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Size and charge characterization of lipid nanoparticles for mRNA vaccines

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ABSTRACT:

Messenger RNA vaccines have come into the spotlight as a promising and adaptive alternative to conventional vaccine approaches. The efficacy of mRNA vaccines relies on the ability of mRNA to reach the cytoplasm of cells, where it can be translated into proteins of interest allowing to trigger the immune response. However, unprotected mRNA is unstable, susceptible to degradation by exo and endonucleases, and its negative charges are electrostatically repulsed by the anionic cell membranes. Therefore, mRNA needs a delivery system that protects the nucleic acid from degradation and allows it to enter into the cells. Lipid nanoparticles (LNPs) represent the non-viral leading vector for mRNA delivery. Physicochemical parameters of LNPs, including their size and their charge, directly impact their *in vivo* behavior and, therefore, their cellular internalization. In this work, Taylor Dispersion Analysis (TDA) was used as a new methodology for the characterization of the size and polydispersity of LNPs, and capillary electrophoresis (CE) was used for the determination of LNPs global charge. The results obtained were compared with those obtained by dynamic light scattering (DLS) and Laser Doppler Electrophoresis (LDE).

27 INTRODUCTION

28 mRNA (messenger ribonucleic acid) was discovered in 1961, eight years after DNA
29 (deoxyribonucleic acid).¹ In the first decades after its discovery, the main focus was on
30 understanding its structural and functional aspects. In 1990, Wolff et al. demonstrated that direct
31 injection of *in vitro* transcribed (IVT) mRNA into mice skeletal muscle resulted in the expression
32 of the protein encoded by the mRNA.² For the first time, the possibility of using IVT mRNA for
33 therapeutic purposes was shown. Unlike strategies based on plasmid DNA, mRNA does not need
34 to enter into the cell nucleus but only into the cytoplasm to be translated into proteins of interest
35 by ribosomes, allowing to trigger the immune response.³ Since then, mRNA has shown promise
36 for the treatment of a wide range of diseases and different types of cancer.⁴⁻⁷ These advances
37 have been enabled by a better understanding of the structure of IVT mRNA, as well as the
38 development of new delivery system.⁸ Lipid nanoparticles (LNPs) are currently the most
39 clinically advanced non-viral delivery vehicle for RNA-based drugs.⁹ The efficacy of mRNA-
40 based vaccines highly depends on the ability of LNPs to enter cells and deliver mRNA. The
41 physicochemical parameters of LNPs including their size, shape, charge and surface composition,
42 directly impact their cellular internalization.¹⁰

43 The size of nanovectors is known to affect intracellular delivery and therefore vaccine
44 efficacy.^{11,12} Indeed, size has a significant contribution to many functional parameters, in
45 particular it determines the entry route of particles into the cells.¹³⁻¹⁶ Furthermore, LNPs size can
46 be optimized to target certain organs.¹⁷⁻²⁰ Size requirements are therefore highly dependent on
47 the route of administration and the targeted organ. In all cases, LNPs size must be finely
48 characterized. Regulatory agencies such as the World Health Organization²¹, the European
49 Commission²² and the U. S. Food and Drug Administration²³ qualify LNPs size and size
50 distribution as Critical Quality Attributes (CQA) of messenger RNA vaccines products.

51 Moreover, the regulatory agencies recommend the use of orthogonal methods for LNPs size
52 determination to address technique-related differences.^{21,24}

53 DLS is a rapid method based on the study of Brownian motion of particles in a liquid.
54 However, critical points can limit the precision of the results obtained by DLS: (i) the samples
55 must often be strongly diluted, which can destabilize some formulations; (ii) the size derived
56 from the scattered light intensity gives more weight to large particles because the scattered
57 intensity scales as the hydrodynamic radius to the power 6²⁵ and (iii) the size distributions are
58 calculated assuming some pattern of particle shape, which is not always accurate. Therefore, DLS
59 is generally only recommended for the control of samples with relatively low polydispersities.
60 Nanoparticle Tracking Analysis (NTA) is another method also based on the analysis of Brownian
61 motion of particles.²⁶ Many particles are analyzed individually and simultaneously, their
62 hydrodynamic diameters are calculated using the Stokes-Einstein equation. NTA appears to be
63 more accurate than DLS for polydisperse particles and for detecting the presence of aggregates.²⁷
64 However, NTA that is based on the same principle as DLS, also shares some similar limitations,
65 such as important dilutions required and restricted resolution for small particles. Otherwise,
66 Cryogenic Transmission Electron Microscopy (Cryo-TEM) has become a leading technology for
67 imaging nanoscale biological samples such as LNPs.²⁸ Cryo-TEM can reveal detailed size,
68 morphology and structure information at high resolution. Although Cryo-TEM analysis has
69 obvious advantages, the large adoption of Cryo-TEM for the characterization of nanoparticles is
70 hampered by several factors, such as the high cost of imaging systems, the complex sample
71 preparation and the low image acquisition and analysis rate.²⁵ Moreover, Cryo-TEM images
72 suffer from high background noise, sometimes non-uniform lighting and artefacts, which makes
73 automatic particle identification difficult.

74 Charge is another crucial physicochemical parameter cited by the regulatory agencies²¹⁻²³.
75 Charge plays a key role in cellular internalization, dictating the transport of objects across
76 biological barriers.^{29,30} The charge of the nanocarriers is controlled by the lipids present in the
77 LNPs, in particular ionizable lipids, and by the composition of the aqueous buffer. Ionizable
78 lipids must be positively charged at low pH allowing mRNA complexation during formulation,
79 LNPs self-assembly and endosomal release (likely). In the meantime, these ionizable lipids must
80 be neutral at physiological pH to avoid potential toxic effects due to unwanted interactions.³¹⁻³³
81 Moreover, studies have shown that the charge of nanoparticles can be optimized to target
82 different organs.^{34,35} The charge of nanoparticles is mostly determined by Laser Doppler
83 Electrophoresis (LDE) and generally expressed in terms of Zeta potential, which represents the
84 potential difference between the dispersion medium and the stationary layer of fluid attached to
85 the particle.³⁶ The Zeta potential strongly depends on the pH, temperature and ionic strength of
86 the medium^{37,38}, and it was reported that the surface charge density can be instead preferably
87 reported^{39,40}. Capillary electrophoresis (CE) is another method for determining the surface charge
88 density of nanoparticles.⁴¹ Several studies have focused on the study of the electrophoretic
89 mobility of liposomes.⁴²⁻⁴⁵ It is worth noting that LDE and CE are both based on the
90 determination of electrophoretic mobilities, which are then transformed into Zeta potential and/or
91 surface charge density using electrophoretic mobility modelling. The determination of
92 electrophoretic mobilities by CE requires small sample volumes and the analyses have the
93 advantage of being easily automated. In addition, CE is a separative method allowing getting
94 information on charge density distributions.

