

***In vitro* Analytical Characterization
based Quality and Potency
Assessment of mRNA Vaccines**

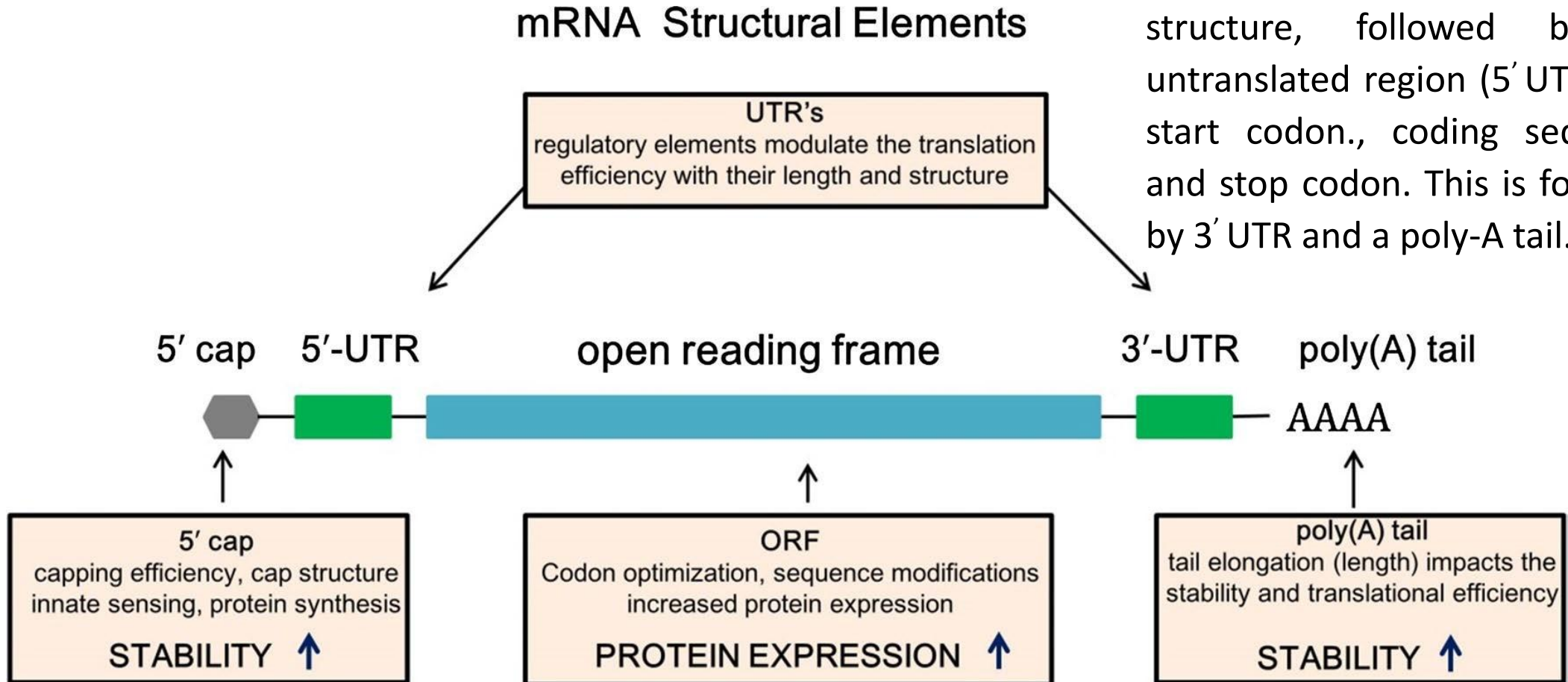
**Gautam Sanyal
Vaccine Analytica, LLC**

Animal Testing Replacement for Vaccines

2025-December 2-4, Bangkok, Thailand

Structural Elements of an mRNA Vaccine: ORF Encoding Protein Antigen of interest, Flanked by 5' and 3' UTRs, 5' Cap Structure and 3' Poly(A) Tail

Structure of in vitro transcribed (ivt) mRNA starts with a cap structure, followed by an untranslated region (5' UTR), the start codon., coding sequence and stop codon. This is followed by 3' UTR and a poly-A tail.



Self-simplifying RNA (saRNA) and Circular RNA (circRNA)

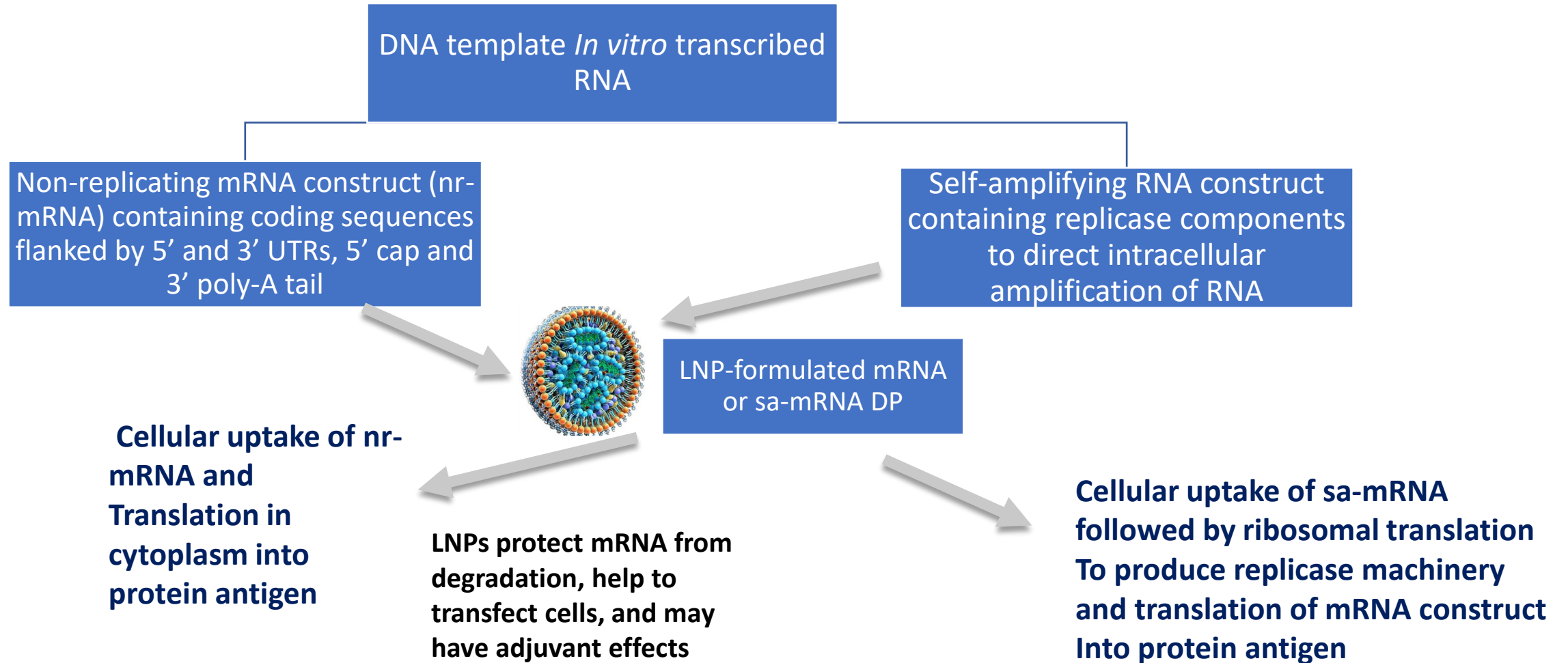
saRNA

- A single strand RNA containing a 5' methylguanylate cap and a 3' polyA sequence
- The saRNA vector contains two Open Reading Frames (ORFs):
 - the first ORF codes for non-structural proteins of a viral replicase (e.g., from Alphavirus).
 - The second ORF codes for antigens of interest, e.g. SARS-CoV-2 antigen(s)
- saRNA also includes
 - 5' and 3' UTRs
 - A packaging signal (PS), and a subgenomic promoter (sgPr) between both ORFs

circRNA

- More stable than linear mRNA
- Ribosome recruitment through Internal Ribosomal Entry Site (IRES)
- Transfects cells and translates to encoded protein antigen the same way as mRNA

Antigen Production Pathways for Non-Replicating and potentially Dose-Sparing Self-Replicating mRNA Vaccine Constructs



CMC Considerations: mRNA DS and mRNA-LNP DP

K. Peden (CBER-FDA) at Public Workshop on FDA Guidance to Industry on Nanomaterial – October 11, 2022

- **Drug Substance**
 - **Assessing mRNA integrity**
 - **Quantifying the proportion of RNA that is:**
 - **Capped**
 - **Polyadenylated**
 - **Full length**
 - **Identity of mRNA (DNA template sequence, RNA sequence, PCR)**
 - **Purity of mRNA (smaller products, dsRNA, residual components of the IVT)**
- **Drug Product**
 - **Evaluation of the LNP structure (*e.g.*, particle size distribution; charge)**
 - **Determining percentage of total mRNA encapsulated**
 - **Identifying and quantifying impurities**
- **Stability of DS and DP (transportation and storage)**

Potency Determination for mRNA-LNP Vaccine Candidates

K. Peden (CBER-FDA) at Public Workshop on FDA Guidance to Industry on Nanomaterial – October 11, 2022

- **How to evaluate potency when the vaccine type is new**
- **Correlate of protection against pathogen is not known**
- **It is not known what type of immune response is necessary to elicit**
- **Potency depends on uptake of the LNP-mRNA by cells and the expression of the antigen**
- **Potency assays:**
 - **Cell-culture-based (read-outs: ELISA, Western, Flow Cytometry)**
- **After demonstrating the vaccine is immunogenic, animal potency assays were not considered practical, desirable, or able to be validated**
- **Dosing is done by the amount of RNA**

EMA Draft Guideline on the Quality Aspects of mRNA Vaccines

27 March-2025

Potency

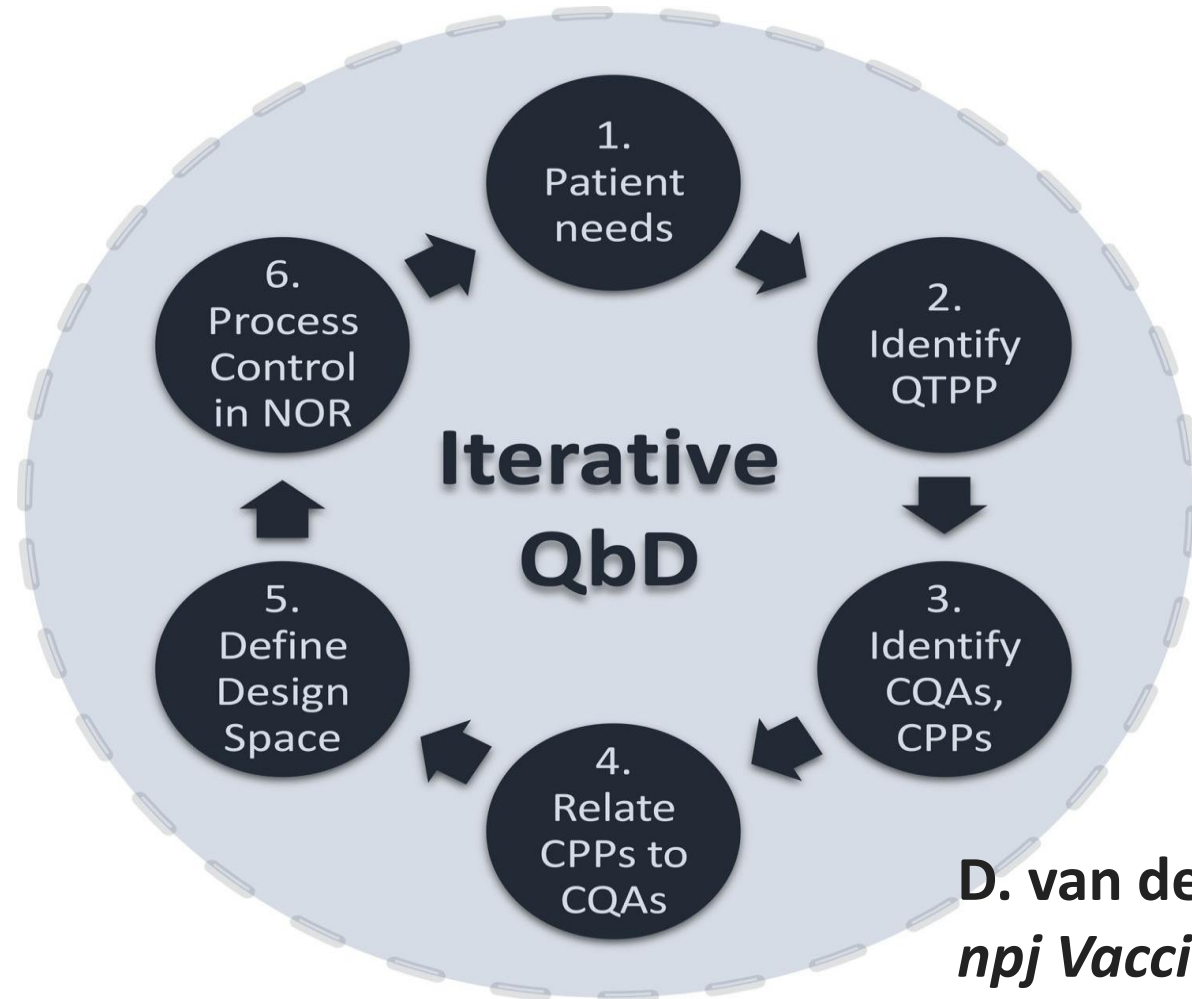
- **Potency of the finished product is determined by a combination of tests including evaluation of RNA content, mRNA integrity and efficiency of mRNA encapsulation.**
 - **Potency testing of the finished product should be performed at release and at shelf-life.**

