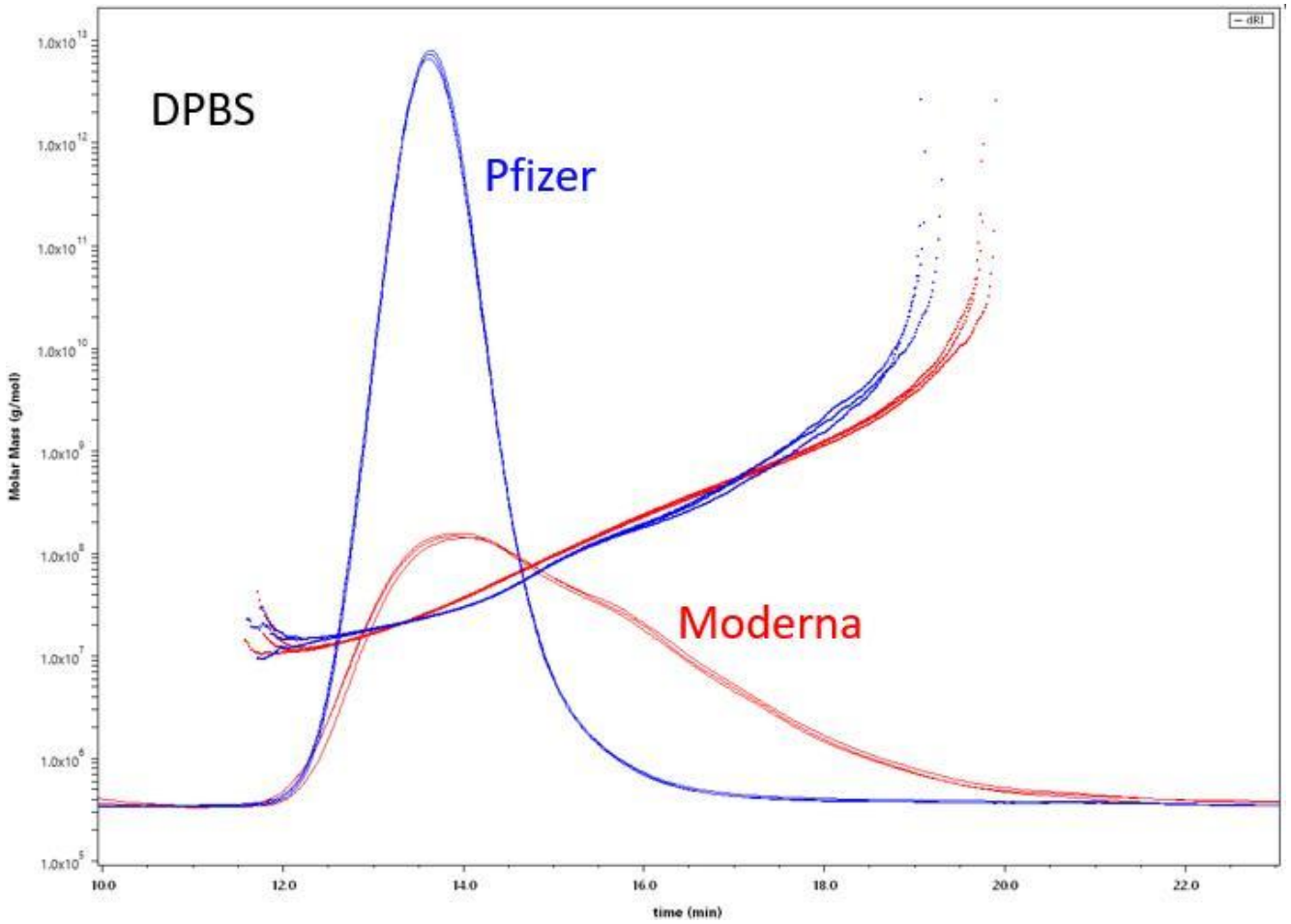
Attribute
✔ RNA integrity
LNP size
LNP distribution
LNP number
Physical stability
LNP morphology
Encapsulation efficiency
mRNA concentration
Lipid concentration

Effect of mRNA concentration and mobile phase



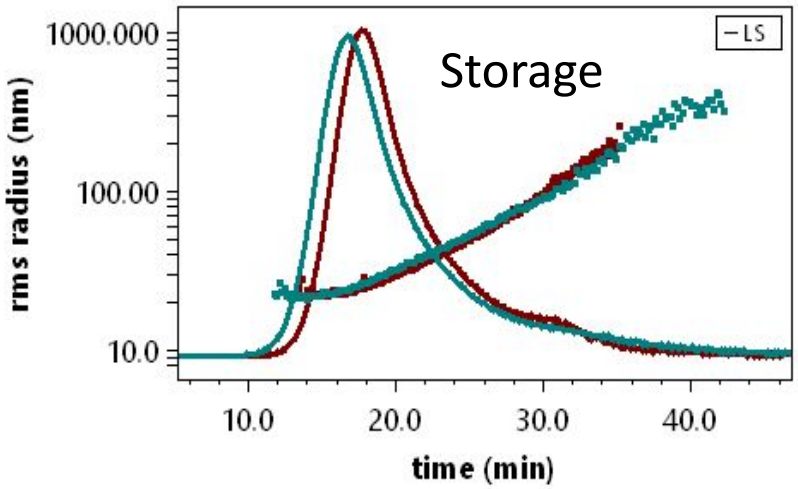
LNP size and polydispersity by FFF-MALS

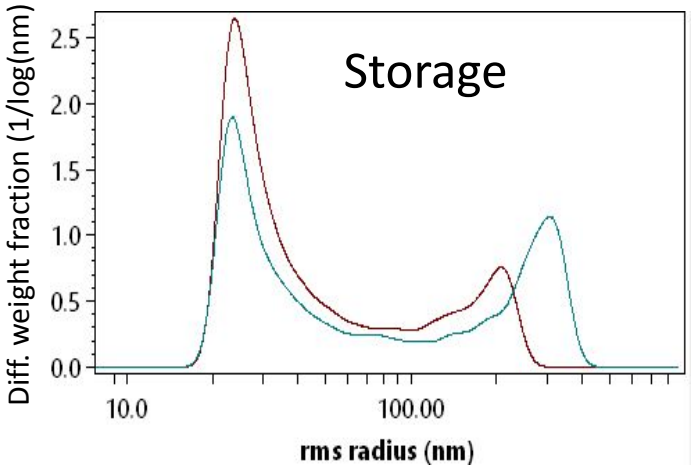
Attribute
✔ RNA integrity
✔ LNP size
✔ LNP distribution
✔ LNP number
Physical stability
LNP morphology
Encapsulation efficiency
mRNA concentration
Lipid concentration