95 In this work, Taylor Dispersion Analysis (TDA) was used as a new alternative method to
96 determine the size and size distribution of LNPs encapsulating mRNA. TDA is an absolute
97 method which requires very little sample, is easily automated and has the advantage to be

98 performed without any alteration of the sample (analysis performed in the sample matrix).
99 Chamieh et al. used TDA to determine the size of micelles and microemulsions,⁴⁶⁻⁴⁸ while
100 Franzen et al. used TDA to determine the size of PEGylated liposomal formulations.⁴² Moreover,
101 the size distribution of nano-objects can be derived from the experimental Taylorgram using
102 Regularized Linear Inversion (CRLI) approach.⁴⁹ To our knowledge, TDA has never been used
103 for the characterization of lipid nanoparticles encapsulating mRNA. This work presents an
104 optimized TDA methodology to access to the size and size distribution of LNPs and a
105 comparison with the results obtained by DLS. Using the same equipment, the charge of the lipid
106 nanoparticles was determined by capillary electrophoresis and the results were compared with
107 those obtained by LDE.

108

109 **EXPERIMENTAL SECTION**

110 **Chemicals and Materials.** FLuc (Cyanine 5 Firefly Luciferase) mRNA (1929
111 nucleotides) was purchased from TriLink BioTechnologies (San Diego, USA). DLin-MC3-DMA
112 ((6Z,9Z,28Z,31Z)-heptatriacont-6,9,28,31-tetraene-19-yl 4-(dimethylamino)butanoate) was
113 purchased from Sai Life Sciences Ltd. (Telangana, India). DSPC (1,2-distearoyl-sn-glycero-3-
114 phosphocholine), Chol (cholesterol), DMG-PEG-2000 (1,2-dimyristoyl-rac-glycero-3-
115 methoxypolyethylene glycol-2000), trisodium citrate dihydrate $\text{HOC}(\text{COONa})(\text{CH}_2\text{COONa})_2 \cdot$
116 $2\text{H}_2\text{O}$, ($M_w = 294.1 \text{ g/mol}$), citric acid monohydrate $\text{HOC}(\text{COOH})(\text{CH}_2\text{COOH})_2 \cdot \text{H}_2\text{O}$
117 ($M_w = 210.1 \text{ g/mol}$) and Hellmanex III were purchased from Sigma-Aldrich (St. Quentin
118 Fallavier, France). PBS (phosphate-buffered saline) 10X buffer was purchased from Eurobio
119 Scientific (Les Ulis, France). Absolute ethanol was purchased from Carlo Erba Reagents (Val de
120 Reuil, France). Bare fused silica capillaries were purchased from Molex Polymicro Technologies
121 (Phoenix, USA). $\mu\text{SIL-FC}$ (fluorocarbon polymer), PVA (polyvinyl alcohol), DB-1

122 (polydimethylsiloxane) and DB-WAX (polyethylene glycol) coated capillaries were purchased
123 from Agilent Technologies (Santa Clara, USA). Ultratrol LN was purchased from Target
124 Discovery (Santa Clara, USA). PEO (poly(ethylene oxide), $M_v \sim 4,000,000$ Da), DLPC (1,2-
125 dilauroyl-sn-glycero-3-phosphocholine) and DDAB (dimethyldioctadecylammonium bromide
126 salt) were purchased from Sigma-Aldrich (St. Quentin Fallavier, France). DMF
127 (dimethylformamide) was purchased from Carlo Erba Reagents (Val-de-Reuil, France).
128 Deionized water was further purified with a Milli-Q system from Millipore (Molsheim, France).

129 **Formulation of LNPs.** The formulation of LNPs involved the rapid mixing of an ethanolic
130 phase composed of DLinMC3-DMA:DSPC:Chol:DMG-PEG-2000 (50:10:38.5:1.5 molar ratio,
131 see Figure S1 for the molecular structure of the LNP constituents) with a 50 mM citrate aqueous
132 phase at pH 4 containing the mRNA at 0.25 mg/mL. Stock solutions of lipids were previously
133 prepared in absolute ethanol at the following concentrations: DLinMC3-DMA 100 mg/mL,
134 DSPC 30 mg/mL, Chol 18 mg/mL, DMG-PEG-2000 30 mg/mL. The mixture was carried out
135 using a NanoAssemblr® system from Precision NanoSystems (Vancouver, Canada) at 4 mL/min
136 total flow rate with 3:1 (aqueous phase:ethanolic phase) flow rate ratio. After formulation, 3
137 dialysis steps were carried out in order to remove ethanol from the formulation and raise the pH
138 to physiological in PBS buffer, 3.2 mL of mRNA-LNP solution were recovered after the last
139 dialysis step.

140 **Taylor Dispersion Analysis (TDA).** All experiments were carried out on a 7100 CE
141 Agilent system (Waldbronn, Germany). This system is equipped with a diode array detector
142 (DAD). All the measurements were performed at 200 nm. The temperature of the capillary
143 cartridge was set at 25°C. The LNPs formulations were injected without prior dilution into the
144 capillaries filled with PBS buffer. TDA experiments were performed using 60 mbar mobilization

145 pressure of a sample plug injected at 20 mbar for 6 s. The elution peak obtained was fitted using
 146 the sum of two Gaussians according to equation (1) using a home-developed Excel spreadsheet
 147 and Excel solver:

$$148 \quad S(t) = \frac{A_1}{\sigma_1\sqrt{2\pi}} \exp\left[-\frac{(t-t_0)^2}{2\sigma_1^2}\right] + \frac{A_2}{\sigma_2\sqrt{2\pi}} \exp\left[-\frac{(t-t_0)^2}{2\sigma_2^2}\right] \quad (1)$$

149 where $S(t)$ is the absorbance signal, t_0 is the average elution time, σ_1 and σ_2 are the temporal
 150 variances and A_1 and A_2 are two constants that depended on the response factor and the injected
 151 quantity of solute. σ_1 , σ_2 , A_1 and A_2 are four adjusting parameters obtained by nonlinear least
 152 square regression. t_0 is directly obtained from the position of the maximum absorbance. One of
 153 the two populations (the largest in size) represents the LNPs, and the other (the smallest)
 154 represents the presence of detected small molecules.