Functionality

- **An antigen-specific cell-based functionality assay should be developed to confirm the LNP uptake, mRNA escape from the endosome and translation of the mRNA into encoded protein/antigen.**
 - **Due to the limitations of such methods, this assay can be semiquantitative**
 - **Functionality of the finished product should be tested at release and at shelf-life**

Quality-by-design (QbD) Framework Development Cycle for Bioprocess Optimization

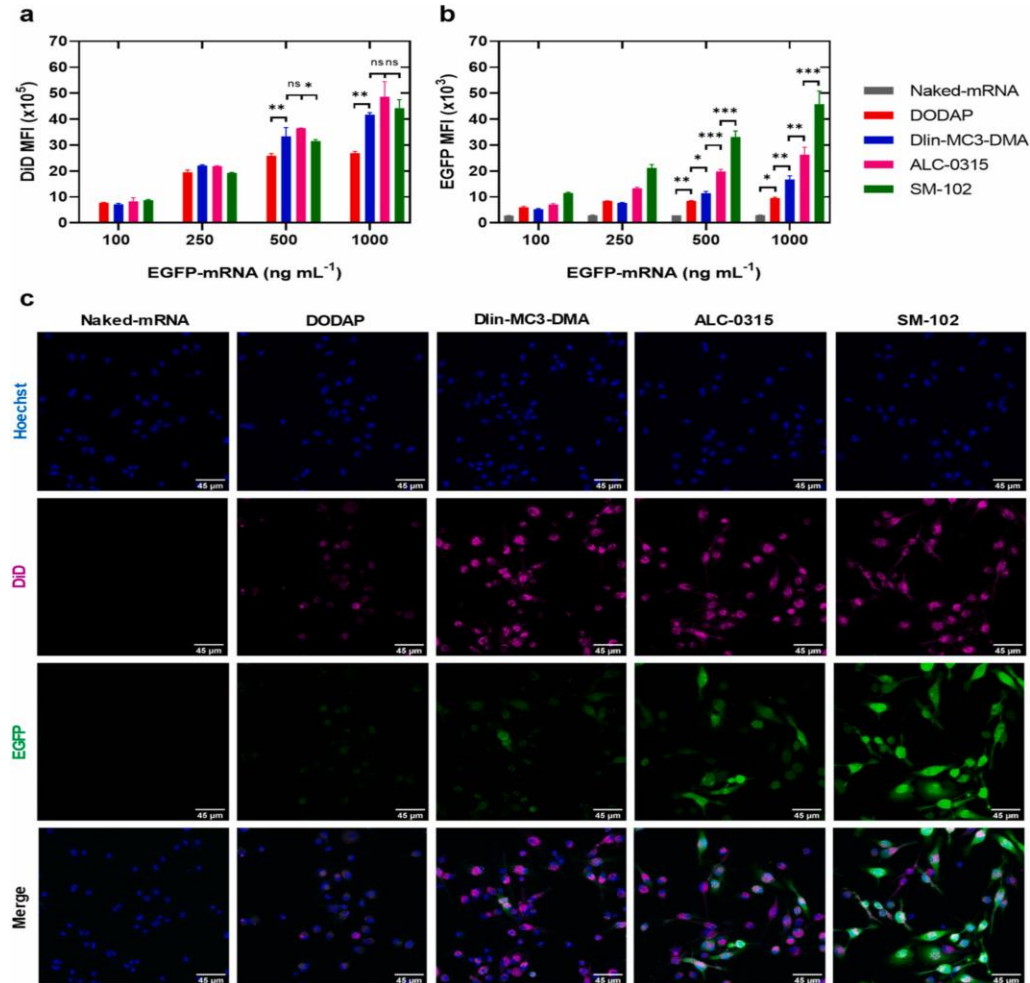
In-process measurements of Critical Quality Attributes (CQAs) drive optimization of critical process parameters (CPP) and the normal operating range (NOR) for a process yielding desired quality target product profile (QTPP)



D. van de Berg et al.,
npj Vaccines 6: 65
(2021)

In Vitro Protein Expression from mRNA Encoding eGFP – Different Expression Levels using LNPs constituted of Different Ionizable Lipids

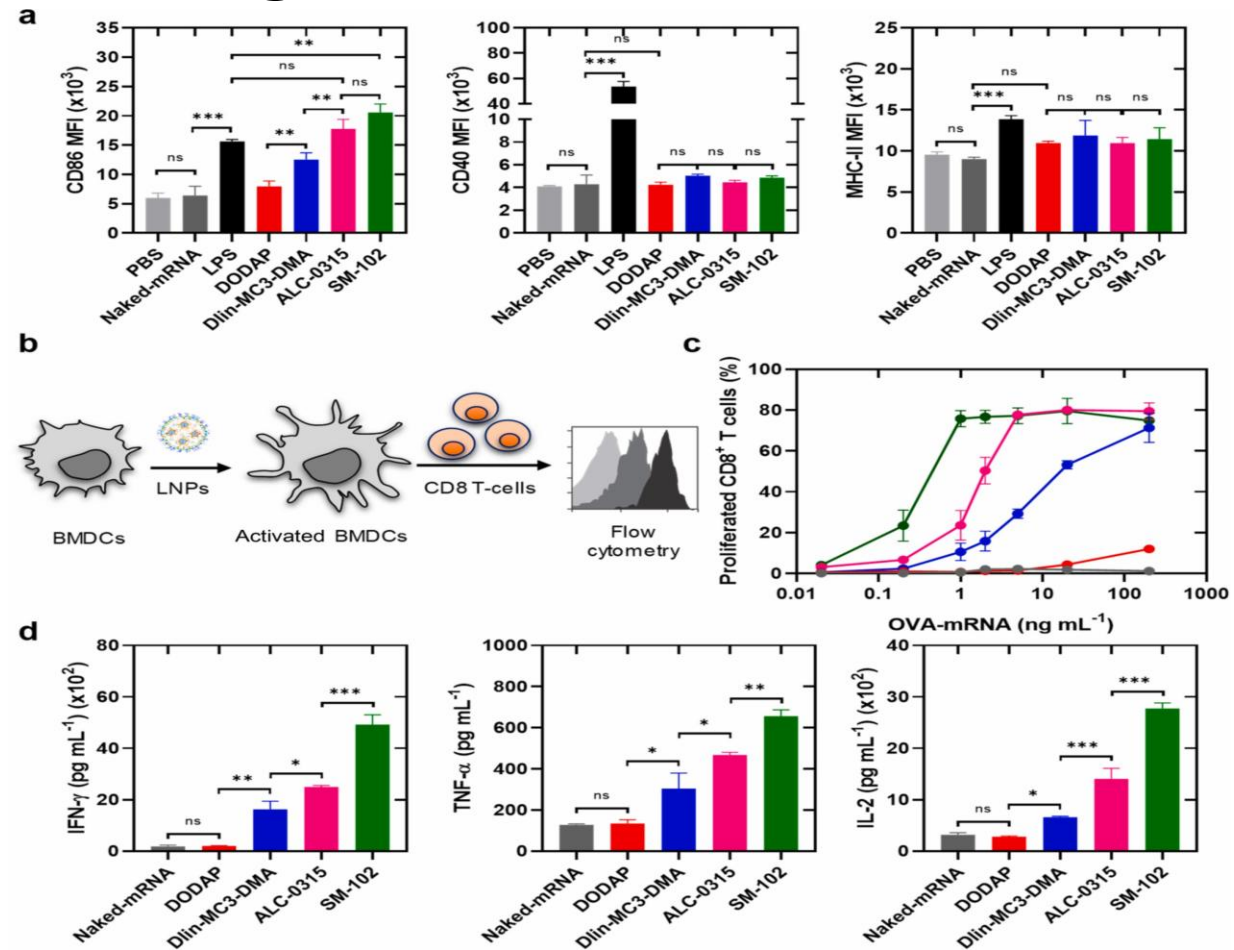
In vitro cellular uptake and transfection of the mRNA-LNPs in primary BMDCs. a) Cellular uptake and b) eGFP expression after exposing BMDCs with DiD-labeled eGFP-mRNA-LNPs for 24 h, as determined by flow cytometry. Cellular uptake and eGFP expression efficiencies were higher for SM-102 than ALC-0315, c) Confocal microscopy images of BMDCs showing cellular uptake (magenta: DiD-labeled LNPs) and protein expression (green: eGFP)



SM-102 > ALC-0315 > Dlin-MC3 for *in vitro* protein expression (eGFP)

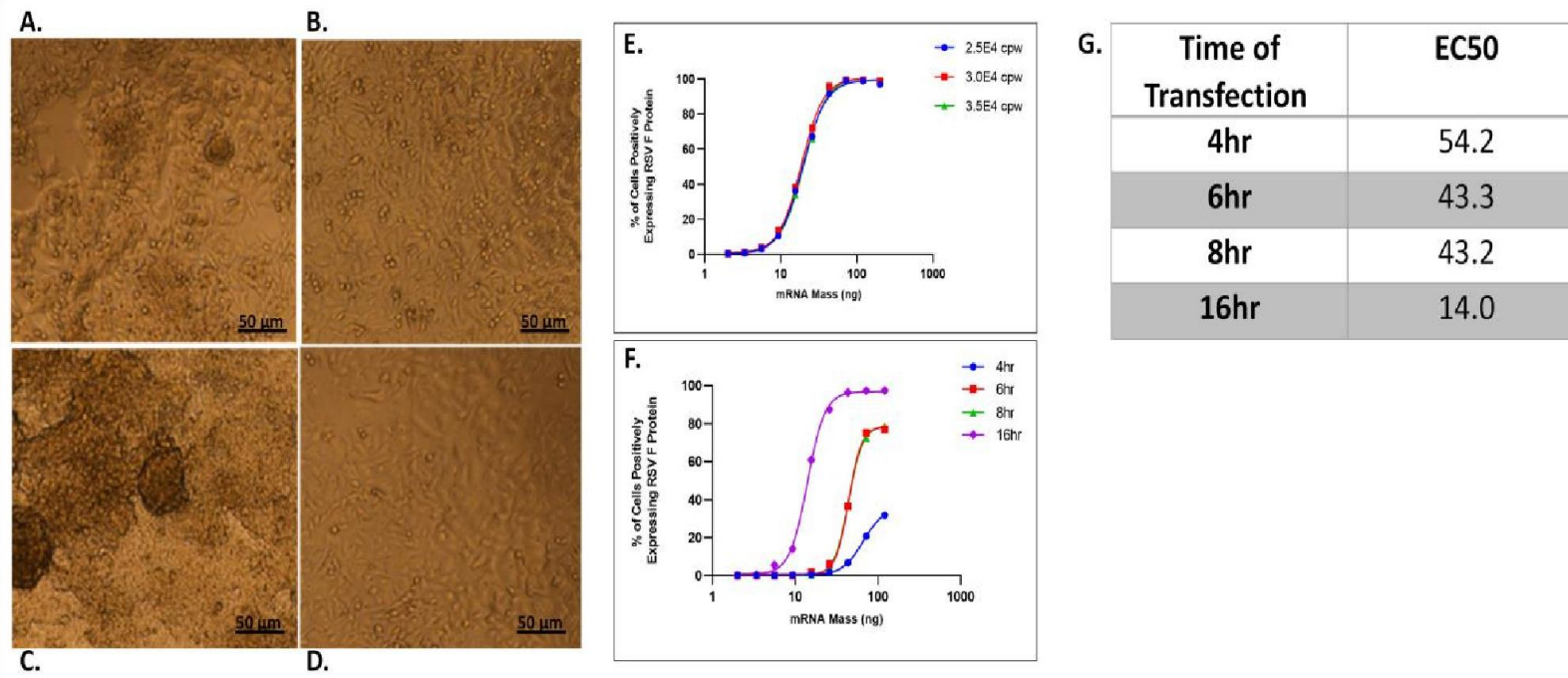
“*In vitro* and *in vivo* Evaluation of ionizable cationic Lipids Shows Divergent Results between mRNA Transfection and Vaccine Efficacy”? Different Antigens used *In Vitro* & *In Vivo*”

Ex vivo proliferation of antigen-specific T cells induced by OVA-mRNA-LNPs. a) Levels of the surface markers; CD86, CD40 and MHC-II in BMDCs after treating with the four OVA-mRNA-LNP formulations (OVA-mRNA concentration; $1 \mu\text{g mL}^{-1}$) for 24 h, as determined by flow cytometry. b) Schematic of ex vivo assay to evaluate T cell activation and proliferation. c) Percentages of proliferated OVA-specific CD8 \rightarrow T cells after co-cultured with the OVA-mRNA-LNP-treated BMDCs for 72 h and d) cytokines levels in the culture supernatant of the stimulated T cells, as determined by flow cytometry and ELISA