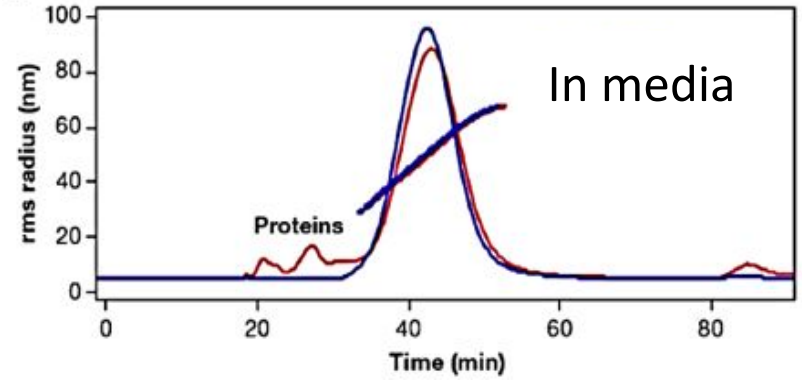


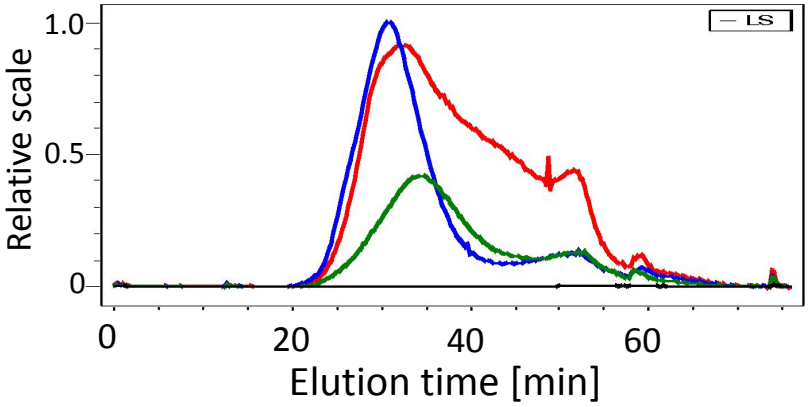
Physical stability

Attribute
✔ RNA integrity
✔ LNP size
✔ LNP distribution
✔ LNP number
✔ Physical stability
✔ LNP morphology
Encapsulation efficiency
mRNA concentration
Lipid concentration









Graph courtesy of Dr. Fanny Caputo, SINTEF

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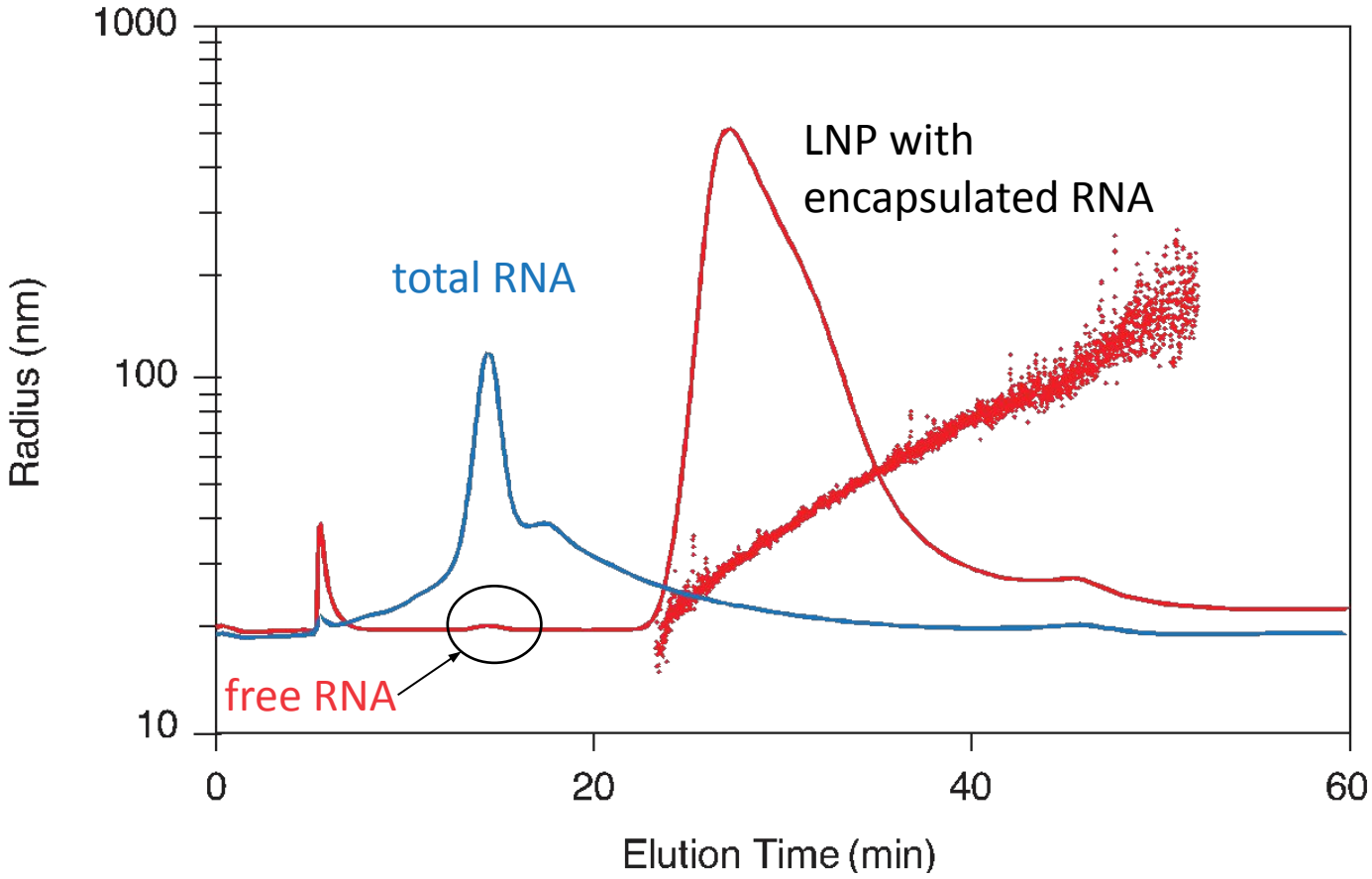
Encapsulation efficiency (EE)