155 The temporal variance σ_i derived from the elution profile allows to calculate the molecular
 156 diffusion coefficient D_i of each population according to equation (2), from which the
 157 hydrodynamic diameter $D_{h,i}$ can be determined using Stokes–Einstein equation (3):

$$158 \quad D_i = \frac{R_c^2 t_0}{24\sigma_i^2} \quad (2)$$

159 where R_c is the capillary radius.

$$160 \quad D_{h,i} = \frac{k_B T}{6\pi\eta D_i} \quad (3)$$

161 where k_B is the Boltzmann constant, T is the temperature, and η is the eluent viscosity.

162 To go further in the data analysis of the Taylorgram, the elution profile was fitted using a second
 163 approach based on Regularized Linear Inversion (CRLI) algorithm⁴⁹ to get a continuous
 164 distribution of the diffusion coefficient or of the hydrodynamic radius according to equation (4):

$$165 \quad S(t) = \int_0^\infty CM(D)\rho(D)\sqrt{D} \exp\left[-\frac{(t-t_0)^2 12D}{R_c^2 t_0}\right] dD \quad (4)$$

166 where C is an instrumental constant, $M(D)$ and $\rho(D)$ are the mass and the molar concentration of
167 the objects with the diffusion coefficient D , respectively. The polydispersity of the sample can be
168 determined using equations (5) and (6):

$$169 \quad \sigma_{D_h}^2 = \frac{\int_{D_{hmin}}^{D_{hmax}} (D_h - \bar{D}_h)^2 P(D_h) dD_h}{\int_{D_{hmin}}^{D_{hmax}} P(D_h) dD_h} \quad (5)$$

$$170 \quad PDI = \left(\frac{\sigma_{D_h}}{\bar{D}_h} \right)^2 \quad (6)$$

171 **Dynamic Light Scattering (DLS) and Laser Doppler Electrophoresis (LDE).** All
172 experiments were carried out on a Malvern Panalytical Zetasizer Nano ZS system (Palaiseau,
173 France). For both analyses, the temperature of the cuvettes was set at 25°C. For DLS
174 measurements, the LNPs formulations were diluted by adding 10 μ L of the formulation into 1 mL
175 PBS buffer prior to analysis. DLS experiments were performed using 173° measurement angle.
176 Cumulant fit was used to fit the experimental data of the autocorrelation function. For LDE
177 measurements, the LNPs formulations were diluted by adding 100 μ L of the formulation into 400
178 μ L PBS buffer prior to analysis.

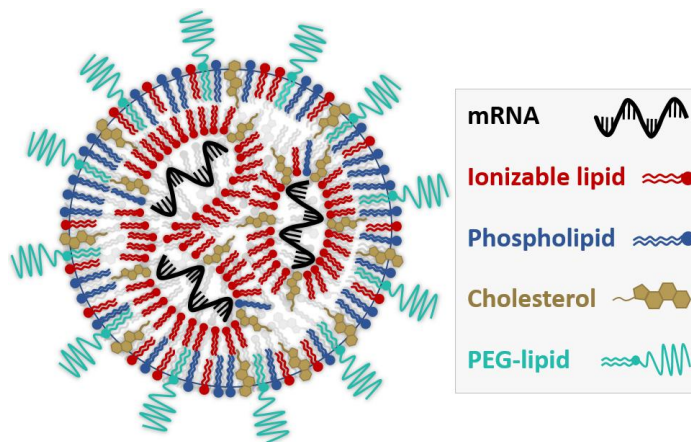
179 **Capillary Electrophoresis.** All experiments were carried out on a 7100 CE Agilent system
180 (Waldbronn, Germany) equipped with a diode array detector (DAD). μ SIL-FC capillaries of 50
181 cm total length (41.5 cm to the UV detector) \times 50 μ m i.d. were used. The temperature of the
182 capillary cartridge was set at 25°C. The capillaries were presaturated before use with LNPs
183 formulations for 10 min at 60 mbar. Before each sample injection the capillaries were flushed for
184 2 min with Mili-Q water at 960 mbar followed by 2 min 10 mM PBS at 960 mbar. LNPs samples
185 were hydrodynamically injected with 20 mbar pressure for 6 s. CE experiment were performed
186 applying 14 kV voltage and 7 mbar pressure. The UV detection was performed at 200 nm.

187

188 **RESULTS AND DISCUSSION**

189 Lipid nanoparticles (LNPs) are usually composed of four lipids serving to encapsulate
190 mRNA: an ionizable lipid, a phospholipid, cholesterol, and a PEG-lipid, as schematically
191 depicted in Figure 1. The ionizable lipid used in this study was DLin-MC3-DMA, which is often
192 used as a reference after its success in the first RNA-based drug (named ONPATRO®
193 (patisiran)) approved by the Food and Drug Administration (FDA) and the European Medicines
194 Agency (EMA) in 2018.⁵⁰ This drug uses siRNAs encapsulated into LNPs for the treatment of
195 hereditary transthyretin-mediated (hATTR) amyloidosis.^{51,52} Ionizable lipids are positively
196 charged at low pH, allowing mRNA complexation, and are neutral at physiological pH reducing
197 potential toxic effects.⁵³ The phospholipid used in this study was DSPC, whose role is to increase
198 the stability of the LNPs.⁵⁴ The PEG-lipid used was DMG-PEG-2000, whose incorporation in
199 LNPs increases their colloidal stability and their resistance to *in vivo* opsonization and
200 clearance.⁵⁴ Finally, cholesterol provided structural integrity to the LNPs.^{33,54} The inclusion of
201 cholesterol in nanoparticle formulations has also been shown to improve efficacy, potentially due
202 to improved membrane fusion allowing endosomal release.^{33,55,56} The chemical formulas of the
203 different lipids are given in Figure S1.

