Analytical Technologies in the Biopharmaceutical Industry,
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Capillary electrophoresis and Taylor dispersion analysis for the characterization of mRNA vaccines

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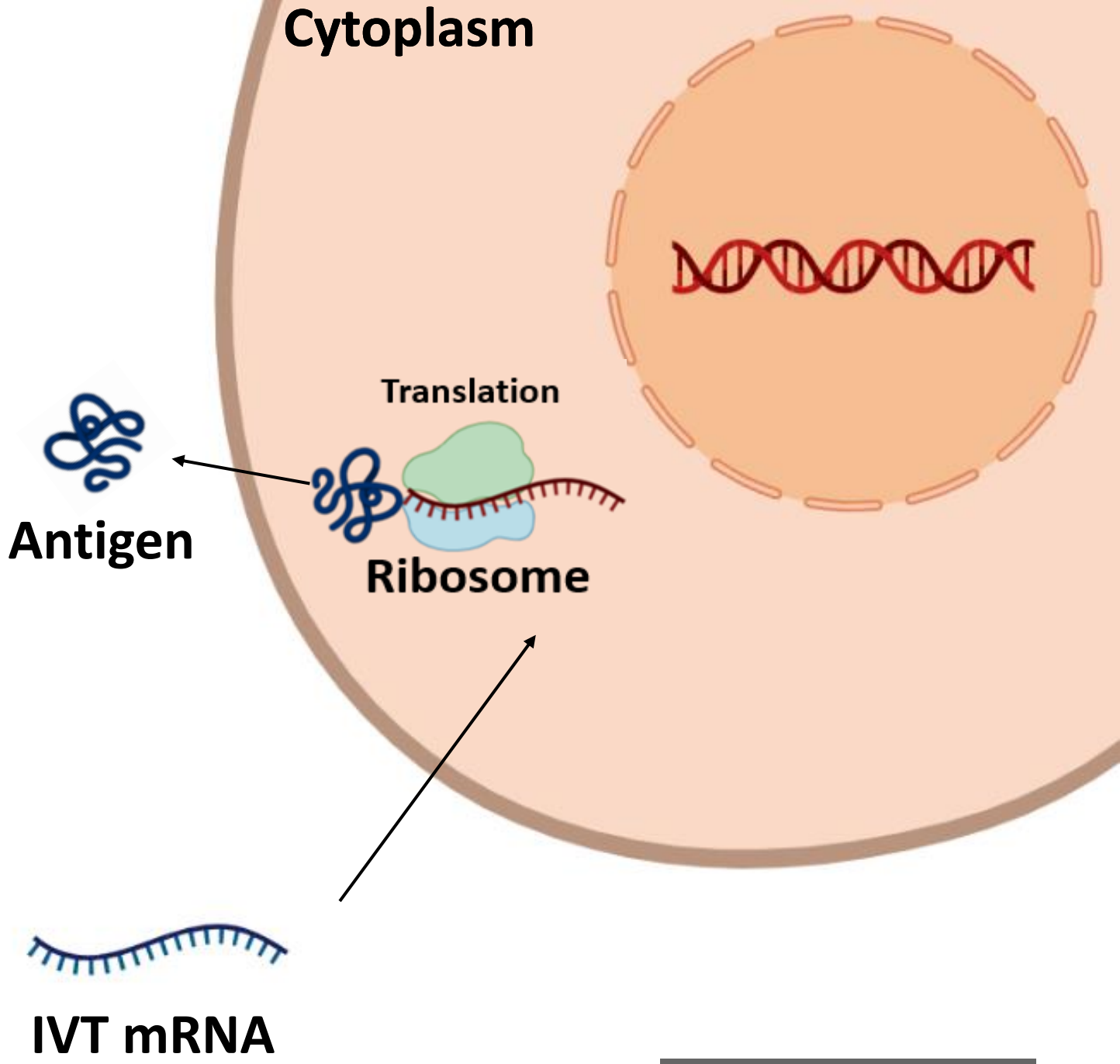
Context

In Vitro Transcribed (IVT) mRNA:

synthesized from a purified linear DNA template

The efficacy of mRNA vaccines relies on the ability of mRNA to reach the cytoplasm of cells

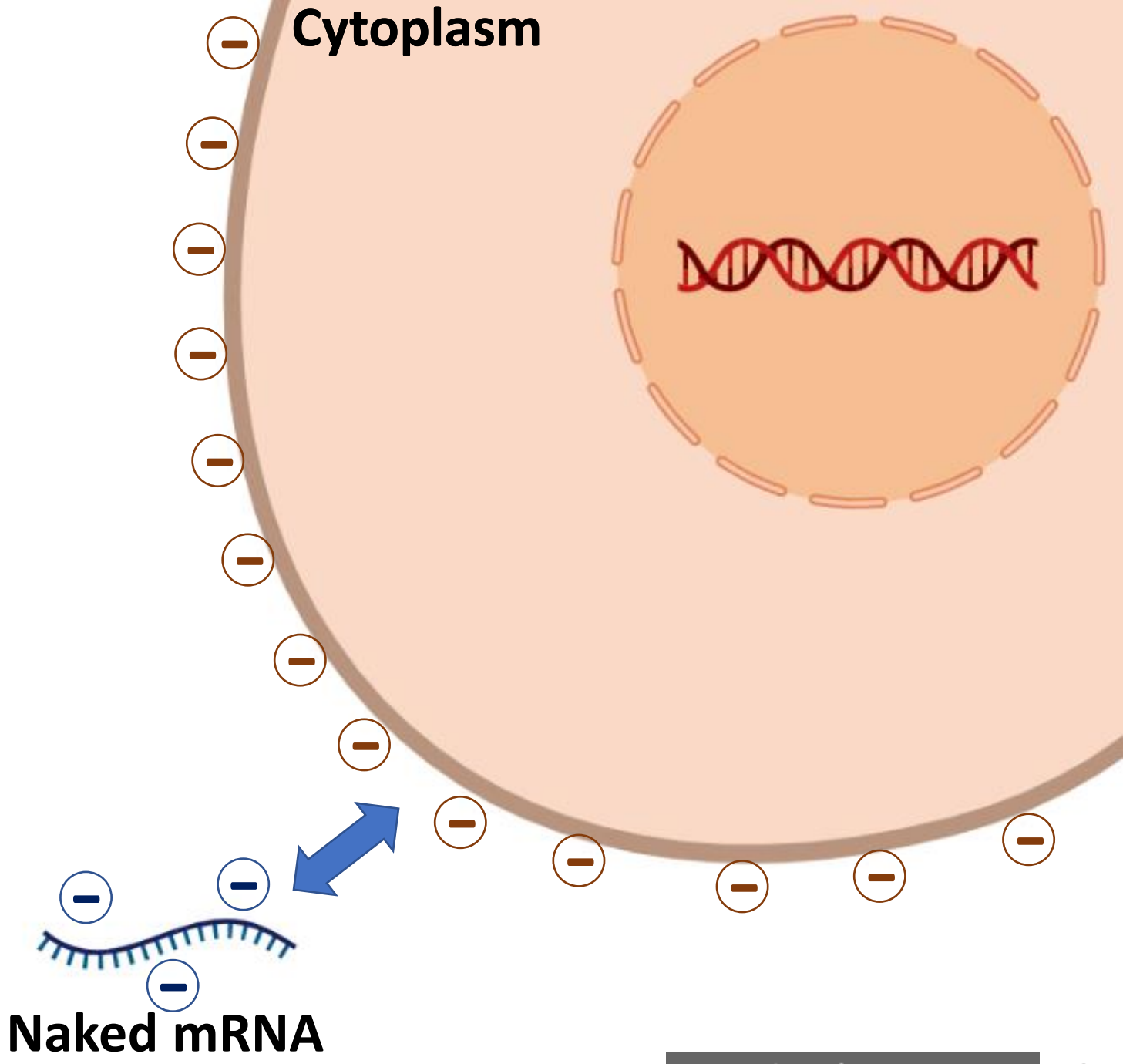


Context

Naked mRNA restrictions:

- instability, vulnerability to nucleases
- inefficient intra cellular delivery

  (global negative charge)



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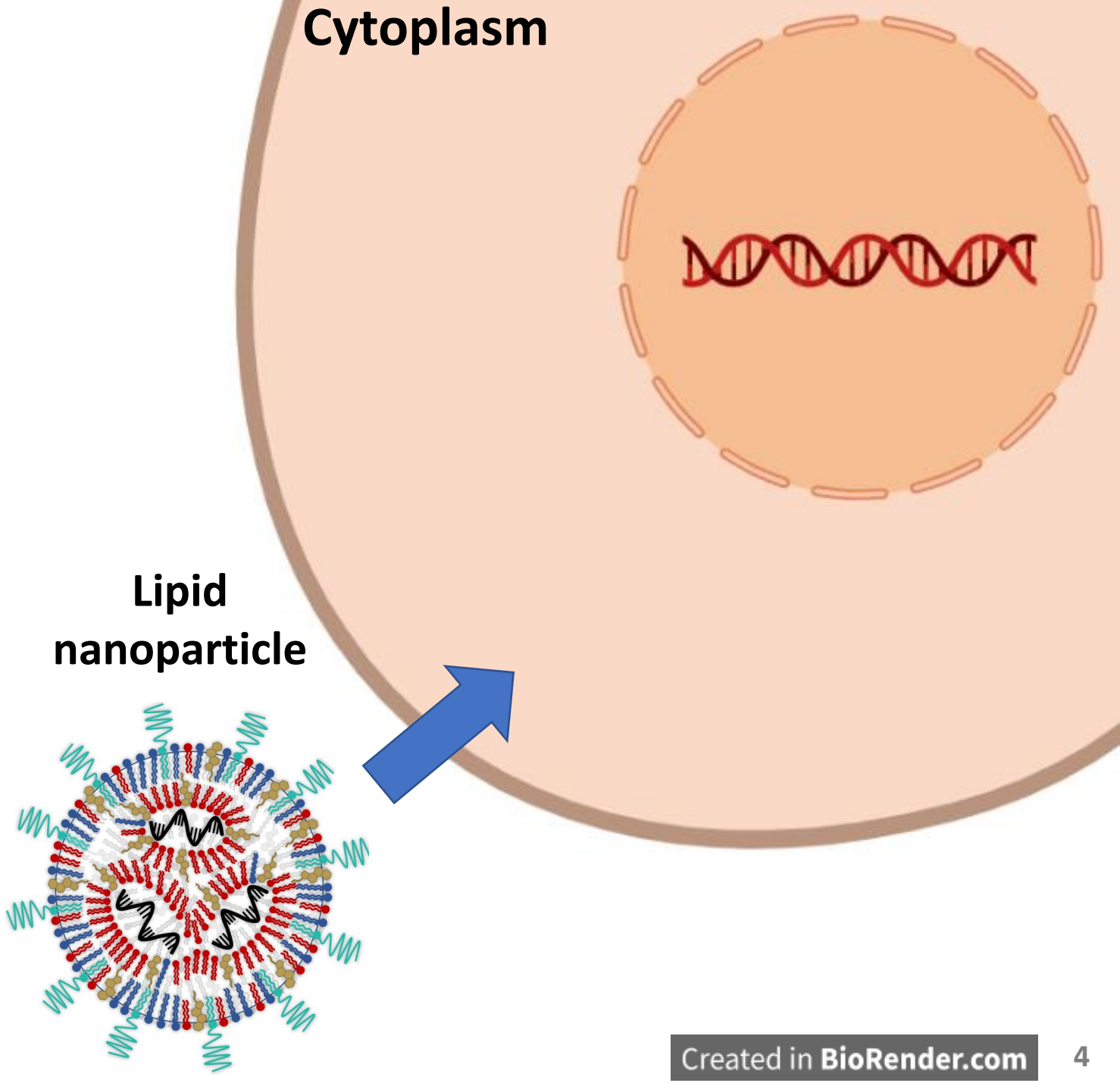
Context

Naked mRNA restrictions:

- instability, vulnerability to nucleases
- high innate immune activation
- inefficient intra cellular delivery (global negative charge)