Attribute
✔ RNA integrity
✔ LNP size
✔ LNP distribution
✔ LNP number
✔ Physical stability
✔ LNP morphology
✔ Encapsulation efficiency
mRNA concentration
Lipid concentration

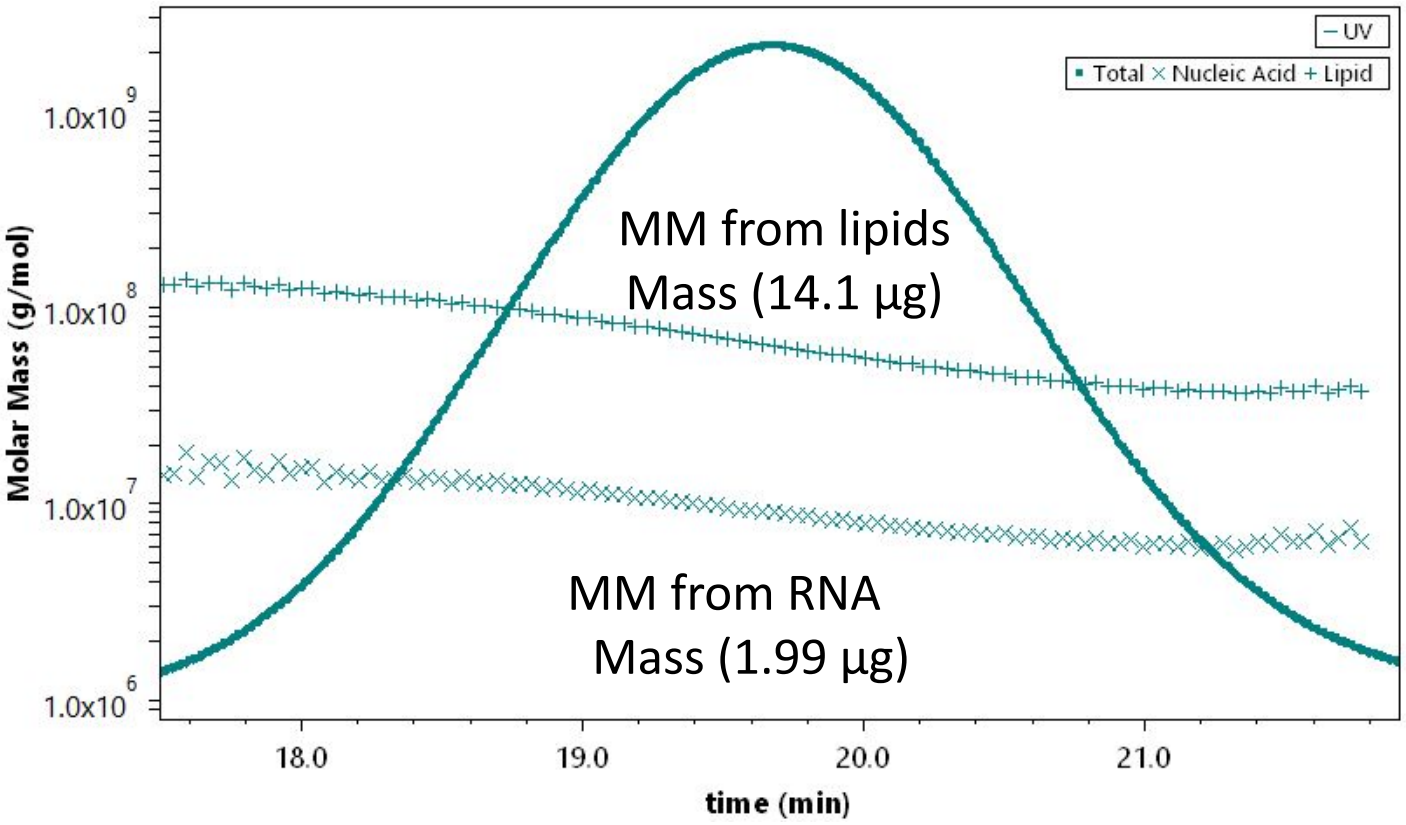
$$EE = (c_{\text{total RNA}} - c_{\text{free RNA}}) / c_{\text{total RNA}}$$



LNP and nucleic acid concentration

Attribute
✔ RNA integrity
✔ LNP size
✔ LNP distribution
✔ LNP number
✔ Physical stability
✔ LNP morphology
✔ Encapsulation efficiency
✔ mRNA concentration
✔ Lipid concentration