SM-102 =
ALC-0316
= Dlin-
MC3 for *in vivo*
protein
expression
(eGFP) in
Zebrafish
embryous

***In Vitro* Cell-based Potency Assay Optimization for an mRNA Vaccine Candidate Encoding RSV Pre-Fusion Protein**

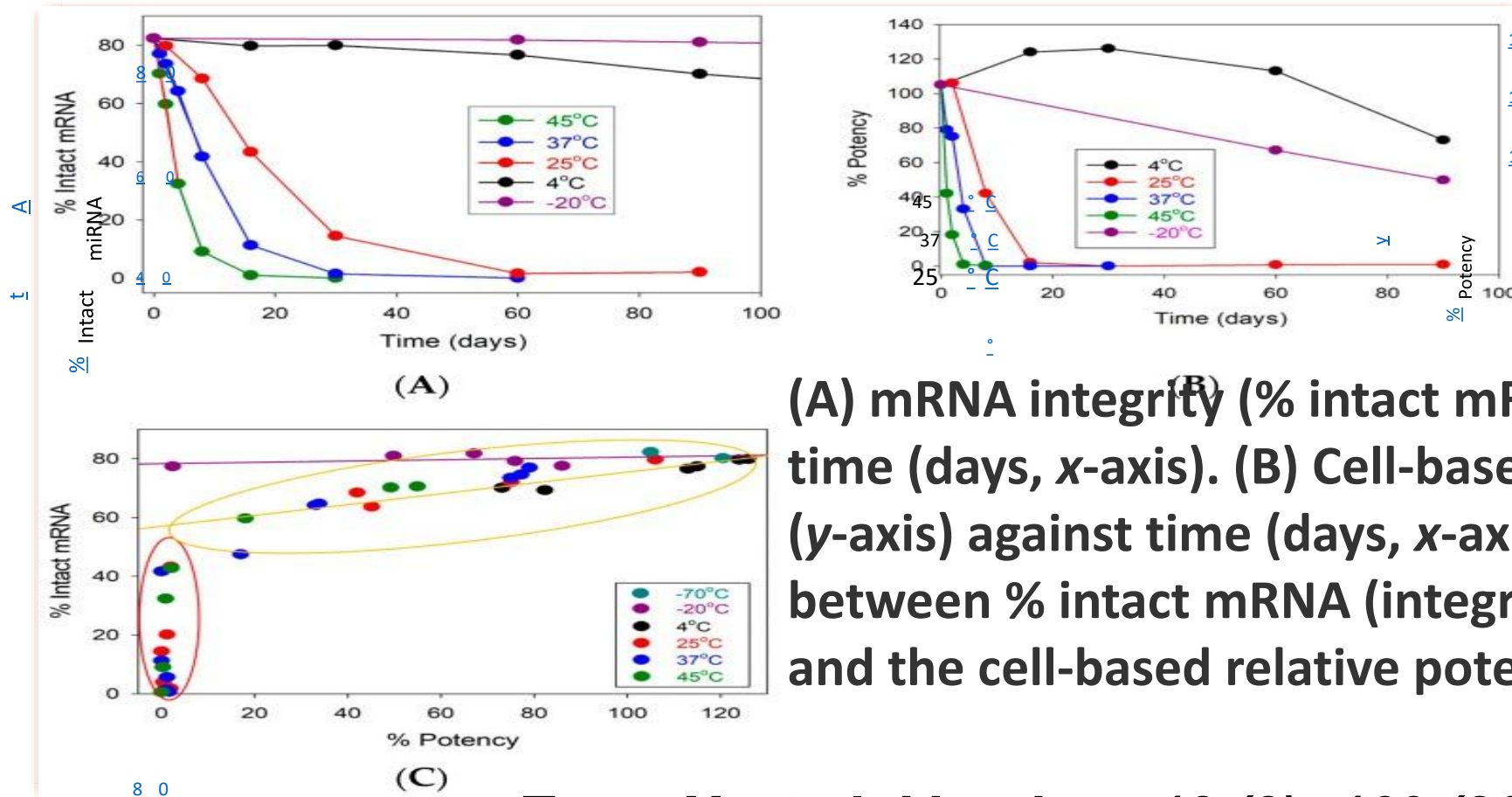


Optimization of cell plating parameters and transfection kinetics. Morphological characteristics of HepG2 monolayers on tissue culture plates and collagen I-coated plates at different cell seeding densities along with transfection kinetics of mRNA-LNP encoding RSV-F protein

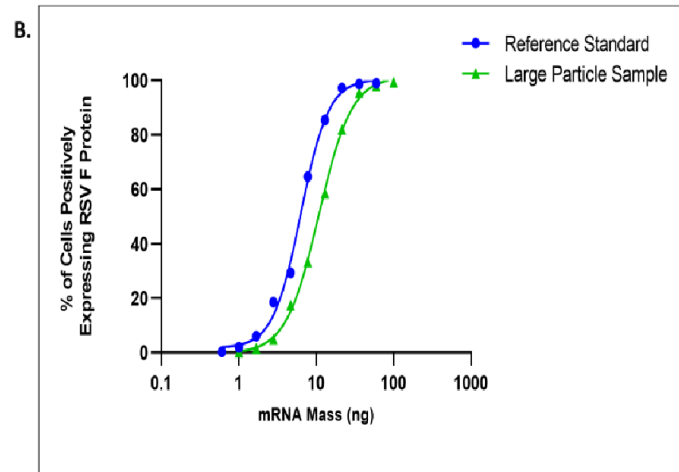
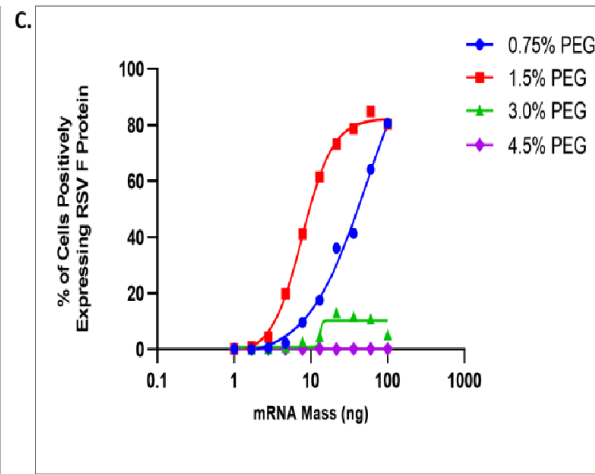
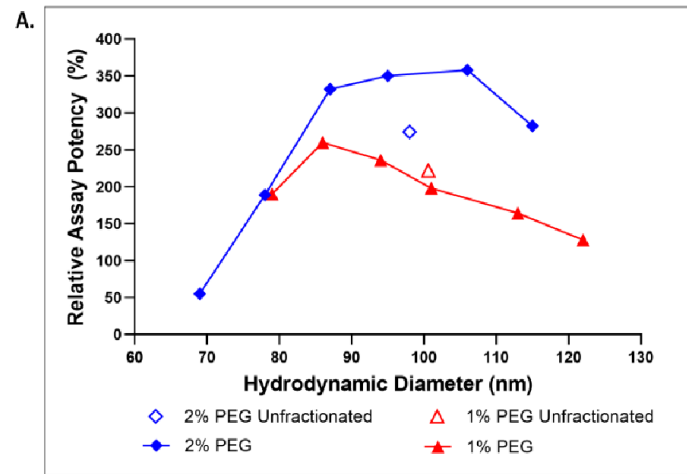
Patel, N. et al. Vaccines 2023, 11, 1224.

<https://doi.org/10.3390/vaccines11071224>

Correlation between mRNA Integrity as Measured by Capillary Electrophoresis and Cell-based *In Vitro* Potency



RSVpreF mRNA-LNP Transfection in HepG2 Cells is Optimal within a Narrow LNP Size and PEGylation Range



D.

Sample ID	Particle Size (d.nm)
0.75% PEG	136
1.5% PEG	92
3.0% PEG	71
4.5% PEG	66

(A) Relative potency (%) for 1% and 2% PEG-DMG unfractionated samples compared to samples after density gradient centrifugation resulting in 6 differing size fractions. (B) RSV-F expression efficiency for sample lots with differing mean LNP size. (C) RSV-F expression efficiency for LNPs with 0.75, 1.5, 3.0 and 4.5% PEG and (D) Associated particle size.

Patel, N. et al. *Vaccines* 2023, 11, 1224

<https://doi.org/10.3390/vaccines11071224>

RSVpreF-Binding Activity (ELISA)- Seroconversion of Animals Post-Dose 1 of mRNA-LNP Encoding RSVpreF Protein

Dose (μg mRNA)	Lot 1	Lot 2	Lot 3	Lot 4	Lot 5	Lot 6
0.125	0	0	0	0	0	0
0.25	0	0	0	1	0	0
0.5	0	0	0	6	4	2
1	1	1	3	9	9	8
2	9	9	9	10	10	10
4	10	10	10	10	10	10
8	10	10	10	10	10	10
12	10	10	10	10	10	10
ED50	1.41	1.41	1.23	0.47	0.58	0.71
95% CI	(1.10, 1.81)	(1.10, 1.81)	(0.93, 1.63)	(0.34, 0.64)	(0.43, 0.77)	(0.53, 0.94)

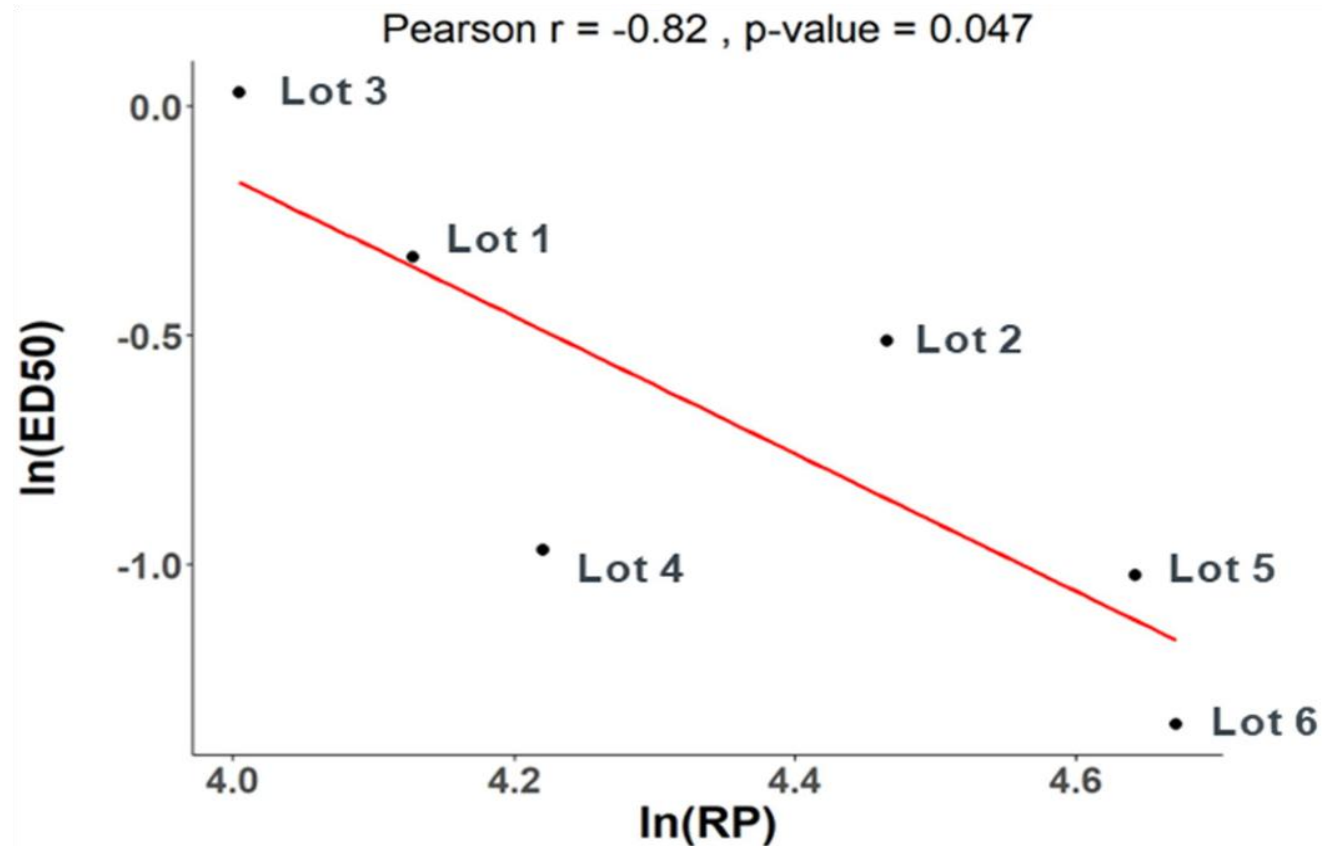
Feller, K. et al. *Vaccines* 13, 326 (2025)

RSV Pseudovirus Neutralizing Activity - Seropositive Animals Post-Dose 2 of mRNA-LNP Encoding RSVpreF Protein

