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1 **Cationic lipid nanoparticle production by microfluidization for siRNA**  
2 **delivery**

3 Xiaojing Liu<sup>a</sup>, Badr Bahloul<sup>b</sup>, René Lai Kuen<sup>c</sup>, Karine Andrieux<sup>a</sup>, Caroline Roques<sup>a</sup>,  
4 Daniel Scherman<sup>a, d</sup>

5 <sup>a</sup> Université de Paris, UTCBS, CNRS, INSERM, F-75006 Paris, France

6 <sup>b</sup> Laboratory of Pharmaceutical, Chemical and Pharmacological Drug Development  
7 LR12ES09, Faculty of Pharmacy, University of Monastir, Tunisia

8 <sup>c</sup> Université de Paris, CNRS, F-75006 Paris, France

9 <sup>d</sup> Corresponding author

10 Email address of corresponding author: [daniel.scherman@u-paris.fr](mailto:daniel.scherman@u-paris.fr)

11

12 **Abstract**

13 Microfluidization has been investigated as a new, scalable, and basic component saving  
14 method to produce cationic lipid nanoparticles, in particular for the delivery of short  
15 interfering RNAs (siRNAs). The design of experiment (DoE) allowed to reach optimized  
16 characteristics in terms of nanocarrier size reduction and low polydispersity. The  
17 structure of cationic liposomes and siRNA-lipoplexes was characterized. The optimized  
18 preparation parameters were identified as three microfluidization passages at a pressure  
19 of 10000 psi, with a thin film hydration volume of 4mL. Microfluidized liposomes mean  
20 size was 160nm, with a polydispersity index of 0.2 to 0.3 and a zeta potential of + 40mV  
21 to + 60mV. Positive versus negative charge ratio between the charges of the cationic lipid  
22 and the phosphate charges of the siRNAs is a key factor determining the structure and  
23 silencing efficacy of siRNA lipoplexes. At a (+/-) charge ratio of 8, a proportion of 88%

24 of the siRNA was associated to microfluidized lipoplexes, which remained stable for one  
25 month. These lipoplexes exhibited moderate cytotoxicity and gene silencing efficacy,  
26 which should be further optimized.

27

## 28 **Keywords**

29 Cationic liposome; lipoplex; siRNA; interfering RNA; gene silencing; microfluidization;  
30 thin film hydration; design of experiment.

31

32 **Running Title:** Microfluidized siRNA lipoplexes

33

## 34 **1. Introduction**

35 RNA interference (RNAi) is a promising strategy for the treatment of a large variety of  
36 disorders, including neurodegenerative diseases <sup>[1]</sup>. Short interfering RNAs (siRNAs) are  
37 19-21 base pairs double-stranded RNAs that lead to the enzymatic degradation of a  
38 targeted mRNA through the RISC complex <sup>[2]</sup>. Cationic liposomes have been extensively  
39 used as siRNA delivery vectors, since they efficiently interact with negatively charged  
40 siRNAs and with the cell extracellular membrane, forming so-called lipoplexes <sup>[3]</sup>. Such  
41 lipoplexes are presently in clinical practice for the treatment of transthyretin amyloidosis  
42 (Patisiran) <sup>[4]</sup>.

43 Microfluidization has been widely used to produce liposomal formulation <sup>[5], [6]</sup>, that  
44 encapsulate nucleic acids <sup>[7]</sup>, proteins <sup>[8]</sup> and drugs <sup>[9]</sup> exhibiting diverse physicochemical  
45 characteristics. The optimization of the manufacturing process of liposomal formulations  
46 can be obtained using a quality by design approach<sup>[10]</sup>. Microfluidization for the

47 preparation of cationic liposomes of appropriate size, distribution, and zeta potential  
48 might provide a scalable and economical platform for the delivery of siRNAs, which  
49 could meet the requirement for good manufacturing practice (GMP). The high pressure  
50 and shear force of the microfluidization process effectively decrease the size and  
51 polydispersity index (PDI) of nanoparticles. Besides, the limited number of parameters  
52 involved in microfluidization optimization, i.e. pressure and number of passages, saves  
53 development costs <sup>[11]</sup>, as compared to other techniques such as sonication (which  
54 depends on time, amplitude, pulse-on/pulse-off time, ice replenish frequency), and high-  
55 pressure homogenization (which depends on pressure, number of cycles, and flow) <sup>[12]</sup>.  
56 <sup>[13]</sup>.

57 At optimized size and zeta potential, cationic liposomes have been shown to efficiently  
58 encapsulate siRNA and functionally in a large number of cells in vitro, and also in vivo in  
59 liver by intravenous administration <sup>[14], [15]</sup>. In the present study, we have used the thin  
60 film hydration method to form cationic lipids multilamellar vesicles, which were  
61 subsequently microfluidized in order to obtain reduced small unilamellar vesicles. We  
62 used a three lipid composition containing the cationic lipid 2-{3-[Bis-(3-amino-propyl)-  
63 amino]-propylamino}-N-ditetradecyl carbamoyl methyl-acetamide (DMAPAP), a neutral  
64 colipid, and a PEG-lipid. The DMAPAP lipid contains three positive amine charges in its  
65 head group and has proved its efficiency for lipoplexes formulation and efficient siRNA  
66 delivery in a series of precedent studies <sup>[16], [17]</sup>. The most important physicochemical  
67 lipoplexes characteristics (mean size, polydispersity and zeta potential value) were  
68 optimized by varying experimental conditions and using a design of experiment (DoE)

69 approach. The siRNA-lipoplexes encapsulation efficiency was evaluated, and the  
70 cytotoxicity and silencing efficacy were tested in vitro.

71

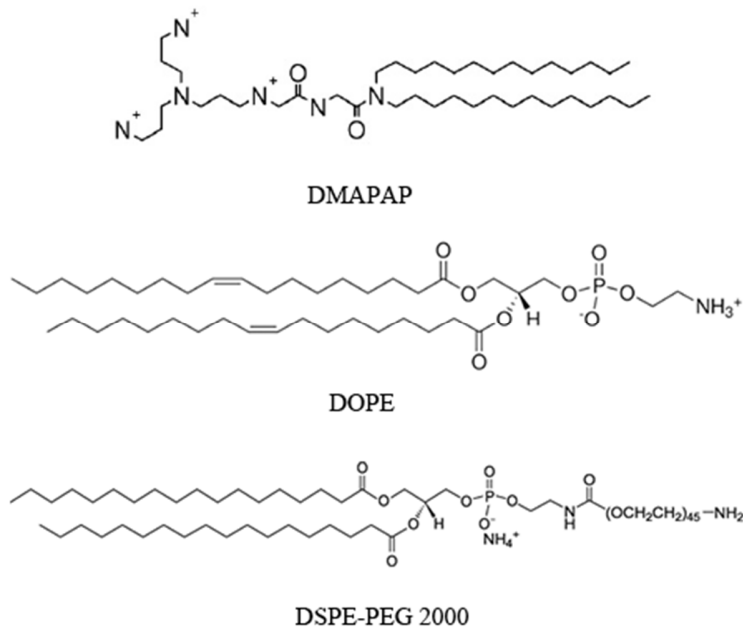
## 72 **2. Materials and Methods**

### 73 **2.1 siRNA.**

74 Luciferase siRNA (CUUACGCUGAGUACUUCGA sense) and scramble siRNA  
75 (UUCUCCGAACGUGUCACGU sense) were purchased from Eurogentec.