204



205

206
207 **Figure 1.** Hypothetical structure of mRNA-LNPs. The PEG-lipid along with DSPC resides
208 primarily on the LNPs surface, while the ionizable lipid and cholesterol are distributed
209 throughout the LNPs.^{57,58}
210

211 After formulation, the first step of this work was to perform a screening of different

212 coated capillaries in order to select the appropriate coating for the analysis of LNPs. Bare fused

213 silica and DB-1 (polydimethylsiloxane), DB-WAX (PEG), μ SIL-FC (fluorinated polymer) and

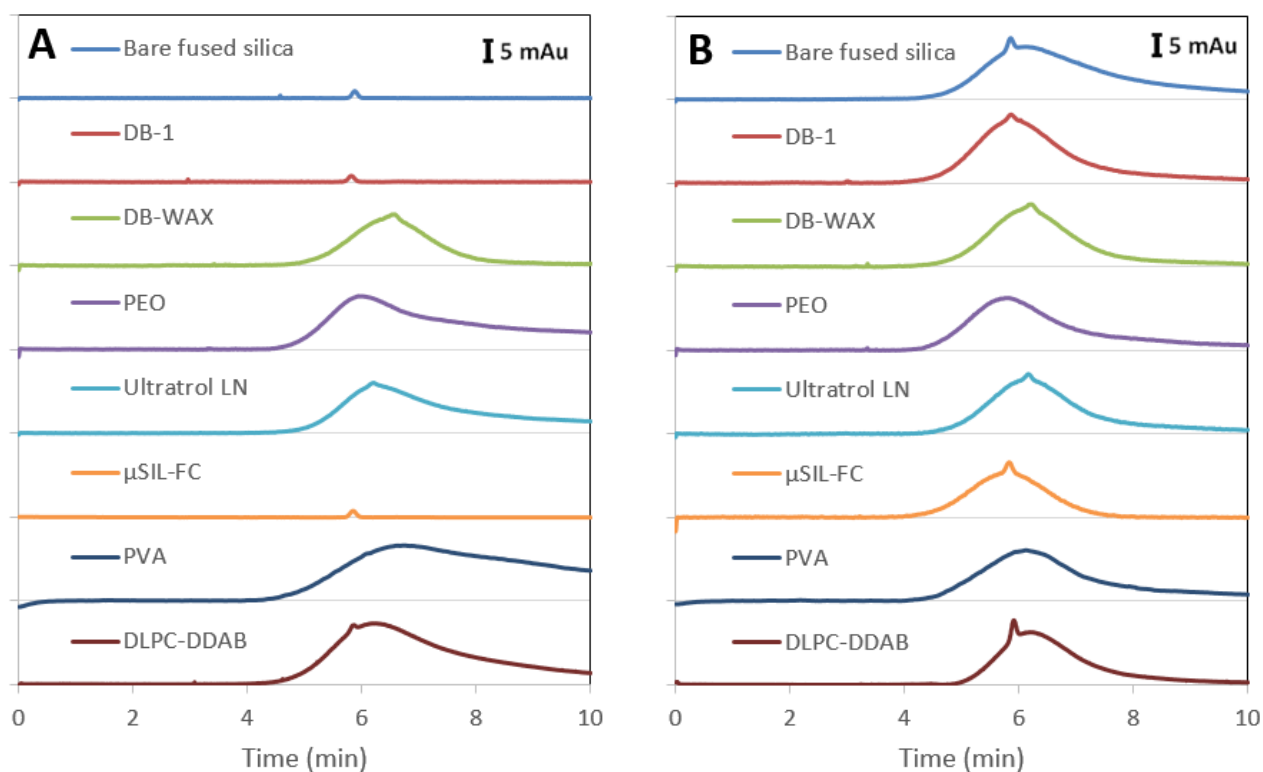
214 PVA coated capillaries are commercial capillaries. PEO, Ultratrol LN and DLPC-DDAB coated

215 capillaries were dynamically produced according to the protocols described in Section 2 of the

216 Supporting Information. A plug of LNPs formulation was first injected into the different

217 capillaries after simply rinsing the capillaries with PBS buffer. Results are presented in Figure

218 2A. It appears that without presaturation of the capillary with the LNPs formulation, DB-WAX
219 coating was the only capillary coating that allowed to observe a symmetrical Taylorgram. Yet,
220 this coating was not retained, due to relatively poor repeatability of the measurements. A very
221 important absorption was observed on all the other capillaries. It is interesting to note that no
222 peak was observed on silica, DB1 and μ SIL capillaries, suggesting that the injected sample was
223 totally adsorbed on the capillary wall. On the contrary, after presaturation of the capillaries by
224 performing a pre-frontal injection of LNPs formulations (60 mbar for 10 min), a peak was
225 observed on all the different capillaries (see Figure 2B). The presaturation allows to saturate the
226 interaction sites within the capillary surface and creates a dynamic coating inside the capillary.
227 For all the studied coated capillaries, the peak appeared more symmetrical after presaturation,



228 suggesting a reduced LNP adsorption. The
229 coating which was selected for the rest of the study was the μ SIL-FC coating, as it gave the most
230 symmetrical gaussian peak and the best baseline return after presaturation (see Figure 2B).

231

232 **Figure 2.** Screening of different capillary coatings without capillary presaturation (A) and with
233 capillary presaturation (B). Experimental conditions: 50 cm total length (41.5 cm to the UV
234 detector) \times 50 μm i.d. capillaries. Buffer: PBS, pH 7.4. Capillary conditioning: H₂O for 2 min at
235 960 mbar followed by 2 min PBS at 960 mbar (A), H₂O for 2 min, LNP for 10 min at 60 mbar
236 followed by 2 min PBS at 960 mbar (B). Injection of LNP: 20 mbar, 6 s. Mobilization pressure:
237 60 mbar. Sample: lipid nanoparticles (DLin-MC3-DMA:DSPC:Chol:DMG-PEG-2000 at
238 50:10:38.5:1.5 molar ratio) encapsulating Firefly Luciferase (FLuc) mRNA. UV detection: 200
239 nm. Temperature: 25°C.