Dose (µg mRNA)	Lot 1	Lot 2	Lot 3	Lot 4	Lot 5	Lot 6
0.125	1	1	0	0	0	1
0.25	0	0	0	3	2	4
0.5	0	4	3	6	9	9
1	8	7	4	10	9	10
2	10	10	9	10	10	10
4	10	10	10	10	10	10
8	10	10	9 *	10	10	10
12	10	10	10	10	10	10
ED50	0.72	0.6	1.03	0.38	0.36	0.26
95% CI	0.52–0.99	0.43–0.83	0.71–1.49	0.29–0.50	0.27–0.48	0.20–0.35

Feller, K. et al. *Vaccines* 13, 326 (2025)

Correlation between Cell-based Relative Potency of RSVpreF encoding mRNA-LNP and RSV Pseudovirus Neutralization ED50



- Six production lots were used, all with reasonably good *in vitro* potencies, were tested for *in vivo* antibody induction in mice
- Sera from vaccinated mice were tested in a GFP-expressing RSV pseudovirus neutralization assay
- Narrow range of potencies
- No structural or functional destabilization method was used to create a wide range of relative potencies

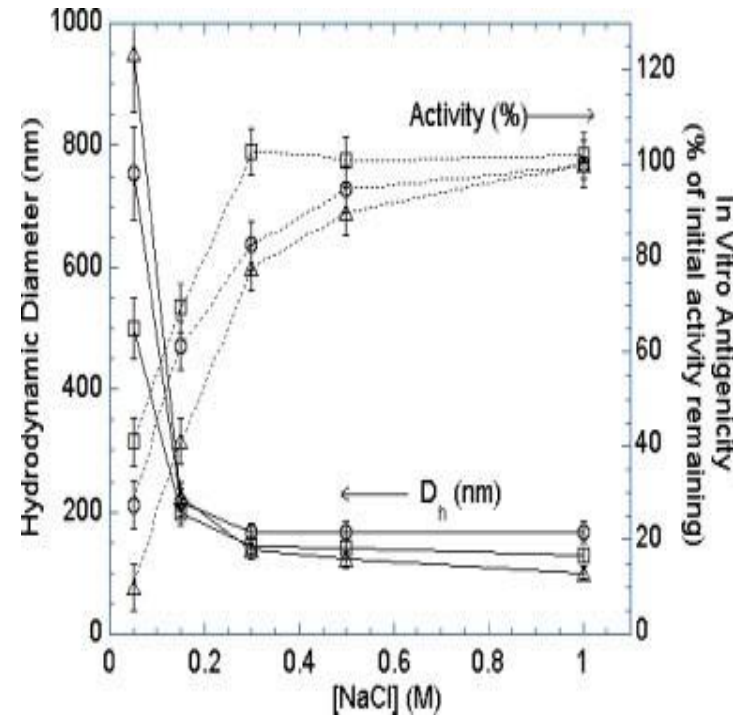
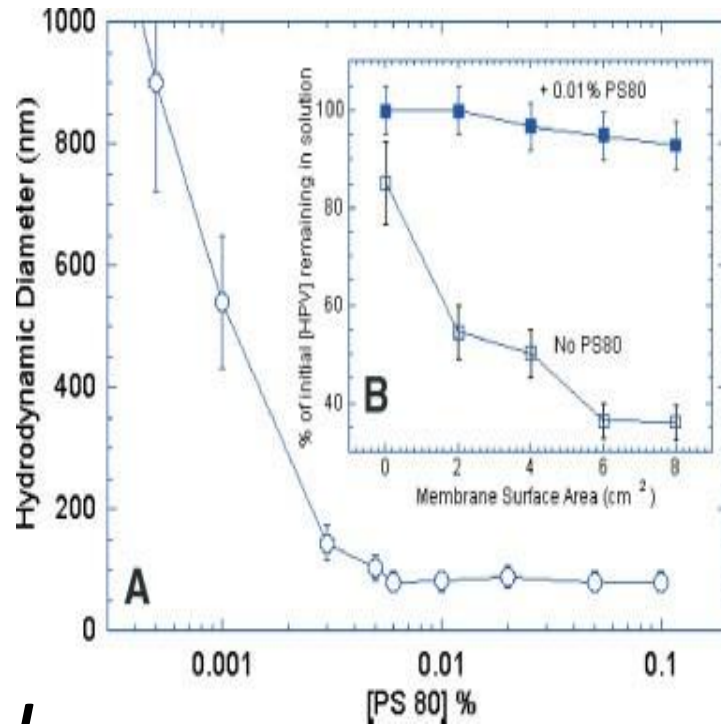
Feller, K. et al. *Vaccines* 13, 326 (2025)

Achieving QTPP by Downstream Process Optimization for a Recombinant Protein based VLP Vaccine

- QTPP of human papilloma virus (HPV) vaccine was to create a non-infectious recombinant protein-based virus like particle (VLP) mimicking the viral capsid
- The capsid of HPV spontaneously forms a T=7 icosahedral particle containing 72 L1 protein pentamers (capsomeres) held together via disulfide bonds between Cys175 and Cys184 and non-covalent associations of surface residues
-
- Uncontrolled aggregation to sizes higher than 72 capsomeres during purification as observed by off-line analytics led to process and formulation modifications:
 - --Introduction of dithiothreitol to disassemble scrambled and aggregated VLPs down to capsomeres
 - --Reassembly in the absence of DTT to target VLPs
 - --Process in presence of non-ionic surfactant and relatively high salt
 -
- As a result of these optimizations, QTPP was achieved in DS
-

Learnings from Structure-Function Correlation based Potency Assay Development of Recombinant HPV Protein-based VLP Vaccine

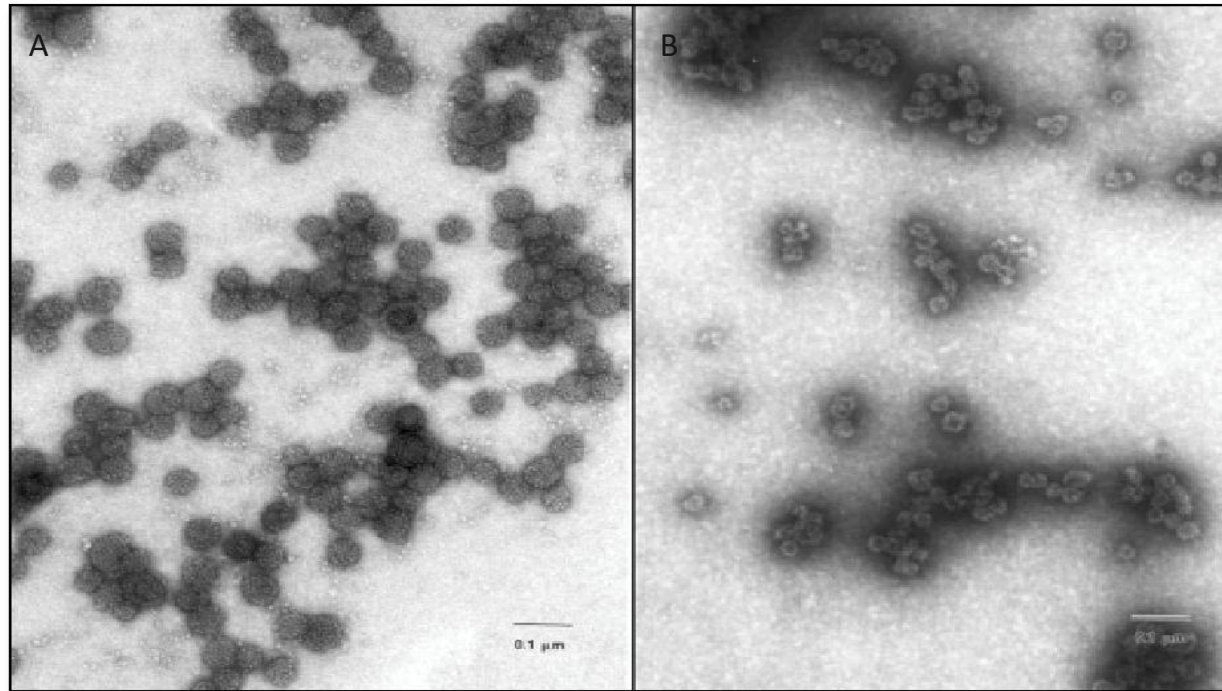
Prevention of VLP aggregation by salt and surfactant combination allows retention of biological activity



- Hydrodynamic Diameter Measured by Dynamic Light Scattering
- Biological Activity Measured as IVRP

L. Shi et al., *J. Pharm. Sci.*, 2005

Downstream Process Modification Prevents Aggregation of HPV16 Antigen-based VLPs

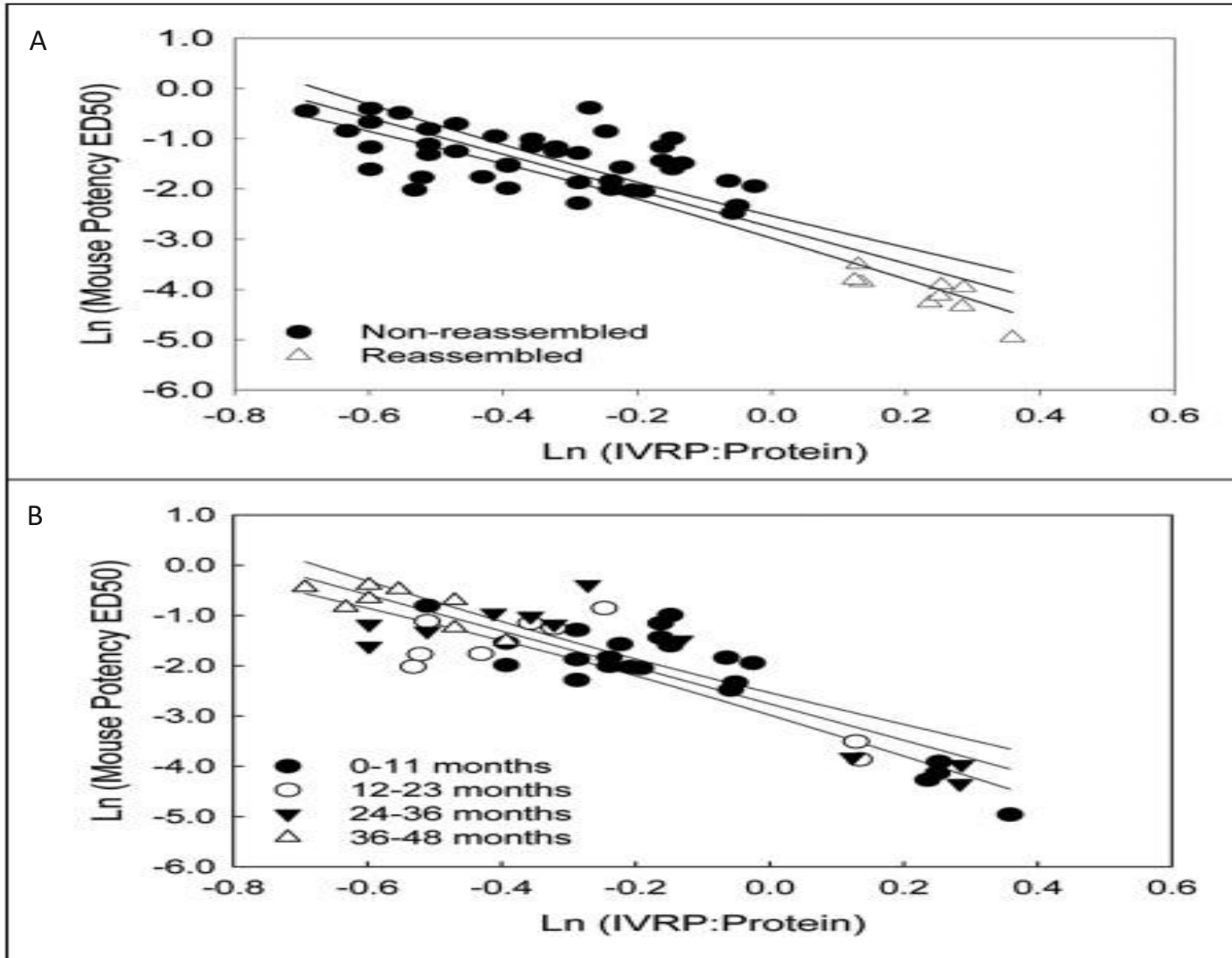


Shank-Retzlaff, M. et al., *Human*

Vaccines 1: 191-197
(2005), DOI:
10.4161/hv.1.5.2126

Transmission Electron Micrographs of HPV Type 16 VLPs. (A) Reassembled samples and (B) Non-reassembled samples

Prevention of VLP Aggregation Leads to Increased *In Vitro* and *In Vivo* Potency of HPV16 VLP Antigen



Correlation between the mouse potency and *In Vitro* Relative Potency (IVRP) results for samples containing HPV 16 VLPs. (A) Results plotted by sample type: non-reassembled and reassembled samples. (B) Result plotted by age of the sample at the time of testing. Solid lines indicate the results of linear regression analysis and the corresponding 95% confidence interval. All samples were stored at 2–8 degrees C.