### 76 **2.2 Liposomes preparation by thin film hydration method.**

77 The cationic lipid 2-{3-[Bis-(3-amino-propyl)-amino]-propylamino}-N-ditetradecyl  
78 carbamoyl methyl-acetamide or DMAPAP (Figure 1) was synthesized as previously  
79 described [16,17]. The helper lipid DOPE (1,2-dioleoyl-sn-glycero-3-  
80 phosphoethanolamine) and the DSPE-PEG 2000 lipid (1,2-diacyl-sn-glycero-3-  
81 phosphoethanolamine-N-[methoxy (polyethylene glycol)-2000] were purchased from  
82 Avanti Polar Lipids (Figure 1). The DMAPAP, DOPE and DSPE-PEG 2000 lipids were  
83 dissolved in chloroform at molar ratio 50/45/5 by reference to previous works [18-20],  
84 evaporated under vacuum at 40°C for 1 hour to form a thin film, and left to dry for 2  
85 hours. The film was then hydrated with different volumes of Milli-Q water overnight at  
86 room temperature.



87

88 **Figure 1.** Molecular structures of DMAPAP, DOPE, DSPE-PEG2000 lipids <sup>[20], [28], [36]</sup>.

89

90 **2.3 Liposomes' size reduction**91 **2.3.1 Extrusion method**

92 The hydrated liposomal suspension (hydration volume 4mL) was passed through  
 93 polycarbonate filters of 0.4  $\mu\text{m}$  pore size for 10 passages, and of 0.2  $\mu\text{m}$  for 10 additional  
 94 passages in a Mini-Extruder (Avanti Polar Lipids) at room temperature.

95 **2.3.2 Microfluidization method**

96 After hydration, the liposomal suspension was injected into a microfluidizer (LV1,  
 97 Microfluidic Corp., Newton, MA, USA) by 5ml/10ml syringe in a Y-type chamber <sup>[21]</sup>  
 98 and processed with various microfluidization pump pressure, and number of passages  
 99 conditions. Cooling coil temperature was maintained at 0°C in ice during the process.

100 **2.4 Selection of factors and responses in the experimental design.** X1 pressure

101 (ranging from 5000 psi to 20000 psi) and X2 number of passages (ranging from 1 to 5)

102 were selected according to the microfluidizer operation guide <sup>[21]</sup>. The factors ranges were  
 103 set up based on preliminary results, on the targeted value of response, and on reference to  
 104 the literature <sup>[22]</sup>. The thin film hydration volume was included as variable X3 (ranging  
 105 from 4ml to 16ml), since it was observed in preliminary results to be a key factor  
 106 determining liposomes size and PDI, in agreement with the literature <sup>[23,24]</sup>. Other factors  
 107 for thin film hydration such as lipid composition and concentration, vacuum pressure,  
 108 evaporation time, heating bath temperature, hydration time, and rotation speed were fixed  
 109 at a constant value, based on our previous results <sup>[18-20]</sup>. Three responses: Y1 (size in the  
 110 present text), Y2 (polydispersity index PDI) and Y3 (zeta potential), were selected to  
 111 define the liposomes and lipoplexes characteristics. Target values of responses were  
 112 optimized for effective delivery and stability of nanocarrier <sup>[25-27]</sup> (Table 1).

113 **Table 1.** Factors and responses used in the design of experiment.

	Level (coded)	
	Low (-1)	High (+1)
<b>Factors</b>		
X1: Pressure (psi)	5000	20000
X2: Number of passages	1	5
X3: Hydration volume (mL)	4	16
<b>Responses</b>		
Y1: Size (nm)	Target value	
Y2 PDI	150	
Y3: Zeta potential (mV)	0.2-0.3	
	+40-+60	

114

## 115 **2.5 Modelization.**

116 Customized design of experiment with the three factors X1 (pressure), X2 (number of  
 117 passages), and X3 (hydration volume) was performed. The analysis and optimization

118 procedure were conducted using the statistical software STATGRAPHICS Centurion  
 119 (Version 15.1.02, USA). The polynomial equation used was:  $Y = b_0 + b_1X_1 + b_2X_2 +$   
 120  $b_3X_3 + b_4X_1X_2 + b_5X_2X_3 + b_6X_1X_3 + b_7X_1^2 + b_8X_2^2 + b_9X_3^2$ , linking the  
 121 dependent variables, Y1 and Y2, to the independent variables, X1, X2 and X3, and  
 122 wherein b0 was the intercept and b1 to b9, were the regression coefficients. We used a 2-  
 123 levels, 3-factors design as a model to improve the quality of predicted results using the  
 124 preliminary preformulation data. Our approach involved the use of prior results to build a  
 125 common design customized for the optimization procedure (Table 2). The optimization  
 126 procedure was performed using a desirability function with the aim to obtain an optimum  
 127 liposome formulation characterized by a 150 nm target size and a + 60 mV zeta potential.  
 128 Validation tests were carried out by triplicates of each experiment conditions of  
 129 optimized batch and optimum value obtained by math model.

130 **Table 2.** Experimental matrix of customized design of experiment.

<b>Run</b>	<b>X1</b>	<b>X2</b>	<b>X3</b>
<b>1</b>	20000	1	16
<b>2</b>	20000	3	16
<b>3</b>	20000	5	16
<b>4</b>	15000	1	16
<b>5</b>	15000	3	16
<b>6</b>	15000	5	16
<b>7</b>	5000	1	16
<b>8</b>	5000	3	16
<b>9</b>	5000	5	16
<b>10</b>	20000	1	4
<b>11</b>	20000	3	4
<b>12</b>	20000	5	4
<b>13</b>	15000	1	4
<b>14</b>	15000	3	4
<b>15</b>	15000	5	4
<b>16</b>	10000	1	4
<b>17</b>	10000	3	4
<b>18</b>	10000	5	4
<b>19</b>	20000	1	8
<b>20</b>	20000	3	8
<b>21</b>	20000	5	8



<b>22</b>	15000	1	8
<b>23</b>	15000	3	8
<b>24</b>	15000	5	8
<b>25</b>	5000	1	8
<b>26</b>	5000	3	8
<b>27</b>	5000	5	8

131

132 **2.6 Lipoplexes preparation and characterization.** Lipoplexes were formulated by  
 133 mixing in 150 mM NaCl an equal volume of cationic liposomes and of siRNA solution  
 134 (anti-luciferase or scramble siRNA as a negative control), vortexing for 1 minute and  
 135 allowing to form lipoplexes for at least 30 min at room temperature. Different charge  
 136 ratios were studied and calculated using the molar ratio of positive charges (3 positive  
 137 charges per molecule of DMAPAP) to negative charges of siRNA (3.03 nmole of  
 138 negative phosphate charges/ $\mu$ g of siRNA) [16, 18, 28].