240

241 Capillary conditioning: H₂O for 2 min at 960 mbar followed by 2 min PBS at 960 mbar.

242

243 In order to improve the repeatability of the measurements, rinsing steps were also optimized.

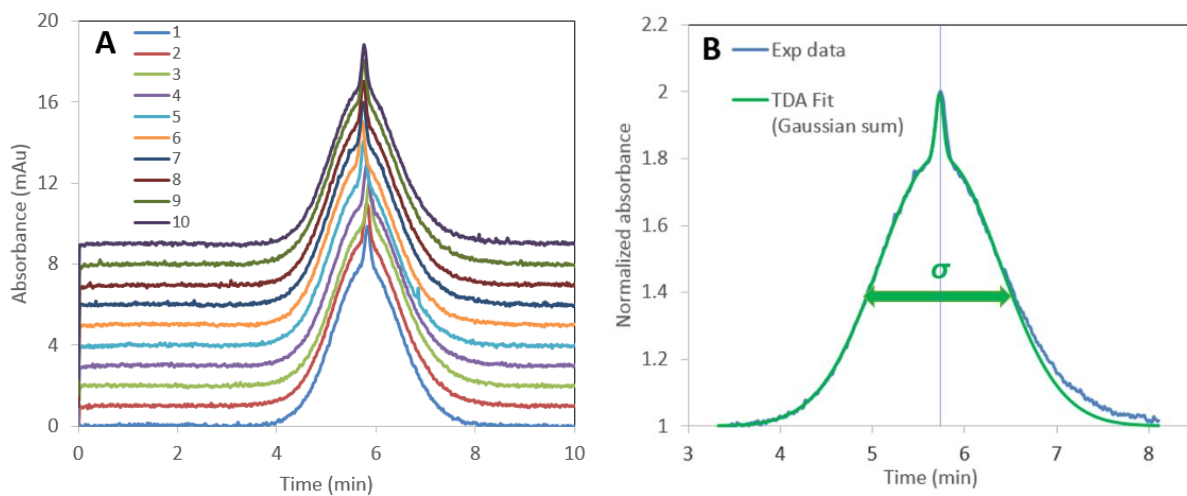
244 Between two successive runs, capillaries were simply rinsed for 1 min with water and 2 min with

245 PBS buffer. The repeatability of 10 successive runs was shown in Figure 3A. After each analysis

246 sequence capillaries were rinsed for 5 min with 1% Hellmanex in water, 10 min with isopropanol

247 and 5 min with water. Capillaries were presaturated again at the start of each new sequence by

248 injecting a front of the sample (60 mbar for 10 min).



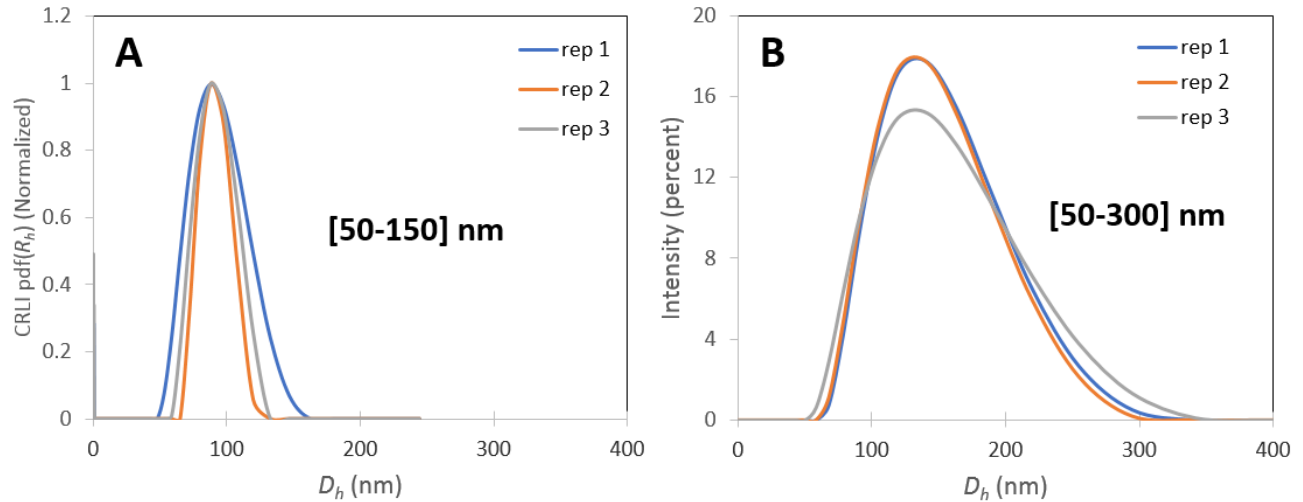
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251 **Figure 3.** Repeatability on 10 successive runs (A) and an example of two-Gaussian fit (B) of the
252 Taylorgrams obtained for LNP size characterization. Experimental conditions: μ SIL-FC coated
253 capillaries of 50 cm total length (41.5 cm to the UV detector) \times 50 μ m i.d. Buffer: 10 mM PBS,
254 pH 7.4, $\eta = 0.9 \times 10^{-3}$ Pa.s. Capillary presaturation: LNP for 10 min at 60 mbar. Capillary
255 preconditioning: H₂O for 2 min at 960 mbar followed by 2 min PBS at 960 mbar. Injection of
256 LNP: 20 mbar, 6 s. Mobilization pressure: 60 mbar. Sample: lipid nanoparticles (DLin-MC3-
257 DMA:DSPC:Chol:DMG-PEG-2000 at 50:10:38.5:1.5 molar ratio) encapsulating Firefly
258 Luciferase (FLuc) mRNA. UV detection: 200 nm. Temperature: 25°C.

259
260 Figure 3B displays an example of two-Gaussian fit obtained for one repetition of the LNP
261 sample. The two-Gaussian fit was performed only on the left half of the elution peak to avoid any
262 impact of peak tailing on the size measurement. Two-Gaussian fit was required due to the
263 presence of UV absorbing small molecules in the LNP sample, appearing as the small peak on the
264 top of the signal. The calculation of the LNP hydrodynamic diameter requires to know the
265 viscosity of the analysis buffer, which can be easily derived by measuring the elution time of UV
266 absorbing marker in a prefilled capillary (see section 3 in Supporting Information for more
267 details). Using the determined viscosity of PBS ($\eta = 0.90 \cdot 10^{-3}$ Pa.s⁻¹), the hydrodynamic diameter
268 obtained by Gaussian fit for the LNPs analyzed by TDA was 86 ± 1 nm (average over the 10 runs
269 presented in Figure 3).

270 A more advanced data processing of the Taylorgram based on Regularized Linear Inversion
271 (CRLI) algorithm⁴⁹ was performed to get a continuous distribution of the diffusion coefficient
272 (see Figure S3 for example of experimental fit). The size distribution could be determined by
273 CRLI as exemplified in Figure 4A.