Shank-Retzlaff, M. et al., *Human Vaccines*, 1: 191-197 (2005)

Characterization-based Process Modification Leads to Higher Potency

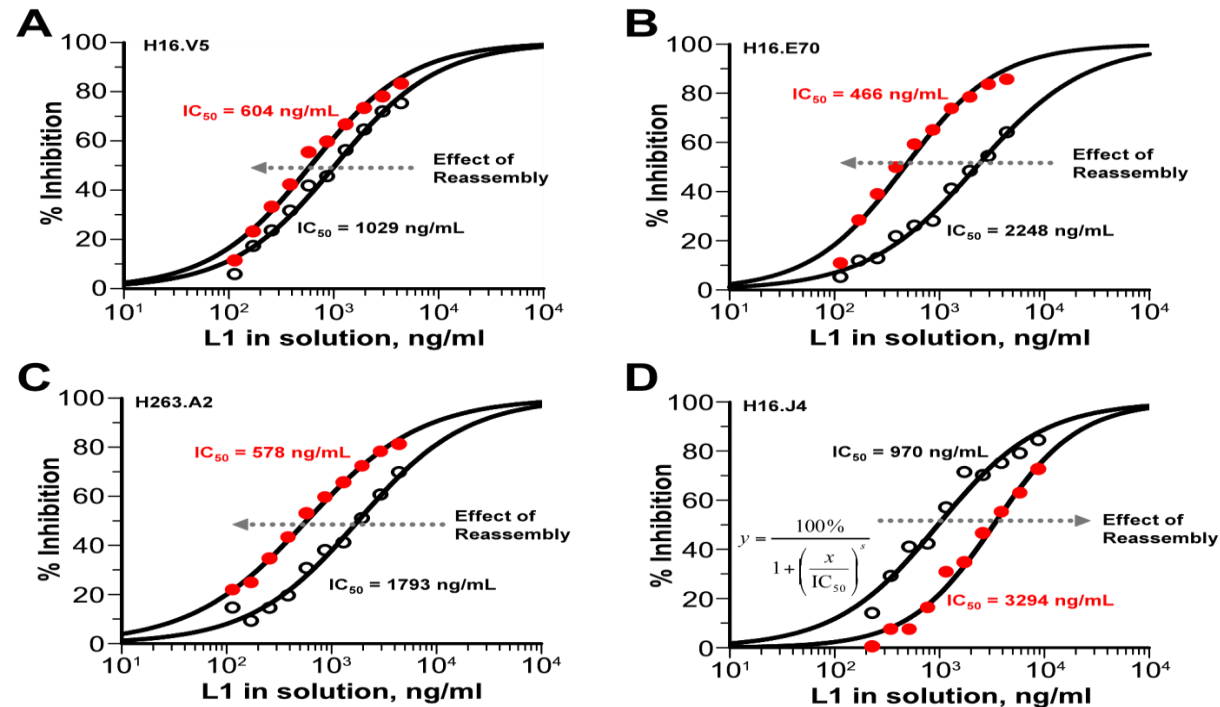
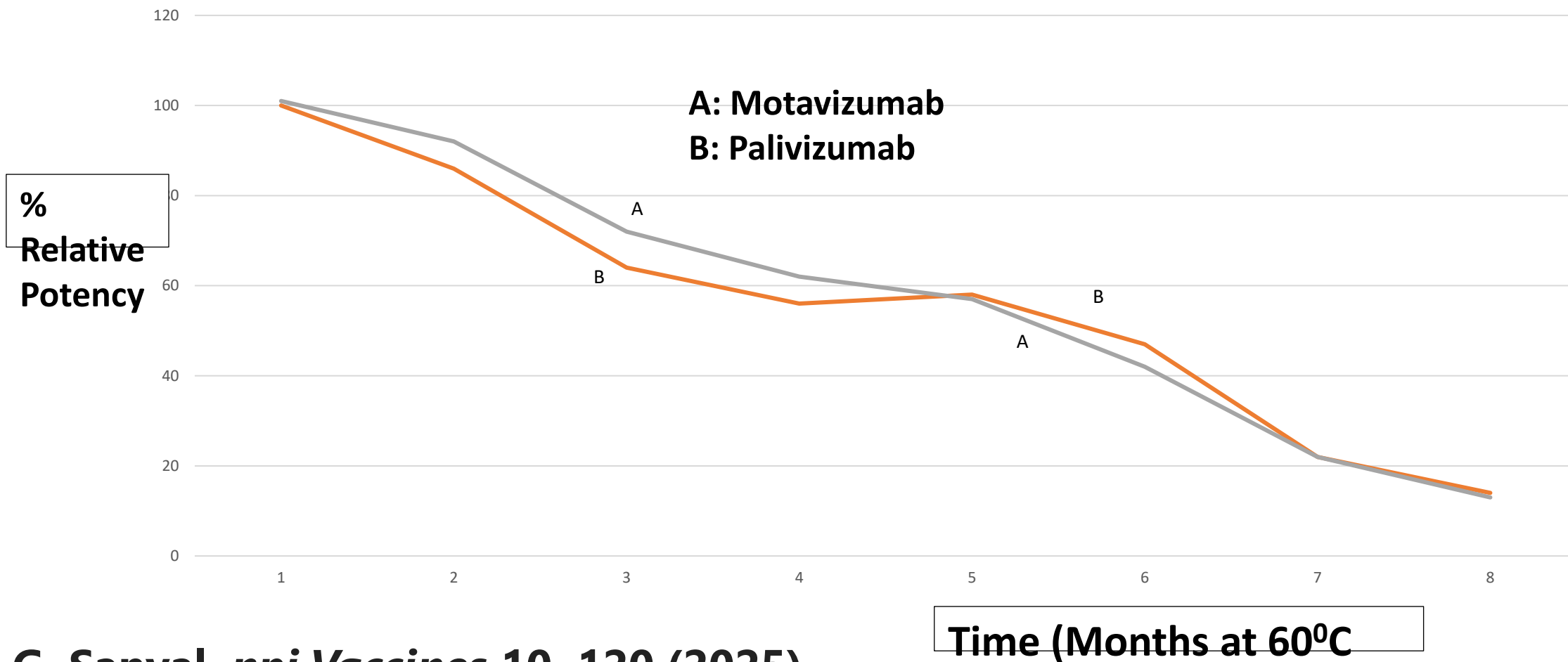


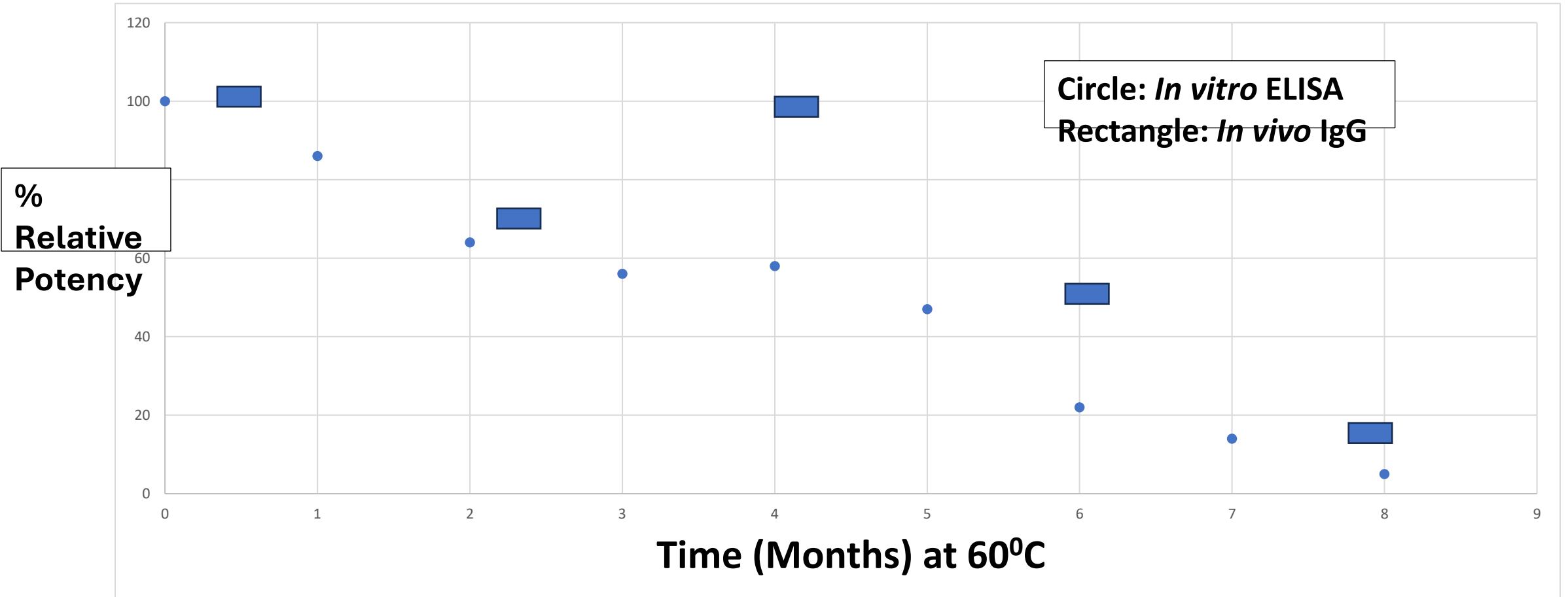
Figure 1 Competitive fluorescence ELISA curves for H16.V5 (A), H16.E70 (B), H263.A2 (C) (conformational epitope and neutralizing) and H16.J4 (D) (linear epitope and weakly neutralizing) on a pair of pre- and post-D/R HPV 16 L1 VLP lots. Fitted curves and the IC_{50} values were obtained with GraFit (20). Open dots correspond to pre-D/R VLPs; closed dots correspond to post-D/R VLPs. The relative IC_{50} values (normalized to a reference lot) for multiple full-scale lots are listed in Table 2.

Development of *in vitro*-*In vivo* Potency Correlation for a Subunit Protein Vaccine: *In vitro* Relative Potency Measurements of RSV Postfusion F Protein (RSV-F) by a Sandwich ELISA using MAB8262 as the Site IV mAb and (A) Motavizumab and (B) Palivizumab as the Site II mAb



G. Sanyal, *npj Vaccines* 10, 120 (2025)

***in vitro*-*In vivo* Potency Correlation for a Subunit Protein Vaccine:
In vitro Relative Potency Data using Motavizumab and MAB8262 are
Compared with Total Antigen Specific Antibody found in Sera of Mice
Immunized with RSVsF Protein Antigen**

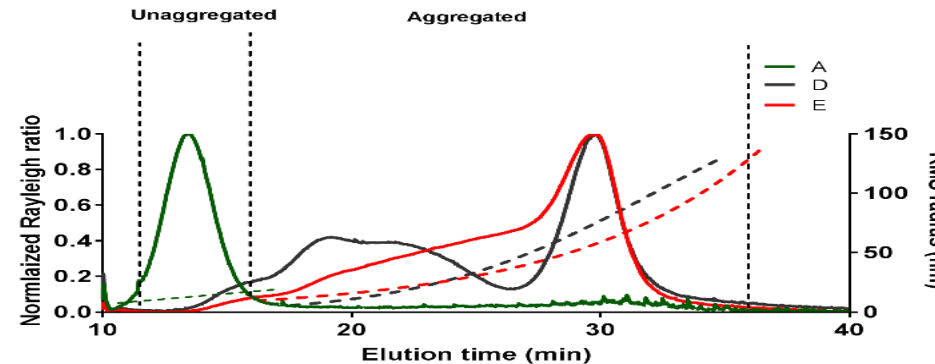
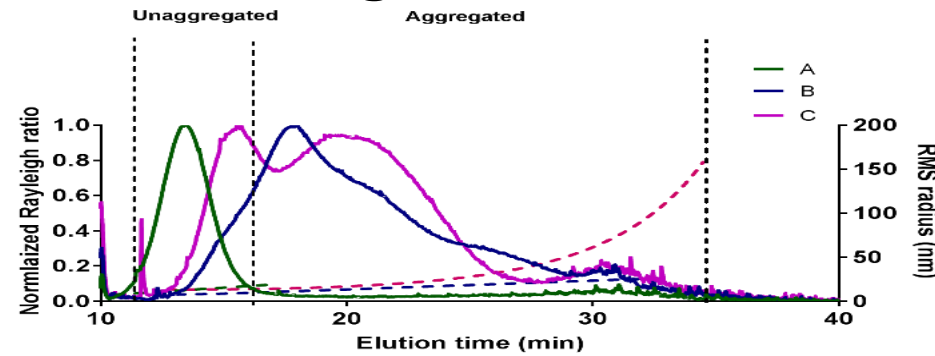


G. Sanyal, *npj Vaccines* 10, 120 (2025)

Structure-Function Correlation: Protein Aggregation causes Loss of Potency

Evaluation of RSVsF Aggregation by aF4-MALS : Rayleigh Scattering intensities (solid lines), Normalized to a Maximum of 1.0, and Calculated Apparent RMS Radii (dotted lines) are Plotted Against Elution Time.