139 Size and zeta potential were determined by dynamic light scattering (DLS) using a  
 140 Zetasizer Nano ZS (Malvern Instruments). Blank liposomes (final lipid concentration 0.1  
 141 mM) and lipoplexes (final siRNA concentration 37.5nM) were suspended in Milli-Q  
 142 water for short term storage. The DLS condition was set up as: water dispersant  
 143 (viscosity, refractive index), detector position 173°, at temperature 25°C.

144 For transmission electron microscopy (TEM), a drop (20  $\mu$ l) of liposomes or lipoplexes  
 145 dispersion (siRNA concentration 375.9 nM) was first applied onto a Formvar/carbon 200  
 146 mesh copper grid for 2 min, blotted with filter paper. Then a drop (20  $\mu$ l) of 1% uranyl  
 147 acetate (UAC) was applied on the copper grid for 2 min, blotted with filter paper and  
 148 dried for 10 min. Analyses were performed on a microscope JEOL, JEM 100S as  
 149 described [18],[19].

150 **2.7 Liposomes/lipoplexes storage and stability.** Cationic liposomes and lipoplexes  
 151 suspensions were stored at 4 °C in 1.5 mL Eppendorf tubes. The size, PDI and zeta

152 potential were measured by DLS within 3 days and weekly within one month after  
153 formulation.

154 **2.8 Gel retardation assay.** Lipoplexes were prepared as described above (0.3 µg  
155 siRNA/sample/10 µl) at different charge ratio. Samples were mixed with 2 µl dye (6X)  
156 and electrophoresed on 1.5% agarose gel in Tris-Acetate-EDTA (TAE 1X) buffer at 80 V  
157 for 30 min <sup>[18]</sup>. Then, the gels were placed into ethidium bromide (BET) bath for 15  
158 minutes and free siRNA band was visualized on UV transilluminator. The digital images  
159 were acquired using Bio-Capt Version 12.6 for Windows and were analyzed by image J  
160 software.

161 **2.9 Cell culture.** Mouse melanoma cells (B16-F10, ATCC CRL6475) were grown on  
162 DMEM with glutamax (Gibco), 10% fetal calf serum (Sigma-Aldrich), streptomycin (100  
163 µg/ml from ThermoFisher scientific), and selection antibiotic geneticin (2 mg/ml, Gibco).  
164 Cells were trypsinized and passaged twice weekly for transfection.

165 **2.10 Cytotoxicity determination.** B16-luc cells were plated on 96-well plates at a  
166 density of 4,000 cells per well in 100 µl of culture medium. After 24 hours plating, 100 µl  
167 of lipoplexes were prepared as described above, mixed with culture medium, and added  
168 to the cells in triplicate experiments. After a 48h exposure period, cell viability was  
169 assayed using the MTT test as previously described <sup>[19]</sup>. Control wells with DMEM and  
170 150 mM NaCl with MTT were run to subtract background absorbance. Results were  
171 expressed relative to non-transfected cells.

172 **2.11 Gene silencing efficiency.** Cell transfection was performed as above. Transfection  
173 medium was replaced by fresh medium at 24 hours after transfection and incubated for an  
174 additional 24 hours. The transfected cells were washed twice with PBS and lysed with

175 200  $\mu$ l cell culture lysis reagent (Promega). The luciferase assay was carried out  
176 according to the manufacturer's protocol. Light emission from luciferase activity was  
177 measured in white 96-microwell plates using a Wallac Victor2 microplate reader  
178 equipped for luminescence. Total protein concentration was determined using the Pierce  
179 BCA assay (Thermo Scientific). The results were calculated in cps (count per second),  
180 normalized to the total protein concentration of each sample, and expressed relatively to  
181 non-transfected control cells <sup>[19]</sup>.

182 **3. Statistical analysis and computation.** All results were expressed by mean value  $\pm$   
183 standard deviation (SD). The adequacy of the polynomial model for the responses was  
184 verified by ANOVA, multiple correlation ( $R^2$ ) and Durbin-Watson (DW) statistical tests.  
185 The desirability function D was calculated by the STATGRAPHICS CENTURION  
186 (Version 15.1.02, USA) software. Two-ways ANOVA multiple comparisons and  
187 Bonferroni test were used to test the significance between groups by Prism 9 (version  
188 9.0.0, GraphPad).

## 189 **4. Results and discussions**

### 190 **4.1 Customized design of experiments (DoE)**

191 Twenty-seven runs were performed for design of experiment analysis. The observed  
192 results are shown in Table 3. Liposome's size varied from 96.75 nm to 310.5 nm, PDI  
193 from 0.17 to 0.45, and zeta potential from 54.2mV to 86.4 mV. The polynomial equations  
194 relating the responses Y1, Y2, Y3 and independent variables are listed in Table 4. The  
195 adequacy of this model was verified by ANOVA, multiple correlation ( $R^2$ ) and Durbin-  
196 Watson (DW) statistical tests. The P values of separated effects for responses Y1, Y2 and  
197 Y3 were found to be less than 0.05. The Durbin-Watson (DW) statistical tests for size and

198 PDI were used in order to determine significant correlations between the Table 3 data.

199 When P values were less than 0.05, there was an indication of serial correlation at the

200 0.05 significance level. R<sup>2</sup> values for responses were > 80%. Thus, it can be concluded

201 that the responses fitted well the quadratic model.

202 **Table 3.** Experimental domain and observed values of the customized DoE analysis.