→ Need for a delivery vehicle to protect mRNA and allow it to enter cells



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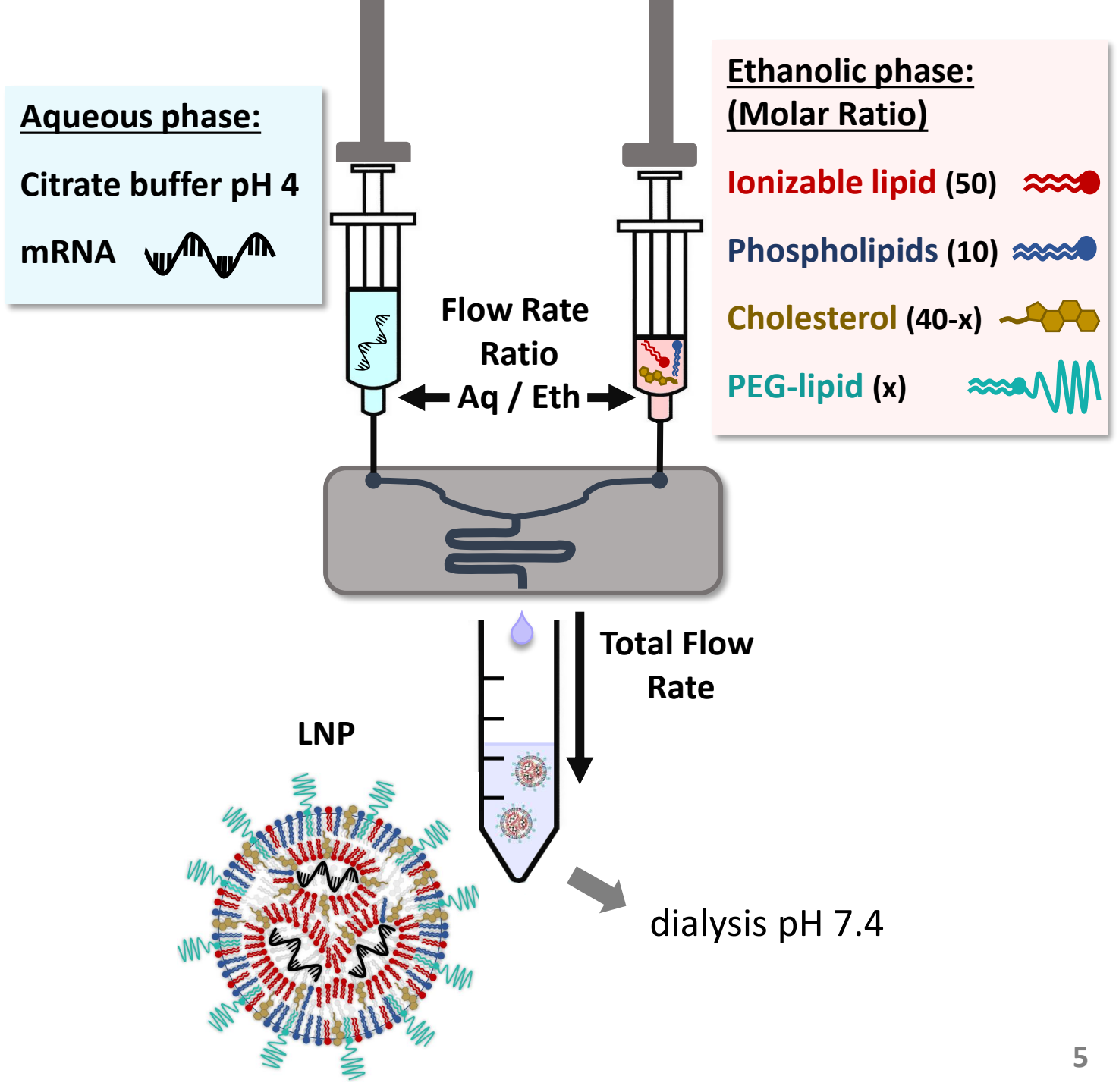
LNPs formulation

Self-assembly of LNPs :

- electrostatic interactions
between anionic mRNA
and ionizable lipids
⇒ LNPs nuclei

- hydrophobic interactions
between lipids
and aqueous phase
⇒ LNPs envelope

+ hydrogen bonding



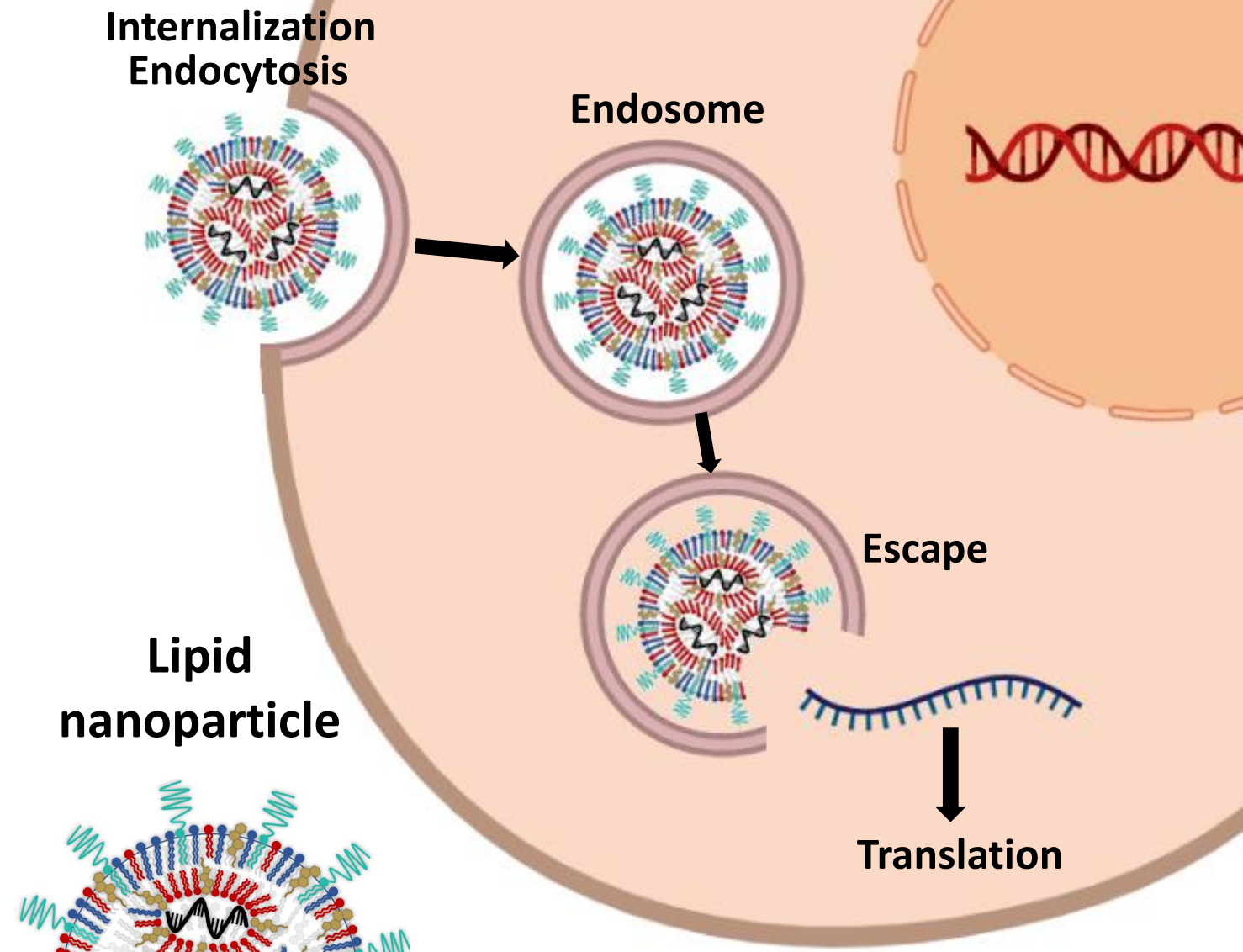
Intracellular delivery

Lipid nanoparticles are taken up by cells via endocytosis. Yet only a small fraction of nucleic acid reaches the cytoplasm.[1]

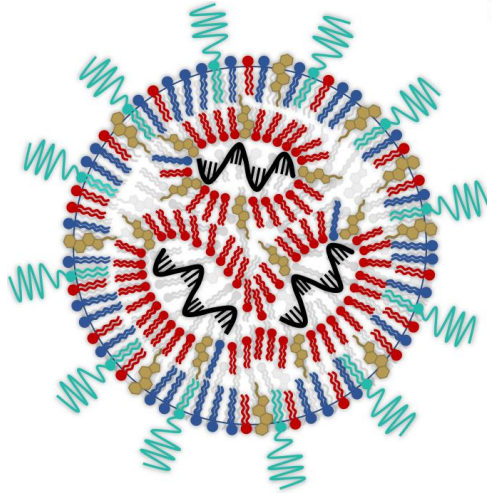
The **physicochemical properties** of LNPs, such as :

- **size**
- **charge**
- **surface composition**
- **shape**

affect intracellular delivery.[2]



Lipid nanoparticle



[1] Gilleron, J. *et al. Nat. Biotechnol.* 31, 638–646 (**2013**)
[2] Kim, J et al. *Adv. Drug Deliv. Rev.* 170, 83–112 (**2021**)

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Regulatory Guidelines

Size and size distribution are **Critical Quality Attributes (CQAs)** of mRNA-LNP drug products

The use of **complementary methods** is recommended

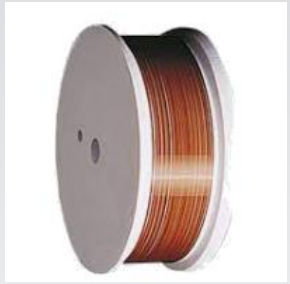
Objectives

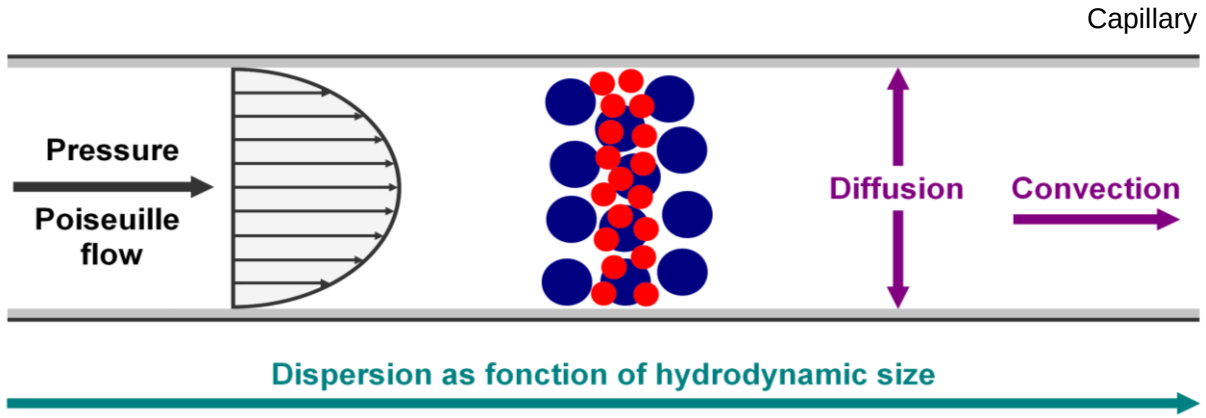
Develop a new analytical method for **size and size distribution** of **mRNA-LNP** drug products

Taylor dispersion analysis (TDA) principle

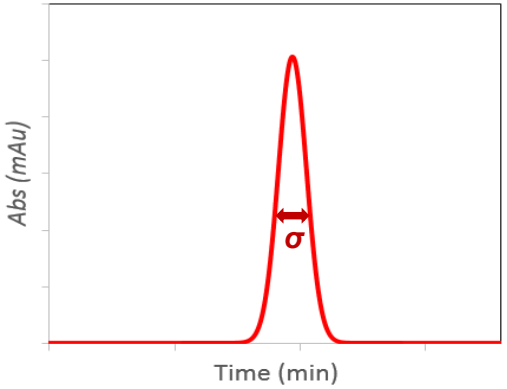
- TDA is based on the analysis of the dispersion of a solute plug in a capillary under a laminar Poiseuille flow.
- The combined action of convection and molecular diffusion results in a special form of elution profile named Taylor-Aris dispersion.

measures with capillary electrophoresis device

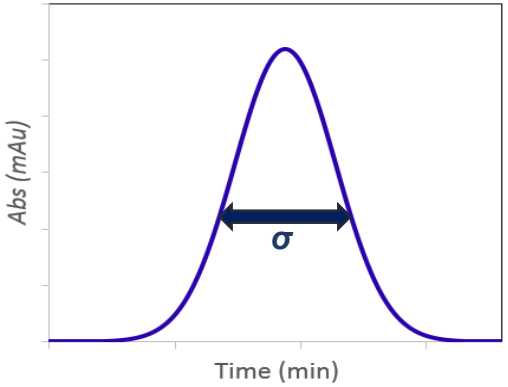




Small molecules diffuse quickly → thin peak



Large molecules diffuse slowly → broad peak



Diffusion coefficient:

$$D = \frac{R_C^{\,2}\, t_0}{24\,\sigma^2}$$

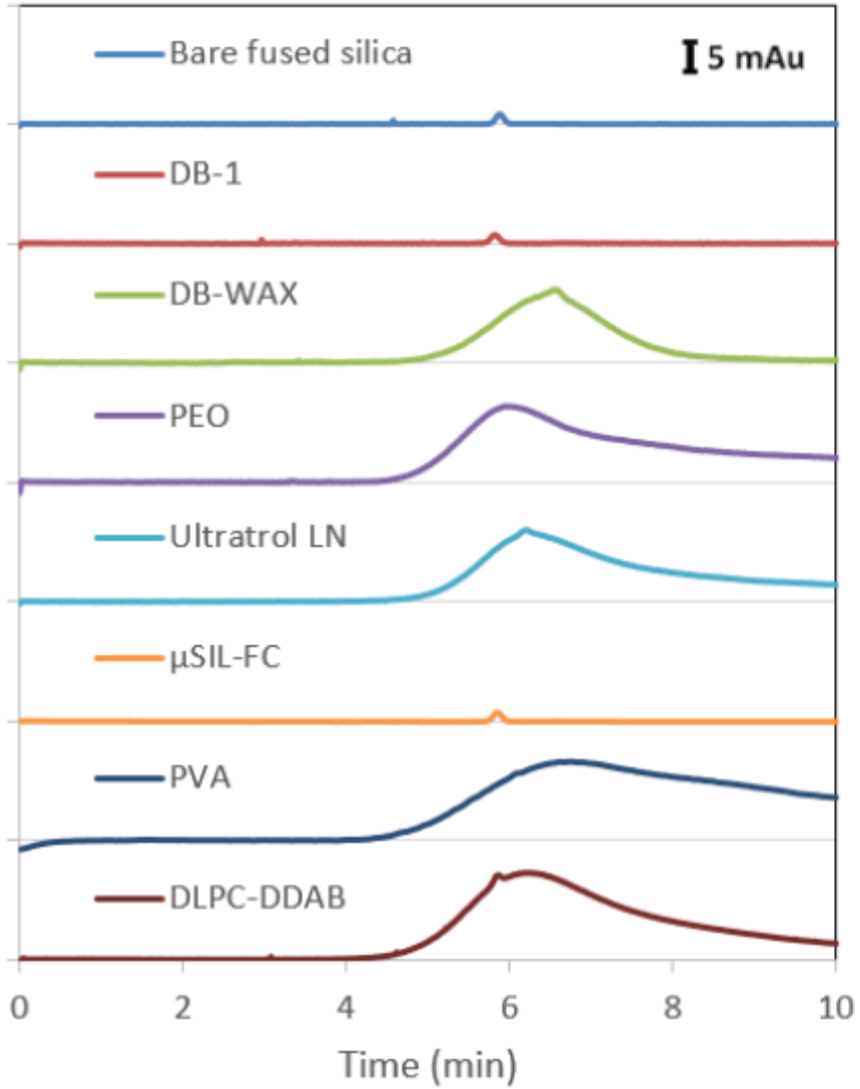
Hydrodynamic diameter:

$$D_h = \frac{2 k_B T}{6\pi\eta D}$$

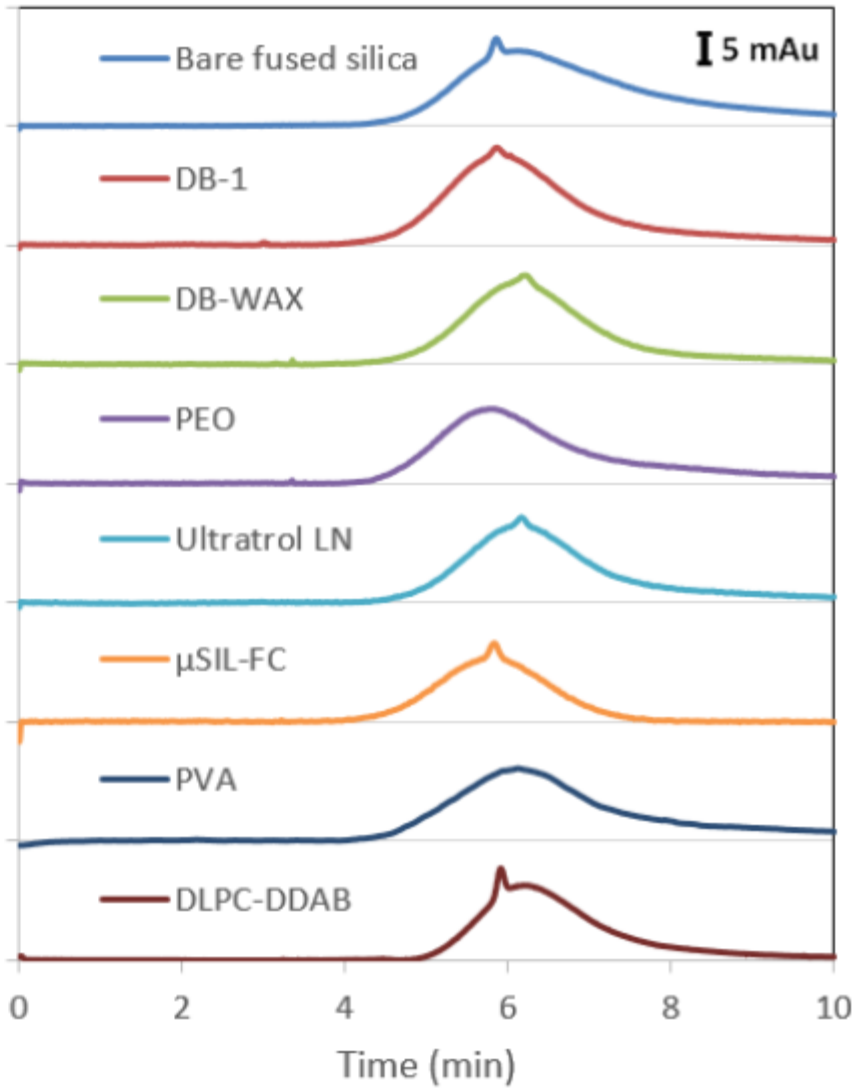
Capillary coatings (presaturation impact)

C. Malburet et al., Anal. Chem., 2022, 94,11, 4677-4685

Without presaturation



With presaturation



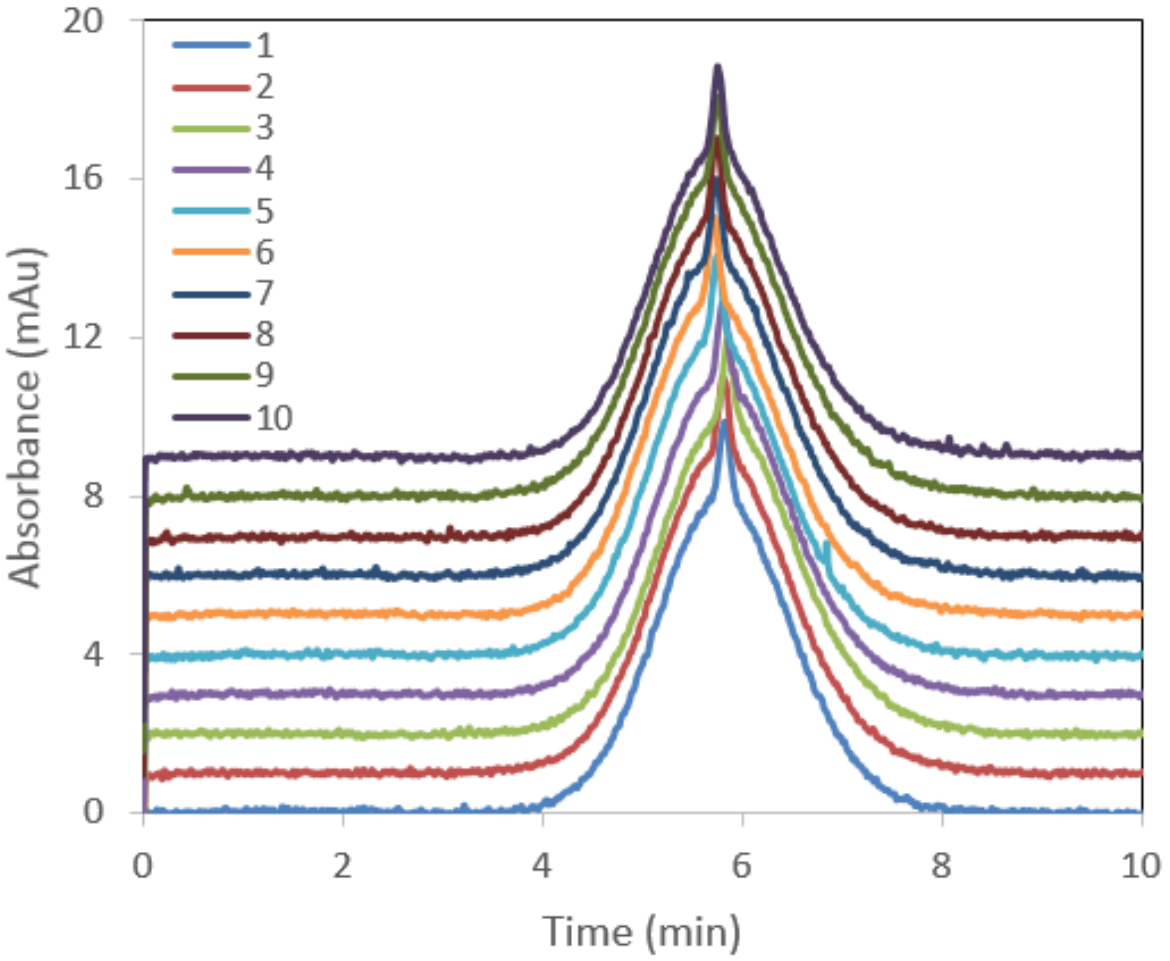
Experimental conditions:
40 cm to the detector × 50 µm i.d
Buffer: PBS, NaCl, pH 7.4
Injection: 20 mbar, 6 s
Mobilization : 60 mbar
UV detection: 200 nm

Presaturation ≈ 4 µL

Significant impact of presaturation

Method Repeatability

C. Malburet et al., Anal. Chem., 2022, 94,11, 4677-4685



Experimental conditions:

µSIL-FC capillary, 48.5 cm total length (40 cm to the detector) × 50 µm i.d. Buffer: PBS 10 mM, NaCl 137 mM, pH 7.4. Hydrodynamic injection: 20 mbar, 6 s. Mobilization pressure: 60 mbar. UV detection: 200 nm. Temperature: 25 °C.

The capillary is rinsed after each series of analysis:

- 10 min Isopropyl alcohol

- 10 min Hellmanex® III 1% into water

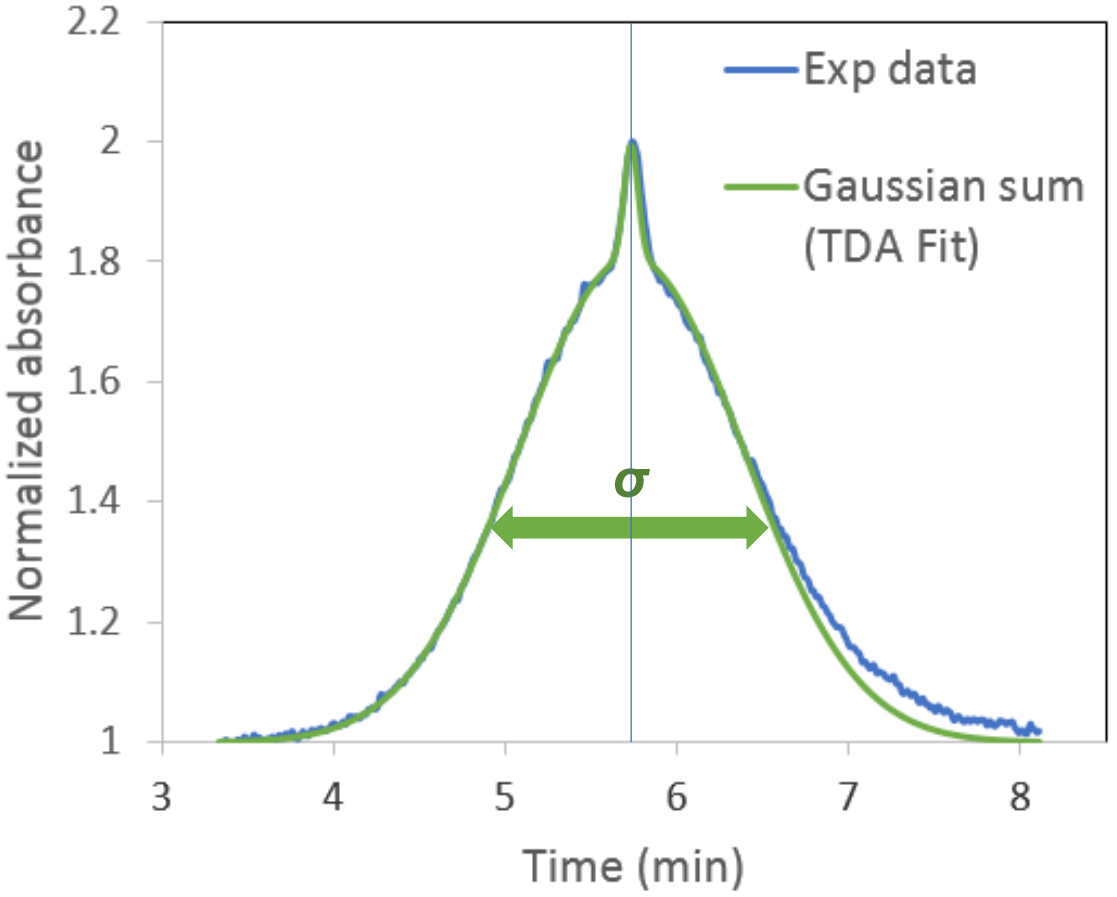
Good repeatability

TDA size

µSIL-FC capillary
40 cm to the detector × 50 µm i.d.
Inj : 20 mbar 6 sec
Mobilization : 60 mbar

C. Malburet et al., Anal. Chem., 2022, 94,11, 4677-4685

Formulation : DSPC/Cholesterol/DMG-PEG/MC3 molar ratio 10/38.5/1.5/50,
mRNA (Trilink) Cyanine5 Firefly luciferase (Fluc)



$$S(t) = \frac{A_1}{\sigma_1\sqrt{2\pi}} exp \frac{-(t-t_0)^2}{2{\sigma_1}^2} + \frac{A_2}{\sigma_2\sqrt{2\pi}} exp \frac{-(t-t_0)^2}{2{\sigma_2}^2}$$