274

275

276 **Figure 4.** Size distributions (three repetitions) obtained by TDA (A) and by DLS (B). Same
 277 experimental conditions for TDA as in Figure 3. Experimental conditions for DLS: 10 μ L LNP
 278 solution into 1 mL of 10 mM PBS, pH 7.4, $\eta = 0.9 \times 10^{-3}$ Pa.s. Measurement angle: 173°C.
 279 Temperature: 25°C.

280

281 The size distributions obtained by TDA (CRLI) were compared with those obtained by DLS

282 using Cumulant fit (Figure 4B). The size distributions obtained by DLS [50-300 nm] were wider

283 than those obtained in TDA [50-150 nm]. Accordingly, the mean hydrodynamic diameter

284 obtained by DLS was higher than the one obtained by TDA (128 ± 1 nm vs 86 ± 1) and the

285 polydispersity index was higher in DLS than in TDA (0.112 vs 0.011). These differences can be

286 explained by the fact that the two techniques are not sensitive to the same size distributions.

287 Intensity average distributions obtained by DLS give more weight to the larger entities (see

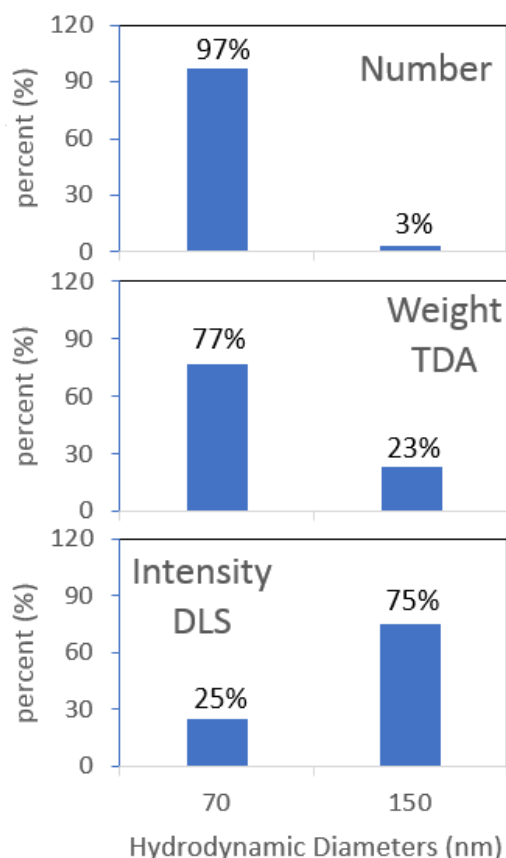
288 Figure 5), since the distribution is weighted by the scattered intensity which basically scales as

289 the hydrodynamic radius to the power 6. In contrast, the size distribution derived from TDA is

290 basically related to the weight-average distribution, which means that each LNP should

291 contribute to the distribution in proportion to their mass content in the mixture. To stress the

292 differences between the two methods, an example of the differently weighted distributions is
293 provided in Figure 5 taking as an example a bimodal mixture containing 5% in number of large
294 particles (200 nm) and 95% of smaller one (80 nm), which could mimic an example of LNP
295 mixture containing a minor content of larger particles. Clearly, the intensity-based distribution
296 (DLS) is weighting more the larger particles than the weight counterpart (TDA). These
297 differences demonstrate the complementarity of the two techniques which do not determine the
298 same size distributions. Of course, for truly monodisperse samples, both techniques should
299 converge; otherwise the discrepancy between the average values and the distributions are
300 indicative of the sample size heterogeneity. However, it should be noted that it is generally not
301 straightforward to convert the intensity based DLS distribution into a mass-weighted (or a
302 number-weighted) size distribution because the scattered intensity is depending on the size and
303 the shape of the solutes⁵⁹. For such transformations, strong assumptions are required such as the
304 spherical shape and the homogeneity of the solutes, and may be restricted to a limited range of
305 sizes. Moreover, due to the intensity based response of the light scattered intensity, the signal due
306 to the smallest solutes can be so weak that it is not detected, especially in the case of polydisperse
307 samples. This is a reason why orthogonal sizing methods are requested by the regulation
308 authorities in the case of polydisperse samples.



309

310 **Figure 5.** Distributions in number, in weight (as determined by TDA for mass sensitive
 311 detection) and in intensity (as determined by DLS) considering a bimodal mixture containing 3%
 312 in number of large particles (150 nm) and 97% of smaller ones (70 nm). The mass proportion,
 313 obtained in TDA for mass sensitive detection, is proportional to the number of particles
 314 multiplied by the hydrodynamic size to the power 3 (volume). We assume as a first
 315 approximation that the density of the particles is the same for both particle sizes. The scattered
 316 intensity obtained in DLS is proportional to the number of particles multiplied by the
 317 hydrodynamic size to the power 6.

318 Finally, the charge of the LNPs was studied by capillary electrophoresis (CE) using a
 319 μ SIL coated capillary as for TDA analysis. Figure 6A displays a Taylorgram (no electric field,
 320 see blue trace) where the marker (DMF at 0.1 g/L in water) is detected at the same elution time as
 321 the LNPs, but with a narrower profile compared to the broad LNP elution profile. By applying an
 322 electric field and reducing the mobilization pressure from 30 mbar (orange trace) to 7 mbar
 323 (green trace), it was possible to separate the two peaks and to calculate the effective
 324 electrophoretic mobility of the LNPs ($\mu_{ep} = -1.86 \times 10^{-9} \text{ m}^2\text{V}^{-1}\text{s}^{-1}$). The applied co-pressure (7

325 mbar) allows to speed up the analysis with a limited impact on the peak broadening of the LNPs
 326 peak, and without changing the LNP effective mobility. Knowing the hydrodynamic diameter
 327 and the electrophoretic mobility of LNPs, it was possible to calculate the electric charge density (σ_{OWO})
 328 using the O'Brien-White-Ohshima (OWO) mathematical model^{39,60,61}, according to
 329 equation (7):

$$330 \quad \sigma_{owo} = \frac{2\varepsilon_0\varepsilon_r\kappa k_B T}{e} \sinh\left(\frac{e\zeta}{2k_B T}\right) \times \left[1 + \frac{1}{\kappa R_h} \frac{2}{\cosh^2\left(\frac{e\zeta}{4k_B T}\right)} + \frac{1}{(\kappa R_h)^2} \frac{8 \ln(\cosh\left(\frac{e\zeta}{4k_B T}\right))}{\sinh^2\left(\frac{e\zeta}{2k_B T}\right)} \right]^{\frac{1}{2}} \quad (7)$$