Run	X1 Pressure (psi)	X2 Passages	X3 Hydration volume (mL)	Y1 Size (nm)	Y2 PDI	Y3 Zeta potential (mV)
1	20000	1	16	297.5	0.42	80.7
2	20000	3	16	214.4	0.23	83
3	20000	5	16	191.6	0.25	76.1
4	15000	1	16	271.2	0.35	70.5
5	15000	3	16	310.5	0.34	73
6	15000	5	16	283.8	0.31	72
7	5000	1	16	307	0.31	69.8
8	5000	3	16	298.8	0.31	73
9	5000	5	16	286.23	0.35	80.4
10	20000	1	4	134	0.37	62.7
11	20000	3	4	176.4	0.3	68
12	20000	5	4	168.1	0.45	63.1
13	15000	1	4	96.75	0.39	54.2
14	15000	3	4	147	0.36	63.3
15	15000	5	4	242.9	0.32	63.7
16	10000	1	4	167.7	0.2	61.9
17	10000	3	4	164.2	0.2	59.6
18	10000	5	4	162.5	0.23	58.6
19	20000	1	8	175.5	0.22	69.7
20	20000	3	8	175.9	0.21	86.4
21	20000	5	8	159.8	0.19	82.6
22	15000	1	8	178.4	0.21	65.8
23	15000	3	8	169.1	0.17	75.1
24	15000	5	8	167.9	0.18	78.8
25	5000	1	8	178.2	0.23	67.7
26	5000	3	8	194.5	0.25	70.9
27	5000	5	8	197.8	0.24	77.3

203

204

205

206

**Table 4.** Polynomial equations for the responses.

Y1	$Y1 = 11.8986 + 0.0109046X1 + 28.383X2 + 9.19764X3 - 3.05845E-7X1^2 - 0.000538838X1X2 - 0.000346733X1X3 + 0.0705556X2^2 - 2.24116X2 X3 + 0.612531X3^2$
Y2	$Y2 = 0.386851 + 0.00000307474X1 - 0.00512917X2 - 0.0487927X3 + 3.9516E-10X1^2 - 0.00000146083X1X2 - 6.93261E-7X1X3 + 0.006X2^2 - 0.00166944X2X3 + 0.00319514X3^2$
Y3	$Y3 = 34.0186 - 0.00127128X1 + 6.57042X2 + 6.10165X3 + 7.8605E-8X1^2 - 0.00010275X1X2 + 1.58306E-7X1X3 - 0.590278X2^2 - 0.0433333 X2X3 - 0.245029X3^2$

207

## 208 4.2 Coefficient estimates and standardized main effects

209 Using our preliminary results, the coefficient estimates with their corresponding p values

210 in the form of polynomial equations were deduced in Table 5. The respective weight of

211 factors (X1, X2, X3) and their interactions on the responses (Y1, Y2, Y3) were calculated

212 by the polynomial equations of Table 4 and were shown in a Pareto chart (Figure 2). It can

213 be deduced from the Pareto chart that the main and quadratic effects of hydration volume,

214 the main effect of the number of passages, and the interaction between these two factors

215 (X2, X3, X2X3, X3<sup>2</sup>) had the most important weight of all factors on Y1, Y2 and Y3. In

216 Table 5, Y1 (size) was significantly affected by the synergistic effect of X2 (number of

217 passages) and the interaction X3X2 (number of passages and hydration volume) with p

218 values of 0.0499 and 0.0035, respectively.

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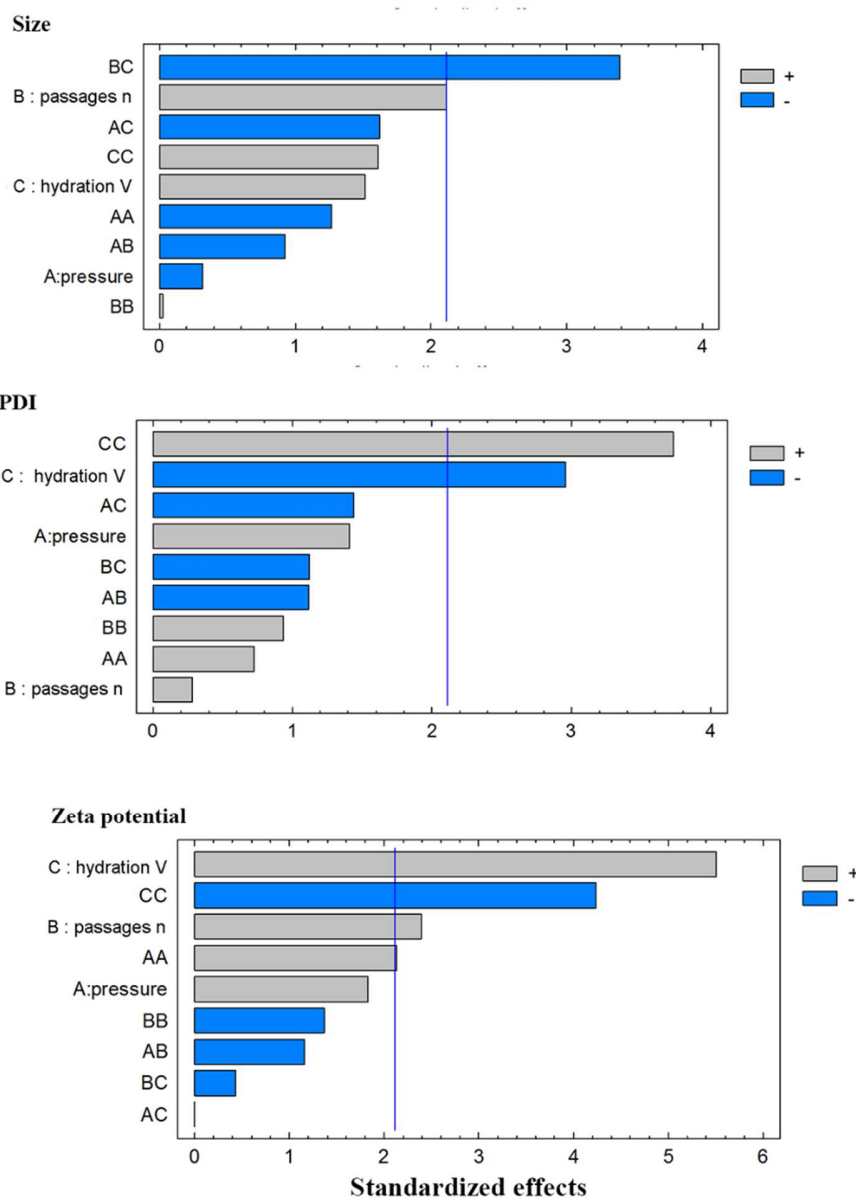
224 **Table 5.** Standardized main effects of the factors and their corresponding p values on the Y1, Y2  
 225 and Y3 responses.  
 226

Source	Factors	Size Y1		PDI Y2		Zeta Y3	
		Estimate	P-Value	Estimate	P-Value	Estimate	P-Value
A: Pressure	X1	0.0109046	0.7561	0.0307	0.1762	-0.00127128	0.0848
B: Passages	X2	28383	0.0499*	-0.00512917	0.7813	657042	0.0287*
C: Hydration volume	X3	919764	0.1483	-0.0487927	0.0088*	610165	0.000*
AA	X1 <sup>2</sup>	-3.06E-07	0.2235	3.95E-10	0.4781	7.86E-08	0.0475*
AB	X1X2	-0.00053884	0.3683	-0.0146	0.281	-0.00010275	0.2624
AC	X1X3	-0.00034673	0.1235	-6.93E-07	0.1681	1.58E-07	0.9962
BB	X2 <sup>2</sup>	0.0705556	0.9805	0.006	0.3618	-0.590278	0.1901
BC	X2X3	-224116	0.0035*	-0.00166944	0.2781	-0.0433333	0.6723
CC	X3 <sup>2</sup>	0.612531	0.126	0.00319514	0.0017*	-0.245029	0.0006*