Fit on the left side of the taylorgram

Diffusion coefficient:

$$D = \frac{R_C^{\,2}\, t_0}{24\,\sigma^2}$$

Hydrodynamic diameter:

$$D_h = \frac{2 k_B T}{6\pi\eta D}$$

Size : D_h TDA = 86 nm

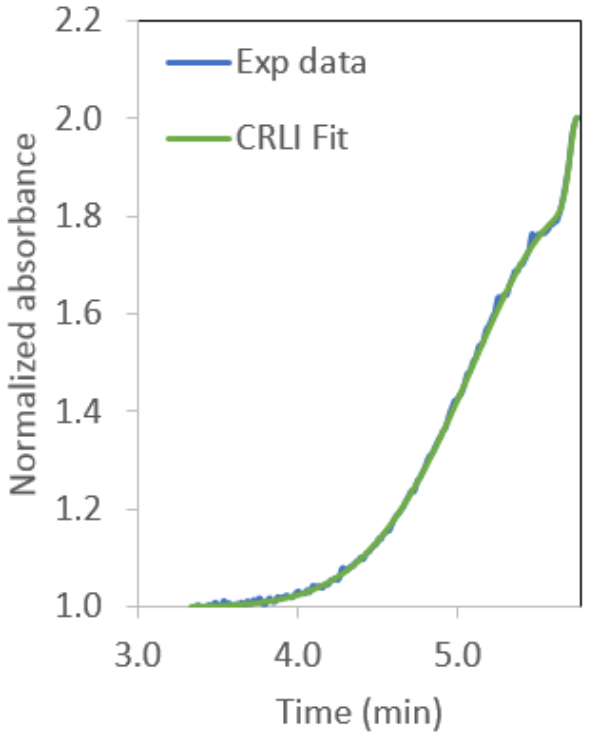
Size distribution and polydispersity

C. Malburet et al., Anal. Chem., 2022, 94,11, 4677-4685

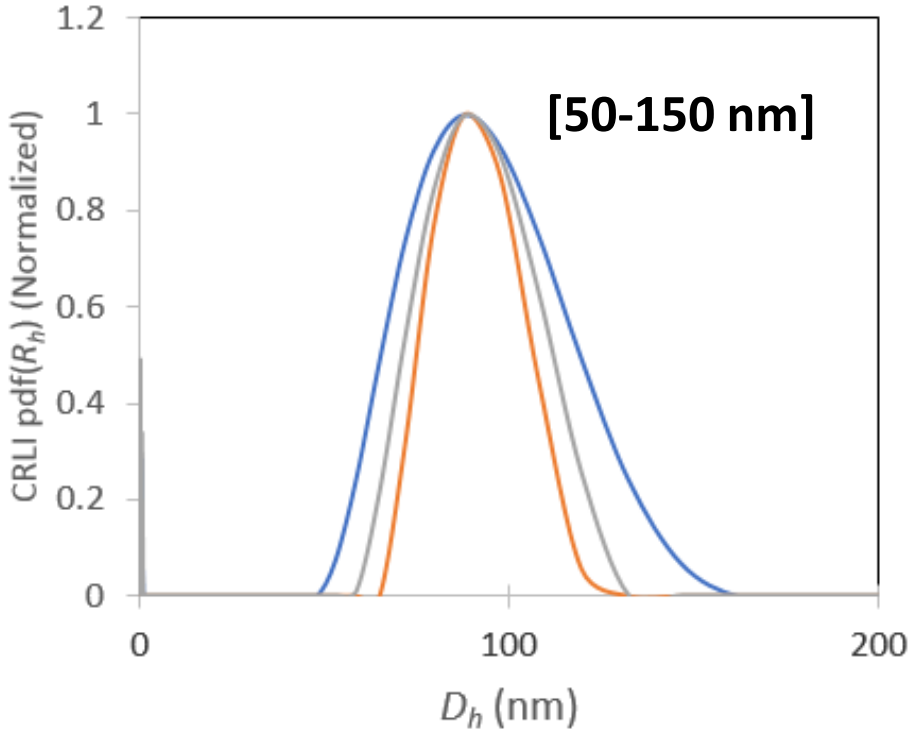
Fit with an infinite number of Gaussians

$$S(t) = \int_0^{\infty} CM(D)\rho(D)\sqrt{D}\ \exp\left[-\frac{(t-t_0)^2 12D}{R_c^2 t_0}\right] dD$$

Fit CRLI



Distribution of hydrodynamic diameters



Polydispersity index calculations

$$\sigma_{D_h}{}^2 = \frac{\int_{D_{h\min}}^{D_{h\max}} (D_h - \overline{D_h})^2 P(D_h) dD_h}{\int_{D_{h\min}}^{D_{h\max}} P(D_h) dD_h}$$

$$PDI = \left(\frac{\sigma_{D_h}}{\overline{D_h}}\right)^2$$

$$PDI = 0.011$$

TDA

Simultaneous determination of:

LNP

→ Size

→ Size distribution

→ Polydispersity

Formulation parameters impacting LNPs size

C. Malburet et al., Gene Therapy, submitted

Aqueous phase:

Citrate buffer pH 4

mRNA

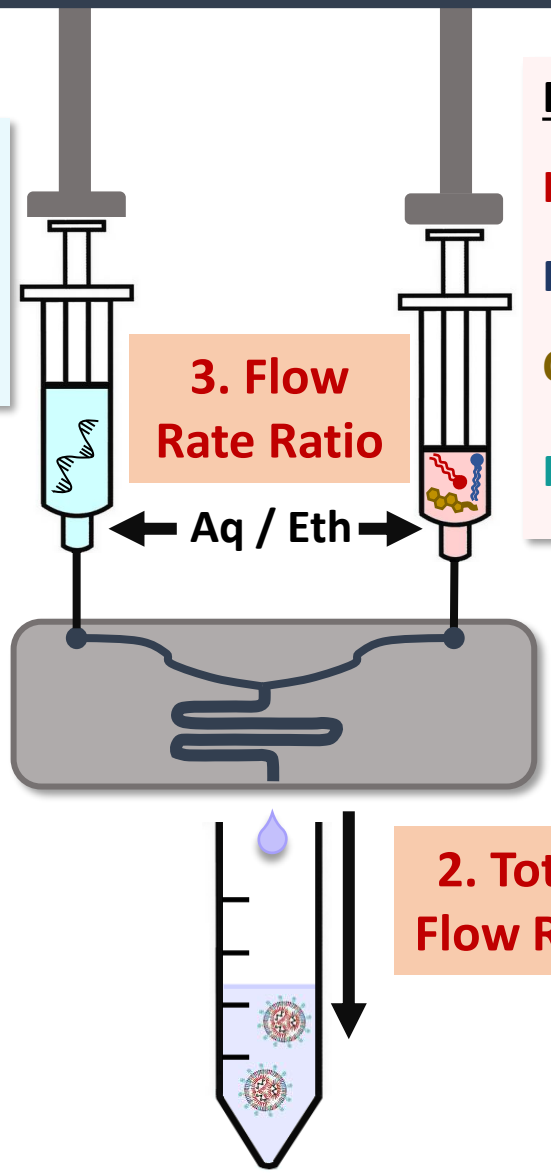
Ethanolic phase:

Ionizable lipid (50)

Phospholipids (10)

Cholesterol (40-x)

PEG-lipid (x)



3. Flow Rate Ratio

1. PEG Molar Ratio

2. Total Flow Rate

Formulation parameters impacting LNPs size

C. Malburet et al., Gene Therapy, submitted

Aqueous phase:

Citrate buffer pH 4

mRNA

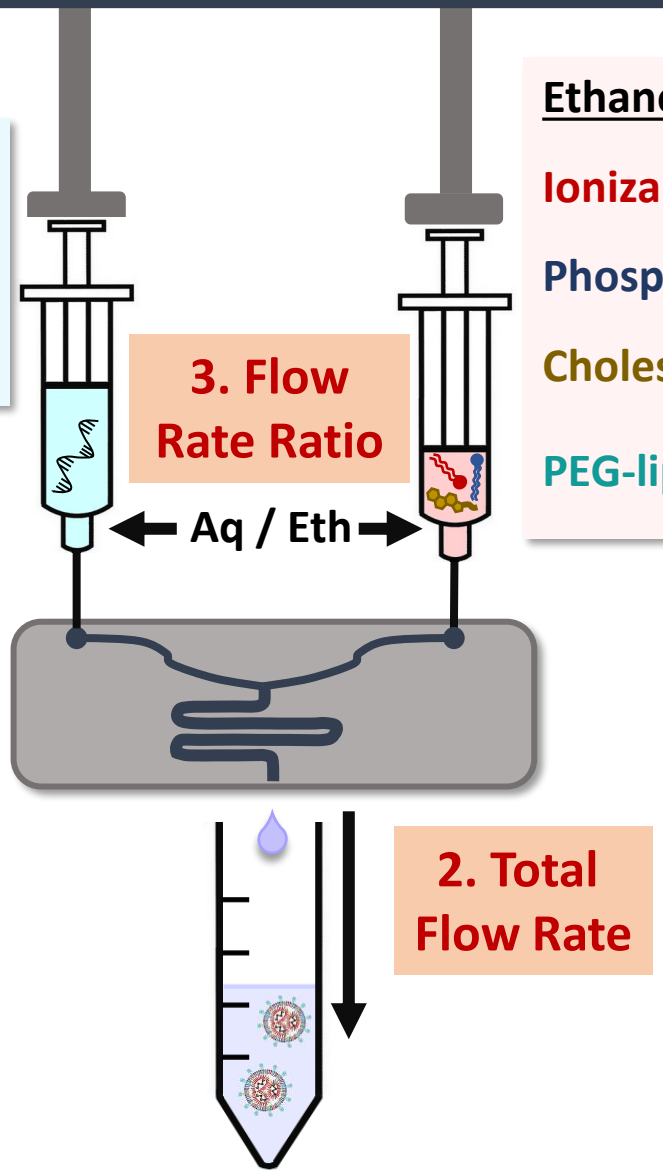
Ethanolic phase:

Ionizable lipid (50)

Phospholipids (10)

Cholesterol (40-x)

PEG-lipid (x)



3. Flow Rate Ratio

1. PEG Molar Ratio

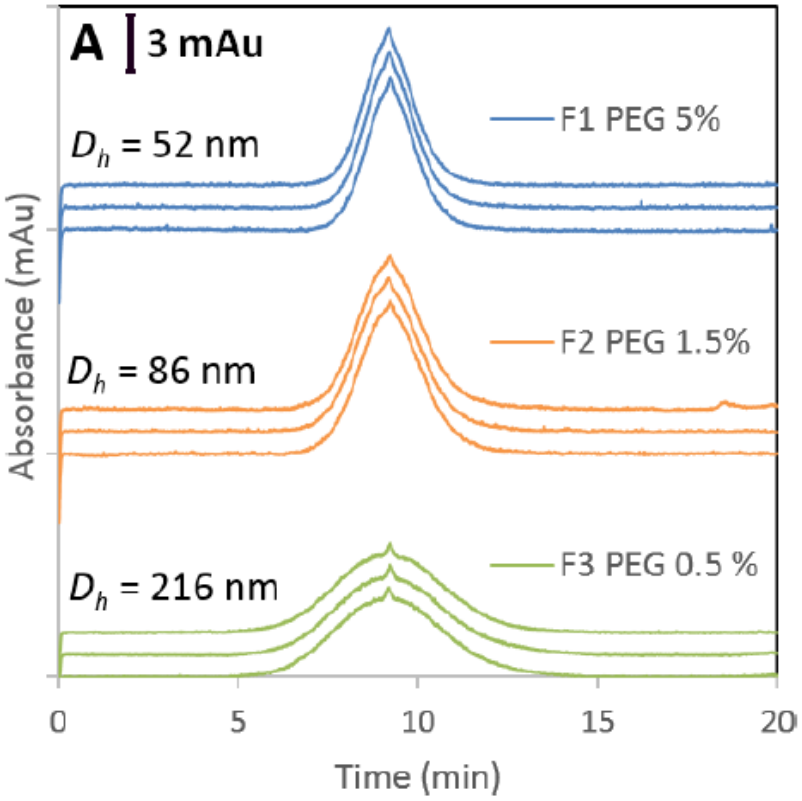
2. Total Flow Rate

target size: <200 nm

Formulation parameters impacting LNPs size

C. Malburet et al., Gene Therapy, submitted

PEG-lipid ratio
(with mRNA)

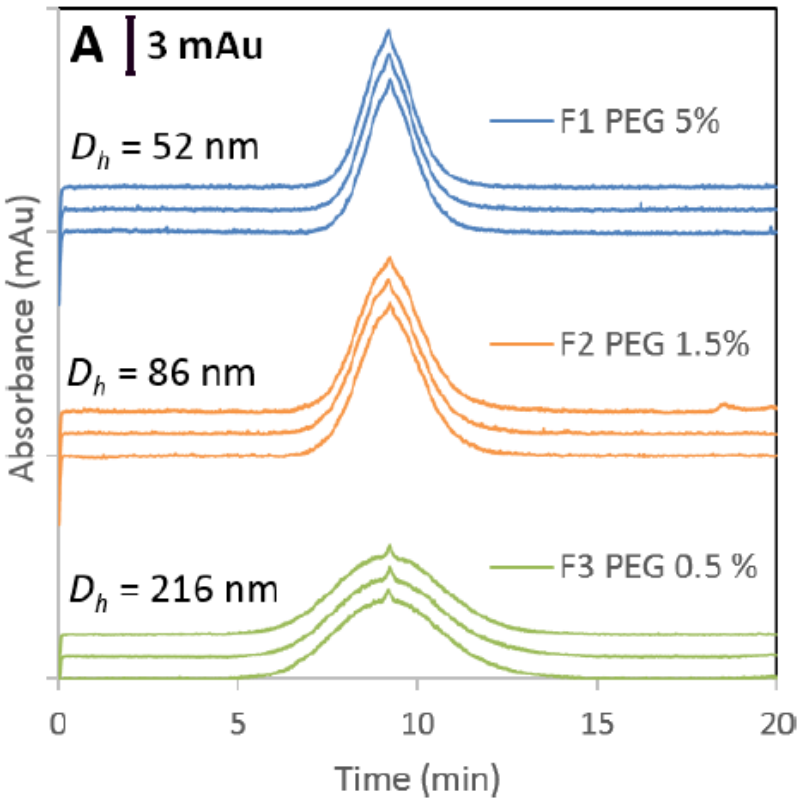


→ The size decreases by increasing the PEG%

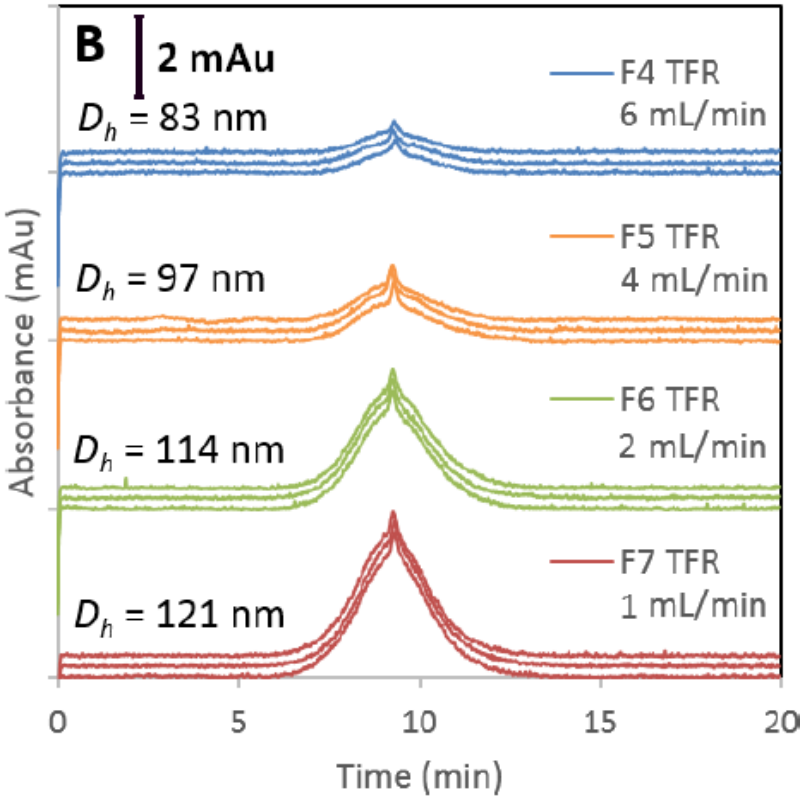
Formulation parameters impacting LNPs size