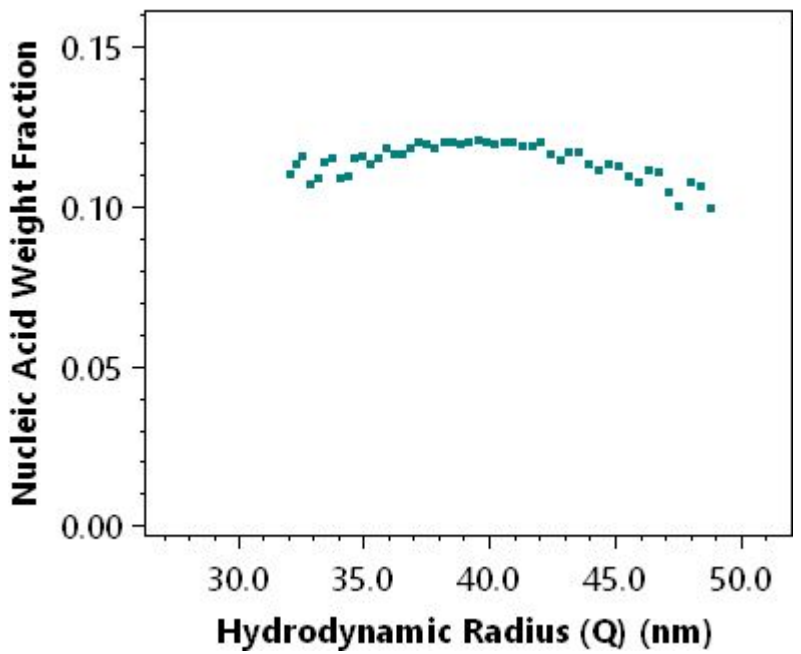
New LNP analysis for measuring payload

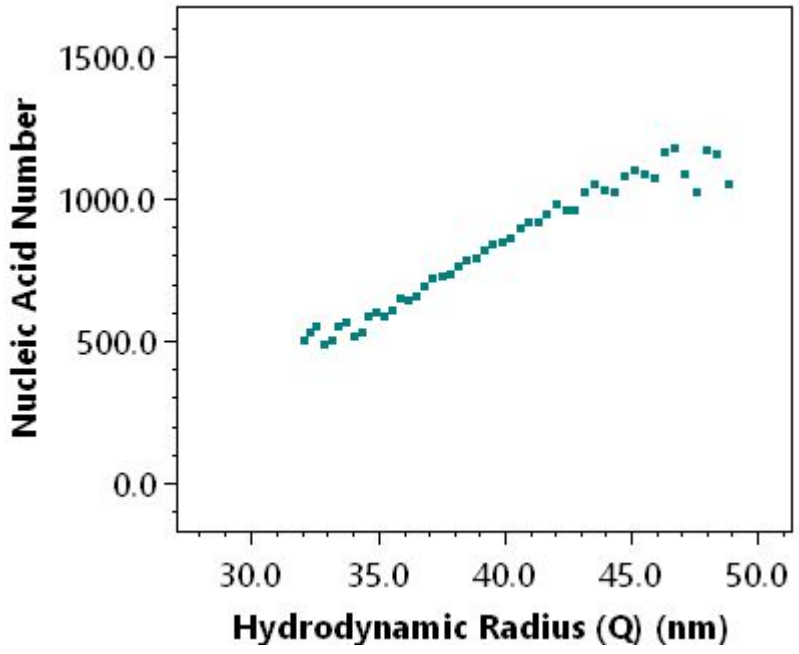


Additional quantitation: Nucleic acid payload

Attribute
✔ RNA integrity
✔ LNP size
✔ LNP distribution
✔ LNP number
✔ Physical stability
✔ LNP morphology
✔ Encapsulation efficiency
✔ mRNA concentration
✔ Lipid concentration

LNP analysis for measuring payload

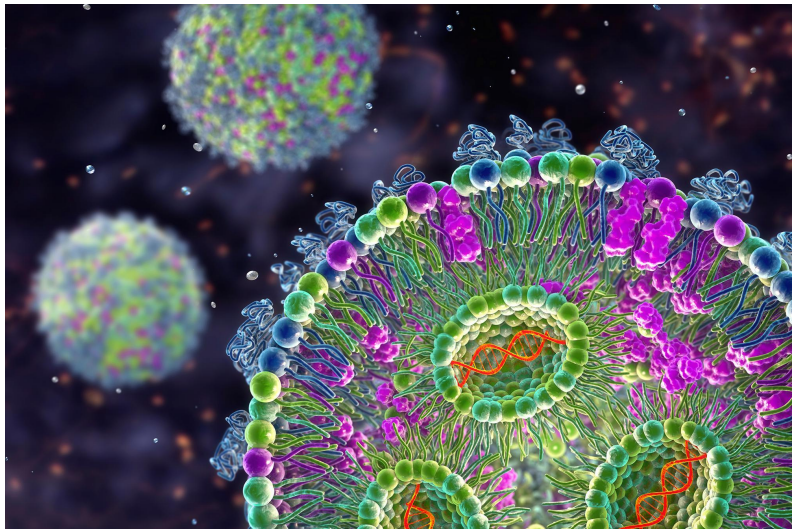




	Mw (Nucleic Acid) (kDa)	Lipid Concentration (mg/ml)	Nucleic Acid Concentration (mg/ml)	Encapsulation Efficiency (%)	Nucleic Acid Number
F1a)	12778.2 (±0.5%)	10.67	1.42	97.8	819.1 (±0.5%)
F2a)	8805.0 (±1.3%)	7.36	0.97	96.8	564.4 (±1.3%)
F3a)	13180.6 (±0.9%)	7.95	1.07	97.5	844.9 (±0.9%)

Online LNP payload analysis principle

- RNA-LNP is a nanoconjugate.
- NP radius > 20 nm (radius): scattering contribution to UV extinction becomes non-negligible.
- Nanoconjugate analysis:
    - Remove UV scattering
    - Analyze payload with MALS, UV at 260 nm (after correction), and dRI signals (U.S. patent pending)