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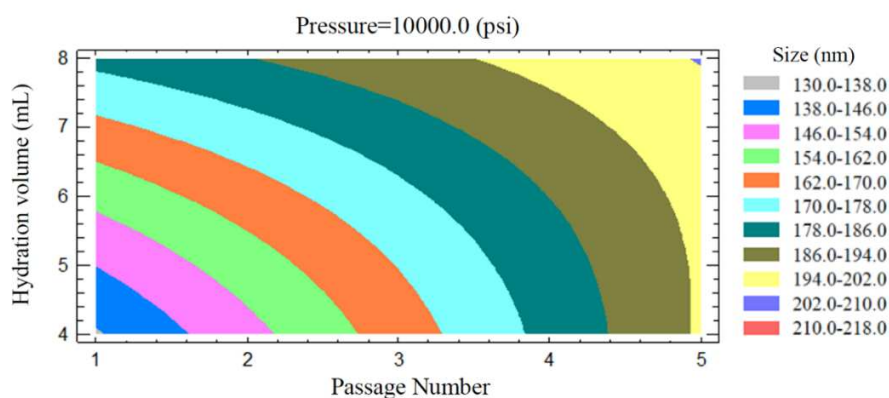
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231 **Figure 2.** Standard Pareto chart showing the effect of the independent variables X1, X2  
 232 and X3 and their combined effects on Y1 (size); Y2 (PDI); and Y3 (zeta potential). (Colored)  
 233

### 234 4.3 Influence of pressure and number of passages on liposomes size

235 The effect of X1 (pressure), X2 (number of passages) and their interaction on Y1 (size) at  
 236 a fixed level of X3 (hydration volume) is given in Figure 3. At low number of passages,  
 237 Y1 increased from 138 nm to 186 nm when the X3 increased from 2ml to 8 ml. However,  
 238 at high number of passages, Y1 did not increase significantly, showing that the effect of

239 the hydration volume is only detectable for a low number of passages, and suggesting that  
 240 hydration volume is the key factor influencing liposome size. The reason could be that the  
 241 number of bilayers of the multilamellar vesicles (MLVs) formed during the film hydration  
 242 spontaneous process can be tuned by varying the initial properties of the lipid droplet [29],  
 243 such as evaporation/hydration volume, flask size, lipid composition and concentration  
 244 [30,31]. These factors also determine the final size of liposomes during the subsequent  
 245 microfluidization.

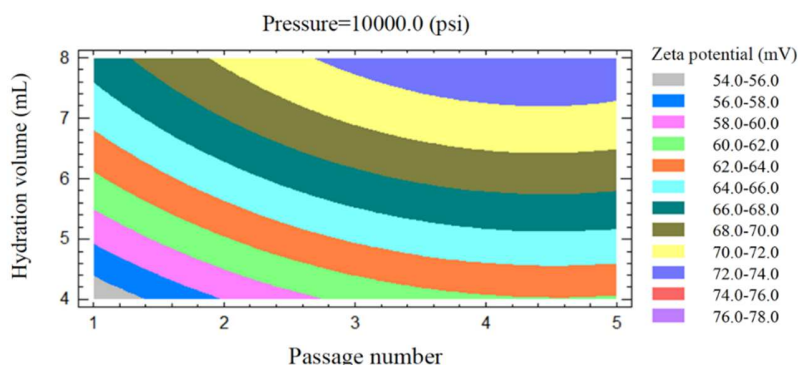


246 **Figure 3.** Response surface plots showing the effect of X2 (passage number) and X3  
 247 (hydration volume) on response Y1 (size). (Colored)  
 248  
 249

#### 250 4.4 Influence of number of passages and hydration volume on zeta potential

251 The effect of X2 (number of passages) and X3 (hydration volume) and their interaction on  
 252 Y3 (zeta potential) at a fixed X1 pressure (10000 psi) was shown in Figure 4. At low X2  
 253 value, Y3 increased from 54mV to 68 mV when X3 increased from 4 ml to 8 ml. Similarly,  
 254 at high value of X2, Y3 increased from 60mV to 72 mV when X3 increased from 4ml to 8  
 255 ml.





256  
257 **Figure 4.** Response surface plots showing the effect of X2 (number of passages) and X3  
258 (hydration volume) on response Y3 (zeta potential). (Colored)  
259

#### 260 4.5 Optimization using a desirability function and validation

261 This procedure helped to determine the combination of experimental factors which  
262 simultaneously optimized several responses by maximizing a desirability function. The  
263 target value for size was 150 nm and for zeta potential was 60 mV. The scale of the  
264 desirability function ranged from  $D = 0$  for a completely undesirable response to  $D = 1$  if  
265 the response corresponds to the most desirable value. The desirability function calculated  
266 at each point in the design is summarized in Table 6. Maximum desirability was achieved  
267 at run 17 with a satisfaction rate of 92.14%. The predicted factor combinations to achieve  
268 optimum value are shown in Table 7.

269 Triplicate validation tests showed that the experimental condition predicted by the  
270 mathematical model did not reach the size value and narrow distribution required for the  
271 cationic lipid nanoparticle (data not shown). The run 17 experiment was observed to be  
272 closer to target size and chosen for in vitro silencing efficacy assay.

273 In the present work, the size of microfluidized-cationic liposomes was larger than that  
274 observed with other types of cationic liposomes prepared under similar conditions  
275 (pressure and number of passages) [22,32]. This discrepancy could be explained by the fact

276 that the DMAPAP molecule has three positive charges and thus possesses a larger  
 277 headgroup volume and identical lipidic moiety volume, as compared to DOTAP or DC-  
 278 Chol, which carry only one positive charge in their headgroup.

279 **Table 6.** Predicted and observed desirability at each point of the design.