C. Malburet et al., Gene Therapy, submitted

PEG-lipid ratio
(with mRNA)



Total Flow Rate (TFR)
(without mRNA)



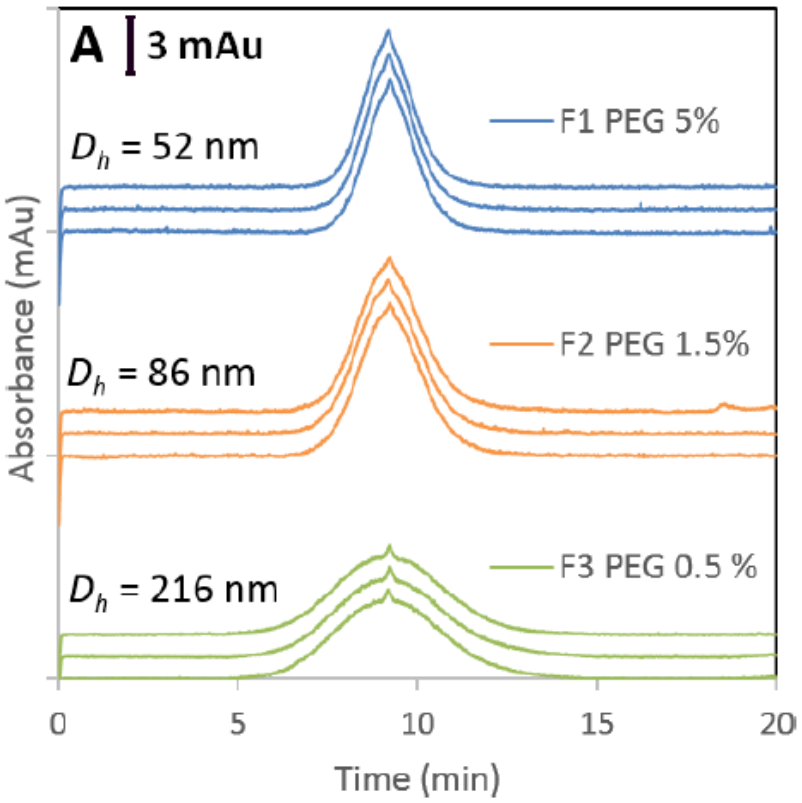
→ The size decreases by increasing the PEG%

→ The size decreases by increasing the TFR

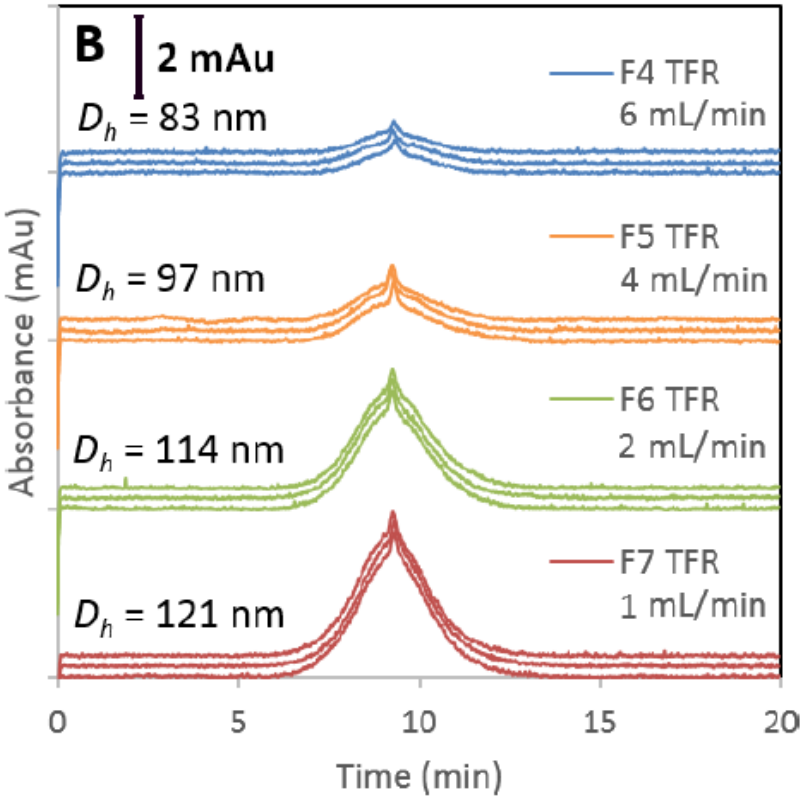
Formulation parameters impacting LNPs size

C. Malburet et al., Gene Therapy, submitted

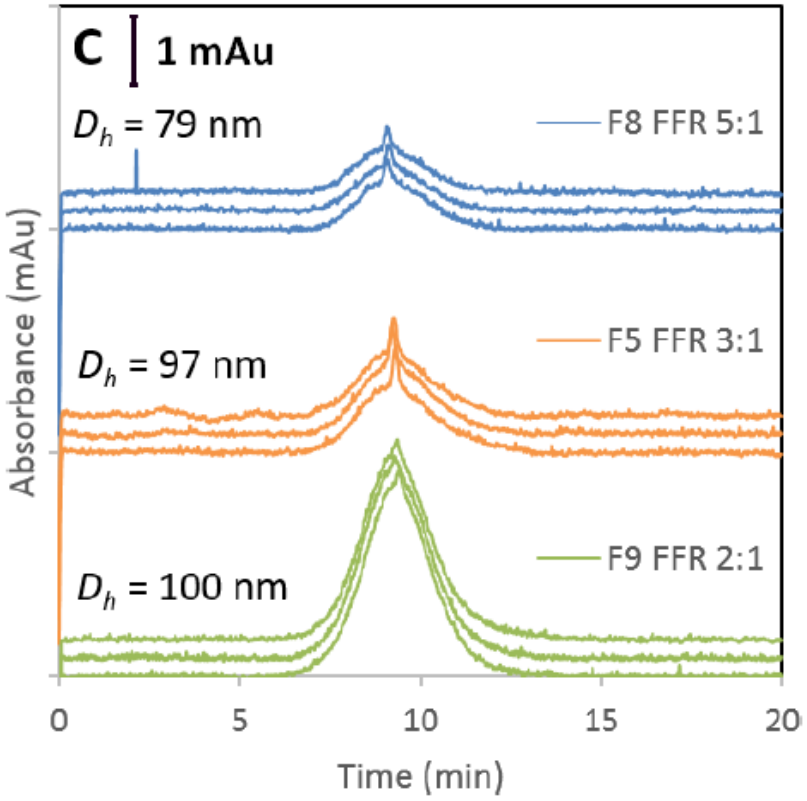
PEG-lipid ratio
(with mRNA)



Total Flow Rate (TFR)
(without mRNA)



Flow Rate Ratio (FRR)
(without mRNA)



→ The size decreases by increasing the PEG%

→ The size decreases by increasing the TFR

→ The size decreases by increasing the FRR

Comparison TDA/DLS : size

TDA η_{PBS} = 0.90 x 10^{-3} Pa/s
T = 25°C

C. Malburet et al., Gene Therapy, submitted

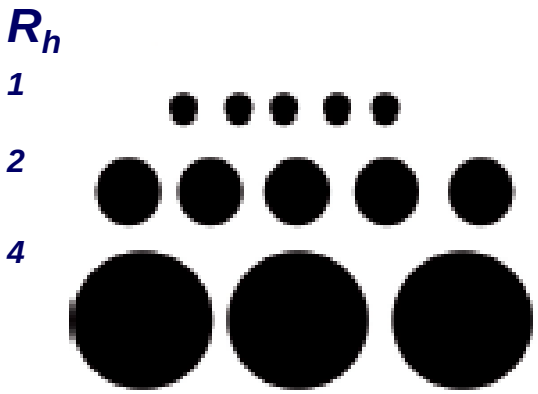
	F1 PEG 5%	F2 PEG 1.5%	F3 PEG 0.5%	F4 TFR 6 ml/min	F5 TFR 4 ml/min	F6 TFR 2 ml/min	F7 TFR 1 ml/min	F8 FFR 5:1	F5 FFR 3:1	F9 FFR 2:1
TDA D_h (nm) weight	55	86	198	83	97	114	121	79	97	102
DLS D_h (nm) intensity	91	109	278	98	105	142	156	89	105	181

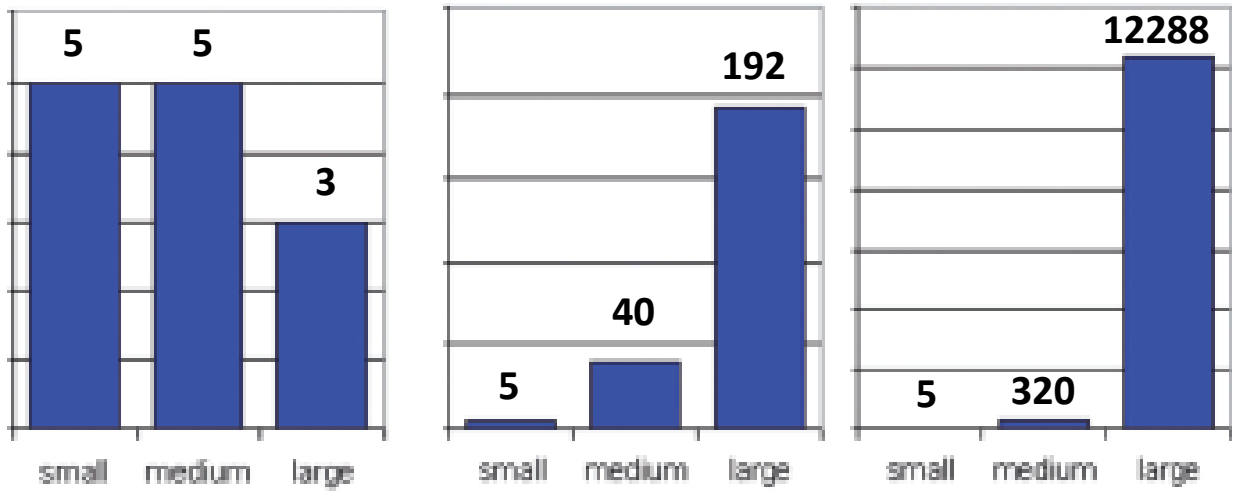
DLS provides an intensity-averaged size distribution (weights larger particles more than smaller ones)

TDA provides a weight-averaged size distribution

Comparison TDA/DLS : size

T. Linsinger et al., JRC Report on nanomaterials, EUR 25404 EN, 2012.





Distributions:

Number-based

Mass-based
TDA
⇨ weight average

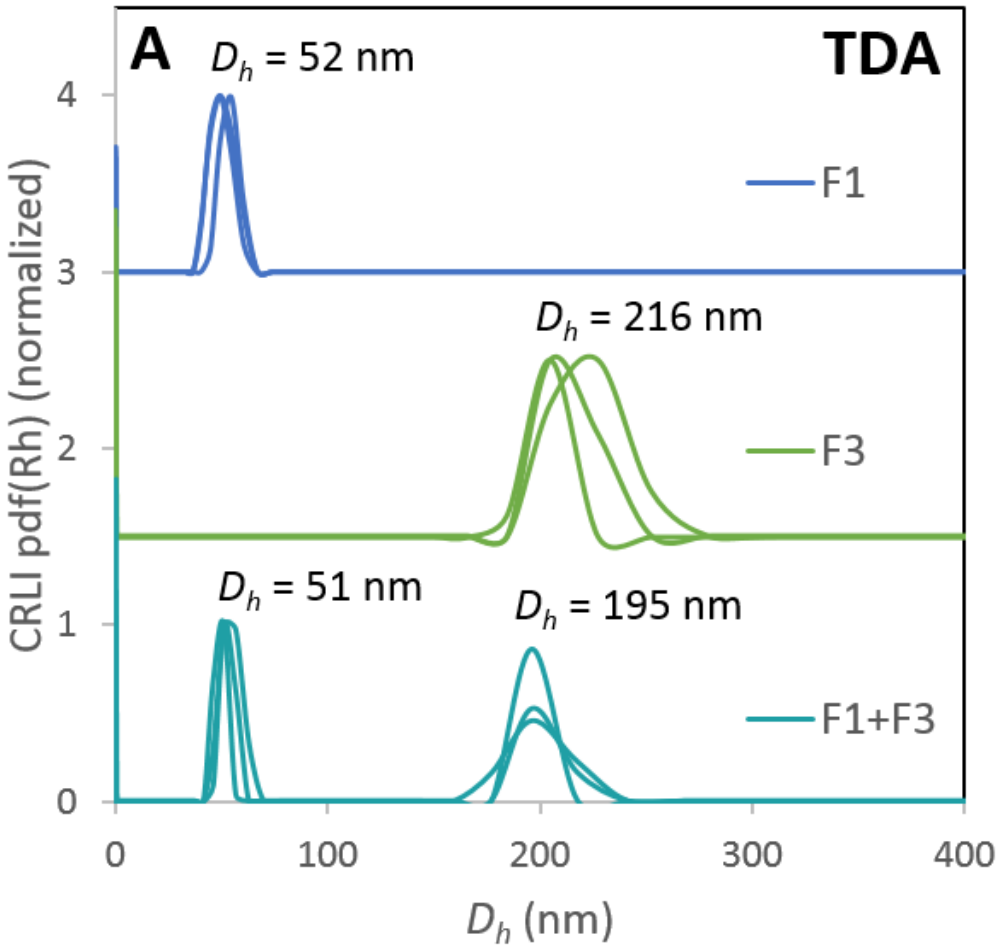
Intensity-based
DLS
⇨ intensity-average

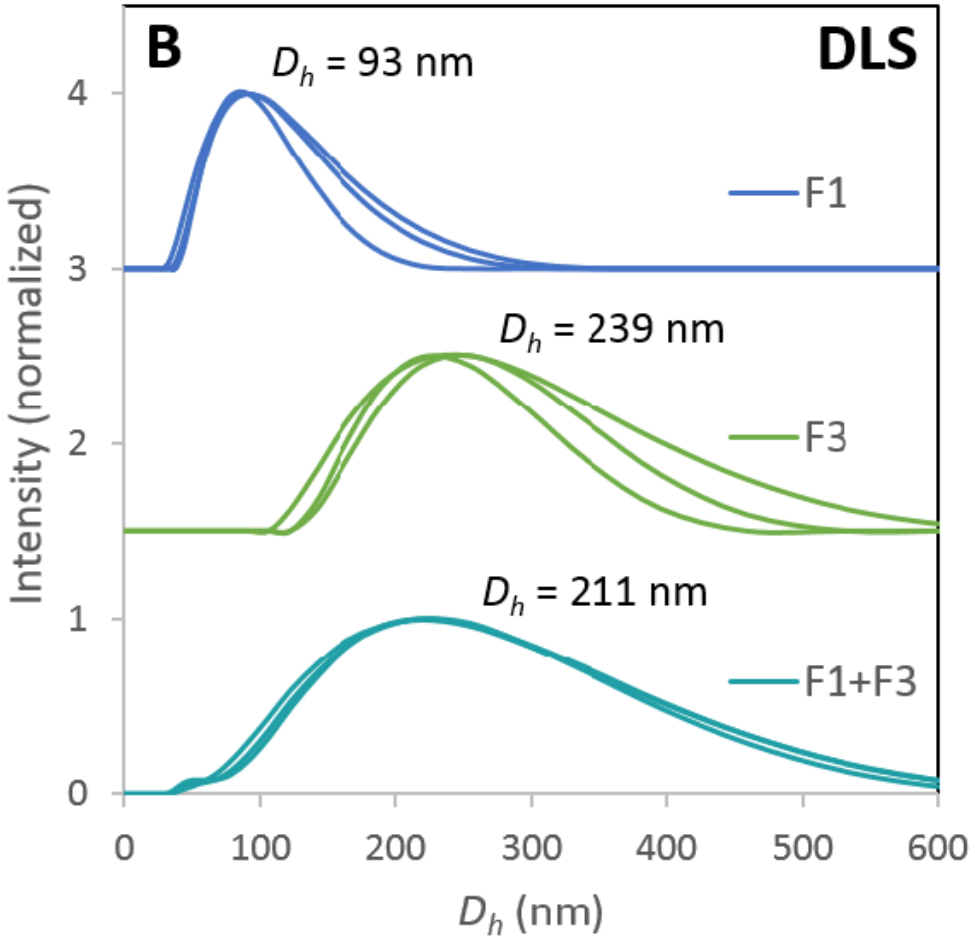
DLS provides an intensity-averaged size distribution (weighs larger particles more than smaller ones)

TDA provides a weight-averaged size distribution

Comparison TDA/DLS, 50:50 mix of F1 and F3

C. Malburet et al., Gene Therapy, submitted



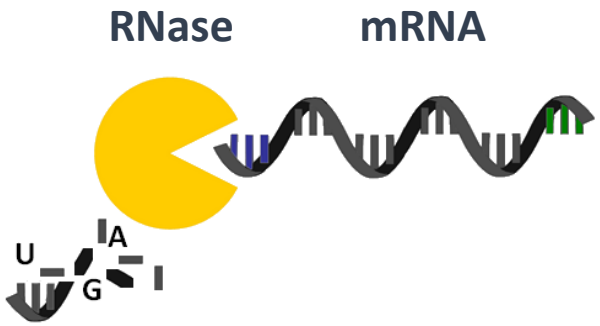


TDA allows to differentiate the two populations present in the mixture

RNAse degradation

C. Malburet et al., Gene Therapy, submitted

mRNA Cleavage



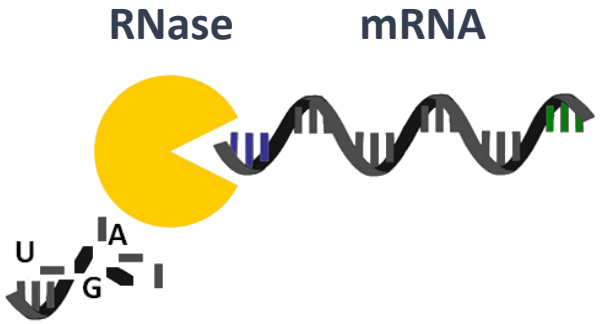
Degradation products

RNAse degradation

61 cm total length × 50 µm i.d.
Inj : 20 mbar 6 sec
Mobilization pressure: 60 mbar
Detection UV 200 nm

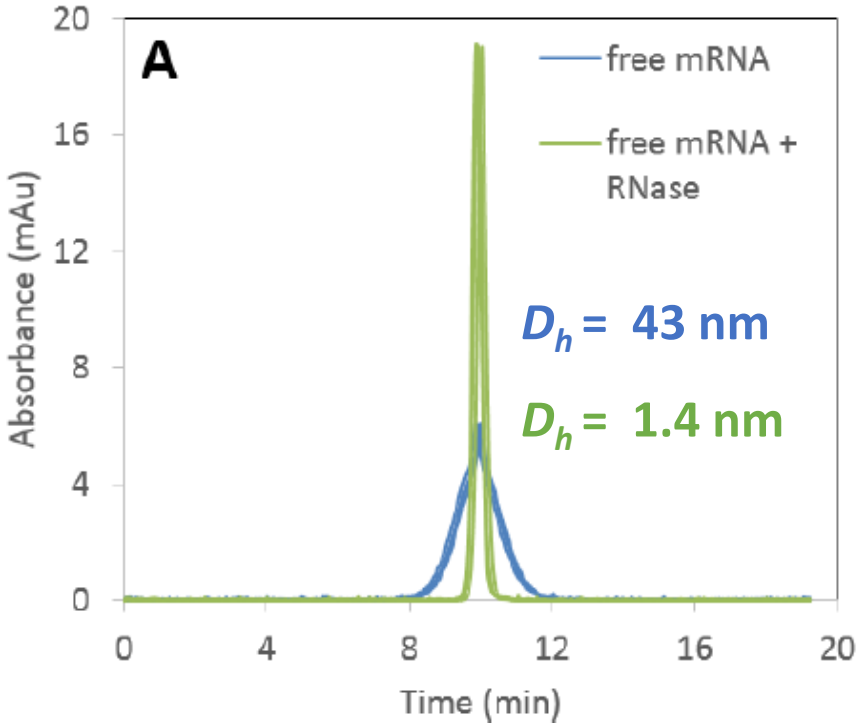
C. Malburet et al., Gene Therapy, submitted

mRNA Cleavage



Degradation products

mRNA + RNase (0.005 mg/mL)



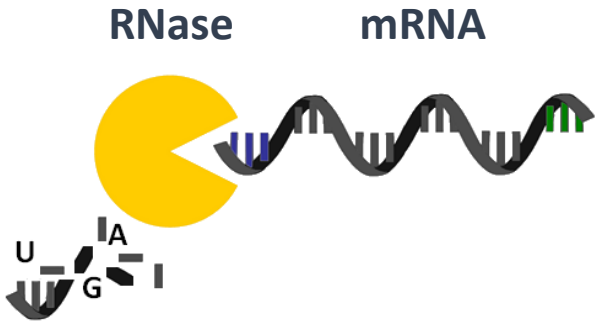
→ Total degradation

RNAse degradation

61 cm total length × 50 µm i.d.
Inj : 20 mbar 6 sec
Mobilization pressure: 60 mbar
Detection UV 200 nm

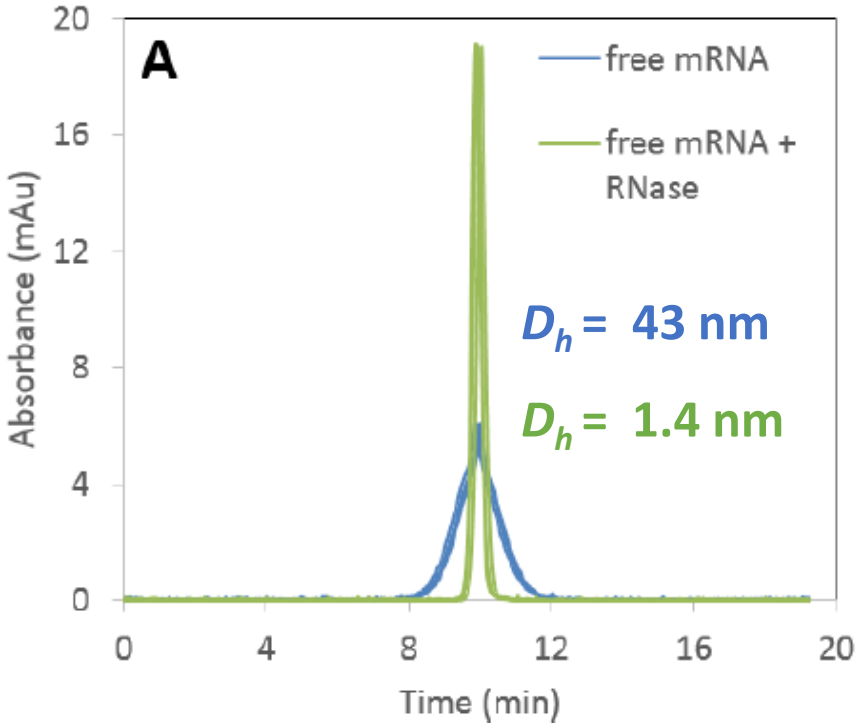
C. Malburet et al., Gene Therapy, submitted

mRNA Cleavage

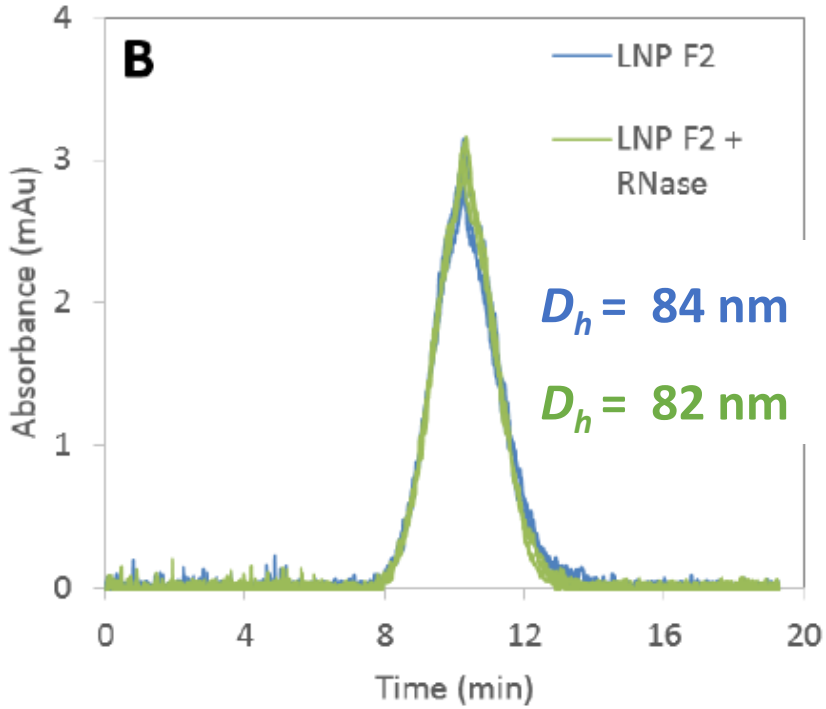


Degradation products

mRNA + RNase (0.005 mg/mL)



LNP + RNase (0.005 mg/mL)