Larger sized particles elute at longer times



Djagbare, M.D. et al. *Vaccine* 36, 1673-1680 (2018), supplemental data

Heat-stress. Top panel: A, B and C represent exposure times of 0, 2, and 4 months; respectively, at 60°C. Bottom panel: A, D and E represent exposure times of 0, 6 and 8 months, respectively, at 60°C

Summary of Observations for RSV-F Antigen

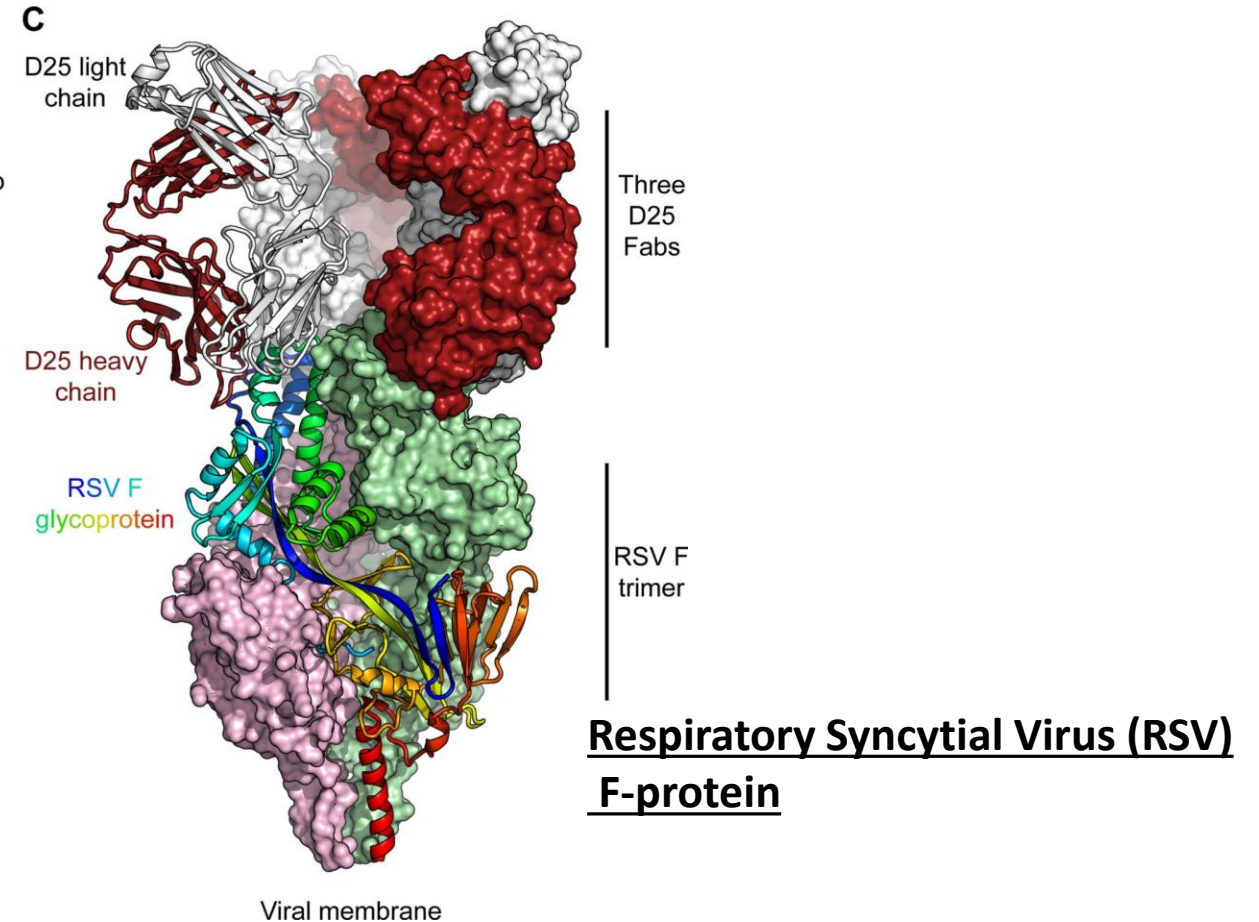
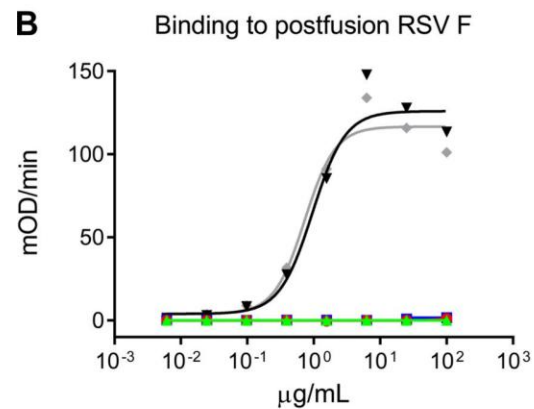
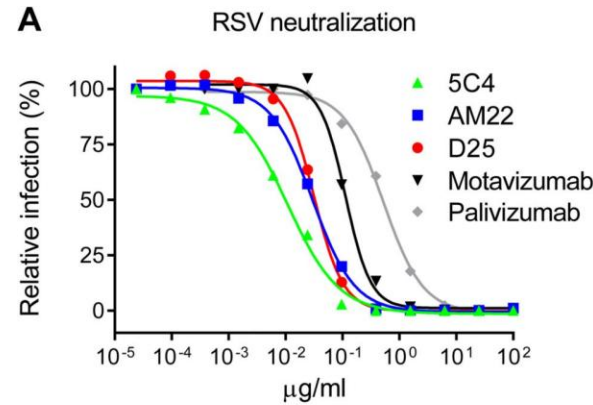
The *In vitro* Relative Potency assay offers a more reliable and stringent lot release cut off for RSV-F antigen than *in vivo* serological F-IgG and pseudovirus neutralization assays

With availability of selective antigenic site (epitope) –specific antibodies, *in vitro* assays can quantitate contributions of individual epitopes to the potency of the vaccine

Finally, RSV post-fusion F did not prove to be the most immunogenic antigen

RSV Pre-fusion F protein (RSVpreF) containing additional antigenic sites and mRNA encoding this antigen have delivered three efficacious vaccines in the past two years

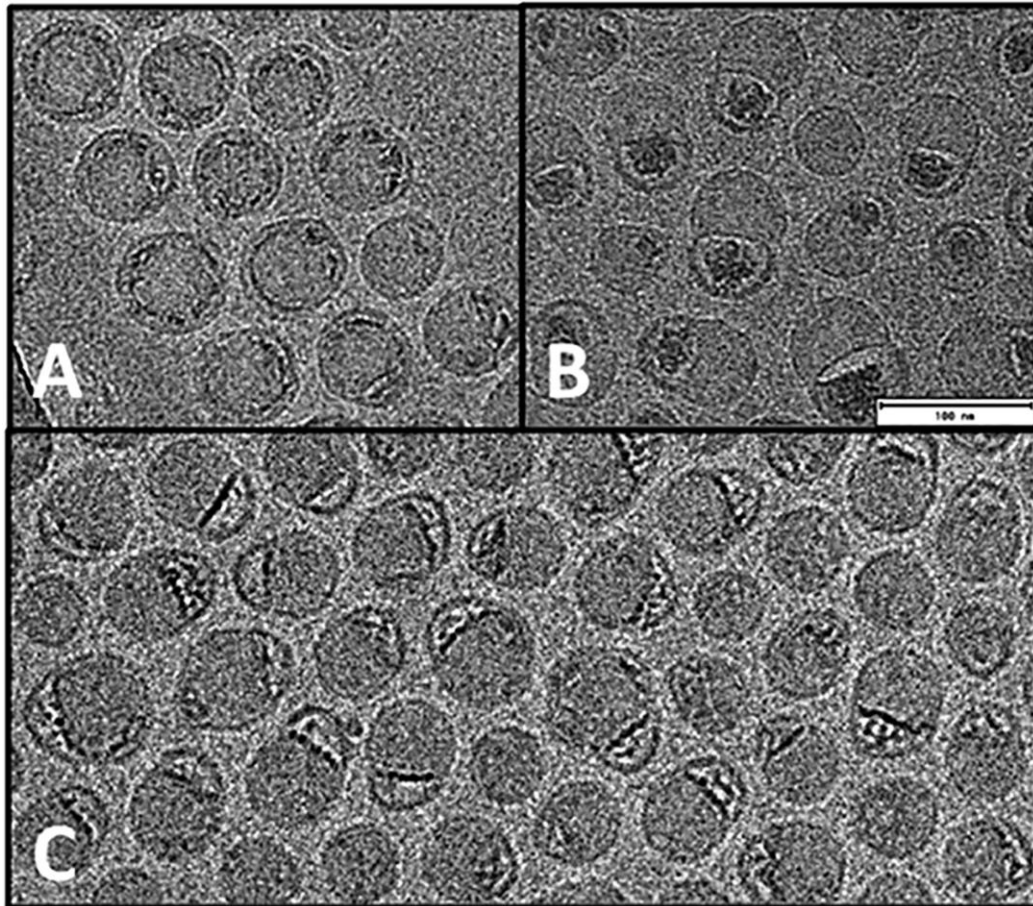
Structure-Guided Vaccine Development: RSV Neutralization, F-glycoprotein Recognition, and Crystal Structure of Human Antibody D25 in Complex with Prefusion RSV- F Trimer



Single-Particle vs. Ensemble Analysis

	Type	What It Measures	Nature of Signal
Mass Photometry	Single-particle	Individual molecule's mass via light scattering	Individual landing events
SEC-MALS	Ensemble	Average molar mass over a chromatographic peak	Bulk light scattering
Native Mass Spectrometry	Can be single-particle or ensemble	Gas-phase ion masses	Individual ion detection (similar in concept)
Cryo-Electron Microscopy	Single-particle	Structure of individual particles	Images of individual molecules

Morphological Characterization of mRNA-LNP Associated States by Cryo-EM – Single Particle Visualization – A Particle by Particle View

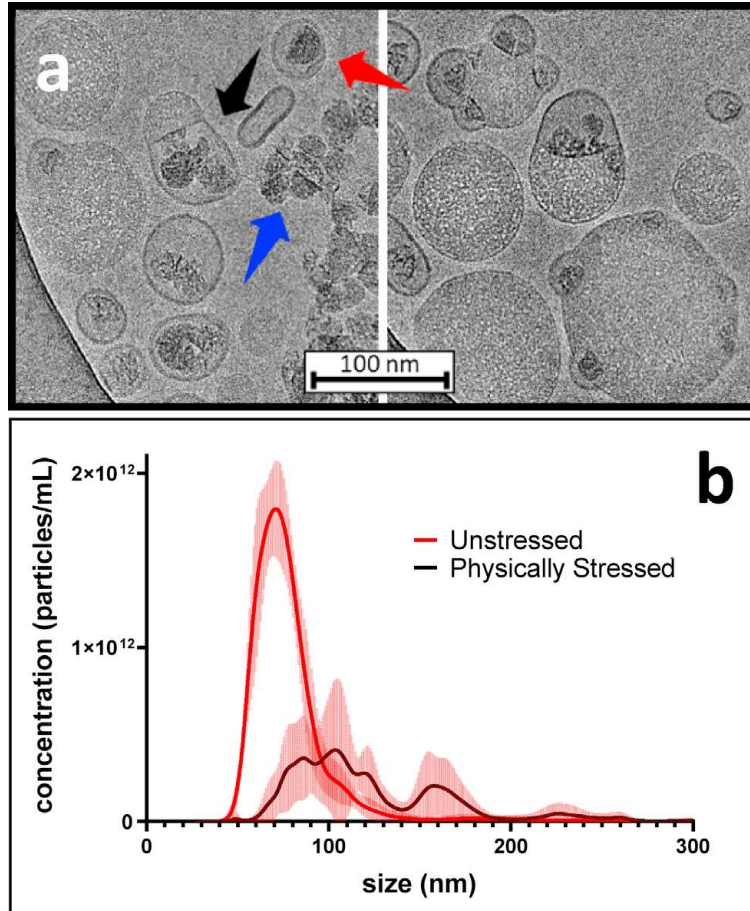


mRNA was highlighted with a permeating cationic dye. Panels A and B show extremes of highly spherical versus nonspherical morphologies where, in the latter, mRNA is dissociated from the solid lipid body of the nanoparticle to reside in a bleb compartment. Panel C shows significant heterogeneity of mRNA-lipid association states ranging from those in panels A and B.

Brader et al. *Biophys. J.* 2021, 120 (14), 2766–2770

M. Kloeziwiak et al., *Mol. Pharmaceutics* 19, 2022-2031 (2022)

Degradation Effects of Multiple Freeze-Thaw on mRNA-LNP: Orthogonal Comparison between Single Particle and Ensemble Analysis



(a) LNP physical degradation pathways. (a) Dye-stained mRNA-LNP sample subjected to multiple freeze-thaws. Cryo-EM shows aggregation (right), liberated mRNA (blue arrow), and liposomal structures containing mRNA (red arrow). The black arrow indicates an LNP that is ready to burst.

(b) Nanoparticle Tracking Analysis size distribution profiles corresponding to the stressed and unstressed sample.