			<i>Predicted</i>	<i>Observed</i>
<i>Row</i>	<i>size</i>	<i>zeta</i>	<i>Desirability</i>	<i>Desirability</i>
1	297.5	80.7	0.2058	0.1081
2	214.4	83.0	0.3221	0.3746
3	191.6	76.1	0.4817	0.6130
4	311.0	73.0	0.0000	0.0000
5	310.5	73.0	0.2318	0.0133
6	283.8	72.0	0.3896	0.2353
7	307.0	69.8	0.0000	0.0624
8	298.8	73.0	0.0728	0.1299
9	286.23	80.4	0.1981	0.1694
10	134.0	66.7	0.7558	0.7145
11	176.4	68.0	0.8845	0.7922
12	168.1	63.1	0.8193	0.8856
13	96.75	54.2	0.7796	0.0178
14	147.0	63.3	0.8980	0.9235
15	242.9	63.7	0.7866	0.5177
16	167.7	61.9	0.3901	0.9004
17	164.2	59.6	0.9242	0.9179
18	162.5	58.6	0.7757	0.8749
19	175.5	69.7	0.6947	0.7737
20	175.9	86.4	0.6029	0.0000
21	159.8	82.6	0.6333	0.5347
22	178.4	65.8	0.7518	0.8100
23	169.1	75.1	0.6624	0.7128
24	167.9	78.8	0.6413	0.6370
25	178.2	67.7	0.8389	0.7883
26	194.5	70.9	0.6762	0.6795
27	197.8	77.3	0.5805	0.5687

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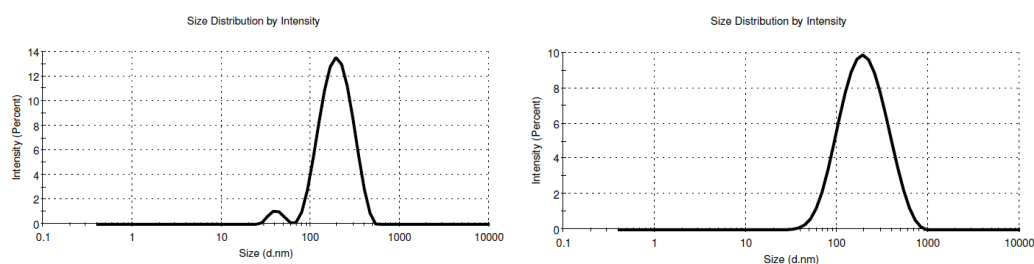
283 **Table 7.** Experiment conditions and validation of run 17 and optimum value (triplicates).

	Pressure (psi)	Passage number	Hydration volume (mL)	Size (nm)	PDI	Zeta potential (mV)
Run 17	10000	3	4	160±9	0.2±0.0	52±9
Optimum	9543	1	5.3	213±53	0.3±0.1	48±10

284

285 **4.6 Physicochemical characteristics of liposomes and lipoplexes**

286 The size and PDI of blank liposomes and lipoplexes were similar, and the lipoplexes zeta  
 287 potential was lower than that of blank liposomes, likely because of the blank cationic  
 288 liposome complexation with the negatively charged siRNA (Figure 5 and Table 8). The  
 289 morphology of blank liposomes and lipoplexes was studied by TEM. Blank liposomes  
 290 were spherical with a blank core. Lipoplexes at charge ratio 8 exhibited unsmooth surface  
 291 and dense core, indicating that the siRNA molecules have been complexed with  
 292 liposomes and formed a structure neither lamellar nor hexagonal, thus different from our  
 293 previous formulations and from the literature [18-20, 33, 34] (Figure 6).



294

295 **Figure 5.** Size and PDI of blank liposomes (left panel) and lipoplexes (right panel) of charge ratio  
 296 8 as assessed by dynamic light scattering (DLS).  
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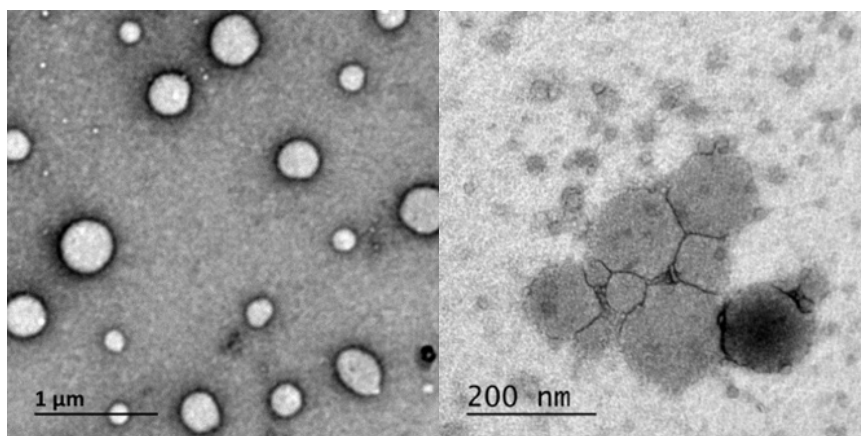
301

302

302 **Table 8.** Average value of size, PDI and zeta potential of blank liposomes and lipoplexes. (Mean  
 303  $\pm$  SD of three separate experiments)  
 304

Formulations	Size (nm)	PDI	Zeta potential (mV)
Blank liposome	160 $\pm$ 9	0.28 $\pm$ 0.0	52 $\pm$ 9.8
Lipoplexe charge ratio 8	165 $\pm$ 1	0.21 $\pm$ 0	36 $\pm$ 3.3

305



306

307

308 **Figure 6. TEM micrographs of blank liposomes and siRNA-lipoplexes.** TEM micrographs of  
 309 blank liposomes (left, spherical shape) and siRNA-lipoplexes at (+/-) charge ratio of 8 (right,  
 310 unsmooth surface and dense core) were performed using negative staining.

311

#### 312 **4.7 Gel retardation assay**

313

Nine samples were prepared and electrophoresed on agarose gel: free siRNA, blank

314

liposomes, and lipoplexes with a charge ratio ranging from 0.5 to 10. The charge ratio

315

between cationic lipid positive amines and siRNA negative phosphate was calculated as

316

described in the Methods section. The result illustrated the efficient siRNA encapsulation

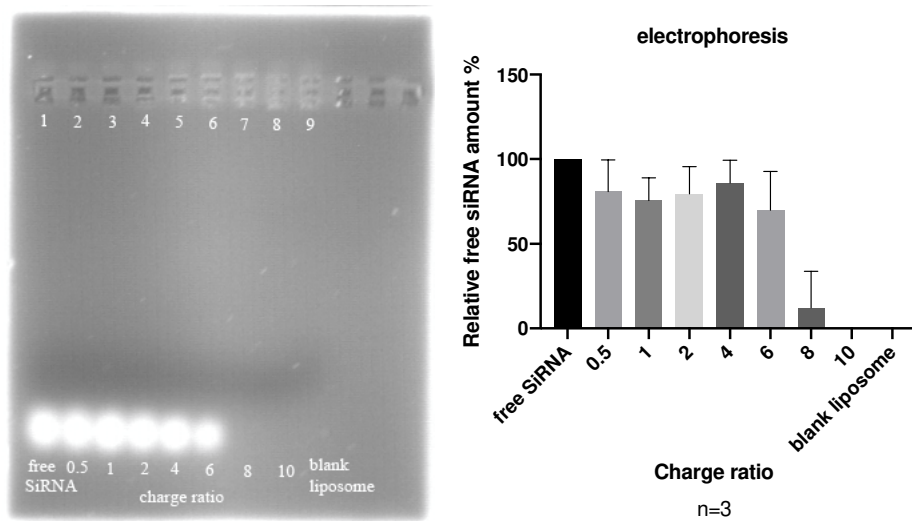
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at a charge ratio of 8, with encapsulation yield of 88%, and at charge ratio of 10, with

318

complete encapsulation (Figure 7).