331 where ε_r is the relative electric permittivity, ε_0 is the electric permittivity of vacuum, e is the
 332 elementary electric charge, ζ is the Zeta potential and κ is the Debye–Hückel parameter ($\kappa = 1.28$
 333 nm^{-1} , 165 mM ionic strength in the present work). The surface charge density is the total surface
 334 charge of the particle divided by the total surface of the particle at the plane of shear. The
 335 determination of σ_{owo} requires the determination of the Zeta potential ζ knowing μ_{ep} and D_h .
 336 This can be done using graphical representations of equation (8):

$$337 \quad \mu_{ep} = \frac{2\varepsilon_0\varepsilon_r\zeta}{3\eta} \left[f_1(\kappa R_h) - \left(\frac{e\zeta}{k_B T}\right)^2 f_3(\kappa R_h) - \frac{m_- + m_+}{2} \left(\frac{e\zeta}{k_B T}\right)^2 f_4(\kappa R_h) \right] \quad (8)$$

338 m_+ and m_- are dimensionless ionic drag coefficients, accessible from the limiting conductances
 339 of the cation Λ_+^0 and the anion Λ_-^0 in the electrolyte considered:

$$340 \quad m_{\pm} = \frac{2\varepsilon_0\varepsilon_r k_B T N_A}{3\eta z \Lambda_{\pm}^0} \quad (9)$$

341 where N_A is the Avogadro's number and $m_+ = 0.263$, $m_- = 0.172$ for NaCl electrolyte. Equation
 342 (8) takes the relaxation effect into account and is valid for $\zeta \leq 100$ mV. f_1 , f_3 and f_4 are functions
 343 given by equations (10), (11) and (12):

$$344 \quad f_1(\kappa R_h) = 1 + \frac{1}{2 \left[1 + 2.5 / \left\{ \kappa R_h (1 + 2e^{-\kappa R_h}) \right\} \right]^3} \quad (10)$$

$$345 \quad f_3(\kappa R_h) = \frac{\kappa R_h (\kappa R_h + 1.3e^{-0.18\kappa R_h} + 2.5)}{2(\kappa R_h + 1.2e^{-7.4\kappa R_h} + 4.8)^3} \quad (11)$$

$$346 \quad f_4(\kappa R_h) = \frac{9\kappa R_h (\kappa R_h + 5.2e^{-3.9\kappa R_h} + 5.6)}{8(\kappa R_h + 1.55e^{-0.32\kappa R_h} + 6.02)^3} \quad (12)$$

347 Thus, for the formulation of LNPs studied in this work, the Zeta potential determined by
 348 graphical representation was $\zeta_{LNP} = -2.5$ mV (see Figure S4 with $\kappa R_h = 55$), which allowed to
 349 calculate the lipid nanoparticles electric charge density, $\sigma_{LNP} = -2.4 \times 10^{-3} \text{ Cm}^{-2} = -0.015$
 350 elementary charge per nm^2 . This negative charge at physiological pH is relatively low but is
 351 desired to reduce unwanted interactions during circulation in the body. Such surface charge
 352 density corresponds to an overall effective charge of about 350 elementary charges per LNP
 353 entity. Because the charge density is low, relaxation phenomena is expected to be negligible, and
 354 it is therefore possible to apply the approximated Henry equation, which is a more
 355 straightforward way to determine the Zeta potential from the effective electrophoretic mobility
 356 using eq. (13)⁶²:

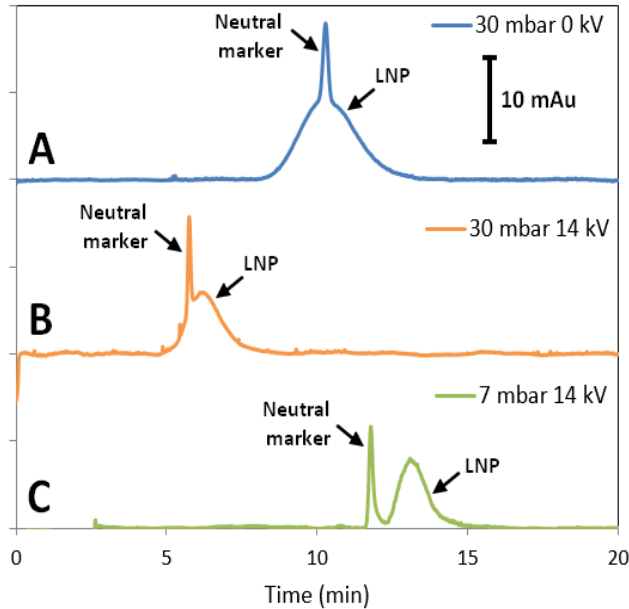
$$357 \quad \mu_{ep} = \frac{2\varepsilon\zeta}{3\eta} \times \left(1 + \frac{1}{2 \left(1 + \frac{\delta}{\kappa R_h} \right)^3} \right) \quad (13)$$

358 where $\delta = \frac{2.5}{1 + 2 \exp(-\kappa R_h)}$. Numerical application to LNP leads to $\delta=2.5$, $\mu_{ep} = \frac{0.958\epsilon\zeta}{\eta}$, ζ_{LNP}
359 = -2.47 mV and finally to the same values of $\sigma_{LNP} = -2.4 \times 10^{-3} \text{ Cm}^{-2} = -0.015$ elementary charge
360 per nm^2 as those previously obtained with OWO modeling.

361 It is worth noting that the surface charge density is a much more robust parameter to characterize
362 solute charge compared to Zeta potential, as previously demonstrated for nanoparticles and
363 nanolatexes.^{39,40} Indeed, Zeta potential is strongly dependent on the ionic strength due to the
364 compaction of the electrical double layer with increasing ionic strength, which is not the case for
365 surface charge density^{39,40}. The electrophoretic mobility value obtained by CE can also be
366 compared to the one determined by Laser Doppler electrophoresis (LDE) $\mu_{ep} = -5.00 \times 10^{-9} \text{ m}^2\text{V}^{-1}\text{s}^{-1}$,
367 which is of the same order of magnitude as the one determined by CE ($\mu_{ep} = -1.86 \times 10^{-9} \text{ m}^2\text{V}^{-1}\text{s}^{-1}$)
368 and allows to determine the Zeta by graphical representation using OWO model $\zeta_{LNP} = -6.5$
369 mV, see Figure S4. The difference between the two values can nevertheless be explained by the
370 fact that the electrophoretic mobility determined by LDE strongly depends on the other ions
371 present in the matrix since LDE is a non-separative method.