M. Brader et al.,
Biophys. J. (2021)

Novel Methods for Single Particle Analysis in Solution

Convex Lens Induced Confinement (CLIC) Microscopy

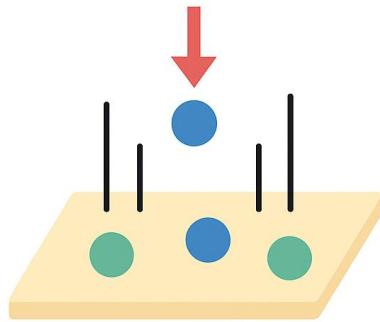
- CLIC microscopy uses a convex lens to create a wedge-shaped nanoscale confinement between a plano-convex lens and a coverslip for enhanced background rejection in wide-field imaging

Cylindrical Illumination Confocal Spectroscopy (CICS) based Microscopy

- CICS employs planar cylindrical illumination to illuminate an entire microchannel cross-section with a planar, non-Gaussian beam and single-molecule fluorescence spectroscopy for continuous, quantitative analysis.

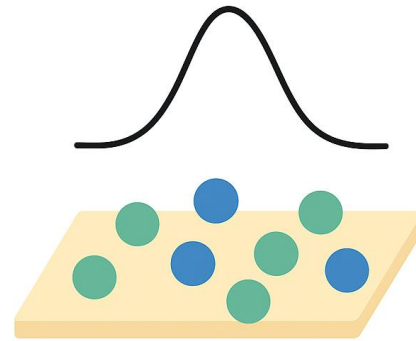
Mass Photometry-A Single Particle Analysis Method for Biomolecules

Single-Particle Analysis



Individual particle masses

Ensemble Detection



Average mass

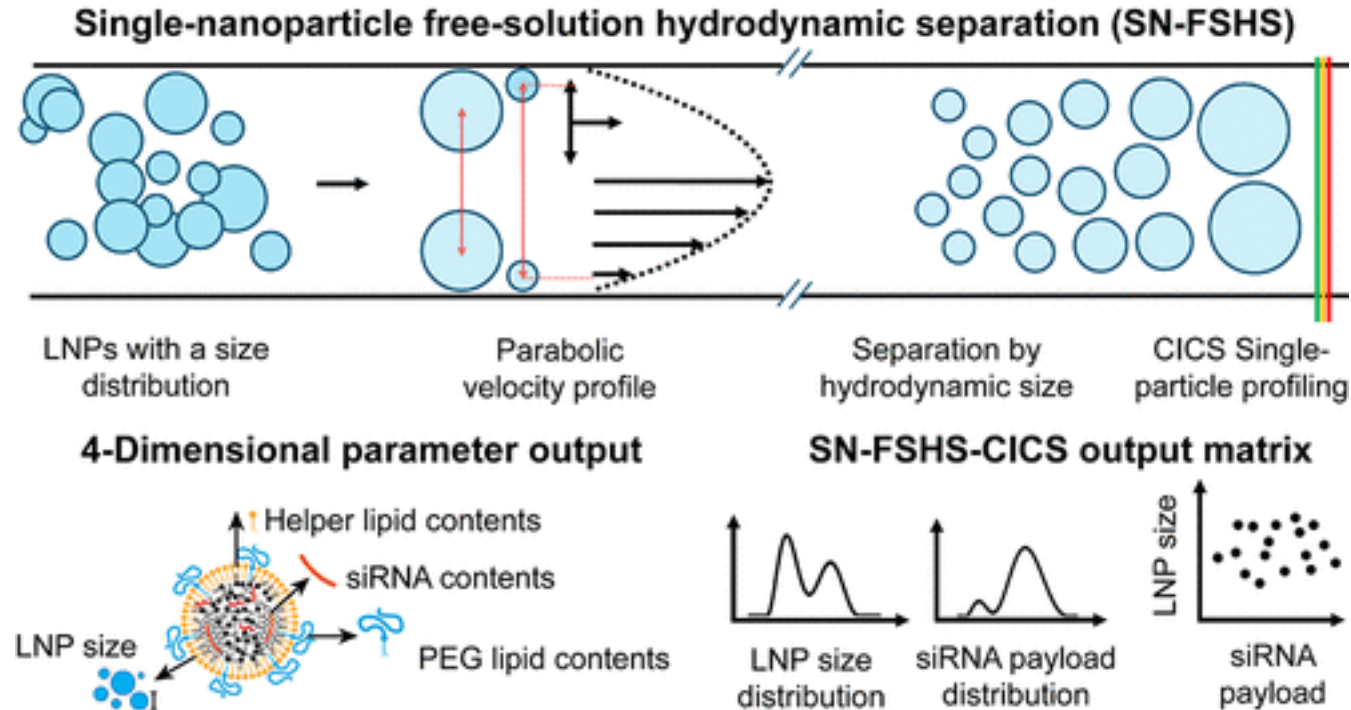
Mass photometry measures the mass of single molecules by detecting the interference between light scattered by a molecule on a measurement surface and light reflected by that surface, with the interference signal (contrast) scaling linearly with the molecule's mass

Compositions of mRNA- LNP Formulation at 20 µg/mL mRNA and N/P of 6 DLin-MC3-DMA:cholesterol:DSPC:DMG-PEG2000= 50:38.5:10:1.5

mRNA Loading in LNPs (Measured by CICS*)	<u>LNPs Before Dialysis at pH 4.0</u>	<u>After Dialysis at pH 7.4 –Final DP</u>	CICS Application
Number-average payload (mRNA copy per particle)	Lipophilic complexes: 3.43 ± 0.38 Non-lipophilic complexes: 1.34 ± 0.20 All nanoparticles: 2.51± 0.24	2.80 ± 0.41	
Most abundant of mRNA payload	Lipophilic complexes: 2 Non-lipophilic complexes: 1	2	
Populations	34%± 8% for lipophilic complexes 25% ± 4% for non-lipophilic complexes <u>41% ± 10% empty LNPs</u>	23%± 8% mRNA-loaded LNPs <u>77% ± 8% empty LNPs</u>	
Encapsulation Efficiency & Z-average Diameter			
Encapsulation efficiency	Not determined	94.2%± 3.6% by RiboGreen 85.6%± 5.1% by CICS*	
Average particle size	106.3 ± 13.0 nm	120.5± 6.0 nm	
Zeta-potential	+45.1± 0.9mV	-6.3 ± 1.3 mV	

Li, S. et al. *Nature Comm.* <https://doi.org/10.1038/s41467-02233157-4>

Hydrodynamic Size based Separation of LNPs in Solution and Single Particle Analysis by CICS Technique



Simultaneous visualization of RNA and lipid components using different fluorescent probes

Correlation between heterogeneous RNA payload distribution in LNPs and heterogeneity in LNP sizes?

Li, S. et al. [ACS Nano 2024, 18, 15729-15743](#)

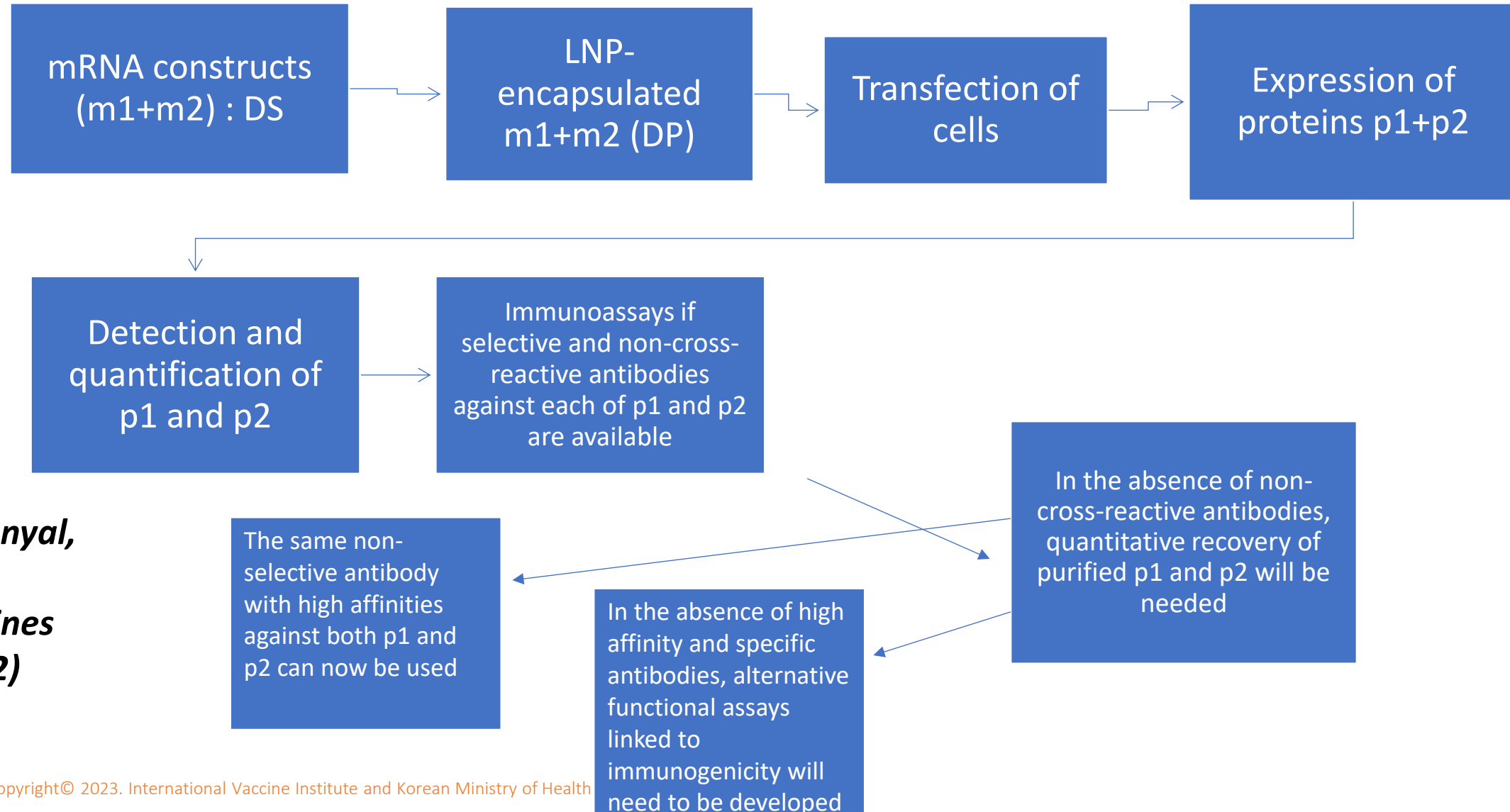
Analytics for Multivalent mRNA Vaccines

- **The dose of each construct in the final DP cannot be determined by a non-specific assay such as Ribogreen fluorescence. This can be accomplished by**
 - **Quantitative RT-PCR**
 - **Separation and simultaneous quantitation of each construct by techniques with adequate resolution such as IP-RP-HPLC**
 - **Capture of each mRNA construct by a complementary oligonucleotide and detection by a covalently linked fluorophore**
- **Integrity of each mRNA by RP-HPLC or IP-RP-HPLC and LC-MS**
 - **Purity in terms of percentage fragmentation, LNP lipid induced RNA modification and ds-RNA may be quantified simultaneously by a single method**

Analytics for Multivalent mRNA Vaccines

- **Protein expression (translation) efficiency, i.e., *in vitro* potency, of each mRNA construct**
 - **High affinity, selective / non-cross-reactive antibodies should be developed to allow multiplexed potency assays**
- **In the absence of non-cross-reactive antibodies, functional assays linked to immunogenicity need to be developed**

Detection and Quantification of Individual Proteins from a Bivalent mRNA-LNP DP after Cell Transfection