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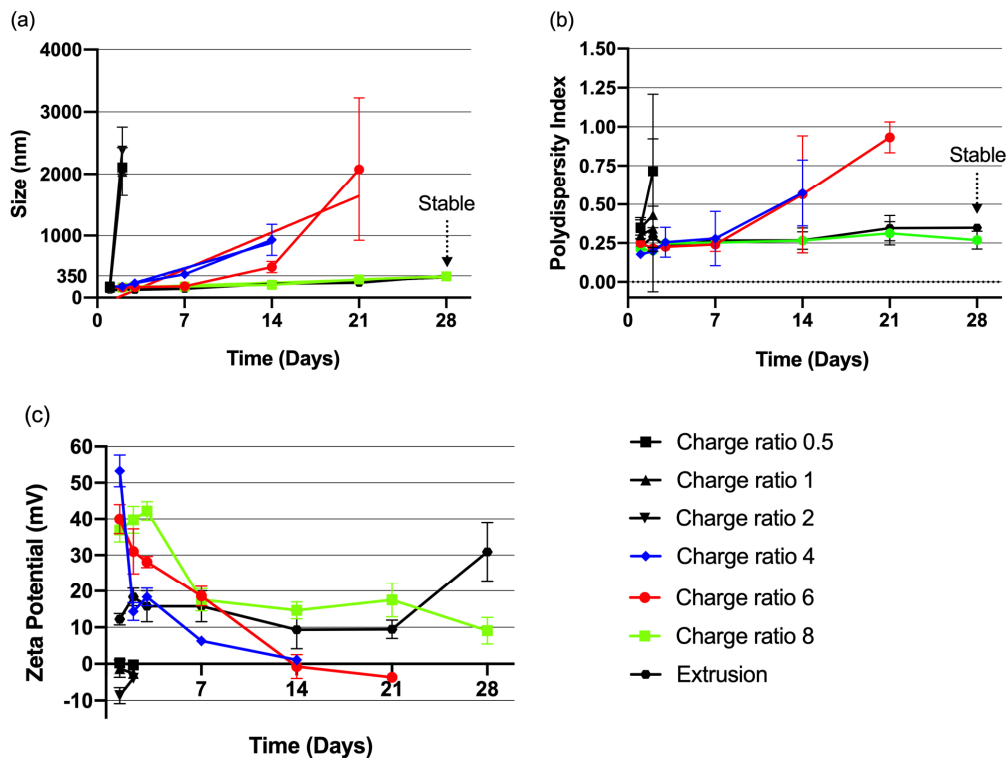


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**Figure 7. Gel retardation assay of lipoplexes.** Lipoplexes with different charge ratio from 0.5 to 10 (0.3  $\mu\text{g}$  siRNA/lane), free siRNA and blank liposomes were tested. Samples were deposited onto 1.5% agarose gel and the electrophoresis was run at 80 V for 30 mins. Free siRNAs were visualized following incubation with BET through fluorescence under UV light. (Charge ratio = nmol of cationic lipid (DMAPAP) / $\mu\text{g}$  of siRNA).

#### 4.8 Stability

329 The stability of lipoplexes prepared either by microfluidization (at different charge ratios  
330 from 0.5 to 8) or by extrusion (charge ratio of 4 as in previous work from our team) was  
331 assessed over one month. The extruded lipoplexe preparation was stable in terms of size,  
332 PDI, and zeta potential. The microfluidized lipoplexes were stable only at the higher  
333 charge ratio of 8. Size, PDI of these two formulations were similar, zeta potential of  
334 microfluidized lipoplexes at charge ratio 8 was higher at 37.0mV compared to extruded  
335 lipoplexes, finally decreased to 12.2mV over one month (Figure 8). At charge ratio 10,  
336 zeta potential revealed unacceptable variability, particle sediment and aggregation (data  
337 not shown), indicating that the high charge ratio 10 resulted in instability.



338

339 **Figure 8. Lipoplexes stability assessment.** Stability of microfluidized lipoplexes with charge  
 340 ratio range 0.5 to 8 in terms of size (a), PDI (b) and zeta potential (c) over one month. Extrusion  
 341 lipoplexes had a charge ratio of 4. (Colored)

342

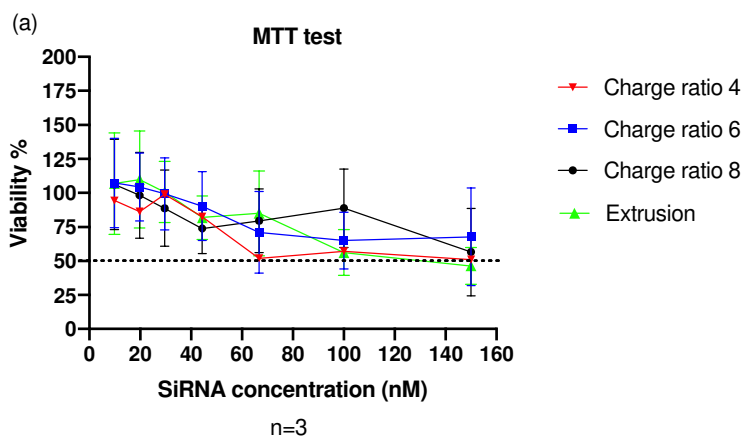
#### 343 4.9 Cytotoxicity and silencing efficiency

344 Cytotoxicity was evaluated by MTT test at anti-luciferase siRNA concentration ranging  
 345 from 10 nM to 150 nM. For siRNA concentrations of 10 nM to 20 nM, cell viability  
 346 attained 98%-100%. However, when the siRNA concentration increased up to 150 nM,  
 347 cell viability decreased down to 50%. Microfluidized lipoplexes with a charge ratio of 4  
 348 to 8 and extrusion reference lipoplexes (charge ratio 4) presented approximately the same  
 349 level of cytotoxicity (Figure 9a).