→ Total degradation

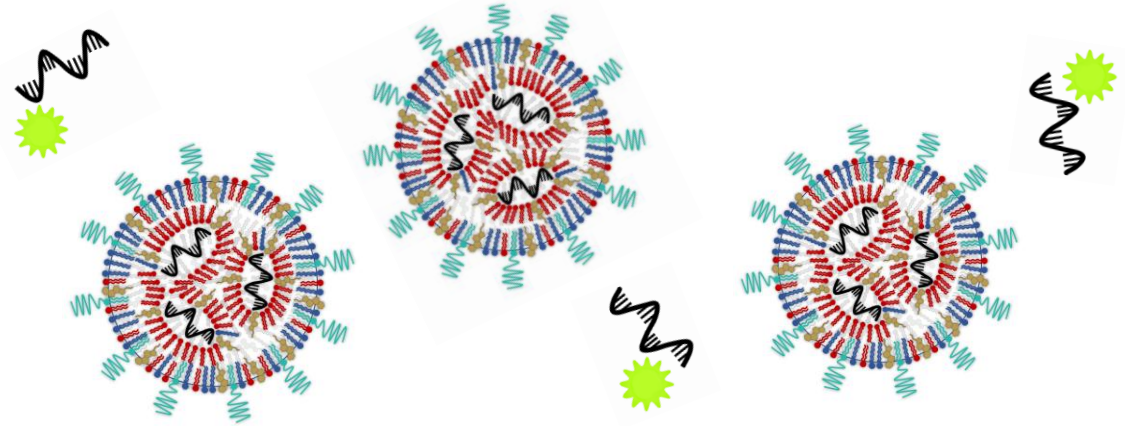
→ No visible impact

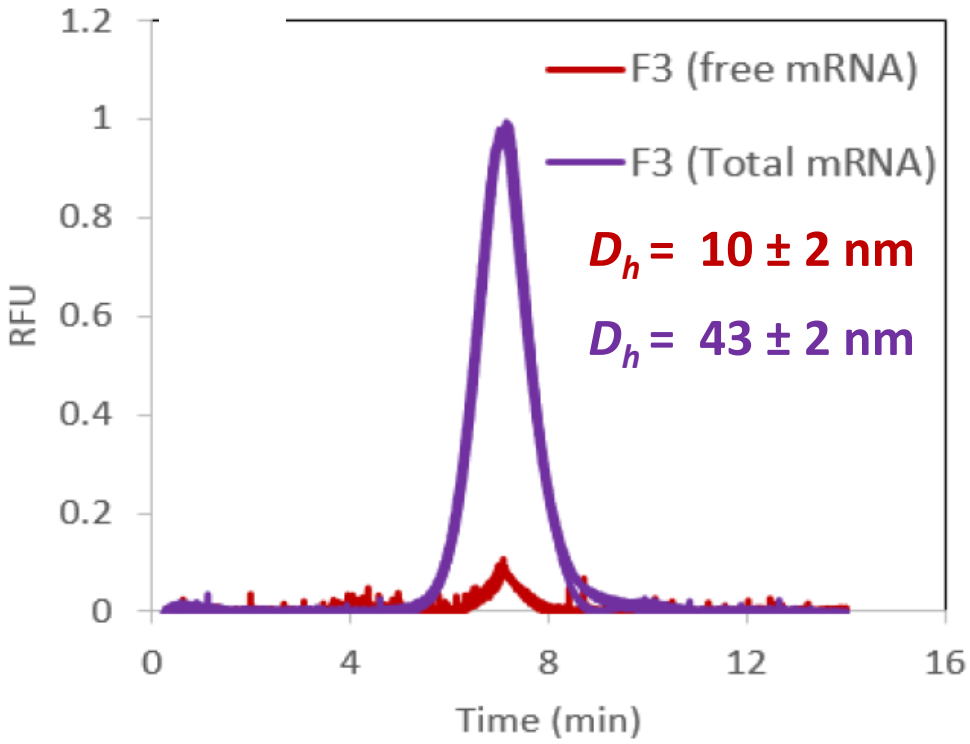
Degree of mRNA encapsulation and integrity

61 cm total length × 50 µm i.d.
Inj : 20 mbar 6 sec
Mobilization pressure: 60 mbar
Detection FLUO ex 480 nm

C. Malburet et al., Gene Therapy, submitted

Formulation + Ribogreen



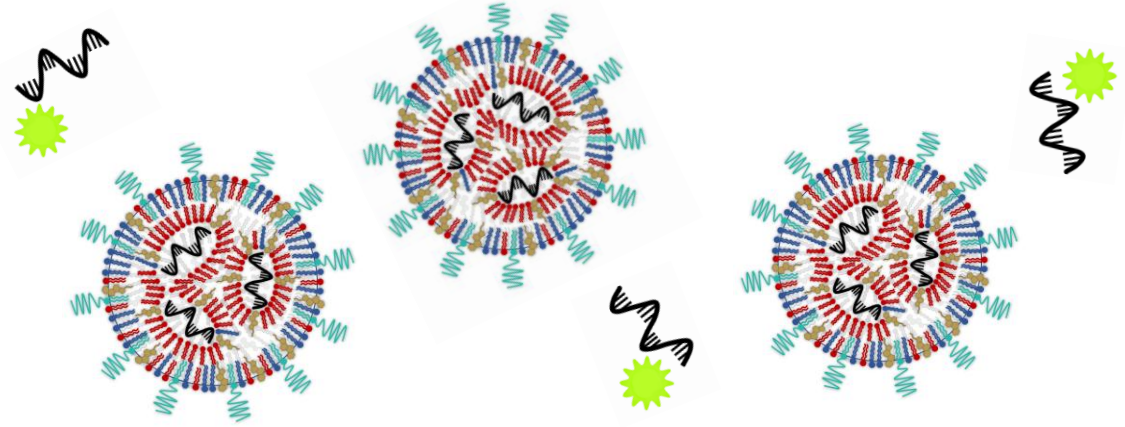


Formulation + Ribogreen + TRITON

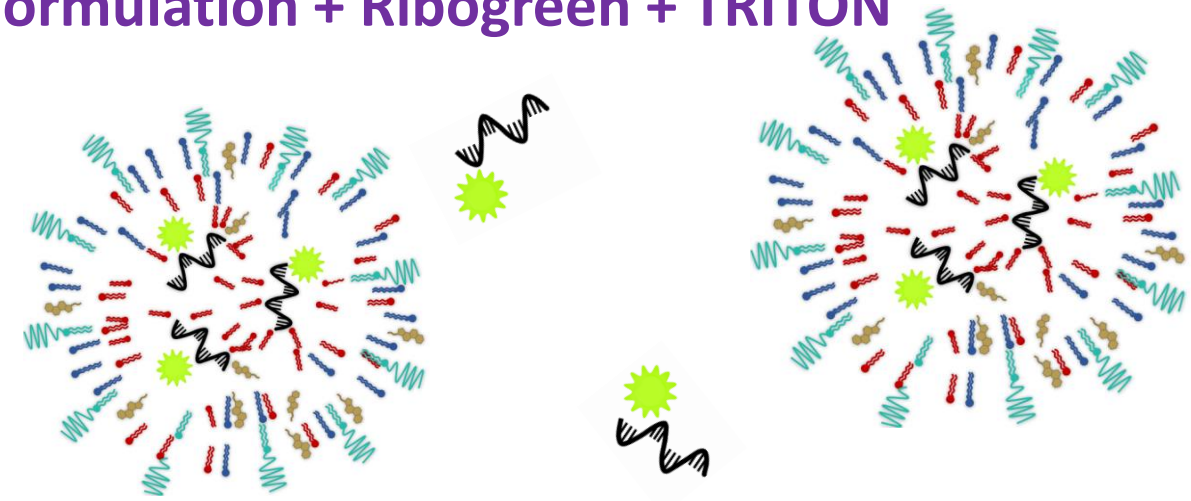
Degree of mRNA encapsulation and integrity

61 cm total length × 50 µm i.d.
Inj : 20 mbar 6 sec
Mobilization pressure: 60 mbar
Detection FLUO ex 480 nm

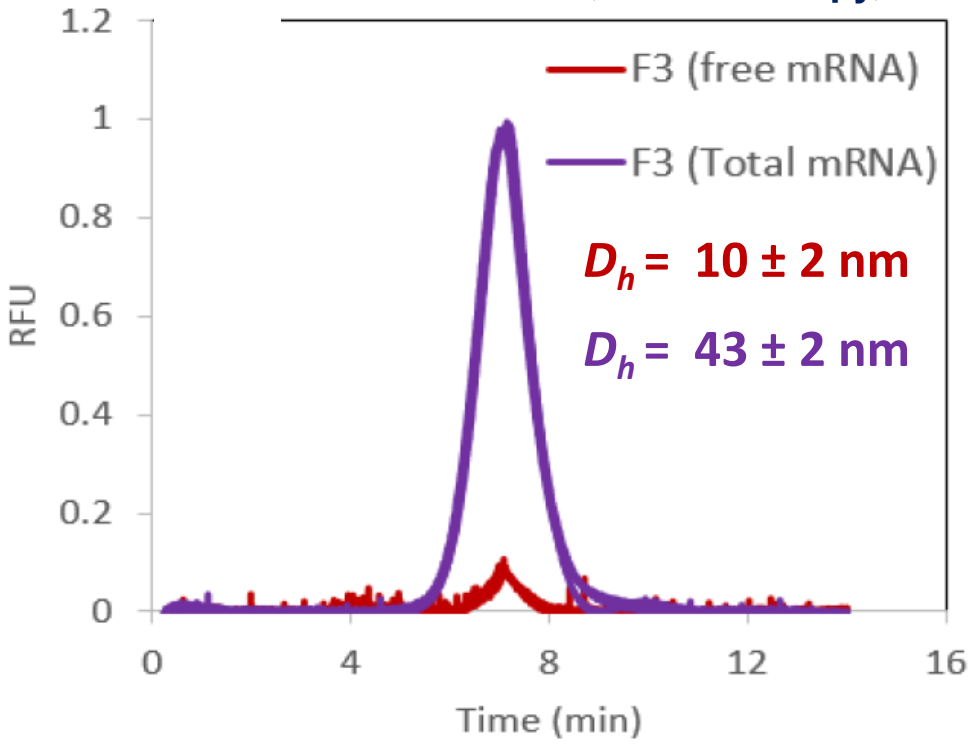
Formulation + Ribogreen



Formulation + Ribogreen + TRITON



C. Malburet et al., Gene Therapy, submitted

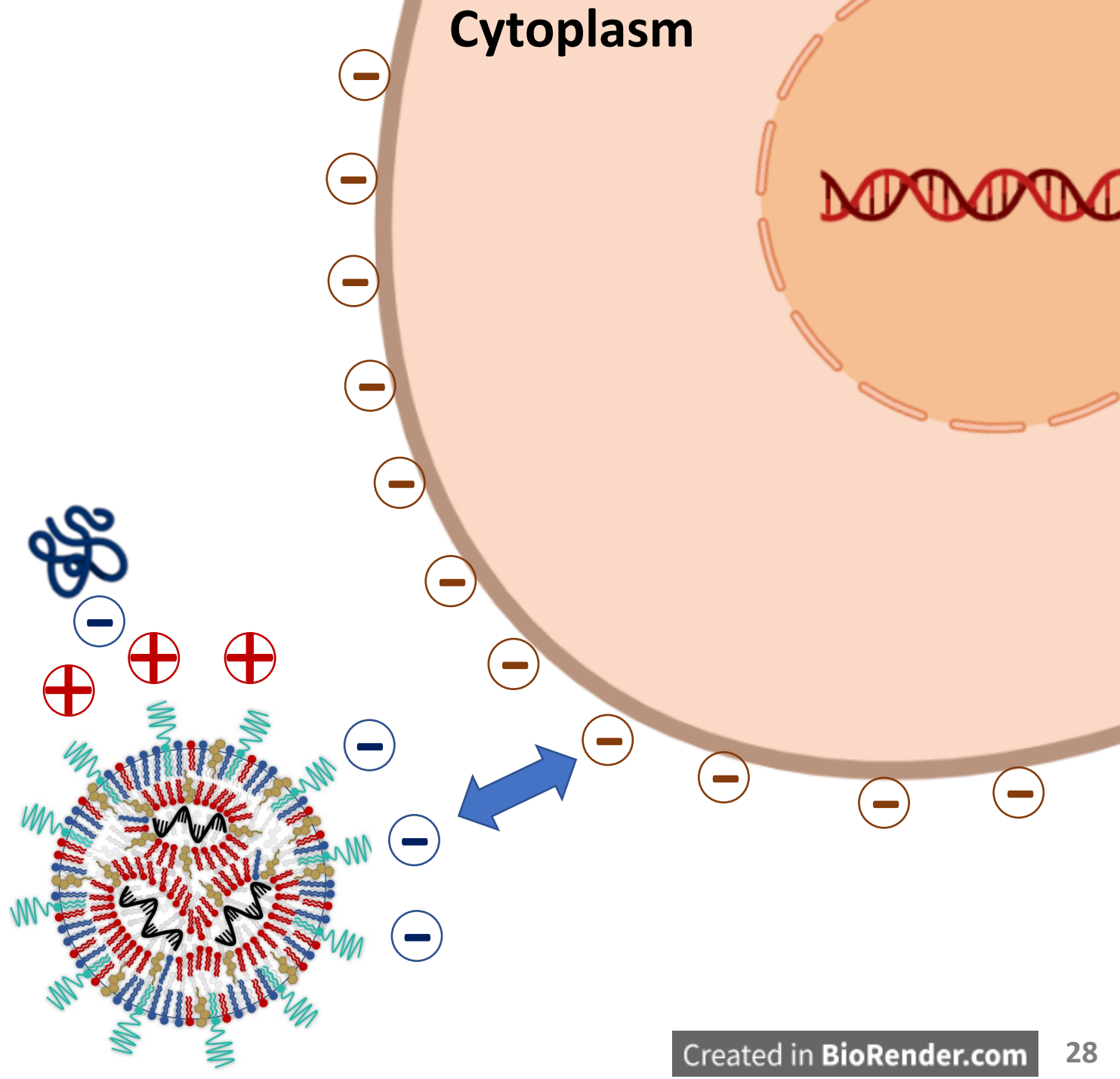


	F3
Encapsulation Rate	95 %
D_h free mRNA	10 nm
D_h total mRNA	43 nm

Charge

Charge is another crucial physicochemical parameter

Charge plays a key role in cellular internalization, dictating the transport of objects across biological barriers.