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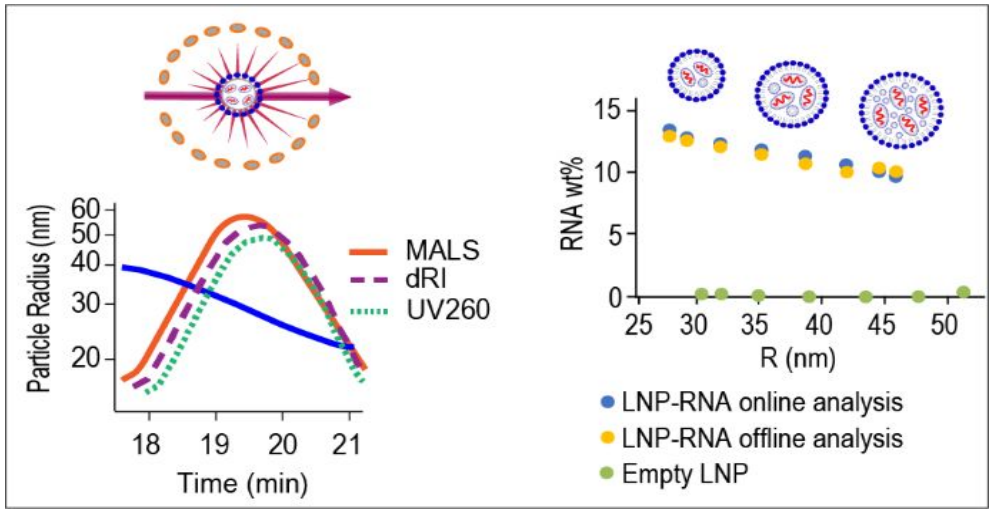
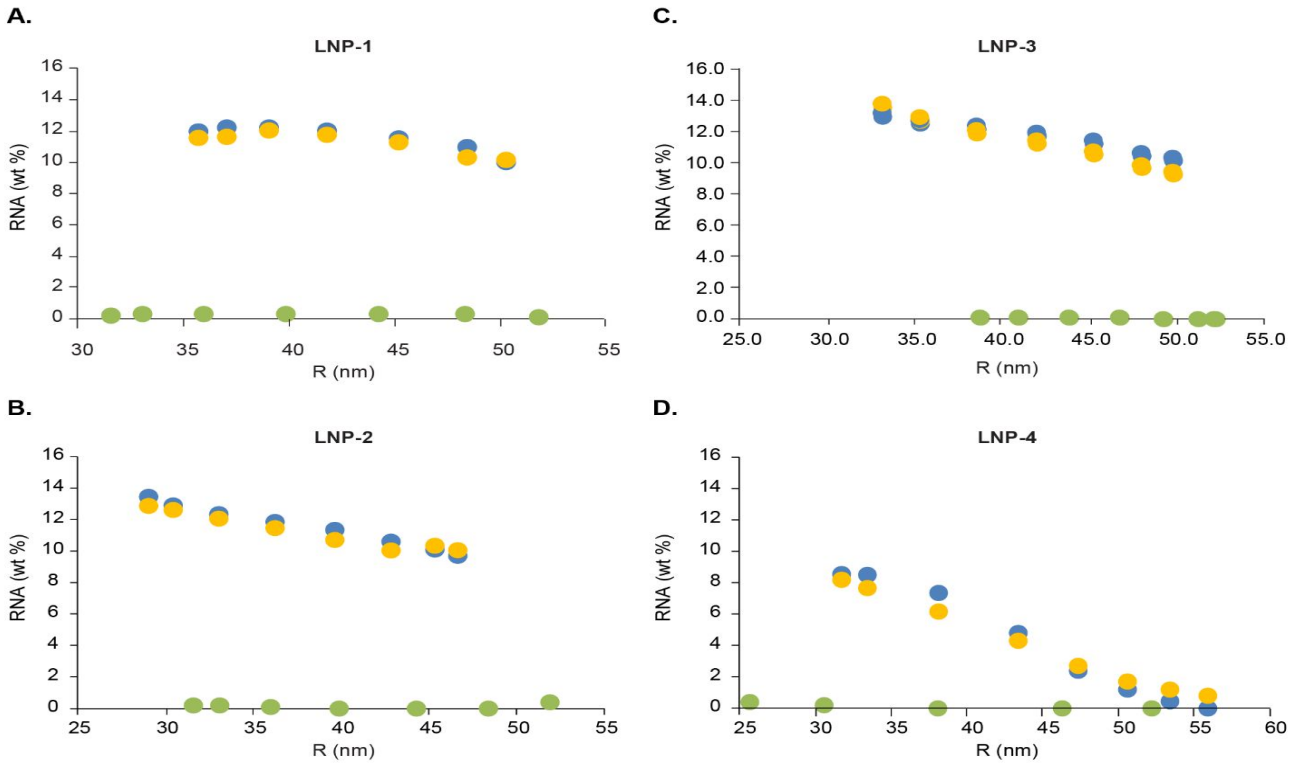
Cross-verification (Merck): LNP-siRNA by SEC-MALS-UV-dRI



X. Jia, et al, "Enabling online determination of the size-dependent RNA content of lipid nanoparticle-based RNA formulations", X. Jia, et al., *Journal of Chromatography B* 1186 (2021): 123015. https://doi.org/10.1016/j.jchromb.2021.123015