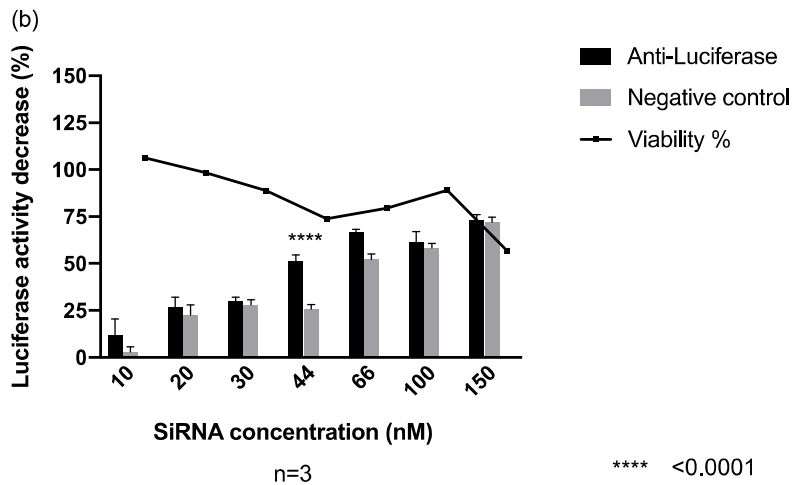
350 For silencing activity assessment, a luciferase assay was performed on a reporter cell line  
 351 [18], [38]. With microfluidized lipoplexes at a siRNA concentration of 44 nM at a charge  
 352 ratio of 8, we observed a 51% of inhibition of luciferase (LUC) gene expression, with

353 30% cytotoxicity, against only 25% of Luc gene expression observed when using the  
 354 scrambled unmatched siRNA (Figure 9b). This difference was statistically significant.  
 355 At a siRNA concentration of 66 nM, the silencing of Luc expression reached 66%, with  
 356 30% cytotoxicity. However, since 52% Luc expression was observed when using the  
 357 scramble siRNA, it can be deduced that, at this high siRNA concentration, the non-  
 358 specific effect of siRNA lipoplexes became predominant. The LUC expression silencing  
 359 observed with microfluidized lipoplexes at charge ratios 4 and 6 was not significant (data  
 360 not shown). A higher silencing efficacy has been described in previous studies and from  
 361 the literature. It can be suggested that the present formulation could be improved by  
 362 reducing the PEG lipid content to less than 5% [18],[19],[22],[28].

363



364



365

366 **Figure 9. Cell viability and Luc silencing efficiency at various siRNA lipoplexe**  
 367 **concentration.** (a) Cytotoxicity was measured with the MTT test and expressed  
 368 relatively to non-transfected cells (microfluidized lipoplexes of charge ratio 4-8,  
 369 extrusion lipoplexes of charge ratio 4, siRNA concentration 10nM-150nM). (b)  
 370 Luciferase silencing efficiency of microfluidized lipoplexes (charge ratio = 8) expressed  
 371 as percentage of control non-transfected cells (mean +/- SD; n=3).  
 372

### 373 5. Conclusions

374 Overall, we have identified a cationic liposome preparation obtained by microfluidization  
 375 combined with thin film hydration method which, at a lipoplexes charge ratio of 8, leads  
 376 to gene silencing efficiency comparable to the more classical extrusion technique. The  
 377 cytotoxicity was similar as compared to reference lipoplexes obtained by extrusion. In the  
 378 present study, PEG lipid was used to decrease the size of liposomes and increase blood  
 379 circulation time for further *in vivo* use. However, PEGylated lipids might also limit the  
 380 silencing efficiency of the present lipoplexe formulation. Indeed, the lipid-PEG shell is  
 381 recognized to weaken the electrostatic adhesion between cationic liposomes and  
 382 negatively charged cell membrane, thus decreasing endocytosis and lipoplexe  
 383 transfection efficiency [35].



384 The present results provide device-design approach using a fitted DoE (with parameters  
385 constrained by the device as well as the chosen raw material) to produce cationic  
386 liposomes, using a microfluidization process which is lab-scalable and potentially  
387 meeting the scale-up industry requirements. The chosen methodology and its fitted design  
388 can be used for other and more recent nanocarriers formulation<sup>[10],[39]</sup>. Ultimately, the  
389 parameters of different size reduction methods with design<sup>[9], [10], [39]</sup> or without design<sup>[22]</sup>  
390 can be used to determine the key factor influencing the physicochemical characters and  
391 functions of nanoparticle formulations.

## 392 **6. Acknowledgements**

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394 University for the helps of instructing the in vitro part.

395

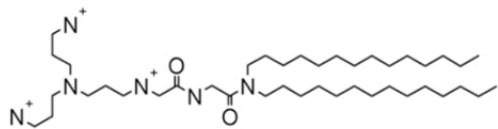
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DMAPAP

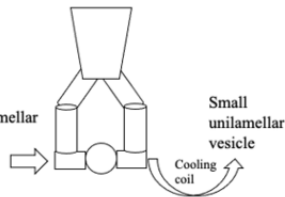
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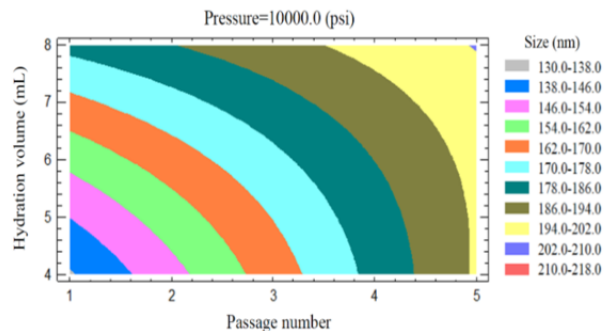
Thin film hydration

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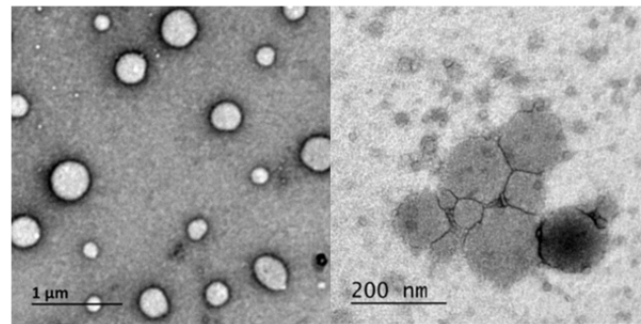
Large multilamellar vesicle



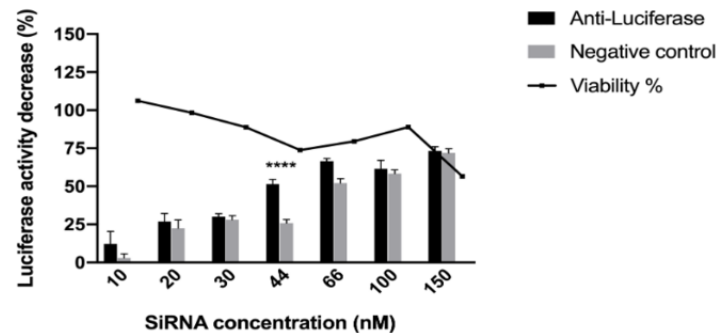
Microfluidization



Design of Experiment (DOE)



siRNA-lipoplexes



In-vitro tests

\*\*\*\* <0.0001