372 It is worth noting that it is also important to know which electrophoretic modelling is used to
373 convert the effective mobility into Zeta potential and at which ionic strength the measurement
374 was performed. Strong differences can arise depending on the retained modelling (Nernst-
375 Einstein, Hückel, Smolukowski, Henry, OWO, modified Yoon and Kim modelling) depending on
376 the solute's characteristics. OWO and modified Yoon and Kim modelling are the most accurate
377 one since they take into account both the electrophoretic effect (counter ion friction) and the
378 relaxation effect (distortion of the counterion cloud)⁶¹. We also recommend, for the reasons

379 previously explained, to express all the results in terms of surface charge density using equation
380 (7) instead of Zeta potential.



381
382 **Figure 6.** Taylorgram (A) and electropherograms (B, C) obtained for a mixture of LNP and
383 DMF. Experimental conditions: μ SIL-FC capillaries of 50 cm total length (41.5 cm to the UV
384 detector) \times 50 μ m i.d. Buffer: 10 mM PBS, pH 7.4, $\eta = 0.9 \times 10^{-3}$ Pa.s.. Capillary presaturation:
385 LNP for 10 min at 60 mbar. Capillary preconditioning: H₂O for 2 min at 960 mbar followed by 2
386 min PBS at 960 mbar. Injection of LNP: 20 mbar, 6 s. Mobilization pressure and separation
387 voltage: as indicated on the graph. Sample: lipid nanoparticles (DLin-MC3-
388 DMA:DSPC:Chol:DMG-PEG-2000 at 50:10:38.5:1.5 molar ratio) encapsulating Firefly
389 Luciferase (FLuc) mRNA. UV detection: 200 nm. Temperature: 25°C.

390
391 **CONCLUSIONS**

392 This work aimed at developing a new methodology to characterize the average size and the size
393 distribution, as well as the charge density, of lipid nanoparticles (LNPs) used as delivery vehicle
394 for mRNA vaccines. The TDA method developed in this work was found to reach this goal with
395 direct injection of the samples (no dilution, no filtration). It appeared that the presaturation of the
396 capillary with the LNP sample and the capillary rinsing were crucial steps that significantly
397 decreased the adsorption of LNPs onto the capillary wall and considerably improved the

398 repeatability. The comparison between TDA and DLS shows that the hydrodynamic diameters
399 obtained by TDA were smaller (mass-weighted) than those obtained by DLS (intensity-
400 weighted), as expected. Both methods are therefore complementary and allow a better
401 characterization of the LNP size. TDA shows promise to be widely used as it is an absolute
402 method (no calibration) that consumes little sample volumes, it is easy and straightforward to use,
403 and it can be easily automated. TDA performed on a CE equipment can also easily provide the
404 viscosity of LNPs formulations, which can be useful when low amounts of product are available.
405 Finally, CE, implemented on the same equipment as TDA, can be used for the charge
406 characterization of LNPs using O'Brien-White-Ohshima or Henry electrophoretic mobility
407 modelling. Thus, a single equipment can give access to important information on critical LNPs
408 physicochemical parameters.

409

410 **ASSOCIATED CONTENT**

411 **Supporting Information.** Chemical formula of the 4 lipids used in the formulation of the lipid
412 nanoparticles. PEO, Ultratrol LN and DDAB-DLPC capillary coatings procedures. Calculation of
413 the viscosity of PBS and LNP solutions. CRLI Fit.

414

415 **DISCLOSURES**

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418

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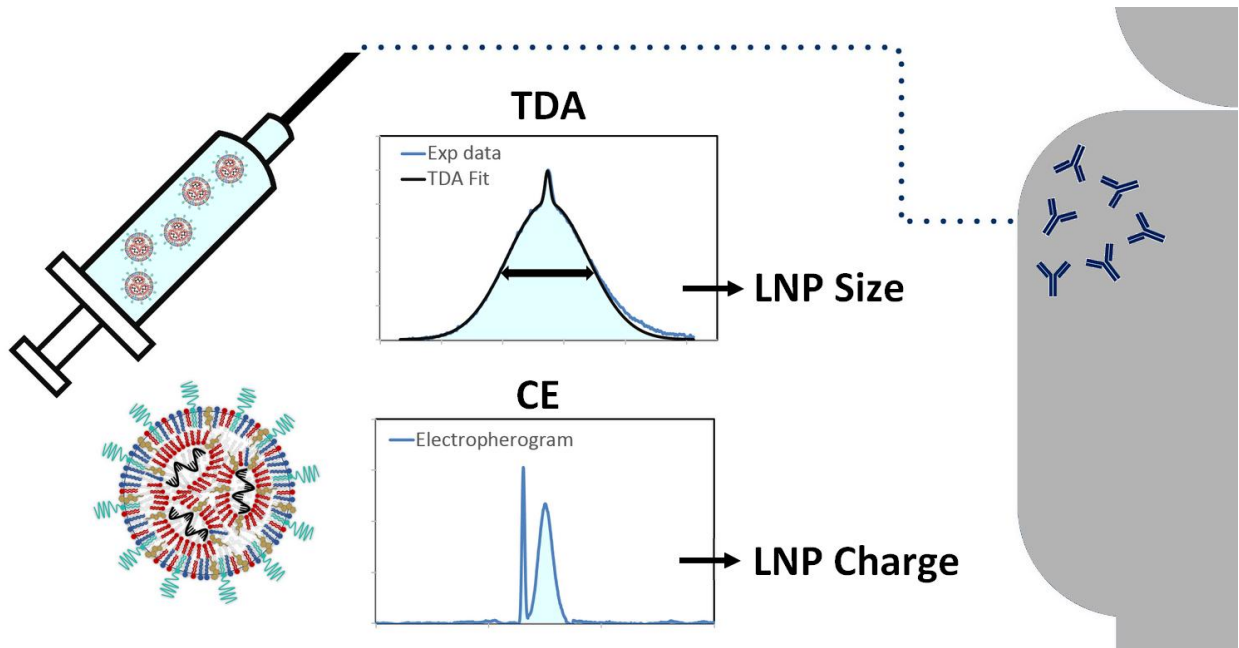
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