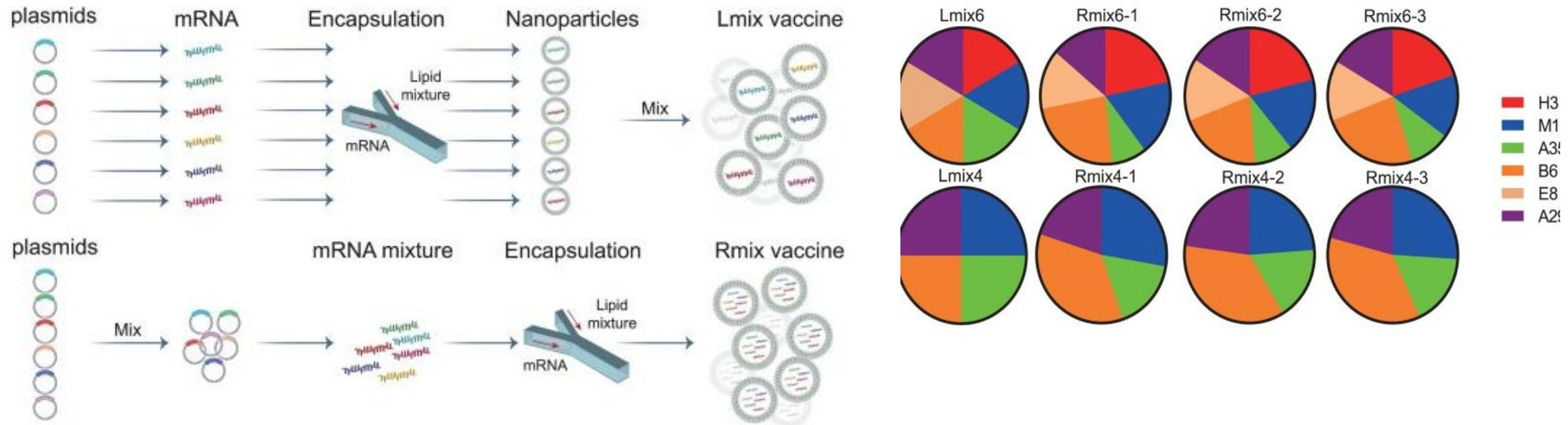


G. Sanyal,
npj
Vaccines
(2022)

Multi-Antigen mRNA Candidate Vaccines against Monkeypox (MPOX) Virus

- Two multi-antigen mRNA vaccine candidates were designed, termed as mix4 (M1, A29, B6, and A35) and mix6 (M1, A29, H3, E8, B6, and A35).
- Individual mRNA was separately transcribed and further encapsulated with lipids to form LNPs .
- These different LNPs were mixed in equal mRNA mass into the vaccine candidate formulations, named Lmix4/6.
- In a second formulation procedure, parent DNA plasmids were mixed in equal mass and transcribed to form mRNA as a whole, which was then encapsulated in LNPs to formulate the vaccine candidates, named Rmix4/6.
- The mRNA encapsulation efficiency was 96.4% for B6, 96.7% for A35, 96% for A29, 96.2% for M1, 96.7% for H3, 97.2% for E8, 96.1% for Rmix4, and 96.7% for Rmix6.
- Quantitative real-time PCR was used to detect proportions of different mRNA components in Rmix4/6. The results showed no significant difference between the three batches of Rmix4/6.
- Compared to Lmix, the ratios of different mRNAs in Rmix4/6 were slightly different

Construction and Characterization of MPOX Multi-Antigen mRNA Vaccine Candidates

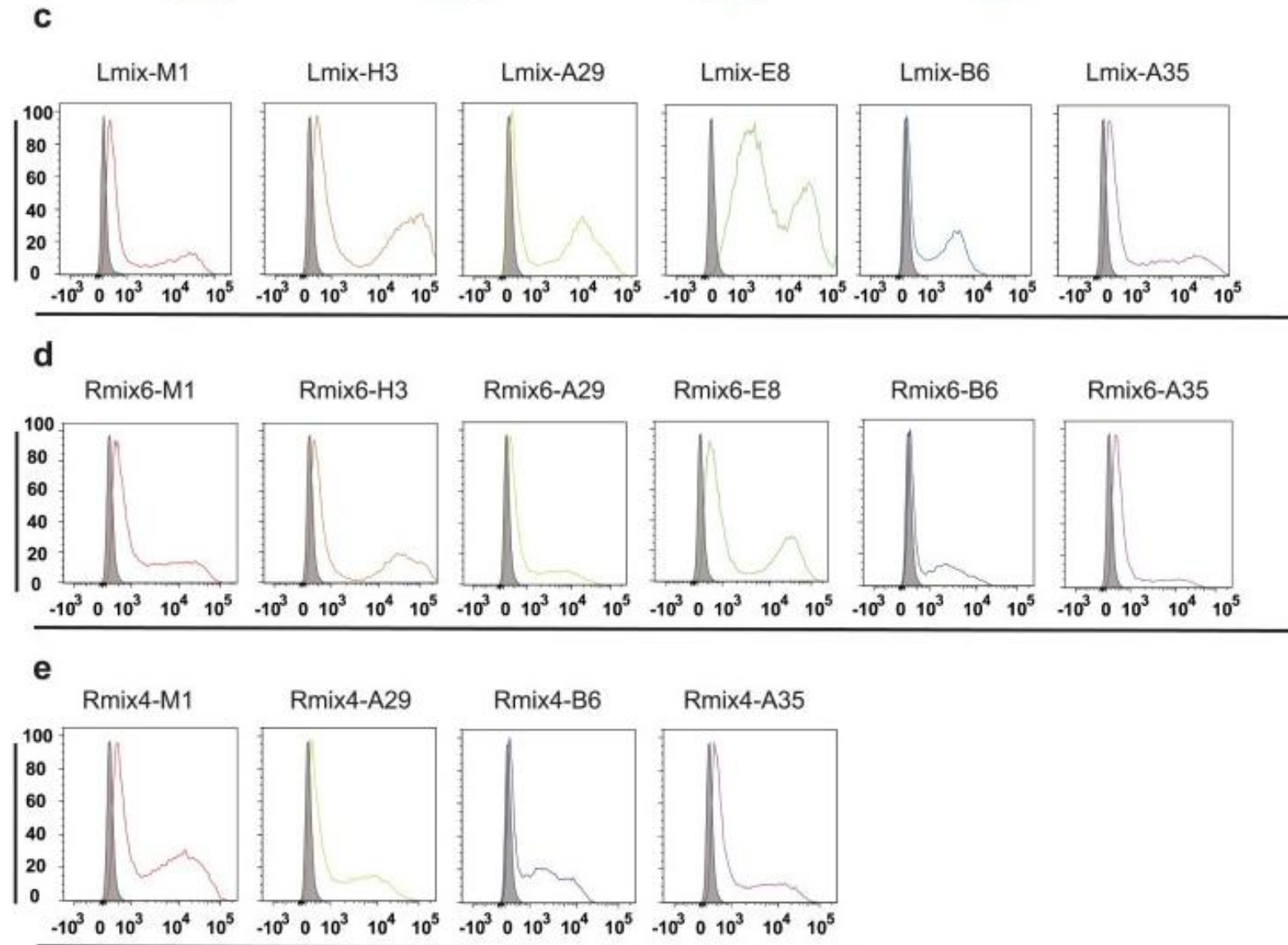


Schematic diagram of formulation procedure for multi-antigen mRNA vaccine candidates

Proportions of each antigen mRNA in three batches of Rmix4/6 and Lmix4/6

Characterization of MPOX Multi-Antigen mRNA Vaccine Candidates

Flow cytometric images of antigen expression after transfection of HEK293T cells with antigen-encoding (c) individual mRNA constructs, (d) Rmix6 combination and (e) Rmix4 combination of constructs. HEK293T cells M1-transfected cells were stained with His antibody PE. The other groups of HEK293T cells were stained with each antigen-specific mAb and analyzed by flow cytometry. The bindings between antigens and their specific mAbs are shown in different colors. Grey shade indicates negative binding.

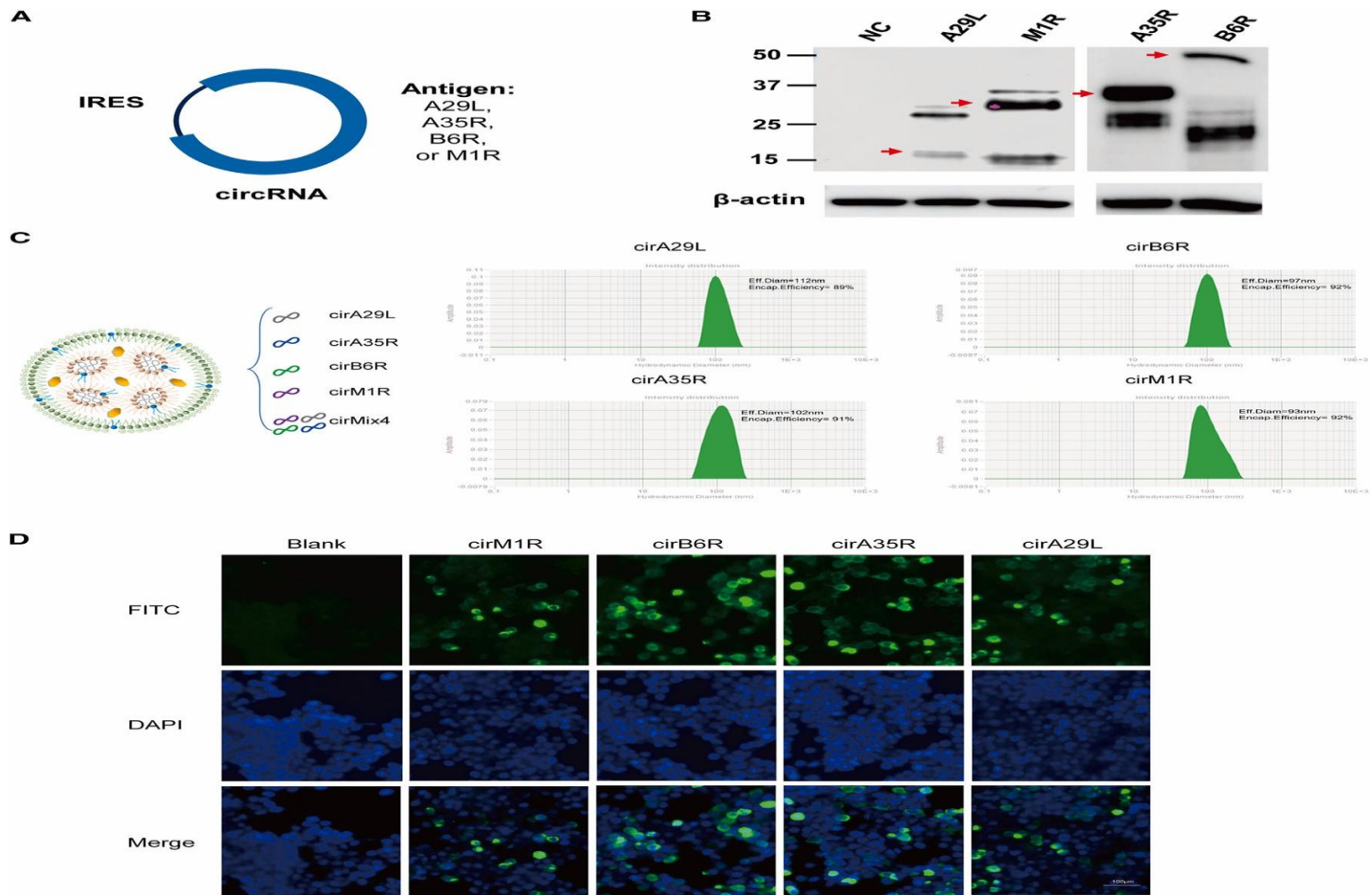


Multivalent Circular RNA Encoding 4 MPOX Antigens– Cell

Transfection based Protein Expression Zhou, J. et al, Mol. Therapy

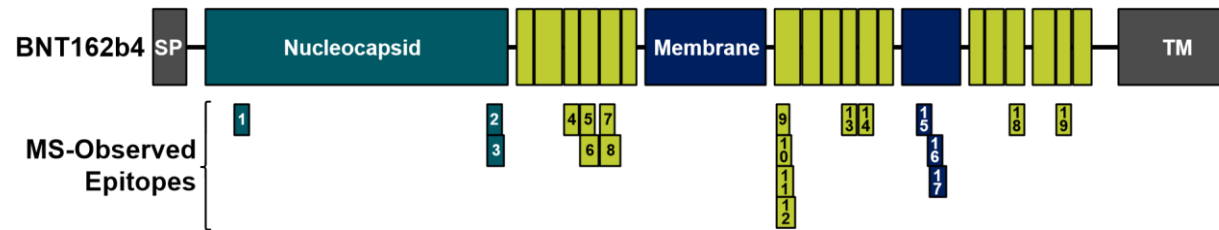
Vol 32 June 2024

(A) circRNA design. The circRNA construct expresses the MPXV-specific antigens A29L, A35R, M1R, and B6R. (B) The expression of these MPXV-specific antigens in HEK293T cells were detected by western blotting. (C) The particle diameter and encapsulation efficiency of each circRNA component. (D) IFAs were used to measure the expression of the MPXV-specific antigens A29L, A35R, M1R, and B6R in HEK293T cells.



A T-Cell Directed mRNA Vaccine Construct: Combining Molecular Engineering with Mass Spectrometric Identification of Epitopes

mRNA construct encoding conserved non-spike antigens protects animals from severe SARS-CoV-2 infection



BNT162b4 is a source of Human Leukocyte Antigens (HLA)-presented epitopes as directly measured by mass spectrometry

19 unique BNT162b4-derived epitopes were detected by targeted mass spectrometry, with at least one epitope coming from each encoded SARS-CoV-2 antigen

C. M. Arieta et al. *Cell* 186: 2392–2409 (2023)

Questions Still to be Resolved for Characterization of Multivalent mRNA Vaccines

Applicable to Circular and Self-Amplifying RNA Modalities

- Is each antigen of a multivalent mRNA DP expressed to the same extent as it is in the monovalent mRNA vaccine?**
- Does each antigen of a multivalent mRNA vaccine get expressed in functionally intact form?**
- Is there any interaction between antigens of a multivalent vaccine that may affect integrity and potency?**
- For a multivalent vaccine containing closely related variants of a parent pathogen, how does one measure potency of each antigen in the DP (final vial)?**
- Is encapsulation efficiency of a particular mRNA construct affected when combined with other mRNA constructs in a multivalent vaccine?**

Need for Further Progress in Developing *In Vitro* Potency Tests

- *In Vitro* demonstration of adjuvant effects for vaccines that require novel adjuvants in addition to antigens for stimulating protective immune response
- *In Vitro* demonstration of cell mediated immunity

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