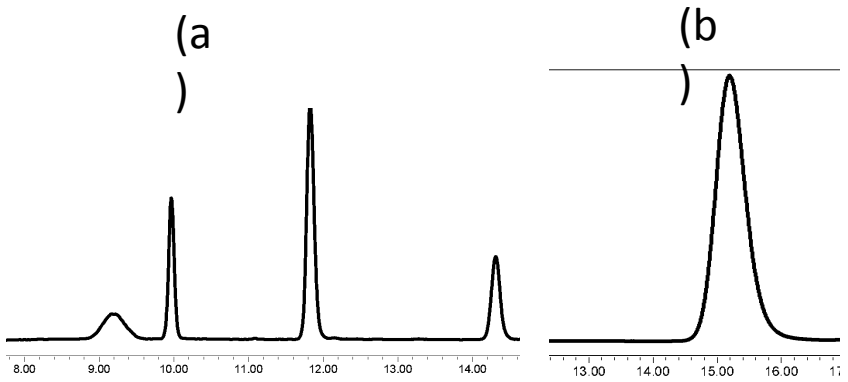
LNP-RNA, RNA wt% (online)
LNP-RNA, RNA wt% (offline)

empty LNP, RNA wt% (online)

Total RNA and lipids quantitation in the same run

sample ID	SEC + LC-CAD (mg/mL)		SEC-MALS (mg/mL)		%diff	
	RNA	Lipids	RNA	Lipids	RNA	Lipids
LNP-1	1.06	8.22	1.12	8.16	5.5	-0.7
LNP-2	1.07	8.30	1.12	8.24	4.6	-0.7
LNP-3	0.98	7.36	1.03	7.43	5.0	1.0

Data courtesy of Merck



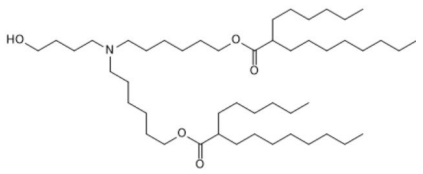
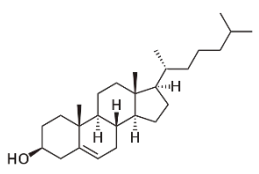
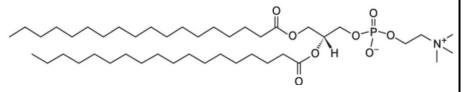
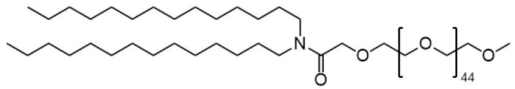
Offline (a) LC-CAD analysis of lipids;
(b) SEC-UV analysis of total RNA

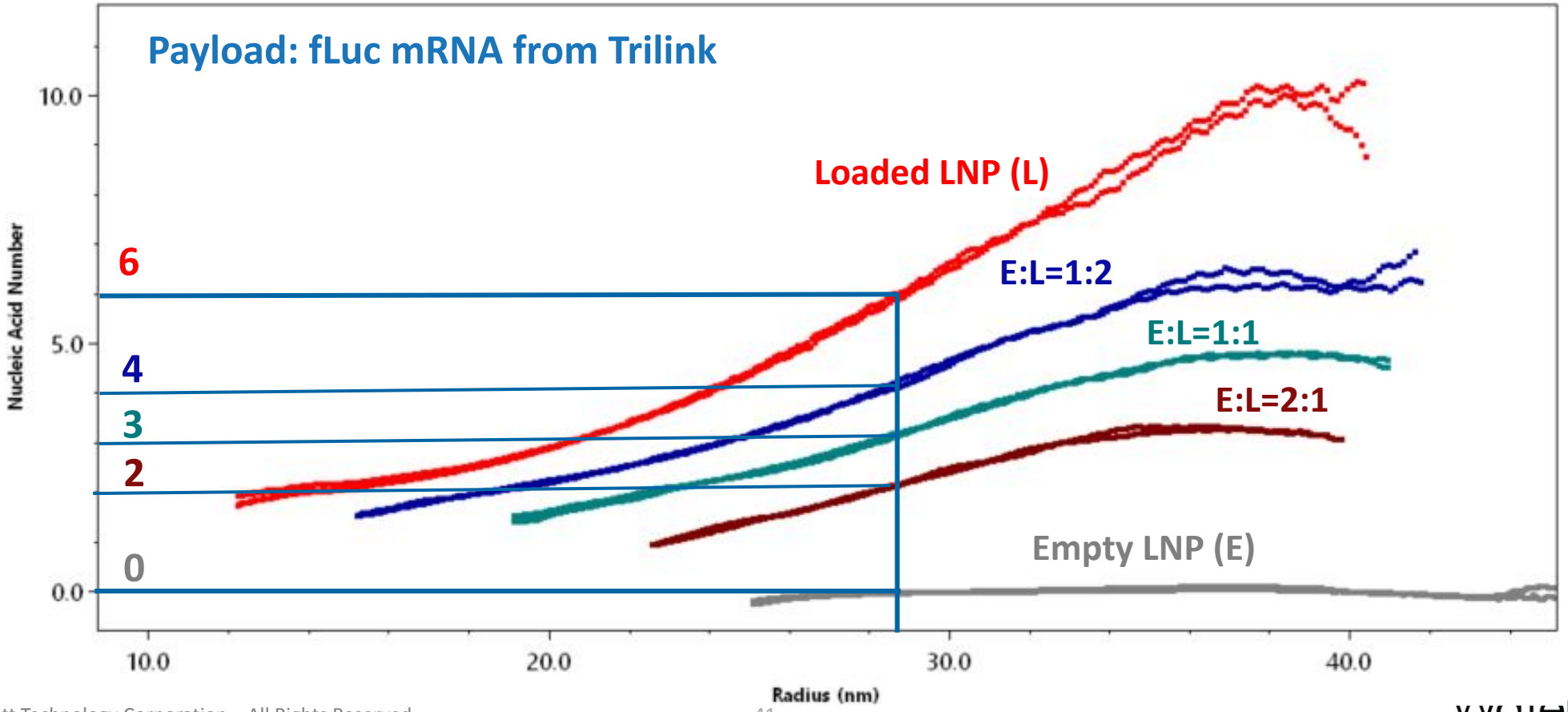
- Simultaneous online analysis of total lipids, RNA and other chemo-physical attributes
- Excellent agreement between online and offline approaches