Created in BioRender.com

Charge : CE

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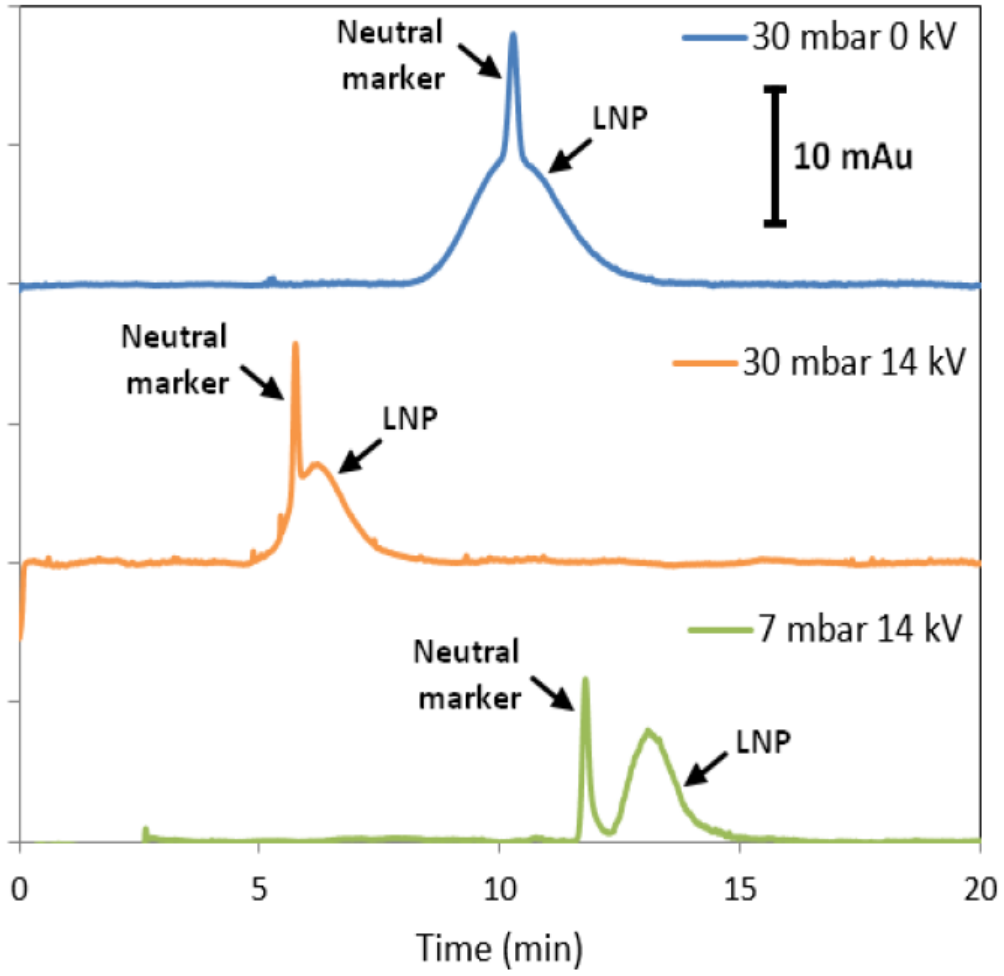
PBS Buffer, pH 7.4



µSIL-FC capillary

40 cm to the detector × 50 µm i.d.

Inj : 20 mbar 6 sec

Detection UV 200 nm

CE Effective electrophoretic mobility :

μ_{ep} **= -1.9×10^{-9} m^{2}V^{-1}s^{-1}**

Laser Doppler electrophoresis (LDE) :

μ_{ep} **= -5.0×10^{-9} m^{2}V^{-1}s^{-1}**

Zeta potential: electrophoretic mobility modeling

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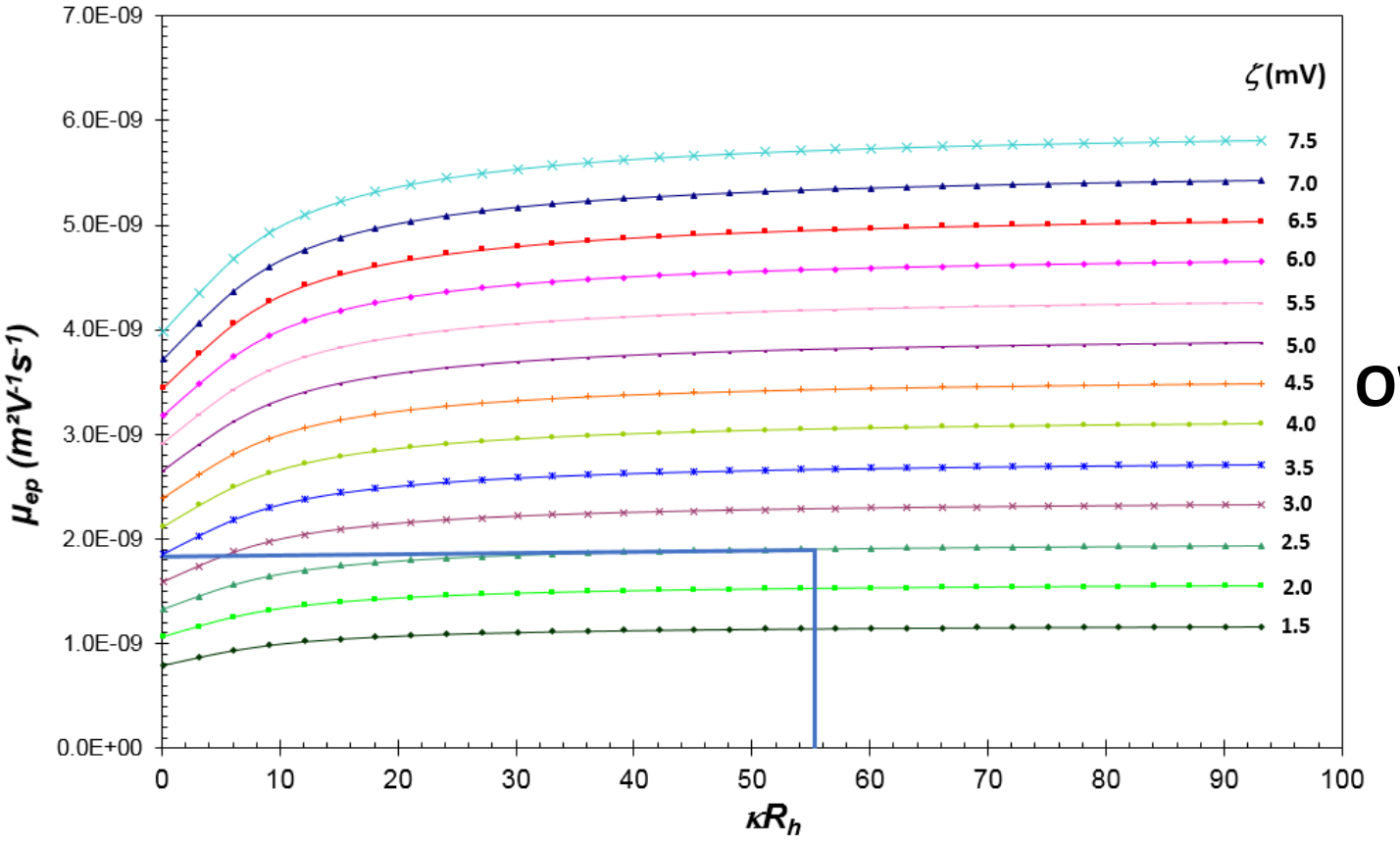
Electrophoretic mobility μ_{ep} as a function of κR_h for different zeta potential (ζ)



μ_{ep} ***CE*** = -1.86×10^{-9} m^{2}V^{-1}s^{-1}

R_h ***TDA*** = 43 nm

κR_h = 55

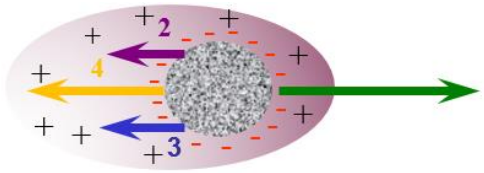
OWO (electrophoretic + relaxation effects)

ζ = - 2.5 mV

Henry Model (electrophoretic effect only)

$$\mu_{ep} = \frac{2\varepsilon\zeta}{3\eta} \times \left(1 + \frac{1}{2\left(1 + \frac{\delta}{\kappa R_h}\right)^3} \right)$$

ζ = - 2.5 mV



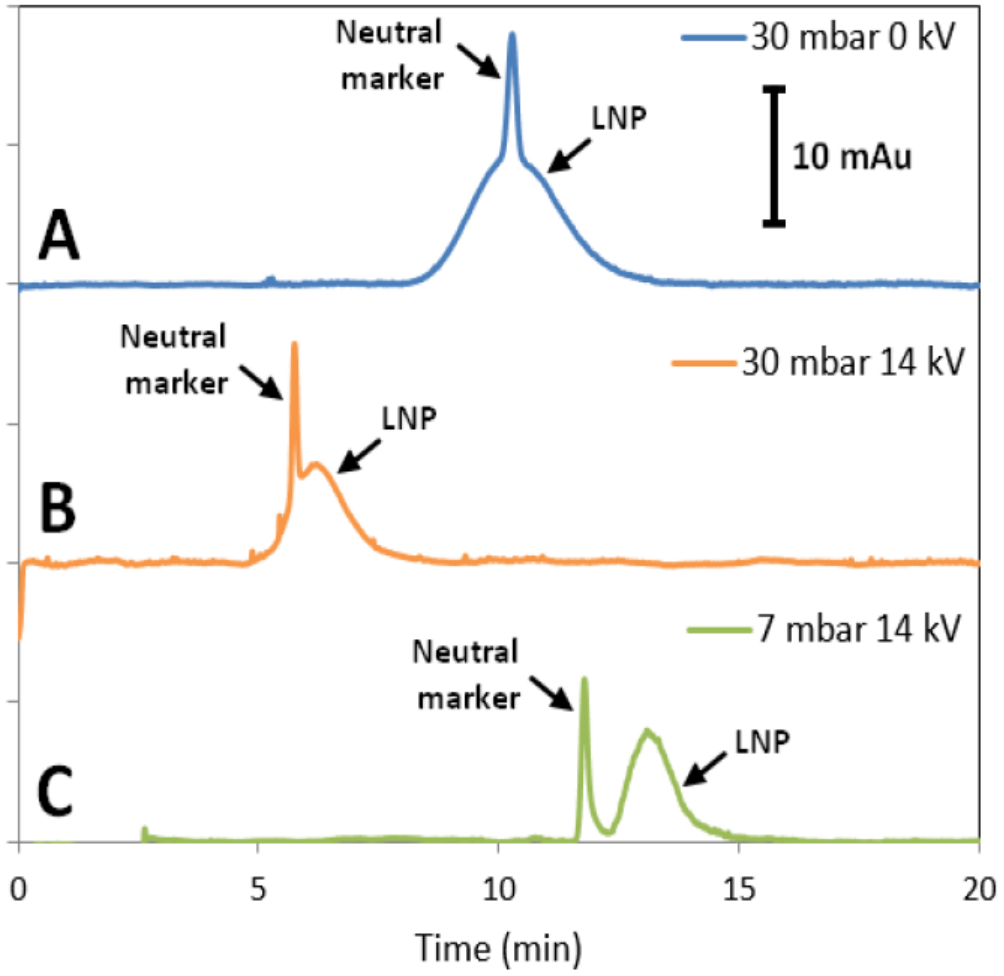
Electrophoretic effect : counter ion friction

Relaxation effect : distortion of the counterion cloud

Charge : electrophoretic mobility modeling

61 cm total length × 50 µm i.d.
Inj : 20 mbar 6 sec
Detection UV 200 nm

C. Malburet et al., Anal. Chem., 2022, 94,11, 4677-4685



Electric charge density (σ) :

$$\sigma_{OWO} = \frac{2\varepsilon_0\varepsilon_r\kappa k_B T}{e}\sinh\left(\frac{e\zeta}{2k_BT}\right)\times\left[1+\frac{1}{\kappa\frac{D_h}{2}}\frac{2}{\cosh^2\left(\frac{e\zeta}{4k_BT}\right)}+\frac{1}{\left(\kappa\frac{D_h}{2}\right)^2}\frac{8\ln\left(\cosh\left(\frac{e\zeta}{4k_BT}\right)\right)}{\sinh^2\left(\frac{e\zeta}{2k_BT}\right)}\right]^{\frac{1}{2}}$$

σ_{OWO} = - 2.4×10^{-3} C.m^{-2}

σ_{OWO} = -0.015 elementary charge per nm^2

Z_{eff} = -350 elementary charges per LNP

This weak negative charge at physiological pH is expected to reduce unwanted interactions during blood circulation.

Conclusion

Characterization of mRNA vaccines

TDA & CE → Access to many parameters:

- LNP **Size, size distribution** and **polydispersity**
- mRNA **encapsulation rate** and **integrity**
- LNP **Charge**

TDA Advantages	TDA limitations
- absence of calibration - low sample consumption - high degree of automation - Remarkable trueness and precision	- adsorption on capillary walls - buffer mismatch effects - does not allow seeing small amounts of large aggregates

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