Powerful yet simple online method for dosing and toxicity studies

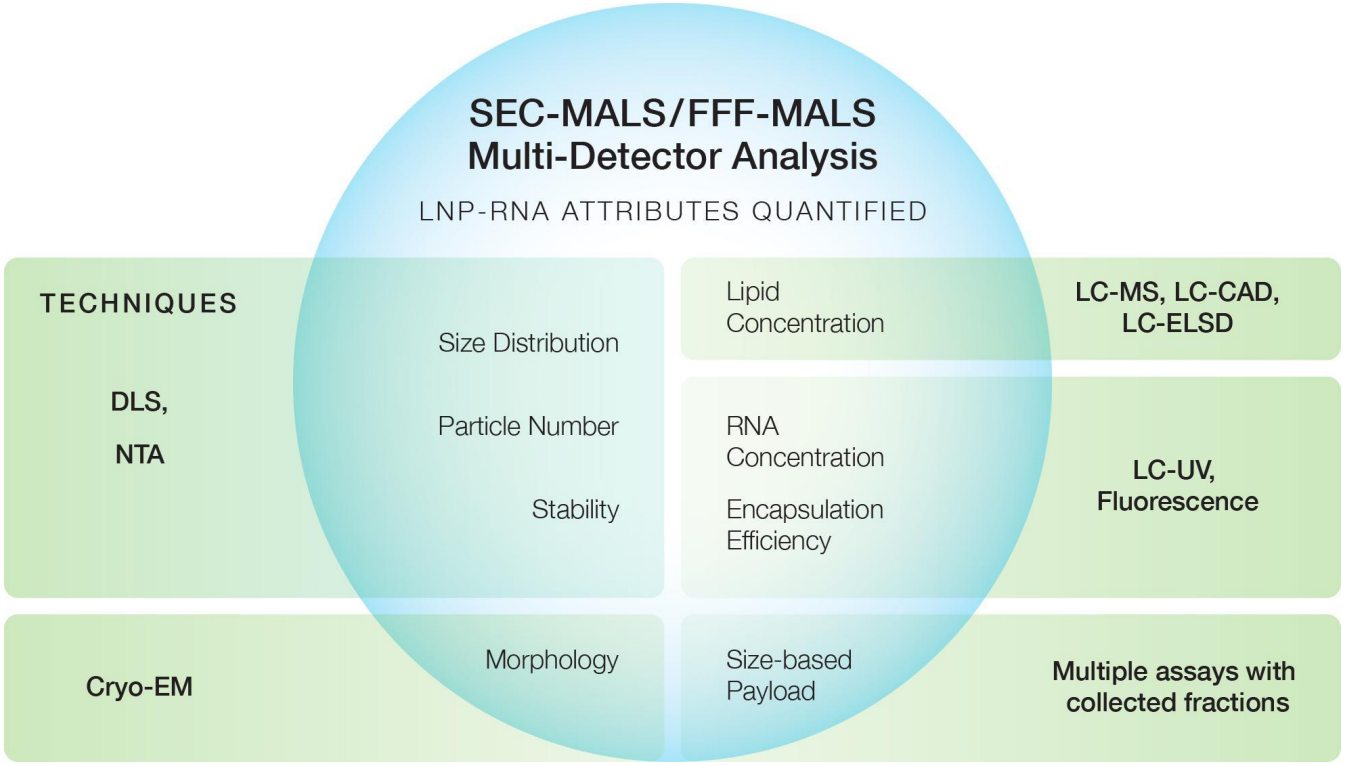
Self-consistency study

Pfizer lipid platform

ALC-0315	Cholesterol	DSPC	ALC-0159
			



SEC/FFF-MALS-UV-dRI: A comprehensive method for LNP-MAQ



FFF-MALS: "Improved multidetector asymmetrical-flow field-flow fractionation method for particle sizing and concentration measurements of lipid-based nanocarriers for RNA delivery", Mildner, R., et al., *Euro. J. Pharm. Biopharm.* 163 (2021): 252-265. https://doi.org/10.1016/j.ejpb.2021.03.004

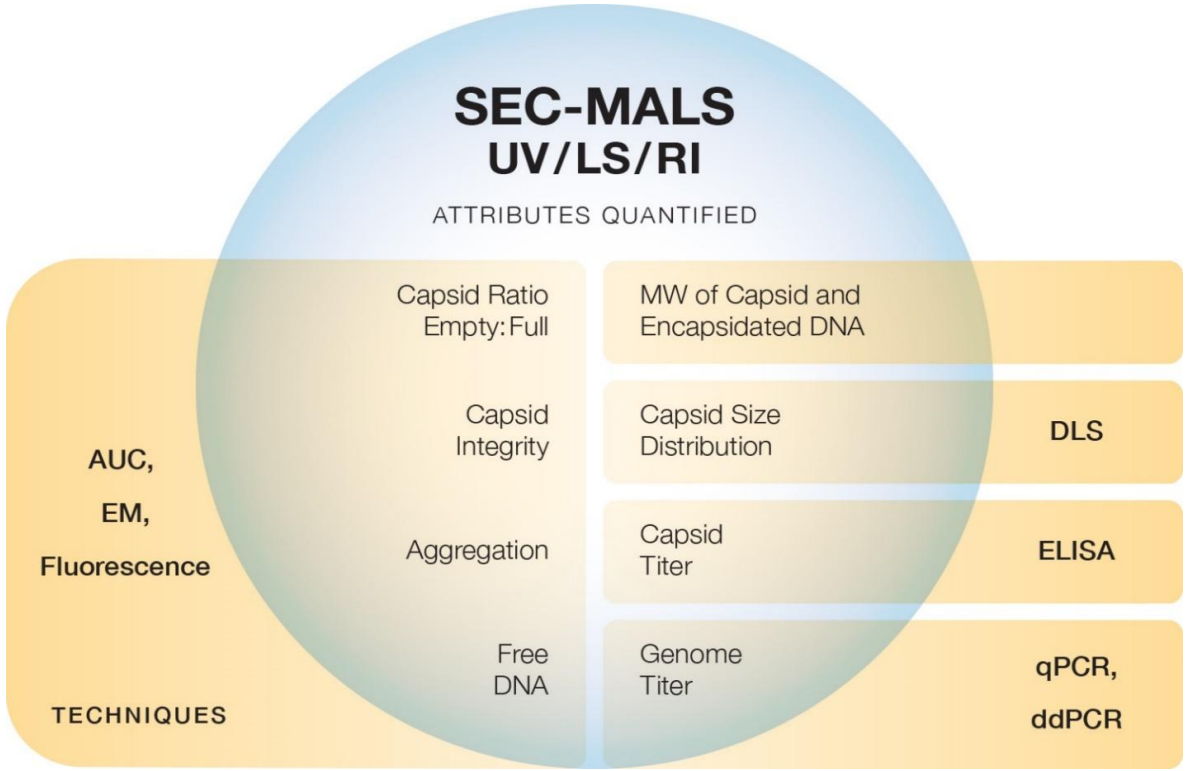
SEC-MALS: "Polydispersity characterization of lipid nanoparticles for siRNA delivery using multiple detection size-exclusion chromatography", Zhang, J. et al., *Anal. Chem.* 84(14), 6088-6096 (2012). https://doi.org/10.1021/ac3007768

Key LS methods for proteins, AAVs, and LNPs

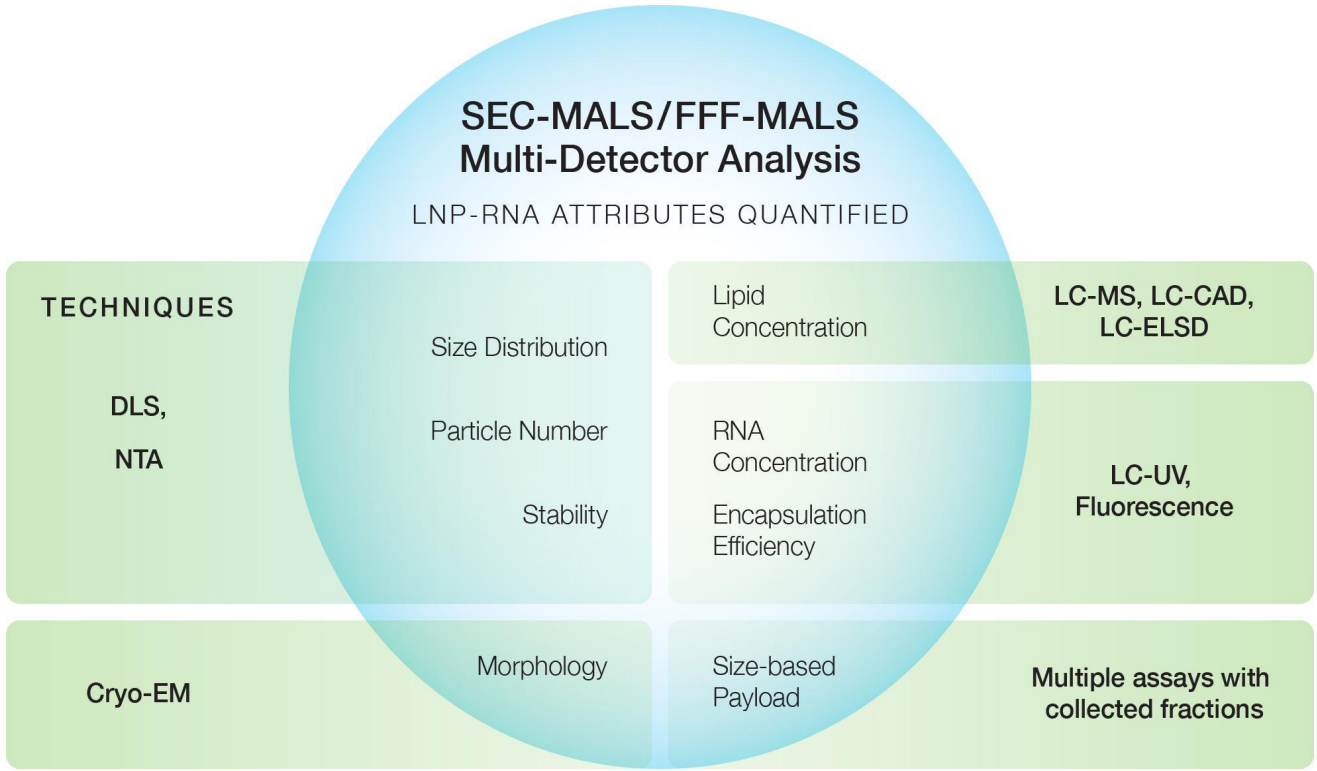
	DLS	SEC-MALS	FFF-MALS
Proteins	▪ Developability & formulation studies ▪ Aggregate screening	Platform method for: MW, composition, aggregation, etc.	▪ Troubleshooting
AAVs (VVA Module)	▪ Size, polydispersity ▪ Titer ▪ Aggregation	Platform method for MAQ: ▪ Titer ▪ E/F ▪ Aggregation ▪ Extended characterization	▪ Quantify all AAV aggregates
LNPs (LNP Module)	▪ Size, polydispersity ▪ Particle concentration ▪ Aggregation	Primary sized-based separation and characterization tool for LNPs	Platform method for MAQ: ▪ High-res size distribution ▪ Size-based payload distribution ▪ Online lipid conc. ▪ RNA or DNA HOS ▪ Isolation of narrowly distributed fractions

Thank you for your attention! Questions?

SEC-MALS for AAV-MAQ



FFF-MALS for LNP-MAQ



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